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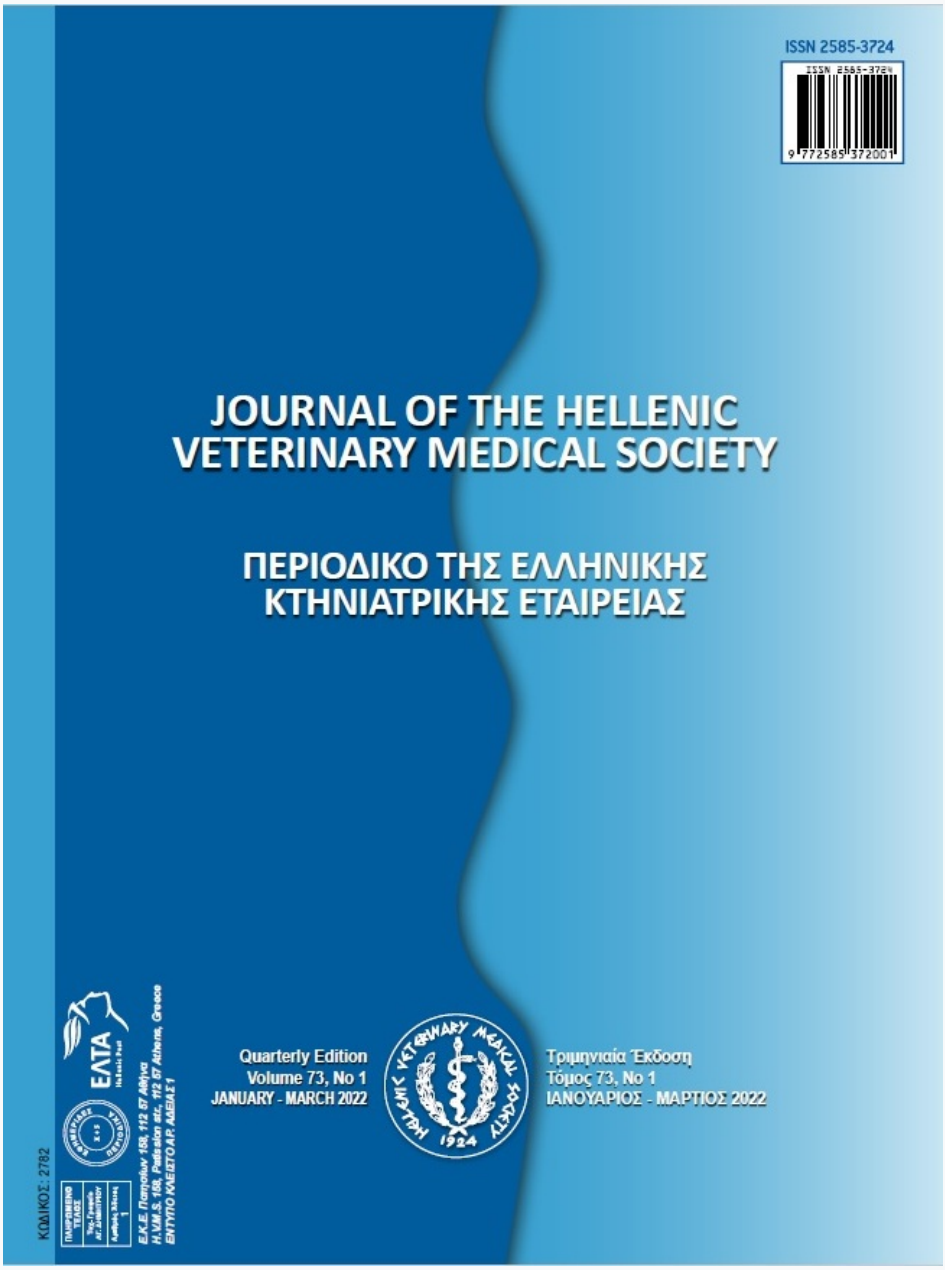

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ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ

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Αριθμός Άδειας 1

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				Number of specialist veterinarians active in Greece Αριθμός ειδικευμένων κτηνιάτρων εργαζόμενων στην Ελλάδα
1		ECAR	European College of Animal Reproduction	2
2		ECAWBM	European College of Animal Welfare and Behavioural Medicine	2
3		ECAAH	European College of Aquatic Animal Health	3
4		ECBHM	European College of Bovine Health Management	3
5		ECEIM	European College of Equine Internal Medicine	0
6		ECLAM	European College of Laboratory Animal Medicine	0
7		ECPHM	European College of Porcine Health Management	3
8		EPVS	European College of Poultry Veterinary Science	4
9		ECSRHM	European College of Small Ruminant Health Management	12
10		ECVAA	European College of Veterinary Anaesthesia and Analgesia	1
11		ECVCN	European College of Veterinary Comparative Nutrition	0
12		ECVCP	European College of Veterinary Clinical Pathology	1
13		ECVD	European College of Veterinary Dermatology	3
14		ECVDI	European College of Veterinary Diagnostic Imaging	2
15		ECVECC	European College of Veterinary Emergency and Critical Care	0
16		ECVIM-ca	European College of Veterinary Internal Medicine-companion animals	0
17		ECVN	European College of Veterinary Neurology	2
18		ECVO	European College of Veterinary Ophthalmology	0
19		ECVP	European College of Veterinary Pathology	0
20		ECVPH	European College of Veterinary Public Health	5
21		ECVPT	European College of Veterinary Pharmacology and Toxicology	1
22		ECZM	European College of Zoological Medicine	1
23		ECVS	European College of Veterinary Surgery	1
24		EVDC	European Veterinary Dentistry College	0
25		EVPC	European Veterinary Parasitology College	3

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History of the Hellenic Veterinary Medical Society

The Hellenic Veterinary Medical Society (HVMS) is one of the oldest Scientific Societies in our Country. It was founded in 1924 and its first scientific journal was published in 1926. Prompter, Founder and Animating Spirit of HVMS was the General Ioannis D. Petridis (1870-1947), first President and for many years Honorary President of the HVMS. Among the 49 founding members of the HVMS there was also the memorable professor Konstantinos Livadas, the founder of the Veterinary School of the Aristotelian University in Thessaloniki. In spite of the disagreements, the HVMS contributed greatly to the foundation of Veterinary School.

During that time there was only one Scientific Society in Greece, the Medical Society of Athens, which was founded in 1835 and published its first scientific journal in 1922. The HVMS dealt not only with scientific but also with professional topics, like the establishment of the invoices for the veterinarians' payment, taxes, insurance etc. Also, at that period, the accession of the Veterinary Branch in the Hygienists' Pension and Self Insurance Treasury (TSAY) was achieved.

The first post-war assembly of the HVMS took place in the private medical office of Petros Kiappe, on Peta Street in Athens. With its post-war first president Konstantinos Melanidis, the HVMS has been working by implementing its old memorandum of association and has been located in the premises of the Veteri- nary Microbiological Institute of Votanicos, from where all mem- bers of the Governing Board and the Editorial Board of the Journal of the HVMS, were coming from. There, the first «nucleus» of the Library of the HVMS, has been created. That is the reason, this second period of the HVMS successor of the «Petridis period», used to be called «Votanikos period, 1944-1965».

Because HVMS's income was very small, it will remain homeless for many years. Looking for a meeting place the HVMS will find positive response from several services and societies (State Veterinary Offices, Greek Chemical Society, Hellenic Agricultural Society, Medical Society of Athens, Insti- tute of Agricultural Studies, State Veterinary Service of Athens, National Organization of Greek Handwork), which during the following years are going to offer its premises, while in the mid 1958 and for a short period, depending on its financing capabil- ities, the HVMS will rent its own room.

In 1944, the HVMS writes down its first post-war Member Book and in 1948 has already acquired its first 74 regular mem- bers. Also, HVMS is actively working with scientific subjects during regular meetings and public seminars, analyzing current veterinary issues, members' proposals and so on. On 29th May 1947 Mr Petridis presented in the Academy of Athens an issue for veterinary science and its contribution to the progress of the agricultural production and safeguard of Public Health. Also, it should be pointed out, that because there was no professional body, the HVMS is also dealing with issues related to the exe- cution of the veterinary profession.

Furthermore, the role of the HVMS has been determinative on the decision making of the Ministry of Agriculture on veter- inary legislation, on the organization of the Veterinary Service in the Ministry of Agriculture as well as on livestock topics. In the decade of 30s the Supreme Veterinary Advisory Council was created mainly dealing with scientific issues and other aims like promotion, publicity and consolidation of the veterinary science and the veterinary profession in our country and internationally.

The Hellenic Veterinary Medical Society publishes a quarterly scientific journal called Journal of the Hellenic Veterinary Medical Society (J Hellenic Vet Med Soc), as well as other scientific publications, organizes Congresses, Symposiums, Meetings, Lectures etc and generally and almost exclusively it has undertaken for life the Continuing Education of the Greek veterinarians and the students of the two Veterinary Schools.

Nowadays, the Hellenic Veterinary Medical Society is governed by a 9 member Governing Board which is elected every 3 years and has 3 branches:

- **Branch of Companion Animals**
- **Branch of Food Hygiene and Public Health**
- **Branch for Farm Animals**

The HVMS collaborates with the Supreme Educational Foundations, the Technological Educational Institutes, the Veterinary Services, and the Veterinary Associations as well as with Scientific Societies and the Greek and Foreign Chambers.

- **The HVMS is member of the:**
- **Worldwide Veterinary Society**
- **Worldwide Veterinary Society for Companion Animals**
- **Federation of European Veterinary Societies for Compan- ion Animals (founding member)**
- **Veterinary Society of the Balkan and the Black Sea (found- ing member)**

The HVMS has a total of 1220 members many of which have been distinguished in the scientific field (University Profes- sors, Researchers), in the Public Administration, in the Army as well as in the Professional Veterinary Societies and Chambers, in Greece and abroad.

Since 29 May 2001, having signed the contract and since 15 December 2002 the date on which the official opening celebration took place, the Hellenic Veterinary Medical Society is housed in its private premises in a beautiful and majestic one-floor apartment, on the 7th floor of a building in the centre of Athens at 158, Patission street, of 265m2 area, including main lobby (14m2), secretary (13m2), lecture room (91m2), the President's office (22m2), the Governing Board meeting room & library (44m2), the kitchen (18m2), two big baths, a storage room and a large veranda. Al the actions performed for possessing this new private office for the HVMS were performed during the presidency of Dr Theodoros Cl. Ananiadis and the following Governing Board:

President:	Theodoros Cl. Ananiadis†
Vice-President:	Veniamin Albalas
General Secretary:	Athanassios E. Tyrpenou
Spec. Secretary:	Konstantinos Chandras
Treasurer:	Olga Sabatakou
Member:	Emmanuel Archontakis
Member:	Apostolos Rantsios

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Review article
Ανασκόπηση

Non-accidental injuries in dogs and cats: review of post-mortem forensic assessment and the social significance of small animal practice

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ABSTRACT: The co-existence of animal abuse cases and domestic violence are well established today. Many studies worldwide have identified that pet dogs and cats are commonly harmed or killed by an abuser who may be a member of the family. In fact, the abuse of pets is an indicator that human members of a family are also at risk for interpersonal abuse. The abusers may show a variety of motivations and mental health disorders, resulting in a variety of abuse forms. The animal victims may be presented alive or dead to a veterinary clinic. The post-mortem differential diagnosis and forensic evaluation of pet dogs and cats with non-accidental injuries (NAI), caused by the physical violence of the abuser and items commonly found in a household environment are presented in the current review, according to the main type of abuse: a) blunt force trauma; b) sharp-force injuries; c) gunshot injuries; d) asphyxiation and drowning; e) thermal injuries; and f) poisoning. The recognition and mandatory report of pet cruelty in the family is a complex issue, causing ethical dilemmas for veterinarians concerning professional confidentiality to the client, obligation to protect the human probable victims and the probability of prosecution when the law has been broken. The key-role of veterinarians in the identificationand report of pet abuse to appropriate state authorities for animal and human welfare is discussed. Also, ethical issues are highlighted in this paper.

Keywords: *Dogs, cats; non-accidental injuries; necropsy; veterinary forensics, intrafamily violence*

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INTRODUCTION

Over the last half-century, the role of companion animals in human society has changed rapidly. Where these animals were once seen as replaceable objects, they have now come to be viewed as "members of the family" by most people who have developed strong emotional bonds with their pets in the household environment (Newberry, 2016; Rollin, 2018).

Animal abuse is defined as a socially unacceptable and illegal behavior that causes unnecessary distress, suffering, pain, injury and/or malicious killing of an animal (Vermeulen and Odendaal, 1993; Ascione et al., 1997; McGuinness et al., 2005).This umbrella term encompasses physical, sexual, and emotional abuse as well as neglect (Tong, 2016; Almeida et al., 2018).It is also reported that, more than one type of abuse can occur concurrently in pets (Almeida et al., 2018).

The term of non-accidental injuries (NAI) is used as a synonym of physical abuse, including any bodily trauma or injury caused by malicious actions of a person (McGuinness et al., 2005; Tong, 2016, Almeida et al., 2018). Wounds may be single or multiple, concentrated in an anatomic region, or present throughout the body (McEwen, 2017; Almeida et al., 2018).

Although research into NAI in pets is increasing, current data are still limited compared to human forensics (de Siqueira et al., 2012; Almeida et al., 2018; Newland, et al., 2019). Types of NAI which may be encountered by veterinarians have been recorded as: asphyxiation or drowning, burns or scalds, blunt force trauma (e.g.bruising or abrasions, fractures, head trauma, ocular injuries, internal thoracic and abdominal injuries), sharp-force injuries (e.g.knife wounds), gunshot injuries, embedded collar or binding injuries, injuries due to dog fighting, poisoning and starvation. Physical injuries inflicted on sexual organs, the anus or rectum must be considered as potential cases of sexual abuse. Repetitive traumas at different stage of healing are a strong indication of chronic cases of NAI (Tong, 2016; Almeida et al., 2018; Newland, et al., 2019).

Many cases of smallanimal abuse take place in the family environment of the abuser (Hensley et al., 2011; Van Wijk et al., 2018). The co-existence of companion animal abuse and intrafamily violence has been the focus of much international research, especially in recent years. The link between animal abuse and violence against humans is now well established, and highlights that animal abuse, child abuse, elder abuse, and intrafamilyviolence frequently coexist (Onyskiw, 2007; Monsalve et al., 2017). The abuse of pets is an element of the complex network of domestic violence and may be an indicator that members of the family are also at risk (Newberry, 2016; Monsalve et al., 2017).

Studies worldwide have identified that: a) dogs and cats are commonly harmed or killed as a form of family violence, andb) a significant number of women and their children remain in intrafamily abusive relationships due to concerns for the safety of their pets (Roguski 2012; Monsalve et al., 2017). Moreover, severalintrafamilyviolence victims reported that companion animals were one of their main sources of support, and many chose to stay in an abusive relationship because shelters for human victims of domestic violence did not have the facilities to house their pets (Newberry, 2016).

Thus, taking into consideration the above-mentioned data, veterinarians in companion animal clinics will be confronted at some time in their careers with pet abuse cases. A pet victim with NAI may be presented alive or dead to a veterinary clinic by the perpetrator or another family member (who may be a victim himself/herself), or a third party under some coercion from the person who caused the injury (Munro and Thrusfield, 2001c; de Siqueira et al., 2012; Arkow, 2015). The common types of physical abuse of small animals inside household may include injuries due to punching, kicking, throwing (e.g.out of windows, against walls), stabbing, beating with instruments, burning and asphyxiation. Sometimes abuse is performed by the domestic equipment such as a microwave oven into which the pet is placed (Munro and Thrusfield, 2001a &2001b; Henderson et al., 2011; Almeida et al., 2018).

The current review is focused on the post-mortem diagnosis and forensic evaluation of pet dogs and cats with NAI, caused byphysical strength of the abuser and/or items commonly found in a household environment. Nevertheless, data for NAI cases concerning stray companion animals, illegal commercial exploitation (e.g. dog fighting, puppy mills) or working dogs (e.g. shepherd dogs, hunting dogs) abuse are not included.

THE ABUSERS OF FAMILY DOGS & CATS

Just as for any crime, people have different motives for abusing animals. Moreover, animal cruelty is a sign of psychopathology of the perpetrator who may be either a child or an adult member of the family (Schwartz et al., 2012; Bright et al., 2018). The companion animal veterinarian may act as a forensic veterinarian. For that, it is helpful to be aware of the range of animal abusers'motives to better generate scenarios to evaluate during a veterinary forensic investigation (Lockwood and Arkow, 2016).

Pet abuse by children family members

Non-adult animal abuserscould bedivided into three categories, in accordance with their age (Hensley et al., 2011).The first category includes preschool children who were poorly supervised and not trained in the care of pets. These children are exploratory or curious animal abusers (Hensley et al., 2011; Doukas et al., 2018).

The second category includes school aged children and young adolescents (Hensley et al., 2011). Animal abuse among them is more indicative of conduct disorder and/or exposure to domestic violence. These youngabusers are more likely to abuse animals in the family and the community.They may also engage in cruelty to animals out of imitation, because they have been desensitized to violence, have decreased empathyor lack of attachment, are perpetrators or victims of bullying in their school or even because they find amusement in these acts. In extreme cases, children living in a violent home may kill their pet to preventitfrom sustaining further injury (Bright et al., 2018; Doukas et al., 2018; Johnson 2018).

The third group is primarily composed of adolescents who may have been under the influence of alcohol and/or drugs while committing acts of animal cruelty. This categoryincludes delinquent animal abusers who often need judicial and clinical interventions (Hensley et al., 2011; Doukas et al., 2018).

Pet abuse by adult family members

The most of adult animal abusers are characterized by an antisocial or other personality disorders. As far as their motivations, both male and female adult perpetrators use threats to harmor harm and kill pets in order to control human members of the family. They commit acts of physical abuse to incite and perpetuate fear and submission in their human victims (Schwartz et al., 2012; Johnson, 2018; Newland et al., 2019).Family petperpetrators tend to engage in more severe forms of violence including intimate partner violence**,** elder abuse, child maltreatment, and emotional violence. They also utilize more controlling behaviors and economic extortion. Some men abuse pets in order to confirm their power over others such as to terrorize; teach submission; and to discourage their partner from leaving the relationship. Jealousy of partner's relationship with the pet as well as the perceived misbehavior of the pethas also been identified as motive. Moreover, some of animal abusers are sadists. They may abuse the animals before moving to human victims or concurrently. Many human victims of intrafamily violence report that pets were harmed or killed by their abuser (Febres et al., 2014; Bright et al., 2018; Johnson 2018).

Less commonly, animal abuse by adult perpetrators has been linked to addictive disorders orMunchausen syndrome by proxy, in which the animal caregiver causes a non-fatal injury in his/her pet, in order to obtain sympathy and attention of other people (Munro and Thrusfield, 2001c; Doukas et al. 2018).

RECOGNIZING NON-ACCIDENTAL INJURIES DURING FORENSIC NECROPSY

The companion animal veterinarian should consider that it is not necessarily a single incident leading to a raised index of suspicion, but rather a variable combination of factors, pattern of actionsand behaviors, or injuries which are not clearly or adequately explained, e.g. repetitive injuries or injuries affecting more than one pet in a single household (Munro and Thrusfield, 2001a &2001b; McGuinness et al., 2005; Arkow, 2015). In animals which have been presented alive to veterinary clinics with repetitive physical trauma e.g. burns, the veterinarian may suspect NAI during the clinical examination, and moreover, based on medical records, anamnesis and the observation of relationship between animal and the client (Newbery and Munro, 2011; Arkow, 2015; Tong, 2016). In animal cases presented dead, either dying during first aid care or euthanized because of severe injuries, a veterinaryforensic necropsy should be performed to evaluate whether the physical trauma lesions present accidental or non-accidental patterns (Almeida et al., 2018; de Siqueira et al., 2012; McEwen, 2017). A proper veterinary forensic investigation of a suspicious violent death requires a full necropsy, in order to clarify the cause, mechanism and manner of death.

The information required for a complete veteri-

nary forensic investigation, in order to find the cause and manner of death, should include not only the evidence from the dead body during necropsy, but also details of the animal's environment and items of evidence present on the crime scene (Touroo and Fitch, 2016).However, it is rare or impossible for a forensic veterinarian to attend a crime scene, due to absence of an infrastructure like the medical forensic examiner system. Therefore, in a suspect case of animal abuse, it is up to the veterinarian to evaluate the complete history or animal medical records in order to start his/her effort to differentiate accidental injuries from NAI (Almeida et al., 2018; de Siqueira et al., 2016; McEwen, 2017).

Aside from the presence of injuries that seemed consistent with physical abuse (e.g. gunshot wounds, stab wounds, cigaretteburns), multiple factors raise the suspicion of NAI in companion animals. These include: (1) the behaviorand comments of the client and/or other family member; (2) the behaviorof the pet, if it has been presented alive; (3) a historythat is vague or incompatible with the types of injury; (4) a history of pets that disappeared or died suddenly in the past or from families with known history of interpersonal violence; (5) a conspicuous lack of concern of the injuries in animals' body or a delay in seeking veterinary care for them; and (6) the presented injured animal has also been examined by other veterinarian (-s) and the owner seems to be greatly dissatisfied with the diagnosis (Munro and Thrusfield, 2001a&2001c; McGuinness et al., 2005; McEwen, 2017).

Especially, the evaluation of NAI lesions during the veterinaryforensic necropsy can confirm the suspicions of physical abuse of small animals, whose case histories give rise to concern that their injuries are not purely the result of an accident (Almeida et al., 2018; de Siqueira et al., 2012).

Blunt force trauma

Non-accidental blunt force trauma is a very frequent form of physical animal abuse with single or repetitive cases.In general, actions of blunt force violence may cause many types of injuries (abrasions, fractures, external and internal contusions and lacerations), showing diverse degree of severity (mild, moderate, or severe). Depending upon the tissue affected and the energy imparted by the force, injuries may resolve, repair, or may kill the animal (Munro and Thrusfield, 2001a; Munro and Thrusfield, 2001b; McEwen, 2017).

Cutaneous and subcutaneous traumaticlesions are very common in blunt force trauma cases.The typical lesion patterns are abrasions, contusions, and lacerations (Ressel et al., 2016). However, the thick hair coat, thick skin, and epidermal pigmentation of many animals may minimize or obscure external injuries. The forensicveterinarian should search meticulously for evidence of superficial blunt force trauma. Therefore, the entire skin should be removed from the body in order to identify traumaticlesions in the subcutis. Sometimes, clipping of the hair coat is also needed to reveal abrasions (Gerdin and McDonough, 2013; Ressel et al., 2016; McEwen 2017).

Muscle trauma is also a common lesion in blunt force trauma cases. The most common lesions in muscles are contusions and lacerations. Grossly, muscle trauma can range from small focal bruises between muscle fibers to large bruises with hematoma formation.Considering the resistance that the heavily haired skin of domestic animals offers, careful examination of the underlying muscles can often clarify whether a severe blunt trauma has been applied (Ressel et al., 2016; McEwen 2017).

As far as thoracic cavity, acuminated blunt objects can perforate the intercostal muscles, causing severe deep damage such as pneumothorax or hemothorax. Moreover, when the abdomen is forcefully compressed by heavy objects that produce high intra-abdominal pressure laceration of the diaphragm can result in herniation of abdominal organs. The liver is the abdominal organ that is most commonly and most severely affected by blunt force trauma, showing lacerationswhich lead to life-threatening abdominal hemorrhage. Depending on the applied blunt force, spleen trauma could be seen as subcapsular tears only or, inthe extreme, complete laceration (Ressel et al., 2016).

Typically, whole body radiographies should be applied in order to evaluate any skeletal trauma due to blunt force violence (Intarapanich et al., 2016). The following five radiographic findings should raisethe index of suspicion and support a diagnosis of NAI: (1) multiplefractures; (2) fractures occurring on more than one body regions; (3) transverse fractures; (4) fractures presenting at a later stage ofhealing (fractures with delayed presentation), and (5) multiple fractures at different stages of healing (Tong, 2014).

In many veterinaryforensic cases blunt force injuries involve not only animal's body, but also head

anatomical structures. Skull fractures may be seen or be absent.Hemorrhages in brain are the most characteristic gross lesions resulting from blunt force trauma, but contusions and lacerationscouldalso be seen (Ressel et al., 2016).Eyes of animal victim can be the direct target of blunt force trauma. Ocular injuries and hemorrhages are common findings. In some cases, these types of injuriesmay be the only external gross evidence of a blunt force trauma to the head. On the other hand, in cases withsevere cranial injuries eye bulb protrusion (traumatic proptosis) and multifocal orbital ecchymoses are seen (Ressel et al., 2016; McEwen 2017).

NAI should be differentiated from accidental injuries. Accidental blunt force injuries in companion animals are common due to struck by motor vehicles, falls from heights and, less commonly, due to bites from aggressive dogs or predators (Finnie, 2016; Ressel et al., 2016).

Motor vehicle traumasare differentiated from NAIby the location and distribution of the fractures and in the context of the reported clinical history and examination findings (Finnie, 2016; Intarapanich et al., 2016). Fractures of the skull, teeth, ribs, and vertebrae, as well as injuries to the claws are more often associated with NAI, whereas fractures of the pelvis, pulmonary contusions, and pneumothorax are more often secondary to a motor vehicle accident. Rib fractures may occur more frequently in NAI than motor vehicle accidents, because the surface area of the body to which force is applied is generally smaller in cases of NAI than in motor vehicle accidents, as the animal is usually punched, kicked, hit with a flexible object, or swung into or thrown against an object. When rib fractures do occur from a motor vehicle accident, the distribution is primarily on one side of the body. On the contrary, bilateral rib fractures predominate in NAI cases (Intarapanich et al., 2016).

Feline high-rise syndrome is characterized as a fall from the second or higherfloor, associated with facial, thoracic, and orthopedic trauma. Death in high-rise syndrome has been determined to be related to shock and respiratory distress from thoracic trauma (Liehmann et al., 2012, Bonner et al., 2012). Orofacial findings include bilateral epistaxis, facial soft tissue injury, hard palate fracture, tear and bruising of soft palatal tissue, mandibular fracture and/or mandibular symphyseal separation, dental trauma, tongue and other oral soft tissue injuries (Bonner et al., 2012). Thoracic trauma typically involves rib fracture, sternum fracture, and pneumothorax. Extremity injuries include fractures of the limbs, pelvis and vertebrae, and luxation of joints (Bonner et al., 2012). Moreover, lacerations of the heartmay be a rare consequence of blunt force trauma in animals.Heart lacerationsare seen via dissipation of an applied force to the sternum, such as falls from a considerable height or motor vehicle accidents (Ressel et al., 2016; Piegari et al., 2018). High-rise syndrome can also lead to hemoabdomen and other abdominal trauma, including pancreatic rupture. In live survived cats, pancreatic rupture results in severe pancreatitis and peripancreatitis (Liehmann et al., 2012)

Sharpforce injuries

Sharp force injuries are caused by a mechanical force using sharp shaped objects against the skin, subcutaneous tissuestilltomuscles and bonesor internal organs of animals. A variety of instruments causing sharp-force injuriescould be used by perpetrators. These instruments are not only knives, but also axes, machetes, scissors, screwdrivers, needles, barbecue forks, broken glass.In general, stab wounds injuries in animals seemed consistent with NAI.Sharp-force injuriesmay or may not be lethal, and this can be determined only by performing a detailed forensic necropsy with a careful external and internal examination. The cause of death should be stated as a sharp injury with an indication of the topographical region (s) affected (Munro and Thrusfield, 2001b; de Siqueira et al., 2016).In a study of NAIfound in dogs and cats, the thorax and the abdomen were the most frequently areas with sharp-force traumas (Munro and Thrusfield 2001b). In addition, sharp-force injuries in the neck and thorax are most likely to be fatal (de Siqueira et al., 2016).

In small animalcases where there is suspicion of abuse, the forensic veterinarian should consider radiographic examinationfor bone fractures, which may be found commonlyin such cases, and alsoexaminationfor blade fragments, which may exist if the blade entered thebony structure. Radiographic investigationmay also indicate distinct tool marks, which may match or indicate the crime weapon (de Siqueira et al., 2016).

During forensic necropsy, each open wound should be individually photographed before and after shaving the skin in the affected body regions. The underlying tissues and muscles should be assessed by reflecting the skin. An analytical description of the in-

juriesshould include the edges, shape, color and marks of lesions have been causedby sharp instruments.In contrast to blunt force injuries, sharp force injuries do not showlacerations or bridges of soft tissue between the edges of the wound. In fact, the edges of sharp injuries are linear or angular (de Siqueira et al., 2016; Ressel et al., 2016).

The cut of stab or incised wound have been caused by a knife may exhibit characteristics that identify the direction the knife was moved in, because the cut may be more superficial at the terminal segment of the wound. The shape of the resulting skin wound is affected by the nature of the tissue, the movement of the animal victim, the force of thepush, and the sharpness of the instrument. The angle of the lesion may indicate the positioning between the animal and the perpetrator. In some cases, the attacker may cause superficial marks or miss the stab due to the movement of the animal (de Siqueira et al., 2016). In each body region with sharp force injuries the coat looks matte with presence of dry blood material (McEwen, 2017). Moreover, when a stab wound occurs deeply in the neck body region, cutting larynx or trachea, the mechanism of death may be asphyxiation due to bloodaspiration (de Siqueira et al., 2016; McEwen, 2017). Deep stab wounds affecting the lungs may lead to hemothorax and/or pneumothorax. Deep stab wounds may also affectthe heart or some of abdominal organs e.g. the liver and gut (de Siqueira et al., 2016).

Chop wounds are showing characteristic patterns primarily seen on bones. The used instruments may cause striations on bones that are unique to each type of weapon. Axes cause crushing of the bones, while machetes produce small bony fragments inside wider and irregular wounds. When multiple chop wounds are present, they may exhibit distinctive depths, sizes, and shapes.For example, barbecue forks with 2 or 3 prongs can cause sharp injuries, which are identified as groups of 2 or 3 wounds, with regular or irregular distances between them caused by each prong depending on the angle of the stabbing. Finally, when wounds produced by glass are suspected, these wounds should be inspected for glass fragments (de Siqueira et al., 2016).

Gunshot injuries

Gunshot injuries are almost invariably evidence of NAI, and since a weapon must be made ready, such injuries always involve a degree of premeditation (Lockwood and Arkow, 2016). High-velocity bullets usually produce a perforating trauma, showingboth entry and exit wounds. On the other hand, lower-velocity bullets often produce a penetrating trauma, with the projectile being retained within soft tissues or bones (Finnie, 2016).

Before performing a forensic necropsy, animal victims should routinely undergo full body radiography to reveal the details of the presenting injuries (Lockwood and Arkow, 2016).The objectives of necropsy are to (1) identify the entry wounds and, if present, exit wounds; (2) document the extent of internal injury; (3) recover the bullets and theprojectile (s) if there is no exit wound; and (4) if a firearm was used, determine the direction and range of fire.

Projectile wounds may be perforating (i.e. pass completelythough the body) or penetrating, with the projectile coming to rest in the body, where the forensic veterinarian may recover it. An entry wound isoften characterized by a perfectly circular to oval shape, with finely abraded margins, whilean exit wound may showany shape (round, oval, stellate, crescent, etc). Also, an exit wound is generally irregular in outline without abrasion of the skin margins. Finally, the exit wound is often, but not always, slightly larger compared with the entry wound (Gerdin and McDonough, 2013).

Asphyxiation and drowning

According to the mechanism of injury, death due to asphyxia can occur with manual or ligature strangulation, hanging, suffocation, and mechanical asphyxia (McEwen, 2016). All forms of intentional asphyxia involve close contact with the companion animal victim. Often this includes binding, taping, or other restraint. Evidence of such actions can indicate a high degree of premeditation. Frequently, small animals with ligatures or those that have been manually strangled also have localized abrasions and contusions, other lesions of physical (blunt force trauma, sharp force trauma, projectile wounds) and/or sexual abuse (Lockwood and Arkow, 2016; McEwen, 2016).

The interpretation or opinion that the death was due to asphyxia requires definitive and compelling evidence from history, death scene investigation and forensic necropsy of the dead companion animal. Unlike medical examiners, veterinarians rarely attend a crime scene and therefore may lack some of the crucial information required to confirm death due to an asphyxia mechanism. Animals dying due to various

types of asphyxia may or may not have macroscopic lesions. It is also possible that in some cases of asphyxia some gross lesions may be due to other causes, e.g. blunt force trauma to the head, and they could be considered as a contributing cause of death (McEwen, 2016; Almeida et al., 2018).

Obstructive asphyxia due to accidental inhalation of foreign bodies, e.g. food or toys, inside larynx or trachea is usually a straightforward postmortem diagnosis. The diagnosis may be problematic, if theforeign body was removed prior to postmortem examination, although there may be intraluminal trace evidence of the object, focal edema, congestion, hemorrhages, erosions, or ulceration at the obstructed site (Roach & Krahwinkel 2009; McEwen, 2016).

In cases of manual strangulation, the intermittent pressure and release on the neck increases the likelihood of petechiae. Contusions, hemorrhages, congestion are seen subcutaneously as well as in muscles of the neck. The jugular veins and thecarotid arteries should be examined in situ for lacerations. Pulmonary edema, congestion, hemorrhage, and/or atelectasis may occur. Scleral congestion and/or conjunctival petechiae in eyes may be observed (McEwen, 2016).

In cases of suspected ligature strangulation and hanging, edema of the proximal neck and head may be visible as well as scleral congestion (Gerdin and McDonough, 2013; McEwen, 2017). Contusions, abrasions, hemorrhages of the neck and base of the head involving the subcutaneous tissue, musculature, airway, and esophagus and/or disruption of thyrohyoid-thyroid cartilage junctions are observed (McEwen, 2016; Almeida et al., 2018). Many types of material, e.g. collars, leashes, ropes, chains, cords, clothing, towels, fabric, and wire, may have beenused as ligatures. The ligature mark is the most relevant lesion in ligature strangulation and hanging, although it may be absent depending on the type of ligature used, the duration, and the characteristics of the animals' fur (Gerdin and McDonough, 2013; McEwen, 2016). The skin should be shaved as the indentation made by the ligature may be more apparent. In practice, it is easier to identify the ligature placement following reflection of the skin as a dark line of compression (McEwen, 2016).

External pressure on the chest and/or lungs pressure by an acquired posture are types of mechanical asphyxia, resulting in respiration restriction and non-specific lesions. If the companion animal has been crushed, fractures, lacerations, and internal hemorrhage due to trauma are likely to be present (McEwen, 2016).

Finally, in cases of suspected drowning, the diagnosisis usually one of exclusion, requiring information from the deathscene (e.g. a swimming pool), recovery scene, the medical history or accounts given by reliable witnesses. Distended, heavy, congested and edematous lungs that fail to collapse and stable froth in the trachea, larynx, nasal passages or mouth are characteristic gross lesions, but they are not specific. Right ventricular distension, pulmonary hemorrhages, pulmonary emphysema and mucus at the base of the tongue or in larynxhave also been observed in some cases. Additionally, contusions or bruising in skin or subcutaneous tissuesand other traumatic lesions e.g. fractures may be seen in cases of forcible submersion of the animal (McEwen and Gerdin, 2016).

Thermal injuries

Thermal injuries in companion animals may are due to exposure to fire and radiant heat, contact with hot items including hot liquids or steam, inhalation of hot air, and exposure to extremely high orcold temperatures. Forensic questions concerning thermal injuries represent relatively infrequent animal cases, concerning mainly alive victims of suspected cruelty (Tong, 2016; Wohlsein et al., 2016). However, the forensic evaluation of live animals is not the aim ofthe present review and only few examples are mentioned.

In general, besides detailed information from the history about the circumstances under which the pethas been injured, a thorough gross and forensic histopathological investigation must be performed with respect to the patterns of lesions that may bedue to thermal injuries (Wohlsein et al., 2016).For example, cigarette burns on live animals in most cases represent acts of NAI. In some of them, because of the presence of long fur and skin pigmentation, it could be relative difficult to observe cigarette burns (de Siqueira et al., 2012).Acute lesions are characterized by circular, well circumscribed hyperemic areas of 6 to 8 mm diameter often with a full-thickness necrosis of the skin, resulting in a crater-like lesion. Hairs are singed and show yellowish discoloration. Chronic lesions exhibit thin scar tissue on the surface of the sunken area. On the contrary, accidental cigarette burns are typically superficial lesions due to brief contact (Wohlsein et al., 2016).

In pets thatwere put inside a microwave oven, tissue damages are directly related to the tissues water content as well as the duration of exposition and the power of the microwaves. At necropsy, the skin may show fragility, splitting with well-defined edges, hair and claw loss, crumpling and reddening of the tips of the pinnae, but no charring or singeing of hairs. Histopathological examination may show abrupt transition of affected and unaffected tissues depending on their water content; for example, an unchanged subcutaneous fatty tissue in contrast to coagulative necrosis of the adjacent musculature and skin may be seen. Pulmonary congestion with or without edema, and internal organs with a cooked appearance and odor due to coagulative necrosis are also common post-mortem findings (Wohlsein et al., 2016).

Poisoning

There are few data examining the links between companion animal poisoning cases and crimes of interpersonal and especially intrafamily violence, although some dog and cat poisoning cases have been reported to occur inside the household environment. Intentional poisoning includesmalicious intent or misuse of a toxic substance. However, it is necessary to consider that intentional poisoning may be mistakenly assumed when in fact the animals are exposed accidentally to household items with toxic action (Lockwood and Arkow, 2016; Almeida et al., 2018).

Forensic evaluation of dead animals suspected of being poisoned should entail performance of a thorough necropsy examination and toxicological analysis of biological (e.g. stomach content) in a Toxicology Laboratory (Volmer and Meerdink, 2002; Gwaltney-Brant 2016). The diagnosis of intentional in contrast to accidental poisoning should be based on laboratory results in conjunction with the findings in the companion animal's environment. However, the investigation of a household as a possible crime scene by the veterinary forensic examiner is not possible in practice. Forensic evidence most appropriate for risk assessment of perpetrators of intentional companion animal poisoningis more likely to be based on the number of cases attributed to a single offender i.e., serial pet poisoning cases into the same family (Volmer and Meerdink, 2002; Gwaltney-Brant 2016; Lockwood and Arkow, 2016).

ETHICAL ISSUES &CONCLUDING REMARKS

Animal abuse is not only a crime. It is part of the spectrum of intra-family and community violence which should be viewed as a leading worldwide public health problem (Arkow, 2015).

Veterinarians must be aware of aspects of abuse, and especially the interaction between animal abuse and domestic violence (Gallagher et al 2008; Williams et al., 2008; Woolf, 2015). The link between human violence and companion animal abuseis well documented, and a history of animal abuse can be a predictor of current and future violence directed against people. This connection between human violence and companion animal abuse makes it clear that animal abuseis a problem that affects more than just pets and emphasizes the importance of reporting instances of animal abuse and punishing the perpetrators (Babcock and Neihsl, 2006; Monsalve et al., 2017).

Veterinarians have dual obligations, both to their animal patients and their human clients (Fawcett, 2016). Given that human victims of domestic violence often seek veterinary first aid for their pets, veterinarians may act as frontline health professionals in the recognition of the link between companion animal abuse and domestic violence (Babcock and Neihsl, 2006; Newland et al., 2019). In fact, veterinarians, like human physicians, are often the ones to become aware of the abuse and the only ones in a unique position to identify and report it when their human clients are unwilling to do so (Benetato et al., 2011; de Siqueira et al., 2012).

Veterinarians are bound by a professional code of ethics and encouraged by professional organizations to report animal abuse (Benetato et al., 2011; Almeida et al., 2018). In some states of USA, veterinarians have already a legal duty to report suspected cases of animal abuse to the proper authorities. These states have enacted legislation to protect veterinarians from potential civil or criminal liability arising from reporting suspected animal cruelty. Interest in efforts to draft similar legislation in other states is growing (Babcock and Neihsl, 2006). In addition, other countries worldwide are moving in the same directions (Robertson, 2010).

Moreover, a discovered animal abuse case may cause an ethical dilemma for veterinarians between the professional confidentiality to the client and the duty to protect victim (or victims) and facilitate prosecution when the law has been broken (Babcock and Neihsl, 2006; Robertson, 2010; de Siqueira et al., 2012; Lachance, 2016). Veterinarians are also con-

cerned that reporting animal cruelty will have an impact on their practice and business (Babcock and Neihsl, 2006).The barriers for a veterinarian to reporting to the police or other specific authorityan animal abuse case may include: fear of losing income, perceptions that no action will be taken as a result of reporting, fear that reporting may compromise the safety of the alive animal victim or other animals and lack of knowledge of the link between animal abuse and the other forms of intrafamily and interpersonal violence (Green and Gullone, 2005; Williams et al., 2008; Robertson, 2010).

In a recent survey in USA, the most of responding veterinarians were unaware of the current legislation status in their state regarding animal abuse reporting. They have reported that the most common reasons for reporting cruelty must be ethical beliefs and the need to protect the abused companion animal, as well as other animals in the household. On the contrary, they have reported that the most common reasons for not reporting cruelty were their uncertainty that the pet had been abused, their belief that the animals' injuries were accidental versus intentional, and belief that client education would be a better way of action (Kogan et al., 2017).

Therefore, the recognition and mandatory report of NAIin a family pet is a complex issue (Robertson, 2010; Fawcett, 2016). Mandatory reporting can only be supported in a context in which veterinary professionals are (1) trained to recognize the signs of animal abuse; (2) trained to elicit a history sensitively from clients - who may themselves be victims of abuse - or supported in doing so; (3) supported by appropriate, responsive authorities; (4) and reasonably protected from legal recriminations (Robertson, 2010; Woolf, 2015; Fawcett, 2016).

Veterinarians could have an important educative role in promoting animal welfare (Green and Gullone, 2005).They could be an essential part of One Health and One Welfare approaches to break the cycles of violence affecting animals and human members of the family and community (Arkow, 2015; Pinillos et al., 2016). Information gathered from veterinary forensic examination of companion animal victims can help illuminate possible motives for the abusive action and aid in assessing the potential risk for other family pets and family persons posed by perpetrators (Lockwood and Arkow, 2016).Therefore, the One Welfare approach could help to reduce the incidence of crime and violence internationally in a common concern for all vulnerable, victimized, and at-risk creatures (Arkow, 2015; Pinillos et al., 2016).

The need for state veterinary and humane-law enforcement organizations to increasing communication and education efforts on animal protecting legislation and animal abuse recognition and reporting by veterinarians has been recognized (Kogan et al., 2017). Beyond the vital role of veterinarians in detection of problems in the human-animal bond, changes to policies for domestic violence prevention are needed (Newberry, 2016; Monsalve et al., 2017). Policies must enable sharing of information on suspected pet and human abuse cases in the family context between medical professionals (veterinarians, physicians), police, state authorities for family protection and animal welfare organizations. Additionally, policies must enable increasing of public awareness in order to encourage the human victims to overcome fear and ask for help both for them and their pets (Onyskiw 2007; Roguski 2012; Newberry, 2016; Doukas et al., 2018).

However, animal abuse is not always emphasized in the education programme of Veterinary Schools. As a result, not all veterinary students and graduates feel comfortable to recognizeclinical signs and/or lesions during necropsy of an abusedanimal and may not be aware of the resources that are available to them when considering animal cruelty reporting (Englar, 2018). Veterinary undergraduate student education is needed to prepare veterinarians for their response to companion animalabuse and intrafamilyviolence in practice (Woolf, 2015; Englar, 2018; Newland et al., 2019).

Conclusively, further publications are required to emphasize the link between pet abuse and domestic violence, both delivering content in the veterinary student education programme and expanding the veterinarians' knowledge/ attitudes. In our opinion, more specific guidelines and/or brochures regarding the recognition of NAI and reporting of pet abuse in connection to intra-family domestic violence within the veterinary literature are needed.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

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Review article
Ανασκόπηση

Inactivation of foodborne viruses by the cold plasma technology

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ABSTRACT: Cold plasma (CP) is an innovative non-thermal food processing method. CP refers to a partially or completely ionized gas containing reactive chemical species, which are active against microorganisms, including viruses or enzymes of foods. CP has a minimal effect on the quality attributes of foods and can also elongate the shelf life of foods. Foodborne outbreaks caused by viruses have been increased in various countries in recent years. The research works on the inactivation effect of CP against viruses including foodborne viruses have been also increased in recent years. The most important foodborne viruses are human norovirus (HuNoV) and hepatitis A virus (HAV), involved in several outbreaks worldwide. Human astrovirus (HAstV), human adenovirus (HuAdV), Aichi virus (AiV), sapovirus (SaV) and enterovirus (EV) are also notable foodborne viruses and were associated in sporadic cases. The CP treatment proved efficient for the inactivation of foodborne viruses such as HuNoV and HAV. The present work reviews the CP as a non-thermal food processing technology and present the published data on the effect of CP process on foodborne viruses in foods.

***Keywords*:** Cold plasma, foodborne viruses, novel food processing technologies

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INTRODUCTION

In recent years, foodborne virus outbreaks have increased worldwide and have become an important concern for health authorities (Pexara and Govaris, 2020). Among recorded foodborne outbreaks, those caused by viruses reached 20.4% and 45% in EU (in 2014) and USA (in 2018), respectively (Yeargin and Gibson, 2019).

Human norovirus (HuNoV) and hepatitis A virus (HAV) are the most common etiological agents of recorded foodborne virus outbreaks worldwide (Yeargin and Gibson 2019). HuNoV has been also found as the leading cause of foodborne illness worldwide, with the highest range of deaths and highest incidence in children of less than 5 years old (Pires et al., 2015). HAV has been also found responsible for ca 5% of recorded foodborne outbreaks, and is the leading cause of viral hepatitis, with 1.4 million of new cases annually (Adefisoye et al., 2016).

Although hepatitis E virus (HEV) is primarily transmitted by contaminated water, it can also cause infections by the consumption of contaminated food, particularly undercooked meat or meat products. HEV has been associated with sporadic cases and small outbreaks, but it is characterized by severe symptoms in infected individuals (King et al., 2018).Other enteric viruses such as human rotavirus (HRV), human astrovirus (HAstV), human adenovirus (HuAdV), Aichi virus (AiV), sapovirus (SaV) and enterovirus (EV) are also notable foodborne viruses, causing sporadic outbreaks worldwide (Pexara and Govaris, 2020).

In foods, foodborne viruses are typically highly stable and can survive for a long time without any loss of infectivity (Sánchez and Bosch, 2016). In addition, traditional preservation methods such as drying, acidification or salting may not be efficient to inactivate foodborne viruses (Sánchez and Bosch, 2016). Thermal processing is an effective traditional method for virus inactivation since viruses are effectively killed at temperatures higher than 80 °C (Zhang et al., 2019). However, thermal processing can also change the physicochemical and organoleptic properties of foods and cause a reduction of the food quality characteristics (Aadil et al., 2019).

Since consumers demand high quality and safe foods, food industry tends to explore alternative preservation methods and replace the traditional processing methods of foods (Petrescu et al., 2020). Amongst novel, non-thermal food processing technologies is the cold plasma (CP) method.

CP is generated, when an electrical energy source is applied to a gas, resulting in the production of several reactive species such as ultraviolet photons, charged particles, radicals and other reactive species of nitrogen, oxygen, and hydrogen. CP has been found effective to inactivate enzyme and microorganisms and remove toxins or pesticide compounds in foods (Varilla et al., 2020). Although that most CP studies were initially focused on the inactivation of foodborne bacteria, recent studies on the effect of this technology on the inactivation of foodborne viruses has been showing promising results. This article reviews the CP as a non-thermal food processing technology and focuses on its effectiveness on foodborne viruses' inactivation.

COLD PLASMA (CP)

Plasma is the fourth state of matter. The term "plasma" was initially coined in 1927 by Irving Langmuir, a Nobel Prize awarded chemist from USA (Rajvanshi, 2008). CP refers to a partially or completely ionized gas containing reactive chemical species such as ions, electrons, neutral molecules, atoms, and charged species. The free electric charges (electrons and ions) make plasma electrically conductive, internally interactive, and strongly responsive to electromagnetic fields (Pignata et al., 2017).Generally, the two types of plasma are thermal or equilibrium and cold plasma. In thermal or equilibrium plasma, all particles have roughly the same temperature (average kinetic energy of random motion). In cold plasma (CP), the light electrons have much higher temperatures compared to heavy atoms and molecules, which often remain close to room temperature (Filipić et al., 2020). CP can be further classified into low pressure, which is also recognized as vacuum plasma, and atmospheric pressure plasma (Filipić et al., 2020). Typically, CP is generated at 1 atmospheric pressure with electron temperatures generally between 1 and 10 eV (Misra and Jo, 2017).

Various apparatus types have been used for the generation of CP such as corona glow discharges, dielectric barrier discharges, radio frequencies, gliding arc discharges, atmospheric glow discharges, inductively coupled plasmas and microwave induced plasmas (Guo et al., 2015).The most important active species generated by plasma discharge are neutral or excited molecules and atoms, UV photons, negative and positive ions, free radicals and electrons. The

presence of these active agents depends also on the plasma source, but the majority of reactive species are vibrationally and electronically excited oxygen and nitrogen, reactive oxygen species (ROS) such as singlet oxygen $^1O_2$, atomic oxygen O, superoxide oxygen O_{2-} and ozone O_3, reactive nitrogen species (RNS) such as atomic nitrogen N, nitrogen dioxide (●NO_2), nitric oxide (●NO), or peroxynitrite ($ONOO^-$). Also, if humidity is high, electrically charged components such as OH^- anion, H_2O^+, OH• radical, or H_2O_2 are present (Scholtz et al. 2015). The antimicrobial activity of CP is due to these active compounds (López et al., 2019).

CP has been examined by several scientists in recent years and has been used in different scientific fields such as medicine, agriculture, environmental protection and food industry due its ability to inactivate microorganisms as viruses, bacteria, spores, yeast or fungi (Niedźwiedź et al., 2019). According to recent research data, the plasma activated solutions (PASs) of H_2O, NaCl, and H_2O_2 are effective against microorganisms found in foods (Pignata et al., 2017).

Foodborne bacteria inactivation by CP

The antibacterial activity of CP has been examined by several researchers in recent years (Niedźwiedź et al., 2019).CP has been also found to possess antibacterial activities against major foodborne pathogens, such as *Listeria monocytogenes*, *Salmonella Typhimurium*, *Escherichia coli* O157:H7, *Campylobacter jejuni*, and *Salmonella* spp. (Leeet al., 2017).CP is active against foodborne bacteria at ambient temperature with no additional heat treatment (Varilla et al., 2020). CP is also able to inactivate different spores of spore forming bacteria, as reported by several workers (Varilla et al., 2020). The microbicidal properties of CP against foodborne bacteria have been found in various foods such as meat and meat products, eggs and eggshell, vegetables, fruits or spices (Apostol et al., 2015; Leeet at al., 2017; Pignata et al., 2017).

The inactivation of bacteria by CP is achieved through three modes of action: reactions between CP reactive species and positive or negative charge compounds of bacteria, the destruction of the membranes and the cell functions of bacteria, and destruction of or alteration DNA or RNA of bacteria (Moisanet al., 2002).

Effect of CP treatment on food quality

The effects of the CP treatment on the quality characteristics of foods have been extensively investigated (Surowsky et al., 2015; Misra et al., 2016; Pankaj et al., 2018). Among quality characteristics, the color of CP treated foods was examined in several studies. The color changes of meat, meat products, eggs, fruits and vegetables was dependent on CP treatment conditions (input voltage, time, power, working gas) (Bae et al., 2015; Surowsky et al., 2015; Pankaj et al., 2018; Roh et al., 2020). The product type (solid or liquid) and storage conditions are critical factors affecting the color. Overall, it is generally accepted that CP processing has a minimal effect on the color of food products at lower treatment times (Thirumdas et al., 2016; Pankaj et al., 2018). Yong et al. (2017) reported a similar red color in a pork jerky treated by CP to this achieved by supplementing sodium nitrite.

According to several studies, the CP treatment resulted in pH changes of food products (Fröhling et al., 2012; Kim et al., 2013; Almeida et al., 2015; Lee et al., 2016). CP buffering capacity can affect pH of CP treated foods (Pankaj et al., 2018).

CP can also adversely affect the quality properties of foods and should be properly applied to avoid a loss of food quality. CP treatment can enhance lipid oxidation in foods rich in lipid components. The CP ROS could interact with food lipids and initiate the oxidation process, especially when foods rich in unsaturated fatty acids are treated eg fish (Gavahian et al., 2018). However, conflicting results have been published on the lipid oxidation of fish, meat or dairy products caused by CP treatment (Bae et al., 2015; Pankaj et al. 2018). CP can also result in degradation of polymerized oligosaccharides in juices and the mechanism of this degradation should be examined in future studies (Almeida et al., 2015; Varilla et al., 2020).

The food enzymes, which can deteriorate the quality attributes of foods, are susceptible to degradation during CP treatment (Han et al., 2019). Food enzymes such as polyphenoloxidase, peroxidase, superoxide dismutase and lysozyme were also inactivated by CP treatment (Attri et al., 2015; Misra et al. 2016; Han et al., 2019). During CP treatment sensitive vitamins, such as riboflavin (B2), pyridoxine (B6), biotin or vitamin C, are usually stable during CP treatment (Pankaj et al. 2018). The structure of allergens, which are protein components, may be changed by reactive species produced by CP, while further studies are re-

quired to elucidate this beneficial effect of CP treatment (Gavahian and Khaneghah, 2020).

According to European Union regulations, CP treatment has been approved as an electronic preservative practice for organic foods (EC, 2014).

CP EFFECT ON FOODBORNE VIRUSES

Foodborne virus inactivation by CP

Published data on the effect of CP treatment on foodborne viruses are presented in Table 1. CP treatment resulted in a successful decrease of enteric viruses and their surrogates in aqueous solutions (Aboubakr et al., 2016; Aboubakr et al., 2018; Nayak et al., 2018), liquid media (Takamatsu et al., 2015), but also on food surfaces (Bae et al., 2015; Min et al., 2016; Lacombe, 2017; Aboubakr et al., 2020; Roh et al., 2020).

Table 1. Inactivation of foodborne viruses/surrogates in CP treatment

Virus/ surrogatestested*	Plasma Type	Plasma treatment parameters	Matrix	Reduction	References
HuNoV (GII.4)	Microdischarge	1 kHz/8.5 kV/ 30 mW/cm$_2$/Air/ 0.5, 1, 2, 3, 4, 5, 10, or 15 min	Medium	1.23 log genomic equivalents/ml after 10 min, 1.69 log genomic equivalents/ml after 15 min	Ahlfeld et al. (2015)
	A two-dimensional array of integrated coaxial microhollow dielectric barrier discharge	20 Hz/10.8 kV/ 14.5 W/ 16.4 slm air flow rate/ 10 cm exposure distance, wet-surface treatment	Romaine lettuce	~2.6 $\log_{10}$ in genome copy number after 5 min	Aboubakr et al. (2020)
HAV	Atmospheric pressure plasma jet	28.5 kHz/3.5 kV/ N2 (99.9%) / 6 slm flow rate flow/ 10 s-20 min	Fresh meats (beef loin, pork shoulder and chicken breast)	90% reduction (1 $\log_{10}$ PFU/ml) after 5 min	Bae et al. (2015)
FCV	Plasma jet	16 Hz/9 kV/ 10 W/ various gas species, including Ar, O_2, N_2, CO_2 mock air, and mixtures of these gases/ gas flow rate 1 L/min	Medium	>6 log TCID50/10 µl within 5 min (CO_2), >6 log TCID50/10 µl within 3 min (N_2)	Takamatsu et al. (2015)
	Radio frequency atmospheric pressure plasma	20 kHz/2.5W/, different gas mixtures (Ar, Ar + 1% O_2, Ar + 1% air, Ar + 0.27% H_2O) / 1.5 slm flow rate	Distilled water, Liquid medium	> 6.5 $\log_{10}$ TCID50/0.1 ml after 15 s (Ar + 1% O_2 plasma), after 30 s (Ar and Ar + 1%)	Aboubakr et al. (2016)
	Plasma jet	20 kHz/2.5W/99% Ar + 1% O_2/1.5 slm flow rate/15, 30, 60, and 120 s/ distance 11.25 mm	Distilled water	>6.5 $\log_{10}$ TCID50/100µl after 15 s	Aboubakr et al. (2018)
	A two-dimensional array of integrated coaxial microhollowdelectric barrier discharge	20 Hz/10.8 kV/ 14.5 W; Dry Air, Ar + 20% O_2/16.4 slm flow rate	Distilled water	>5 $\log_{10}$TCID50/100µl after 3 min (dry air)	Nayak et al. (2018)

	A two-dimensional array of integrated coaxial microhollow dielectric barrier discharge	20 Hz/10.8 kV/ 14.5 W/ 16.4 slm air flow rate/ 10 cm exposure distance, wet-surface treatment	Romaine lettuce	>5 log_{10}TCID50 after 3 min	Aboubakr et al. (2020)
MNV-1	Atmospheric pressure plasma jet	28.5 kHz/3.5 kV/ N_2 (99.9%) / 6 slm flow rate flow/ 10 s-20 min	Fresh meats (beef loin, pork shoulder and chicken breast)	99% reduction (2 log_{10} PFU/mL) after 5 min	Bae et al. (2015)
	Atmospheric pressure plasma jet	47 kHz/549 W/Air	Blueberries	5 log PFU/g after 90 s	Lacombe et al. (2017)
TV	Dielectric barrier discharge	47.6 kV and 1 A for 5min/electrode distancewas 30 mm	Romaine lettuce	1.3 log PFU/g after 5 min (rigid package)	Min et al. (2016)
	Atmospheric pressure plasma jet	47 kHz/549 W/Air	Blueberries	3.5 log PFU/g after 120 s	Lacombe et al. (2017)
	Atmospheric dielectric barrier discharge	32 kV, 3.5 min/Air In-package	Chicken breast	2.2 PFU/cube after 3.5 min,	Roh et al. (2020)
CVA7	Plasma jet	16 Hz/9 kV/ 10 W/ various gas species, including Ar, O_2, N_2, CO_2 mock air, and mixtures of these gases/ gas flow rate 1 L/min	Medium	<2 log TCID50/10 µl within 10 min (CO_2) >6 log TCID50/10 µl within 10 min (N_2)	Takamatsu et al. (2015)
MS2	Plasma jet	20 kHz/6 kV/gas mixture of 0.0-1.0% and 100.0-99.0% helium/ 2 slm flow rate, 1- 9 min/distance 10 mm	Medium	3 log_{10} after 5 min, >7 log_{10} reduction after 9 min. (helium/oxygen: 99.25%/ 0.75%)	Alshraiedeh et al. (2013)
	Dielectric barrier discharge	30 V/20, 24, 28 W/ Air, 98% Ar + 2% O_2, 98% He + 2% O_2/ flow rate 2.5 liters/ min/ for up to 3 min	Distilled water	>0.69 log PFU/m^3 for all tested gas carriers and power levels for > 30 s	Wu et al. (2015)

* HuNoV, Human noroviruses; HAV, Hepatitis A virus; FCV, Feline calicivirus; MNV-1, Murine norovirus 1; TV, Tulane virus; CVA7, Coxsackie virus; MS2, bacteriophage MS2.

The majority of CP treatment studies was focused on the susceptibility of HuNoV since this virus has been associated with several food outbreaks and is a major problem in the food industry (Pexara and Govaris, 2020). A CP treatment for 5 min showed a reduction of 99% (2 log PFU/ml) of MNV-1, a surrogate of HuNoV, and 90% reduction (1 log PFU/ml) of HAV in fresh meat (beef loin, pork shoulder and chicken breast) (Bae et al., 2015). Ahlfeld et al. (2015) reported that HuNoVwas decreased by 1.23 log and 1.69 log in fecal samples treated by CP for 10 and 15 min, respectively. Alshraiedeh et al. (2013) studied the effect of CP on bacteriophage MS2, a surrogate for HuNoV, ingases (0-1% oxygen in helium) for the plasma generation. Overall, the highest reduction of MS2 was observed for treatments with 0.75% oxy-

gen, while MS2 was reduced by 4 log and 7 log, after 5min and 9 min treatments time, respectively. Aboubakr et al. (2015) reported a6 log reduction of Felinecalicivirus (FCV), a surrogate for HuNoV, with CP plasma generated in 1% oxygen for 90 s. In a CP treatment on blueberries at refrigerated temperature for 2 min, MNV-1 and Tulane virus (TV), two surrogates of HuNoV, were reduced by 3.5 log and 5 log PFU/g, respectively (Lacombe et al., 2017). Aboubakr et al. (2020) reported that CP treatment on stainless-steel surface and lettuce leaves caused almost the same reduction of HuNoV (ca 2.6 log) on both surfaces. The FCV on a stainless-steel surface was completely inactivated by CP treatment after 3 min (Nayak et al., 2018).Takamatsu et al. (2015 reported the inactivation of FCV in a nitrogen CP treatment in contrast to Coxsackie virus (CVA7) in laboratory media (Vero cells).

Mechanisms of foodborne virus inactivation by CP

The specific mechanisms of viruses' inactivation by CP have not yet been elucidated. The studies carried out so far, demonstrated that exposure to CP can lead to the modification or degradation of proteins and lipids of viral envelopes as well as nucleic acids of viruses (Pradeep and Chulkyoon, 2016).

The virucidal activity of CP is particularly affected by formed ROS and RNS. However, the formation of virucidal reactive agents vary and are highly dependent on the experimental conditions, such as the gas for the CP generation, the food matrix, or the virus type (Pignata et al., 2017).Among reactive species, UV radiation and charged particles (e.g. ions, electrons) may also have an important role in the inactivation of viruses by the CP treatment (Filipić et al., 2020).

According to recent studies, $^1O_2$ was the most significant ROS for the inactivation of FCV (Aboubakr et al., 2016; Aboubakr et al., 2018, Yamashiro et al., 2018) and phage T4 (Guo et al., 2018). Aboubakr et al. (2018) reported that $^1O_2$ causes oxidative modification of histidine residues and a shift in molecular mass of methionine residues. Guo et al. (2018) revealed that O_2 also reacts with cysteine, tyrosine, and tryptophan, and oxidizes guanine. The O_3 was one of the most important factors (Nayak et al., 2018) or an additional factor in FCV inactivation (Aboubakr et al., 2016; Aboubakr et al., 2018). The O_3 may also have a role in the inactivation of bacteriophages of MS2 (Xia et al., 2019) and HuAdV (Zimmermann et al., 2011).H_2O_2had a secondary role in inactivation of HuAdV (Sakudo et al., 2016) and FCV (Aboubakr et al., 2016). RNS such as ONOOH (in an acidic environment) (Aboubakr et al., 2016; Yamashiro et al. 2018), $ONOO^-$ (Yamashiro et al., 2018), or NOx (Nayak et al. 2018) have been found as the principal inactivation factors for the FCV surrogate. In future, the development of accurate methods for activity measurement of RONS and UV intensity will clarify viral inactivation by CP treatment in a better manner (Filipić et al., 2020).

The CP reactive species can also react with the capsid protein, leading to protein peroxidation and destruction of the virus. According to previous studies the damage of the capsid was the most important factor for the inactivation of bacteriophages λ (Yasuda et al., 2010), MS2 (Wu et al., 2015), T4 (Guo et al., 2018), and FCV (Aboubakr et al., 2018). The reactive species can damage or eliminate the viral nucleic acid and reduce gene expression (Pignata et al., 2017). The nucleic acid degradation was proved as an important mode of HuAdV inactivation (Sakudo et al., 2016). Yamashiro et al. (2018) reported that nucleic acid degradation was found to a significant factor for FCV inactivation. The damage of DNA in parallel to viral protein degradations are also responsible for foodborne viruses' inactivation by the CP treatment (Pignata et al., 2017).Additional studies are needed to clarify the mechanisms of viruses' inactivation by CP (Liao et al., 2017).

CONCLUSIONS

CP is an innovative non-thermal food processing method which has a minimal effect on the quality characteristics of foods and can also elongate the shelf life of foods. Several studies indicated the inhibition or inactivation activities against foodborne viruses of the CP process in foods. The CP treatment proved efficient for the inactivation of HuNoV and HAV, which are the major viraletiological agents of food-borne outbreaks worldwide.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Review article
Ανασκόπηση

A mini-review of toxicokinetics and toxicity of heavy metals in marine and freshwater fish

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ABSTRACT: The aquatic environment continues to be polluted by anthropogenic activities that cause heavy metal contamination of fish.These trace metal contaminants are present in abundance in the aquatic environment because their inputs also originate from several natural processes. In addition, they are bioaccumulative, persistent and non-bio-degradable over time.Therefore the present mini-review aims to assess the bio-kinetics and known effects of the heavy metals and their toxicity in fish. Among the most toxic metals are arsenic (As), cadmium (Cd), lead (Pb) and mercury (Hg), and they have been the primary focus of many aquatic ecotoxicological studies in recent years. According to the previous studies analyzed; heavy metals bioaccumulate in the body of fish, whose kinetic activity depends on the metal and also varies from one fish species to another, and can cause irregular and sometimes devastating effects in different organs and systems of the body, acute or chronic, depending on the duration of exposure and the dose of metal assimilated through water or food. Metal uptake can affect all life stages of fish, and these effects are a function of the concentration of the metal in the surrounding environment, its chemical form and also the type of the water in which it lives; fresh or marine.

***Keywords*:** Fish, Heavy metals, Bio-kinetics, Bioaccumulation, Toxicity

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INTRODUCTION

Fish consumption has increased in recent years because it is recognized as an important part of a balanced human diet containing high levels of many essential nutrients that are not present in other foods such as proteins of high biological value, vitamins (A, D, E, K and water - soluble vitamin B and C), iodine, selenium and calcium (Kara et al., 2020; Mehouel et al., 2019). Beneficial polyunsaturated fatty acids (omega-3 fatty acids) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are found in large quantities in fish tissue. These polyunsaturated fatty acids have the ability to both prevent and cure certain diseases such as cancer, heart disease, rheumatoid arthritis and inflammation (Ayanda et al., 2019). For this reason, most European dietary guidelines recommend a minimum of two servings of fish per week to ensure the supply of essential nutrients (EFSA, 2014). However, many natural and anthropogenic activities pollute the environment with various chemical contaminants such as heavy metals and metalloids that accumulate in aquatic organisms, especially fish (Micheline et al., 2019).

Heavy metals are important components of a wide range of contaminants in the aquatic environment. This is due to their toxicity, accumulation and ability of some to biomagnify throughout the food chain (Ayanda et al., 2019). Lead, cadmium, arsenic, and mercury are the main non-essential elements that contribute to risks to human health through food consumption and especially in the case of drinking water for arsenic. And they are not required for the human body and their toxic effects have been recorded at extremely low concentrations; hence they are of primary concern on all lists of toxic substances (Ince et al., 2021; Ooi et al., 2015; Chahid et al., 2013).Hence the importance of monitoring their concentrations in fish and comparing them to international regulatory limits regulating the presence of these contaminants in fish to protect consumer health (ANZFSC, 2011; EU, 2006).

Exposure to metals can damage several organs in humans, even at low levels of exposure. Mercury can accumulate in the brain, the intestines, kidneys, liver and placenta and cause many dysfonctions and cancer. Prolonged exposure to cadmium is toxic to various tissues, including the kidneys, liver, bones, the central and peripheral nervous system, cardiovascular system, immune system and reproductive system whichcause irreversible damage and different types of cancer. Exposure to low doses of lead has been associated with neurological, immunological, cardiovascular, renal, digestive, reproductive and developmental effects. Exposure to arsenic causes a variety of complications in organ systems, *e.g.*the integumentary, nervous, respiratory, cardiovascular, hematopoietic, immune, nervous, endocrine, hepatic, renal and reproductive systems (Genchi et al., 2020; Tamele et al., 2020; Al-Saleh and Abduljabbar, 2017).

The aim of this global review is to investigate the most critical aspects of arsenic, cadmium, lead and mercury contamination of fish and examine their toxicokinetics and toxic effects based on experimental works.

HEAVY METALS

Arsenic in fish

Arsenic is a metalloid widespreadin the environment. Its presence in aquatic waters is due to anthropogenic sources such as industrial effluents, and to natural sources which are essentially volcanic eruptions, fluvial contributions and erosion of rocks. This element causes toxic effects in fish and other aquatic organisms via oxidative stress (Ince et al., 2018; Arslan-Acaroz et al., 2017; Kumari et al., 2017).

The concentration of arsenic is generally very high (1-10 µg/g) in marine fish (Zhang et al., 2016).Arsenite (As^{3+}) and arsenate (As^{5+}) are the inorganic forms, whereas monomethyl arsenic acid, dimethyl arsenic acid, arsenobetaine, arsenocholine and different arsenolipids and arsenosugars are the important organic forms (Jabeen and Javed, 2011). In fish arsenobetaine is the most dominant form and contributes 95% of the total arsenic which is non-toxic, while the more toxic inorganic forms contribute only 1-4% (Avigliano et al., 2020; ; Kollander et al., 2019; Amlund et al., 2006).

Toxicokinetics

Fish absorb inorganic arsenic via water and food whereas organic arsenic (arsenobetaine) is only accumulated from food (Hong et al., 2014). Arsenobetaine accumulates in the major organs (liver, kidney) and at a higher rate in muscle tissue (Amlund et al., 2006). In contrast, inorganic arsenic accumulates more in the liver and stomach and at low concentrations in the gills and muscle tissue (Ferreira et al., 2019). The principal routes of excretion are through the kidney (urine), liver (bile), gills and skin (Amlund et al., 2006; Özcan et al., 2006).

Toxicity

The toxicity of inorganic arsenic (As (III) and As (V)) is more toxic than the organic forms (Avigliano et al., 2020; Kim et al., 2018). Acute exposure to arsenic is usually generally fatal to most fish, while the chronic exposure is sublethal and can result in a multitude of molecular events (Chen et al., 2019; Kumar et al., 2019). Arsenic in aquatic organisms is highly dependent on the chemical form in which it occurs. Arsenic modulates antioxidant enzymes such as glutathione reductase (GR) and glutathione S-transferases (GST), it also leads to lipid peroxidation and loss of DNA integrity by inducing the generation of reactive oxygen species and by depressing the functions of the antioxidant defense system (Ince et al., 2018; Arslan-Acaroz et al., 2017; Ventura-Lima et al., 2009). The reduction of AsV to As III may allow the fixation of arsenic in the body through the interaction with thiol groups (Ince et al., 2018; Arslan-Acaroz et al., 2017). For higher trophic level species, sensitivity to arsenic is much less critical, and concentrations in the range of 100 to 500 µg/L are necessary to observe the first toxic effects on macro-algae, shrimp or fish (IFREMER, 1993). Toxicity manifests itself by affecting different systems and functions such as growth, reproduction, ion regulation, smoltification, gene expression, immune function, enzyme activities and histopathology of fish (Kumari et al., 2017).

Cadmium in fish

Cd is a non-essential metal widely present in the aquatic environment as a result of various industrial and mining activities (Renieri et al., 2017). It can be toxic to fish, even in trace amounts, of which free ions (Cd^{+2}) are the most bioavailable and toxic and are found either in free form or in a variety of complexes with suspended particles and in sediments (Renieri et al., 2017; Kovarova and Svobodova, 2009). Hence Cd is available for bioaccumulation by fish via a number of dissolved Cd and food ingestion pathways (Renieri et al., 2017; Kovarova and Svobodova, 2009).

Toxicokinetics

Metal ions dissolved in water are absorbed through the gills and other permeable surfaces of the body, while metals bound to solid particles are ingested and then detached from their carrier particles in the digestive system and absorbed through the intestinal epithelium (Kovarova and Svobodova, 2009). Cadmium is transported in the blood, where it is distributed mainly in the liver and kidneys, which are the main organs of accumulation during acute and chronic exposure, and to a lesser extent in the visceral mass (Ferain et al., 2018; Verge, 2006). The cadmium content in muscle tissue is generally very low (Bremner, 2002). In the cytoplasm uptake is supported by metallothioneins, which are proteins rich in sulfur amino acids and poor in aromatic amino acids, and they facilitate a stable bonding with cadmium ions (Cd^{+2}) (Verge, 2006). The elimination of cadmium is low and takes place mainly through the urine. This renal elimination seems to depend only on the intensity of exposure, in fact, it is independent of metabolic activity or the size of the individual (Le Croizier et al., 2019; Verge, 2006).

Toxicity

Even at low ambient concentrations, cadmium can accumulate in fish causing several toxic effects, particularly in the early life stages (Safari, 2015; Maunder et al., 2011). The acute toxicity of cadmium generally affects ionic homeostasis, in particular that of calcium, by concurrence on absorption sites (Dave and Kwong, 2020; Olsvik et al., 2016). Chronic toxicity can affect a wide variety of physiological processes and functions (Olsvik et al., 2016). Cadmium can indirectly generate oxidative stress all and free radicals that may be caused by overproduction of reactive oxygen species (ROS) or by depletion of cellular antioxidant levels; furthermore it can also lead to DNA damage and blocking of DNA repair (Zhang and Reynolds, 2019; Malarvizhi et al., 2018). In addition, cadmium is responsible for endocrine and ionoregulatory disruption, histopathology and depression of the immune system, all of which can affect the growth, reproduction and survival of fish (Le Croizier et al., 2019; Sierra-Marquez et al., 2019).

Lead in fish

Toxicokinetics

Ionic lead (Pb^{+2}) enters the body of fish through the gills, the surface of the body and also by direct ingestion. Absorption from contaminated water is more important than absorption from food (Macirella et al., 2019; Bibi and Ahmed, 2010). Bioaccumulation is well documented in skin, gills, stomach, muscles, intestines, liver, brain, kidney and gonads, however the main target organs are liver, kidney and muscles (Bibi and Ahmed, 2010).In contrast to inorganic lead compounds, tetra-alkyl lead is readily absorbed by fish and rapidly eliminated after the end of exposure (Casas and Sordo, 2011).

Toxicity

Exposure to lead causes a wide range of toxic effects on the physiological, behavioral and biochemical functions of fish. It also damages the central and peripheral nervous system, the immune, hematopoietic, and cardiovascular systems as well as organs such as the liver and kidneys (Lee et al., 2019).Lead also leads to reproductive disorders, growth and developmental problems (Lee et al., 2019; Kim and Kang, 2015). Exposure to this metal can be lethal, even at low concentrations, due to efficient bioaccumulation (Lee et al., 2019).

Lead is one of the most toxic metals in the aquatic environment because of its ability to mimic biologically important metals, mainly calcium, iron and zinc (Company et al., 2011). It can enter the cell because of its similarity to calcium by using calcium's transport pathways (Amadob et al., 2012). It can also reduce the unidirectional influx of sodium (Na^+), frequently correlating with the inhibition of Na^+/K^+ATPase, an enzyme essential for the maintenance of cell volume (Amadob et al., 2012). Lead can interact with a variety of cellular lipids by changing the composition of the cell membrane, a process which results in disruptions in membrane integrity, permeability and function thereby increasing the sensitivity to lipid peroxidation (Taylor and Maher, 2012). Lead accumulation can also cause the production of reactive oxygen species (ROS) which leads to depletion of cellular antioxidant defense systems, destabilization of cell membranes and DNA damage. Furthermore its accumulation in mitochondria and other organelles can cause serious disturbances in the energy balance of tissues and eventually cell death (Taylor and Maher, 2012).

Mercury in fish

Mercuryis a highly toxic chemical contaminant (Custódio et al., 2020). Its presence in the environment comes from both natural and anthropogenic sources. It can be released into the aquatic environment in its inorganic form where it can undergo various biochemical processes (oxidation, reduction, methylation and demethylation) which result in producing the more toxic methyl mercury species (Donadt et al., 2021; Kim et al., 2016). Elemental mercury, inorganic mercury and organic mercury are the three main forms of this metal found in the aquatic environment (Vasanthi et al., 2019). The total mercury content of fish may consist of a combination of its organic and inorganic forms, with methylmercury being the most toxic and abundant organic form. The latter is obtained by methylation of inorganic mercury via aquatic bacteria which makes it more bioaccumulatable in aquatic food chains, accounting for 75-100% of the total mercury in fish, and with the potential for biomagnification in certain fish species especially large predatory fish (Sadeghi et al., 2018; Bosch et al., 2016; Kim et al., 2016).

Toxicokinetics

The different forms of mercury that come into contact with aquatic organisms originate from the surrounding water, sediment and food (Cambier, 2009). Inorganic mercury crosses biological barriers less easily than methylmercury; it has an absorption rate through the intestinal barrier on the order of 10% when contamination occurs via the trophic route (Cambier, 2009). However, the intestinal barrier as well as the gill epithelium are highly permeable to methylmercury (Cambier, 2009). Methylmercury is absorbed and accumulated more than other forms, with 99% of the bioaccumulated mercury in this methylated form (methylmercury) (Bridges and Zalups, 2017). After contamination via the direct route (water), gills and muscle show the highest concentrations (Dominique, 2006) ; however, after trophic contamination, bioaccumulation in the gills is low, while the liver, brain and muscle tissue show high concentrations (Dominique, 2006). Inorganic mercury is mainly found in the liver and kidneys from where it is excreted (Dominique, 2006). Although aquatic organisms have mechanisms for intestinal and renal demethylation and excretion, the loss rate of methylmercury remains low compared to the entry velocity, thereby bioaccumulating over a lifetime of certain species, particularly in top trophic level predators (Amiard, 2011).

Toxicity

Exposure to mercury can cause a variety of adverse effects in fish at the physiological, histological, biochemical, enzymatic and genetic levels (Morcillo et al., 2017). Induced toxicity is influenced by various factors such as species, age, environmental conditions, concentration and duration of exposure (Morcillo et al., 2017). The chemical form of mercury plays an important role in its toxicity, for example, the methylated form is 50 to 100 times more toxic than the initial inorganic form of mercury (Meyer et al., 2004).

The acute toxicity of mercury following 24 h of exposure to 0.73 ppm leads to erratic swimming, ab-

normal posture, sluggishness, imbalance in posture, increase in surface activity, opercular movement, gradual loss of equilibrium and spreading of excess of mucus all over the surface of the body, and mortalities were also observed (Vasanthi et al., 2019). Chronic exposure to mercury especially affects the embryonic and larval stages which are the most sensitive periods of growth. By affecting the development of organs and also the metabolic activity of fish, it can also leads to slow development, morphological abnormality, dysfunction and eventually death. In general, this metal causes neurological, hepatological and reproductive damages that are always worrying even at low concentrations (*e.g.*0.02 ppm) (Zheng et al., 2019).

Summary

Given the large number of experimental studies which have addressed the problem of heavy metal contamination in both marine and freshwater fish, we have summarized in Table 1 the basic toxicokinetic data and ancillary information that has been produced from toxicokinetic experiments following exposures to As, Cd, Pb and Hg. The findings highlight the exposure times, tissue distribution of the metal, target organs and elimination routes in both juvenile and adult marine and freshwater fish.

With respect to aspects of metal toxicity, in Table 2 the toxic effects of the four contaminant metals are summarized for similar types of teleost fish following experiments using a wide range of different exposure doses of As, Cd, Pb and Hg. The results underscore the very different effects which arise from acute and chronic exposures.

CONCLUSIONS

In this overview we have examined the toxicokinetics and distribution and potential effects of four of the most toxic heavy metals (As, Cd, Pb, and Hg) in fish and, based on the results of previous experimental studies, the degree of toxicity of these metals is ordered as follows : As<Pb<Cd<Hg. Each metal has a specific affinity for certain organs and tissues.Previous studies on the toxicokinetics of heavy metals in fish have shown differences even for the same metal, and within the same species of fish variations are found according to the stage of development of the fish and the aquatic environment in which it lives, i.e. marine or freshwater. Their toxicity also depends on several factors; *e.g.* for a given metal certain chemical forms are more toxic than others and for fish, toxicity depend mainly on the exposure dose which can lead to either acute or chronic effects.Toxicity also depends on the stage of development of the fish and the physico-chemistry of the water in which it lives. It would therefore be of interest to conduct further detailed studies on both the toxicokinetics and toxicity of these metals for the most abundant physico-chemical forms of the metals and for the most toxic to freshwater and marine species, including investigating these aspects at all stages of development from hatching to adulthood. It would also be important to conduct toxicokinetics and toxicity studies of these metals based on the route of dietary or water exposure and especially to evaluate the degree of toxicity of these four metals for the same species of fish after acute and chronic exposure. Such information will be vital in improving and refining our assessments of the potential for biomagnification of Hg in various species of fish.

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CONFLICT OF INTEREST

None declared by the authors.

Table 1. Summary of toxicokinetic studies of heavy metals (As, Cd, Pb and Hg) in fish

Metals	Species	Developmental stage	Habitat type	Time of exposition	Distribution in Tissues	Target organ	Elimination	References
As	Tilapia (*Oreochromis mossambicus*)	Adult	Freshwater	7 days	Gill, liver, muscle, intestine, stomach, whole body	Stomach	Intestine	(Liao et al., 2004)
	Tilapia (*Oreochromis mossambicus*)	Adult	Freshwater	7 days	Gill, liver, alimentary canal, carcass	Liver, Alimentary canal	Liver Alimentary canal	(Tsai and Liao, 2005)
	Tilapia (*Oreochromis niloticus*)	Juvenile	Freshwater	7days	Gill, liver, stomach, muscle	Stomach, liver	Liver, stomach	(Ferreira et al., 2019)
Cd	Zebrafish (*Danio rerio*)	Adult	Freshwater	3 days	Gill, liver, intestine, gonad, carcass, brain	Liver	-	(Zhang et al., 2019)
	Rainbow trout (*Oncorhynchus mykiss)*	Adult	Freshwater	100 days	Gill, liver, kidney, Whole body	Kidney	-	(McGeer et al., 2019)
	Olive flounder (*Paralichthys olivaceus*)	Juvenile	Seawater	50 days	Gill, intestine, kidney, liver, muscle	Intestine	Intestine	(Kim et al., 2004)
Pb	Zebrafish (*Danio rerio*)	Adult	Freshwater	3days	Gill, liver, intestine, gonad, carcass, brain,	Gill	-	(Zhang et al., 2019)
	Prussian carp (*Carassius gibelio*)	Adult	Freshwater	24 months	Muscles, hepatopancreatic gland, kidney, intestine, gill.	Kidney	Kidney	(Łuszczek-Trojnar et al., 2013)
	Tilapia (*Coptodon zillii*)	Adult	Freshwater	10 days	Liver, gill, brain, muscle tissues	Gill	Gill and liver	(Kalay and Canli, 2000)
MeHg	White sturgeon (*Acipenser transmontanus*)	Juvenile	Freshwater	2 days	Gastro-intestinal tract, kidney, spleen, gill, heart, liver, brain, white muscle	Gastro-intestinal	Kidney	(Huang et al., 2012)
	Sheepshead minnows (*Cyprinodon variegatus*)	Adult	Freshwater	35 days	Intestine, blood, liver, gill, rest of body	Intestine	Kidney	(Leaner and Mason, 2002)
	Wild rabbitfish (*Siganus canaliculatus*)	Adult	Seawater	21 days	Gill, liver, intestine, muscle and whole body	Muscle	Intestine	(Peng et al., 2016)
Hg (II)	Wild rabbitfish (*Siganus canaliculatus*)	Adult	Seawater	21 days	Gill, liver, intestine, muscle and whole body	Gill, intestine	Gill	(Peng et al., 2016)
	Sea bream (*Diplodus sargus*)	Adult	Seawater	14 days	gills, eye wall, lens, blood, liver, brain and bile	Gill	gills, blood and liver	(Pereira et al., 2015)
	Flounder (*Platichthys Flesus*)	Adult	Seawater	4 months	Liver, kidney, muscle	Liver, kidney	Fillet	(Riisgård and Famme, 1988)

Table 2. Summary of toxic effects of heavy metals in fish experimentally exposed to selected concentrations of As, Cd, Pb and Hg

Metals	Fish species	Developmental Stage	Habitat type	Exposure dose of metal	Toxic effect		
					Actue toxicity	Chronic toxicity	
As	Tilapia (*Oreochromis mossambicus*)	Adult	Freshwater	328.05 mg/L	Lethargy, mouth and operculum wide open, body slimy, mortality after a few minutes of exposure		(Ahmed et al., 2013)
	Teleost fish (*Channa punctatus*)	Adult	Freshwater	5 mg/L		Distortions in the cell organelles, DNAfragmentation, activity enzymatic inhibited, fish survived more than 100 days after exposure	(Das et al., 2012)
Cd	Red sea bream (*Pagrus major*)	Juvenile	Seawater	3mg/L	Cardiac edema, degenerated, hooked tails, fin lesions, spinal curvature with skeletal deformities, mortality also observed.		(Cao et al., 2009)
	Sole (*Solea senegalensis*)	Adult	Seawter	25µg/g		Alteration of the whole-body	(Le Croiziez et al., 2019)
Pb	Rainbow trout (*Salmo gairdneri*)	Juvenile and adult	Freshwater	0.20 g/L	Mortality after 5 days of exposure		(Davies et al., 1976)
	Rainbow trout (*Salmo gairdneri*)	Juvenile and adult	Freshwater	64µg/L		Black tails noted, fish exhibited spinal curvatures and eroded caudal fins, paralysis and atrophy of the flexed portion of the body, little interest for food observed one month after exposition,	(Davies et al., 1976)
Hg	Nile Tilapia (*Oreochromis niloticus*)	Adult	Freshwater	0.1453 mg/L	Mortality after 72h of exposure, external abnormality : namely pale gills, anemic eyes, whitish body color, internal lesions affecting the gills, liver, and hepatopancreas also observed		Suhendrayatna et al., 2019
	Salmon (*Salmo salar*)	Adult	Freshwater	0.10 mg/kg		Diverse abnormalities observed in kidney, brain and liver in the 4 months of exposure	(Berntssen et al., 2003)

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Review article
Ανασκόπηση

Vitamin E and Its Impact on Poultry Health and Production: An Update

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ABSTRACT: The main goals of recent poultry production sectors are to enhance the immune response of the birds, improve the performance, reduce mortalities and reduce stressors. These goals are achievable with dietary supplementationof vitamins. Vitamin E is one of the fat-soluble vitamin that has been used from last decades for different poultry production types. The inoculation level of vitamin E in the diet of poultry depends on several factors. Low or high level of vitamin E can induce severe adverse economic losses in poultry industry. Vitamin E has been regarded as a potent chain-breaking antioxidant as well as immuno-stimulator for both cell-mediated and humoral immunity. Vitamin E is added to the diet of broilers, layers and breeders especially those under heat stress conditions. Inbroilers, vitamin E can improve the health conditions, feed efficiency and immunity. However, in layers and breeders, it enhances the egg's quantity and quality as well as the fertility; respectively. Moreover, vitamin E proves its efficacy in modifying the carcass trait or meat quality of broilers. Therefore, this review article aimed to investigate the forms and inoculation levels vitamin E, the role of this vitamin in the biological process as well as its effect on different poultry production types, carcass qualityand hematological parameters.

***Keywords*:** Birds, vitamin E, antioxidant, immunity, carcass quality

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INTRODUCTION

The best and efficient strategy to enhance the production performance, disease resistance and the immune response of poultry is the nutrients modification of poultry farming (Liu et al., 2014).Vitamins are organic substances with a complex nature that are found in a very small amount in feed. They are very important for the performance and body's physiological functions. Vitamins supplied to poultry ration to improve the viability, reduce stressors and enhance the growth performance parameters and antioxidant properties (Attia et al., 2017; Surai, 2020). Vitamins are divided into fat and water soluble types.

Vitamin E is considered as a fat-soluble vitaminthat has been discovered in1920s (Evans and Bishop, 1922). It is crucial for humans and animals and poultry species.The natural form of vitamin E (Dα-tocopherol) is the most common and superior form in being retained in serum and tissues (Yang et al., 2009). The inoculation levels of vitamin E in poultry diets is recommended by NRC (NRC, 1994), however, the ideal levels are still controversial due to several factors (Kuttappan et al., 2012). Either deficiency or excess levelsof vitamin E is associated with severe adverse economic losses in poultry industry.

Vitamin E is necessary for the functions of immune, reproductive, nervous, respiratory, muscular and circulatory systems.Moreover, dietary supplementation of this vitamin is a common in poultry practice to improve both cell-mediated and humoral immunity (Konieczka et al., 2017; Pompeu et al., 2018) as well ascounteract the deteriorative effects of oxidative stress (Surai et al., 2019; Pirgozliev et al., 2020). It has been recorded that vitamin E plays an important role for broilers production (Pal, 2017; Pitargue et al., 2019) as well as for layers and breeders production and reproduction (Asl et al., 2018; Nawab et al., 2018; Aamir et al., 2019). The carcass trait and meat quality are also positively affected by inoculation of vitamin E in broiler diet during rearing (Fellenberg and Speisky, 2006; Rey et al., 2015; Pitargue et al., 2019).

Accordingly, the objectives of the current review article were to investigate the forms and inoculation levels vitamin E, the role of this vitamin in the biological process as well as its effect on different poultry production types, carcass quality and hematological parameters.

Forms of vitamin E

Chroman-6-ols collectively tocochromanols (tocopherols and tocotrienols**)** are emerged as vitamin E molecules. However, 8 substances have been detected to have the activity of vitamin including 4 tocopherols (α-, β-, γ- and δ-tocopherols) and 4 tocotrienols (α-, β-, γ- and δ-tocotrienols) (Panda and Cherian, 2014) ; only tocopherol can met the requirements of animals to vitamin E. They present in the fat sources of the diet as they absorbed in the intestine after ingestion and transformed into non-esterified form (Colombo et al., 1998). The form α-tocopherol is considered as the most common studied form of vitamin E, while tocotrienols form is still under investigations. Vitamin E is commonly added to poultry diet in the natural form (Dα-tocopherol) that being retained in the blood and tissue (Yang et al., 2009). The form of vitamin E determines its bioavailability. Vitamin E is measured in international units by defining one mg of all-rac-α-tocopherol acetate as 1 IU, as D-α-tocopherol has a bioactivity of 1.49 IU (Machlin, 1991).

Recommended vitamin E levels in poultry diets

Several factors as bird's physiological and metabolic functions as well as the environmental stressors can control the actual requirements for vitamin E. The fatty acid contents, pelleting and storage conditions of the diets can mainly affect on the required amount of vitamin E. In addition, genetic variations among birds that lead to differences in vitamin E absorbability and degradation in the intestinal tract. Therefore, the ideal inclusion levels of vitamin E in poultry diets are still controversial (Kuttappan et al., 2012).

Poultry cannot synthesize vitamin E. Birds can obtain their requirements for this vitamin from fat sources and then stored by the body, so there is no need to be consumed daily (Colombo, 2010). Vitamin E is regarded as one of the most expensive vitamins for poultry. Under normal conditions, the standard recommended dose of vitamin E for poultry according to NRCranges from 5-25 IU/kg of feed (NRC, 1994). To meet the poultry requirements, 10 IU/kg of the ration is also suitable. Studies of Rebolé et al. (2006) ; Singh et al. (2006) ; Hashizawa et al. (2013) ; Habibian et al. (2014) and Ismail et al. (2014) successfully used the basal level of vitamin E as adequate or to marginally exceed the minimum requirements of broilers. However, vitamin E requirements may increase especially in broilers to alleviate the negative effects of high temperature condition that affects on feed efficiency (Guo et al., 2003; Niu et al., 2009). Liu et al. (2014)

suggested that using of vitamin E as 25 times up to NRC requirement to enhance the antibody titer in turkeys. It has been suggested that poultry fed on 100 mg vitamin E/kg diet may prevent vitamin E deficiency (Aamir et al., 2019).The recommended dietary level of vitamin E to maintain bird's fertility differs according to the age, breed and the health of the bird as well as the composition of vitamin E. However, a concentration of 10 mg /kg of diet vitamin E is beneficial to maintain the fertility (Biswas et al., 2007; Hooda et al., 2007; Pekmezci, 2011; Khan et al., 2012b).

Deficiency of vitamin E in poultry

Vitamin E deficiency produces severe adverse economic losses in the poultry industry.There are some interaction of vitamin E and other nutritional elements aspolyunsaturated fatty acids (PUFAs), sulfur-containing amino acids and selenium. The deficiency of vitamin E with PUFAsis associated with nutritional encephalomalacia in chicks, while with selenium and sulfur-containing amino acids deficiencies induce exudative diathesis and enzootic muscle dystrophy; respectively (Beck, 2007; Guetchomet al., 2012; Michalczuket al., 2016). Reproductive disorders, hock disorders and retardation of growth are also forms of vitamin E deficiency in poultry (Niu et al., 2009). Besides, depletion of lymphocytes (Dietert et al., 1983) and growth depressant effect of thymus, bursa and spleen (Marsh et al., 1986) have been recorded as a result of vitamin E deficiency.

Hypervitaminosis with vitamin E in poultry

High dietary levels of vitamin E resulting in reticulocytosis, decreased hematocrit value, lowered thyroid activity and increased vitamins D and K requirement in chicks (March et al., 1973). Also, supplementationwith high levels of tocopherol alleviated hypervitaminosis with vitamin A in chicks (Mc Cuaig and Motzok, 1970; Sklan and Donoghue, 1982). Though, decreases the level of vitamin A in the blood and liver may adversely affects on the bone ash and plasma calcium level of birds (Aburto and Britton, 1998).

The role of vitamin E in the biological process

Antioxidant

Vitamin E plays a major antioxidant role by prevention of lipid peroxidation of PUFAs in plasma membranes of cells and sub-capsular organs (Fusco et al., 2007; Khan et al., 2012a; SuraiandKochish, 2019), therefore protecting cells from free radicals toxicity (free radicals scavenger) during normal metabolic status and inflammation (Colombo, 2010; Khan, 2011; Rizvi et al., 2014). Vitamin E can mediate free radicals signal transduction and finally modulates the genes expression that are regulated by free radical signaling (Packer and Suzuki, 1993). In addition, ithas a negative effect on the production ofreactive oxygen species (ROS) which activate unsaturated phospholipids and critical sulfhydryl group oxidation (Traber and Atkinson, 2007). Particularly, phospholipids membranes are more prone to oxidative stress, being positively correlated with thedegree of PUFAs. Vitamin E has been classified as a reducing agent for ROS molecules. Nowadays, the bioactive contents of some phytobiotics plant react synergistically with vitamin E toenhance the antioxidant potential of vitamin E (Sonam and Guleria, 2017).

Immunity

It has been documented that vitamin E is essential for the ontogeny of the bird's immune response (Gore and Qureshi, 1997; Silva et al., 2011). Vitamin E significantly increased Sephadex-elicited inflammatory exudate cells as well as the macrophages percentage of chickens in a dose of 10 IU (Gore and Qureshi, 1997). Dietary vitamin E increases the T helper cells, and in turn improves responsiveness to immunologic stimuli (Erf et al., 1998). The dietary level of vitamin E may alter the innate cellular oxidative immunity (Perez-Carbajal et al., 2010). Besides, vitamin E is regarded as immuno-potentiator via delaying the production of ROS in lipid membranes (Pekmezci, 2011; Tufarelliand Laudadio, 2016; Aslet al., 2018).It has been shown that vitamin E reduces the generation of MDA, decreases the total antioxidant capacity levels in the liver which is consistent with enhancing hepatic α-tocopherol content; resulting in improvement of the antioxidant capacity (inhibit lipid peroxidation) of immunosuppressed broilers (Cheng et al., 2017).

Vitamin E acts on the immune organs either directly or indirectly through the affection of metabolic and endocrine parameters (Gershwin et al., 1985; Marsh et al., 1986; Leshchinsky and Klasing, 2001; Lohakare et al., 2005; Pompeu et al., 2018). As an antioxidant, vitamin E may reduce plasma concentrations of corticosterone (Puthpongsiriporn et al., 2001). It can modulate cyclooxygenase and lipoxygenase pathways which reflects on the synthesis of leukotrienes and prostaglandins (Leshchinsky and Klasing, 2001, 2003).

It is not exactly known whether vitamin E directly increases production of antibodies by altering B cells or indirectly through T cells (Lee and Han, 2018).

As vitamin E acts as an antioxidant, it may prevent the oxidation of arachidonic acid involved in the biosynthesis pathway of prostaglandins which has immunosuppressive effects at elevated levels (Sheffy and Schultz, 1979). Modulation of arachidonic acid metabolism via cyclo-oxygenase and lipoxygenase pathways lead to synthesis of prostaglandins and leukotriens, respectively (Leshchinsky and Klasing, 2001).The inhibition of lipid peroxidation and protection of mitochondria and microsomes of the liver against oxidative stress may be another possible immunomodulatory role of vitamin E (Leshchinsky and Klasing, 2001). In addition, Gore and Qureshi (1997) suggested that higher levels of vitamin E may maintain the integrity of macrophage membrane that needed for phagocytosis. Broilers fed on excess vitamin E showed an increase in the phagocytosis process of peritoneal macrophages as a result of increasing the expression of Fc receptors of antibodies on macrophages membranes (Konjufca et al., 2004). Elevated numbers of macrophages displayed an increased ability to opsonize sheep red blood cells (SRRCS). Khan et al. (2014) suggested that vitamin E may affect macrophage cell viability and function by regulating levels of free radicals to maintain normal cell functions.

It has been appeared that vitamin E can boost booth cell mediated and humoral immune response to various antigens. It enhances IFN-γ production, induces proliferation of immune cells and modulates chemotaxis and bactericidal properties of polymorphonuclear cells (Boxer, 1986). Quantitatively and qualitatively augments of lymphocyte and monocyte mediated responses have been shown after dietary supplementation with vitamin E. For instance, feeding of broiler chickens with 80 IU/kg or 40 IU/kg of vitamin E following vaccination with infectious bursal disease virus (IBDV) vaccination induced significant increase in peripheral blood CD4+ and CD8+ T cells (Abdukalykova et al., 2008). Similarly, an increase in lymphocytes populations of the thymus as well as the number of plasma cells in spleen, cecal tonsils and ileum of broiler chickens have been observed following feeding on higher levels of vitamin E (Khan et al., 2008). Dalia et al. (2018) detected that inclusion of vitamin E (100 mg/kg) along with inorganic selenium (0.3 mg/kg) effectively improved the immune system through regulation of some cytokines expression and immunoglobulin levels.

Vitamin E can benefit the immune response of poultry via anti-inflammatory effects. It has an essential role in balancing cytokine responses, which could be critical in cases of inflammation. It has been found that broiler chickens fed on 220 IU/kg of vitamin E showed significant decrease in the level of IL-6 mRNA in spleen (Kaiser et al., 2012) as vitamin E controls inflammatory responses when pro-inflammatory cytokine production is elevated. Broilers fed on vitamin E supplemented feed (100 mg/kg) and kept under heat stress showed significant decrease in liver expression IL-6 and heat shock protein 70 (Jang et al., 2014). Recent study of Pitargue et al. (2019) revealed that broiler chickens received vitamin E showed decrease in inflammatory (IFN-γ, IL-1β and IL-6) and anti-inflammatory (IL-4, IL-10 and TGF-β) cytokines in the intestine. Inclusion of arginine in a vitamin E-supplemented diet in broiler chickens enhanced responses to phytohemagglutinin as assessed by the cutaneous basophil hypersensitivity test (Abdukalykova and Ruiz-Feria, 2006).

The findings of Lin and Chang (2006) suggested that moderate supplementation of vitamin E may enhance immune responses to selective antigens in breeders. Supplementation with vitamin E at level of (100IU/kg) to the diet of broilers breeders resulting in enhancing the immune response to bronchitis virus vaccine (Khan et al., 2014). In the same context, broiler chickens supplemented with 200IU/kg of vitamin E and 0.2 mg/kg of selenium and vaccinated with Newcastle disease virus (NDV) vaccine developed significant higher vaccine-specific antibodies when compared with control (Singh et al., 2006). Similarly, Ismail et al. (2014) demonstrated significant increase in the titers of antibodies against NDV and avian influenza disease virus in the plasma of vitamin E supplemented broiler chickens (300 mg/ kg diet). Significant elevation of antibody titers was observed in broilers after primary and secondary immunization with SRBCS and feeding on vitamin E (Niu et al., 2009; Habibian et al., 2014).Chickens infected with IBDV and fed on vitamin E (178 IU/kg) showed reduced mortalities and high body weight gain (McIlroy et al., 1993).

Broiler chickens received a diet containing 100 IU/kg of vitamin E and infested with *E. tenella* oocysts revealed significant resistance indicated by decreasing in mortalities and increasing in body weight gain (Colnago et al., 1984). Perez-Carbajal et al. (2010)

demonstrated that supplementation of chickens with vitamin E and arginine improved the phagocytic activity of heterophils and monocytes. Similar effects were also seen when chickens were challenged with *Salmonella enterica* serovar Typhimurium (Liu et al., 2014).Recently, Liu et al. (2019) assessed the effects of feeding laying hens on 30 IU/kg of vitamin E on antibody levels, pro-inflammatory cytokines and mortalities after challenge with *Salmonella enteritidis*. The results proved increasing IgA, IgM and IgY levels, while decreasing in IL-1β, IL-6 and mortalities at 2 weeks post-challenge.

Feeding of vitamin E to breeder hens can passively transferred antibody-mediated response against diseases in their progeny. When broiler breeder hens supplemented with vitamin E (150 IU/kg) or (450 IU/kg) in feed before inoculation with *Brucella abortus* antigens, their chicks that received more vitamin E showed higher antigen-specific antibody titers (Jackson et al., 1978). Supplementation of breeders on 0.03% total vitamin E in their diet for 3 weeks prior to immunization with Newcastle disease virus vaccine induce high antibody levels in their progeny at 1 and 7 days old as compared with controls (Haq et al., 1996). Inoculation of 10 mg of vitamin E in embryonated chicken eggs increased cellular and humoral immunity in newly hatched chickens with (Gore and Qureshi, 1997). In addition, these chicks showed higher phagocytic activity when inoculated with sheep red blood cells at 7 days of age as well as higher antibody titers to SRBC were also detected at 14 and 21 days of age. The same research also tested the effect of inoculating three doses of vitamin E into embryonated turkey eggs 3 days prior hatching. The results revealed that inoculation of 20 and 30 IU of vitamin E resulting in significant reduction of hatchability, while 10 IU induced slight higher hatchability. Furthermore, 7 days old turkey poults showed higher level of IgM antibodies against sheep red blood cells than controls at 7 and 14 days post-inoculation. The number of phagocytic macrophages at 7 weeks post hatch were also significantly higher in the group inoculated with 10 IU of vitamin E.

The effect of vitamin E supplementation in poultry production

Broilers

Improvement in feed efficiency has been recorded in broilers after feeding on vitamin E at levels of 60, 90 and 120 IU/kg of diet (Serman et al., 1992). Addition of both vitamins E and C at levels of 150 mg/kg and 200 mg/kg ration, respectively enhanced chicken's growth and immune response to vaccination (Rajmane and Ranade, 1994).Moreover, improvement in broilers feed efficiency has been observed after addition of 75 ppm of vitamin E/kg in diet (Aravind et al., 2001). Villar-Patino et al. (2002) recorded an enhancement of the live body weight of broilers supplemented by 75 mg of vitamin E/kg of diet.

Erf et al. (1998) reported that inoculation of vitamin E at levels beyond those needed to enhance the optimal growth is efficient forincreasing the immuno-competence of growing broilers. It has been documented that supplementation of vitamin E induced significant increase in the relative weight of spleen which indirectly has a benefit for the broilers' immune system (BasmacıoğluMalayoğlu et al., 2009). Konieczka et al. (2017) detected an increase in the relative weight of bursa Fabricius of chickens supplemented with dietary 300 IU/kg vitamin E as compared to those fed diets containing 50 IU/kg. Moreover, vitamin E at 100 and 200 mg/kg of the diet could improve the performances and have immune potentiating effect in broiler chickens (Desoky, 2018).Vitamin E has a significant role in enhancing the health conditions through the positive influence on both humoral and cell-mediated immune response of birds (Zhao et al., 2011; Lu et al., 2014; Rizvi et al., 2014). Moreover, it can induce protective immunity in broiler chickens through amelioration of the immuno-suppressive effect of lipopolysaccharide (Zhang et al., 2010), *Escherichia coli*, *Eimeriatenella*, T2 toxins (Jaradat et al., 2006), as well as heat stress (Niu et al., 2009).

It has been documented that heat stress increases the serum and liver concentrations of malondialdehyde (MDA), while vitamin E decreases the production of MDA in the liver by acting against lipid peroxidation and cell damage (McDowell, 2012) and results in the enhancement of the bird's performance (Sahin and Kucuk, 2001; Sahin et al., 2001). Several reports showed the positive effect of vitamin E supplementation alone or with other elements on broiler performance under heat stress condition. Both vitamin E and vitamin C at levels of 250 mg/kg of the dietinduced the highest productive performance of Japanese quails reared under heat stress (Sahin and Kucuk 2001; Sahin et al., 2003). Habibianet al. (2014) confirmed that heat stressed broiler chickens supplemented with combined levels of vitamin E and selenium at 250 mg/kg and 0.5 mg/kg, respectively

showed an improvementof both health and immune response to sheep red blood cells (SRBCs). Broiler chickens fed on dietary vitamin E at 30-50mg/kg under heat stress pressure showed reduced lipid peroxidation that can be detected by reduced levels of MDA (Dalólio et al., 2015). Furthermore, dietary concentration of zinc at 30-60 mg/kg has synergistic positive action with vitamin E on the productive performance of broilers under heat stress climate (Kim et al., 1998; Salgueiro et al., 2000).

Other literatures revealed no effect of vitamin E on broiler performance. Sosnowka-Czajka et al. (2005) found that dietary supplementation of broilers with both 40 mg/kg of vitamin C and 70 mg/kg of vitamin E failed to increase the resistance of birds to high temperature stressor. This result may be due to low doses of the vitamins or presence of factor interfere with the vitamins bioavailability. Feeding of dihydroquercetin (antioxidant) or vitamin E improved different parameters of antioxidant status of broiler chickens, although it did not affect growth performance parameters and energy or nutrient availability (Pirgozliev et al., 2020).

Layers

Laying hens supplemented by 6% semi-refined sunflower oil and 150 mg/kg vitamin E showed significant increase in egg production performance (Narimany-Rad et al., 2011).A concentration of 60 IU vitamin E /kg feed revealed an increase in egg production, yolk and albumin weights, and vitelline membrane strength of layer chickens (Parolini et al., 2015). In addition, vitamin E at levels 125-300 mg/kg has been found to minimize the egg production losses, eggshell density and feed efficiency (Cherian, 2015). Dietary supplementation of 125 to 300 mg vitamin E /kg feed improved the feed efficiency rate, egg production and egg shell thickness of layers (Karadas et al., 2017).

It has been observed that addition of vitamin E to the diets of layer hens appeared to be beneficial especially during the heat stress, probably, due to its concurrent function as fertility factor (Bollingier-Lee et al., 1999; Sahin et al., 2002a; Attia et al., 2016). Numerous studies have investigated the beneficial effects of vitamin E supplementation in laying hens under heat stressed conditions. For example, Kirunda and Scheideler (2001) found that vitamin E supplementation in the diet of heat stressed hens was able to alleviate egg quality deterioration. Ciftci et al. (2005) found that vitamin E can improve the egg quantity and quality of laying chickens reared under heat stress conditions. It has been demonstrated that vitamin E at 250 mg/ fed of layer hens may decrease the harmful stress effects of high temperature (Chung et al., 2005). Besides, dietary concentration of zinc at 30-60 mg/kg has synergistic positive action with vitamin E on the health and egg production of laying hens (Onderci et al., 2003; Sahin and Kucuk, 2003; Kucuk et al., 2008).Sahin and Kucuk (2001) observed a greatest performance of Japanese quails after supplementation with a combined treatment with vitamin C (200 mg) and vitamin E (250 mg) under chronic heat stress. Also, supplementation with150 mg vitamin C and/or 150 mg vitamin E to the diet improved the production performance in heat stressed layer chickens (Joachim Ajakaiye et al., 2011).Dietary vitamin E and vitamin C at levels of 65 IU/kg and 1, 000 ppm; respectively enhanced the *in vitro* lymphocyte proliferations of layer hens under bad environmental conditions (Jiang et al., 2013).

The role of vitamin E in improving the egg production under heat stress may be through the protection of liver from lipid peroxidation and damage of cell membrane that resulted in increasing in plasma egg yolk precursors as very low density lipoprotein and vitellogenin (Bollingier-Lee et al., 1999).Addition of vitamin E to diets containing high levels of PUFAs may prevent feed oxidation as well as may contribute to egg formation as these evidenced by increasing in the egg/bird/day and improving the feed intake and efficiency. Moreover, vitamin E protects the tissue from lipid peroxidation due to production of ROS and consequently affects the egg quality in layers (Lin et al., 2004; Khan et al., 2017).

Breeders

There are several factors that have a great hazardous effect on the semen and sperm quality (Rengarajet al., 2015; Nawabet al., 2018).Antioxidant feed supplementationreduces these effects by lipid peroxidation (Richard et al., 2008).As a result of neutralization of free radicals and inhibition of lipids membranes oxidation, vitamin E is regarded as chain-breaking antioxidant (Raederstorff et al., 2015). Vitamin E reduces the production of ROS molecules in the cells at their initial phase with destruction of thousands of PUFAs molecules (Anwar et al., 2016). It has been found that ROS damages hydroxyl radical, superoxide anion radical, singlet oxygen and hydrogen peroxide that produced during aerobic cellular metabolism (Anwar et al., 2016). Nevertheless, these oxidative

radicalsinducedestruction of healthy cells if they are not eliminated.Thus, it is necessary to add vitamin E to poultry ration to increase antioxidant metabolites in sperms andsemen and consequently helps in improving the quality and motility of sperms (Khan et al., 2017). Vitamin E reduces the defects in the DNA of spermsthroughdecreasing free radicals production and consequently increase the semen volume, sperm motility and sperm capacity in fertilizing eggs (Anwar et al., 2016).Biswas et al. (2009) demonstrated that birds supplemented with high doses of vitamin E (100mg/kg diet) showed good quality semen and spermatozoa in comparison with those received 10mg/kg of the vitamin. At a level of 20 mg /kg diet of breeder chickens, vitamin E significantly enhanced the immune response of SRBCs in comparison with levels of 0, 80 and 160 mg /kg diet (Lin and Chang, 2006).

Carcass trait

Lipid oxidation is very important process by which deterioration of meat products can occur as it is initiated at the membrane level in the intracellular phospholipid fractions (Buckley et al., 1995; Cortinas et al., 2005). Generally, supplementing birds with high levels of antioxidants in the diets enhances the oxidative stability, sensory quality, shelf life and consequently acceptability of meat (Buckley and Morrissey, 1992). It has been demonstrated that the peroxidation process begins just after slaughter, so the rate of meat spoilage is dependent on the concentration of vitamin E in the tissue (Morrissey et al., 1994). Vitamin E, in the form of α tocopheryl, is regarded as the major antioxidant defense and the lipid-soluble antioxidant that delays and breaks the lipid peroxidation chain in cell membranes, prevents hydroperoxides formation (Halliwell, 1987) and improves the quality of poultry meat (Pompeu et al., 2018). The level of dietary α-tocopheryl acetate in the poultry feed determines its level in the muscle and consequently the oxidative stability of meat (Carreras et al., 2004; Goñi et al., 2007). Previous studies of Gao et al. (2010) and Rey et al. (2015) have suggested that α tocopherol retained in serum and tissues and improved the meat quality of broiler chickens. Increasing the levels of α tocopherol levels in poultry diets significantly improved the feed conversion rate, average body weights, and net income/bird (Kennedy et al., 1992). In addition, the higher levels of α tocopherol resulting in high tissue concentrations, improvement of the cells membranes structure as well as an increase the oxidative stability of meat and meat products (Bartov and Frigg, 1992; Sheehy et al., 1993). It has been found that addition of α tocopherol to turkey's ration can improve meat oxidative stability leading to improving the flavour and colour (Sheldon et al., 1997). Supplementing chickens with 20 mg vitamin E/kg diet doubled the storage time in freezer, however 40 mg vitamin E/kg diet extended storage time by one day in refrigerated broiler carcasses (Coetzee and Hoffman, 2001). The MDA production in the broilers muscles decreased by addition of vitamin E to the ration, which reflects on the lipid peroxidation during the storage of chicken meat (Yesilbag et al., 2011). Brandon et al. (1993) suggested that feeding on 200 mg α tocopheryl acetate/kg of ration (i.e. 20 times higher than the NRC requirement) for at least 4-5 weeks, is essential to obtain the protective benefit of the vitamin in processed meat. However, Nobakht (2012) demonstrated that inclusion of broilers fat until 6% has no adverse effects on performance and carcass percent while, supplementing diet with 150 mg/kg of vitamin E is not recommended.

Hematology

Vitamin E significantly protected erythrocytes against high levels of hydrogen peroxide (Calabrase et al., 1985). In addition, vitamin E prevents oxidation of unsaturated fatty acids as linoleic acid on the membranes of erythrocytes (Bast et al., 1991) so, the deficiency of this vitamin increases erythrocytes' hemolysis (Levander et al., 1977). The effect of different dietary levels of vitamin E (100, 200 and 300) ppm on erythrocyte osmotic fragility and some biochemical parameters were studied in broiler chickens for 7 weeks observation period and the results showed significant decrease in erythrocyte osmotic fragility (Arslan et al., 2001).

Excessive supplementation of vitamin E decreases the plasma cholesterol (Bell, 1971; Clegg et al., 1976) and triglyceride levels and consequently inhibits atherosclerosis in poultry (Donaldson, 1982; Smith et al., 1989). Francini et al. (1988) demonstrated that the addition of vitamin E at level of 325 ppm in the diet of broilers resulting in decrease in cholesterol and triglyceride levels till 49 days of age. Decreasing the level of cholesterol was also found in turkeys fed with vitamin E on the 42nd day (Franchini et al., 1990). Ismail et al. (2014) found no effects on T4, total lipids, total cholesterol and high density lipoprotein cholesterol after inoculation of high levels vitamin E (200, 300 and 400 mg/kg diet) in the diets of broiler

chickens. However, plasma level of T3 increased significantly in response to high level of vitamin E (400 mg/kg diet). Serum concentration levels of of T3 and T4 were higher in birds treated with dietary vitamin E (Sahin et al., 2001, 2002b).

It has been declared that dietary supplementation of birds with vitamin E in high levels increased alkaline phosphatase (ALP) levels. Arslan et al. (2001) demonstrated statistical significant decrease in ALP level by the 7th week after treatment of broilers with 100, 200 and 300 ppm of vitamin E. In the same line, no significant difference was also found in the plasma ALP levels of turkeys supplemented with 30, 90, 180, 360 ppm of vitamin E/kg of ration, however, an increase in plasma ALP levels were increased with increasing the bird's age (Francini et al., 1990).

In addition, the total protein, calcium (Ca), phosphorus (P), aspartate aminotransferase (AST) or alanin aminotransferase (ALT) was not affected by the treatment with vitamin E by the 5th and 7th weeks of age (Arslan et al., 2001). In the same context, no significant differences were found in Ca and P in broilers treatment with 100 and 200 mg/kg of vitamin E (Desoky, 2018). One hundred, day old broilers fed on 25 and 10, 000 IU of vitamin E/kg of the diet showed decrease in plasma P and Ca levels (Murphy et al., 1981). But Francini et al. (1988) found an increase in the levels of ALP, Ca and P in birds treated with excess vitamin E and proposed this result to the osteoblastic activity (Francini et al., 1988).

Although AST level has been increased with the increase in the dietary level of vitamin E in turkey poults, but decreased in older birds (140 days old) (Francini et al., 1990). Desoky (2018) observed significant decline in AST and ALT activities in the group of broilers fed with vitamin E at 200 mg/kg.

Dietary supplementation with vitamin E (250 mg/kg) for Japanese quails under heat stress induce significant increase in lymphocytes (L) numbers and white blood cells counts, whereas, heterophils (H) numbers and H/L ratio was decreased (Abdel Maksoud, 1999; Ipek et al., 2007).

It has been shown that broilers fed on vitamin E at levels of 100 and 200 mg/kg had significant increase in hemoglobin, total proteins and albumin, significant decrease in the level of glucose and while no significant differences were found in globulin (Desoky, 2018)

CONCLUSION

As can be seen, vitamins E has measurable effects as an efficient antioxidant and immuno-stimulant agent. Addition of vitamin E in poultry diet is essential for growth and health parameters as well as maintenance and enhancement of immune system function in broilers. In addition, supplementing layers and breeders with vitamin E has positive effects on the quantity and quality of eggs as well as fertility and hatchability. Improvement of carcass trait and blood parameters are also the other beneficial aspects of vitamin E.

CONFLICT OF INTEREST

The author declares that there are no conflicts of interest.

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Review article
Ανασκόπηση

The gynecological controls in the elderly female dogs

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ABSTRACT: In canine geriatric medicine has many different research areas such as cardiology, endocrinology, dentistry. However, diagnosing many related diseases and complications can be late related to thoughts that spayed dog's genital exams are thought of as unneeded approaches. Reproductive infections, mammary/gynecologic tumoural diseases, and their complications decrease their life quality and have a considerable mortality rate in aged intact and spayed bitches. Therefore, an effective gynecologic health check program supported with different imaging techniques is highly needed in small animal clinics. In this review, it is planned an annual gynecologic examination for older/geriatrics dogsand presented the main steps to early diagnose and prevent genital diseases with high mortality and lower their life quality and longevity.

***Keywords*:** Gynecological controls, diagnosis, aging, dogs.

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INTRODUCTION

With the new diagnostic and therapeutic techniques in canine medicine, dogs' life span is getting longer, and they can accompany human beings much more years. According to their breed size, dogs are called senior and geriatric after 7-10 and 10-15-year-old (Fortney, 2004) ; one-third of them have multi-organ endocrinologic diseases such as diabetes mellitus, hypothyroidism, and hyperadrenocorticism. In the patient portfolios of a pet clinic, it can be seen that the older dogs' records take place 30%; also, 20% of the older dogs have genital diseases approximately (Haydardedeoğlu and Kalınbacak, 2015; Hoskins et al., 2004; Willems et al., 2017).

After 7-8-year-old, the female dog's reproductive physiology starts to slow and attenuate each year. Inter-oestrus duration is getting longer; proestrus bleeding and oestrus behaviors' visibility decrease (Feldman et al., 2015), so some cases cannot be noticed on time. However, early diagnosis of uterine infections or genital tumors is essential for appropriate surgical and therapeutic approaches, especially in elderly ages (Balducci et al., 1986; Waldron and Budsberg, 1989). For example, considering two cystic endometrial hyperplasias (CEH) -pyometra or mammary carcinoma cases in a young daughter and aged mother, the treatment protocols, prognosis, and complication rates would differeven though they have similar genetic, breed, and size features. Although these changes and high risks of genital diseases, the owners can be reluctant to refer their pets to veterinary clinics, especially spayed bitches. Fortunately, setting a well-designed collaboration between owners and veterinarians is possible because many of them have a strong relationship with the dogs as time passes andare more alert to their pets' behavior and health status. Although the female dogs consistof many spayed and senile individuals, genital system checkedup should be performed with clinical examinations and scans during their lifetime at least once a year. Scanning helps detect uterine infectious or hormone-dependent tumoural masses in intact females for early diagnosis and avoiding many late post-spaying complications after the four weeks of proestrus bleeding. It is suitable for the spayed bitches that the gynecologic evaluations once a year at the vaccination visits.

In this review, the main steps of a genital check-up program for older/geriatric female dogs are presented to use at the early diagnosis of different genital diseases with high mortality and lowering their life quality.

THE MAIN STEPS OF THE GYNECOLOGICAL CHECK-UPS

There are five main areas in a gynecological control program that must be evaluated by recording the clinical, radiological findings presented below:

1. Behavioral changes
2. Urinary incontinence
3. Perineal examinations
4. Gynaecological ultrasonography
5. Mammary glands' evaluations

1. Behavioral changes

With aging, various and irreversible changes are seen on many organ systems that affect their daily behavioral patterns. These effects may originate from neurological, endocrinologic, or orthopedical disorders. For example, catecholamine neurotransmitter levels are decreased (Milgram et al., 1993), but free radicals' levels rise in brain tissue (Mecocci et al., 1993). Diabetes mellitus can result in hypoxia by anemia and high blood pressure (Ivy et al., 1994). Loss of appetite and disorders in thermoregulation and orientation functions are remarkable, making the dogs more sedentary during the day (Landsbaerg and Head, 2011).

Moreover, by occurring the neurocognitive deficits, the owners define their pets as unhappy, lethargic, and unresponsive (Fortney, 2004). However, on the other side, anxiety can be seen in some individuals, representing much more physical activity and aggressive reactions to other animals and humans (Vite and Head, 2014). Furthermore, anxiety and protest acts can result in intermittent home soiling issues. Therefore, the differential diagnosis of organic disorders than neurocognitive impairment or protesting is crucial for appropriate evaluation by askingabout the duration of this condition and questions about life changes such as (moving to a new house, death of the owner, participating in a new pet, or any family member) (Landsbaerg and Head, 2011). When considering gynecologic diseases, lethargic and unresponsive dogs are the first group that must be evaluated for infectious and tumoural diseases and post-spaying complications. Suffering from defecation and urination can be originated from the perineal mass related to neoplasms orhernias.

The other behavioral changes can be increased sex-

ual activity and hyper-estrus symptoms.Long-lasting proestrus bleeding and copulation days would point to ovarian cysts or tumors such as granulosa cell tumors (GCT). Therefore, all remarkablebehavioral changes should be recorded for the subsequent clinical examination, even if they seem unrelated to the gynecologic disorders. Effective interventions and perioperative/chemotherapy protocols can be standardized for better health management based on recordings of these changes, especially irreversible ones.

2. Urinary incontinence

The spayed bitches weighing 20 kilograms above are at risk for home soiling problems (Veronesi et al., 2009). Following the surgery, by the irreversible decrease of the oestradiol level, contractility on the muscles of the orificium urethra externa disappears gradually, and urinary incontinence occurs.Studies show that ovariohysterectomised dogs have difficulty holding urine in the bladder as between %5-20 (Okkens et al., 1997; Veronesi et al., 2009). Home soiling caused by estrogenic deficiency is observed in resting/sleeping time, and it should be differentiated from euphoric emotions, protests act, or other neurocognitive deficits (Hoskins et al., 2004).

In differential diagnosis, thoracic and lumbar vertebrae radiographies should be evaluated for spondylitis cases; urine sample tests should be done to eliminate the cystitis case. Ultrasonographically, when the bladder is observed in average dimensions and with homogenous wall echotexture, without any stone or inflammatory signs, it should be suspected of the estrogen deficiency resulting fromspaying operation (Hoskins et al., 2004). Researchers have pointed to this issue that can be seen very long after the ovariohysterectomy until ten years (Okkens et al., 1997).

3. Perineal examinations

The perineal examination consists of three main steps: perineal skin inspection, searching perineal/perivulvar masses, and investigating the vaginal discharges. After recording anamnesis data, inspection and the perineal area's palpation reveal many genital malformations related to aging in bitches. Obesity risk increases with aging, and it causes excessive perivulvar skin folds. In addition, the vulvar atrophy and position change are seen more evidently in ovariohysterectomised females because of basal estrogen level (Graham, 2014). In these cases, owners should keep hygienic their pet's perivulvar area after the daily routines. If not, the poor ventilation and moisture from urine cause the impairment of local bacterial flora; specific and nonspecific agents can quickly contaminate and infect the skin. Intermittent, serous, seromucous vaginal discharge, odor, and itching are common symptoms of the infection that have a high risk of cystitis and pyometra in immunosuppressive individuals (Lightner et al., 2001).

At the perineal area's examination, clinicians should investigate any painless swellings that can push back into the pelvis and possible mass formation, especially the dogs showing urination or defecation changes such as constipation, pain symptoms, etc. Aging is a predisposing factor for weakening the pelvic muscles that support the perineal wall (Hayashi et al., 2016). Ultrasonographic imaging of the mass is a helpful tool as an additional diagnostic procedure. Before deciding the surgical treatment, transdermal usg may reveal the entrapped pelvic organs such as the bladder, uterine horns, intestinal loops (de la Porta Machado et al., 2020).

The other possible gynecologic disorder is the genital tract neoplasms in female dogs. Vaginal, vestibular, and vulvar tumors are the most common tumors in female dogs, excluding mammary gland neoplasia (White and Brearley, 2018). Leiomyomas, fibromas, and the malign forms of the mesenchymal tumors are common gynecologicaltumors in the canine practice. The owners cannot see hidden swelling under the furry tail in the long hairy dogs until showing clinical symptoms. Detailed palpation reveals the small-sized mass formation in the perineal area.

After detecting a mass, B-mode and Doppler usg help define its shape, capsule formation, and imaging vascular map that simulates the mass and proper preparation for a better outcome. According to the tumor's solid or multilobular shape and vascular intensity, performing the appropriate operative approaches are possible. At the exams of genital tract tumors, a possible estrogenic effect caused by the follicular cysts or the functional ovarian tumorsshould be investigated via abdominal usg and vaginal cytology because these tumors are associated with the high level of ovarian estrogen secretion (White and Brearley, 2018).Measuring and monitoring the total ovarian dimensions at the periodical visits play an essential role in gynecological scans of senior/geriatric dogs. Ovarian diameters more than 25 millimeters, cystic anechoic areas are typical findings to perform spaying operations. In the suspected ovarian images, cornified and nonnuclear superficial epithelial cells

at vaginal cytological samples reflect the high serum estrogen levels (Root Kustritz et al., 2010). In these cases, myelosuppressive signs on the hematological tests should be investigatedfor the medical/surgical precautions before surgery.

All vaginal dischargesreflect differentgenital disorders and have clinical importance in spayed and intact dogs of any age. Therefore, even if there is a little spot with various characters (serous, mucous, hemorrhagic, purulent) on the vulvar area that thought the vaginal discharge, detailed evaluations consist vaginal touché, cytology, and abdominal usg should be done a too early diagnosis of genital diseases (Perivulvar dermatitis, vaginitis, transmissible venereal tumor, CEH-pyometra complex, ovarian remnant syndrome, ovarian-uterine-vaginal tumors, etc.).

4. Gynecological ultrasonography

In this check-up program, gynecological usg is one of the most effective steps to diagnosing genital infections, neoplasms, and spaying complications. However, most cases are referred to the veterinary clinics quite late, so they have many complications at the perioperative and chemotherapy stages. In addition, there are many differences in the anesthesia/operation durations and the response to surgery and medical administrations between the infections and tumoural cases diagnosed early and lately for geriatric dogs. Therefore, annual scans of the genital organs help the vets for more detailed and proper genital evaluation.

Few and small cysts (1-2 mm) in the uterine wall, increased uterine horn diameter (>0.9 mm), and anechoic uterine content is the main signs for cystic endometrial hyperplasia cases (Bigliardi et al., 2004; Hagman, 2018; Veiga et al., 2017). In addition, according to researchers, low resistance in the uterine artery reflects uterine infections (Veiga et al., 2017). Therefore, early diagnosing cystic endometrial hyperplasias would avoid surgical complications and help the less drug usage because there is not disturbing in the kidney and liver functions yet, in the early stages of the disease.Regarding the many complication risks of uterine infections in senior and geriatric females, annual uterine scans play an essential role in avoiding septicemia and toxemia. The main target for this control program is the other aseptic forms of uterine disorders, such as hematometra, mucometra, and hydrometra. As time passed following vaginal flora contamination, cystic endometrial hyperplasia results inthe open or closed cervix pyometra formation. The observing vaginal discharge results in an earlier treatment possibility than in closed cervix pyometra cases. However, closed cervix pyometra cases do not show any specific symptoms until disturbingthe kidney and liver functions. In these cases, the higher uterine diameter and the thinner uterine wall cause high intrauterine tension, intraabdominal fluid accumulation with various echogenicity is detected (Hagman 2018). Until showing general symptoms, these patients will well tolerate surgical treatment and respond without complication.Closed-cervix pyometra is a latent and progressive disorder; the owner cannot notice general symptoms such as loss of appetite, polydipsia, polyuria, lethargy, etc. When these signs are remarkable, hepatic and renal function are damaged and need intense medical therapy.

New members of the pyometra risk group are considered that the ehrlichiosis and leishmaniosis positive dogs are having an additional risk for uterine infections (Behera, 2010; Silva et al., 2009). Therefore, clinicians should be much more alert in these dogs'periodic genital checks.

Vaginal cytology for differential diagnosisof uterine infectionsis not the right choice in healthy dogs at the diestrus stage due to similar microscopic results (intermediary cells, neutrophilia, fibrin debris, etc.) in both cases. Nevertheless, only taking the vaginal samples can be helpful in spayed females with Stumph pyometra.Stumph pyometra occurs in the ovariohysterectomised females caused by the hormonal active remnant ovarian tissue (Ball et al., 2010). Both the sagittal and transversal pelvic sections should be performed to detect the infected corpus uteri. Acoustic enhancement resulting from a full bladder may hide the corpus uteri, so; a secondary usg evaluation should be performed after urination. In those cases, ovarian pedicles should be investigated to virtually surgical treatment consisting of ovarian remnant treatment.

Besides uterine infections, abscesses and granuloma formation are the latent issues in spayed females. Early and late spaying operation complications are responsible for many gastrointestinal or urinary disorders in older female dogs. Nonabsorbable suture materials, the lack of aseptic approaches, retained sponges resulting in a granuloma formation can adhere to the abdominal wall and organs (Erdoğan and Yaygıngül, 2020; Goethem et al., 2006). For this reason, all surgical ligation areas should be investigated ultrasonographically for any suspected mass and its connection with the other abdominal organs.

Genital tumors are recorded as %1-2 for ovarian and uterine origins of all neoplasms. Their incidence is higher in intact females not allowed to mate and parturition (White and Brearley, 2018). If they do not produce estrogen, such as non-functional granulosa cell tumors, it is late to diagnose these latent and progressive tumors (Erdoğan et al., 2015). Metastasis to the other tissues and capsule formation, including kidney, bladder, and other vital organs, decreases surgical outcomes. At the ultrasonographic examination of the big-sized tumors, there is a challenge in detecting tumor origin and differentiation. Nevertheless, seeing anechoic cystic areas and vessels and blood flow evaluation with Doppler usg can guide the surgeons for additional medical and instrumental preparation in the preoperative stage.

5. Mammary gland evaluation

Mammary tumors are detected in approximately half of the intact canine female population, and malignity rates are recorded at 50% in dogs older than 10-year-old. The cumulative effect of progesterone triggers mammary neoplasia (Momont and Barber, 2003; Moulton et al., 1970). Informing owners about the risks, the protective effects of spaying on mammary tumors, and reasonable mammary controls at home aresignificant during visits. Because the home checks' findings help monitor the patients and make higher the success of treating the mammary tumors. Owners detect external masses in the simple regular palpations quickly. Every month, simple mammary palpations should be done to catch any suspected swelling. The primary purpose of these checks is to detect all-palpable or nonpalpable masses in the first grade (T1N0M0) and to start surgical and medical procedures immediately.

In literature, the intact or spayed females after 2.5-year-old, having relatives with the mammary tumor, affected with steroid administrations, and obese ones feeding with home-waste foods are in the risk group (Momont and Barber, 2003; Egenvall et al., 2005; Munson and Moresco, 2007). Following monthly checks by owners, inspection and palpation are performed on all five couple mammary lobes. In history, coughing and decreased exercise performance should be recorded for distant organ metastasis. In addition, every asymmetrical swelling should be evaluated for hernia formation, commonly seen in the inguinal mammary lobe areas. Inguinal hernia seen in dogs commonly due to the high diameter of canalis vaginalis is also confused with the tumoural masses (Parkinson et al., 2019; Waters and Stone, 1993). Differential diagnosis by usg reveals additive findings and appropriate surgery (Munro and Stead, 1993).

In small mammary masses (<2 cm), several physical features such as mobility, ulceration-inflammation signs should be recorded. Additionally, palpating of the regional lymph nodes and their usg find out the possible lymphatic metastasis condition. Finally, cytological samples are used for differentiation among other tumoural masses originating from different tissues and prediagnostic approaches between benign and malign neoplasms (Egenvall et al., 2005; Alonso-Diez et al., 2019).

Non-invasive imaging systems are pretty helpful in the monitoring of canine mammary tumors. Areas with more than five millimeters of opacity are suspicious for lung metastasis (Gobello and Corrada, 2001; Momont and Barber, 2003) ; magnetic resonance or computerized tomographical scans are needed. By using more comprehensive imaging, many detailed results can be used to appropriate management. B-contract, contrast-enhanced, and Doppler usg show the morphological changes, dimensions, echogenicity, hemodynamic features of the masses, and regional and abdominal lymph nodes (Wehrend et al., 2001; Marquardt et al., 2003; Momont and Barber, 2003; Nyman et al., 2006a; 2006b). Also, computerized assistant analysis programs can perform quantitative evaluation of the images (Feliciano et al., 2017; Mülazimoğlu et al., 2016). For more certain differentiation of the benign and malign masses, elastography is recommended to measure the changing elasticity and increased hardness of the malign tumor (Kutschker et al., 2008; Feliciano et al., 2017). Besides, infrared thermography may be used in the mass diagnosis and thermal changes due to the intravenous thrombus in the dogs (Kim and Park, 2012; Redaelli et al., 2014).

CONFLICT OF INTEREST

Author declares that there are no conflicts of interest associated with this publication.

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Research article
Ερευνητικό άρθρο

Total and Lipid-bound Serum Sialic Acid and Hematological-Biochemical-Blood Gas Changes in Dogs with Dirofilariosis

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ABSTRACT: The measurement of serum sialic acid concentration is of importance to indicate damage in cells or tissues. *Dirofilaria immitis* causes cardiovascular, pulmonary, hepatic and kidney lesions. Therefore, present study was planned to investigate the potential changes in the levels of serum total sialic acid (TSA), lipid-bound sialic acid (LSA) and hematological-biochemical parameters in the dogs naturally infected by *Dirofilaria immitis*.The patient group included the 7 dogs clinically and parasitologically (Modified Knott and PCR) diagnosed with *Dirofilaria immitis* while 7 healthy dogs were assigned as the control group. The biochemical parameters, blood gas parameters and hematological parameters were tested in the blood samples taken properly according to the guidelines from all the study animals using automated biochemistry analyzer, blood gas analyser and automated hematology analyzers, respectively. Serum TSA and LSA levels were measured spectrophotometrically using Sydow and Katapodis methods, respectively. Compared with the healthy group, the dogs diagnosed with dirofilariosis were found to have significantly increased levels of serum cardiactroponin, triglycerides, VLDL, LDL, BUN, urea and creatinine and enzymatic activities of CK-MB, ALT, AST, ALP and LDH, and statistically significantly decreased levels of cholesterol and HDL ($p<0.05$). A significantly decreased level of RBC and a significantly increased level of WBC was determined in the dogs with dirofilariosis ($p<0.05$). The differential leucocyte count test of the dogs with dirofilariosis indicated statistically significantly increased eosinophil count ($p<0.05$). In the dogs with dirofilariosis, the decreases in the levels of pCO_2, HCO_3 and BE in venous blood were statistically significant ($p<0.05$). The levels of serum TSA and LSA of the dogs with dirofilariosis were found statistically significantly higher than the healthy group ($p<0.05$). As a conclusion, statistically significant differences were identified between the dogs with dirofilariosis and healthy dogs in terms of sialic acid levels and certain biochemical, venous blood gas and hematological parameters. On the other hand, the present study is the first to investigate the serum sialic acid levels in the dogs with dirofilariosis.

Keywords: *Dog, Dirofilariosis, Lipid bound sialic acid, Sialic acid, PCR*

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INTRODUCTION

Dirofilariosis is a nematode disease caused by *Dirofilaria immitis* or *D. repens* that leads to serious systemic disorders in the carnivores, particularly dogs. *D. immitis* adults localize in the right ventricle, pulmonary arteries, right atrium and vena cava in the heart of the host. Their microfilariae are found in the peripheral bloodstream. The excessive work load on the right ventricle leads to congestive heart failure, organ dysfunctions such as particularly heart and kidneys, and various grades of pulmonary embolism and pulmonary hypertension in the dogs with dirofilariosis (Atkins, 2005; Sevimli et al., 2007).

Sialic acid (SA) is the general name of the acyl derivatives of neuraminic acid. N-acetylneuraminic acid (NANA) is the most common form of the sialic acids. Sialic acids are the terminal components of glycoproteins, glycolipids and oligosaccharide units of the proteoglycans. Total sialic acid (TSA) consists of two fragments as lipid-bound (lipid-bound sialic acid, LSA) and protein-bound (protein-bound sialic acid, PSA). The essential lipoproteins that contain LSA are VLDL, LDL, HDL and lipoprotein (a). The changes in the levels of serum SA indicate the damage in the cells or the tissues. The monitoring of these changes provides useful information in diagnosis, differential diagnosis and prognosis of many diseases (Sydow et al., 1988; Taşkın, 2017). For instance; increase of LSA and TSA levels have been demonstrated on babesiosis (Esmaeilnejad et al., 2014), theileriosis, anaplasmosis (Ertekin et al., 2000), leptospirosis (Erdoğan et al., 2008), dermatophytosis (Karapehlivan et al., 2007) and pneumonia (Karapehlivan et al., 2007).

Since this cardiopulmonary disease of the dogs is a zoonosis, the detection, elimination and treatment of the dogs is of great importance for both dog and human health. Besides, increased dog adopting in the recent years elevates risk for transmitting dirofilariosis in the humans. No study that aimed at the levels of TSA and LSA in the dogs with dirofilariosis was found in the literature review. The present study was planned to investigate the potential changes in the levels of serum TSA, LSA and hematological-biochemical parameters in the dogs naturally infected by *Dirofilaria immitis.*

MATERIALS AND METHODS

Parasitological analysis

The study was conducted on 96 male stray dogs between 4 and 5 years old brought to the Animal Care and Rehabilitation Center for castration from Van Central and surrounding districts between May and October 2019. Before being castrated, the animals were fasted for one day and only drank water as the last 12 hours. Negative control samples were obtained from PCR negative dogs with no previous history of dirofilariosis. The approval of the Ethics Committee for this research was obtained from the Animal Experiments Local Ethics Committee of Van Yüzüncü Yıl University (2019/01). Blood samples were drawn from vena cephalica antebrachium of the dogs with 2 mL of blood to heparinized injectors for blood gas analyses, 3 mL of blood to EDTA tubes for hematological analyses and to 5 mL gel polyethylene tubes without anticoagulants for biochemical analyses. Blood samples taken into the EDTA tubes were analyzed by performing modified Knott's (Gioia et al., 2010) and PCR (T100 BioRad, USA) techniques using PCR kit (NorgenBiotek, EP44500). PCR positivity was detected in only 7 of 96 dogs. This study included totally 14 dogs aged 4-5 years old including 7 dirofilariosis-positive and 7 healthy dogs.

Biochemical analysis

Blood samples in serum separator tubes were centrifuged at 3000 rpm for 10 min within 2 h of collection, and the obtained sera were separated, stored at -20 °C. Cardiac troponin I (cTnI), total creatine kinase (CK), creatine kinase-myocardial isoenzyme (CK-MB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total protein, total bilirubin triglyceride, cholesterol, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), urea, blood urea nitrogen (BUN) and creatinine in the sera were analysed with commercially available kits using anautomated biochemistry analyser (Cobas Integra 800, Roche).

Serum TSA and LSA analyses were carried out colorimetrically on a spectrophotometer (Boeco, Germany) using the methods reported by Sydow et al. (1988) and Katopodis et al. (1982) respectively. Briefly, a mixture of 0.2 mL of serum and 1.5 mL of 5% perchloric acid (HClO4) was incubated at 100 ° C for 5 minutes, cooled and centrifuged at 500g for 4 minutes. Then 0.2 mL of Ehrlich reagent was added to 1.0 mL of clear supernatant and heated at 100 ° C for 15 minutes. After cooling, 1.0 mL of distilled water was added to this mixture and the optical density was measured at 525 nm in a spectrophotometer. The amount of TSA was determined using a standard

curve developed from a standard sample of N-acetyl neuraminic acid (Sydow et al. 1988).

For LSA, 44.7 µL of serum was transferred with a capillary pipette to 150 µL of distilled water. Contents were vortexed for 5 seconds. The tube was transferred to crushed ice. Three milliliters of cold (4-5 ° C) 2: 1 (v / v) chloroform: methanol were added to the tube and the mixture was vortexed for 30 seconds. 0.5 mL of cold distilled water was added to this mixture and the tube was closed. The tube was then inverted repeatedly for 30 seconds to mix the contents. After the tube was centrifuged at 500g for 5 minutes at room temperature, 1 mL of the upper layer was transferred to another tube. Fifty microliters of phosphotungstic acid solution (1 g / mL) was added and, after mixing, the tube was left at room temperature for 5 minutes. The tube was centrifuged at 500g for 5 minutes and the supernatant was suction removed. Continuing the researchers's method, the final color obtained was read at 580 nm.The amount of LSA was determined using a standard curve developed from a standard sample of N-acetyl neuraminic acid (Katopodis et al. 1982).

Blood samples collected heparinized injector were delivered to the laboratory with ice within 10 minutes. Blood samples were used for determination of venous blood gases parameters. Acidity (pH), carbon dioxide pressure (pCO_2), partial oxygen pressure (pO_2), bicarbonate ($HC0_3^-$), base excess (BE) were measured using a blood gas analyzer (ABL90 FLEX).

Haematological analysis

The blood samples containing heparin were refrigerated and analysed within 48 h. Blood samples were used for determination of haematological parametrs. Red blood cell (RBC), haemoglobin (Hb) hematocrit (Hct), white blood cell (WBC), neutrophil, lymphocyte, monocyte, eosinophil and basophil were measured using a automated haematology analyser (Vet. Wasson MC-1200).

Statistical analysis

SPSS Version 22 Software Package was used for the statistical analysis of the study data. First of all, distribution normality test was performed to assess whether the groups showed normal distribution. The groups were found to show normal distribution since significance value of Shapiro-Wilk test was over statistical significance level of 0.05. Therefore, the statistical differences between the groups were evaluated using Independent Sample T-Test. The obtained results were expressed as X±SE.

RESULTS

The analysis of 96 blood samples taken from the stray dogs by Modified Knott's technique revealed that 5 (5.2%) dogs were dirofilariosis-positive. However, PCR method was applied to all the samples and *D. immitis* was detected in totally 7 (7.2%) dogs (Figure 1). The clinical examination of the infected animals indicated coughing and respiratory distress in 3 and 2 dogs, respectively, whereas other 2 infected animals were asymptomatic.It was determined that TSA, LSA, cTnI, CK-MB, triglycerides, VLDL, LDL, BUN, urea, creatinine levels and AST, ALT, ALP, LDH enzyme activities statistically significantly increased; however, cholesterol, HDL, pCO_2, HCO_3^-, BE levels statistically significantly decreased in dogs naturally infected with *D. immitis* compared to the healthy group ($P< 0.05$). The changes in the total protein and total bilirubin levels with total CK enzyme activity in dirofilariosis group were not statistically significant compared to the healthy group ($p>0.05$) (Table 1). It was detected that the RBC level significantly decreased while WBC level and eosinophils count significantly increased in dogs naturally infected with *D. immitis* compared to the healthy group ($P<0.05$). The changes in Hb and Hct levels with lymphocytes, monocytes, eosinophils and basophils counts were not statistically significant among diroflariosis and healthy groups ($P>0.05$) (Table 2).

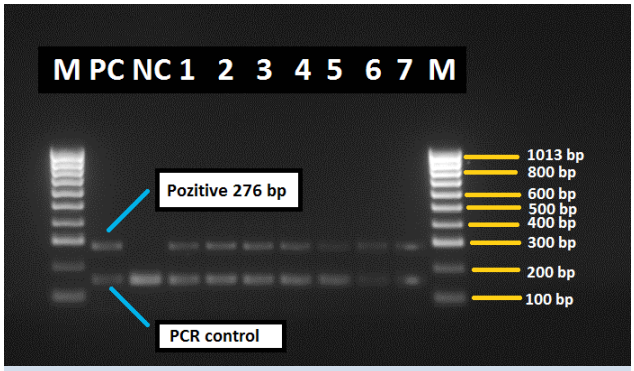

Figure 1. Analysis of PCR products by agarose gel electrophoresis M: 100 bp Marker; 1- 7: Positive samples; PC: Positive control; NC: Negative control.

Table 1. The biochemical parameter levels of healthy dogs and dogs naturally infected with *D. immitis*

Parameters	Healthy group (n=7) (X ± SE)	Dirofilariosis group (n=7) (X ± SE)	Reference valuesa, b, c, d
TSA (mg/dl)	8.25±0.92	11.23±2.65*	-
LSA (mg/dl)	2.00±0.90	4.02±1.13*	-
cTnI (µg/l)	0.09±0.04	1.49±0.49*	0.03-0.11b
CK-MB (µg/l)	0.60±0.14*	2.07±0.53*	0-0.64b
Total CK (u/l)	15.42±62.30	22.84±96.65	5-25a
AST (u/l)	43.59±9.76	80.39±16.70*	23-66a
ALT (u/l)	48.39±16.52	94.92±26.31*	21-102a
ALP (u/l)	60.86±21.84	118.18±34.96*	20-156a
LDH (u/l)	136.71±66.18	279.29±146.70*	45-233a
Total protein (g/l)	63.43±6.40	65.43±4.35	53-73a
Total bilirubin (µmol/l)	0.11±0.03	0.12±0.04	0-6.84a
Triglycerides (mg/dl)	61.85±9.73	97±12.16*	50-100a
Cholesterol (mg/dl)	202.85±28.35	140.14±17.78*	125-250a
VLDL (mg/dl)	11.97±2.2	20.31±3.08*	-
LDL (mg/dl)	61.91±5.05	133.57±28.54*	5-86c
HDL (mg /dl)	79.77±7.18	56.54±4.03*	49-165c
BUN (mg/dl)	26.14±12.60	60.00±31.12*	12-25a
Urea (g/dl)	31.10±11.97	51.89±5.19*	42.8-59.9a
Creatinine (mg/dl)	0.86±0.18	1.47±0.35*	0.5-1.5a
pH (-log H+)	7.31±0.05	7.29±0.01	7.35-7.42d
pCO2 (mm/Hg)	41.84±4.00	39.49±3.65*	29.0-42.0d
pO2 (mm/Hg)	48.87±8.32	44.83±3.99	49.9-54.2d
HCO3- (mm/L)	22.55±2.12	19.16±1.90*	22.2-22.4d
BE (mm/L)	0.73±1.70	-2.61±2.80*	-

*p<0.05 shows the significance between the parameters on the same row. a (Karagül et al., 2000), b (Ok et al., 2010), c (Loria et al., 2020), d. (Anonymous, 2020). Cardiac troponin I (cTnI), creatine kinase (CK), creatine kinase-myocardial isoenzyme (CK-MB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), carbon dioxide pressure (pCO_2), partial oxygen pressure (pO_2), bicarbonate ($HC0_3^-$), base deficit (BE)

Table 2. The hematological parameter levels of healthy dogs and dogs naturally infected with *D. immitis*

Parameters	Healthy group (n=7) (X ± SE)	Dirofilariosis group (n=7) (X ± SE)	Reference valuese
RBC (106/mm3)	6.93±1.11	5.62±0.96*	6.0-9.0
Hb (g/dl)	18.11±2.74	17.72±2.45	10.5-20
Hct (%)	49.41±5.47	43.71±5.96	37-54
WBC (103/mm3)	10.98±3.45	18.77±3.24*	6.0-17.0
Lymphocytes (%)	24.95±12.99	19.26±8.97	12-30
Monocytes (%)	10.33±6.92	6.44±4.53	3-10
Neutrophils (%)	56.65±24.31	66.04±13.76	60-75
Basophils (%)	0.13±1.34	0.23±1.12	0-1
Eosinophils (%)	2.75±1.34	7.34±2.42*	2-10

*p<0.05 shows the significance between the parameters on the same row. Reference, (Yılmaz, 2000), red blood cell (RBC), haemoglobin (Hb) hematocrit (Hct), white blood cell (WBC).

DISCUSSION

Age and gender of dogs are also important as well as environmental circumstances such as presence of mosquitoes and temperature in development of dirofilariosis caused by *D. immitis*. It has been reported that elderly, male and stray dogs carry a higher risk for infection than younger, female and house dogs (Fırat et al., 2005; Taylor et al., 2007). The male stray dogs aged 4-5 years old analyzed in the present study are consistent with the reports in the literature. High-

er infection risk in the elderly stray dogs can be explained by the higher risk of exposure to mosquitoes and longer prepatent period of parasites in these dogs (Balıkçı, 2005).The presence of the findings such as coughing and respiratory distress in 3 and 2 dogs, respectively, as well as 2 asymptomatic dogs in the clinical examination of the infected dogs in our study were consistent with clinical findings reported for that disease (Börkü et al., 1996; Taylor et al., 2007).

The excessive work load on the right ventricle leads to congestive heart failure, organ dysfunctions such as particularly heart and kidneys, and various grades of pulmonary embolism and pulmonary hypertension. Microfilarial diseases involve several findings such as changes in the blood gases, increased enzymatic activities in the liver, reduced hepatic function, proteinuria and uremia accompanied with the lesions in the lungs, heart, kidneys and liver (Atkins, 2005).

The heart is affected both functionally and morphologically in the dogs with dirofilariosis. *Dirofilaria immitis* adults leads to endothelial damage, myocardial infarction and arteriosclerosis by locating in the heart of the dogs (Pasca et al., 2012). The serum levels of cTnI (Gazyagci et al., 2011; Carretón et al., 2011; 2012; 2013; 2014; Yoon et al., 2017) and the enzymatic activities of CK (Voyvoda et al., 1996; Balıkçı, 2005; Meral et al., 2007; Sevimli et al., 2007; Kına, 2009) and CK-MB (Kına, 2009; Carretón et al., 2013) were detected to be significantly increased suggesting myocardial damage in the dogs with dirofilariosis. It was determined also in this study that serum cTnI level and enzymatic activity of CK-MB significantly increased in the dogs with dirofilariosis compared with the healthy group and that this increase was higher than the reference value. Differently from the mentioned reports, an insignificant increase (Sribhen et al., 1999) was determined in the enzymatic activity of total CK in present study. The increased levels of cTnI and CK-MB enzymatic activity may be a consequence of myocardial damagecaused by adult forms of *D. immitis* that localized in the right ventricle of the dog heart.

Increased activities of the liver enzymes are resulted from the ascites and passive congestion of the liver developing secondary to right-sided heart failure in the dogs with dirofilariosis (Tabrizi, 2012). Consistently with the studies that have reported increased liver enzymes in the dogs with dirofilariosis (Goggin et al., 1997; Balıkçı, 2005; Niwetpathomwat et al., 2006; 2007; Sevimli et al., 2007; Kına, 2009; Aslan et al., 2010; Ranjbar-Bahadori et al. 2010; Borthakur et al., 2011; Tabrizi, 2012; Atyha and Alani, 2017), we have encountered in the present study that enzymatic activities of the several eznymes such as AST, ALT, ALP and LDH significantly increased in the group of infected dogs compared with the healthy dogs. Additionally, the increased enzymatic activity levels of AST and LDH were over the reference values. That increased activity levels of these liver enzymes may be caused by the microfilariae detected in the blood samples or the adult parasites that localize in the liver and cause liver injury. Beside that, increased enzymatic activities of AST and LDH may support the presence of cardiovascular injury caused by *D. immitis* in the infected dogs.

In the present study, insignificant elevations were encountered in the levels of total protein (Balıkçı, 2005; Aslan et al., 2010; Ranjbar-Bahadori et al., 2010; Borthakur et al., 2011) and total bilirubin (Sevimli et al., 2007; Borthakur et al., 2011) in the dogs with dirofilariosis supporting the reports of the previous studies. In contrast to that result, significantly increased levels of total protein (Sevimli et al., 2007) and total bilirubin (Goggin et al., 1997; Tabrizi, 2012) were also found.

The development of atherosclerotic cardiovascular disease depends on the state of cholesterol as LDL and HDL in the bloodstream. The high levels of VLDL and LDL and low level of HDL create predisposition for the cardiovascular diseases (Karagül et al., 2000). Jacobs et al. (1992) have reported elevated levels of triglycerides and cholesterol besides the decreased level of HDL in the dogs with dirofilariosis. Another study has reported that the levels of triglycerides, VLDL and LDL increased whereas the levels of cholesterol and HDL decreased (Kına, 2009). That study has also detected that serum triglyceride, VLDL an LDL levels significantly increased whereas HDL and cholesterol levels significantly decreased in the group with dirofilariosis. Additionally, the increased level of LDL was higher than the reference value. The high levels of triglycerides and VLDL may be resulting from lipolysis that stimulates the production of triglycerides and VLDL cholesterol depending on glycolysis. The low level of the cholesterol may be a consequence of impaired cholesterol synthesis in the hepatocytes due to the liver damage caused by the parasite. On the other hand, Kına (2009) has suggested that the high level of LDL may be resulting from the reduced entry of LDL into the cells from the plasma due to the suppression of the LDL receptors caused by

D.immitis. The low level of HDL may be depending on the reduction in the cholesterol synthesis during the disease. Besides, high levels of VLDL and LDL beside low level of HDL may have contributed to the development of the cardiopulmonary diseases due to dirofilariosis. Differently from our results, no difference was encountered in terms of cholesterol level (Aslan et al., 2010; Ranjbar-Bahadori et al., 2010).

The blood levels of urea, creatinine, BUN and uric acid are usually tested as the indicators of the renal function. Blood urea nitrogen (BUN) and serum creatinine determinations are related to the amount of nitrogenous residues removed by the kidney. Serum creatinine and urea analysis are used as a kidney function test. BUN may increase in cases such as bleeding, fever, corticosteroid administration, burns, hunger, infection, tetracycline administration, decrease in protein intake, severe liver failure (Karagül et al., 2000). Kidney injuries such as glomerulonephropathy, glomerulosclerosis, chronic interstitial nephritis and amyloidosis have been identified in the dogs infected by *D. immitis* (Niwetpathomwat et al., 2007). It has been stated that formation of immune complexes by accumulation of the microfilarial antigens on the glomerular basal membrane causes glomerulonephropathy (Balıkçı, 2005). It was determined in this study supporting the findings of many researchers that the levels of serum urea (Şahal et al., 1997; Balıkçı, 2005; Atyha and Alani, 2017), creatinine (Börkü et al., 1996; Niwetpathomwat et al., 2006; 2007) and BUN (Niwetpathomwat et al., 2006; 2007; Sevimli et al., 2007; Tabrizi, 2012) significantly increased in the dogs with dirofilariosis compared with the healthy dogs. At the same time, the increase in the level of BUN was found to be higher than the reference value. Differently from the results of our study, some studies demonstrated that no difference was present in terms of urea, creatinine and BUN levels (Ranjbar-Bahadori et al., 2010; Atyha andAlani, 2017).

Parasitic agents leads to anemia in the host by causing decreased circulatory erythrocyte count or decreased erythrocyte count per unit volume of blood or reduced hemoglobin concentrations (Atyha and Alani, 2017). The dogs with dirofilariosis have various grades of anemia as a consequence of the changes in the RBC, Hb and Hct levels (Niwetpathomwat et al., 2007; Sevimli et al., 2007). Although, its etiological mechanism is not exactly clear yet, anemia was reported to be resulting from the haemolysis of the circulatory erythrocytes with increased fragility due to the obstruction of bloodstream depending on the presence of numerous adult infectious agents in the veins during the infection period (Atyha and Alani, 2017). The related studies have encountered significant (Kitagawa et al., 1993; Borthakur et al., 2011) and insignificant (Şahal et al., 1997) declines in the RBC, Hct and Hb levels of the infected dogs. In this study, a significant decline in the RBC level (Balıkçı, 2005; Sarıtaş et al., 2005; Ranjbar-Bahadori et al., 2010; Atyha and Alani, 2017) and insignificant declines in the Hct and Hb levels (Ranjbar-Bahadori et al., 2010; Atyha and Alani, 2017) in the dogs with dirofilariosis compared with the healthy group (Ranjbar-Bahadori et al., 2010; Atyha and Alani, 2017).

It was determined in this study that WBC level significantly increased in the dogs with dirofilariosis compared with the healthy dogs (Şahal et al., 1997; Balıkçı, 2005; Sarıtaş et al., 2005; Niwetpathomwat et al., 2007; Sevimli et al., 2007) and this increase was over the reference value. The development of leukocytosis in the infected dogs may be resulting from the reduced resistance against the other infectious diseases and secondary infections such as pneumonia and nephritis. In contrast to this result, Ranjbar-Bahadori et al. (2010) and Atyha andAlani (2017) have determined that no difference was encountered in the level of WBC.

Although, increased eosinophil and basophil counts are considered as the general characteristic findings for dirofilariosis according to the differential blood count, nevertheless, it has been reported that these findings may be obtained also in the infections caused by other intestinal parasites and were not specific for dirofilariosis (Şahal et al., 1997). In contrast to the studies which suggested that no difference was encountered in the eosinophil counts of the dogs with dirofilariosis (Ranjbar-Bahadori et al., 2010; Atyha and Alani, 2017), a significant increase was found in the eosinophil count in this study. The detection of the increased eosinophil count confirms the conclusion that eosinophil may have a supportive diagnostic value in dirofilariosis. In our study, the changes in the neutrophil, basophil, lymphocyte and monocyte counts were found insignificant. Differently from this result, significant increases and/or decreases were determined in the neutrophil, basophil, lymphocyte and monocyte counts in the other studies (Şahal et al., 1997; Balıkçı, 2005; Niwetpathomwat et al., 2007; Sevimli et al., 2007).

In the severely infected dogs with dirofilariosis; thoracic radiography displays right ventricular enlargement, prominent major pulmonary arteries,

edema in the caudal lung lobes, haemorrhage and parenchymal impairments (Atkins, 2005; Meral et al., 2007). The pathological impairments in the lungs lead to respiratory distress and coughing in the infected dogs (Börkü et al., 1996). Respiratory distress causes impairments in the pulmonary gas exchange functions (Sarıtaş et al., 2005). The significantly low levels of arterial and venous pO_2 and pCO_2 levels in the blood gas analysis are the sensitive criteria in the diagnosis of pulmonary embolism in the dogs infected with dirofilariosis (Kitagawa et al., 1993). In this study, significant declines were detected in the levels of pCO_2, HCO_3 and BE in the dogs with dirofilariosis compared with the healthy dogs. Besides, reduction in the level of HCO_3 was found lower than the reference value. An insignificant decline was encountered in the levels of pH and pO_2. Hypoxemia develops in the clinical picture of the cardiopulmonary disorders in the dogs, the body, under this circumstance, produces buffering by accelerating respiration to increase the alveolar O_2 and excreting HCO_3 to balance alkalosis caused by the decreased levels of CO_2 in the blood (Balıkçı, 2005). In this study, significantly low levels of pCO_2, HCO_3 and BE in the infected dogs indicate the buffering capacity of the organism. In the infected dogs, the low levels of HCO_3 and BE despite absence of a difference in blood pH compared with the healthy group points out the development of metabolic acidosis in dirofilariosis.

Sialic acids are the terminal components of the oligosaccharide units of the glycoconjugates (glycoproteins, glycolipids, proteoglycans). Sialic acids have an important impact with respect to host recognition by the pathogens, intercellular interactions, hormone-receptor relationships and protection of the cell from proteolysis due to carrying negative charge. Sialic acids are diffusely found on the outer surface of the biological membranes. The changes in the serum SA levels demonstrate the cellular or tissue damages. The monitoring of these changes provides useful data in diagnosis, differential diagnosis and prognosis of many diseases (Sydow et al., 1988; Taşkın, 2017). No study that aimed at the levels of TSA and LSA in the dogs with dirofilariosis was found in the literature review. Increased levels of sialic acid were detected in the distemper disease of dogs (Altıntaş et al., 1989), hydrocortisone and dexamethasone injections in the dogs with distemper (Engen, 1971) and those with tumoral and non-tumoral different diseases (Thougaard et al., 1998). Another study that investigated the correlation between TSA and serum α 1-acid glycoprotein (AGP) levels identified a positive correlation between AGP and TSA concentrations (Thougaard et al., 1999) in the dogs with tumors.

In this study, serum TSA level was found significantly higher in the dogs with dirofilariosis compared with healthy dogs. That increase may be resulting from the increased synthesis of acute phase proteins (APPs) that contain sialic acid residues at the terminal position of the lateral oligosaccharide chain. Because, it has been reported that the levels of APPs increase both to meet the increased need for oxygen and as a response to the potential tissue damage in the dogs with dirofilariosis (Carretón et al., 2014). In our study, LSA levels of the dogs with dirofilariosis were detected to be significantly higher than the healthy dogs. Approximately 85-90% of the sialic acid are bound to the proteins. The remaining 10-15% portion is bound to the lipids. The increase in the level of APPs leads to only increased level of sialic acid fraction bound to the proteins in dirofilariosis, however, shows no impact on the sialic acid fraction bound to the lipids. Thus, increased level of serum LSA encountered in dirofilariosis may a consequence of the increased synthesis of lipoprotein in the liver that contains sialic acid. Accordingly, increased levels of VLDL and LDL were found to increase in our study in the dogs with dirofilariosis. Besides, the increase in the level of LDL was determined to be higher than reference value. On the other hand, the sialic acid residues released from oligosaccharides in the damaged cell and cell membranes due to the increased oxidative stres (Aslan et al., 2010) may be responsible for the increased levels of TSA and LSA in dirofilariosis.

CONCLUSION

We concluded in the light of the present study data that statistically significant differences were identified between the dogs with dirofilariosis and healthy dogs in terms of sialic acid levels and certain biochemical, venous blood gas and hematological parameters. On the other hand, the present study is the first to investigate the serum sialic acid levels in the dogs with dirofilariosis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Postoperative pain assessment with concurrent administration of intraperitoneal tramadol and incisional lidocaine following ovariohysterectomy in dogs

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ABSTRACT: This study aimed to assess postoperative pain with concurrent administration of intraperitoneal tramadol and incisional lidocaine following ovariohysterectomy in dogs. A group of twenty mixed breed female dogs, aged 1-2 years, weighing 19.5 ± 0.8 kg were used. Initially, dogs were sedated with intramuscular administration of acepromazine 1%. Anaesthesia was induced with diazepam (0.5 mg/kg) and ketamine (10 mg/kg) and maintained with 1.5% Isoflurane. Ketoprofen (2 mg/kg) was administered intravenously just before the initiation of the surgery. Five minutes before midline incision, saline (0.2 ml/kg) was injected to the muscles and subcutaneous space around the incision in the tramadol-saline (TS) group. Also, in the tramadol-lidocaine (TL) group, lidocaine 2% with epinephrine (1.5 mg/Kg) was administered subcutaneously. Ovariohysterectomy was performed and before closing the linea alba, tramadol (4 mg/kg) was splashed on the abdominal viscera in both groups. Cortisol, vital signs and pain scoring systems were evaluated at different time points. Heart rate, respiratory rate and rectal temperature changes were not statistically significant between groups. Cortisol level showed a significant difference between groups at 1, 3 and 6 hours after surgery ($p \leq 0.05$). Both UMPS and CMPS-SF pain scores in the TL group were significantly lower than the TS group at 30 minutes, 1, 3 and 6 hours after surgery ($p \leq 0.05$) and also at 12 hours after surgery ($p \leq 0.05$). It seems that intraperitoneal administration of tramadol (4 mg/kg) along with administration of incisional lidocaine with epinephrine (1.5 mg/kg) is recommended following ovariohysterectomy in dogs.

***Keywords*:** dog, intraperitoneal tramadol, incisional lidocaine, ovariohysterectomy.

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INTRODUCTION

Pain is an unpleasant sensation associated with actual or potential tissue damage (Wright & Aydede 2017). Postoperative pain has many adverse effects such as decreased food intake, increased protein catabolism, impaired respiratory function, irregular heart rhythm, increased central sensitivity to painful stimuli, increased postoperative stress, suppressed immune system, increased arterial blood pressure, delayed wound healing, and inconsistent behaviors, including self-attack (Gwendolyn and Carrol, 1996; Gaynor, 1999; Flecknell and Watermen-Pearson, 2000). Effective and better pain management is achieved when multiple analgesics are used to target multiple pain pathways (Hellyer et al., 2007; Reuben et al., 2007). Post-operatively abdominal pain usually occurs within the first 24 hours (Golubovic et al., 2009). In the other, ovariohysterectomy is one of the most common surgeries in small animals. Pain after ovariohysterectomy is classified as mild to moderate (Hardie et al., 1997; Carpenter et al., 2004). A way to induce appropriate analgesia in ovariohysterectomy is to combine local anaesthesia with systemic analgesics (Slingsby et al., 2008; Gurney et al., 2012). Intraperitoneal administration of local anaesthetics or analgesics is a valuable and proven method for pain control after abdominal surgery in human and veterinary medicine (Golubovic et al., 2009; Chilkoti et al., 2019). Although intraperitoneal analgesia can block pain transmitters in visceral structures, it does not prevent pain transmission to areas close to the skin. Similarly, local anaesthesia around an incision that prevents pain in the superficial areas cannot block visceral pain (Ng et al., 2002). As topical analgesics have mild systemic effects, concomitant intraperitoneal administration of analgesia in abdominal surgery can optimally control pain with minimal side effects (Marks et al., 2012). Tramadol is a synthetic analogue of codeine, narcotic drug used for analgesic purposes after surgery in small animals (Mastrocinque and Fantoni, 2003; Morgaz et al., 2013). Its analgesic effect is the one-tenth of morphine and it less affects the respiratory function than morphine (Mastrocinque and Fantoni, 2003). Numerous clinical studies have shown sutiebale postoperative pain management concomitant administration of opioid drugs such as tramadol intraperitoneally and local anaesthetics, following open or laparoscopic abdominal surgery in humans (Karsli et al., 2003; Memis et al., 2005; Golubovic et al., 2009). Local anaesthesia is very diverse due to its clinical applications. This group of drugs usually anaesthetizes a block of nerves or the spinal through infiltration, by inhibiting pain transmitters (Skarda and Tranquilli, 2007). Lidocaine is a local anesthetic which blocks the sodium-calcium channel (Fozzard et al., 2005) and inhibits neurons associated with visceral pain (Ortega and Cruz, 2011). Various studies have shown that intraperitoneal administration and/or around the incision site of lidocaine or bupivacaine is sufficient for analgesia after ovarian hysterectomy in dogs (Carpenter et al., 2004; Campagnol et al., 2012; Guerrero et al., 2016; Lambertini et al., 2018; Chilkoti et al., 2019). Using peripheral and intraperitoneal lidocaine in cat ovarian surgery, it was showed that this method enhances postoperative analgesia (Zilberstein et al., 2008). According to the literature, there is no published data about the analgesic effects of concomitant administration of intraperitoneal tramadol and lidocaine around the incision site. Therefore, this study aimed to evaluate the effectiveness of concomitant administration of lidocaine around the incision and tramadol intraperitoneally to manage post-operatively pain after ovariohysterectomy in dogs.

MATERIALS AND METHODS

The project was approved by the local Committee of the Institutional Animal Care and Use of Shahid Chamran University of Ahvaz, Iran.

Animals

This study was carried out on twenty clinically healthy female mixed-breed dogs ranged in age from 1-2 years and weighted 19.55 ± 0.8 kg. The health status of all animals confirmed with clinical examination and blood cell counts, and total protein level. The animals were kept in the same conditions and had access to enough water and food. The animals were randomly assigned to two equal groups, tramadol-saline (TS) and tramadol-lidocaine (TL) groups. Food was withheld for 12 hours and water for 2 hours before the experiment. They were housed individually and fed on a commercial diet.

Procedure

Initially, dogs were sedated with intramuscular administration of 0.05 mg/kg acepromazine 1% (Alfasan, Neatherland) (Grimm et al., 2015). Thirty minutes later, an intravenous catheter (No. 20) was inserted into the cephalic vein and the abdominal area was clipped to perform ovariohysterectomy. After 10 minutes (40 minutes after sedation), anaesthesia was induced through titration with diazepam (0.5 mg/kg)

and ketamine (10 mg/kg) (Grimm et al., 2015). Then, endotracheal intubation was performed. To maintain anaesthesia, the animals were connected to an inhaled anaesthetic device equipped with an isoflurane vaporizer. Isoflurane was administered at a concentration of 1.5% and an oxygen flow at a rate of 1.5 litres. Anaesthesia was maintained until the skin was closed. During the anaesthesia period and to be aware of the patient's condition and the depth of anaesthesia, the vital parameters were evaluated every five minutes but they were not recorded as results of the work. Furthermore, ketoprofen (2 mg/kg) (Lemke et al., 2002) and cefazolin (10 mg/kg) were administered intravenously immediately before surgery. Ringer's solution was also administrated during the surgery at a rate of 10 ml/kg/hr.

Five minutes before midline abdominal incision, saline (0.2 ml/kg) (Campagnol et al., 2012) was administered to the muscles and subcutaneous space around the incision in the TS group. Also, in the TL group, lidocaine 2% containing epinephrine (1.5 mg/Kg) was administered with the same route as in the TS group. The final volume of the injections was 0.2 ml/kg (Vicente et al. 2012). Then, ovariohysterectomy was performed by a regular team blinded concering the groups. Before closing the white line, tramadol (4 mg/kg) with the final volume of (0.2 ml/kg) (Campagnol et al., 2012) was splashed on the viscera of the abdominal area in both groups. After surgery, cefazolin (10 mg/kg intramuscularly) was given every 12 hours for 3 days.

The pain was scored and vital signs (respiratory rate, heart rate and rectal temperature) were recorded and at 30 minutes, one, three, six, 12 and 24 hours after extubation. To assess patients' sedation status, a score range of zero (without sedation) to three (deep sedation) was used (Lambertini et al. 2018). The following pain scoring systems were assessed: a modified form of subjective pain assessment system (Sammarco Method) (Sammarco et al., 1996; Groppetti et al., 2011), descriptive pain assessment methods Simple descriptive score (SDS), the University of Melbourne pain scale (Saberi Afshar et al., 2017) and short-form Glasgow Composite Measure Pain Scale (CMPS-SF) (Reid et al., 2007). Dogs with CMPS-SF score more than six out of 24 or five out of 20 (Lambertini et al., 2018), were administered with morphine intramuscularly at a dose of 0.5 mg/kg as a rescue analgesia (Campagnol et al., 2012). If necessary, an analgesic dose was given, and the animal data of the recipient were included in the study results. It should also be noted that to prevent any individual error, scoring was recorded by two investigators who were blind to the treatments. To measure the serum levels of cortisol (ELISA method, Commercial Kit, Monobind Inc, Germany), glucose and total protein (Colorimetric assay kits, Pars Azmun, Iran), blood samples were taken at different time points including before sedative administration, before intraperitoneal administration, one, three and six hours after extubation. The sera were stored at -70 ^{0}C until the day of evaluation.

Statistical analysis

IBM SPSS Version 23 (SPSS Inc.; IL, USA) was used for data analysis. An independent samples t test and Mann-Whitney U test were used to compare the physiologic values and sedation scores between treatments, respectively. A repeated measure analysis of variance test and Wilcoxon signed rank test were used to analyze the physiologic data and sedation scores within each treatment, respectively. Data were presented as mean ± standard error. The level of significance was defined as $p < 0.05$.

RESULTS

The age of all animals was selected in the range of 1-2 years. The weight of the animals, the duration of surgery and anaesthesia recovery did not show a statistically significant difference between the groups (Table 1). There was not a significant difference in the heart rate between the study groups and also in the study times in each group, in the study times after surgery compared to the baseline time (Table 2). Comparing respiratory rates between the studied groups did not show a statistically significant difference (Table 2). The increase in the respiratory rates on each study group was significant in the first hour after surgery compared to the baseline in both groups (TS group, $p = 0.038$ and TL group, $p = 0.041$) (Table 2). The changes in rectal temperature between the study groups and also in the study times on each group was not statistically significant in the study times after surgery compared to the baseline time (Table 2). According to the data, a significant decrease in serum cortisol levels in the tramadol-lidocaine (TL) group compared to the tramadol-saline (TS) group was observed at times 1 ($p = 0.049$), 3 ($p = 0.047$) and 6 ($p = 0.023$) hours after surgery (Table 2). In tramadol-saline (TS) group, there was a significant increase in serum cortisol levels at 5 minutes before peritoneal

administration (p = 0.044) and at times 1 (p = 0.001), 3 (p = 0.010) and 6 (p = 0.024) hours relative to baseline time was observed (Table 2). In the tramadol-lidocaine (TL) group, despite the significant increase in serum cortisol levels, these changes were not significant at any time point compared to baseline (Table 3). There was no statistically significant difference in serum glucose levels in the evaluation and comparison between groups in all studied times (Table 3). In the tramadol-saline (TS) group, an increase in serum glucose levels was observed at all times compared to baseline, which was statistically significant only at 6 hours after surgery compared to baseline (p = 0.005) (Table 3). In the tramadol-lidocaine (TL) group, an increase in serum glucose levels was observed at all times points compared to baseline, which was statistically only at 1 (p = 0.010) and 6 (p = 0.035) hours after surgery compared to baseline (Table 3). Total protein changes were not significant between groups and also at different time points in each group. SDS and Sammarco pain scores were not significant between groups and also at different time points in each group (Table 4). UMPS pain scores in the TL group were significantly lower than the TS group at 30 minute (p=0.043), 1 (p=0.04), 3 (p=0.041) and 6 (p=0.04) hours after surgery (Table 4). Also, CMPS-SF pain scores in the TL group were significantly lower than the TS group at 30 minute (p=0.041), 1 (p=0.043), 3 (p=0.043), 6 (p=0.043) and 12 (p=0.046) hours after surgery (Table 4). Sedation scores did not show a significant difference between groups (Table 4). There was no need for morphine administration at any time points regarding CMPS-SF pain scores.

Table 1. Mean ± SE of Weight, surgery duration and recovery duration in twenty dogs before and after *ip* administration of 4 mg kg^{-1} Tramadol with SC administration of 0.2 ml kg^{-1} Saline (TS) or 1.5 mg kg^{-1} lidocaine with epinephrine (diluted to 0.2 ml kg^{-1}) (TL) undergoing ovariohysterectomy

Groups / Parameter	Weight (kg)	Surgery duration (min)	Recovery duration (min)
TS	19.7 ± 1.4	30.3 ± 2.9	76.6 ± 8.8
TL	19.6 ± 1.1	27.0 ± 2.6	86.7 ± 3.3

Table 2. Vital signs result as mean ± SE in twenty dogs before and after *ip* administration of 4 mg kg^{-1} Tramadol with SC administration of 0.2 ml kg^{-1} Saline (TS) or 1.5 mg kg^{-1} lidocaine with epinephrine (diluted to 0.2 ml kg^{-1}) (TL) undergoing ovariohysterectomy

Parameters		Before surgery (baseline)	After surgery					
	Group/ Times	30 minutes	30 minutes	1 hour	3 hours	6 hours	12 hours	24 hours
Hear rate (rate/min)	TS	113.0 ± 4.7	105.0 ± 7.6	114.3 ± 8.3	112.0 ± 6.1	99.6 ± 8.9	106.6 ± 6.6	104.6 ± 5.6
	TL	115.3 ± 6.3	112.0 ± 7.0	115.2 ± 2.3	104.6 ± 12.4	110.0 ± 6.1	109.0 ± 3.7	97.0 ± 6.2
Respiratory rate (breath/min)	TS	19.3 ± 1.3	22.6 ± 0.6 †	20.6 ± 0.6	21.0 ± 0.5	18.0 ± 1.1	18.0 ± 1.2	20.0 ± 1.2
	TL	18.7 ± 1.7	22.0 ± 1.1 †	21.3 ± 2.6	20.7 ± 1.8	20.0 ± 1.2	19.3 ± 0.7	19.3 ± 1.3
Rectal Temperature (^{0}C)	TS	37.90 ± 0.30	37.20 ± 0.26	36.80 ± 0.51	36.1 ± 0.54	37.36 ± 0.17	37.93 ± 0.31	37.83 ± 0.29
	TL	38.44 ± 0.55	36.46 ± 0.12	37.10 ± 0.36	37.2 ± 0.35	37.00 ± 0.30	38.23 ± 0.20	38.10 ± 0.17

† indicates a significant difference compared to baseline (P <0.05).

Table 3. Blood parameters result as mean ± SE in twenty dogs before and after *ip* administration of 4 mg kg^{-1} Tramadol with SC administration of 0.2 ml kg^{-1} Saline (TS) or 1.5 mg kg^{-1} lidocaine with epinephrine (diluted to 0.2 ml kg^{-1}) (TL) undergoing ovariohysterectomy

Parameters		Before surgery (baseline)	Before ip administration	After surgery		
	Group/Times	30 min	5 min	1 hour	3 hours	6 hours
Cortisol (µg/dl)	TS	8.27 ± 0.95	13.49 ± 0.53 †	12.82 ± 0.87 * †	12.30 ± 0.97 * †	13.54 ± 0.71 * †
	TL	8.67 ± 0.67	12.16 ± 0.44	9.61 ± 0.74	9.37 ± 0.35	10.56 ± 0.41
Glucose (mg/dl)	TS	73.33 ± 3.71	83.00 ± 6.24	94.33 ± 8.83	103.00 ± 9.53 £	90.00 ± 4.04 †
	TL	82.66 ± 6.17	89.66 ± 4.63	95.66 ± 3.75 † £	102.33 ± 5.04	94.33 ± 4.97 †
Total protein (mg/dl)	TS	6.53 ± 0.52	5.70 ± 0.30	5.46 ± 0.38	6.03 ± 0.29	5.96 ±0.26
	TL	6.86 ± 0.47	6.40 ± 0.35	6.53 ± 0.28	6.16 ± 0.08	6.33 ± 0.23

*indicates a significant difference between groups (P <0.05).
† indicates a significant difference compared to baseline (P <0.05).
£ indicates a significant difference compared to the time before ip administration (P <0.05).

Table 4. Sedation and pain Scoring results as median (minimum-maximum) in twenty dogs before and after *ip* administration of 4 mg kg^{-1} Tramadol with SC administration of 0.2 ml kg^{-1} Saline (TS) or 1.5 mg kg^{-1} lidocaine with epinephrine (diluted to 0.2 ml kg^{-1}) (TL) undergoing ovariohysterectomy

Parametrs		After surgery					
	Group/Times	30 min (*)	1 hour	3 hours	6 hours	12 hours	24 hours
Sedation	TS	3 (2-3)	3 (2-3)	2 (1-2)	1 (1-2)	2 (1-3)	2 (1-2)
	TL	1 (1-2)	1 (1-2)	2 (1-2)	1 (1-2)	2 (1-2)	1 (1-2)
SDS	TS	0 (0-1)	0 (0-1)	0 (0-2)	0 (0-2)	0 (0-2)	0 (0-1)
	TL	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)
Sammarco	TS	8 (3-9)	6 (2-9)	5 (2-6)	6 (1-10)	6 (1-11)	6 (1-7)
	TL	3 (0-8)	3 (1-6)	4 (2-6)	6 (1-9)	6 (1-9)	6 (1-9)
UMPS	TS	4 (4-6)	4 (3-6)	4 (4-5)	5 (4-7)	5 (3-7)	3 (2-3)
	TL	0 (0-3) *	1 (0-2) *	2 (1-3) *	2 (1-2) *	2 (2-3)	2 (1-2)
CMPS-SF	TS	5 (3-5)	4 (4-5)	4 (3-4)	4 (3-5)	3 (3-6)	2 (1-3)
	TL	1 (1-2) *	2 (1-2) *	2 (1-2) *	2 (1-2) *	2 (0-2) *	1 (0-2)

* indicates a significant difference between groups (P <0.05).

DISCUSSION

In this study, ovariohysterectomy was studied as a causative agent. (Hardie et al., 1997; Carpenter et al., 2004). On the other hand, in this study, the intraperitoneal method was used to prescribe analgesics. It has evaluated that the effectiveness of intraperitoneal administration and have explicitly stated that the intraperitoneal administration of local anaesthetics is a valuable and approved method for controlling postoperative pain (Chilkoti et al., 2019). In this study, lidocaine was administered concomitantly as a common anaesthetic and tramadol were administered intraperitoneally. Intraperitoneal administration of analgesics in combination with topical anaesthetics causes effective analgesia in humans. Hernandes-Palazon et al., (2003) showed that the addition of morphine to bupivacaine intraperitoneally, causes appropriate pain relief after laparoscopic removal of the gallbladder, while the addition of tramadol to bupivacaine intraperitoneally causes appropriate pain relief after laparoscopic uterine ligator surgery in humans (Memis et al., 2005). In several studies, researchers have reported the local anaesthetic properties of tramadol opioid drug with low side effects (Altunkaya et al., 2003; Altunkaya et al., 2004; Robaux et al., 2004). Intraperitoneal administration is a risk-free, inexpensive, non-invasive procedure that can be easily performed. On the other hand, in the intraperitoneal method, a higher dose of drugs can be used with confidence than in the intravenous method, which may, in turn, increase the duration of the drug's effect (Karsli et al., 2003; Wilson et al., 2004). Comparing cortisol levels between the studied groups revealed that there was no statistically significant difference between the groups

at baseline time and five minutes before the drug was administered, while at all time points after the drug administration changes were significant. Cortisol levels in the TL group were significantly lower than that of in other groups at times 1 ($p = 0.049$), 3 ($p = 0.047$) and 6 ($p = 0.023$) hours after surgery ($P < 0.05$). Tissue damage causes pain and increases cortisol levels by activating the hypothalamic-pituitary-adrenal axis. (Fazio et al., 2015; Nenadović et al., 2017). Cortisol is a common marker of surgical stress that has been reported to increase in various surgical procedures as well as in anaesthesia procedures. Increased cortisol levels during surgery are due to tissue damage, which is more common in abdominal surgery than in surface surgery (Fox et al., 1994; Evangelista et al., 2014; Fazio et al., 2015; Nenadović et al., 2017; Gutiérrez-Bautista et al., 2018). The study carried out by Shutt et al., (2003) showed that plasma cortisol levels increase due to postoperative pain, which is consistent with the findings of the present study. A significant reduction in cortisol levels in the TL group at all time points after drug administration may be a sign of less pain perception, more pain suppression with prescribed medications, and greater patient relaxation in this study. Additionally, measuring and evaluating the comparison of glucose levels among the studied groups did not indicate any statistically significant difference. Pain as a stressor can be associated with increased glucose concentrations, and changes in these concentrations can be a means of determining the effectiveness of analgesics (Martins et al., 2010). Pain increases the amount of glucose by upsetting the balance of the hypothalamic-pituitary-adrenal axis and affecting the adrenal glands, so this parameter can also be used as an auxiliary tool to track pain. Increasing glucose levels also reduces pain tolerance (Morley et al., 1984). On the one hand, increasing glucose levels can, in turn, increase cortisol levels (Cradock and Hawthorn, 2002). However, this study considered glucose to be an interfering factor which might make the results more valuable.

Moreover, the purpose of measuring the amount of the total protein in this study was to assess the status of possible hydration/dehydration as an intervening factor so that, if reduced, call into question the increase in other parameters. In a way, measuring this parameter is a component of the patient's health sign. Measurement of total serum protein showed no statistically significant difference between the studied groups and, as mentioned earlier, indicated the appropriate state of hydration/dehydration and confirmed the appropriateness of other parameters. On the other hand, one of the side effects of pain is an increase in protein catabolism (Mastrocinque and Fantoni, 2003; Morgaz et al., 2014), so lack of significant changes in this parameter indicates relatively stable pain condition. Comparision of the heart rate displayed no statistically significant differences, furthermore, in this study, changes in the respiratory rates did not show any statistically significant differences between groups. Also, changes in anal temperature in this study did not have any statistically significant difference between the groups under study. In the current study, no statistically significant differences were observed between the groups considering the changes in pain scoring with SDS and Sammarco methods. On the other hand, changes in pain scoring with the UMPS and Glasgow methods in this study showed a significant reduction in pain scores in the TL group compared to the TS. Carpenter et al. (2004) used two visual analogue scales (VAS) and CPS to assess pain. They found that VAS was a simple way to assess pain, allowing the supervisor to make treatment decisions while CPS was not. Another Melbourne pain assessment scale (UMPS) was used by Hellyer et al, which is a more objective method because it uses some physiological data, such as heart rate and respiration. Hellyer et al., (2007) claimed that the Melbourne scale was highly effective for clinical use. The Glasgow Pain Scale is a behavioural approach to assessing acute pain. This scale is more accurate than other methods of pain assessment because it is based on the principles of animal behaviour (Reid et al., 2007). For this reason, in several studies, the ultimate criterion for assessing pain and administration or not administration analgesics has been the CMPS-SF pain scale (Gutiérrez-Bautista et al., 2018; Lambertini et al., 2018). No need to rescue analgesic showed that the overall postoperative status of the dogs was considered to be suitable.

CONCLUSION

According to the obtained results and obtaining less pain score (better condition) in TL group than TS group, it seems that intraperitoneal administration of tramadol (4 mg/kg) along with administration of incisional lidocaine with epinephrine (1.5 mg/kg) is recommended following ovariohysterectomy in dogs.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Research article
Ερευνητικό άρθρο

Ameliorative effects of L-carnitine on florfenicol-induced hepatotoxicity in broilers

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ABSTRACT: L-carnitine is a non-essential amino acid derivative naturally occurring and widely distributed in nature. It received a growing interest in its potential uses as a medicinal agent possess protective effects that postulated to be related to its antioxidant action. This study was aimed to evaluate the ameliorative role of L-carnitine on florfenicol induced hepatic toxicity in broilers. A total of 150 broiler chicks were grouped into 6 groups each of 25 chicks. Group one was kept as a control group, while group two and three were treated with florfenicol and L-carnitine, respectively. Group 4 was pre-treated with L-carnitine for three days before florfenicol administration. Groups five and sex were cotreated with L-carnitine and florfenicol and post-treated with L-carnitine for three days after florfenicol administration, respectively. The biochemical analysis, liver indices, antioxidant profile, and histopathological examination were performed to evaluate its ameliorative effects. Results emphasized that florfenicol induced hepatic toxicity in broilers and L-carnitine can ameliorate its action when its usage preceded the florfenicol or when they were used together which reflected by an enhancement in liver indices, antioxidant profile, and histopathological findings. As far as we know this the first study confirming the ameliorative potency of L-carnitine on florfenicol-induced hepatotoxicity.

Keywords: Antioxidant Profile, Florfenicol, Hepatotoxicity, L. carnitine, Liver Indices.

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INTRODUCTION

Antibiotics are used in the poultry industry to improve performance by increased feed conversion, growth rate promotion as well as for the treatment and prevention ofa wide range of avian diseases (Mehdi et al., 2018). According to an estimate, more than 60% of worldwide antibiotic production was intended for livestock production, including poultry (Van Boeckel et al., 2015). Owing to its superior spectrum of activity with greater potency besides its fewer adverse effects than other members of amphenicol group of antibiotics; florfenicol has been increasingly used in livestock for treating bacterial diseases replacing chloramphenicol (Wei et al., 2016). Florfenicol is a fluorinated derivative of chloramphenicol where the hydroxyl group at C-3 site issubstituted with a fluorine atom (sams, 1994). Florfenicol is primarily bacteriostatic functions through binding irreversibly to the 50_sribosomal subunit preventing protein synthesis (Dowling, 2013). However, the uncontrolled usage of florfenicol for the treatment and prevention of infectious diseases in animal husbandry has contributed to many impacts as hepatotoxicity (Amacher1998; Hassaninet al., 2014; Jiaoet al., 2009). Consequently, concerns have been put forward to discover hepatoprotective agents that can counteract these impacts.

L-carnitine (LC) is a non-essential amino acid derivative naturally occurring and widely distributed in nature compound which can be obtained either through endogenous biosynthesis or from exogenous sources (Surai, 2015). L-carnitine received growing interest in its potential uses as a medicinal agent possess protective effects which postulated to be related to its antioxidant action (Hassanin et al., 2014). In poultry industry, L-carnitine was purposed for growth promotion, immune system strengthening and antioxidant actions (Adabi et al., 2011). L-carnitine has protective effects against lipid peroxidation by reducing the formation of hydrogen peroxide and buffering of excess acetyl-CoA, which in itself can cause free radicals formation and potentially toxic to the cells (Agarwaland Said, 2004; Bayraktar et al., 2008). L-carnitine acts by reducing the availability of lipids for peroxidation through transportation of fatty acids into the mitochondria for β-oxidation to produce ATP energy (Nouboukpo et al., 2010). L-carnitine acts as an antioxidant in the protection of glutathione peroxidase, catalase and superoxide dismutase enzymes from further per oxidative damage (Kala Iselvi and Panneerselvam, 1998) and by restoration of the endogenous antioxidant enzymes (SOD, CAT, GSH-Px, GR and GST) and non-enzymatic antioxidants (vitamins E and C) in the liver and other tissues of stressed animals, increased intracellular concentration of GSH in liver and other tissues and decreased lipid and protein oxidation (Surai, 2015). Therefore, this trial aimed to evaluate the potential ameliorative role of L-carnitine on florfenicol-induced hepatotoxicity in broilers before, with, and after florfenicol administration through assessment of liver indices including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin (T.Bil). Furthermore, its antioxidant profile was estimated by the evaluation of superoxide dismutase (SOD), glutathione reductase (GSH), glutathione peroxidase (GPx) and malondialdehyde (MDA) levels. To the best of our knowledge, this is the first study elucidate the hepatoprotective effect of L-carnitine against florfenicol-induced hepatotoxicity.

MATERIALS AND METHODS

Drugs

Florfenicol (Floribiotic10%) ® was obtained from ATCOpharma, Co, Egypt. L-carnitine® was obtained from IDPCO for feed additives, Egypt.

Experimental Birds and protocol

A total of 150 one day old broiler chicks (Erbo plus) were used in this trial. They were accommodated in separated pens in experimental construction rooms on deep litter, maintained under natural environmental conditions (25-30 °C), free access to feed, water and continuous lightening program. Chicks were divided into 6 equal groups, each of 25 chicks; group (1) : kept as a control group, group (2) : chicks in this group were given (60 mg/kg b.wt.) florfenicol orally (Marwa et al., 2013) on day 18 of age once daily for 3 successive days, group (3) : chicks in this group were given (50 mg/kg b.wt.) L-carnitine orally (Baumgartner and Blum, 1997) once daily on day18 of age for 3 successive days, group (4) : chicks in this group were given (50 mg/kg b.wt.) L-carnitine orally on day 15 of age old once daily for 3 successive then were given (60 mg/kg b.wt.) florfenicol orally on day 18 of age old once daily for 3 successive days, group (5) : chicks in this group were (60 mg/kg b.wt.) florfenicol together with (50 mg/kg b.wt.) L-carnitine orally once daily on day 18 of age for 3 successive days, group (6) : chicks in this group were given (60 mg/kg b.wt.) florfenicol orally on day 18 of age once daily for 3 successive days then were given (50 mg/kg b.wt.) L-carnitine orally on day 21 of age once dai-

ly for 3 successive days. All experimental protocols were approved by the Committee on Animal Experiments, Faculty of Veterinary Medicine, Kafrelsheikh University.

Biochemical analysis

After the last day of florfenicol administration (day 20), five birds from each group were randomly selected after five days (day 25), seven days (day 27), and nine days (day 29) of the experiment and blood samples were obtained from their wing vein without anticoagulant and placed in a slant position then centrifuged at 3000 r.p.m. for 20 minutes to obtain serum.

Liver indices

Serum ALT and AST activities were assayed by using commercial kits that were obtained from Diamond Diagnostic Company, Egypt, according to the method of (Murray and Kaplan, 1984). ALP activity was assayed according to (Belfield and Goldberg, 1971) by using commercial kits that were obtained from Vitro Scient, Company, Egypt. The concentration of serum total bilirubin was determined according to the method described by Jendrassik and Grof (1938) by using commercial diagnostic kits that were supplied by cell biolabs, USA.

Preparation of tissue homogenate for antioxidant profile assay

From liver samples, 0.5gm was homogenized in 5ml of distilled water using an electrical homogenizer, centrifuged at 3000r.p.m. for 15 minutes. The supernatant was collected and used for the estimation of SOD, MDA, GPx, and GSH. Tissue homogenates were preserved at -20°C until performing the investigations (Sakeran et al., 2014).

Histopathological examination

The livers from all groups were obtained, kept in 10% formalin and processed in paraffine wax. Sections of five-microns thickness were stained with Hematoxylin and Eosin (H&E) and examined microscopically according to Bancroft and Gamble (2008).

Statistical Analysis

The obtained data were statistically analyzed through one way (ANOVA) using the software statistical program (SPASS, ver.16.00, USA). Data are expressed as the mean± SE, and the results were statistically significant at$P$≤0.05.

RESULTS

Changes in Liver indices

Our results showed that administration of florfenicol in a dose of 60 mg/kg b.wt. orally for 3 successive days caused hepatotoxicity in broilers as indicated by a significant increase ($P \leq 0.05$) in serum AST, ALT, ALP, and total bilirubin levels compared to the control group. This elevation significantly ($P \leq 0.05$) decreased in previously treated with the L-carnitine group and co-treated with L-carnitine group (G4and G5) compared with florfenicol treated group (G2). AST, ALT, ALP, and total bilirubin levels in co-treated florfenicol with L-carnitine groups (G4 and G5) were significantly decreased (P≤0.05) when compared with post-treated florfenicol with L-carnitine group (G6), (Table1). Our results confirmed that there are no statistical differences between the results after five days (day 25), seven days (day 27), and nine days (day 29) of florfenicol administration (data not shown).

Table 1. The effect of L-carnitine (50 mg/kg, oral) on florfenicol (60 mg/kg, oral) induced hepatotoxicity in broilers on day nine after florfenicol administration (day 29)

Parameters	Groups					
	G1	G2	G3	G4	G5	G6
AST (IU/L)	16.94±0.15	29.94±0.11*	17.94±0.11#	19.94 ± 0.29#	20.08 ± 0.29#	27.10± 0.31*
ALT (IU/L)	45.54±0.37	54.98 ± 0.45*	44.9 ± 0.25#	49.34 ± 0.22#	48.32 ± 0.20#	51.76 ± 0.08*
ALP (IU/L)	88.54±0.41	140.12±0.41*	95.79±0.27#	121.30±0.49#	121.84±0.14#	138.96± 0.38*
T. Billirubin	0.81 ± 0.03	1.40 ± 0.00*	0.85±0.01#	0.98 ± 0.00#	0.89 ± 0.01#	1.18 ± 0.01*

AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase and T.Bil: total bilirubin. G1, control group; G2, florfenicol group; G3, L-carnitine group; G4, previously treated with L-carnitine; G5, Co-treated group with L-carnitine; G6, Post treated group with L-carnitine. Data are presented as (mean ± SE) with *significant at ($P \leq 0.05$) as compared with control group, # significant at ($P \leq 0.05$) as compared with florfenicol treated group.

Changes in hepatic SOD, GSH-Rd, GPx and lipid peroxidation levels

A significant ($P \leq 0.05$) increased in the liver MDA of florfenicol treated group (G2) and post-treated group with L-carnitine (G6) when compared with the control and L-carnitine groups (G1 and G3). At the same time, the data declared significant decreased ($P \leq 0.05$) in the liver SOD, GSH-Rd, and GPx levels of the florfenicol treated group (G2) and post-treated with L-carnitine (G6) as compared with the control and L-carnitine (G1 and G3) groups. As well, the importance of treatment with L-carnitine groups (G4 and G5) has been shown significantly decreased in the MDA and a significant increase in the liver SOD, GSH-Rd, and GPx levels when compared to the florfenicol group (G2) and post-treated groups (G6), (Table 2).

Table 2. The effect of L-carnitine (50 mg/kg. oral) on florfenicol (60 mg/kg, oral) induced hepatotoxicity in broilers on day nine after florfenicol administration (day 29)

Parameters	Groups					
	G1	G2	G3	G4	G5	G6
SOD (u/g)	386.53±0.70	176.64±3.92*	378.05±3.09#	324.35±2.76#	326.33±1.46#	199.38±0.62#
GSH (mg/g)	57.77±0.29	45.55±0.23*	57.86±0.32#	51.18±0.25#	51±0.21#	47.97±0.30*
GPx (u/g)	59.14±0.23	19.15±0.28*	58.35±0.23#	35.58±0.11#	33.82±0.21#	20.52±0.19*
MDA (nmol/g)	9.17±0.28	12.26± 0.22*	9.15±0.11#	9.95±0.08#	11.05±0.24#	12.33±0.36*

SOD: Superoxide dismutase, GSH: Reduced glutathione, GPx: glutathione peroxidase and MDA: Malondialdehyde. G1, control group; G2, florfenicol group; G3, L-carnitine group; G4, previously treated with L-carnitine; G5, Co-treated group with L-carnitine; G6, Post treated group with L-carnitine. Data are presented as (mean ± SE) with *significant at ($P \leq 0.05$) as compared with control group, # significant at ($P \leq 0.05$) as compared with florfenicol treated group.

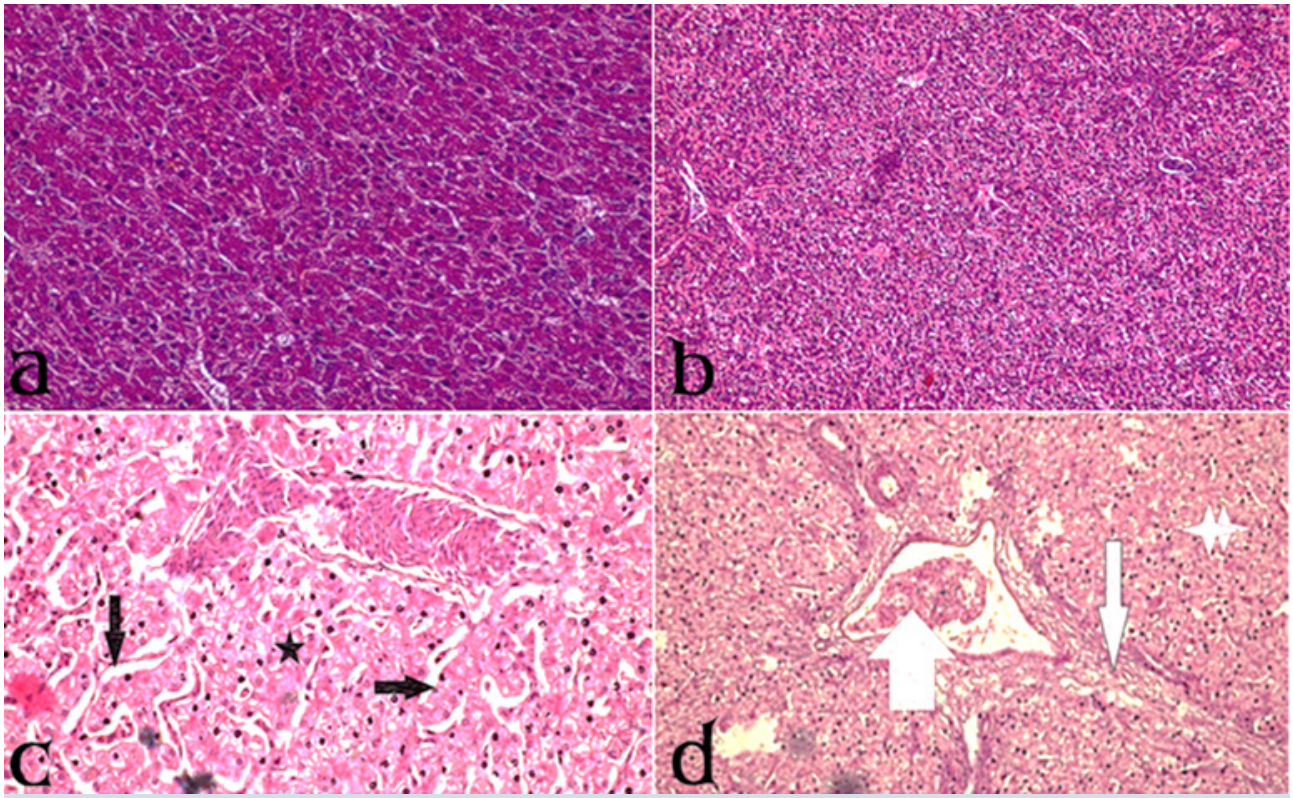

Fig. 1. (a) A liver section of broiler showing normal architecture H&E (X400 (. (b) A liver section from a broiler treated with L-carnitine revealing normal hepatocyte H&E (X200 (. (c) A liver section from a broiler treated with florfenicol showing severe edema in the hepatic sinusoid (black arrows) and hepatocyte showing necrosis (asterisk) H&E (X400). (d) A liver section from a broiler treated with florfenicol revealing periportal fibrosis (thin arrow), portal blood vessel showing dilatation and engorged with blood (thick arrow), and hepatocytes revealing vacuolar degeneration (asterisk) H&E (X400).The histopathological findings were adapted from the liver of the broilers on day nine after florfenicol administration (day 29).

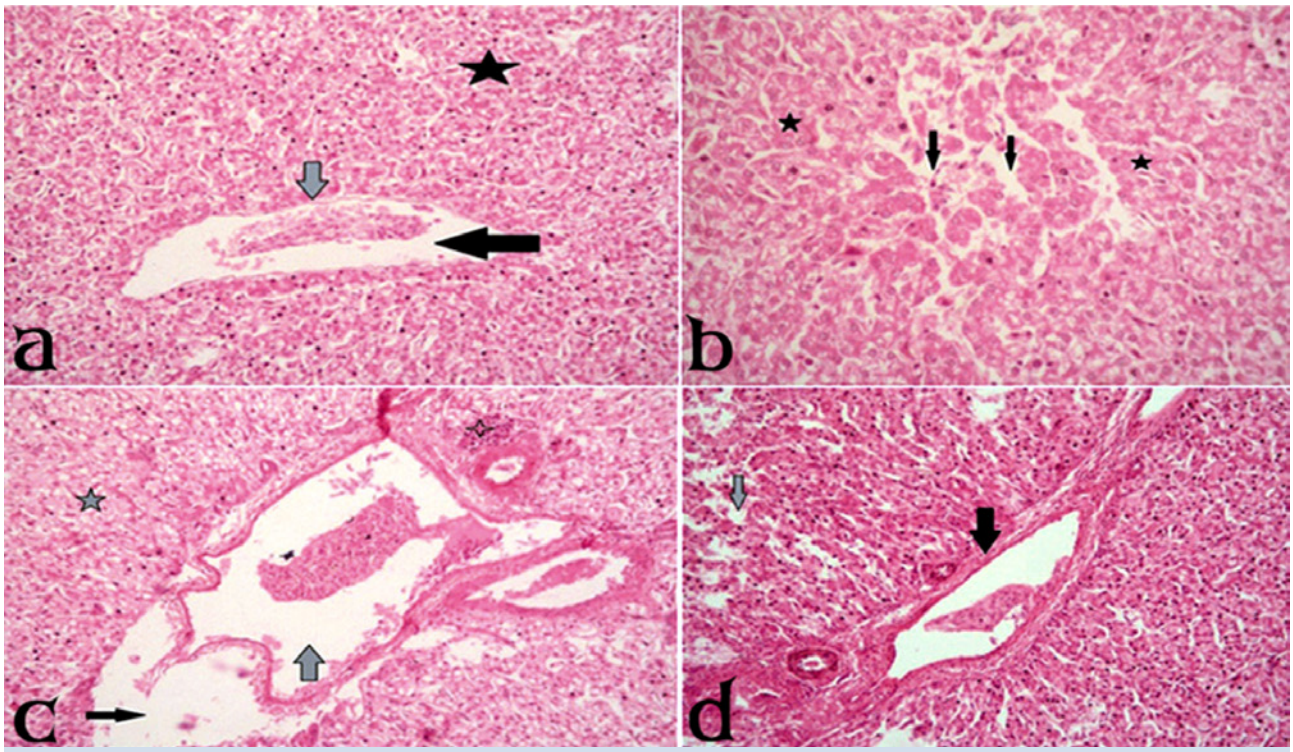

Fig. 2. (a) A liver section from a broiler previously treated with L-carnitine showed mild dilatation of central vein (black arrow), mild pericentral hepatocytic necrosis (grey arrow), and most of hepatocyte revealing vacuolar degeneration (asterisk) H&E (X200). (b) A liver section from a broiler previously treated with L-carnitine showing mild necrosis in the hepatocyte (asterisk) and edema in the hepatic sinusoid (arrows) H&E (X400). (c) A liver section from a broiler co-treated with L-carnitine showed periportal edema accompanied by focal inflammatory cell infiltration (black arrow, black asterisk) with mild necrosis of the hepatocyte (grey asterisk) H&E (X200). (d) A liver section from a broiler post-treated with L-carnitine showed periportal fibrosis (black arrow), dilatation, and edema in the hepatic sinusoid (grey arrow) H&E (X400). The histopathological findings were adapted from the liver of the broilers on day nine after florfenicol administration (day 29).

Histopathological changes

The histopathological changes in the liver of the broilers in the experimental groups are shown in (Fig. 1 and Fig. 2). The broiler liver sections in the control group (G1) and L-carnitine treated group (G3) showed normal hepatocyte and sinusoidal architectures (Fig. 1a, 1b). The liver sections in the florfenicol treated group (G2) showed severe edema in hepatic sinusoid, periportal fibrosis, and hepatocyte showing necrosis and vascular degeneration (Fig. 1c, 1d). The liver sections in previously treated with L-carnitine group (G4) (Fig. 2a, 2b) showed a good degree of improvement in hepatocytes where mild necrosis of hepatocyte and dilatation in hepatic sinusoid. The liver sections in the co-treated group with L-carnitine (G5) (Fig. 2c) showed periportal edema accompanied with focal inflammatory cell infiltration and mild necrosis of the hepatocytes. The liver sections in post treated with L-carnitine group (G6) (Fig. 2d) showed periportal fibrosis, dilatation, and edema in the hepatic sinusoid.

DISCUSSION

Florfenicol is a fluorinated structural analogue of thiamphenicol, is a synthetic broad-spectrum antibacterial drug with a range of activity similar to that of chloramphenicol, commonly used in veterinary medicine (Dowling, 2013). However, high dosages of florfenicol may give rise to hepatotoxicity (Amacher, 1998; Jiao et al., 2009; Hassanin et al., 2014). L-carnitine is a non-essential amino acid derivative widely distributed in nature and had received growing interest in its potential uses as a medicinal agent possess protective effects that postulated to be related to its antioxidant action (Hassanin et al., 2014). This is the first study confirming the hepatoprotective effects of L-carnitine against hepatotoxicity induced by florfenicol.

The previous reports confirmed the complete withdrawal of florfenicol from the serum of broiler after six days in healthy chickens and seven days in infected ones (EL-Banna et al., 2007). To ensure the hepatoprotective effect of L-carnitine after complete withdrawal of florfenicol from the serum of broiler,

we present our results on day nine after florfenicol administration (day 29). In agreement with the previous records, our study revealed that administration of florfenicol induced hepatic damage which was evidenced by increased levels of AST, ALT, ALP, and total bilirubin (Marwa et al., 2013; Er and Dik, 2014). Furthermore, our findings are in agreement with the results of experimental studies of other authors, who reported that elevated levels of these parameters due to their release into the circulation after the cellular damage has occurred as evidence of liver toxicity (Amacher, 1998; Sakeran et al., 2014). The obtained histopathological observations basically supported the results obtained from the serum enzyme assay (Fig. 1c, 1d).

In the current study, supplementation of L-carnitine was found to alleviate the changes in liver indices which induced by florfenicol, where, the group which previously treated with L-carnitine had the more prominent effect (G4) than the co-treated florfenicol with L-carnitine group (G5). The ameliorative effect of L-carnitine was confirmed by the histopathological findings (Fig. 2a, 2b, 2c).Our results were in the same line with some recent studies (Ahmed, 2016; Mousah et al., 2016).The ability of L-carnitine to significantly improve liver function enzymes may be due to its ability to act as a radical scavenger, leading to protection of membrane permeability (prevent the leakage of intracellular enzymes by its membrane stabilizing activity (Augustyniak and Skrzydlewska, 2009).

Oxidative stress is an indicator of the damage that results from a change in the balance between oxidants and antioxidants in favor of oxidants. If the delicate balance between oxidants and antioxidants cannot be maintained in tissues, many pathological changes extending to cellular damage occur (Jahovic et al., 2004). SOD, GPx, and GSH-Rd play a major role in the first line of antioxidant defense (El-Demerdash et al., 2009).SOD prevents the inhibition of glutathione by scavenging superoxide radicals and glutathione in turn prevents the inhibition of SOD by scavenging H_2O_2. MDA is the most important oxidation by-product of lipid breakdown which can show the extent of lipid peroxidation in many organs (Del Rio et al., 2005). In the current study, MDA levels in the florfenicol group were significantly increased, with SOD, GSH-Rd, and GPx levels were significantly decreased when compared with the control group. The current results were similar previously reported by other investigators (Ren et al., 2014; Yuxuan et al., 2019). The ability of florfenicol to produce oxidative stress is by inhibition of antioxidant enzymes or as a result of the generally impaired physiological state of an organism (Wu et al., 2011).Our histopathological result is agreed with Khalil et al., (2012) who stated that such portal fibrosis infiltrated with few lymphocytes, seemed to be due to direct toxic effects of florfenicol.

Our result clearly demonstrated that L-carnitine increased the levels of hepatic SOD, GSH-Rd, and GPx associated with a reduction in MDA levels with no significant difference between previously treated with L-carnitine (G4) and co-treated with L-carnitine (G5). On the other hand, there was a significantly increase in the MDA and significant decreased in the liver SOD and GPx levels in post-treatment with L-carnitine (G6) when compared to co-treatment with L-carnitine groups (G4 and G5).These effects of L-carnitine may result directly from the antioxidant effects against free oxygen radicals (Bayraktar et al., 2008), or from the restoration of the endogenous antioxidant enzymes and non-enzymatic antioxidants in the liver enhanced biosynthesis of enzymatic antioxidants (Surai, 2015).Our results were in the same line with Çekınet al., (2013) who reported that administered of L-carnitine for 4 days prior to ischemia-reperfusion injury in male Westar-Albino rat induced marked protection as detected by a significant elevation in the liver reduced glutathione (GSH) and significant decrease in MDA. Furthermore, the present study agreed with Dokmeci et al., (2006) who recorded that pretreatment with L-carnitine before irradiation, resulted in a marked decrease in MDA values of liver tissue and a significant increase in glutathione (GSH) level in liver tissue. These results indicated that pre-treatment with L-carnitine may potentially protect from florfenicol induced hepatotoxicity, which is also agreed with Abu-El-Zahab et al., (2019) who reported that administration of L-carnitine decreases oxidative stress by reducing lipid peroxidation and increasing GPx and SOD activities in the liver. All these enhancements were confirmed by the histopathological finding where the treatment with L-carnitine showed moderate to good degree of improvement in hepatocytes in (G4 and G5) (Fig. 2a, 2b, 2c). While liver sections in post-treated florfenicol with L-carnitine showed marked disturbance of the hepatocytes, where periportal fibrosis and edema in the hepatic sinusoid were observed (Fig. 2d).

CONCLUSIONS

The current study suggested that oxidative stress

plays a major role in florfenicol induced hepatotoxicity. Administration of L-carnitine preceded the florfenicol usage or when they were used together had a protective effect against florfenicol-induced hepatic damage in broilers more prominent than the post-treatment with L-carnitine. The hepatoprotective effect of L-carnitine is most probably due to its ability to scavenge free radicals and enhance the antioxidant systems. Our study elucidates for the first time the significant importance of admiration of L-carnitine with florfenicol to prevent its undesired hepatotoxic action in broilers.

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CONFLICT OF INTEREST

There is no declared conflict of interest.

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Research article
Ερευνητικό άρθρο

Determination of Nutritional Value of Alfalfa Varieties and Lines by Using the In Vitro Method and Gas Production Technique

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ABSTRACT: This study was organized to determine the nutritional value of different alfalfa varieties and lines selected within the scope of the "Cukurova Region Alfalfa Breeding Project", conducted at the Eastern Mediterranean Agricultural Research Institute, by using the in vitro gas production technique. In the project, Nimet was certified as a new variety. The used alfalfa lines were YSH 26-12, YSH 23-9, YSH 21-1, YSH 27-9, YSH 37-12, YSH 35-11, YSH 28-6, YSH 16-11, YSH 14-3, and YSH 11-4. The certified Nimet variety was used as a control. Alfalfas have been harvested in April, at the beginning of blooming. Chemical composition, metabolic energy (ME), net energy lactation (NEL), and organic matter digestibility (OMD) of the certified Nimet variety with 10 different alfalfa lines used in the experiment were determined by Hohenheim in vitro gas production technique. The incubation times in the Hohenheim gas production technique are 3, 6, 9, 12, 24, 48, 72 and 96th hours. Crude protein (CP), crude cellulose (CC), NDF, and ADF contents of the alfalfa varieties and lines ranged between 19.06-22.40%, 24.90-33.30%, 33.16-45.73%, and 30.77-39.75%, respectively. After 96-hour incubation, the highest total gas production (GP) was found at the YSH 11-4 line (45.32 ml) ($P < 0.05$). While ME, OMD and NEL contents were found to be high at the YSH 11-4 line, ME and OMD values were statistically different from the YSH 28-6, YSH 16-11, YSH 14-3, and YSH 21-1 lines. On the other hand, NEL values were determined different from YSH 23-9, YSH 21-1, YSH 28-6, YSH 16-11, YSH 14-3 lines.

Keywords: Alfalfa, variety, Line, Nutrient chemical composition, In vitro gas production, Digestibility

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INTRODUCTION

One of the most important problems to solve in our country's animal husbandry is the difficulties in finding quality and cheap roughage that the animals need. In order to deal with these problems, it is necessary to use meadows and pastures properly, to reclaim them, and to meet the needs of animals by increasing the production areas of forage crops (Ünalp, 2015).

One of the good-quality roughage sources is alfalfa too. Alfalfa is an important forage crop both in terms of CP and in terms of yield. The harvest of alfalfa 6-7 times a year in Adana conditions and the changing of the nutrient material depending on the harvest time are the issues to be taken into account for those who prepare rations in animal feeding. In addition, the fact that the alfalfa is dry and its fresh grass is higly palatable is a preference reason for animals (Alvaro et al., 2019).

In the Eastern Mediterranean Agricultural Research Institute, studies are being carried out within the scope of the "Cukurova Region Alfalfa Breeding Project". Clonal reproduction of promising alfalfa genotypes is needed at different stages of alfalfa breeding studies. Within the scope of the Alfalfa Breeding Research Project, the appropriate method for clonal reproduction of 16 different alfalfa genotypes selected for the aim of variety development was investigated (Avcı et al., 2017).

The Nimet variety, which was developed at the Research Institute and registered, is a variety that is grown successfully in Mediterranean and Southeastern Anatolia regions, contains 18-20% CP, 32-36% ADF, 40-44% NDF, and whose seed yield is 2-2.5 tons/da (Anonim, 2019).

The YSH 26-12, YSH 23-9, YSH 21-1, YSH 27-9, YSH 37-12, YSH 35-11, YSH 28-6, YSH 16-11, YSH 14-3, YSH 11-4, which were used in the study, are alfalfa lines under development at the Institute.

In this study, using in vitro gas production technique in Adana conditions, it was aimed to identify the nutritional value of different alfalfa varieties and lines (Nimet, YSH 26-12, YSH 23-9, YSH 21-1, YSH 27-9, YSH 37-12, YSH 35-11, YSH 28-6, YSH 16-11, YSH 14-3, YSH 11-4) selected within the context of the "Cukurova Region Alfalfa Breeding Project" carried out in the Eastern Mediterranean Agricultural Research Institute.

MATERIALS AND METHODS

Animal Material

In the present study, rumen liquids and contents obtained from the slaughterhouse were brought in thermoses to the Zootechnical Laboratory of Cukurova University Faculty of Agriculture and used in the *in vitro* gas production system.

Forage Material

Different alfalfa varieties and lines found at the Eastern Mediterranean Agricultural Research Institute was used as forage material. The used forages were analyzed in a way that they were three-repetition.

In Vitro Gas Production Technique and Forage Analyses

100:1 ml (Fortuna, Germany) gas syringes were used in the application of the *in vitro* gas production technique. While the analyses of DM, CP, CF, CC and CA in forage materials used in the experiment were conducted as reported by AOAC (2001), analyses of the ADF and the NDF were conducted as reported by Van Soest and Wine (1967). On the other hand, the values of nitrogen-free extract (NFE) were determined by calculation. All chemical analyses were performed as three-repetition.

In vitro gas production technique was applied in determining the total gas quantities of forages (Menke et al., 1979; Ørskov and McDonald, 1979; Blümmel and Ørskov, 1993). In the experiment, first of all, nutrient material analyses were made in alfalfa varieties and lines in a way that it was 3-repetition. Then, OMD was determined by the in vitro gas production technique. OMD, ME and NEL values of alfalfa varieties and lines were calculated based on 24-hour gas production values, and gas production parameters and pH values were also determined as a result of 96-hour incubation.

Application of the In vitro Gas Production Technique

The alfalfa samples were grounded in a way that they can pass through a 1 mm sieve and the dry forage matter (200 mg DM) was weighed in approximately 250 mg air and placed at the bottom of the injector. Just before the rumen liquid was taken, the medium prepared by mixing 0.1 mL micro-mineral solution, 200 mL rumen buffer solution, 200 mL macro-mineral solution, 1.0 mL resazurin solution and 40 mL

reduction solution into 400 mL pure water was kept in a water bath at 39 ºC under CO_2. The rumen liquid taken from the slaughterhouse was transported to the laboratory in thermoses in a short span of time and the rumen liquids were filtered through two layers of cheesecloth into a 2-liter erlenmeyer flask, which was heated at 39 °C and fed by carbon dioxide. One-part rumen liquid was mixed with two parts medium, and carbon dioxide gas was constantly given into the mixture. 30 mL from the rumen liquid and medium mixture was added to each injector. Readings were performed at the 3, 6, 9, 12, 24, 48, 72, and 96th hours. In addition, after 96 hours of incubation, determination of pH was carried out in the liquid remaining in the injectors.

Gas production parameters were calculated according to the following model with the help of the PC package program called NEWAY.

$$GP=a+b\ (1-e^{-ct})$$

where;
a: amount of gas (ml) consisting of the immediately soluble fraction,
b: the amount of gas formed based on time (ml),
a: gas production speed,
a+b: potential gas production (ml),
t: incubation period (hours),
GP: gas production in "t" time

Organic matter digestibility (OMD, %) was calculated by using gas production quantity (GP) at the 24th hour, CP (g / kg DM), and CA (g / kg DM) values according to the following formula (Menke et al., 1979).

$$OMD, \% = 14.88 + 0.889\ GP + 0.45\ CP + 0.065\ CA$$

In order to determine the NEL (MJ / kg DM) (Menke and Steingass, 1988) and ME (MJ/kg DM) (Close and Menke, 1986) contents of the forages, the following equation was used.

$$NEL= 0.075\ GP + 0.087\ CP + 0.161\ CF + 0.056\ NFE - 2.422$$

$$ME= 1.06 + 0.157\ GP + 0.00884\ CP + 0.022\ CF - 0.0081\ CA$$

where;

GP: Gas production after 24-hour incubation (ml / 200 mg DM), CP: g / kg DM, CF: g / kg DM, CA: g / kg DM, NFE: g / kg DM

Statistical Analysis

To determine the differences between the means for the aim of statistical evaluation of the data obtained from the research, the analysis of variance (General Linear Model) was performed using the SPSS (1999) package program. The Duncan's multiple comparison test was also used to determine the significance levels of the differences.

RESULTS

While nutrient analysis of alfalfa varieties and lines are given in Tables 1 and 2, their average gas productions at different times are presented in Table 3. On the other hand, whereas GP, ME and OMD of the alfalfa varieties and lines are given in Table 4, their effect on gas production is shown in Figure.

Table 1. Crude ash, crude protein of alfalfa varieties and lines

Varieties and lines	CA, DM %	CP, DM %
Nimet Variety	10.055	21.935
YSH 26-12 line	10.185	22.395
YSH 23-9 line	9.54	21.40
YSH 21-1 line	9.14	19.06
YSH 27-9 line	9.36	20.09
YSH 37-12 line	9.00	20.00
YSH 35-11 line	9.96	20.00
YSH 28-6 line	9.82	20.33
YSH 16-11 line	9.59	19.75
YSH 14-3 line	8.90	18.89
11-4 line	10.26	22.26

CA: Crude ash; CP: crude protein; DM: Dry matter

Table 2. ADF, NDF, Crude cellulose and hemicellulose contents of alfalfa varieties and lines

Varieties and lines	ADF, DM %	NDF, DM %	CC, DM %	HES, DM %
Nimet Variety	31.92	37.32	24.90	5.40
YSH 26-12 line	34.21	37.09	26.82	2.88
YSH 23-9 line	32.85	36.92	27.02	4.07
YSH 21-1 line	39.75	45.73	33.31	5.98
YSH 27-9 line	36.27	40.30	29.82	4.03
YSH 37-12 line	39.00	43.70	31.91	4.70
YSH 35-11 line	32.30	37.30	26.08	5.00
YSH 28-6 line	35.95	38.08	28.52	2.13
YSH 16-11 line	38.51	42.15	31.25	3.64
YSH 14-3 line	38.84	42.63	32.50	3.79
11-4 line	30.77	33.16	23.90	2.39

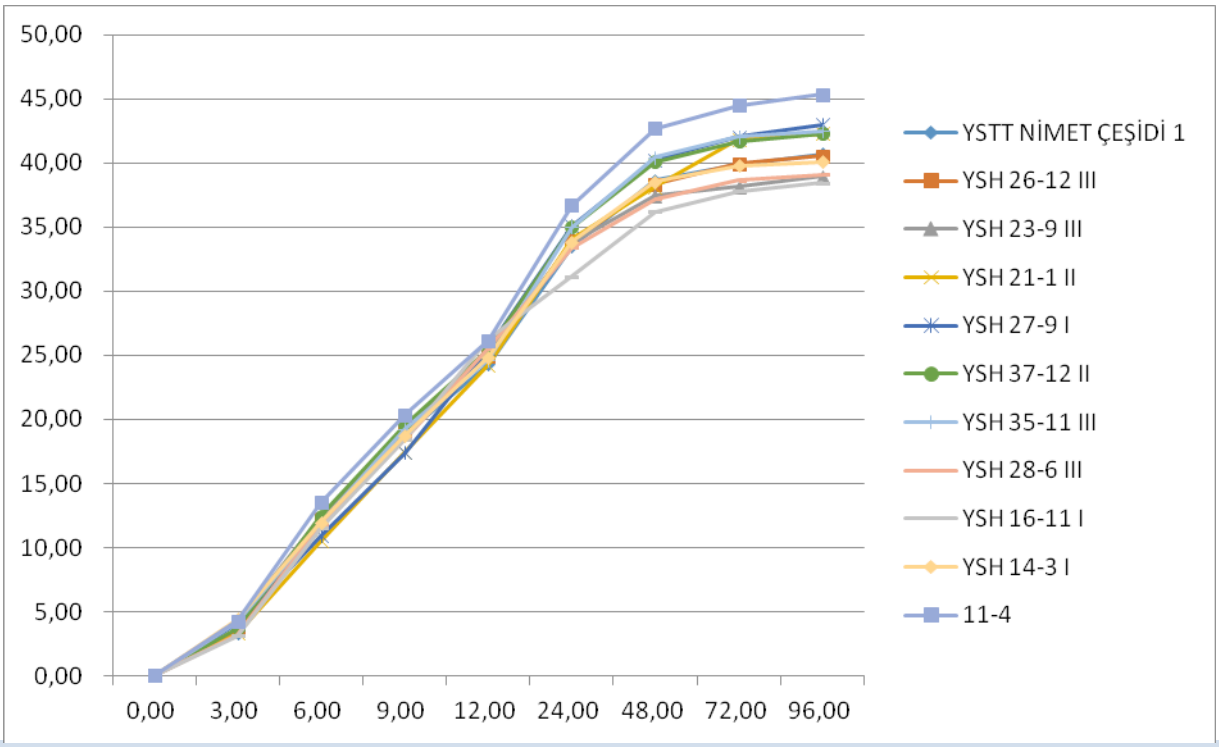

Figure 1. Effect of alfalfa varieties and lines on gas production (ml)

Table 3. Average gas production of alfalfa varieties and lines at different times

Varieties and lines	0.00	3.00	6.00	9.00	12.00	24.00	48.00	72.00	96.00
Nimet Variety	0.00	3.37	11.86	18.87	24.31	33.52	38.64^{bc}	39.90^{bc}	40.71^{bc}
YSH 26-12 line	0.00	3.63	12.25	19.07	24.90	33.88	38.34^{bc}	39.92^{bc}	40.54^{bc}
YSH 23-9 line	0.00	4.22	12.43	19.18	25.26	33.82	37.46^{bc}	38.17^{bc}	38.97^{bc}
YSH 21-1 line	0.00	3.34	10.50	17.45	24.26	34.09	38.13^{bc}	41.84^{abc}	42.28^{abc}
YSH 27-9 line	0.00	4.09	10.93	17.40	25.55	35.11	40.23^{ab}	42.02^{ab}	43.01^{ab}
YSH 37-12 line	0.00	3.70	12.47	19.60	25.52	35.00	40.08^{abc}	41.68^{abc}	42.30^{abc}
YSH 35-11 line	0.00	4.23	11.94	19.25	24.63	35.01	40.44^{ab}	42.04^{ab}	42.49^{abc}
YSH 28-6 line	0.00	3.16	11.74	18.52	25.53	33.40	37.22^{bc}	38.64^{bc}	39.08^{bc}
YSH 16-11 line	0.00	3.14	11.53	18.45	26.32	31.16	36.21^{c}	37.81^{c}	38.43^{c}
YSH 14-3 line	0.00	4.42	11.95	18.74	24.85	33.83	38.56^{bc}	39.81^{bc}	40.08^{bc}
11-4 line	0.00	4.28	13.52	20.37	26.18	36.67	42.71^{a}	44.51^{a}	45.32^{a}
SEM	0.00	0.38	0.85	0.88	0.92	1.04	1.18	1.22	1.25
Significance level	0.00	0.17	0.56	0.50	0.83	0.13	0.04	0.02	0.02

Table 4. Gas production, metabolic energy and organic matter digestibility of alfalfa varieties and lines

Varieties and lines	pH	a	b	c	RSD	OMD	ME, MJ / kg DM	NEL, MJ / kg DM
Nimet Variety	6.850	-7.69^{abc}	47.597	0.09^{abcd}	.693	56.32^{abc}	8.15^{ab}	4.90^{ab*}
YSH 26-12 line	6.833	-7.86^{abc}	47.610	0.09^{abcd}	.773	56.65^{abc}	8.20 ^{ab}	4.86^{ab}
YSH 23-9 line	6.810	-8.07^{abc}	46.493	0.1^{abc}	.820	56.32^{abc}	8.16^{ab}	4.75^{b}
YSH 21-1 line	6.843	-7.42^{ab}	48.667	0.08^{d}	1.490	55.23^{bcd}	8.04^{bc}	4.70^{bc}
YSH 27-9 line	6.833	-7.47^{ab}	49.673	0.08^{cd}	1.433	56.71^{abc}	8.24^{ab}	4.9^{ab}
YSH 37-12 line	6.830	-7.833	49.367	0.09^{abcd}	.790	56.43^{abc}	8.20^{ab}	4.89^{ab}
YSH 35-11 line	6.845	-6.61^{a}	48.607	0.087$_{bcd}$	.593	58.26^{ab}	8.43^{ab}	4.98^{ab}
YSH 28-6 line	6.818	-9.63^{bc}	48.047	0.10^{ab}	1.190	55.26^{bcd}	8.02^{bc}	4.78^{b}
YSH 16-11 line	6.827	-9.91^{d}	47.177	0.11^{a}	1.780	52.95^{d}	7.67^{c}	4.41^{c}
YSH 14-3 line	6.850	-6.84^{a}	46.547	0.09^{abcd}	.717	54.88^{cd}	7.99^{bc}	4.73^{b}
11-4 line	6.848	-6.62^{a}	51.123	0.08^{cd}	.763	59.32^{a}	8.60^{a}	5.14^{a}
SEM	.012	.732	1.311	.006		.925	.142	.106
Sig	.361	.058	.370	.057		.009	.017	.013

*a, b: P<0.05

DISCUSSION

In our study, the CP values of the alfalfa varieties and lines were between 18.89% (YSH-14-3) and 22.40% (YSH-23-9). It has been reported that the Nimet variety contains 90.41 % DM, 19.83% CP, 1.64% CF, 23.47% CC, 48.39% NDF, 37.06% ADF, 11.29% ADL (Acid detergent lignin), 60.04% DDM, and 115.4% relative feed value in its structure (Central Directorate of Seed Registration and Certification, 2016).

Engin and Mut (2017), who investigated the nutrient content of the Nimet variety grown under Yozgat conditions, stated that the Nimet variety contained 23.3-25.1% CP, 41.3-42.2% NDF, and 28.8-29.7% ADF in its structure. Whereas the average CP (21.935%) obtained in our study was consistent with the CP (20.62-23.76%) determined by Töngel and Ayan (2010), it was lower than the CP rate identified as 22.67% by Saruhan and Kuşvuran (2011), and it was higher than the CP rates determined by Lemes et al., (2016) 17.37%; Kır and Soya (2008) (17.86-20.26%), Avcı et al., (2009) (17-18%), and Yılmaz (2011) (17.53%). Gökalp et al., (2017) also stated that the average CP rates of alfalfa varieties varied between 17.51% and 18.00%. The differences between the CP values found in the literature and the CP values found in this study might have been due to the different ecological conditions of the place where the study was conducted, the delay of harvest time, and the differences in total precipitation and temperature during the vegetation.

In their study, in which they described 16 pieces of alfalfa hay produced in Kırıkkale region as good and bad quality, Güngör et al., (2008) found DM, CP, CF, CC, CA, ADF and ADL values of the good-quality alfalfas as 92.87, 20.26, 2.33, 24.71, 8.74, 33.52, and 8.26%, respectively, while they found the same values for the bad-quality alfalfas as 91.79, 12.11, 1.47, 30.62, 10.57, 39.64, and 9.92%, respectively.

In our study, alfalfa varieties and lines were not subjected to crude fat (CF) analysis since the instrument was out of order. While Şeker (2002), Abaş et al., (2005) and Kara and Deniz (2005) found the CF amounts in alfalfa hay as 1.48, 2.34, and 1.75%, respectively, Dale and Batal (2005) reported the same value as 1.7-3.5%. On the other hand, Ünalp (2015) stated that the CF in alfalfa differed according to different reaping times and vegetative periods. He found that the CF content of alfalfas at the beginning of blooming and during the full blooming and pod setting stages varied between 1.39 and 1.52%. Whereas the CA value for alfalfa varieties and lines was the lowest with 8.90% at the YSH 14-3 line, line 11-4 had the highest CA value with 10.26 %. In a research, it was stated that CA value of alfalfa was 8.40-9.52% (Kavut and Avcıoğlu, 2017).

The ADF is an indication of the digestibility of roughages and is determined in forages according to Van Soest (1963). ADF value (31.92%) of the variety in our study was consistent with the ADF values found in alfalfa by various researchers (Kamalak, 2005), (27.36%) ; Basbağ et al., (2009), (16.8%-33.3%)). However, it was also found that ADF value determined in this study was lower than the values found by some researchers (Kır and Soya, 2008), (35.16-36.03%)) and it was also higher than the values found by some

other researchers (Canbolat et al., 2013) (26.60%)). Erbeyi (2017), who reported that the ADF values of different alfalfa varieties vary by year, found that the ADF rates ranged between 21.55%-25.87% and 26.81%-30.12%. In addition, it was expressed that the forage materials examined in terms of ADF were in compliance with the results of alfalfa dried fodders (28-37%) in the internationally accepted reference tables (Perry *et al.,* 2004). Stavarache et al., (2012) stated that the ADF rates in the whole plant differed by the time of fertilization and harvest, and they observed that the ADF rates ranged between 42.3% and 52.8%.

Although NDF contains cellulose, lignin and hemicellulose in its structure, digestion of it is difficult in terms of ruminant animals. Low NDF values in forages are a desired feature for animals and are associated with the increasing of the animal forage consumption. Engin and Mut (2017) reported that the NDF, ADF and HES contents of the Nimet variety received values of 41.3-42.2%, 28.8-29.7%, and 11.6-13.4%, respectively. Avcı et al., (2011) stated that the NDF values (43.6-48.7%) of alfalfa varieties and lines received an average value of 46.7% and found that the lines with the highest NDF values among the lines had a progressive morphological development. The same researchers stated that the average NDF contents of 2 types of alfalfa and 32 selected lines were 45.5% and 47.1%, respectively. Erbeyi (2017) found that NDF rates differed by years and this value ranged between 36.69-40.64% and 38.07-41.87%. The differences observed in terms of NDF among studies in the literature are due to ecological conditions, differences in terms of variety, and applied cultural practices.

In the study, CC values of alfalfa varieties and lines showed statistically significant differences and CC ranged between 24.90 and 33.31%. Özyiğit and Bilgen (2006) determined that CC value in alfalfa varied according to reaping times and it was 18.88% on average. Çerçi et al., (2004) found the CC value in alfalfa to be 23.13%. Ünalp (2015) reported that CC received values between 28.99% and 41.86% according to the harvest times.

The crude ash rates received values between 8.90 and 10.26% in our research. In a research on this subject, it was found that CA value in alfalfa varies according to the harvest, cutting times, but its average was 9.07% (Özyigit and Bilgen, 2006). Ünalp (2015) stated that in different cutting times and vegetation periods, CA content of alfalfa samples was statistically significant during full bloom and pod setting periods and that CA values were between 7.18 and 10.95%.

Hemicellulose values (HES) showed variance between 2.13% and 5.98% in alfalfa varieties and lines. Nikolova et al., (2018) found the HES value in alfalfa to be between 6.41% and 8.52%. The difference between the HES value we obtained in the experiment and the HES values of the researchers in the literature is due to the differences in the application of biological products to alfalfa.

The average gas production of alfalfa varieties and lines at different times is shown in Table 2. In Hohenheim gas production technique, incubation times are 3, 6, 9, 12, 24, 48, 72 and 96th hours. There was no difference between alfalfa varieties and lines in terms of gas production until the 48th hour. After the 48th hour, while the highest gas production was detected on the YSH 11-4 line, the lowest gas production was detected on the YSH 16-11 line with 36.21 ml / 200 mg DM. The 96-hour gas production of the forages ranged between 38.43 and 45.32 ml, and the difference between alfalfa varieties and lines was found to be statistically significant. 96-hour gas production received the highest value (45.32 ml) at the YSH-11-4 line, while it received the lowest value (38.43 ml) at the YSH-16-11 line. The reason that the gas production value of an alfalfa variety or line is low is that the NDF and ADF values of the alfalfa are high.

The pH values, a and b values of alfalfa varieties and lines did not differ statistically. c is the gas production rate of insoluble fractions; while time-dependent gas production of YSH-16-11 (c) was found the highest with 0.11, it was found the lowest (0.08) at YSH-21-1, 11-4, and YSH-27-9 alfalfa lines (Table 3). Aksoy and Yilmaz (2003), who investigated the OMD in some alfalfa varieties, stated that OMD values in the first cutting varied between 39.04-47.67% and OMD values in the second cutting varied between 48.15-54.21%. On the other hand, in our experiment, OMD ranged between 52.95 and 59.32%. In this difference, the variety used and the line types, the cutting time of the alfalfa, and the fact that regions from which alfalfa samples were taken were different played an active role. Çerçi et al., (2004) stated that alfalfa's dry and organic matter digestion values obtained at the end of 24-hour incubation by enzyme technique were 44.19% and 43.27%. Çerçi et al., (2004), who investigated the OMD of forages by nylon bag technique, expressed that OMD of alfalfa at the 48th hour was 61.72% and this value was 60.85% at the 24th hour.

The increase in the amount of gas produced by the 11-4 alfalfa line in the 24th hour together with CP content increased the OMD level (59.32%). The fact that alfalfa lines such as YSH-16-11 and YSH-21-1 were rich in nutrients such as NDF and ADF, which are difficult to dissolve in rumen, suppressed the microbial fermentation and reduced the amount of OMD.

Metabolic energy is a parameter obtained using criteria such as CP, CF, and CA. In our study, the ME value in alfalfa varieties and lines was found as 7.67-8.60 MJ / kg DM. The ME values that we obtained in the experiment were lower than the values of 13.73-19.54 MJ/kg DM determined by Aksoy and Yilmaz (2003) for alfalfa varieties. The harvest time of the alfalfa, analysis method, and regions were effective on this difference. Whereas the NEL value was found highest on the 11-4 alfalfa line, it was found lowest at the YSH-16-11 line. The reason for the low ME and NEL content in YSH-16-11 alfalfa can be explained by the fact that the CP and in vitro gas production amount of these forages are lower than other alfalfa varieties and lines.

CONCLUSION

In this study, determination of nutrient content of different alfalfa varieties and lines and determination of their in vitro GP, OMD, ME, and NEL values were the main objective. In the study, it was seen that the differences between the characteristics of alfalfa varieties and lines in terms of average GP, gas production rate, OMD, ME, and NEL were statistically significant.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Staphylococci and zoonotic potential: oral carriage and antibiotic susceptibility in healthy dogs and cats in Algeria

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ABSTRACT: This study aimed at identifying species of staphylococci isolated from the oral cavity of dogs and cats, in Algeria, and to determine their antibiotic susceptibility.

Oral swabs were collected from 70 healthy animals (35dogs and 35cats) and were grown in Mannitol Salt Agar medium. Isolates were identified using API staph commercial kits and then confirmed with MALDI-TOF MS associated with SARAMIS software. Coagulase-positive staphylococci (CoPS) were tested for antibiotic susceptibility by disk diffusion method.

On the 70 sampled animals, 59 were carriers of staphylococci in the oral cavity and more than one species was detected in 11 of them. Seventy (70) staphyloccoccal isolates were obtained belonging to 10 different species. Coagulase-negative staphylococci (CoNS) were most prevalent (81.42%), of which the dominant species was *S.xylosus* (40%). Other coagulase-negative species, such as *S, simulans (14.28%), S, sciuri (11.42%), S.saprophyticus (10%), S.capitis* (2.85%), *S, cohniisubspcohnii* (1.42%), *and S, epidermidis (1.42%)* were also isolated. The remaining n.13 CoPS included : *S.intermedius* (2.85%), *S.pseudintermedius* (8.57%) and *S. intermedius* group (SIG) (4.28%). No strain of *S. aureus* was found.

Results of antimicrobial resistance showed that 61.53% of CoPS isolates were resistant to at least two drugs. The highest rate of resistance was observed against penicillin, ampicillin and tetracycline (53.84% for each drug), while amoxicillin-clavulanate was active on most isolates.

In Algeria, stray dogs and cats are carriers of many staphylococci species in the oral cavity, including multidrug resistant CoPS, which could be transmitted to humans through bites.

***Keywords*:** Algeria, Antibiotic susceptibility; bites, Dog and cat; Oral cavity; Staphylococci.

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INTRODUCTION

Worldwide dogs and cats are responsible for the most animal bites (Damborg et al. 2016; Morzycki et al. 2019; Owczarczak-Garstecka et al.2019) and for around 1% of all emergency department visits per year (Damborg et al.2016; Goldstein et al.2018; Morzycki et al.2019).The most common complication of animal bites is secondary bacterial infection, requiring medical attention (Muniz et al.2013; Oh et al.2015; Tabaka et al.2015; Rossi at al.2017), which imply high costs to public health (Muniz et al.2013 ; Holzer et al.2019).

It is estimated that about 3-18% of dog bites and 20-80% of cat bites can lead to infections (Tabaka et al. 2015; Damborg et al. 2016), through the skin flora of the victim itself, but above all through the animal's oral flora that bites (Rothe et al.2015; Damborg et al.2016; Bula-Rudas and Olcott2018) ; wounds are usually polymicrobial (Rothe et al.2015; Goldstein et al.2018; Bula-Rudas and Olcott2018) and can contain up to five different bacterial species (Rothe et al.2015; Bula-Rudas and Olcott2018).

The natural oral flora of dogs and cats comprises abundant and complex bacterial species, including opportunistic pathogens (Muniz et al.2013; Oh et al.2015), such as *Staphylococcus* spp. (Muniz et al. 2013; Misic et al.2015; Rossi et al.2017) which may be transmitted to humans through bites (Muniz et al. 2013; Rotheet al.2015; Rossi et al.2017).

Members of the genus *Staphylococcus* are widely spread in the environment (Paharik and Horswill 2016). They are common inhabitants of the skin and nasal mucosa in humans (Misic et al.2015; Paharik and Horswill 2016), while the principal carriage sites are the nares, mouth and perineum in animals, inculding dogs and cats (Misic et al.2015; Iverson et al.2015).

Among staphylococci species, *Staphylococcus aureus*.and other coagulase-positive staphylococci (CoPS) may be opportunistic pathogens (Paul et al.2014; Lozano et al. 2017; Agabou et al.2017). Several reports suggest that CoPS are the most commonly detected aerobic bacteria in infected bite wounds (Abrahamian and Goldstein 2011; Muniz et al. 2013; Katica et al. 2019), accounting for 10-50% of bacterial isolates (Abrahamian and Goldstein, 2011; Katica et al. 2019).

The choice of effective treatment for this microbial infection focuses on the knowledge of staphylococcal species present.in the mouth of dogs and cats (Muniz et al.2013; Rossi et al.2017), as well as their antibiotic susceptibility (Rossi et al.2017).

In Algeria, .the number of stray dogs and cats roaming freely through the streets and urban areas, has been steadily increasing in recent years (Kardjadj and Ben-Mahdi2019) exposing people to the risk of bites..Each year about 120'000 people are exposed to animal bites, 80% of which are bitten by stray dogs (Yahiaoui et al. 2018).Despite this, no study has yet investigated the oral carriage of staphylococci in dogs and cats.

For all these reasons, the aim of this study was to identify staphylococci species found in the oral cavity of stray dogs and cats collected in Algeria, and to determine their susceptibility to antibiotics.

MATERIAL AND METHODS

Study area

The study was conducted in the department of Algiers situated on the central coast of Algeria between 3°2'31.09'' east longitude and 36°45'9'' north latitude. There are 57 districts in this department, covering a total area of 1190km^{2}and a population of over 2.9 million inhabitants. Algiers is bordered from the north by the Mediterranean Sea, from the south by the department of Blida, from the east by the department of Boumerdes, and from the west by the department of Tipaza.

Ethics statement

The protocol was approved by the ethics committee and decision board of Public Industrial and Commercial Company (P.I.C.C.) and Urban Hygiene and Environmental Protection (U.H.E.P.) of Algiers (application no.01/2018). P.I.C.C-U.H.E.P. is an affiliate of the Algerian Ministry of Water Resources and Environment. It controls zoonoses and vector-borne diseases such as rabies and leishmaniosis.

In 1996, an Algerian Rabies Prevention Program was launched (Yahiaoui et al.2018).It is in this context that the P.I.C.C-U.H.E.P.catches stray dogs and cats in the 57 districts of the department of Algiers (Yahiaoui et al.2018; Kardjadj and Ben-Mahdi2019). During the legal period (7 days) before euthanasia, Captured animals are then sheltered in the dog-pound of El-Harrach (Zaidi et al.2018). The geographical position in Algiers of the dog-pound and the animal capturing

radius is shown in Fig.1.

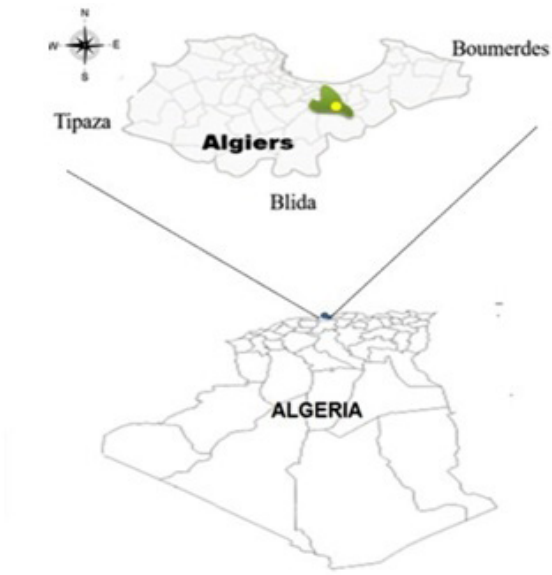

Fig.1 Map of Algeria showing the location of the study area in Algiers

Samples collection

In the period from January 2018 to July 2019, oral swabs were collected from 70 stray animals (35 dogs and 35 cats) which were randomly selected without distinction of age, sex, or breed. All sampled animals were caught by the P.I.C.C-U.H.E.P. from the 57 municipalities of Algiers during the study period, they were apparently healthy and didn't receive any previous antibiotic.

Before swabbing, retaining clip was placed at the animal's neck to immobilize. Subsequently, a dry and sterile cotton swab (without transport medium) was inserted in the oral cavity and it was rubbed over the sublingual area for few seconds to obtain oral fluid.

All swabs were put in the icebox (+4°) and brought back to the microbiology laboratory, within 60 minutes after collection.

Isolation and biochemical identification of staphylococci

Once in the laboratory, each oral swab was immediately put in Brain-Heart Infusion Broth (BHIB) (Oxoïd, Basingstoke, UK) and incubated at 37°c for 24 h befor isolation on Mannitol Salt Agar (MSA) (Oxoïd, Basingstoke, UK). From BHIB 10 µl with a loopful were streaked on MSA and then incubated aerobically for 24 h at 37°C.

After purification, presumptive staphylococci colonies were identified using Gram stain (Gram-positive cocci), catalase test (positive) and API-staph commercial kits (Biomerieux, Marcy Etoile, France).

Each recovered staphylococcal isolate was tested for coagulase activity using rabbit plasma (BioRad) and finally placed in eppendorf tubes containing BHIB and 15% of glycerol and stored at -80°C until use.

Proteomic identification (MALDI-TOF MS)

The API identification was confirmed by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) as described in other studies (Lee et al. 2015; Kaspar et al. 2018).

Frozen isolates were subcultured on Nutrient Agar (Biolife, Milan, Italy) supplemented with 5% sheep blood (Biolife, Milan, Italy) and incubated aerobically for 24 h at 37°C.

From the obtained culture, a loopful (1 µl loop) from a single colony of each isolate was smeared onto the FlexiMass MALDI target plates containing 48-well sample spots (bioMerieux, Firenze, Italy).

The well containing the colony was covered with 1 µlof CHCA solution (alpha-cyano-4-hydroxycinnamic acid dissolved in 500 mL/L acetonitrile, and 25 mL/L tri-fluoracetic acid) (bioMerieux, Firenze, Italy) and air-dried for about 5 minutes at room temperature for the co-crystallization (CHCA solution and microbial material).Each strain was analysed 3 times in distinct and separate runs.

As required by the instrument the *Escherichiacoli* ATCC 8739 (bioMerieux, Firenze, Italy) was used as calibrator and internal ID control and inoculated on distinct and separate calibration spots (G3 and G4 position). This strain was grown on Nutrient Agar (Biolife, Milan, Italy) supplemented with 5% sheep blood (Biolife, Milan, Italy) and incubated aerobically for 24 h at 37°C.

The mass spectra, ranged from 2.000 to 20.000 Da, were evaluated using a VITEK MS Axima Assurance mass spectrometer (Biomerieux, Firenze, Italy) in positive linear mode at a laser frequency of50 Hz with an acceleration voltage of 20 kV with an extraction delay time of 200 ns.

The obtained mass spectra were automatically transferred into the SARAMIS software (Spectral Ar-

chive and Microbial Identification System - Database version V4.12- Software year 2013, bioMerieux, Firenze, Italy) where they were compared to the database hosting the reference spectra and Super Spectra of bacteria, giving a results reported as confidence level, represents specific peaks between the generated spectrum and the database spectra of their similarity in terms of presence or absence. For confidence levels ranging from >60% to 99.8%, the identification was considered a "good ID" because the spectrum was adequately close to that of a reference spectrum, while for a value of <60%, "no identification" (no ID) was given.

Antimicrobial susceptibility testing

CoPS were tested for susceptibility to five antibiotics frequently recommended for bite- wounds treatment. Resistance to Amoxicillin/clavulanate 30µg (Oxoïd, Basingstoke, UK), Penicillin10I.U. (Oxoïd, Basingstoke, UK), Ampicillin 10µg (Oxoïd, Basingstoke, UK), Tetracycline 30µg (Oxoïd, Basingstoke, UK) and Erythromycin 5µg (Oxoïd, Basingstoke, UK) was determined on Muller-Hinton agar (Oxoïd, Basingstoke, UK) using the standard Kirby-Bauer disk diffusion method.

Statistical analysis and Map conception

Pearson's Chi-square test and Fisher exact test (α=5%) were applied to compare staphylococci prevalence, using Microsoft excel 2007.Differences were statistically significant when P value was less than 0.05 ($p \leq 0.05$).

Map of the study area was designed using Microsoft PowerPoint 2007, available in Microsoft Office software.

RESULTS

Of the 70 sampled animals, 59 (84.28%) carried *Staphylococcus* spp. in oral cavity, and among these, 48 (68.57%) carried only one *Staphylococcus* species, while the remaining n. 11 (15.71%) carried two different species (Table 1).

In the 59 positive animals (28 dogs and 31 cats) a total of 70 strains of *Staphylococcus* were isolated (35 in dog and 35 in cat), with a prevalence of CoNS (n. 57 - 81.42%) compared to CoPS, represented by only 13 isolates (18.57%) (Table2).

Among CoNS, *S.xylosus* was the most frequently isolated (45.71% in dog and 34.28% in cat), followed by *S.simulans* in dog (14.28%) and *S.saprophyticus* and *S.sciuri* in cat (17.4% for each specie).

CoPS were represented by *S.pseudintermedius* (8.57%), *S. intermedius* group (SIG) (4.28%) and *S.intermedius* (2.85%).No *S.aureus* strain was isolated.

The isolation rate of *S.intermedius* was similar in dog and cat (2.85%), while *S.pseudintermedius* and SIG were isolated with higher frequency in dog (14.28%) compared to cat (5.71%).

Regarding the results of the antibiotic sensitivity, the majority (61.53%) of isolated CoPS were resistant to at least two drugs, while only five CoPS isolates (38.46%) were susceptible for all tested drugs.Multidrug resistance profiles (to three or more drugs) was observed in 46.15% (n=6/13) of the isolates (Table3).

Almost all strains were susceptible to amoxicillin-clavulanate and erythromycin (92.3% and 84.61% respectively), while a high resistance to penicillin, ampicillin, and tetracycline was found (53.84% for each) (Table 4).

Table 1. Prevalence of staphylococci in the oral cavity of dogs and cats

Characteristics	Total population		Dog		Cat	
	No	%	No	%	No	%
Sampled animals	70	100	35	100	35	100
Animals negative for staphylococci	11	15.71	7	20	4	11.42
Sataphylococci carriers	59	84.28	28	80	31	88.57
1 specie	48	68.57	21	60	27	87.09
2 species	11	15.71	7	25	4	12.9

Table 2. Distribution of staphylococcal species isolated from oral cavities of dogs and cats

Staphylococci	Total population		Dog		Cat	
	No	%	No	%	No	%
No of isolates	70	100	35	100	35	100
CoNS	57	81.42	27	77.14	30	85.71
S, capitis	2	2.85	2	5.71	-	-
S, cohnii subsp cohnii	1	1.42	1	2.85	-	-
S.saprophyticus	7	10	1	2.85	6	17.14
S, epidermidis	1	1.42	-	-	1	2.85
S, simulans	10	14.28	5	14.28	5	14.28
S, sciuri	8	11.42	2	5.71	6	17.14
S, xylosus	28	40	16	45.71	12	34.28
CoPS	13	18.57	8	22.85	5	14.28
S, intermedius	2	2.85	1	2.85	1	2.85
S, pseudointermedius	6	8.57	5	14.28	1	2.85
S, pseudointermedius/delphini/intermedius (SIG)	3	4.28	2	5.71	1	2.85
Not identified	2	2.85	-	-	2	5.71

Table 3. Antibiotic resistance patterns of CoPS isolated oral cavites in dogs and cats

Antibiotic resistance patterns	Total (n=13)	Dog (n=8)	Cat (n=5)
S^{ble} (%)	5 (38.46)	4 (50)	1 (20)
Pen-Amp	1 (7.69)	-	1 (20)
Ery-Tet	1 (7.69)	-	1 (20)
Pen-Amp-Tet	4 (30.76)	2 (25)	2 (40)
Pen-Amp-Tet-AMC	1 (7.69)	1 (12.5)	-
Pen-Amp-Tet-Ery	1 (7.69)	1 (12.5)	-

Pen penicillin, *Amp* ampicillin, *Ery,* erythromycin, *Tet* tetracycline, *AMC* amoxicillin-clavulanate

Table 4. Antibiotic resistance of CoPS isolates among dog sand cats

Staphylococci isolates	Total (n=13)		Isolates from dogs (n=8)		Isolates from cats (n=5)	
Antimicrobial agents	**R (%)**	**S (%)**	**R (%)**	**S (%)**	**R (%)**	**S (%)**
Penicillin	7 (53.84)	6 (46.15)	4 (50)	4 (50)	3 (60)	2 (40)
Ampicillin	7 (53.84)	6 (46.15)	4 (50)	4 (50)	3 (60)	2 (40)
Amoxicillin-Clavulanate	1 (7.69)	12 (92.3)	1 (12.5)	7 (87.5)	-	5 (100)
Erythromycin	2 (15.38)	11 (84.61)	1 (12.5)	7 (87.5)	1 (20)	4 (80)
Tetracycline	7 (53.84)	6 (46.15)	4 (50)	4 (50)	3 (60)	2 (40)

DISCUSSION

Staphylococci are common inhabitants of the skin, nasal and oral mucous membranes in humans and many mammals, including dogs and cats, (Iverson et al. 2015; Paharik and Horswill 2016)

Oral carriage of *Staphylococcus* spp. among healthy dogs and cats was confirmed in this study, due to the high incidence of strains isolated from the mouth of the studied animals (84, 28%).This is consistent with other studies that isolated *Staphylococcu*s spp. from the oral cavity of more than 60% of dogs and cats (Paul et al.2014; Muniz et al.2013; Bean and Wigmore2016; Rossi et al.2017)

Unlike the CoPS commonly associated with clinical infections (Natsis and Cohen2018), CoNS are very common in healthy animals, including dogs and cats. (Muniz et al. 2013; Rossi et al. 2017; Kaspar et al. 2018). It was therefore not surprising, that the rate of CoNS (51.42%) detected in this study was 4 times higher than that of CoPS (18.57%). This result was compatible with that reported by Muniz et al. (2013) showing a high rate of CoNS (89.6%) in healthy cats, compared to CoPS (10.4%).

Likewise, *S.xylosus* is known to be widely spread in the environment and animals (Malik et al.2005), including healthy dogs and cats (Muniz et al.2013; Rossi et al.2017 ; Kaspar et al.2018). As shown in this study, *S.xylosus* was the most commonly found in dogs and cats among CoNS (45.71% and 34.28% respectively). This result is in agreement with Hariharan et al. (2011), who reported a frequency of 33.33% in feral cats, but much higher than that reported by Rossi et al. (2017) in pet cats (5.3%).

Muniz et al. (2013) recorded a higher frequency (50.9 %) of *S.xylosus* in pet cats in Brazil, while Kaspar et al. (2018) found a lower prevalence (23.1 %) in pet dogs.

In addition to *S.xylosus*, dog and cat were found to be carriers of several other CoNS specis: *S.simulans* (14.28%), *S.siuri* (11.42%), *S.saprophyticus* (10%), *S.cohniisubspcohnii* (1.42%) *and S. epidermidis* (1.42%). Statistical analysis showed no significant difference between dog and cat in the distribution of CoNS species. Those staphylococci species were already detected in several previous studies (Hariharanet al. 2011; Muniz et al.2013; Misic et al.2015; Rossi et al.2017), whose frequencies and distributions vary from one report to another, depending on several factors such as: age, diet, environment, geographic location and study methodology (Paul et al.2012).

It is well known that CoNS are not as pathogenic as CoPS (Natsis and Cohen, 2018), but recent studies (Nemeghaireet al.2014; Tous Romero et al.2016; Shields et al.2016; Natsis and Cohen2018) showed that certain species of CoNS such as *S.sciuri* and *S. simulans*, with multiple virulence factors and genes of resistance, can cause serious infections in humans, such asendocarditis, septicemia, urinary tract, soft tissue and osteoarticular infections. Thus, a high proportion of CoNS in dog and cat saliva may be a potential risk to public health.

Among staphylococci species, *S.aureus* is known to be the most significant pathogen (Gharsa et al.2015b; Velázquez-Guadarrama et al.2017), causing a broad variety of infections in humans and animals (Gharsa et al.2015b; Agabou et al.2017).Neither the dog nor the cat harboured *S.aureus* in the mouth in this research. In contrast to our finding, Muniz et al. (2013) and Rossi et al. (2017), recorded very low *S.aureus* prevalence in brazilian pet cats (4.7% and 2.66%, respectively). In a study by Kaspar et al. (2018), *S.aureus* was found in the oral cavity of 4.68% and 5.33% of pet dogs and cats, respectively. According to Misic et al. (2015), *S.aureus* is optional in pets, especially dogs that are colonized by other coagulase-positive staphylococci such as *S.pseudintermedius*. In addition, *S.aureus* is a common inhabitant of the skin and nares in humans, that can be transmitted to pets through close contact (caressing, kissing, licking the skin) with the owners. Thus, *S.aureus* is significantly more abundant in pets compared to stray animals (Bierowiec et al.2016). All of the animals in our sample are stray, have no regular contact with humans, and that may explain our finding without *S.aureus*.

Other than *S.aureus,* some CoPS are also recognized as opportunistic pathogens, such *S.intermedius* group members, which include: *S.intermedius*, *S.pseudintermedius* and *S.delphini* (Gharsa et al.2015a; Kaspar et al.2018). These bacterial species are zoonotic agents, especially *S.pseudintermedius*, whose transmission between dogs and owners has been already documented (Gharsa et al.2015a; Magleby et al.2019). In this study, *S. Pseudintermedius* was detected in the oral cavity of 5/35 (14.28%) dogs, accounting for 62.5% of all CoPS isolates in this animal. This result is in agreement with the literature (Paul et al.2012; Paul et al. 2014; Magleby et al. 2019) stating that *S.pseudintermedius*is the most common species of all coagulase-positive staphylococci in dogs colonizing puppies immediately after birth (Paul et al.2014). The most relevant sites of colonization are the oral cavity and perineum, with a carriage rate between 23% and 92% (Paul et al.2012; Paul et al.2014; Kaspar et al.2018). Several authors (Muniz etal.2013; Rossi et al. 2017; Kaspar et al.2018) did not find any *S. pseudintermedius* in pet cats. In our research, only one cat was found to bear this bacterium in the oral cavity. According to Gandolfi-Decristophoris et al. (2013), *S.pseudintermedius* is more common in healthy dogs than in healthy cats. Thus, dogs are considered to be the primary source of *S.intermedius* for humans.

In addition, *S.pseudintermedius* is a zoonotic agent, that is frequently transmitted to humans by canine bites or by daily dog contact (Paulet al. 2014; Gharsa et al. 2015a; Lozano et al. 2017; Magleby et al. 2019), causing local or systemic infections such as bacteremia, brain abscess, endocarditis or pneumonia (Lozano et al. 2017).

Antibiogram results showed that the majority (61.53%) of isolated CoPS were resistant to at least two drugs. Resistance to penicillin, ampicillin and tet-

racycline was the most common (53.84% for each). This finding is in agreement with several studies (Muniz et al.2013; Bean et al.2016; Rossi et al.2017; Kaspar et al.2018) reported that dog and cat staphylococci isolates have become resistant to several antibiotics in recent years, including, ampicillin, penicillin and tetracycline. Although these molecules are the most recommended drugs for the treatment of infected bite wounds (Muniz et al.2013; Bula-Rudas and Olcott2018), the results of this study show that in the case of infections involving staphylococci, the risk of resistant strains is very high, leading to ineffective antibiotic therapy. In contrast, almost all CoPS were sensitive to amoxicillin-clavulanate (92.30%). This finding is in accordance with that reported by Muniz et al. (2013) in Brazil (81.81%). ß-lactamases are the most common resistance mechanism to ß-lactam antibiotics, such as penicillins (Klachbrenner 2017), therefore the use of amoxicillin combined with clavulanic acid which is a beta-lactamase inhibitor is recommended for multidrug-resistant staphylococci (Klachbrenner 2017; Bula-Rudas and Olcott 2018).

Staphylococci carry several plasmid-encoded antibiotic resistance genes (Muniz et al.2013; Rossi et al.2017), which may be transferred horizontally (conjugation) to other bacteria, including zoonotic pathogens (Muniz et al.2013; Velázquez-Guadarrama et al. 2017; Rossi et al. 2017; Kaspar et al. 2018;).

Dogs and cats carrying antibiotic-resistant staphylococci pose therefore a possible danger to humans being in close contact with these animals and their saliva (Gandolfi-Decristophoris et al.2013; Muniz et al.2013; Bierowiec et al.2016; Kaspar et al.2018).

CONCLUSION

This study indicates that there are many species of staphylococci in the oral cavity of stray dogs and cats in Algeria, including multidrug resistant CoPS, which could be a potential risk to public health.

S.aureus which is considered to be the most pathogenic among staphylococci species, was not revealed in this report. However, *S.pseudintermedius*was common in dogs.

Amoxicillin-clavulanate is recommended for the treatment of infected bites due to staphylococci due to the high sensitivity of staphylococci species to this drug. In people with penicillin allergies, high tetracycline resistance discourages the treatment of infected bites.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Research article
Ερευνητικό άρθρο

The comparison between AgB-ELISA and a new method of Nano-ELISA for diagnosis of hydatidosis in sheep

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ABSTRACT: Hydatidosis is one of the most important zoonotic diseases, and it is transmitted via dogs to intermediate hosts such as humans and domestic animals including sheep and cattle. Epidemiological studies and genetic investigations indicate that the sheep strain is the most common species causing hydatid cysts in humans. The prevalence and incidence of this disease are increasing. According to surveys, economic losses due to this parasite in intermediate hosts are significant. In this survey, 25 serum samples were obtained from newborn lambs as negative control and obtained 25 serum samples from infected sheep to hydatidosis as the positive control. Antigen B isolated from hydatid cysts fluid was used for designing ELISA methods. Using Antigen B in ELISA design for hydatidosis diagnosis has attracted researchers in recent years. During this study, an Iranian native B antigen was used to design the specific detection of hydatidosis in sheep using a specific ELISA technique. The first method used the anti-Sheep conjugate (SIGMA, at 1:3000 dilution), and the second method used gold nanoparticles in combination with anti-sheep conjugate. According to the results, sensitivity and specificity in sheep of the AgB-ELISA method were both 92% and for the Nano-ELISA with Gold Nanoparticles 100% and 96%, respectively. Moreover, results indicated that using Antigen B in ELISA design is valuable but specificity and sensitivity will increase significantly, especially in lower titer, when gold nanoparticles with anti-sheep conjugate are used.

***Keywords*:** Nano-ELISA, Antigen B, hydatid cysts.

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INTRODUCTION

Hydatidosis (infection with the larval stage of the *Echinococcus granulosus* parasite) is transmitted via dogs to intermediate hosts such as humans and other domestic animals including sheep and cattle. The parasite develops to hydatid cyst in the body of the intermediate hosts (Bhutani and Kajal, 2018). According to the latest reports of WHO, *Echinococcus granulosus* is endemic in South America, Eastern Europe, Russia, the Middle East and China with a relatively high incidence rate (Brunetti et al., 2010).

Genetically, distinct species of *Echinococcus granulosus* parasites are identified in the globe, of which, two strains are specified for sheep (G1 and G2), two strains for cattle (G3 and G5), a horse species (AG4), a camel strain (G6), a pig strain (G7), two cervid strains (G8 and G10), and G9 strain which was reported in a swine in Poland. However, it should be noted that some of these species require further investigation to determine more details on the hosts, geographic region, and genetic characteristics (Grosso et al., 2012).

Epidemiological studies and genetic investigations indicate that the sheep strain is the most common species causing hydatid cysts in humans. Moreover, G1 strain is common in sheep, goats, cattle, and camels of Iran (Sharafi et al., 2014).

In Iran, the prevalence of hydatidosis in Human is 5% (Mahmoudi et al., 2019) and in domestic animals in different parts of Iran is reported to be between 3.1 to 16.4 percent (Azami et al., 2013). In Ilam province of Iran, the rate of infection was reported to be 23% in sheep. In addition, the infection rate is reported to be between 0.8% and 57.7% in Iran buffaloes (Paykari et al., 2007). Recent reports indicate that there is a severe rate of infection in humans in different parts of Iran and its health complications (Fasihi-Harandi et al., 2012). Infection in the intermediate host depends on several factors including the average number and activity of the parasite eggs (Jawad et al., 2018).

Slaughterhouse studies are known as useful references for evaluating the epidemiological aspects of some diseases, especially parasitic diseases. Considering the presence of unsanitated livestock slaughterhouses in many parts of Iran, as well as the lack of accurate and systematic recording of data on the infected livestock, seroepidemiological investigations seems to be necessary in some cases. Using the ELISA method can be very efficient because of the precision and rapid results it provides. During this study, an Iranian native B antigen was used to design the specific detection of hydatidosis in sheep using a specific ELISA technique.

MATERIALS AND METHODS

Sera

A total of 50 sheep sera samples were investigated in this study. 25 samples from new-born lambs were used as negative, and 25 others were obtained from slaughterhouses as positive to infection with hydatid cysts and were entered the study. All sera were collected from the sheep in the East Azerbaijan province of Iran.

Extraction of antigen B

In order to extract the antigen B from the hydatid cyst fluid, the modified method was implemented (Shirazi et al., 2016). In the first stage, 300 ml of hydatid cysts' fluid were extracted from the sheep, then 100 ml of the obtained hydatid fluid were transferred to a dialysis bag and were placed in a container containing polyethylene glycol (PEG) 4000 for 1 hour. This stage leads to the condensation of AgB in the hydatid cyst fluid. After that, the fluid was filtered using a 0.2 microfilter, and the resulting liquid was centrifuged at 1500g for 30 minutes. The extracted fluid was dialyzed for one night, and after that, the contents of the dialysis container were centrifuged with a refrigerated ultracentrifuge with 30000g in a vacuum condition and at a temperature of 4 ° C for 30 minutes. The resulting precipitate was dissolved in 10 ml of 0.2M phosphate buffer with pH 8 and the solution was saturated with 40% ammonium sulfate. The solution was centrifuged for 30 minutes at 3000g afterward. The supernatant resulting from salting out was placed in a boil-water bath for 15 minutes. The mixture was centrifuged for one hour using the ultracentrifuge, and finally, the supernatant-soluble antigen B was collected. After filtration with Millipore (0.2 microns) and the addition of 2% sodium azide (NaN3), the mixture was stored at a temperature of -70 ° C until next use.

Finally, Bradford protein analyzes method was used to measure the protein content of the prepared solutions. In addition, the solution containing the prepared antigen B was evaluated using SDS-PAGE.

Designing the ELISA method with Antigen B

In order to design this method, the most suitable level of serum dilution and the most desirable con-

centration of antigen B that should be attached to the wells (Coate) should be determined in the first step. To achieve this purpose, different levels of antigens and serum concentrations were tested.

In order to prepare serum dilutions, a robust positive serum and a negative serum were used. Then, dilutions of 1:100, 1:200, and 1:400 were prepared.

Steps for designing ELISA method with antigen B

• Binding of antigens to the wells (Coating)

The used wells in this study were polyester (Nunc, Denmark), and in order to achieve antigen binding, 100 µl of each antigenic concentration was added to each well and stored in the refrigerator for one night in order to complete the binding process of antigen to the wells.

• Sealing the gaps and empty areas of the wells (Blocking)

To this purpose, a neutral protein such as Skim Milk 5% was used. To block the wells containing 100 µl of antigen solution, they were drained and washed three times using PBST, and after drying the wells (by tapping onto a filter paper), 250 µl of blocking buffer was added to the wells and they were placed in a humid condition for 75 minutes in a 37 ° C incubator.

• Transferring serums

Wells were emptied and then, 100 µl of positive and negative serums were transferred into the wells with pre-prepared dilutions after three to four times of washing and drying. Then, they were incubated in a humid environment for 75 minutes at 37 ° C.

• Conjugation of solution

Wells were emptied and 100 µl of the anti-Sheep conjugate (manufactured by SIGMA USA at 1: 3000 dilution) was prepared in wells after five to six times of washing and drying and incubated for 75 minutes in a humid environment at 37 ° C.

• Addition of substrate (Chromogen - Substrate)

Wells were emptied and washed five to six times and were dried. In the next stage, 100 µl of BM Blue POD (Roche Company, Germany) was transferred to the wells and placed in a dark environment for 12 minutes.

• Stop Solution

The stop solution includes sulfuric acid 1M, of which 50 µl is transferred into each well, and then, the wells should be immediately read.

In order to measure the optical absorbance of each well, an ELISA reader with a 450 nm filter was used and the absorbance of all wells was read. The results for positive and negative samples are presented in the results section.

Due to the high sensitivity of the extracted antigen (AgB) in this study, a dilution of 0.5 µg / ml and the serum dilution of 1:400 were used.

Designing the Nano-ELISA method using antigen B

In order to design the method, the most suitable concentration of gold nanoparticles conjugate should be determined initially. The concentration of antigen B is fixed, and 0.5 µg/ml of antigen is bound to the wells.

In order to prepare serum dilutions, a robust positive and a negative serum were used. According to the hypothesis of this study, nanoparticles increase the sensitivity of the test. In this stage, serum diluted of 1:500 was used instead of 1:400 dilution.

Steps for designing Nano-ELISA with antigen B

• Binding of antigens to the wells (Coating)

The wells were polyester made (Nunc, Denmark). 100 µl of antigen B with a concentration of 0.5 µg/ml was added to each well and stored in the refrigerator for completing the antigen binding process.

• Sealing the gaps and empty areas of the wells (Blocking)

A neutral protein such as Skim Milk 5% was used for this purpose. In order to block the wells containing 100 µl of antigen solution, they were emptied and washed three times using PBST, and after drying the wells (by tapping onto the filter paper), 250 µl of blocking buffer was added to the wells and incubated in humidity for 75 minutes at 37 ° C.

• Transferring serums

Wells were emptied and then, 100 µl of positive and negative serums were transferred into the wells with a dilution of 1:500 after three to four times of washing and drying. Then, they were incubated in a humid environment for 75 minutes at 37 ° C.

• Conjugation with gold nanoparticles (Conjugation of Solution)

Preparing gold conjugate

Ready gold nanoparticle colloid (Plasma Chem, Germany) was used in this study. In order to find the

most suitable dilution for conjugate five different concentrations of conjugate were prepared with four different dilutions (1, 1:2, 1:4, 1:8). Five containers were labeled and 1 ml of gold nanoparticles were transferred to them. In the next step, anti-sheep conjugates (SIGMA, USA) were combined with these particles. In order to achieve this step, containers with gold nanoparticles were combined with different amounts of anti-sheep conjugates on the shaker and inside ice for 30 minutes (Fig 1).

Fig 1: vials contain sheep conjugate and gold nanoparticles

In addition, 100 µl of the gold conjugate (Gold nanoparticle combined with anti-sheep conjugate) with dilutions of 1, 1:2, 1:4, and 1:8 were transferred to wells and stored in a humid environment for 75 minutes in a 37 ° C incubator.

• Addition of substrate (Chromogen - Substrate)

Wells were emptied and washed five to six times and were dried. In the next stage, 100 µl of BM Blue POD (Roche Company, Germany) was transferred to the wells and placed in a dark environment for 9 minutes.

• Stop Solution

The stop solution includes sulfuric acid 1M, of which 50 µl is transferred into each well, and then, the wells should be immediately read.

In order to measure the optical absorbance of each well, an ELISA reader with a 450 nm filter was used and the absorbance of all wells was read. Results of implementing this step showed that gold conjugate Number 4 with a dilution of 1:8 was very suitable.

In the next step, all positive and negative serums were prepared with a dilution of 1:500 and tested with the designed Nano-ELISA method with antigen B concentration of 0.5 µg/ml and a concentration of 1:8 for gold conjugate Number 4 (Table 1). Then, the Cut off was calculated.

Table 1: Dilutions of Gold nanoparticles conjugated

No.	1	2	3	4	5
Anti-Sheep (µL)	2	4	6	8	10
DDW (µL)	98	96	94	92	90
GNP (ml)	1	1	1	1	1

RESULTS

AgB was measured by Bradford assay and it concentrations was 0.7 (mg/ml) and SDS-PAGE result shows that bands on 20 and 24 KDa subunits of AgB (Fig 2).

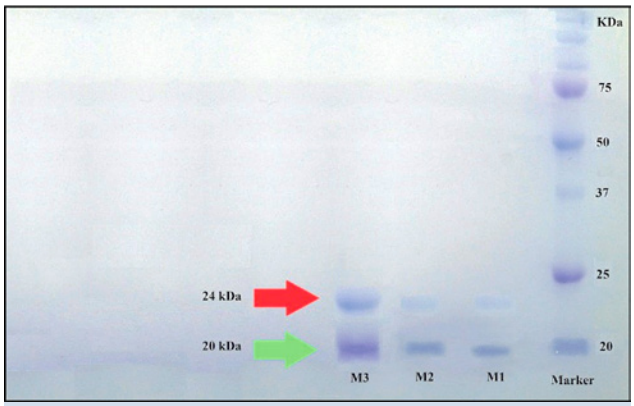

Fig 2: SDS-PAGE bands of Antigen B (M3)

At first, 0.5 µg /ml AgB concentration and also 1:3000 the sheep's conjugate were used. In order to find the best serum dilution, 2 sera of sheep with hydatidiosis and 2 sera without infection (new-born lamb) were used. According to the results of Table 2, the best dilution of serum was determined at a dilution of 1:400.

In the next step, for finding the cut off, definitive positive and negative sheep sera were examined by ELISA method. According to the results, it was equal to one. In other words, the sera with up to one, positive and below one, optical absorption was considered negative. Also according to the results of this study, the sensitivity and specificity of the ELISA designed with AgB for detecting hydatidosis in sheep determined 92%.

Table 2: Result of sheep sera dilatation with AgB (450 nm)

Serum	**Positive Serum**		**Negative Serum**	
Serum Dilution	1	2	1	2
1/100	2.455	2.048	1.383	1.583
1/200	2.201	1.839	1.032	1.185
1/400	1.946	1.457	0.445	0.578

AgB concentration was determined to 0.5 µg / ml for binding to the end of the wells, in AgB-ELISA method. In order to minimize the interfering variables in this study although we didn't want to change the

amount of coated antigen, but it was assumed that gold nanoparticles would increase the optical absorption and increase the quality of detection so serum dilution 1:500, was selected.

According to the results of Table 3, the suitable dilution for gold nanoparticles conjugate was conjugate No.4 with dilution of 1:8 that made the best difference between positive and negative sera optical density (OD).

In the next step, for finding the cut off, definitive positive and negative sera were tested by a nano-ELISA method. Results showed that, cut off was equal to 0.8. In the other words, the sera with up to 0.8, positive and below 0.8, optical absorption was considered as negative. Accordingly, all sera of sheep with positive hydatidosis, and one of the non-infected sheep serum, showed absorbance higher than 0.8.

According to the results the sensitivity and specificity of the gold nano particles ELISA designed with AgB for detecting hydatidosis in sheep determined 100% and 96%, respectively (Table 4).

DISCUSSION

Hydatid cyst is a common zoonotic international disease that is recognized as an important disease in endemic areas, especially in North Africa, South America, China and the Middle East (Mandal, 2011). Recently, the World Health Organization (WHO) recognized *Echinococcus granulosus* in a subgroup of selected Neglected Tropical Diseases (Fasihi-Harandi et al., 2012).

Epidemiologic studies across Iran indicate that the disease has an increasing trend (Fasihi-Harandi et al., 2012). The common serological methods for diagnosing the infection to hydatid cyst are as follows according to sensitivity and specificity: ELISA, indirect immunofluorescence, indirect hemagglutination, Counter immunoelectrophoresis, and Complement fixation test (Weinberg test) (Barnes et al., 2012).

In humans, serologic methods are generally used to confirm radiological findings. Therefore, the ELISA method is an initial screening method to test the serum (Rokni, 2009). Consequently, antigens used to prepare the ELISA kit must be specific, and one of these antigens is antigen B which is recommended by WHO (Shirazi et al., 2016).

Antigen B is used in several hydatidosis seroepidemiological studies in Iran. In 2013, the researchers developed an ELISA kit using antigen B and investigated the contamination with hydatidosis in different provinces of the country. Moreover, hydatidosis has been confirmed with this antigen (AgB-ELISA) in some cities of Iran such as Shiraz and Ark. (Asghari et al., 2013).

Due to the high sensitivity and specificity of AgB-ELISA method and the increasing trend in utilizing this method in seroepidemiological investigations, and regarding the fact that this method is still used only in research facilities, using correct methods for extracting this antigen is very important.

In contrast to humans, a few types of research have been conducted to improve the immunological methods for diagnosing hydatidosis in domestic animals such as sheep and cattle. The diagnosis of hydatidosis in natural hosts is basically done during autopsy (Beard, 1973). The accurate serologic diagnosis of nfection in the livestock is difficult due to cross-reactivity

Table 3: Results of different dilutions of gold nanoparticles with 1:500 sera dilution (450 nm)

No	1		2		3		4		5	
	Pos. Serum	Neg. Serum	Pos. Serum	Neg. Serum	Pos. Serum	Neg. Serum	Pos. Serum	Neg. Serum	Pos. Serum	Neg. Serum
Crude	2.745	0.832	3.124	1.103	3.123	1.154	3.789	1.002	3.321	1.003
1/2	2.129	0.612	2.451	0.596	2.703	0.879	3.310	0.701	3.001	1.234
1/4	1.746	0.451	1.989	0.435	2.012	0.568	2.981	0.671	2.987	0.820
1/8	0.814	0.320	0.879	0.376	1.412	0.381	2.209	0.399	3.110	0.509

Table 4: The comparison between AgB-ELISA and Nano-ELISA in sheep sera

		ELISA		Nano-ELISA	
Case	Serum	No. of Positive	No. of Negative	No. of Positive	No. of Negative
Infected	25	23	2	25	0
Non infected	25	2	23	1	24
Total	50	25	25	26	24
Sensitivity		92%		100%	
Specificity		92%		96%	

with other cestodes such as *Taenia hydatigena* and *Taeniaovis*. In addition, animals produce a very weak antibody response in contrast to the high level of specific antibody produced in human. In order to detect the infection in intermediate hosts, using diagnostic methods such as CT scan and radiology requires expensive equipment, which is not available everywhere. However, using serological methods including ELISA is very simple, beneficial, and cost-effective (Rokni, 2009).

Recently, Nano-biotechnology is being used to improve the existing common methods for diagnosis of various diseases (Ambrosi et al., 2010; Cho et al., 2013). However, these studies are limited in parasitology and only a few studies are available on the subject. During a study, gold nanoparticles and antigen B were used to design a Dot-Immuno-gold Staining (Dot-IGS) method, which showed that this method could be a rapid and reliable method. However, it cannot express sensitivity and specificity (Jahani et al., 2014). During another study implemented in 2015, gold nanoparticles and EPC1 recombinant antigen were used to isolate anti-*Echinococcus granulosus* Ig Gantibodies in dogs contaminated with *E. granulosus*. ELISA and Dot-Immuno-gold Filtration Assay (DIGFA) were used in this study, and the results showed that the ELISA method had a higher sensitivity and specificity (Kord Afshari et al., 2015).

It should be noted that the follow-up on sheep showed that there was no specific ELISA kit available for detecting hydatidosis in sheep in Iran. Therefore, the comparison was conducted between the ELISA designed with antigen B and the ELISA design with antigen B along with a conjugate combined with gold nanoparticles. The results showed that Nano-ELISA method was more sensitive and more specific compared to the method without gold nanoparticles. The reason for increased sensitivity and specificity in the Nano-ELISA method in this study is probably the high-level surface to volume ratio in gold nanoparticles, which causes more antibodies to enter the antigen-antibody complex to aid nanoparticles and provide better pigmentation.

CONCLUSIONS

Using an effective and accurate method can be very helpful in seroepidemiological studies. Regarding the fact that there is no commercially ELISA Kit available for sheep, using Nano-ELISA designed with native antigen B can be very effective in evaluating the immune responses of intermediate hosts such as sheep, as well as epidemiological studies according to high sensitivity and specificity. Future studies can make progress in improving diagnosis of hydatidosis in humans using nanoparticles, as well as improving the diagnosis of other diseases.

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CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Effect of the locational relationship between the 1st wave dominant follicle and the corpus luteum on conception rate after embryo transfer: data analysis of 297 embryo transfers from a commercial embryo production program

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ABSTRACT: The ovarian structures identified at the time of ET are important for the selection of recipients. In previous report, the first-wave dominant follicle, located ipsilateral to the corpus luteum on the ovary, was associated with reduced conception rate after artificial insemination. Thus, a similar locational relationship may affect conception rate during embryo transfer. Data from 297 transfers of fresh embryos to virgin heifers were analyzed aiming to check the effect of first-wave dominant follicle location in relation to the corpus luteum on conception rate using a multivariable logistic regression model with six confounders.The location of the first-wave dominant follicle in relation to the corpus luteum location had no significant effect on conception rate, suggesting that it is not necessary to consider the first-wave dominant follicle size and location in the ovaries for recipient selection.

***Keywords*:** corpus luteum; embryo transfer; first-wave dominant follicle; ipsilateral; recipient

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INTRODUCTION

Since the 1990s in Japan, the production of Japanese Black (JB; otherwise known as Wagyu) via embryo transfer (ET) in dairy cattle has rapidly increased. Since the demand for meat fromJB is increasing annually in Japan and abroad, JB beef has recently positioned as an important export resource for Japan. Currently, 100, 000 JB embryos are annually being transferred to recipients in Japan (MAFF, 2016). As a result, about 8% of JB calves are produced annually through ET, which also supports the demand for JB calves (Oro, 2019). However, during the past 20 years, the conception rate (CR) of ET in Japan has remained stagnant at 51% for fresh embryos and 45% in frozen embryos (MAFF, 2016). Further improvement of CR associated with ET is important to allow a more efficient production management of JB cattle.

Recipient selection is an important factor affecting CR of ET in cattle. The corpus luteum (CL) diameter (Gonella-Diaza et al., 2013) and the presence of the first-wave dominant follicle (W1DF) ≥ 10 mm (Nishigai, 2003) at the time of transfer affects CR, which indicates that the ovarian state at the time of transfer is an important indicator for recipient selection. Furthermore, our previous study demonstrated that the W1DF located ipsilateral to the CL in the ovary was associated with reduced CR through artificial insemination (AI) in both lactating cows and dairy heifers (Miura et al., 2014), suggesting that the location of the CL in relation to the W1DF affects cattle fertility. Therefore, the location of ovarian structures should be considered during ET recipient selection. According to the recently published report, the location of the CL in relation to the W1DF did not affect the CR after ET (Pugliesi et al., 2019). However, the CRs in ET are affected by several factors, other than the ovarian state of the recipient, such as embryo stage, quality, state (frozen or fresh), etc. (Hasler, 2001; Peixoto et al., 2007). These confounding factors, which can affect CR during ET, were not considered in the aforementioned study (Pugliesi et al., 2019), and this may have masked the effects of the location of the CL in relation to the W1DF on CR.

In the present study, we aimed to investigate whether ovarian state can be considered as an indicator for recipient selection, and the effect of the relationship between the CL and the W1DFlocationon the CR in Holstein-Friesian heifer recipients during JB fresh embryo transfer.

MATERIALS AND METHODS

This study was conducted in accordance with the guidelines for the care and use of laboratory animals at Obihiro University of Agriculture and Veterinary Medicine.

The study was carried out in the area of Tokachi-region, which is located in Hokkaido, in northern Japan and has a subpolar climate. All ET recipients were Holstein-Friesian heifers with an average age of about 14.3±1.9 (average ± standard deviation) months old. These recipients were all kept under equal conditions, with a self-producedgrass silage and management system at the Naitai Plateau public ranch in the Tokachi-region. Donor animals were superovulated with 20 AU of FSH administered twice daily in decreasing doses (5, 5, 3, 3, 2, 2).Recipients were prepared by natural estrus or estrous synchronization using hormone drug treatment prior to transfer. Synchronization treatments for recipients were performed witheither a single intramuscularinjection of PGF2α (0.15mg; Dalmazine; d-cloprostenol, Kyoritsuseiyaku, Tokyo, Japan), oran intravaginal progesterone-releasing device (OVAPRON; Kyoritsuseiyaku, Tokyo, Japan) for 8 days and an intramuscularinjection of estradiol (2 mg; OVAHORMON, estradiol benzoate; ASKA Animal Health, Tokyo, Japan) at progesterone device insertion.The recipients received PGF2α intramuscularinjections two days prior to OVAPRON removal. The end of synchronization treatments was at the time of the last FSH injection.After the end of treatments, heifers were observed for signs of estrus twice per day, for two days; estrus was detected based on clinical signsand rectal palpation.At 6-7 days after the onset of estrus (day 0), one day prior to ET, examination of ovarian structures (CL and W1DF diameter) was performed through trans-rectal ultrasonography (HS-101V; FHK, Tokyo, Japan, with 5MHz linear probe). Clinical findings on the vulva or uterus were also considered during ovarian structures assessment.Heifers with apparent uterine contractions or swollen vulvaat the time of the ultrasoundwere excluded from the study. Embryos were then transferred non-surgicallydeep into the uterus horn ipsilateral to the CL at day 7 or 8 after the onset of estrus using a disposable ET catheter (YT gun; YAMANETECH, Nagano, Japan). Pregnancy diagnosis was performed 53 days following ET using ultrasonography. All embryos in this study were non-surgically collected at day 7 (day 0= estrus) from superovulated JB cows inseminated with frozen JB semen (sex-unsorted). All embryos were evaluated and classified based on the coding system of the Inter-

national Embryo Transfer Society (IETS) for developmental stage and for quality.Among them, the quality code 1 (IETS code 1) embryos were classified into the 'excellent' or 'good' categories, the quality code 2 (IETS code 2) embryos were classified into 'fair' categories.Fresh embryos were encapsulated in a straw and immediately non-surgically transferred to recipients.Data from 297 transfers of JB fresh embryos to virgin Holstein-Friesian heifers, performed between July 2019 and October 2019 by a private company (Zen-noh Embryo Transfer Center), were analyzed. The outcomes were summarized using summary statistics and statistically analyzed by multiple logistic regression model. For analyses with a single variable, the mean valuesof continuous variables between the two groups (W1DF ipsilateral to CL vs W1DF contralateral to CL) were compared using t-test. Comparisons between categorical variables were performed using the chi-square test and the Cochran-Armitage trend tests by using data from analysis of variance and contingency table analyses among groups. The dependent variable in the logistic regression model was the conception status. The independent variables were W1DF ipsilateral to CL (No or Yes), CL diameter (≤ 20mm or > 20mm), W1DF diameter (≤ 10mm or > 10mm), synchronization treatment before transfer (Non-treated or Synchronized), technician code (1, 2, 3, or 4), embryonic quality (Excellent, Good, or Fair) and embryonic stage (Morula, Early blastocyst, or Blastocyst). In the analyses, we categorized CL and W1DF diameter on the basis of the population median. Interactions between the two categorical independent variables were included in this model, but insignificant interactions were removed from the final models ($P \geq 0.05$). Values were considered statistically significant if $P < 0.05$. All statistical analyses were performed using SAS version 9.4 (SAS Institute Japan Ltd., Tokyo, Japan).

RESULTS

In this study, the W1DF was confirmed in 268 of 297 heifers (90.2%). Additionally, W1DF was confirmed in the ovary ipsilateral to CL in 137 (51.1%) and contralateral to CL in 131 (48.9%) out of 268 heifers. As for the size of ovarian structures and recipient age, there were no significant differences between the W1DF ipsilateral to CL group and the W1DF contralateral to CL group; the result of this comparison (t-test) is presented in Table 1.Conceptionrates in the category groups of each confounding variable are shown in Table 2. A total of 211 conceptions (78.7%) were confirmed after ET. As concernembryonic quality, there was a significant tendency for the rate of conception to improve as the embryo quality improved. There were no significant differences regarding the other categorical variables. The relationships between W1DF location in relation to the CL on theovaries and the CRwasanalyzed using multivariable logistic regression model with six confounding variables (Table 3). Our analysis showed that the W1DF location in relation to the CL on ovaries had no significant effect on conception status. A significant association was found between the conception status and embryonic quality.

Table 1. Size of ovarian structures and recipients' age in the heifers having W1DF ipsilateral to CL or W1DF contralateral to CL

	W1DF ipsilateral to CL		
	No (n=131)	**Yes (n=137)**	P
CL diameter (mm)	20.7±2.5* (13.0-30.0)**	20.6±2.2 (16.0-25.0)	0,534
W1DF diameter (mm)	10.7±2.3 (5.0-18.0)	10.6±2.2 (5.0-18.0)	0,547
		DF	
Age (month)	14.4±1.8 (11.5-19.1)	14.3±1.9 (11.5-18.4)	0,601

*: Average ± standard deviation
**: Range
P: t-test

Table 2. Conception status in the category groups of each confounding variable

Variable	Category	N	Conception		*P*	*P*-trend
			n	%		
W1DF ipsilateral to CL	No	131	101	77,1	0,523	-
	Yes	137	110	80,3		
CL diameter	≤20mm	152	118	77,6	0,615	-
	>20mm	116	93	80,2		
W1DF diameter	≤10mm	196	152	77,6	0,436	-
	>10mm	72	59	81,9		
Synchronization treatment before transfer	Non-treated (Natural estrus)	165	132	80,0	0,521	-
	Synchronized	103	79	76,7		
Technician code	1	10	9	90,0	0,276	-
	2	14	13	92,9		
	3	100	74	74,0		
	4	144	115	79,9		
Embryo quality	Excellent	149	125	83,9	0,068	0,031
	Good	77	56	72,7		
	Fair	42	30	71,4		
Embryo stage	Morula	46	37	80,4	0,849	0,589
	Early blastocyst	181	143	79,0		
	Blastocyst	41	31	75,6		

N: number of recipients
n: number of conceptions
P: $\chi 2$
P-trend: cochran-armitage trend test
W1DF: 1st wave dominant follicle
CL: corpus luteum

Table 3. Association between conception status after embryo transfer and the locational relationship between the 1st wave dominant follicle (W1DF) and the corpus luteum (CL) on the ovaries (multiple logistic regression model with six confounding variables)

Variable	Category	EV	SEM	*P*	OR	95% CI
Independent variable						
W1DF ipsilateral to CL	No	-0,065	0,154	0,674	0,878	0.480-1.608
	Yes	Ref				
Confounding variable						
CL diameter	≤20mm	-0,078	0,162	0,630	0,856	0.454-1.613
	>20mm	Ref				
W1DF diameter	≤10mm	-0,109	0,192	0,568	0,804	0.379-1.703
	>10mm	Ref				
Synchronization treatment before transfer	Non-treated (Natural estrus)	0,089	0,164	0,587	1,195	0.629-2.270
	Synchronized	Ref				
Technician code	1	0,448	0,853	0,600	2,266	0.266-19.331
	2	0,732	0,837	0,382	3,011	0.369-24.580
	3	-0,810	0,418	0,053	0,644	0.345-1.204
	4	Ref				
Embryonic quality	Excellent	0,555	0,218	0,011	2,400	1.037-5.558
	Good	-0,234	0,224	0,296	1,091	0.464-2.569
	Fair	Ref				
Embryonic stage	Morula	0,144	0,296	0,626	1,491	0.508-4.377
	Early blastocyst	0,111	0,225	0,623	1,441	0.612-3.391
	Blastocyst	Ref				

EV: estimated value
SEM: standard error of means
P: probability of the reference category in the variable
OR: odds ratio
95% CI: 95% confidence intervals

DISCUSSION

In the present study, the effects of the location of the CL in relation to the W1DF on CR after ET were examined using multivariable logistic regression analysis, which included confounding variables that can affect CR. Miura et al (2014) reported that the development of the W1DF in the ovary ipsilateral to the CL was associated with reduced CR during AI. However, the results from the present study show that the W1DF located ipsilateral to the CL in the ovary did not affect CR after ET. Different results between AI and ET may be related to differences in the environment for early embryo development. Unlike AI, the embryo transferred into uterus is not affected by the oviductal environment. The functions of the oviduct and uterus, which regulate the environment for early embryo development, are controlled by steroid hormones secreted from the ovaries (Chen et al., 2013; Lonergan, 2011). Wijayagunawardane et al (1998) reported that the highest estradiol or progesterone concentration was observed in the oviduct ipsilateral to the pre-ovulatory dominant follicleor to the functional CL, respectively. These resultssuggested that the oviduct environment differs depending on the location of ovarian structures such as W1DF.Therefore, the oviductal environment affected by the W1DF may have an effect on the CR during AI. The present results show that the locational relationships between the W1DF and the CL seems to have no effect on the uterine horns' environment.

It should be noted that only high-quality fresh embryos (characterized as fair, good or excellent) were transferred in this study. After AI, the quality of embryos that reach the uterus varies depending on the quality of the ovum, the state of fertilization, and the environment of the oviduct, which may affect embryo quality.Therefore, in the previous study (Miura et al., 2014) differences in the uterine environment related with differences in the location of ovarian structures may have affected low-quality embryos produced after AI and, consequently, CR.Since, only high-quality embryos are transferred in this study, the effect of the differences in uterine environment on conception rate may be have been masked.In order to clarify this, it may be necessary to include the results of ET using low-grade embryos, such as embryos graded as IETS code-3, in future studies. In addition, this study has shown that the differences in the size of W1DF and CL have no effect on CR. Gonella-Diaza et al. (2013) concluded that CL diameter is an important factor affecting pregnancy ratesafter transfer of in vitro produced embryos (CR 31.4%, after 17, 521 ETs). In our study, CR was very high (78.7%) because we used in vivo produced embryos and this differenceof embryo produced process might account for the conflicting findings. Nishigai (2003) reported that a tendency towards decrease in the CR was observed in recipients having poorly developed CL, high ratio of estradiol-17β toprogesterone in blood plasma, and the coexistence of a follicle ≧10mm in diameter with a CL; however, this decrease was not observed in the cases of good CL development. The present study suggests that the W1DF size does not affect CR under the presence of a functional CL. Our results supported the results of Pugliesi et al (2019) who reported that locational relationship of W1DF and CL has no effects on conception rate. However, there are some differences between their study and ours, recipient's parity, breed or embryo type (in vitro vs in vivo). In the future, we could include more cases, and confounding factors such as recipient's parity, recipient's breed and embryo type to make the analysis model more realistic.

CONCLUSION

In summary, our results showed that the CL and the W1DF diameter and the locational relationship between W1DF and CL on the ovaries did not affect CR after the transfer of fresh high quality JB embryos to Holstein heifers. This suggests that if the recipient heifer has a functional CL, it is not necessary to consider the size and location of the W1DF on the ovaries during recipient selection for the transfer of fresh embryos. This study provides useful information in increasing the efficiency of recipient selection during ET.

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CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

ABSTRACT: The current research aims to explore the effects of body condition score (BCS) reduction on some reproductive factors such as pregnancy rate, number of artificial inseminations (AI) per pregnancy, clinical endometritis, and ovarian activity in 1613 lactating Holstein cows, and also the effects of the number of calving and its difficulty, on changes in the BCS. Cows were divided into 6 groups from 1 (no changes in the BCS) to 6 (1.25 units' changes and more). The reproductive system examination and diagnosis of the pregnancy were conducted using an ultrasound machine. To evaluate the endometritis, the vaginal evaluation was performed after external examining. Grading of vaginal internal mucus was done from 0 to 3 based on the color and amount of pus in secretions. Presence or absence of cyst in ovaries and also ovaries status including static, follicles >1cm, or an active corpus luteum were evaluated. All cows were monitored until 33±3 and 65±5 days of a new pregnancy. Data analysis shows that with a further decrease in BCS the first month after parturition, the percentage of non-pregnant cases and the number of cows with inactive ovaries increases significantly. By decreasing 0.5 units or more in BCS, the rate of endometritis significantly increases. The number of inseminations necessary for creating pregnancy in cows with a reduction of 0.5 units or more in BCS was significantly higher. The odds ratios of developing clinical endometritis, inactive ovaries, and Pregnancy with more than three inseminations in cows with BCS changes> 0.5 were significantly greater**.** BCS changes of cows in calving 3 and 4 were significantly higher than cows in the first calving. Regarding the type of delivery only dystocia has been associated with significant BCS changes ($P<0.05$). According to these results, it can be concluded that a further reduction in BCS was associated with a reduction in reproductive performance.

Keywords: *Body condition score (BCS) ; Reproductive performance; Dairy cows; Endometritis.*

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INTRODUCTION

A favorable reproductive period and faster conception of cows are important goals in the dairy herd industry. One calf is expected to be born from each cow every year to have such a profitable system. In this matter, energy metabolism is a factor affecting the reproductive performance of dairy cows, which is critical in the transition period (Carvalho et al., 2014). The transition period refers to 3 weeks before and 3 weeks after calving, which is a challenging period for dairy cows because it increases milk production and food intake. The lack of optimal coordination between these two processes can lead to the creation of negative energy balance (NEB), mobilization of adipose tissues, and reduction of the body condition score (BCS), and the body weight (Carvalho et al., 2014). The energy metabolism status can be controlled by assessing the BCS of herds since the evaluation of BCS is a simple and available evaluation method for managing cow feeding based on their physiological conditions (Delgado et al., 2004).Therefore, the current research aims to explore the main effects of reducing the BCS on some reproductive factors such as pregnancy rate, number of inseminations per pregnancy, clinical endometritis, and ovarian activity. This research also investigates the effects of factors, such as the number of calving and dystocia, on changes in the BCS.

MATERIALS AND METHODS

This study was performed for about 24 months on 1613 lactating Holstein cows, in a large commercial dairy herd in Fars province, located in the south of Iran. We recorded the data of each cow, including the number of calving, type of calving (normal, with a little help, dystocia), ovarian status (static, presence of follicle, presence of corpus luteum, existence of cyst), pregnancy status, number of inseminations per pregnancy, presence or absence of endometritis, as well as the BCS. Cows had a TMR diet and were fed two times a day with a diet formula adjusted by a nutritionist using NRC 2001 software. The cows were housed in a free-stall barn with sand bedding, and milking was done 3 times a day at intervals of 8 hours. The average milk production in the cows was 45 kg on 30±3 days after calving. Estrus synchronization was established in all cows after clean test on 30±3 days in milk (DIM) by using the Presynch-heatsynch method. Cows that did not pregnant after the first insemination synchronized by using the heatsynch program in the absence of uterine infection. Estrus was detected three times a day by a technician and all animals presenting signs of standing estrus were artificially inseminated by an AI technician. Pregnancy diagnosis was performed 33±3 days after AI via trans rectal ultrasonography (Easyscan, BCF, UK) and it was reconfirmed 65±5 days postAI.

The evaluation of their BCS was performed at two different times (the first time around the calving and the second time on 30±3 days after calving) and each time by two expert technicians, and finally the mean scores were recorded. To assess the BCS, the amounts of subcutaneous fat in the relevant areas of the cows' body were estimated and it was rated 1 to 5 (at intervals of 0.25 units) based on the severity of obesity and thinness of the cows (Radostits et al, 2010) and finally, after calculating the amounts of changes in the BCS, cows were divided into 6 groups, The groups included 1 (unchanged cases), 2 (decrease of 0.25 units), 3 (decrease of 0.5 units), 4 (decrease of 0.75 units), 5 (decrease of 1 unit), and 6 (decrease of 1.25 units and more).

Clean test for examination of the reproductive system of the cows after parturition was done by using an ultrasound machine (Easyscan, BCF, UK) and rectal palpation at 30±3DIM.The vaginal examination was also used to diagnose the postpartum endometritis in the clean test. After inspection for the presence of fresh discharge around the vulva perineum or tail, if the discharge was not visible externally, a vaginal examination was performed. After cleaning the cow's vulva with a dry paper towel, a clean, lubricated, and gloved hand was inserted through the vulva. By palpation of all directions in the vagina, mucous contents of the vagina were withdrawn manually for examination. According to the color and proportion of pus in mucus, the vaginal discharge was scored on a 0inatione (Sheldon et al., 2006; Williams et al., 2005), 0 = normal uterine discharge, 1 = flakes of purulent exudates in the uterine discharge, 2 = >50% of the uterine discharge is made up of purulent exudates and 3 = hemorrhagic uterine discharge mixed with purulent exudates (Sheldon et al., 2006, Williams et al., 2005).

Ultrasonographic scanning was performed using a 5 MHz rectal linear probe to assess ovarian

structures, the diameter of the uterus, echotexture, the thickness of the uterine wall, and intraluminal fluid accumulation in all cows. The ovaries and uterus were clinically examined via rectal palpation and ultrasonographic scanning based on our previous study (Makki, Ahmadi et al. 2017). Cows were divided into 2 groups in terms of ovarian status, including 1: static (where there was no follicle more than 10 mm and active corpus luteum on the ovary, and the animal was in the anestrus status) and 2: cases with follicles over 10 mm, or cases with an active corpus luteum. In terms of the presence or absence of cysts, cows were divided into two general groups.

Statistical analysis

We finally examined the results using SPSS 21. The Chi-square test was also used to evaluate the rate of re-pregnancy and different degrees of endometritis and its relationship with different degrees of changes in BCSs. Furthermore, we used the Chi-square test and then the odds ratio to examine the relationship between factors such as endometritis, ovarian activity, ovarian cysts, and the number of inseminations with changes in the BCS. To this end, the cows were grouped into the groups with endometritis and healthy cases, cows with ovaries without significant structures, and cases with ovaries with follicles larger than one centimeter or active corpus luteum, cows without ovarian cysts and ovarian cysts, and finally cows pregnant with maximum three inseminations, and the cows with a need for more than three inseminations for pregnancy, also all the cows were divided into two groups of changes≤ 0.5 and >0.5 in terms of BCS from delivery to 30±3 days of lactation. Furthermore, we utilized the double logistic regression test to examine the relationship of changes in BCS with the number of calving as well as the calving difficulty. We used the one-way ANOVA and the LSD post hoc test to compare the mean number of DO (days open) and the number of inseminations leading to pregnancy in different groups with reduction of the body scores. Finally, we took the advantage of Cox regression and Kaplan-Meier survival plot to examine the chances of re-pregnancy in different groups of changes in the BCS.

RESULTS

We obtained the following results after evaluating the BCS of the cows at the target times and also examining the cases in them as follows separately. The results of chi-square test in Table 1 show that all cows had either reduced scores or were unchanged, and no cows had increased scores.Based on these results with a further decrease in BCS between delivery and clean test, the percentage of non-pregnant cases in the herd (regardless of the number of inseminations) increases significantly (P <0.05) (Table 1).Table 2 shows that the more the amount of reduction in the BCSs of the livestock at a certain time interval, the more number of inseminations are needed to pregnancy (P <0.05).

Table 1. Relationship between BCS lostand induction of pregnancy regardless of the number of inseminations in the Holstein cows

BCS Chages	Non pregnant	Pregnant	Total
0	1 (1.6%)	61 (98.4%)	62 (100%)
0.25	7 (2.9%)	234 (97.1%)	241 (100%)
0.5	9 (1%)	900 (99%)	909 (100%)
0.75	8 (2.5%)	308 (97.5%)	316 (100%)
1	4 (5.7%)	66 (94.3%)	70 (100%)
1.25	1 (6.7%)	14 (93.3%)	15 (100%)
Total	30 (1.9%)	1583 (98.1%)	1613 (100%)

Table 2. Relationship between BCS lost and number of AI

BCS	AI									
change	1	2	3	4	5	6	7	8	9	10
0	17 (27.4%)	20 (32.3%)	10 (11.2%)	7 (11.3%)	5 (8.1%)	2 (3.2%)	0 (0%)	1 (1.6%)	0 (0%)	0 (0%)
0.25	94 (39%)	59 (24.5%)	27 (21.8%)	30 (12.4%)	14 (5.8%)	9 (3.7%)	2 (0.8%)	6 (2.5%)	0 (0%)	0 (0%)
0.5	259 (28.5%)	241 (26.5%)	198 (20.9%)	101 (11.1%)	52 (5.7%)	31 (3.4%)	17 (1.9%)	5 (0.6%)	4 (0.4%)	1 (0.1%)
0.75	76 (24.1%)	67 (21.2%)	66 (20.9%)	50 (15.8%)	30 (9.5%)	15 (4.7%)	8 (2.5%)	2 (1.4%)	1 (0.3%)	1 (0.3%)
1	13 (18.6%)	18 (25.7%)	11 (15.7%)	11 (15.7%)	8 (11.4%)	3 (4.3%)	3 (4.3%)	1 (6.7%)	2 (2.9%)	0 (0%)
1.25	4 (26.7%)	1 (6.7%)	2 (13.3)	3 (20%)	1 (6.7%)	0 (0%)	2 (13.3)	1 (6.7%)	1 (6.7%)	0 (0%)
Total	463 (28.7%)	406 (25.2%)	314 (19.5%)	202 (12.5%)	110 (6.8%)	60 (3.7%)	32 (2%)	16 (1%)	8 (0.5%)	2 (0.1%)

Table 3 presents different degrees of endometritis in different groups of changes in the BCS. The rate of endometritis significantly increases by increasing negative changes in BCS by 0.5 units or more (P<0.05). The table also indicates that the number of cows with inactive ovaries significantly increases by increasing negative changes in the BCS (*P*<0.05). The number of AI for pregnancy in two groups by a decrease in BCS less than and equal to 0.5 units and the group with a decrease of more than 0.5 units of BCS was also associated with a statistically significant difference (*P*<0.05) so that a further decrease in BCS has been associated with a higher number of AI.

Table 3. Relationship between BCS changes and number of AI, ovarian structure, and endometritis

	BCS change	
	≤0.5	>0.5
AI≤ 3	925 (76.32%)	258 (64.33%)
AI>3	287 (23.67%)	143 (34.87%)
Total	1212 (100%)	401 (100%)
Static ovary	194 (16.1%)	96 (24%)
Cyclic ovary	1011 (83.9%)	304 (76%)
Total	1205 (100%)	400 (100%)
Endometritis	413 (34.07%)	185 (46.13%)
Clear	799 (65.92%)	216 (53.86%)
Total	1212 (100%)	401 (100%)

Table 4 presents the odds ratios of cows for pregnancy with three and less than 3inseminations, clinical endometritis, and inactive ovaries, and ovarian cyst status, up to about 30 days after calving in both groups of BCS change≤ 0.5 and BCS change>0.5 changes. As shown, the odds ratios of developing clinical endometritis and inactive ovaries in cows with BCS changes <0.5 were significantly greater than the other group (P<0.05). In addition, odds ratios of pregnancy with finally 3 AI in cows with changes in BCS≤ 0.5 were significantly different from cows with higher BCS changes (P<0.05).Table 5 compares two factors of the number of calving and dystocia as predisposing factors to reduce BCS and the odds ratio of reducing BCS by more than 0.5 units per cow according to the factors.

The relationship between the number of calving and the rate of decrease in BCSat an interval between calving and clean test

Comparing multiparous cows with the primiparous indicated that there was no significant difference between parity 1 and parity 2, while the BCS lost in parity 3 and 4weresignificantly higher than parity1. (P<0.05) (Table 5).

Table 4. Relationships between changes in BCS>0.5and odds ratio of cows in terms of pregnancy with more than 3 AI, clinical endometritis, inactive ovaries, and ovarian cyst in the cows

Factor	RR (relative risk)	Cl 95% (confidence BCS change interval)	*p*-value
Clear	1.140	1.071 - 1.213	0.000
Endometritis	0.688	0.581 - 0.814	
	Odds ratio=1.657	1.317- 2.084	
Static	0.870	0.798 - 0.948	0.000
Cyclic Ovaries	1.432	1.183 - 1.733	
	Odds ratio=0.608	0.461 - 0.801	
Normal Ovaries	0.955	0.853 - 1.070	0.457
Cystic Ovaries	1.163	0.774 - 1.748	
	Odds ratio=0.821	0.488 - 1.382	

Table 5. Relationship between parity and parturition type on the odds ratio of declining BCS in Holstein cows

		Odds ratio	95% confidence interval	*p*-value
Number of parity	Parity 1	Reference		
	Parity 2	1.172	0.828 - 1.658	0.371
	Parity 3	3.129	2.237 - 4.376	0.000
	Parity 4	10.643	6.351 - 17.833	0.000
parturition type	Normal Parturition	Reference		
	Parturition with help	1.121	0.838 - 1.498	0.442
	Dystocia	5.177	3.439 - 7.793	0.000

The relationship between parturition type and the rate of BCS lostat30±3 DIM

Comparing the changes in BCS in different calving models in the present study, as mentioned in the method section, indicated that changes in BCS in cows that gave birth with some help compared to those that gave birth easily and without dystocia were not significant, and their odds ratios in declining the BCS were similar. However, this change in cows with calving difficulty was significantly different from cows with easy calving in a way that cases with dystocia had greater odds ratios for reducing the BCS. (P<0.05) (Table 5). Table 6 compares the average number of days open in different groups of BCS reduction in the first 30 days after calving, and it is found that the BCS reduction more than 0.75, increased the average days open. Besides, the average number of AI in a group with a decrease in BCS of 1.25 was significantly higher than other groups (*P*<0.05).

Survival plot

As shown in the Fig. 1, the blue and green lines, which represent the cases without change in BCS and the least decrease in BCS, respectively, are at the lowest level of the plot compared to other cases, indicating that they were associated with fewer days open and got pregnant earlier. Medians for survival time of Days Open of different BCS change group were 123.41 (108.23-138.60) days (*n* = 62, 1.6% censored), 125.38 (116.00-134.77) days (*n* = 241, 2.9% censored), 132.35 (127.81-136.88) days (*n* = 909, 1.0% censored), 143.72 (135.51-151.94) days (*n* = 316, 2.5% censored), 146.88 (128.39-165.38) days (*n* = 70, 5.7% censored) and 178.06 (124.79-231.34) days (*n* = 15, 6.7% censored) in 0, 0.25, 0.5, 0.75, 1.0 and 1.25 BCS change groups, respectively.

Table 6. Relationship between days open and different degrees of BCS reduction and comparison with unchanged cases in the Holstein cows

BCS Change	N	Days open Mean	AI Mean
0.00	62	122.61 ± 58.75 [a]	2.58±1.55 [a]
0.25	241	123.76 ± 70.53 [a]	2.48±1.73 [a]
0.50	909	132.05 ± 69.06 [a]	2.62±1.60 [a]
0.75	316	142.56 ± 72.05 [a]	2.97±1.72 [ab]
1.00	70	144.41 ± 75.06 [a]	3.30±2.00 [b]
1.25	15	174.53 ± 100.58 [b]	4.00±2.69 [c]
Total	1613	133.44 ± 70.41	2.71±1.69

a, b, c Values with different superscript letters within a column differ significantly at P<0.05

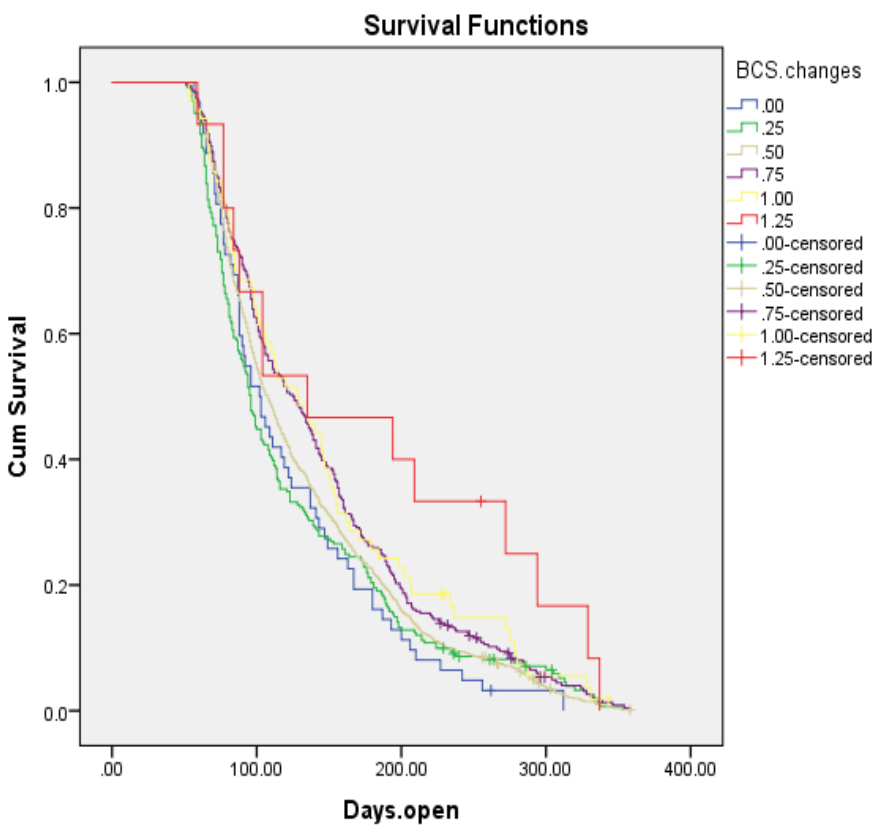

Fig. 1. Kaplan-Meier survival curve for days open of different BCS change group

DISCUSSION

A profitable system for maintaining dairy cows depends mainly on their reproductive performance (Delgado et al., 2004). Numerous factors affect the fertility of cows, including BCS. The effect of BCS has been mentioned in various articles, but the evaluation of BCS has been done at different times, and in general, no specific coherence and effect have been reported in these studies. It is noteworthy that, unlike other studies which focused more on the BCS of each cow, the present study examined the effects of changes in BCS at an interval of a month after calving on the reproductive performance.

BCS changes and fertility

For instance, Delgado et al., 2004found that cow fertility could be affected by the BCS during calving and its changes. Some researchers have found that BCS at calving time does not affect the reproductive performance (Gillund et al., 2001; Buckley et al., 2003; Waltner et al., 1993), while other researchers have reported specific effects of this issue (Roche et al., 2007). According to some researchers, the differences in the above studies may be due to the difference in the number of the samples or the number of times of the BCS evaluation (Delgado et al., 2004). Also, the difference in results of different previous articles may be due to their greater focus on the cow's BCS at a particular time rather than its changes.

Mulliniks et al. (2012) reported that the BCS did not affect the overall pregnancy rate. Ciccioli et al. (2003) also reported that the BCS at calving time did not affect the estrus, ovarian function, or reproductive performance. As we know, the negative energy balance of the early lactation will disappear with the progress in lactation days, and the cows will be in the energy balance status, but the consequences of this imbalance can affect the reproductive performance (McArt et al., 2013; Vanholder et al., 2006), which is difficult to be assessed because other variables can be involved in this issue in addition to nutritional factors. NEB and loss of the BCS are associated with a reduction of progesterone concentrations during the pre-ovulatory period and lower pregnancy rates. In general, a NEB leads to lower fertility in dairy cows. NEB also leads to defects in the secretion of LH and causes negative effects on ovulation because the frequency of LH pulse sis significantly lower during the first postpartum follicle, leading to no ovulation in the dominant follicle (Butler, 2003). On the other hand, the ovary responses to LH stimuli also decrease in such conditions. It should also be emphasized that the NEB is associated with lower insulin and glucose (Butler, 2001). The existence of insulin is important to increase the ovarian follicular response to gonadotropins through the reset of LH receptors. Furthermore, the plasma concentration of IGF1, which is directly related to energy status, is vital for the growth of ovarian follicles (Butler, 2003).Blood insulin levels are lower than normal in cows with a reduction of BCS.

In general, the ability of the follicles to produce enough estradiol for ovulation during NEB generally depends on the availability of insulin and IGF1 in serum and changes in energy balance profiles. Under normal conditions in herds, it is impossible to directly assess the NEB in each cow, but a change in the BCS makes an indirect assessment possible. A further reduction in the amount of BCS will further decrease the pregnancy rate (Butler, 2001).Buckley et al. (2003) reported significant effects of body weight changes from breeding time up to 90 days later on the pregnancy rates at the first insemination. About changes in the BCS, Bulter and Smith (1989) found that cows lost their BCS from 0.5 to 1 unit at the time interval from calving and the first service, gaining 35% pregnancy rate in the first service, while the rate was17% in cows that lost more than one unit of BCS. Results of a study by Buckley et al. (2003) emphasized the importance of the BCS in achieving appropriate reproductive performance. Their results were consistent with the present study because it seems that changes in BCS and its reduction indicate the intensity of the NEB that occurs in the early lactation. Most studies also indicate a negative relationship between changes in the BCS and reproductive performance (Stockdale. 2001).Various studies have found that there is a 17-38% higher risk of lower fertility in cows at a rate of 1 unit or more BCS in a5-point system in the early lactation. It seems that the obvious reduction of the BCS by more than 1.25 units is associated with a reduction of more than 50% in successful conception in the first insemination compared to cows with a lower reduction in the BCS (Gillund et al., 2001).

Gillund et al. (2001) studied the Norwegian dairy cows and claimed that cows with a significant reduction in the BCS in the early lactation got pregnant with a probability of 50% in the first service in comparison with those with a moderate BCS reduction. They also found that the reduction of BCS in the postpartum period was associated with an increase in the

interval between calving and pregnancy. Pryce et al. (2001) found that a one unit increase in BCS at week 10 of lactation was associated with a 6.2-day reduction in the interval between calving to the first service and a 9% greater probability of pregnancy in the first service; and a one unit increase in the BCS during the week 1 to 10 of lactation was associated with a decrease in the interval between calving and the first service by 10.6 days, and a 9% increase in the probability of pregnancy in the first estrus.

Lopez-Gatius et al. (2003) found that the reduction of BCS by 0.5-1 units in the early lactation was not associated with specific effects on the number of days open, but the number of days open increased by 10.6 days in cows with a severe reduction of BCS more than one unit in comparison with cows with lower reduction of the BCS. Drackley (1999) found that the moderate changes in BCS (both decrease and increase) had no specific effects on the number of days open, but a sharp decrease in the postpartum period led to a sharp increase in the number of days open (Drackley, 1999). In a study by Garnsworthy (2006), it was also reported that the loss of 0.5-1 units of BCS was equal to an increase of days open by 3.5 in cows compared to those that lost 0.5 units of BCS. In the present study, as the decrease in body score became higher than 0.75, the number of days open also changed significantly compared to cows without any changes in the BCS, and cows showed a lower probability of getting pregnant than the group without changes.

BCS changes and clinical endometritis

For the impact of the BCS on the occurrence of clinical endometritis, it should be noted that the prevalence of clinical endometritis in cows with lower and equal to 2.5 BCS was higher than cows with higher body scores (Carneiro et al., 2014). Of course, the role of BCS on the occurrence of subclinical endometritis has also been mentioned in several studies (Bacha & Regassa, 2010).Priest et al. (2013) reported that the rates of BCS were lower in cows with endometritis. Dubuc et al. (2010) stated that subclinical endometritis was more seen in cows with BCS of less than 2.75. Giuliodori et al. (2013) also reported that cows with a NEB had a higher risk of developing clinical endometritis, and the NEB had detrimental effects on neutrophil function, and thus the uterine health. The above studies have only paid attention to the BCS of cows at the endometritis examination stage, but the present study emphasized the amount of changes in BCS so that the probability of developing endometritis was significantly higher in cows with a reduction in BCS higher than 0.5.

Wathes et al. (2009) reported that the numbers of WBC and lymphocytes were lower in cows with severe NEB in the postpartum period than cows with mild NEB, and the inflammatory responses in the uterus of cows with severe NEB were still active at 2 weeks after pregnancy while the endometrium of cows with mild NEB largely returned to normal. Roche et al. (2013) found that the number of multinucleated cells in uterine secretions was higher in cows with low or medium BCS than those with higher BCS at the first 3 weeks after calving, indicating a higher risk of developing uterine diseases. Çolakoğlu et al. (2019) also mentioneda change in BCS before calving and stated that the change of more than 0.25 units in BCS was accompanied by an increase in BHBA on the 30th day after calving, and as we know, the higher BHBA was associated with a weaker immune response and lower reproductive performance. Furthermore, Galvão et al. (2010) reported that NEFA was higher in cows with subclinical endometritis and metritis than healthy cows. As mentioned earlier, the poor body condition was associated with a higher probability of uterine problems, including clinical endometritis, but it should be noted that none of the above studies investigated the changes in the BCS during the transitional period after delivery like the present study. Results of the present study indicated that postpartum changes could also affect the occurrence of clinical endometritis.

Parturition type and BCS reduction

There is little information about the impact of calving difficulty on the postpartum BCS in dairy herds (Berry et al., 2007). Drennan and Berry (2006) studied the broiler cows and reported a greater reduction of BCS in cows with calving difficulty than those without any pregnancy help. It was also reported that the higher BCS during calving and NEB along with a sharp reduction of BCS after calving was associated with an increase in cases of calving difficulty, retained placenta, metritis, etc. (Roche et al., 2009; Garnsworthy, 2006).According to studies, the rate of decrease in BCS after calving was very clear and significant in the group with dystocia. According to Ingvartsen et al., the stress caused by dystocia as well as possible subsequent problems such as retained placenta, metritis, and lameness could affect the food intake of livestock and predisposed it to a sharp decline in BCS

(Ingvartsen et al., 2003).

Parity and BCS changes

The present study also considered the relationship between the parity number and the amount of loss in the BCS on the first 30 days of lactation, as addressed in various studies, and the results of the present study were consistent with the results of most of them. However, it should be noted that in most of the studies cows are often divided into two groups (the first and multiple calving), while in the present study cows were divided into 4 groups (first, second, third, and fourth calving). In general, in cows of third calving onwards with complete growth period, the results were similar to previous studies. In a study by Lee and Kim (2006) and according to the divisions, it was found that the rate of reduction in the BCSs in calving 3, 4, and 5 was significantly higher than the first calving. According to a study by Ettema and Santos (2004), the depletion and recovery of body reserves in the first calving cows were less severe than those with multiple calving. Sakaguchi (2009) found that the parity number could affect BCS changes after calving so that the reduction of BCS at an interval between calving and about 3 weeks later was higher in multiparous cows than primiparous. Piñeyrúaet al. (2018) found that the energy balance was more affected in the multiparous cows, and the amounts of postpartum blood NEFA and BHBA were lower in the primiparous cows than the multiparous. Primiparous cows tended to produce less milk than multiparous cows probably due to the fulfillment of their growth needs. According to a report by Piñeyrúaet al. (2018), the curves of NEFA and BHBA were different in multiparous cows with the primiparous cows as the multiparous cows had 2 peaks in the curve (weeks 1 and 4), while there was only one peak in the primiparous cows probably due to the continuity of fat transfer in multiparous cows in the early lactation (Piñeyrúa et al., 2018).On the contrary to the above content which uses the TMR system, some studies with the grazing system reported that the primiparous cows had a more metabolic problem, less BCS, and lost higher BCS than multiparous cows. (Meikle et al. 2004; Cavestany et al., 2009). In the grazing system, receiving energy and protein were lower than feeding by TMR (Piñeyrúa et al., 2018).

CONCLUSION

According to the present study results, a further reduction in BCSwas associated with a reduction in reproductive performance, but there is a need for more studies to investigate the possible reasons for this effect.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

The biological potential of a product containing *Pythium oligandrum* against *Uncinaria stenocephala* (Railliet, 1884) larvae

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ABSTRACT: *Pythium oligandrum* is an oomycete commonly used in the biological control of plant and vegetable pests. In veterinary medicine is used to treat dermatophytosis produced by *Microsporumcanis*. It acts as an antagonist, negatively influencing the development of other fungi. Through hydrolytic enzymes, such as kinase and cellulase, it destroys the cell wall and the internal cytoplasmic content. Many fungi are considered nematophagous. This potential has not been exploited for *Pythium oligandrum*, which is why in this study the potential larvicidal action against *Uncinaria stenocephala* larvae was investigated.

Ecosin® product, which contains *P. oligandrum* and other excipients, was used. A solution was prepared according to manufacturer. The *Uncinaria stenocephala* larvae were exposed to this substance and the action was investigated after 7 days. The results obtained showed 37.23% larval reduction.

Being an eco-friendly product, further studies are needed to improve the protocol for its use in cleaning spaces and surfaces in veterinary clinics, where various parasitic forms (larvae or eggs) are found.

Keywords: Pythium oligandrum, *Uncinaria stenocephala*, larvicide, dog parasites.

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INTRODUCTION

Pythium oligandrum is a common fungus used in agriculture due to the various actions that offer plants pest resistance. It can act through mycoparasitism, antibiosis and through the competitive mechanism regarding the nutrition reserves and space necessary for the development of other fungi (Bradshaw-Smith et al., 1991; Benhamou et al., 2012). The most frequent localization of *Pythium oligandrum*, under natural conditions, is at the root of the plant. Multiplication only occurs at the epidermal or at the most cortical level, without affecting the integrity of the morphological components of plants. Unlike other fungi, *Pythium oligandrum* multiplies in the hyphae and produces different hydrolytic enzymes (kinase, cellulase) that destroy the cell wall and internal cytoplasm (Picard et al., 2000). Through cellulolytic enzymes, *Pythium oligandrum* destroys other oomycetes or fungi. The mechanism of the antibiosis consists in the production of secondary toxic metabolites with antagonistic effect on other fungi (Gerbore et al., 2014).

The action of plant protection against various pathogens is conferred by *Pythium oligandrum* through some eliciting proteins, such as: oligandrine and elicitin (Takenaka and Tamagake, 2009). *Pythium oligandrum* is used in silviculture in the biological control of various pests attacking plants and vegetables. In veterinary medicine *Pythium oligandrum* is used against the dermatophyte *Microsporum canis* but has antagonistic action for about 50 fungi and oomycete species (Gabrielová et al., 2018). Through chemotropism, *P. oligandrum* develops and forms a network of hyphae around the targeted agent (fungus). Binding to the pathogenic fungus also occurs in the hyphae, through cell surface receptors. Penetration inside cells takes place after the hydrolytic enzyme release. Once inside, it continues to multiply and to form papillary structures. The multiplication process is favored by the need of carbon provided through the gates created after cell wall degradation. Where *P. oligandrum* develops, the cell contents are neutralized and only wall fragments remain (Benhamou et al., 2012; Horner et al., 2012).

No data has been found on its potential action against various parasitic forms in carnivores or other animals.

The present study follows the action of Ecosin®, containing *Phytium oligandrum*, on *Uncinaria stenocephala* larvae. The purpose of this study is to investigate the biological potential of *P. oligandrum*, so that it can be used as an agent in cleaning various surfaces in current veterinary practice.

MATERIALS AND METHODS

The source of fungus

In this experiment the Ecosin® (Galenicka laborator Ostrava, Obrancumiru 234/41, 703 00 Ostrava - Vitkovice, Czech Republic) product was used, whose components are: citric acid, sodium bicarbonate, sorbitol, silicon dioxide, *Panicum miliaceum, Pythium oligandrum*, polyethylene glycol (PEG 6000) and sodium carbonate. This product comes in the form of tablets weighing 3 g. The indicated therapeutic dose was chosen to see the effect that may be observed on *U. stenocephala* larvae: one tablet was dissolved in 2 l of hot water (approximately 34°C).

The source of larvae

Fresh feces were collected from several adult dogs from the public shelter. Each sample was initially examined using the Willis method to identify possible eggs from the *Ancylostomatidae* family. From each positive sample, larval cultures were made using the Harada-Mori method.

Identification of larval species (*Uncinaria stenocephala*)

The PCR reaction was performed according to the technique described by Silva et al. (2006) and Gasser et al. (1996), with some minor changes. The actual amplification was performed by classical PCR and was based on the creation of several copies of an ITS gene sequence, 8850-bp in size, for *Ancylostoma* ITS region.

The primers used were: NC5 (forward 5′-GTAGGTGAACCTGCGGAAGGATCATT-3) and NC2 (reverse 5′-TTAGTTTCTTTTCCTCCGCT-3′) for the first PCR; NC1 (forward 5′- ACGTCTGGTTCAGGGTTGTT-3′) and NC2 (reverse 5′-TTAGTTTCTTTTCCTCCGCT-3′) for the second PCR.

The amplification was done according to the protocol described in the article, modified according to mixture requirements. For the PCR reaction a MyTaqTM Red Mix Master Mix (BIOLINE®) was used. The final volume used in the PCR reaction was 25 µl: 12.5 µlMyTaqTM Red Mix (BIOLINE®), 1 µL primer 1F, 1 µl primer 2R (diluted to 10 pmol / µl accord-

ing to protocol described by the manufacturer), DNA extracted from the test sample and ultrapure water.

The amplification program was performed using a fast My Cycler (BioRad®) thermocycler. The program used for the first and second PCR, included: the DNA denaturation steps at 95 °C for 1 minute; 32 denaturation cycles at 95 °C, for 30 seconds; a hybridization at 50 °C for 30 seconds and extension at 72 °C, for 30 seconds; followed by incubation at 4 °C.

The analysis and amplicons control were done using horizontal electrophoresis in a submerged system of 1.5% agarose gel electrophoresis, adding Midori Green (Nippon Genetics® Europe) fluorescent staining, at 120 V and 90 mA, for 60 minutes.

The 100 bp Ladder DNA marker was used in the first well in the gel.

After the sample migration in agarose gel, the image with the migrated DNA fragments was captured using a UV photo documentation system (UVP®).

To identify the species, the PCR products were sequenced and compared with those available in the GenBank database, using BLAST alignment. *Uncinaria stenocephala* (Railliet, 1884) has been identified.

Exposure of *Uncinaria stenocephala* larvae to *Pythium oligandrum* fungus

Thirty Petri dishes with 9 cm diameter were prepared, in which *Uncinaria stenocephala* larvae were placed (about 100 larvae in each plate), together with 3 ml of physiological serum. In 15 of the Petri dishes (considered the treated group), 7 ml of Ecosin® solution were added. In the control samples (n = 15) another 7 ml of physiological serum were added. According to the manufacturer, after 2 days of exposure, 5 ml of Ecosin® solution were removed and re-added. The same operation was performed after another 2 days to ensure that all the indicated therapeutic protocol steps were followed. The solution was put on media several times, due to its inactivation after 24 hours. The samples were sputtered daily to provide oxygen. They were examined daily and after 7 days of exposure, the percentage of larval reduction in the treated samples was evaluated, according to the following equation:

$$\% \text{ Reduction} = \frac{\text{(average of recovered L3 from control-average of recovered L3 from treated)}}{\text{average of recovered L3 from control}} \times 100$$

Statistical analysis

The average and standard deviation for treated and control group were determined. The statistical interpretation of the results was made in GraphPad Prism program, QuickCalcs, using two-tailed Fischer's exact test to obtain the P-value.

RESULTS

After 7 days, mobile and immobile larvae were identified in the treated samples. There were no morphological changes at the cuticular level due to the multiplication of the oomycete *Pythium oligandrum*. The percentage of larval reduction was 37.23%, the result being encouraging.

Extremely statistically significant differences were observed between the treated and control samples ($P < 0.0001$). The average number of L3 recovered in the treated samples was 52.8 (±12.89) compared to 84.13 (±8.97) found in the control samples (Table 1).

Table 1. The number of viable larvae recovered from control and treated samples

Treated samples	Recovered L3/ Total	Control samples	Recovered L3/ Total
1	56/100	**1**	73/100
2	48/100	**2**	82/100
3	70/100	**3**	90/100
4	62/100	**4**	90/100
5	32/100	**5**	75/100
6	70/100	**6**	77/100
7	47/100	**7**	82/100
8	70/100	**8**	82/100
9	44/100	**9**	90/100
10	54/100	**10**	90/100
11	56/100	**11**	100/100
12	47/100	**12**	100/100
13	49/100	**13**	78/100
14	60/100	**14**	71/100
15	27/100	**15**	82/100

DISCUSSION

Being the first study on the biological potential of Ecosin® in controlling the viability of *U. stenocephala* larvae, no other results have been identified in the literature to be compared.

About 99, 000 fungi species have been described (Kirk et al, 2008). Many of these are found in soil and are saprophytic but can become predatory agents in contact with various parasitic forms (eggs or larvae). In general, fungi are not resistant in the external environment and require a nutrient substrate to form spores or to develop mycelium (forms of resistance). *Duddingtonia flagrans*, *Monacrosporium* spp. and *Arthrobotrys* spp. have larvicidal action in hookworms and roundworms (Saumell et al., 2016). Satisfactory results were observed in experiments using *Duddingtonia flagrans*. Chlamydospores determined a percentage of larval reduction (*Ancylostoma* spp.) between 57 - 79.4% (Maciel et al., 2010) or 4.5 - 63% (De Mello et al., 2014). The percentages varied depending on chlamydospore number to which the larvae were exposed. The best effect was observed using 25.000 *Duddingtonia* chlamydospores. Thus it can be a limiting agent in the development of infesting larvae from *Ancylostomatidae* family (De Mello et al., 2014).Other studies suggest that *Duddingtonia flagrans* has no larvicidal effect against *Muellerius capillaris* (first-stage larvae), in small ruminants (goats) (Paraud et al., 2005).*Duddingtonia* can also be used to control the viability of L1 *Angiostrongylus vasorum*. The raw extract of *D. flagrans* determined 53.5% (after 24h) and 71.3% (after 48h) larval reduction (Braga et al., 2009). Similar results were obtained after using raw extract of *Monacrosporium thaumasium*. Conidia used in coprocultures determined 40% larval reduction.Higher percentages were observed in water-agar media, 74.5% respectively (Soares et al., 2015).

Other authors observed a high larvicide action (73.84%) using *Arthrobotrys robusta* (isolated I-31) preserved on silica gel against L3 of *Haemonchus contortus* in ruminants (Braga et al., 2014).Good results were also observed after *Drechmeria coniospora* (Santos and Charles, 1995) or *Harposporium anguillulae*use (Charles et al., 1996).

The fungi action used in the treatment of various diseases is also due to its secondary metabolites. The ones with medical use are as following: alkaloids, quinones, peptides, terpenes, polyketides, coumarins and sterols (Costa et al., 2016). Among the alkaloids, diketopiperazine alkaloids produced by various fungi showed antileishmanic action (Metwaly et al., 2015). The quinones (cercosporin) have antiparasitic action in the case of *L. donovani* and *T. cruzi* (Martínez-Luis et al., 2011). The peptides can be used in parasitism with *T. gondii*, *N. caninum* and *Cryptosporidium parvum* due to the inhibitory action reflected on histone diacetylase, thus intervening in DNA transcription and affecting the reproduction of the parasite (Darkin-Rattray et al., 1996). The sesquiterpene molecules produced by different fungi have antiprotozoal action (*Leishmania* spp., *T. gondii* and *N. caninum*) (Loo et al., 2017). Triterpenoids produced by basidiomycete fungi are also good agents in controlling certain parasites (Nyongbela et al., 2013). In case of malaria, coumarin products, such as dihydroisocoumarins, have worked effectively (Kongsaeree et al., 2003).

The advantage of identifying fungi that can be used in the control of various parasites is related to their less devastating effect on the ecosystem. Thus, it is desired to use natural biotic agents, with localization and development in the external environment, to be reused in the same context, only as a protection against harmful agents to animals, plants, etc.

CONCLUSIONS

The results obtained in this study indicate a potential larvicidal action of Ecosin®, containing *P. oligandrum* (37.23% larval reduction). However, further studies are needed to improve the protocol for the use of Ecosin® product as a potential biological agent in the control of parasites.

CONFLICT OF INTEREST

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of paper.

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Research article
Ερευνητικό άρθρο

Nutritional evaluation of the agro-industrial by-products and waste fruits - vegetable for sustainable ruminant nutrition

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ABSTRACT: The growing consumption of ruminant animal products gives rise to a huge demand of animal feed in growing countries.By-product feeds, waste fruits-vegetables, and crop residues should be considered as a valuable alternative feed resource in ruminant nutrition. This wastecan be reutilized and converted by ruminants to valuable products for human benefits as a new resource and in return to increase the effectiveness of limited feed sources. But, there are limited new information and research regarding the nutritive value of this waste for ruminants. For this purpose, the experiment was conducted to evaluate the nutritional potential of some agro-industrial by-products, waste fruits-vegetable, and crop residue for ruminants specifically. Fourteen by-products, waste fruits-vegetable and crop residue were collected from the west part regions of Turkey. Nineby-product feeds (whole cottonseed, rice bran, soybean hull, apple pomade, citrus pulp, grape pomade, tomato pomade, grape stalk, rice hull), three waste fruits (dry grape, dry fig, carrot), one waste vegetable (potato) and also one crop residue (cornstalk) were analyzed for nutritional composition and metabolizable energy values were calculated by crude nutrients for ruminants. Further, energy, DMD, and OMD of these samples were investigated by using the cellulose enzyme method. All samples were analyzed the macro minerals (Ca, P, Na, K, and Mg) and the microelements (Fe, Cu, Mn, and Zn) contents. In the research, all samples regarding the parameters in DM, ash, OM, CP, EE, CF, NFE, NSC, NDF, ADF, starch, sugar, Ca, P, Na, K, Mg, Fe, Cu, Mn, Zn, DMD, OMD, ME_{CN}, and ME_{CEL}were different ($P<0.05$). Thestudy showed that the waste fruits, vegetable, and by-products have valuable sugar (grape, fig, and carrot), starch (potato, rice bran), NSC (citrus pulp), and oil (cottonseed) content that is the main compounds making them high energetic feeds for ruminants. Also, most of these research materials have enough or much more macro and micro mineral concentrations for ruminant nutrition.

***Keywords*:** By-products, Fruit, Vegetable, Ruminant

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INTRODUCTION

The growing world population depends on the consumption and use of limited and diminishing natural resources such as arable land, fuel oil, fertilizer, and water. However, the enlargement of human urbanization and with them increase of agricultural production has caused not only environmental adversities such as climate change, soil, and water degradation but also economic and social concussion (Anguloa et al. 2012). The increased demand for plant and animal foods needed to meet the basic nutritional needs of people while making the already existing pressure on agricultural production even more noticeable (Dou et al. 2016). Also, the prediction byFAO (2011) that the human population will reach 9.5 billion and the world would need 73 percent more meat and 58 percent more milk in 2050 (Wadhwa and Bakshi 2013). Especially in animal production, to meet high feed requirement demands, a huge quantity of feed resources supply and the sustainability of the feed production systems must be considered carefully.Also, there is already a considerable scarcity of feed accessibility in most developing and developed countries for animal production (Godfray et al. 2010).

Therefore, it is necessary to use the limited resources most efficiently and to evaluate the foods obtained as the least waste, while raising the interest in alternative feed materials that can increase the productivity by animal species and reduce the food competition between animals and humans. Considering that approximately 30% of food produced (1.3 billion metric tons) is waste before reaching people (Ajila et al. 2012), and also each day the matters riseas long as the amount of waste produced is greater than the amount of waste.Therefore, serious attention to waste management is essential for sustainable animal production.

Several factors have led to increased interest in by-product feeds and waste foods such as pollution abatement, regulations, increasing cost of waste disposal, and changes in perception of the value of these feed as economic feed alternatives (Belyea et al. 1989). The primary reason for using by-product feeds and waste foods as feed material is to reduce feed cost and also during a drought or when is high fiber forages limited. By-product feeds come from various agro-industrial sources including such as grain processing, extraction of juice processing industry, brewing- wine distillery industry, marketplace or bazaars that are main sources of fruit and vegetable wastes, or crop harvesting, etc. Although many of these feeds have been used for years, others like fruit and vegetable wastes as ruminant feed are relatively new (Bernard 2010; Anguloa et al. 2012). Agricultural and industrial by-products are generally cellulosic in nature, with a high cellulose and hemicellulose content and less efficient to the animal except for ruminants (Agus, 2015; Bernard 2010). Ruminants have a valuable role in sustainable animal production and their rumen serves as a fermentation tank containing the microbial cellulose enzyme that is the only enzyme to digest the fiber fractions rich in the by-products feed stuffs, Oltjen and Beckett (1996).

Livestock is one of the fastest-growing agricultural sectors in developing countries. Also, the demand for animaloriginated foods is rapidly increasing in most developing countries.However, many developing countries have feed deficits. New unconventional alternative feed resources such as agricultural and agro-industrial by-product feeds, fruits, vegetables, and crop residues originated feeds could play an important role in meeting this deficit, Wadhwa and Bakshi (2013). Besides, their use in the ruminant ration can also reduce the cost of feeding, giving higher economic advantages to producers. These by-products feeds, which contain little economic value as foods for human consumption would become considerable sources of dietary nutrients and energy in ruminant nutrition. Their use can also reduce the cost of feeding, giving higher profits to producers. These by-products feeds, which contain little economic value as foods for human consumption would become considerable sources of dietary nutrients and energy in ruminant nutrition.However, there has been little new research regarding the nutritional value of the agricultural and industrial by-products feedstuffs in ruminant nutrition. The aim of this study was to know and reevaluate the nutritional value of some agro and agro-industrial by-products feeds for ruminant nutrition.

MATERIALS AND METHODS

Sample collection

Nine different types of by-product feed; apple pomace, citrus pulp, grape pomace, grape stalks, tomato pomace, cottonseed, rice bran, rice hull, soybean hull; three fruits; carrot, dry fig, dry grape; one vegetable; potato and one crop residue; corn stalk were provided by six different agro-industrial factories, bazaars and harvested corn fields for nutritive

evaluation based on their use in ruminant nutritionin Izmir/Turkey. Each by-product, vegetable, fruits, and crop residue consists of six different samples; each one is analyzed in three replicates one by one for each parameter. Before chemical analyses, all experimental samples were ground through a 1 mm screen in preparation for chemical analysis and stored at 4°C in a refrigerator until analysis.

Nutrient composition

Nutrient contents of air-dry samples were analyzed according to the methods reported in AOAC (1997), and all data were presented on a dry matter basis. All samples were analyzed for dry matter (DM) (method 934.01), ash (method 942.05), crude protein (CP) (method 990.03), ether extract (EE) (method 920.39), crude fiber (CF) (method 962.09). The sugar content of the materials was determined by the Luff-Scroll method and the starch determination by the polarimetric method, AOAC (1990). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined using the methods by Van Soest et al. (1991). Nitrogen-free extracts (NFE) were calculated as 100-% (moisture+CP+EE+CF+ash). Non-structural carbohydrate content (NSC) was obtained using the following equations as 100-% (NDF+CP+EE+ash). Organic matter (OM) was calculated as OM%=DM%-ash%.

Phosphorus (P) contents of the materials were read by spectrophotometer (model PE General TU-1880 Model Double Beam UV-V15) by calorimetric methods. Atomic absorption spectroscopy (Ultrospec2100 pro UV/visible 106 spectrophotometer) was used for determining calcium (Ca), potassium (K), sodium (Na), magnesium (Mg), iron (Fe), copper (Cu), manganese (Mn), zinc (Zn) concentrations.

Estimates for crude nutrition metabolizable energy (ME_{CN}) as kcal kg^{-1} in DM were based on crude nutrients (protein, fiber, and fat levels) determined from the samples using a prediction equation, TSI-9610 (1991) ;

ME_{CN}kcal kg^{-1}:3260+ (0.455xCP+3.517xEE-4.037xCF) and CP, EE, CF quantities in OM (gkg^{-1}).

All nutritional parameters, mineral contents, and energy values of the samples are given on a dry matter basis.

In vitro digestibility

The in vitro dry matter digestibility (DMD), organic matter digestibility (OMD), and *in vitro* cellulose enzyme metabolizable energy (ME_{CEL}) were determined according to the cellulose enzyme method described by (De Boever et al. 1986) modified from Tilley and Terry (1963). The in vitro OMD was estimated by the equation developed by Weissbach et al. (1999). The enzymatic procedure investigated comprises 3 steps: (1) pepsin (Sigma) in 0.1 M HCl at 40°C for 24 h; (2) starch hydrolysis in the same solution at 80°C for 45 min; (3) cellulase (from *Trichodermaviride, Serva*) at 40°C for 24 h. The *in vitro* digestibility analyzes were serially performed on each sample in triplicate. Values are expressed on a dry matter basis in all equations. Enzymatic DMD, OMD, and *in vitro* ME_{CEL} were calculated using the following equations;

DMD, %= IVDMD %: ((A_n- (A_k - A_o)) / A_n) x 100

OMD, %=IVDOM %:100 x (940-CA-0.62 x EULOS-0.000221 x $EULOS^2$) / (1000-CA)

ME_{CEL} (Mcalkg-^{1}DM) = (1.04 + (0.00001611 x $ELOS^2$) + (0.3724 x EE) - (0.0003674 x ELOS x EE) - (0.0004919 x EE x CF) + (0.01548 x CF)) /4.186

*EULOS; enzyme insoluble OM, A_n; sample weight (g), A_o; crucible tare (g), ash%and EULOS in (g) DM

Statistical analysis

The statistical analysis of the results included a one-way analysis of variance ANOVA using General Linear Models and Duncan's multiple range test, which were applied to the results using the IBM SPSS Statistics 25, SPSS (2016). The model included by-product feed samples as main effects. Differences were considered to be significant based on the 0.05 level of probability.

RESULTS

The means and standard errors for the nutrient composition and mineral content of fourteensamples are given in Table 1 and Table 2 respectively. Also, Table 3 showed the DMD, OMD, ME_{CN}, and ME_{CEL} values of the samples.

The differences among the by-product feed (apple pomace, citrus pulp, grape pomace, grape stalk, tomato pomace, whole cottonseed, rice bran, rice hull, soybean hull) fruits, (carrot, fig, grape), vegetable (potato), and one crop residue (corn stalk) in DM, ash, OM, EE, CP, CF, NFE, NSC, NDF, ADF, starch, and sugar contents were significantly different $P<0.05$. The DM content of the by-product feedswas within the range

from117 to 935 g. In terms of the DMcontent of the by-product feeds had a great variation. Carrot had the lowest DM content, while corn stalk had the highest DM content. The overall average means of the ashes in by-product feeds varied between 24 and 193g. The rice hull had extremely high ash content compared with other by-product feed samples. The average OM content of samples was quite high (806-975g). Rice hull and rice bran, in samples, were lower the OM content other than.The EE content of by-product feeds and other samples were considerably variable; cottonseed, rice bran, tomato pomace, and grape pomace had the highest EE content (177.7, 159.8, 84.5, and 75.7g respectively).On the other hand; potato and rice hull had the lowest average EE content (3.3 and 5.3 g respectively) among the samples. Concerning to the protein average means, in the by-product samples ranged from 219.6g to 61.2g. When samples are compared with each other; cottonseed, and tomato pomace were higher CP content (219.6 and 196.3 g respectively) while corn stalk and fig were lower CP (61.2 and 66.1 g respectively) content.

The NFE values calculated for research samples-ranged the lowest in tomato pomace and whole cotton seed 206.4 and 224.9 g to the highest in fig and potato both the same value 783.9 g.Similarly, NSC means as NFE were the lowest in rice hull, cottonseed, and tomato pomace (16.2, 32.1, and 56.3g) on the other hand; fig, carrot, and potatohad the highest NSC values (731.0, 683.2 and 660.3 g) in the samples.

In the study, the CF content of the by-product feeds had a great variation. The cellulose concentration of the by-product feedswas within the range from 119.4 to 506.2 g. When rice hull, tomato pomace, and soybean hull had the highest CF contents (506.2, 454.2 and 411.7 g respectively), potato, and fig had the lowest average fiber concentration (19.4 and 76.6 g respectively). Except for some fruits and vegetables such as fig, potato, and carrot (129.6, 143.0, and 143.8g) were lower NDF content than all the other by-products feeds and research samples. Cornstalk and rice hull showed the highest NDF concentrations (766.0 and 745.4g respectively). The ADF showed that the potato had a lower mean value (22.1 g) than other by-product feeds means. The highest ADF content was seen in rice hull as 645.3 g.

When the starch content was not determined in grape, fig, carrot, and corn stalk; also rice hull and grape pomace had the lowestmean and both the same value (12.7 g), and potato had the highest starch content (642.0 g) among the samples. The sugar content ranged from 5.9g to 235.1g in by-products feed, fruits, vegetable, and crop residue samples; with the lowest content, in rice hull, tomato pomace, and cottonseed (5.9, 17.2, and 19.7g respectively) and highest value recorded for fig and carrot (235.1 and 235.0 g respectively).

The means and standard errors for the mineral content of by-products feed, fruits, vegetable, and crop residue (apple pomace, citrus pulp, grape pomace, grape stalk, tomato pomace, cottonseed, rice bran, rice hull, soybean hull, carrot, fig, grape, potato, corn stalk) were given in Table 2. All the observed parameters related to mineral contents as Ca, P, K, Na, Mg, Fe, Cu, Mn, and Zn of the feed samples were found significantly different $P<0.05$. When the citrus pulp and grape stalk had the greatest concentration of Ca (17.3and 15.9 g respectively) ; rice bran and potato had the lowest concentrations of Ca (1.4 and 1.9g respectively) compared with all the other feed samples.

The mean P content was the highest in rice bran (6.6g) and the lowest in rice hull (0.1g). The phosphorus concentration was less than 1% in all samples. The potassium means the value of the by-product feedswere within the range from 6.9 to 24.9 g. The K concentration in rice hull and apple pomace (6.9 and 8.6g) is the lowest and significantly lower than all other by-product feeds. Except for the rice bran (8.4g), all the other samples were less than 5.9g mean values for the Mg content. When the Na content was the highest for carrot (5.3g), the lowest for the citrus pulp and corn stalk samples (both the same value 0.5g). The Fe content was the highest for corn stalk, soybean hull, and grape (619, 509, and 372mg), the lowest the fig (141mg). The Mn content was greater than the grape stalk (133 mg) compared with the other by-product feed samples. The Cu concentration was the lowest for the corn stalk, rice bran, fig, and apple pomace (54, 56, 57, and 57 mg) and the other samples ranged 59 to 80mg.The Zn content in terms of micro minerals for research samples ranged from 8.8 to 26.1mg. The Znconcentration mean value was the highest for rice hull (26.1 mg), the lowest for the citrus pulp (8.8mg).

The values of *in vitro* DMD, OMD, ME_{CN}, and ME_{CEL} contents of by-products feed, fruits, vegetables, and crop residue samples are shown in Table 3; and all parameters were observed significantly differences $P<0.05$. In terms of DMD, considerable variation was observed among the samples. The DMD values were obtained in all samples (ranged 10.3 to 96.0%).The

DMD was the highest for citrus pulp and potato (96.0 and 95.8% respectively), the lowest for the rice hull (10.3%). The OMD of the samples were ranged from 22.8% for the rice hull to 95.7% for potato average value. In the study, OMD values of the samples were seen similar to DMD means. The average ME_{CN} value in by-products feed, fruits, vegetable, and crop residue samples was the highest for rice bran and potato (3118 and 3052 kcal/kg) and the lowest for rice hull (640kcal/kg) average mean. The ME_{CEL} value was ranked 723 kcal/kg (rice hull) to 3310 kcal/kg (rice bran).

DISCUSSION

The present study was performed to evaluate the nutrition value of the by-product feeds (apple pomace, citrus pulp, grape pomace, grape stalk, tomato pomace, cottonseed, rice bran, rice hull, soybean hull) fruits, (carrot, fig, grape), vegetable (potato), and one crop residue (corn stalk) for ruminant nutrition. When samples were grouped in four classes (by-products, fruits, vegetables, and crop residue), DM was the lowestin carrotand potato compared with other samples. All by-products and other feed materials showed considerable variation within the DM contents. Findings the DM determined in this study are consistent with the findings of relevant studies (White 1985; Arosemena et al. 1995; Aghsaghali and Sis 2008; Lardy and Anderson 2009; Azevêdo et al. 2012; Eliyahu et al. 2015; Wadhwa et al. 2015). However, the range reported by Gupta et al. (1993) (carrot, potato) and INRA (2004) (rice bran, soybean hull, cottonseed) were lower, when DM contents declared by NRC (2001) (apple pomace, tomato pomace) ; and Filleau et al. (2018) (apple pomace, grape stalk) were exceptionally high value some samples.These differences could be due to different agronomic practices adapted in different regions and also originated by different industrial processing methods. (Lardy and Anderson 2009) indicated that water content may result in excessive effluent losses and reduce ration dry matter content.Also the high DM or low moisture content in ruminant nutrition is very important because of providing easy storage and use for ration. Meanwhile, the high moisture content in by-products and food waste can cause difficulties in balancing ration dry matter content, storageand also increase microbial growth (Kabak et al. 2006; Tretola et al. 2017). When using these types of feed that have low dry matter content such as vegetables, fruits or by-products may have to be used as soon as possible and using together with dry forages to balance the dry matter content of the TMR.

Our finding ofthe ashand calculated OM content of the study samples were found similar with results of relevant studies in general (White 1985; Arosemena et al. 1995; NRC 2001; INRA 2004; Azevêdo et al. 2012; Wadhwa et al. 2015). Except for the rice hull (193g), other samples had a low quantity of the ash contents that were ranged from 24 to 88 g. Although the rice husk has high ash content, it consists of approximately 90% silica while the useful mineral concentration is lowWhite (1985). On the other hand; the low ash and high OM contents of them suggest that they may be valuable feed resources in ruminant nutrition.

The findings about EE content of research samples are consistent with the findings of relevant studies (Arosemena et al. 1995; NRC 2001; INRA 2004; Azevêdo et al. 2012; Wadhwa et al. 2015) but it is lower than the value found by (Kajikawa 1995).In the experiment, cottonseed and rice bran had the greatest concentration of EE compared with all the other selected feed samples.These by-products could be used successfully as a source of energy, protein, and fiber in ruminant ration (Wadhwa and Bakshi 2013). Since the whole cottonseed has high fat and protein contents, it may be defined as a concentrate feed Arieli (1998). Although the high oil content of the cotton seed (about 20% of dry matter) has a suppressive effect on rumen microbial activity, it should be considered as a good energy source for ruminants.

In terms of the CP content by-product feeds, fruits, and vegetable showed the low mean value and variability, except with cottonseed and tomato pomace. The findings about CP contents of experiment samples are consistent with the findings of the relevant studies in general (Kajikawa 1995; NRC 2001; INRA2004; Wadhwa et al. 2015; Filleau et al. 2018). However, some researchers have reported that higher CP content (Belyea et al. 1989; Azevêdo et al. 2012). Based on this study data, the average CP contents of the samples could vary 39.0 from to 219.6 g.The differences in protein content may vary depending on the origin of the by-products, food industrial production process, or used different agronomic production model.The results related to the CP contents suggest that the by-products, fruits, vegetables and crop residue should not be considered as a good source of protein because of the low concentration and origin (Azevêdo et al. 2012; Wadhwa et al. 2015) except the cottonseed and tomato pomadefor ruminant. However, Arieli (1998) concluded that the protein in the cottonseed has high rumen degradability of about 70-77%

and can be use a good protein source in the ruminant ration. Results of NFE and NSC contents agree with findings of NRC 2001; Bernard 2010; Wadhwa et al. 2015. The NFE and NSC concentrations of the potato, fig, grape, carrot, and citrus pulp were similar and these samples had higher mean value than others in the study. On the other hand, the research showed that the tomato pomace, cottonseed, and rice hull had lower contents both of NFE and NSC in accordance previously mentioned by Bernard (2010).

As expected in the study, there was no starch in the fruits (grape, fig, and carrot) and corn stalks, but a very low starch content was found in other by-product feed samples except the potato (642.0 g). These findings agree with the previously reported study results or data by Belyea et al. 1989; Kajikawa 1995; INRA 2004; Filleau et al. 2018. As a result, the potato had a very high starch concentration feeding value equal to cereal grain such as barley or corn grain on a dry matter basis, INRA (2004). Satisfactory results can be obtained in finishing or dairy cattle rations by feeding potatoes as energy source raw feed material like cereals (Nelson et al. 2000; Lardly and Anderson 2009). On the other hand, the research showed that fruits (grape, fig, and carrot) and citrus pulp had higher sugar content than all others. Most of the research or literature has focused on the starch concentration of feed samples, while limited information is available on sugar content.This study showed that sugars constitute an important part of carbohydrates in the fruits samples. As known, carbohydrates are especially starch and fiber as primary nutrition components that contribute up to 70% of the diets, used to dairy cows and beef steers (Allen 1996). Also, sugars may be good alternative energy sources for any adverse effect on rumen fermentation and animal performance. Generally, sugars are known as water-soluble carbohydrates that are readily available in the rumen, and consist of disaccharides, such as sucrose, lactose, and maltose, and monosaccharaides, such as glucose, galactose, and fructose (Oba 2011). Thus, feeding sugar or when sugar replaced dietary starch, improves rumen degradable protein utilization (Broderick 2008) dry matter intake and milk fat content. The fruits waste (grape, fig, carrot), potato, and by-products (citrus pulp) having high sugars, starch, and pectin content, could form a significant part of ruminant ration as energy resource on dry matter basis.

Based on the structural carbohydrate contents such as NDF, ADF, and CF contents of the by-products and other research samples, were significantly different from each other. Waste fruits and vegetable had low NDF, ADF, and CF contents, because these samples were composed primarily of simple or water-soluble sugar or pectin (Wadhwa et al. 2015). This funding agrees with previously reported research that by-product samples characterized by comparable fiber (Kajikawa 1995; NRC 2001; INRA 2004; Wadhwa et al. 2015; Filleau et al. 2018). Fiber is the main carbohydrate fraction of ruminant rations and is necessary to provide adequate amounts of complex carbohydrates to slow digestibility and control the acidity in the rumen for healthy rumen fermentation. Because dairy or beef steers require fibrous feedstuffs in the diet, the ADF and NDF content of the feeds are important fiber fractions that need to be carefully considered in balancing the ration formulation (Varga et al. 1998). Utilizing this kind of waste fruits, vegetables, and by-products for ruminant feeding, ADF and NDF contents should be carefully considered in ration making. Cornstalk and rice hull can be used as straw because of nutrition contents are similar to other cereal residues such as straw.

Considering DMD and OMD values of the research samples have shown considerable variation. The potato, citrus pulp, carrot, and fig had higher digestion values than the other samples. These results compare with previously reported by Azevêdo et al. (2012) and Wadhwa et al. (2015) that fruit waste and vegetables are highly digestible depending on the origin or the mixture used in their preparation. Also, the small variations observed between research and lower digestion rate (DMD and OMD) for the rice hull, grape stalk, and grape pomade which are thought to be due to nutrition composition, especially structural carbohydrate contents (both NDF and ADF) and nonstructural carbohydrate level Wadhwa et al. (2015). So, both the high NDF-ADF and the low nonstructural carbohydrate content significant decrease in the DMD and OMD values of these samples may be interpreted as structural carbohydrate negatively affects the digestion of the feeds comparing with ruminant. On the other hand, fruits and vegetables having higher DMD and OMD values may be seen as related to having high nonstructural carbohydrate fractions.In the study, the reason why rice husk has the lowest DMD and OMD may be thought to be due to its high ash concentration, and this situation also supports the high negative correlation between ash content and digestibility. Also, there may be thought that the DMD and OMD digestibility of the samples had a negative strong correlation with their structural carbohydrate

content (NDF and ADF or CF), and a strong positive correlation with their non-structural carbohydrate content (NFE and NSC).Although soybean has low NSC and high NDF content, its DM and OM digestibility is high. This may be explained by the fact that most of the structural carbohydrate content of the soybean hull consists of highly digestible pectin. Regarding the digestibility results of soybean hull (DMD 81.1% and OMD 79.1%) in this study confirm the mentioned by Bach et al. (1999) that because of high NDF digestibility of soybean hull can be used as a substitute for cereal bran in the concentrated feed fraction of the ration. Taken together, the nutrition composition and energy contents (ME_{CN} and ME_{CEL}) of the by-product feeds, fruits, vegetable, and crop residue were in line with the literature which declared that the content and amount of structural carbohydrates and also the type of non-fiber carbohydrate contents of a feed highly affectits digestibility (McDonald et al. 2012).

Data for concentrations of ME and have indicated that potato, rice bran, and fruits (carrot, fig, and grape) samples contained more ME_{CN} and ME_{CEL} than others, which may be a result of the high concentration of starch, sugar, and oil; and also low content fiber fractions in the by-product (NRC 2001; Wadhwa et al. 2015; Liu et al. 2018).The metabolizable energy values obtained by the calculation systems based on the chemical composition of feeds cannot accurately determine the true energy values, so an *in vitro* energy assessment was performed enzymatically and the experiment samples were similar or even slightly higher than the energy values found by the calculation.ME_{CN} and ME_{CEL} values are consistent with the results of NRC 2001, INRA 2004, and McDonald et al. 2012. As a study result, the rice bran, potato, fig, grape, and carrot had higher energy levels both of them (ME_{CN} and ME_{CEL}) as concentrated feeds on a dry matter basis. Interestingly, the grape pomace and grape stalk were seen haveto low DMD and OMD digestibility, but relatively higher energy values (both ME_{CN} and ME_{CEL}) compared to other samples.This situation can be explained as follows; because of the correlation between the energy values and nutrient contents of feeds, energy values of the samples decrease as the cellulosic or fibrous contents (NDF) increase; non-structural carbohydrate content (sugar or starch) increases as they rise (Weiss 1993). Also, Nicolini et al. (1993) decelerated that winery waste such as grape pomace and grape stalks, as a result of a decrease in lignin through the fungal treatment, the cellulose is better accessible to rumen micro flora and its DM digestibility is similar to forages.

The Ca concentrations of the research samples were observed a little variation in this study, and were similar to previously reported values (Macgregor 2000; NRC 2001; INRA 2004; Wadhwa et al. 2015). The citrus pulp and grape stalks had the highest Ca content than other samples. The contents of P in the by-product feeds, fruits, vegetable and crop residue observed in this study were within the range of previously published values (Macgregor 2000; INRA 2004; Wadhwa et al. 2015). Except for the rice bran and the potato, which contains an insufficient amount (1.4 and 1.9 g respectively) of Ca, all other research samples contained a higherquantity of Ca than the advisedrange for ruminants (0.40-0.80%, NRC 2001; 0.22-0.44%, NRC 1985). Contrary, in the study, the fruits (grapeand fig), by-products (soybean hull, apple pomace, citrus pulp, corn stalks, grape stalks, and rice hull) contained lower levels of P than recommended for dairy cattle (<0.22%, NRC 2001), while the remainder only had sufficient amount of P, exceptrice bran and cotton seed (6.6 and 4.6 g respectively). The dietary Caand P mineral metabolism in ruminant nutrition is closely related to each other. Their absorption and utilization in the animal body depend on the relative proportion (general calcium to phosphorus ratio, 2:1in diet) of the two minerals in the ration. Also, the Ca to P ratio on the absorption of calcium and phosphorus is a wide ratio that is not critical (unless Ca:P ratio of >7:1 or ≤ 1:1). Because this is considered acceptable in dairy cattle ration (NRC 2001). In the present study, the calcium to phosphorus ratio in all research samples wasenough large, except for potato, rice bran, and cottonseed. Thus, the concentrations of K, Mg, and Na relating to samples in the research were in line with the literature decelerated by Arosemena et al. 1995; NRC (2001), and INRA (2004). The corn stalks, citrus pulp, potato, rice bran, apple pomace, cottonseed, and grape pomace were deficient in Na (<0.16%, NRC 2001). In the research, the by-product feeds, fruits, vegetable, and crop residue samples contained a high amount of K, except the rice hull and the apple pomace, which were adequate to reference (<0.38%, NRC 2001) in K. Besides, in ruminants fed rations with high roughage content, this mineral deficiency is not observed, since the roughages contain high levels of potassium. In the research, because the potato, corn stalks and some b-products feeds (cottonseed, apple pomace, citrus pulp, and grape pomace) had a high Ca:P ratio and the lower content of Na; ruminants eating a relatively

large amount of these feeds would need to supply additional P and Na resources to deal with their possible deficiencies. The Mg concentrations of all study samples contained adequate or high quantity of Mg which from the minimum requirement level for dairy cattle by (<0.03%, NRC 2001).

The concentration of the Fe, Cu, Mn, and Zn investigated as micro minerals for samples in this study were close or similar to what is expected in the previous literature byNRC (2001), INRA (2004), and Soni et al. (2014). Among the micro mineral contents of research samples, the contents of Cu wasenough high (<11 mg, NRC 2001) in all study samples. The Zn mean values of the experiment samples in the trial are quite lowreferred to (<40 mg, NRC 2001). All experiment samples contained greater amounts of Fe concentration (>50 mg) than required for ruminants. Similarly, research samples had higher quantities of Mn (>40 mg, NRC 2001), apart fromthe whole cottonseed. These results show that all the by-product feeds, fruits, vegetables, and crop residue samples were rich in many macro-micro minerals.Overall, these by-products, fruits, vegetables, or crop residue to supply to ruminant ration adequate amount of a lot of minerals, except Mg, Fe, Cu, Mn. In general, taking into account the biological functions of minerals such as animal health, reproduction, and growth, rations should be supplemented with deficient minerals to avoid loss of production.

CONCLUSION

The increasing global food and feed require finding alternative energy sources, which has led to researches in the field of non-conventional feed materials as by-product feeds, waste fruits-vegetables, and crop residues. Therefore, based on this research results regarding the nutrition composition of some waste foods and by-product feed indicates that (i) the waste fruits, vegetable and by-products have valuable sugar (grape, fig, and carrot), starch (potato, rice bran), pectin (citrus and pulp) and oil (cottonseed) content that are the main compounds making them high energetic feeds; (ii) a significant portion of the research samples, especially fruits and vegetables, showed at least as much or higher DMD-OMD digestibility and metabolizable energy than roughages; (iii) a result showed that most of these research materials have an enough or much more macro and micro mineral concentrations for ruminant; (iv) using such waste fruit-vegetables or by-product sources as feed helps to reduce waste and minimize the adverse effect on the environment and their use will also decrease food-feed competition; (v) also using these feed sources in ruminant ration will raise the economic profitability and sustainability of the animal production.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Table 1. Nutritional composition of the by-products feeds, waste fruits-vegetable and crop residue (g kg^{-1}, in DM)

	DM	Ash	OM	EE	CP	CF	NFE	NSC	NDF	ADF	Starch	Sugar
Apple Pomace	229^{g}	24^{j}	975^{a}	46.0^{e}	80.5^{ik}	315.8^{e}	533.0^{d}	259.7^{e}	589.1^{cd}	449.2^{d}	22.3^{c}	58.8^{d}
Carrot	117^{j}	79^{c}	920^{h}	10.6^{gh}	82.3^{i}	92.3^{jk}	734.7^{b}	683.2^{b}	143.8^{i}	135.9^{h}	-	235.0^{a}
Citrus Pulp	214^{hi}	44^{h}	955^{c}	14.7^{g}	92.9^{h}	137.6^{h}	710.5^{c}	644.0^{c}	204.2^{h}	174.5^{f}	91.4^{d}	220.6^{b}
Corn Stalk	935^{a}	54^{f}	945^{e}	8.1^{ghi}	61.2^{l}	352.1^{d}	523.8^{d}	109.8^{g}	766.0^{a}	494.0^{c}	-	25.3^{g}
Fig	850^{d}	37^{i}	962^{b}	35.5^{f}	66.1^{l}	76.6^{k}	783.9^{a}	731.0^{a}	129.6^{i}	109.5^{i}	-	235.1^{a}
Grape	858^{d}	69^{d}	930^{g}	12.1^{gh}	75.6^{k}	94.4^{j}	748.2^{b}	575.6^{d}	267.0^{g}	227.0^{e}	-	201.1^{c}
Grape Pomace	491^{e}	55^{f}	944^{e}	75.7^{d}	142.5^{d}	281.4^{f}	445.1^{f}	118.8^{g}	607.7^{c}	550.0^{b}	12.7^{gh}	49.1^{e}
Grape Stalk	293^{f}	74^{d}	925^{g}	24.2	104.3^{g}	252.3^{g}	544.6^{d}	218.5^{f}	578.3^{d}	545.6^{b}	25.7^{fg}	20.3^{gh}
Potato	205^{i}	61^{e}	938^{f}	3.3^{j}	131.6^{e}	19.4^{l}	783.9^{a}	660.3^{c}	143.0^{i}	22.1^{j}	642.0^{a}	31.1^{f}
Rice Bran	912^{c}	88^{b}	911^{i}	159.8^{b}	154.5^{c}	120.3^{i}	476.6^{e}	249.2^{e}	347.7^{f}	143.3^{h}	279.8^{b}	61.1^{d}
Rice Hull	926^{ab}	193^{a}	806^{j}	5.3^{hi}	39.0^{j}	506.2^{a}	255.3^{h}	16.2^{i}	745.4^{a}	645.3^{a}	12.7^{gh}	5.9^{j}
Soybean Hull	919^{bc}	46^{gh}	953^{cd}	15.2^{g}	125.0^{f}	411.7^{c}	401.5^{g}	117.3^{g}	696.0^{b}	538.4^{b}	61.7^{e}	22.2^{gh}
Tomato Pomace	222^{gh}	54^{f}	945^{e}	84.5^{c}	196.3^{b}	454.2^{b}	206.4^{i}	56.3^{h}	608.0^{c}	549.3^{b}	26.6^{f}	17.2^{h}
Whole Cotton Seed	929^{ab}	49^{fg}	950^{de}	177.7^{a}	219.6^{a}	320.5^{e}	224.9^{i}	32.1^{i}	520.8^{e}	447.7^{d}	17.5^{fg}	19.7^{h}
SEM	31	3	4	5	4	14	18	25	21	19	16	8
P	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

^{a,k}Different superscripts indicate differences among the group means in the same row at p<0.05

Table 2. Mineral content of the by-products feeds, waste fruits-vegetable and crop residue

	Ca[1]	P[1]	Na[1]	K[1]	Mg[2]	Fe[2]	Cu[2]	Mn[2]	Zn[2]
Apple Pomace	5.5^{gh}	1.6^{h}	0.8^{f}	8.6^{g}	1.2^{g}	257^{d}	57^{fg}	49^{g}	10.7^{f}
Carrot	6.4^{g}	2.3^{e}	5.3^{a}	24.9^{a}	2.2^{efg}	163^{fgh}	66^{de}	51^{fg}	24.4^{abc}
Citrus Pulp	17.3^{a}	1.2^{i}	0.5^{f}	12.5^{f}	1.4^{g}	175^{efgh}	62^{ef}	71^{defg}	8.8^{f}
Corn Stalk	6.5^{g}	1.1^{j}	0.5^{f}	12.4^{f}	2.5^{ef}	619^{a}	54^{g}	57^{fg}	9.1^{f}
Fig	7.9^{f}	1.1^{ij}	3.3^{c}	14.3^{e}	1.6^{fg}	141^{h}	57^{g}	66^{efg}	20.8^{bcd}
Grape	13.1^{d}	1.8^{g}	4.5^{b}	21.0^{b}	5.9^{b}	372^{c}	75^{b}	81^{cde}	19.7^{cd}
Grape Pomace	10.7^{e}	2.1^{ef}	1.4^{e}	17.7^{c}	1.9^{fg}	234^{def}	71^{bc}	55^{fg}	13.4^{ef}
Grape Stalk	15.9^{b}	2.0^{fg}	4.6^{b}	21.3^{b}	2.9^{de}	241^{def}	72^{bc}	133^{a}	25.2^{ab}
Potato	1.9^{i}	2.5^{d}	0.6^{f}	22.1^{b}	1.6^{fg}	277^{d}	59^{fg}	50^{fg}	16.6^{de}
Rice Bran	1.4^{j}	6.6^{a}	0.6^{f}	14.8^{de}	8.4^{a}	212^{defgh}	56^{g}	115^{ab}	23.1^{abc}
Rice Hull	5.3^{gh}	0.4^{k}	2.6^{d}	6.9^{h}	1.2^{g}	222^{defg}	66^{de}	91^{cd}	26.1^{a}
Soybean Hull	14.6^{c}	1.2^{i}	3.2^{c}	16.1^{d}	3.6^{d}	509^{b}	69^{cd}	72^{def}	19.7^{cd}
Tomato Pomace	8.0^{f}	4.0^{c}	4.4^{b}	17.5^{c}	5.8^{b}	253^{de}	80^{a}	99^{bc}	19.6^{cd}
Whole Cotton Seed	4.7^{h}	4.6^{b}	0.9^{f}	14.8^{de}	4.7^{c}	152^{gh}	67^{d}	15^{h}	16.6^{de}
SEM	0.4	0.1	0.2	0.4	0.2	14	0.8	3	0.6
P	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

[a,k]Different superscripts indicate differences among the group means in the same row at $p<0.05$
[1]g kg^{-1}, in dry matter
[2]mg kg^{-1}, in dry matter

Table 3. Dry and organic matter digestibility and metabolic energy content of the by-products feeds, waste fruits-vegetable and crop residue

	DMD%	OMD%	ME_{CN} kcal[1]	ME_{CEL} kcal[1]
Apple Pomace	75.7^{d}	73.9^{d}	2104^{g}	2391^{f}
Carrot	94.2^{ab}	92.3^{ab}	2702^{cd}	3072^{bc}
Citrus Pulp	96.0^{a}	95.5^{a}	2654^{d}	3112^{bc}
Corn Stalk	44.6^{g}	37.5^{h}	1755^{h}	1858^{h}
Fig	92.3^{b}	91.3^{b}	2983^{b}	3134^{b}
Grape	74.2^{d}	67.3^{e}	2729^{c}	2872^{d}
Grape Pomace	38.1^{h}	26.1^{i}	2275^{f}	2543^{e}
Grape Stalk	43.1^{g}	25.9^{i}	2131^{g}	1857^{h}
Potato	95.8^{a}	95.7^{a}	3052^{a}	2995^{c}
Rice Bran	74.9^{d}	68.5^{e}	3118^{a}	3310^{a}
Rice Hull	10.3^{i}	22.8^{i}	640^{j}	723^{j}
Soybean Hull	81.1^{c}	79.1^{c}	1556^{i}	2171^{g}
Tomato Pomace	66.2^{e}	59.0^{f}	1647^{i}	1296^{i}
Whole Cotton Seed	58.5^{f}	50.9^{g}	2532^{e}	2759^{d}
SEM	2.3	2.4	64	30
P	<0.05	<0.05	<0.05	<0.05

[a,j]Different superscripts indicate differences among the group means in the same row at $p<0.05$
[1]:kcalkg-[1], in dry matter

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Research article
Ερευνητικό άρθρο

Identification of the *Nosema* spp., a microsporidian parasite isolated from the honey bees (*Apis mellifera*) and its association with honey bee colony losses in apiaries of Iran

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ABSTRACT: The aim of this study was to determine the *Nosema* species by microscopic and molecular method and its association with honeybee colony losses (Colony Collapse Disorder) in apiaries of Urmia, Northwest of Iran. For this purpose, honeybee samples were collected from 840 colonies kept in 120 apiaries in five different location of Urmia. The specimens were examined for the presence of *Nosema* spores. After DNA isolation, the 16S rRNA gene was evaluated using multiplex PCR. Total infection prevalence with the microscopic evaluation was 32% while in PCR test was 58.2%. *Nosema* positive samples were evaluated by PCR sequencing. Based on the results of PCR, all identified cases were *N. ceranae*. The obtained sequences were transferred to GenBank/NCBI (samples accession numbers MT001887 and MT001893). The results showed the prevalence of Colony Collapse Disorder like symptoms in the studied honeybee colonies were 13.33%. *N. ceranae* was detected by PCR in 20.28% of honeybee colonies with Colony Collapse Disorder like signs. Our findings showed that there was a significant relation between Colony Collapse Disorder and presence of *N. ceranae*. The results of this study concluded that *N. ceranae* is the only specie that affects the honeybees which may have an important role in the occurrence of collapse of bee families and depopulation of hives in this area.

***Keywords*:** Colony Collapse Disorder, Honeybee, *Nosema cerenae,* Iran.

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INTRODUCTION

Nosema species lead to a very serious disease in adult honeybees which is called nosemosis. The colonies are repeatedly infected, and all members of the colony are susceptible to this infection. The ingestion of spores through food or water is the main source of infection. Spores are germinated in the presence of physical and chemical features of the midgut and the vegetative stage of *Nosema* happens inside midgut cells (Chen et al., 2009). Just in two weeks after the infection, one can find 30-50 million of spores in a bee's midgut (Bailey and Ball, 1997). The spores also spread through the feces of the infected bees and create new source of infection (Chen et al., 2009). This parasite can cause digestive disorders and is a common reason for malnutrition, reduced longevity, and physiological aging and all these side-effects cause a reduction in the amount of honey produced by the bees (Hornitzky, 2008).

Nosema apis has long been known as a parasite which only affects Eurpean honeybees (*Apis mellifera*) (Matheson, 1996), but Asian honeybees, *A. ceranae* are mostly infected with *N. ceranae* parasite (Fries et al., 1996).The study conducted by Fries et al. (1996) reported *N. ceranae* which infects the adults of the eastern honey bee (*A.cerana*) in Beijing, China. In another report, Higes et al. (2006) noted that *N. ceranae* has infected *A. mellifera* in Europe. Other studies have reported *N. ceranae* from Asia and the Americas (Chauzat et al., 2007; Chen et al., 2008; Cox-Foster et al., 2007; Huang et al., 2007; Invernizzi et al., 2009; Teixeira et al., 2013; Emsen et al., 2016; Williams et al., 2008).

Since it is difficult to distinguish various *Nosema* species, various epidemiological and molecular investigations are conducted in order to identify the type of the species (Razmaraii et al., 2013). PCR is the most appropriate method used to detecting this microsporidian infection, since it can detect even the lowest levels of infection and it is also able to distinguish various stages of microsporidian life cycle (Aroee et al., 2017). The difference of *N. apis* and *N. ceranae* is related to their small subunit (16S) rRNA gene sequence (Fries et al., 2006), and this difference helps the researchers distinguish them (Paxton, 2010).

Due to evidence implicates the high colony losses or Colony Collapse Disorder (CCD) imposed by *N. ceranae* in the US and Europe, the occurrence of *N. ceranae* in *A. mellifera* is a one of the main points of interests of researchers working on honeybees all around the word (Chaimanee et al., 2010; Higes et al., 2008; Martín-Hernández et al., 2007; Prodanović et al., 2019).Rapid loss of adult bees from the colony is one of the main symptoms of CCD. There are no dead bees near or inside the colony. The final stages of CCD show that the queen is with a few recently grown up bees. The colonies which are collapsed often have some capped brood and food reserves. The first instance of CCD dates back to 2006, but there some initial reports regarding CCD in 2004. Some researchers hypothesize that CCD is the result of some unidentifiable infections (Coxfoster et al., 2007). In fact, the risk of colony depopulation is six times higher in colonies infected with *N. ceranae* than in uninfected ones (Martín-Hernández et al., 2007).

Previous studies used molecular techniques to investigate the presence of *N.apis* and *N. cerenae* in Iran (Aroee et al., 2017; Nabian et al., 2011; Razmaraii et al., 2013). The study by Nabian et al. (2011) was the first detection of *N. ceranae* in Iran. Previous reports regarding *Nosema* in Iran considered *N. apis* the only specie of *Nosema* which infected the honeybees (Lotfi et al., 2009; Razmaraii and Karimi, 2010; Tavassoli et al., 2009).The objective of this study was to identify *N. apis* and *N. ceranae* using light microscope examination and multiplex PCR in honey bee of Urmia, northwest of Iran.

MATERIALS AND METHODS

Study area

This investigation was done during spring and summer (March-July) of 2017 in the colonies of Urmia, West Azerbaijan province in Northwest of Iran. The study area is in a fertile agricultural region between 37º 32´ N and 45º 04´ E, with an area of 8000 km^2. The amount of rainfall in the area is 73.1 mm and the average relative humidity is 77% during different months of the year. The average temperature of the area varies from -3.8° C to +23.4° C in different seasons. The location is a four-seasoned area: cold (from January to March), spring (from March to June), summer (from July to September), and autumn (from October to December). The area shares border with Iraq and Turkey (Yakhchali and Hosseine, 2006) (Figure 1).The sampled apiaries were all the year in the same location.There was sufficient pollen and nectar in the studied area and the sampling beehives give a production of honey.

Collection of honeybee samples

This study was done in 840 colonies living in 120 apiaries in five different locations of Urmia. Samplings were done in accordance with the guidelines of the Office International des Epizooties (OIE, 2008). At least 60 adult forager bees were obtained from each of the colonies. Each sample was comprised of bees from the same hive. Three to six hives were sampled per apiary/location. Before the initiation of the sampling in each apiaries studies, for each sampled colony it was recorded if the characteristics of CCD (sudden disappearance of adult bees prior to colony death, unattended brood, colony weakness, no dead or trembling bees around the hives) were observed at the time of sampling and in the years afterwards (Stevanovic et al., 2010). Other data such as degree of infestation/apiary, in relation to bee losses, beekeeping manipulations or use of medicines for other diseases, such as *Varroa* and other symptoms like aggression, swarming were recorded for each sampled colony. The samples were immediately transferred to the parasitology laboratory of Urmia University.

Preparation of samples and microscopic assessment

The abdominal contents of twenty adult bees from the same colony were crushed in 4 ml of distilled water. The resulting solution was filtered via a two-layer muslin and then it was centrifuged for 20 minutes at 1000 g in order to remove the supernatant. precipitateof the separated spores were suspended again in 1.5 ml of distilled water and then it was transferred to a fresh tube (TUNCA et al., 2016). One drop of the sample was put under the microscope slide and it was examined for the spores of *Nosema* spp. under the magnification of 400X. Haemocytometer was used to count the number of spores (TUNCA et al., 2016). A software named Image Focus (V 2.0.0.0) was used to analyze spore size.

DNA isolation and PCR amplification

PCR of *N. apis* and *N. ceranae* was conducted on 16S rRNA locus. To this end, genomic DNA was extracted by a commercial kit (MBST, Iran), based on the manufacturer's instructions and isolated DNA was analyzed in order to confirm the *Nosema* species of the spores by Multiplex PCR as previously described using *N. apis* (321 Apis (FOR5'-GGGGGC ATG TCT TTG ACG TAC TATGTA 3', 321APIS REV 5'-GGG GGG CGT TTAAAA TGT GAA ACA ACT ATG-3') and 218 MITOC for *N. ceranae* (FOR 5'-CGGCGA CGA TGT GAT ATG AAA ATATTA A-3') 218MITOC: (REV 5'-CCC GGT CAT TCTCAA ACA AAA AAC CG-3') (Nabian et al. 2011).

It should be noted that multiplex PCR reaction was done in 20 µl of a solution with 10 ng of the template DNA, 10XPCR buffer (Fermentas, Germany), 2.5 mM $MgCl_2$, 0.4 µM of each of the primers, 0.3 mM dNTP mixture and 0.5U of *Taq* DNA polymerase (Fermentas, Germany) and high pure H_2O. The conditions for cycling was as follows: 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min with a final extension step of 72°C for 5 min. 1.5% agarose gel electrophoresis along with ethidium bromide staining and photography were used to investigate PCR products (Nabian et al., 2011). Positive controls were obtained from Tehran University and distilled water was chosen as the negative control for PCR amplification process.

DNA Sequencing

Two positive PCR products resulting from 16SrRNA gene were purified and subjected to sequencing with both forward and reverse primers by Sinaclon Co. (Tehran, Iran). Mega software (ver. 6) was used for the analysis of the sequencing results. The software was also used for comparison of the sequence with the reference sequence existing in the GenBank database which was the result of the BLAST analysis (refer to www.ncbi.nlm.nih.gov). Clustal O program was used to compare the partial nucleotide sequences with available sequences of *Nosema* species in other parts of the world (https://www.ebi.ac.uk/Tools/msa/clustalo).

Statistical Analysis

All data were analyzed by SPSS software, version 26.0 (SPSS Inc, Chicago, IL, USA). The prevalence of *Nosema* between colonies with CCD signs and colonies without CCD signs (or with and without disease) was analyzed by Chi-square. Difference were considered significant when $P < 0.05$.

RESULTS

The microscopic findings

Positive samples of the *Nosema* were found in all the five locations studied during the microscopic examination. The results of this diagnosis are shown in Table 1. *Nosema* spp. spores were found in 32.02% (269/840) hives of five location samples. The observations with light microscopy delineated that the

fresh spores of the *Nosema* were in the shape of a rod or had the oval shape and the average length of them were 4.2 ± 0.31 μm (mean ± SD) and the width were 2.1 ± 0.14 μm (mean ± SD).

The molecular findings

Molecular diagnosis of the honey bee samples were investigated using multiplex PCR for 16S rRNA gene of *Nosema* species. The PCR analysis showed that 488 of 840 beehives (58.09%) were positive for *N. ceranae* (Figure2), however, there were no positive apiaries for *N. apis*.

In this study, we used 16S rRNA gene to detect *Nosema* species successfully. The sequences of 16S rRNA gene were used to investigate diagnosis, and the PCR products were sequenced (Sinaclon Co., Tehran, Iran) and submitted to GenBank/NCBI (accession numbers MT001887, MT001893). The obtained sequences were compared with those of related species received from the GenBank and the diagnosis of *N. ceranae* were confirmed. The nucleotide sequences of the amplification products from the *Nosema* positive honeybee samples were 100% identical with the *N. ceranae* sequence deposited in the GenBank database. *N. ceranae* was reported in all the samples obtained from various locations of the study area.

The findings of Colony Collapse Disorder

The results of this showed that CCD prevalence in the studied honeybee colonies were 13.33% (112/840). The percentage of infected honeybee colonies detected by PCR which had CCD signs were 99/112 (20.28%). This study showed that there is a significant relation between *Nosema* infection and CCD ($p<0.05$) (Table 2).

DISCUSSION

The aim of performing this study was to determination prevalence of species of *Nosema* using microscopic examination and multiplex PCR based on small subunit rRNA (16SrRNA) and screening for significance of its association with CCD in apiaries of Urmia.

Previous studies used molecular techniques to investigate the presence of *N.apis* and *N. cerenae* in Iran (Aroee et al., 2017; Nabian et al., 2011; Razmaraii et al., 2013). The study by Nabian et al. (2011) was the first detection of *N. ceranae* in Iran. Previous reports regarding *Nosema* in Iran (Lotfi et al., 2009; Razmaraii and Karimi, 2010; Tavassoli et al., 2009) considered *N. apis* the only specie of *Nosema* which infected the honeybees. The replacement of *N. ceranae* with *N. apis* has been reported by various scientists all over the world (Martín-Hernández et al., 2007; Paxton, 2010). The difference in these two microsporidians is related to the speed by which *N. ceranae* causes colony death. *N. ceranae* weaken the colony and leads to its death. Furthermore, *N. ceranae* has more pathogenic features compared to *N. apis* and this leads to its expansion in various parts of the world (Paxton, 2010; Paxton et al., 2007; Whitaker et al., 2011).

The current study showed that *N. ceranae* is the only *Nosema* species found in northwest of Iran and no instance of *N. apis* have been found using molecular method from collected samples in years 2016 and 2017. The results are in line with that of Razmaraiiet al. (2013) and Nabian et al. (2011) which reported the species of *Nosema* in some other regions of Iran. The existence of different species is related to the pathogen features, because *N. ceranae* is highly pathogenic compared to *N. apis*. There is another potential reason for higher rate of *N. ceranae* infection. Since *N. ceranae* is mostly asymptomatic, many of the beekeepers do not pay enough attention to it and the infection rate increases in different colonies (TUNCA et al., 2016). The results of the study also suggest the colonization process of *N.ceranae* in this area and show that *N. cerenae* is not limited to its original host and has jumped from *A.ceranat*o *A. mellifera* and hence it has been expanded in the area studied (Martín-Hernández et al., 2007). The same results have been reported in other regions of the world by various researchers (Chen et al., 2008; Klee et al., 2007; Razmaraii and Karimi, 2010).

The analysis with light microscopy revealed that *N. cerenae* spores which are fresh have oval shape and they are rather uniform with respective length and width of 4.2 ± 0.31 μm and 2.1 ± 0.14 μm (mean ± SD). Studies conducted by Fries et al. (Fries et al., 2006; Fries et al., 1996), Higes et al. (2007) and Chen et al. (2009) have investigated the developmental and morphological qualities of *N. ceranae*. The study conducted by Chen et al. (2009) showed that *N. ceranae* spores are approximately 2.2-4.4 mm on fresh smears. The result of our study is in line with that of the Chen et al. (2009).

It should be noted that the prevalence of *Nosema*. spp. was 32% in microscopic examination, while it was measured as 58.2% in PCR. The potential reason

for this higher difference was due to high specificity and sensitivity of PCR amplification for detection and quantification of *Nosema*. spp (Chen et al., 2009). The prevalence of *Nosema* found in this research was lower than that in other previous studies in Iran. In a study conducted by Razmaraii et al. (2013) in East-Azerbaijan province or Iran, the prevalence of infection by PCR and microscopic examination was 67.1% and 58.1%, respectively.

In this study, *N. ceranae* isolate from Urmia region (Acc. No. MT001887 and MT001893) was showing maximum similarity (100%) with the sequence of *N. ceranae* isolate from Argentina (Acc. No. KX024757). Furthermore, in our study, the sequenced amplicons of *N. ceranae* (Acc. No. MT001887 and MT001893) were found to be 99.5% identical with the *N. ceranae* sequences previously published in Lithuania (Acc. No. JQ639308), Italy (Acc. No. HM859896) and North of Iran (Acc. No. JF431546) (Figure3). This was the first study of the type conducted in Urmia, northwest of Iran.

There is no definite answer to whether *N. ceranae* causes CCD per se or in combination with other factors like loss of the habitat or scarcity of floral resources (Paxton, 2010). Higes et al. (2008) were the first to note that *N. ceranae* can lead to collapse of bee colonies and they established a relationship between *N. ceranae* infection and death of honeybees. The weakness of the colony is mostly hidden until the queen loses its ability to replace the loss of the infected bees. Since the incubation period is asymptomatic, there are no signs before the collapse of the colony. Results of Three publications on Spanish honey bee colonies show that the Spanish honeybee colonies suffering from *N. ceranae* collapse in time period of 18 months after being infected (Higes et al., 2008, 2009; Martín-Hernández et al., 2007).

Our study shows that the prevalence of CCD in the studies area was 13.33% (112/840). Studies show that the occurrence of colony depopulation is different in various regions and this is indicated by different clinical and epidemiological patterns in Iran (Mohammadian et al., 2019).The study conducted by Mohammadian et al. (2019) showed that the occurrence of colony collapse in apiaries in various regions of Iran was 26.8%.

In our study, 99 out of 112 colonies affected with CCD were positive for *N. ceranae* (88.39%). Cox-Foster et al. (2007) were also unable to decide about the possibility of *N. ceranae* causing CCD alone or in combination with other factors. The results of our study does not agree with the results of the study conducted by Mohammadian et al. (2019) who believe that *N. ceranea* cannot be the cause of CCD. They concluded that presence or absence of *N. ceranae* does not affect the colony collapse.

CONCLUSION

Based on molecular data, our studies confirm that the microsporidia isolated from Urmia samples of honeybees are *N. ceranae*.The information obtained from this study and other similar studies help us improve management practices and they are also helpful in reducing the production cost. These results are also useful for beekeepers and forces them to use new hygiene policies to create an infection-free environment for bees.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Effect of some medicinal herbs on natural humoral immunity in turkeys

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ABSTRACT: The infectious diseases causes devastating economic losses in poultry industry. It is important to boost factors of natural immunity to improve defense to infectious diseases in birds. The experimental groups (105 turkey poults) allocated to 7 treatment groups, 3 replicates per treatment and 5 birds per replicate with initial body weight 49, 78 ± 0, 2 g) received basal diet plus 1% dry herbs or 0.01% essential oils supplements obtained from the herbs (Chamomilla; Rosmary; Lavender; Oregano; Thyme; St. John‘s Wort). Blood serum lysozyme concentrations, alternative pathway of complement activation (APCA) and betalysin activities were determined. The highest blood lysozyme concentrations were determined among the control group and groups supplemented with dry herbs - rosemary, oregano, and thyme. APCA activity was the highest in thyme-supplemented group followed by the non-supplemented one. Betalysin activities were the highest in groups receiving lavender, thyme, and oregano, while the lowest levels were found out in chamomile-supplemented birds. In groups treated with essential oils (EO), blood serum lysozyme concentration was the highest in the group treated with rosemary EO, followed by control group, while the lowest activity was established in birds that received EO from Saint John’s wort. The highest APCA activity was found in the lavender-treated group, and the lowest - in rosemary-treated turkeys. The highest betalysin activity was found among the groups treated with thyme, oregano and Saint John’s wort, and lowest activity was determined in the control group. It could be concluded that studied herbs possess an important immunomodulating potential in turkeys, which could improve their health and consequently, their productive performance.

Keywords: Betalysin, Complement, Herbs, Lysozyme, Turkeys.

INTRODUCTION

For the last few decades, the ban on using antibiotics in livestock husbandry and poultry farming in particular, has increased the interest to biotechnological and natural products with the purpose to improve birds' productivity, health, quality and safety of produce (Liu et al., 2011). Medicinal plants constitute a new class of growth promoters which recently has gained importance in food industry for production of functional foods. Improved antioxidant status of live animals along with increased oxidative stability of raw meat are considered useful for both consumers and processing industry. Research has been focused mainly on the effect of medicinal and aromatic plants on mortality, stress hormones, blood, and muscle metabolism, and even the immune system function of domestic animals. It is reported that tea (Tang et al., 2000), rosemary (Sevim et al., 2020) and lavender contain high concentrations of antioxidants and applied in various in vitro system models (Dorman et al., 2000) reduce oxidation of muscle fat in chickens. Rasouli et al. (2020) treated chicken broilers with different doses of water extract of salvia (*Salvia officinalis L.*) and found dose dependent enhancement of the immunity response of broilers. They also reported for bactericidal effect of sage extract against *Escherichia coli*. Similar results for positive effect of salvia on immune system in chickens reported Farhadi et al. (2020). At a global scale, numerous studies on effects of herbs and herbal products on various production traits in broiler chickens are published (Ocak et al., 2008; Moorthy et al., 2009; Ali, 2014; Mohamed, 2015). Wallace et al., (2010) demonstrated that plant extracts and different phytobiotics from leaves, roots, tubers or fruits of herbs, spices and other plants were excellent growth promoters in poultry farming. Some researchers reported chamomile effects on specific immunity of broiler chickens (Abdoul-Latif et al., 2011; Roby, 2013; Munir et al., 2014; Stanojevic et al., 2016). Dehkordi et al. (2009) provided evidence for a better antimicrobial effect of natural rosemary extract against Listeria monocytogenes if combined with unheated or heat-treated lysozyme at a low pH = 5. Matouskova et al. (2016) reported that encapsulated extracts of various herbs, including rosemary, had a marked antibacterial effect, which is increased in the presence of lysozyme. Mohamed et al. (2020) deliberates the different practical applications of a few medical herbs to improve the health state of poultry particularly as thermoregulatory and immunomodulatory agents. Yousefi et al. (2020) reported very detailed information on the application of lavender extract (LE) in carps before and after stress. Rostami et al. (2012) reported that oily extracts (OE) from lavender (*Lavandula officinalis*) and lemon balm (*Melissa officinalis*) exhibited high-efficient bactericidal activity against some bacteria. Lillehoj et al. (2011) and Hashemipour et al. (2013) investigated the effect of phytocompounds carvacrol, cinnamaldehyde and Capsicum oleoresin, on translational regulation of genes associated with immunology, physiology, and metabolism in an in vivo model of coccidiosis in chickens. The results provided clear evidence that isolated phytoderivatives had immunostimulating properties in chickens. Boskovic et al. (2015) investigated antibacterial effects of oregano and thyme essential oils (EOs) on *Salmonella Enteritidis, Salmonella Thyphimurium, Staphylococcus aureus, Staphylococcus aureus, Escherichia coli* and *Bacillus cereus* and found that EOs exhibited antibacterial activity against all tested microorganisms. Antimicrobial properties of thyme were discussed also by Rota et al. (2008). Jiang et al. (2012) investigated the effect of methanolic extract from of Saint John's wort on specific humoral immune response in chickens vaccinated against various avian influenza strains. Landy et al. (2012) carried out an interesting experiment for investigation of effects of using dried ground aerial parts from of Saint John's wort as alternative of nutritional antibiotics in poultry farming.

The lack of information about the effects of Saint John's wort (*Hypericum perforatum*), lavender (*Lavandula angustifolia*), chamomile (*Matricaria chamomilla*), thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*) and rosemary (*Rosmarinus officinalis*) on natural humoral immunity in turkeys was the incentive for this research. The aim of the current study was to evaluate blood serum concentrations of lysozyme, activity of the alternative pathway of complement activation (APCA) and betalysin activity in turkeys, whose rations were supplemented with either 1% dry herbs or 0.01% herbal essential oils.

MATERIALS AND METHODS

Experimental Design

Turkeys were reared in the poultry farm of the Agricultural Institute, Stara Zagora. A total of 105, 1 day-old of age female turkey poults were weighed individually and were randomly allocated to 7 treatment groups (3 replicates per treatment and 5 birds per replicate with initial body weight 49, 78 ±

0, 2 g) until 126 day old, namely control (C) and six experimentals. The treatment of turkey poults starts from the 1st day and lasts until 126 days of age. Control group turkeys received basal diet without herbs (nutritional program NRC was used). The composition of the basal diet is presented in Table 1. All diets were in mashed form. The experimental groups received basal diet plus 1% supplemented with dry herbs (see table 2) or 0, 01% essential oils (Eos) (see table 3) obtained from the same herbs (*Matricaria chamomilla*; *Rosmarinus officinalis*; *Lavandula angustifolia*; *Origanum vulgare*; *Thymus vulgaris*; *Hypericum perforatum*). We use commercial Eos (Nature energie LTD, Bulgaria; ALTEYA ORGANICS LTD, Bulgaria). Each group was placed to a clean floor pen, in a brooder ring for the first seven days (5 birds in ring), with equal floor space, one feeder, one drinker and one heating lamp for each ring. After day 7, the rings were removed and the turkeys were reared together as a group. Birds had *ad libitum* access to feed and water and lighting was provided continuous. The experiments were conducted within standard ethical norms and no birds were subjected to undue stress. The minimum requirements for the protection and welfare of experimental animals and the requirements for facilities for their use, keeping and /or supply are set out in Ordinance № 20 of 1.11.2012 on the minimum requirements for protection and welfare of experimental animals and the requirements for sites for use (8.1.2018), breeding and/or delivery, which transposes Directive 2010/63/EU.

Assay methods

At the end of the fattening at 18 weeks of age, blood samples for analyses were collected from *v. subcutanea ulnaris* from 6 turkeys from each group to assay some parameters of humoral innate immunity (totally 42 blood samples). Serum lysozyme concentrations were determined by method of Lie et al. (1985 see Fig. 1), alternative pathway of complement activation (APCA) was evaluated by method of Sotirov (1986) and betalysin activity were assessed by method of Buharin et al. (1977). All these methods were described in detail in our previous publication (Bozakova et al., 2020).

Statistical analysis

Data were processed by one-way analysis of variance (ANOVA) with the fixed effect model using Data analysis tool pack, Microsoft Excel 2016, Microsoft Corporation Ltd. at a level of significance $P<0.05$.

RESULTS

The results from Table 2 demonstrate that the highest lysozyme concentrations were those of control group and groups treated with rosemary, oregano, and thyme. Levels following lavender and chamomile treatments were lower, yet between-group differences were insignificant. The alternative pathway of complement activation (APCA) exhibited the highest activity in the group that received thyme ($P<0.001$), followed by the control group. Relatively lower activities were observed in birds supplemented with oregano ($P<0.01$) and rosemary ($P<0.001$). The results showed that tested herbs had a considerable effect on the activity of this important element of innate immunity in turkeys. Data for betalysin activities showed maximum values in the groups treated with lavender, oregano, and thyme ($P<0.001$), and lowest activity in chamomile-treated birds. Obviously, this parameter was also influenced significantly by tested medicinal herbs.

Table 1. Composition of the basal diet

Growing period	Crude protein (%)	Metabolic energy (kcal/kg)
Starter 1 from 1 to 21 day- old turkey	28	2800
Starter 2- from 22 to 42 day- old turkey	26	2900
Starter 3 - from 43 to 63 day- old turkey	24	3000
Grower 1 from 56 to 84 day old turkey	22	3100
Grower 2 - from 85 to 100 day old turkey	19	3250
Finisher from 101 to 126 day old turkey	17	3350

Table 2. Effect of some dry medical herbs on lysozyme concentrations, APCA and betalysin activity in turkeys

Groups	Investigated traits		
Dry herbs (1%)	Lysozyme (mg/L)	APCA (CH50)	Beta-lysin (%)
Control	4,29 ± 0,46	716,05 ± 8,84	8,94 ± 1,2
Matricaria chamomilla	3,77 ± 0,28	712,35 ± 40,41	7,02 ± 0,9
Rosmarinus officinalis	4,29 ± 1,09	634,47 ± 19,53	11,79 ± 1,2
Lavandula angustifolia	3,44 ± 0,89	707,41 ± 11,63	17,04 ± 1,7
Origanum vulgare	4,38 ± 0,62	661,42 ± 28,06	16,39 ± 0,5
Thymus vulgaris	4,29 ± 0,46	718,94 ± 20,07***	15,42 ± 0,3
Hypericum perforatum	3,46 ± 0,44	573,6 ± 12,19	18,05 ± 1,5***

P < 0,001 - the superscripts within a column indicate statistically significant differences among the groups

Table 3. Effect of essential extracts obtained from some medical herbs on lysozyme concentrations, APCA and beta-lysin activity in turkeys

Groups	Investigated traits		
Essential extracts from herbs (0,01%)	Lysozyme (mg/L)	APCA (CH50)	Beta-lysin (%)
Control	4,29 ± 0,46***	716,05 ± 8,84	8,94 ± 1,2
Matricaria chamomilla	3,7 ± 0,58	612,92 ± 12,85	16,22± 5,6
Rosmarinus officinalis	5,45 ± 2,46	592,24 ± 7,37	15,57 ± 0,8
Lavandula angustifolia	3,49 ± 0,57	779,28 ± 18,84***	14,79 ± 2,02
Origanum vulgare	4,44 ± 0,66	667,86 ± 9,4	19,31 ± 1,1
Thymus vulgaris	3,39 ± 0,35	676,54 ± 14,72	19,79 ± 2,3***
Hypericum perforatum	2,29 ± 0,28	601,67 ± 22,11	19,04 ± 1,8

*** - P < 0,001 - the superscripts within a column indicate statistically significant differences among the groups

Table 3 present the results from supplementation of turkeys with essential oils of the same herbs. The highest serum lysozyme was observed in the group treated with rosemary essential oil, followed by untreated group, and the lowest level of lysozyme was that of Saint John's wort-treated birds. Statistically significant differences were found only between controls and Saint John's wort-treated turkeys (P<0.001). Although the highest mean value was found in the group treated with rosemary essential oil, it was not statistically significantly compared to that of the Saint John's wort group due to the presence of a single individual with exceptionally high serum lysozyme concentration (17.656 mg/L). The highest APCA activity was observed in lavender-supplemented turkeys (P<0.001), whereas the lowest in rosemary-treated birds. With regards to betalysin, the highest activity was found in the groups supplemented with thyme, oregano, and Saint John's wort essential oils (P<0.001), and the lowest one - in control group.

DISCUSSION

Matouskova et al. (2016) reported that encapsulated extract of various herbs, including chamomile had a marked antibacterial effect that is increased in the presence of lysozyme. Our results confirmed that dry chamomile and chamomile essential oil possessed immunomodulating properties on blood serum lysozyme in turkeys (Tables 1 and 2). Primo et al. (2018) let us know that lysozymes are enzymes that break down the bacterial cell wall and disrupt the bacterial life cycle by cleaving the linkage between the N-acetylglucosamine and N-acetylmuramylpentapeptide carbohydrates. So, adding medical herbs to diet of turkeys will increasing the serum lysozyme concentrations and will improve their resistance to infectious diseases. This fact explains the similarities between our results and these one obtained by Matouskova et al. (2016). Other researchers reported chamomile effects on specific immunity of broiler chickens (Abdoul-Latif et al., 2011; Roby, 2013; Munir et al., 2014; Stanojevic et al., 2016) which, in some instances are contradictory. For instance, Mahmmod (2013) reported that chamomile did not influence antibody titers against Newcastle disease, and 4 years later reported the exact opposite results (Mahmmod et al., 2017). In

the available literature, no research studies were found on the effects of dried chamomile and chamomile essential oils on APCA activity. It is acknowledged that APCA is the primary humoral means for control of viruses, virus-infected cells, Gram-negative bacteria, cancer cells etc. (Sotirov et al., 1998; Andonova et al., 2001; Goundasheva et al., 2002; Yotova et al., 2004; Bozakova et al., 2018). Matouskova et al. (2016) reported that encapsulated extracts of various herbs, including rosemary, had a marked antibacterial effect, which is increased in the presence of lysozyme. Our results confirmed that dried rosemary and especially rosemary essential oil exerted an immunomodulating effects on serum lysozyme concentrations in turkeys (Tables 1 and 2). This correspondence in the results can be explained again by the information published by Primo et al. (2018). Ayoub et al. (2019) have investigated the effects of dried and ground leaves from moringa (*Moringa oleifera*), rosemary (*Rosmarinus officinalis*) and curcuma (*Curcuma longa*) on immune parameters of Nile tilapia before and after infection with *Aeromonas hydrophila*. The results showed that serum concentrations of albumin, globulins and total protein were statistically significantly higher compared to those in the control group. Also, serum lysozyme and respiratory activity were statistically significantly higher in treated groups than in control fish. Shokrollahi et al. (2015) investigated the effect of rosemary extract (*Rosmarinus officinalis*) on weight, haematological parameters, and cell-mediated immune response in newborn goat kids. The results confirmed that rosemary essential oil added to the milk of kids exerted a positive effect on their immunity. Franciosini et al. (2016) established positive effect of aqueous extracts of oregano (*Origanum vulgare L.*) and rosemary (*Rosmarinus officinalis L.*) on immune functions and intestinal microbial population of broiler chickens.

Yousefi *et al.* (2020) reported that application of lavender extract (LE) increased total white blood cell counts, plasma globulins, APCA activity, serum lysozyme concentrations in carps before and after stress.

Hashemipour et al. (2013) evaluated the effect of a phytogenic product containing a mixture of equal parts of thymol and carvacrol applied at 4 dose rates (0, 60, 100, and 200 mg/kg) to broiler chickens. The authors concluded that combination thymol and carvacrol improved immune response of broilers. Haghighi et al. (2018) and Ali et al. (2018) investigated the effect of oregano extract on total serum protein, albumin and globulins, respiratory activity, phagocytic activity and serum lysozyme in rainbow and reported improved growth performance, increased serum lysozyme concentrations, increased total antibodies and better survival rate.

Borugă et al. (2014) analyzed the chemical composition and antimicrobial properties of essential oil isolated from thyme (*Thymus vulgaris*), cultivated in Romania and found that thyme essential oil had an antimicrobial effect and could be used as source of natural antiseptic substances. Chun et al. (2001) have carried out a unique experiment that demonstrated anticomplementary activity (inhibition of complement system activation) of a polysaccharide isolated from thyme leaves (*Thymus vulgaris L.*). The isolated polysaccharide has inactivated both pathways of complement activation - the classical and the alternative. The analysis of our results on APCA activity in turkeys treated with thyme (dried and essential oil) presented in Tables 1 and 2 show that APCA in turkeys treated with dried thyme was the highest (718.94 CH50), whereas birds treated with essential thyme oil - average (676.54 CH50) with statistically significant differences ($P<0.05$). The results suggested that possibly, thyme essential oil contained a small amount of the polysaccharide reported by Chun et al. (2001).

Jiang et al. (2012) investigated the effect of methanolic extract from of Saint John's wort on specific humoral immune response in chickens vaccinated against various avian influenza strains. They found that the application of the extract as dietary supplement during the immunization period enhanced the effect from vaccination against avian influenza. Landy et al. (2012) carried out an interesting experiment for investigation of effects of using dried ground aerial parts from of Saint John's wort as alternative of nutritional antibiotics in poultry farming. It was observed that this herb improved feed conversion, increased antibody titer against avian influenza, decreased blood cholesterol concentration compared to chickens treated with a nutritional antibiotic (flavophospholipol). Other researchers (Shang et al., 2012) attempted to treat chickens infected with Gumboro disease virus (IBDV BC-6/85) by applying Saint John's wort extract. According to the results, Saint John's wort extract applied at 1330 and 667.9 mg/kg resulted in statistically significant therapeutic response and improvement of immune functions of infected chickens. Mohammadi et al. (2020) treated Nile tilapia (*Oreochromis niloticus*) juveniles with

extracts of *Hypericum perforatum* (HP), *Origanum vulgare* (ORG), and *Melissa officinalis* (MOF) and found that ORG 0.5 % and MOF 0.5 % groups showed a significant increase in serum total protein, and alternative complement activity (ACH50). Serum lysozyme levels were also increased in all groups fed on phytogenic diets. The MOF 0.5 % group showed the highest activities of skin mucus lysozyme and ACH50. ORG 0.5 % and MOF 0.5 % groups showed the highest protection levels of fish following challenge with pathogenic *Aeromonas hydrophila*. The authors concluded that both ORG and MOF extract at a 0.5 % level can effectively improve the growth, health, and immune status of Nile tilapia juveniles.

Some authors try to explain the mechanisms of immunostimulation of medicinal plants. For example according to Kumar et al. (2012) the main substances of medical plants are alkaloids, polysaccharides, cannabinoids, triterpenoid saponins, glycosides. Mentioned substances which acts on immune system by different ways for example increasing of serum immunoglobulin levels, neutrophil adhesion, phagocytosis, total number of T-helper and T-suppressor cells, activation of macrophages, inhibition of C3 convertase of the classical complement pathway, induction of cytokine (TNF-a, IFN-g), lymphoid cells stimulation, cellular immune function enhancement and nonspecific cellular immune system effect, increase immunoglobulin production, nonspecific immunity mediators and natural killer cell numbers. Sharma et al. (2017) in their review also showed that alkaloids, flavonoids, terpenoids, polysaccharides, lactones, and glycoside products possessed immunomodulatory properties. For example, the root extract of *Astragalus membranaceus* was found to lower IL-6 in in vitro human model (IL-6 is inflammatory and impending deterioration marker). Garlic (*Allium sativum*) is found to lower IL-1 and IL-6, TNF, IL-8 acting as anti-inflammatory inhibitor and boosting effect on IL-10 which is an antagonist to pro-inflammatory cytokines. Megna et al. (2012) tested the effect of Echinacea, Rhodiola and Ginseng on the immune system of athletes especially in endurance sports in relation to exercise and reported that *Echinacea purpurea* is stimulating on all cytokins as to the root, while the leaf has an immunosuppressive action. *Rhodiola rosea* stimulates all the cell lines of the immune system like Echinacea. Trinh et al. (2020) investigated the effect of herbal formulation KM1608 (alcohol extract from *Saussurea lappa, Terminalia chebula*, and *Zingiber officinale*) on RAW264.7 murine macrophages and showed that KM1608 stimulates the expression of immune cytokines (interferon (IFN) -α, β, IL-1β, IL-6, IL-10 in macrophages. Bozakova et al. (2018) reported that preparation Immunobeta (CHEMIFARMA S.p.A., Animal nutrition products, Italy) which consist ß-glucans and mannan oligosaccharides (they are produced by bacteria, yeast, fungi, and many plants) enhanced serum lysozyme concentrations, the activity of alternative pathway of complement activation and betalysine, IgM and IgG immunoglobulins in layer hens. Immunomodulatory effect of ß-glucans and mannan oligosaccharides is based on activation of neutrophils, B cells, T cells, and natural killer cells. They also enhance cytotoxic activity and inflammatory cytokines of primary macrophages and RAW264.7 cell lines (Kim et al., 2011).

CONCLUSION

Based on obtained results, it could be concluded that studied herbs possess an important immunomodulating potential in turkeys, which could improve their health and consequently, their productive performance.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest and disclose that we have not any financial and personal relationships with other people or organizations that might inappropriately influence or bias our work.

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Research article
Ερευνητικό άρθρο

The synergistic effect of probiotic and phytobiotic for improving growth performance and biological indices in broiler chickens

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ABSTRACT: The use of phytobiotics and probiotics show positive effect on the performance in poultry production and can produce healthy protein sources for humans. In this study, the effects of both commercial probiotic and phytobiotic were evaluated on a chicken experimental model. A total of 300 chicks were divided into 4 groups that fed the basal diet, diet containing probiotic (Protexin®), phytobiotic (garlic extract and Tichoke®), and probiotic plus phytobiotic all over the growing period. The growth indices were measured weekly, analyzed at the 21 and 42 days of age. At 42 days of age, blood samples were collected from all chickens. The concentration of liver enzymes, lipid profiles and antioxidant status were measured in blood samples. Results showed that the weight gain was significantly higher and FCR significantly lower in chickens which received probiotic alone or probiotic plus phytobiotic complex in comparison with chickens which received phytobiotic alone or control chickens ($p<0.05$). Furthermore, the addition of phytobiotic plus probiotic showed a significantly increase of blood GPx and TAS level in comparison to chickens that received probiotic or phytobiotic, alone. The level of TG, CHL and LDL were lower and the HDL value was higher in chickens which received phytobiotic or probiotic plus phytobiotic in comparison to chickens fed with probiotic or control chickens ($p<0.05$). The level of AST, ALT and ALP showed a significant decrease in chickens fed with probiotic plus phytobiotic. In conclusion, the continuous administration of the studied probiotic and phytobiotic in broiler diets can induce a synergistic effect on growth improvement, increasing of antioxidant capacity, reducing of serum lipids, and improvement of liver function in broiler chickens.

***Keywords*:** Antioxidant, Chicken, Growth Performance, Phytobiotic, Probiotic.

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INTRODUCTION

Antibiotics are commonly used in the poultry industry to prevent, and treat infectious diseases (Ben *et al.*, 2019). Some antibiotics promote growth of chickens; reduce mortality by reducing the level or activity of gastrointestinal pathogens, ultimately reducing the absorption of bacterial toxins, improving digestion, and absorption of nutrients (Gholami-Ahangaran *et al.*, 2019; Barton, Hart 2001). In addition, some antibiotics, which play a key role in boosting the immune system, are thought to stimulate the immune response (Gholami-Ahangaran *et al.*, 2020).

Probiotics have long been used as a live beneficial microbe in livestock. In poultry diets, it has positive effects improving the microbial balance of the gastrointestinal tract, (Sekhin *et al.*, 2010). According to the WHO (2001), probiotics are living microorganisms that can cause positive effects in the host. Probiotics may contain one or more strains of bacteria or yeast (Sánchez*et al*., 2017). The bacteria species commonly used as probiotics include *Bacillus*, *Bifido bacterium*, *Lactobacillus*, *Enterococcus, Pediococcus, Leuconostoc, Escherichia coli* and *Streptococcus*, whereas the yeast species, mainly include *Saccharomyces cerevisiae* and other *Saccharomyces* species (Pandey *et al.*, 2015; Fijan, 2014, Villena, Kitazawa, 2017). Probiotics benefits include the reduction of the dominance of pathogenic bacteria, balance of microbial populations, and stimulation of immune responses (Sekhon *et al.*, 2010).

Another alternative to the use of antibiotics can be herbs, and their derivatives (Ghoalmi-Ahangaran *et al*., 2020). The use of medicinal plants in poultry production provides beneficial effects for poultry due to the presence of phenolic, terpenoid and alkaloid compounds (Gheisar, Kim 2018). Phytobiotics include a wide range of plant-derived products which are added to poultry diets to enhance growth performance, improve meat quality, showing immunostimulatory, antimicrobial, anti-inflammatory, and antioxidant properties (Windisch *et al.*, 2008; Kumar *et al.*, 2014). The chemical composition and expected effects of the used phytobiotics depends on the botanical family, part of the plant used (flowers, leaves, seeds, etc.), geographical origin, and harvest season (Bakkali *et al.*, 2008).

Phytobiotics can increase growth by improving taste, increasing the secretion of digestive enzymes (Czech *et al.*, 2009), and modulation of enteric pathogenic bacteria (Ghoalmi-Ahangaran *et al*., 2020). The aim of our study was to evaluate the effectiveness of a commercial standard probiotic (named Protexin®) and a phytobiotic (an artichoke extract named Tichoke® plus garlic extract) on several health indices in broiler chickens.

MATERIAL ANDMETHODS

Study design

The study was undertaken with approval from the Islamic Azad University, Shahrekord Branch ethics committee for care and use of animal for research (Ethical No.: 95-711).

In this study, 300 one-day old broiler chicks (Ross 308) were randomly divided into 4 groups with 5 replicates, so that 15 chickens were allocated in each replicate until 42 days of age. All groups of chickens received freely feed, which was balanced according to Ross 308 production manual (Ross 308 Broiler Nutrition Specification, 2019), *ad libitum* sanitized drinking water, reared under same growing condition comprising continuous lighting program, mechanical ventilation, at least 50% air humidity, and comfortable temperature (Starting from 32 ℃ and gradually decreased to 21 ℃ until end of growing period). All chickens received Newcastle disease (ND) vaccines at 7 (Hiprviar B1, B1 strain), 18 (Nobilis ND Clone 30, Clone 30 strain), 35 (Cevac New L, LaSota strain) days of age.

Chickens of the group one (G1) received a commercial probiotic (Protexin®, Probiotics International Ltd., UK) according to the manufacturer's recommendation. Chickens of the group2 (G2) received phytobiotic (complexing of prepared garlic extract 100 mg/L, and artichoke extract; Tichoke®, Goldaru, Iran, 100mg/L). Chickens of the group 3 (G3) received the mentioned probiotic and mentioned phytobiotic by using both mentioned doses. Finally, chickens of the group4 (G4) did not receive any additives in the basal diet and were considered as negative control. The weight gain (WG), feed intake (FI), feed conversion rate (FCR) were measured weekly, and calculated at the 21and 42 days of age.

At the 42 days of age, chickens of the four groups were weighed, and non-heparinized and heparinized blood samples were taken from wing vein. The serum samples obtained from the non-heparinized were utilized for measuring of humoral antibody against ND vaccine by using the hemagglutination inhibition test according to Allan and Gough (1976). For separation of plasma, the heparinized blood samples were cen-

trifuged at 3, 000 × g for 15 min at 4◦C. All samples were stored at -80◦C until analysis was carried out.

The concentration of plasma total protein (TP), triglyceride (TG), cholesterol (CHL), high-density lipoprotein (HDL), low-density lipoprotein (LDL), aspartate transferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) levels were determined by commercial kits (Pars-Azmoon Co., Tehran, Iran) in a spectrophotometer (Technicon RA1000, H83014 model, Technicon Industrial Systems, Tarrytown, NY), according to the instructions of the manufacturer. Furthermore, the plasma total antioxidant status (TAS) was determined using Randox total antioxidant capacity test kit (Randox Laboratories Ltd., Crumlin, UK) as described by Miller *et al.* (1993). The blood superoxide dismutase (SOD) activity was measured by Ransod spectrophotometric kit (Ransod, Randox Laboratories Ltd., Crumlin, UK), according to the method previously described by Woolliams *et al.* (1983). The blood Glutathione peroxidase (GPx) activity was assessed by Ransel spectrophotometric kit (Ransel, Randox Laboratories Ltd., Crumlin, UK) as described by Paglia and Valentine (1967).

Statistical analysis

All data were analyzed with the One-way ANOVA method, using SPSS (version 22) statistical package (SPSS Inc., Chicago, IL, US). Significant differences among the treatments were recognized at $P < 0.05$ using Tukey test.

RESULTS

Growth performance

At the growth indices in 21and 42 days of age there was no statistical differences between the chickens of the different groups during the growing period, while the WG and FCR showed statistically significant differences between both ages.

At the 21 days of age, the weight gain was significantly higher in chickens which received the probiotic, or the combination of the probiotic plus phytobiotic in comparison with chickens which only received the phytobiotic or control chickens (P<0.05).

The comparison of WGs in all treated groups at 42 days of age showed the highest WG and it was obtained in chickens which received both probiotic and phytobiotic that it did not show significant differences with chickens fed with probiotic only.

The FCR data in 21 and42 days of age representsthe same pattern as the lowest FCR was noted in chickens which received both probiotic and phytobiotic, and showing no statistically significant differences with those chickens which received probiotic only (Table 1).

Biochemical parameters

The TP in all treated chickens was higher than the TP in the control group. In chickens which received both probiotic and phytobiotic, the TG, CHL, LDL were lower, and the HDL was higher than those parameters observed in chickens only fed with probiotic or the control chickens (p<0.05) (Table 2).

The comparison of the ALT, AST and ALP between the different treated groups showed that chickens that received diet supplemented with phytobioticor probiotic did not have any significant differences with those parameters of the control chickens or chickens which received both probioticand phytobiotic. The minimal value of the ALT, AST and ALP was noted in chickens which received both probiotic and phytobiotic that was significantly lower than the ones of the control group (P<0.05) (Table 2).

The concentration of SOD in all treated groups did not have any significant difference with SOD in control chickens. The GPx and TAS concentration was significantly higher in chickens fed with probiotic alone, phytobiotic alone and probiotic plus phytobiotic. Indeed, the addition of both phytobiotic and probiotic can significantly increase the activity of GPxand TAS in comparison with chickens that received probiotic or phytobiotic alone (P<0.05) (Table 2).

Finally, The IgG antibody titer against ND vaccine in control group was significantly lower than other groups (P<0.05). There was no significant difference in ND titers between chickens which received probiotic alone, phytobiotic alone and probiotic plus phytobiotic (Table 2).

Table 1. The growth parameters in broiler chickens fed with probiotic and phytobiotic at the 21 and 42 days of age

Index/ Groups	Feed intake (gr)		Weight gain (gr)		FCR	
	21 days of age	42 days of age	21 days of age	42 days of age	21 days of age	42 days of age
Probiotic	858±20[a]	3620±150[a]	729±9[a]	2234±81[a]	1.16±0.04[b]	1.59± 0.03[b]
Phytobiotic	863±31[a]	3510±183[a]	701±15[b]	2001±83[b]	1.22± 0.03[a]	1.73±0.02[a]
Probiotic plus Phytobiotic	873±30[a]	3610±186[a]	743±19[a]	2284±90[a]	1.16±0.04[b]	1.58±0.03[b]
Control	882±18[a]	3588±202[a]	710±18[b]	2055±121[b]	1.24±0.02[a]	1.70±0.03[a]

• The different superscript in each column represents significant differences between treatment group (P<0.05).

Table 2. The biochemical parameters in broiler chickens fed with probiotic, phytobiotic and probiotic plus phytobiotic at the 42 days of age

Index/Group	Probiotic	Phytobiotic	Probiotic plus Phytobiotic	Control
TP (gr/dl)	4.14±0.50[a]	3.61±0.42[ab]	4.11±0.45[a]	3.11±0.22[b]
TG (mg/dl)	94±11[b]	72±15[c]	59±19[c]	125±14[a]
CHL (mg/dl)	153±26[a]	130±20[b]	133±22[b]	169±41[a]
HDL (mg/dl)	69±15[b]	75±23[ab]	82±17[a]	65±23[b]
LDL (mg/dl)	63±19[a]	44±14[b]	43±19[b]	78±20[a]
ALT (U/L)	4.50±0.7[ab]	4.30±0.6[ab]	3.90±0.7[b]	5.00±0.8[a]
AST (U/L)	152±26[ab]	135±43[ab]	130±46[b]	166±32[a]
ALP (U/L)	2.90±0.23[a]	2.77±0.34[a]	2.53±0.23[b]	2.95±0.25[a]
SOD (U/mg Hb)	1145±90[a]	1168±103[a]	1175±127[a]	1106±90[a]
GPx (U/mg Hb)	188±26[b]	139±27[b]	200±20[a]	103±24[c]
TAS (mmol/L)	0.74±0.06[b]	0.77±0.06[b]	0.88±0.08[a]	0.49±0.08[c]
HI titer (NDV)	4.04±1.00[a]	4.22±1.01[a]	4.69±1.54[a]	2.30±1.32[b]

• The different superscript in each line represents significant differences between treatment group (P<0.05).

DISCUSSION

In our study, the continuous administration of probiotics (Protexin®) or probiotic (Protexin®) plus phytobiotic (garlic extract and Tichoke®) in poultry diets increases growth indices by improving FCR and WG. The consumption of this phytobiotic has no effect on growth indices when was administrated alone in broiler chickens in our experimental model.

The current literature related to the effect of probiotics on growth indices showed very diverse results of using probiotic in poultry diets from not affecting on growth indices to improvement in all growth indices of treated birds (Gunal *et al.,* 2006; Shargh *et al*., 2012). Gunal *et al.* (2006) and Shargh et al. (2012) reported that the use of probiotic has no effect on growth indices, while Khosravi *et al.* (2010) showed that it has no effect on feed intake and final weight but increases food efficiency. In another report, Murry *et al.* (2006) stated that *Lactobacillus*-based probiotics increased food efficiency and reduced FI. Also, Awad *et al.* (2009) noted the improvement in all growth indices following dietary supplementation with a *Lactobacillus*-based probiotic in chickens. The wide variation in the results of these studies on probiotics seems to be affected by several factors such as growing conditions, diet ingredients, types of probiotics, gastrointestinal pH, presence of stressors, administrated dose, and period of probiotic administration (Wang *et al.*, 2017).

Addition of phytobiotic complex in broiler diets enhanced growth performance at 21 and 42 days of age. The beneficial effect of phytobiotic complex may be partially related to garlic. In a study, Ziarlarimi *et al*. (2011) stated that garlic powder included many organosulfur compounds such as allicin, alliin, ajoene, diallylsulfide, dithiin, and S-allylcysteine, so that garlic as a natural plant-derived feed additive may be successfully used to improve broiler growth performance. The obtained results are in agreement with the findings of Khan *et al.* (2012) who observed that broiler treated with garlic powder had significantly higher live weight gain than control group. The same trend was also found by Suriya *et al.* (2012) who used 0.5% garlic powder in broiler diet. The better effect of garlic as natural feed additives might be due to increased enzymes activity of pancreas, which offer a better environment for digestion and absorption of nutrients (Ismail *et al.,* 2021).

Various studies have been performed to elucidate

the effects of the artichoke plant in poultry (Nadia *et al.*, 2007; Fallah *et al.*, 2013; Khoramshahi *et al.*, 2015; Mirderikvandi *et al.*, 2016). The role of this plant was previously studied in liver protection of Japanese quails (Nateghi *et al.*, 2013; Khoramshahi *et al.*, 2015), improving the performance index of laying hens (Nadia *et al.*, 2007; Yildiz *et al.*, 2006), and lowering cholesterol (Fallah *et al.*, 2013; Abdo *et al.*, 2007) in chickens. Several studies evaluated the effect of dried-leaf-powder or extract of artichoke on FI, WG and FCR in broilers, but showing a wide range on the effect of artichoke on growth indices. These results varied from no effect on growth indices (Tajodini *et al.*, 2015 Mirderikvandi *et al.*, 2016) to decrease of the FCR at the end of the growing period (Rouzmehr *et al.*, 2014), increase of the body weight (Lertpatarakomo *et al.*, 2015), increase of the WG and FI (Boroumandnia *et al.*, 2014), increase body weight, FI and decrease of the FCR (Shokri *et al.,* 2018), and decrease of final weight (Abdo *et al.*, 2007). However, the results of our study are consistent with previously reported works showing no effect on growth indices after administration of artichoke (Mirderikvandi *et al.,* 2016; Tajodini *et al.,* 2015).Several studies have been performed in order to elucidate the effects of probiotics on the antioxidant system in birds (Cross *et al.* 2002; Erdogan *et al.* 2010). Amaretti *et al.* (2013) showed that the use of probiotics increases antioxidant capacity, decreases oxygen radicals and reduces oxidative stress. In fact, probiotics produce butyric acid, hydrogen, which may play a stimulating role in the production of antioxidants and free radical scavenging (Zheng *et al.*, 2019).In our study, the use of probiotics had no effect on the SOD but increased the GPx, and TAS, significantly. Cross *et al.* (2002) and Erdogan *et al.* (2010) showed that probiotics had no effect on GPx level. In addition, Aluwong *et al.* (2013) showed that the use of yeast probiotics significantly increased GPx activity without affecting SOD in broilers. In another study, Bai *et al.* (2016) reported a *Bacillus subtilis* based probiotic increased antioxidants in broiler breast muscle. This is associated with increased mRNA expression of antioxidant genes, decreased oxidative damage in the pectoral muscle. Later, the same authors (Bai *et al.*, 2017) also demonstrated that the higher expression of SOD and GPx genes in mitochondria of the hepatic cells was directly related to feeding with probiotics in chickens.

In this study, we demonstrated that the use of artichoke extract alone could increase GPx and the combination of the probiotic and phytobiotic (garlic extract and artichoke extract) also have synergistic activity in increasing GPx and TAS, which even showed significant differences with the groups that received probioticor phytobiotic alone. There is scarcity of clinical research studies on the antioxidant properties of artichoke in poultry because most of the published studies have been performed in laboratory animals (Jimene-Escrig *et al.*, 2003). In rats, Jimene-Escrig *et al.* (2003) studied the antioxidant properties of artichoke and highlighted the in vitro antioxidant properties of this plant detecting increased blood GPx and had no effect on catalase and SOD, which is consistent with the findings of our study. In broilers, Mirderikvandi *et al.* (2016) stated that adding 500 mg/L of artichoke extract in drinking water for 2 weeks had no effect on MDA and reduced GPx. However, we found the increase in antioxidant activity after the use of the artichoke extract is related to the level of scavenging activity of AE. The antioxidant property of artichoke extract could be related to RSA (40% of AE content) and phenolic contents (5% of AE content, in the form of chlorogenic acid). The role of chlorogenic acid as a potent antioxidant has been previously demonstrated under *in vivo* and *in vitro* conditions (Sato *et al.*, 2011).

In the chickens received phytobiotic complex, the levels of TAS and GPx were significantly increased while no effect on SOD. Our results agreed those obtained by Nasiroleslami *et al*. (2018) who showed that broiler fed diet supplemented with guanidinoacetic acid significantly improved GPx activity in broiler liver and decreased serum MDA. Furthermore, Alagawany *et al.* (2007) demonstrated that dietary supplementation of garlic in diet had positive impact on SOD and TAS activities.

Several properties of probiotics in chickens have been previously studied (Makled *et al.*, 2019, Yan *et al.*, 2019; Ashayerizadeh *et al.*, 2011). The positive effects of probiotics in chickens are the improvement on growth indices, specific and non-specific immune responses, gastrointestinal health (Makled *et al.*, 2019), laying performance, bone strength (Yan *et al.*, 2019), but there is little information about the effect of probiotics in improving liver health in chickens. In our study, we found that the continuous consumption of the studied commercial probiotic can improve liver enzymatic activity, reduce total cholesterol and increase total protein. Several studies on the effect of probiotics on serum lipids in poultry were previously published (Ashayerizadeh *et al.* 2011; Abdulrahim *et*

al., 1996; Mohan *et al.*, 1996). Ashayerizadeh *et al.* (2011) demonstrated that adding probiotic to broiler diet decrease serum cholesterol level in these animals. In addition, dietary supplementation with probiotic containing *Saccharomyces cerevisiae* was demostrated to decrease cholesterol in both egg yolk (Abdulrahim *et al.*, 1996) andchicken serum (Mohan *et al.*, 1996). Amer and Khan (2012) showed that the supplementation with probiotic containing *Lactobacillus acidophilus*, *B. subtilis*, *S. cerevisiae* and *Aspergillus oryzae* decreases cholesterol in chicken serum after 6 weeks of initiate the addition of probiotics. Further to the ability of probiotic in elimination of lipids, these microorganisms can adsorb, detoxify the microbial toxin in the gastrointestinal tract and prevent the intestinal absorption. Detoxification of poisons in GIT inhibits the effect of toxins on hepatocytes (Markowiak*et al.*, 2019). In our study, the increasing of TP may be related to the influence of probiotic on secretory function of the gastrointestinal tract that leads to increase digestion, adsorption, and subsequently can elevate total protein in plasma. It seems that the positive effects of probiotics on physiological function lead to an improvement in growth indices in chickens.

There are few studies previously reported on the effect of probiotics on liver enzyme profiles (Bityutsky *et al.* 2019; Gholami-Ahangaran *et al.*, 2016). Bityutsky *et al.* (2019) reported that that the use of probiotics in quail could reduce the levels of liver enzymes of ALT and AST. The damage to the liver cell membrane causes these enzymes to be released into the bloodstream (Gholami-Ahangaran *et al.*, 2016). Therefore, the lack of increase in liver enzymes indicates no liver damage following probiotic supplementation.

In this study, AE was able to have effect on serum lipid profile, reducing CHL, TG, LDL and increasing HDL. Regarding the effect of artichoke on lipid metabolism, several studies in humans, mammals and poultry agreed that artichoke can positively affect lipid metabolism. Rouzmehr *et al.* (2014) reported that, although the addition of 200 g per ton of dried artichokes to the diet can increase WG and FI, reduced abdominal fat and blood CHL. Also, Abdo *et al*. (2007) stated that consumption of 6% dried artichoke leaves in the diet causes weight loss. However, AE seems to reduce plasma cholesterol levels by increasing bile secretion and decreasing cholesterol biosynthesis (Edward *et al.*, 2015). In addition, there is evidence that the active ingredients in artichokes have the ability to inhibit HMG-CoA reductase (Gebhardt, 1998). In our study, the decrease of hepatic enzymes including ALT, AST and ALP, and decreased plasma lipid profiles may support this hypothesis because if lipids accumulated in hepatocytes, hepatic complications would manifest as elevated hepatic enzymes in plasma. Certainly, this improve of liver health is in accordance to the increase of antioxidant capacity and it can be due to the increased ability of antioxidants to protect liver cells against toxins and oxidants.

Findings of our study showed that supplementing diets with garlic and artichoke extract decreased blood TG and LDL, and increased TP, and HDL. These results are partially in agreement with Rahimi *et al.* (2011) who found in broilers that blood TG, and LDL concentrations were decreased, while HDL increased when fed enriched-diet with 0.1% garlic powder. At this regard, Issa and Omar (2012) showed that addition of garlic powder at 0.2% and 0.4% reduced significantly concentrations of TG, CHL, LDL in broilers, and increasing the level of HDL compared to control. The potential effect of garlic products may be due to depressing the lipogenic and cholesterogenic activity of liver enzymes such as fatty acid synthase, glucose-6-phosphatase dehydrogenase, malic enzyme, and 3-hydroxy-3-methylglutaryl-CoA reductase, consequently, the mechanism of hypocholesterol and hypolipid syntheses (Mahmoud *et al.,* 2010).

In conclusion, our study demonstrated that the continuous administration of the studied commercial probiotic along with artichoke and garlic extract in broiler chicken diets improved growth indices, increased antioxidant capacity, reduced serum lipids and improved liver function in the experimentally studied broiler chickens.

CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Protective Effect of *Spirulina platensis* against Aspartame Induced Oxidative Stress and Molecular Gene Brain damage in New-Zealand rabbits

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ABSTRACT: *Spirulina* and alpha-lipoic acid have been considered as one of the most effective in antioxidative stress and anti-inflammatory *activity.* Aspartame (ASP) is one of the most widely used (artificial sweeteners) used in a variety of foods and feeds. This study was conducted to evaluate the possible protective effect of *Spirulina* and alpha-lipoic acid against aspartame induced oxidative stress and brain damage in rabbits. Forty two white male New-Zealand Rabbits were classified into seven equal groups. Group I: (Control group) received no drugs. Group II: rabbits administered with aspartame (250 mg/kg b. wt/day). Group III: rabbits received alpha- lipoic acid (100 mg/kg b. wt/day). Group IV: rabbits received *Spirulina platensis* (1500 mg/kg. b. wt/day). Group V: rabbits received aspartame (250 mg /kg b. wt) and treated with alpha- lipoic acid (100 mg/kg b. wt). Group VI: rabbits received aspartame (250 mg mg/kg b.wt) and treated with *Spirulina platensis* (1500 mg/kg b. wt). Group VII: rabbits received aspartame (250 mg mg/kg b.wt) and treated daily with alpha-lipoic acid (100 mg/kg b. wt) and *Spirulina platensis* (1500 mg/kg b. wt) for 8 weeks. At the end of experiment brain tissue was isolated and analyzed for determination of L-malondialdehyde (L-MDA), catalase (CAT) and reduced glutathione (GSH) in addition to anti-inflammatory cytokines: interleukin-10 (IL-10), Activator Protein-1 (AP-1), Bax gene expression and DNA damage. The obtained results showed a significant up-regulation of AP-1, Bax gene expression level and a significant down-regulation of IL-10 and marked increase in L-MDA and DNA damage that was indicated by an increase in tail length and tail DNA percent in brain tissue of aspartame treated rabbits. However, brain CAT activity and GSH concentration were significantly decreased when compared with control group. Co-administration of*Spirulina*and alpha-lipoic acid protected aspartame induced brain damage in rabbits *via* a significant improvement of all previous parameters and attenuate DNA changes. Conclusively, *Spirulina platensis* and alpha-lipoic acid exert a protective effect against DNA damage and oxidative stress in aspartame induced brain through free radical scavenging and anti-inflammatory activities as well as regenerating endogenous antioxidants defense system mechanisms.

Keywords: *Spirulina platensis*, Alpha-lipoic acid, Aspartame, Oxidative stress, DNA damage.

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INTRODUCTION

Aspartame (L-aspartyl-L-phenylalanine methyl ester ASP) is one of the most widely used artificial sweeteners. It is composed of substances normally found in human and animal foods, i.e. the amino acids, aspartic acid, phenylalanine, and the alcohols (i.e. methanol). The metabolism of aspartame provides approximately 4 kcal/g of energy (Gouge et al., 2004).However, this energy is negligible as the high intensity sweetening power of ASP (approximately 200 sweeter than sucrose by weight means that little is needed to be added to foods to achieves wetness (Magnuson et al., 2007) after oral administration to humans and experimental animals, ASP is rapidly and completely metabolized to aspartic acid, phenylalanine and methanol. Aspartic acid is a highly excitatory neurotransmitter (Krebs, 1992). However, the blood-brain barrier precludes influx of aspartate into the brain (Pardridge, 1979). The neutral amino acid phenylalanine is the precursor of the two brain catecholamine neurotransmitters, dopamine and norepinephrine. When phenylalanine concentration in blood plasma is elevated, the uptake into the brain increases at the expense of that of the other neutral amino acids. Thus, an increased phenylalanine level may affect brain levels of dopamine and norepinephrine and thus influence brain functions (Harper, 1984).

In addition, the increased uptake of phenylalanine might reduce the uptake of tryptophan (precursor of serotonin) and hence indirectly influence the biosynthesis level of serotonin in the brain, and thus affect brain function (Pardridge, 1986).The methanol is oxidized in the liver to formaldehyde which is further oxidized to formic acid. Formic acid is converted to CO_2 and water, via formation of 10-formyl tetra hydro folate (Barceloux et al., 2000). However, consumption of 50 mg ASP/ kg body weight would result in ingestion of 5 mg methanol/kg body weight (10% of ASP by weight is methanol), which is less than the amount of methanol formed during consumption of many foods including fruits and vegetables (Garriga, Metcalfe, 1988). Additionally, consumption of aspartame has been reported to be responsible for neurological and behavioral disturbances in sensitive individuals. The adverse neurological effects such as headaches, insomnia and seizures may be attributed to the alterations in regional brain concentrations of catecholamine (Coulombe and Sharma, 1986).Oxidative stress is very important pathophysiology of the central nervous system (CNS) and via regulating of mitochondrial activities, mediation of inflammation mediators, and others (Halliwell and Gutteridge 1999).

Compared to other organs, brain is especially vulnerable to oxidative stress due to the high utilization of oxygen, the large amount of easily oxidizable polyunsaturated fatty acids, the abundance of redox-active transition metal ions and the relative reduction of antioxidant defense systems (Butterfield and Stadtman, 1997). Antioxidant system is involved in the defense system against free radical mediated tissue or cellular damage. An enzymatic antioxidant system included glutathione-dependent enzymes, superoxide dismutase and catalase (Chaudiere and Ferrari-iliou, 1999), in addition to the non-enzymatic antioxidant (glutathione and uric acid). Moreover, GSH acts as the non-enzymatic anti-oxidative defense because it reacts with nitric oxide and protects the cellular system against the toxic effects of lipid peroxidation which produced from induced aspartame (Chaudiere and Ferrari-Iliou, 1999). Kono and Fridorich (1989) suggested that CAT is the main scavenger of H_2O_2 at high concentration. It catalyzed the conversion of H_2O_2 to water and molecular oxygen. Hence, the increase in CAT activity after 6 weeks of ASP treatment could be expected to converted toH_2O_2, which produced, as a result of the enzymatic activity of SOD, to H_2O and molecular oxygen. Also, AP-1 is a pivotal transcription factor that regulates a wide range of cellular processes including proliferation, apoptosis, differentiation, survival, cell migration, and transformation. Accumulating evidence supports that AP-1 plays an important role in several severe disorders including cancer, fibrosis, and organ injury, as well as inflammatory disorders such as asthma, psoriasis, and rheumatoid arthritis. AP-1 has emerged as an actively pursued drug discovery target over the past decade. Excitingly, a selective AP-1 inhibitor T-5224 (Shen et al., 2008).

Additionally, it was demonstrated that chronic ASP consumption (75 mg/kg b.wt/day) for 90 days significantly increased the brain damage revealed to brain markers (BDNF, COX-2 andPGE2) and elevated the production of cerebral cortex cytokines, IL-6 and TNF-a, respectively (Soffritti et al., 2007; Soffritti et al., 2010). Moreover, the same exposure reduced GSH levels, enhanced TNO production as well as ROS generation and LPO of ASP-result in apoptosis. ASP can be triggered by signals arising from the activation of death receptor-mediated (extrinsic) or mitochondrial-mediated (intrinsic) signaling pathways

(Itoh and Nagata, 1993).Extrinsic apoptotic signaling involves the activation of cell surface death receptors belonging to the protein family of tumor necrosis factor receptors (Itoh and Nagata, 1993). The binding of Fas receptor with its cognate ligand, FasL, can result in activation of caspase 8, activating downstream effector caspases (e.g., caspases 3, 6, and 7), resulting in apoptosis (Boldin et al., 1995). Cytochrome c accelerates the activation of caspase 9, initiating a downstream caspase cascade, which ultimately leads to cell death. AIF induces apoptosis via a caspase-independent pathway when cells experience serious oxidative stress. Beg et al., (1993) demonstrated that, cytokine, TNF-α, mediates early-stage responses of inflammation by regulating the production of other cytokines, including IL-1, IL-10 and IL-6. Because TNF-α is the main mediator of several inflammatory toxic responses to chemicals, it represent promising target for the prevention of uncontrolled inflammation. TNF-α has also been reported to induce nuclear factor-kappa beta (NF-$\kappa\beta$) production and this protein is inhibited by the presence of antioxidants.

Alphalipoic acid acts as coenzyme of pyruvate, free radical scavenger, metal chelator, and it protects against oxidative stress both in peripheral tissues and central nervous system (Winiarska et al., 2008).Additionally, *Spirulina* is a widely used microalgae in aqua feeds due to the richness with vitamins, proteins, fatty acids, minerals, amino acids, and carotenoids and it documented as potent antioxidant and antiinflammatory agent. Also, it strongly induces antioxidant enzyme activity, helps to prevent lipid peroxidation and DNA damage, and scavenges free radicals (Abdelkhalek et al., 2015).Accordingly, the present study aims to investigate the harmful effect of aspartame exposure on brain of rabbits, and the potential ameliorating role of *Spirulina platensis* and alpha-lipoic acid against aspartame induced oxidative stress and brain damage in rabbits.

MATERIAL AND METHODS

Experimental animals

Forty two white males New-Zealand rabbits of 4-6 weeks old age of average body weight 800-1200 g were used in the experimental investigation of this study. Rabbits were obtained from Laboratory Animals Research Center Faculty of Veterinary Medicine, Benha University. Rabbits were housed in separated metal cages (6per cage) with well-balanced ration and fresh clean drinking water ad-libitum. Rabbits were kept at a constant environmental and nutritional condition throughout the whole period of experiment. All rabbits were left for 15 days for acclimatization before the beginning of the experiment.

Chemicals and antioxidants

All chemicals were of analytical grade are obtained from standard commercial suppliers. Aspartame was purchased from Al-Ameriya pharma company, Egypt. *Spirulina* microalgae green powder was obtained from National Research Center, Dokki-Egypt.

Experimental design

Alpha-lipoic acid (Thioctic acid) was manufactured by Eva pharma, Egypt. *Spirulina* was freshly prepared by dissolved in distilled H_2O and administered orally using gastric tube in a daily dose of 1500 mg/kg body weight (Collaet al., 2008). DL-α-Lipoic acid was given orally in a daily dose of 100 mg/kg body weight of rabbits according to Şehirliet al., 2008.

After acclimatization to the laboratory conditions, the animals were randomly classified into seven groups, 6 rabbits each, placed in individual cages and classified as following: Group I: (Control group) received no drugs. Group II: rabbits administered with aspartame (250 mg/kg b. wt/day). Group III: rabbits received alpha- lipoic acid (100 mg/kg b. wt/day). Group IV: rabbits received *Spirulina platensis* (1500 mg/kg. b. wt/day). Group V: rabbits received aspartame (250 mg /kg b. wt) and treated with alpha-lipoic acid (100 mg/kg b. wt). Group VI: rabbits received aspartame (250 mg mg/kg b.wt) and treated with *Spirulina platensis* (1500 mg/kg b. wt). Group VII: rabbits received aspartame (250 mg mg/kg b.wt) and treated daily with alpha-lipoic acid (100 mg/kg b. wt) and *Spirulina platensis* (1500 mg/kg b. wt) for 8 weeks.

Tissue samples for biochemical analysis

At the end of the experiment, the brain tissues of rabbits were isolated immediately and weighed. Brain tissue was divided into 2 parts. One part was cleaned by rinsing with cold saline and stored at -20 ºC for subsequent biochemical analysis. All brain samples were analyzed for the determination of reduced Glutathione (GSH), catalase (CAT) activity and L-malondialdehyde (L-MDA) and the second part was kept at - 80°Cfor molecular analysis.

Brain tissue preparation for biochemical analysis

Brain tissues were cut, weighed and minced into

small pieces, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH7.4) to make 10% homogenates. The homogenates were centrifuged at 6000 r.p.m for 15 minutes at 4 ºC then the resultant supernatant was used for the determination of the following parameters: L-MDA and CAT. About 0.2 g of brain tissues were minced into small pieces homogenized with a glass homogenizer in 0.4 ml of 25% metaphosphoric acid (MPA), (ref. No.: 253-433- 4, Sigma-Aldrich, Germany), then 1.4 mL of distilled water was added, mixed, incubated for 1 hour and centrifuged for 10 min at 3, 000 r.p.m then the clear supernatant was removed and used for determination of GSH concentration.

Brain tissue for molecular and biochemical analysis

Brain tissue was immediately excised and frozen in liquid nitrogen and then in -80°C until used for determination of DNA damage using comet assay, Baxgene andIL-10, AP-1, gene expression analysis by qPCR. Brain tissue L-MDA, SOD and GSH were determined according to the method adapted by (Ohkawa et al., 1979), (Nishikimi et al., 1972) and (Beutler et al., 1963), respectively**.** Total RNA was isolated from brain tissue of rabbits using RNA easy Mini Kit (Thermo Scientific, Fermentas, #K0731) according to the manufacturer's protocol. Following determination of RNA concentration and purity by Qwell nanodrop Q5000 (USA), 5 mg of total RNA from each sample was reverse transcribed using Quant script reverse transcriptase. The produced cDNA was used as a template to determine the relative expression ofBax, IL-10), AP-1, gene using StepOnePlus real time PCR system (Applied Biossystem, USA) and gene specific primers. The reference gene, β actin, was used to calculate fold change in target genes expression. The thermal cycling conditions, melting curves temperatures, and calculation of relative expression were done. For the treated groups, assessment of 2-ΔΔCt determined the fold change in gene expression relative to the control. Also, DNA damage was determined by alkaline single-cell gel electrophoresis (comet assay) according to the protocol described by (Singh et al., 1988).

Statistical analysis

The results were expressed as mean ± SE using SAS computerized program v. 9.2 SAS. (2008) program to calculate the analysis of variance. The data were analyzed using one-way ANOVA to determine the statistical significance of differences among groups. Duncan's test was used for making a multiple comparisons with in groups for testing the inter-grouping homogeneity. Values were considered statistically significant when $p<0.05$.

RESULTS

The obtained results presented in table (2) revealed that oral aspartame administration to rabbits for 8 weeks resulted in significant increase in L-MDA concentration, decrease of CAT activity and GSH concentration when compared with control normal group. On the other hand, treatment with *Spirulina* and alpha-lipoic acid to aspartame administered rabbits for 8 weeks shown a significant decrease in serum L-MDA level, increase of CAT activity and GSH concentration when compared to aspartame group. Additionally, no significant changes were observed in L-MDA concentration, CAT activity and GSH concentration in *Spirulina* and alpha-lipoic acid treated normal rabbits groups when compared to control group.

The obtained results in (Table 3 and Figs. 1, 2, 3) revealed significant ($P\leq0.05$) up-regulation of Bax and AP-1 gene expression level in brain of Asp-treated (Asp group) rabbits as compared to the normal control group (Cnt group). In Asp administered rabbits and treated with LA and/ or Spir exhibited a significant down-regulation of Bax and AP-1 gene expression at the following order; Asp+LA+Spir (lowest expression) <Asp+Spir<Asp+LA (highest expression) as compared to Asp group. Additionally, no significant changes in the expression of Bax and AP-1 were noticed among the three groups (Cnt, LA and Spir).

Table 1. Forward and reverse primers sequence used in qPCR.

Gene	Forward primer (/5 ------ /3)	Reverse primer (/5 ------ /3)
Bax	ACACCTGAGCTGACCTTG	AGCCCATGATGGTTCTGATC
IL-10	GTTGCCAAGCCTTGTCAGAAA	TTTCTGGGCCATGGTTCTCT
AP-1	GCACATCACCACTACACCGA	TATGCAGTTCAGCTAGGGCG
β- actin	AAGTCCCTCACCCTCCCAAAAG	AAGCAATGCTGTCACCTTCCC

Table 2. Effect of alpha-Lipoic acid and/or *Spirulina* administrations on brain tissue GSH, L-MDA concentrations and CAT activity in aspartame treated male rabbits

Animal groups	GSH (ng/g. tissue)	L-MDA (nmol/g. tissue)	CAT (U/g. tissue)
Group I (Cont.)	4.63 ± 0.17[a]	4.27± 027[e]	1.77[a]±0.17
Group II (Asp)	1.51± 0.10[e]	9.40 ± 0.58[a]	0.31[d]±0.10
Group III (LA)	4.41± 0.19[a]	4.36± 0.21[e]	1. 51[a]±0.12
Group IV (Spir)	4.75± 0.15[a]	4.09± 0.30[e]	1.75[a]± 0.17
Group V (Asp+LA)	2.84± 0.12[d]	7.51± 0.43[b]	2.84[c]± 0.10
Group VI (Asp+Spir)	3.37± 0.10[c]	6.32± 0.25[c]	3.37[bc]± 0.10
Group VII (Asp+LA+Spir)	3.72± 0.14[b]	5.24± 0.22[d]	3.72[b]± 0.14

Data are presented as (Mean ± SE). SE = Standard error. Mean values with different superscript letters in the same column are significantly different at (P≤0.05).

Table 3. Effect of alpha-lipoic acid and/or *Spirulina*administrations on the relative expression of Bax, IL-10andAp-1gene in brain tissues of Aspartame treated male rabbits

Animal Groups	(Baxgene) Fold change mean ±SEM	(IL-10 gene) Fold change ±SEM	(Ap-1 gene) Fold change ±SEM
Group I (Cont.)	1.00±0.08[d]	1.00 ±0.06[a]	1.00 ±0.09[e]
Group II (Asp)	2.93±0.12[a]	0.18±0.01[e]	3.78±0.01[a]
Group III (LA)	1.04±0.07[d]	1.07±0.07[a]	0.86±0.09[e]
Group IV (Spir)	1.06±0.07[d]	1.03±0.05[a]	0.95±0. 1[e]
Group V (Asp+LA)	2.03 ±0.1[b]	0.39±0.02[d]	2.91±0.09[b]
Group VI (Asp+Spir)	1.84 ±0.01[bc]	0.62 ±0.03[c]	2.20 ±0.07[c]
Group VII (Asp+LA+Spir)	1.56±0.09[c]	0.84 ±0.02[b]	1.74 ±0.08[d]

Data are presented as (Mean ± SEM). SEM = Standard error of mean. Mean values with different superscript letters in the same column are significantly different (P≤0.05).

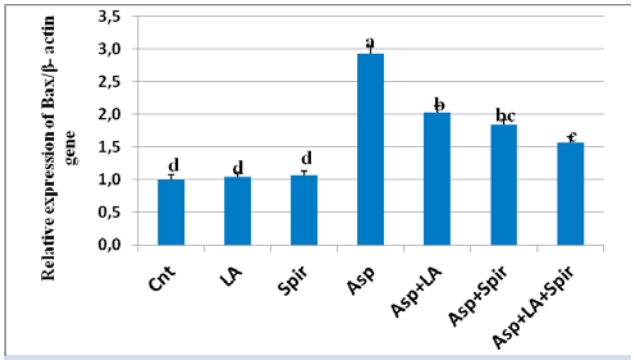

Figure 1. Graphical presentation of real-time quantitative PCR analysis of the expression of Bax gene in brain tissue of Asp administered rabbits following treatment by LA or/and Spir

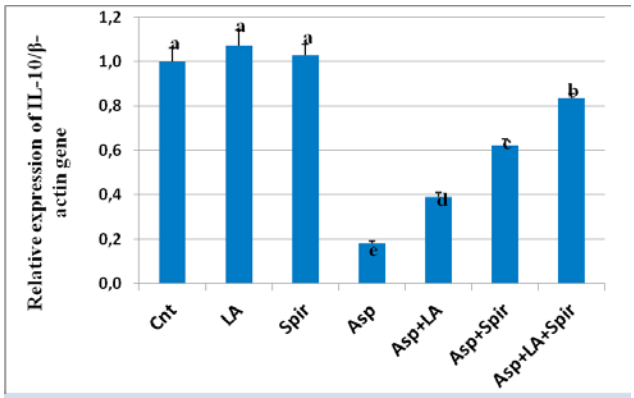

Figure 2. Graphical presentation of real-time quantitative PCR analysis of the expression of IL-10 gene in brain tissue of Asp administered rabbits following treatment by LA or/and Spir

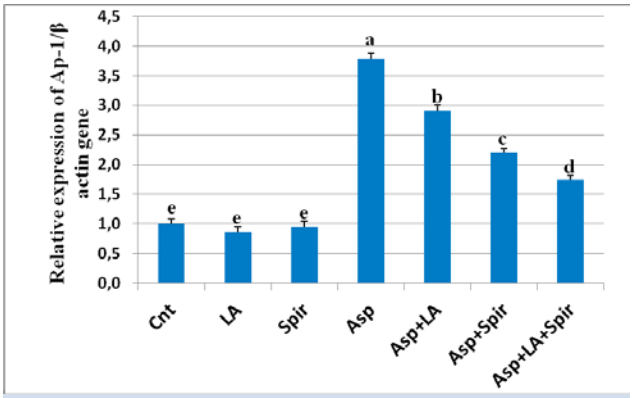

Figure 3. Graphical presentation of real-time quantitative PCR analysis of the expression of AP-1 gene in brain tissue of Asp administered rabbits following treatment by LA or/and Spir

Results of Comet assay analysis

A comet assay was performed to assess DNA damage in brain of Asp administered rabbits after treatment by *Spirulina* and/or LA as compared to the control. The results of comet assay presented in figures (3) and tables (4) shown a significant increase in DNA damage (P < 0.05) that was indicated by increase in tail length, tail DNA% and tail moment was observed in rabbits administrated with aspartame as compared to the control and protective groups (group administration of *Spirulina* and lipolic alone). This increased DNA damage was significantly reduced after

Table 4. Comet assay parameters obtained by image analysis in cells of all rabbits groups after treatment experiment

Animal Groups	Tailed %	Untailed %	Tails length µm	Tail DNA%	Tail moment
Group I (Cont.)	2	98	1.44±0.13[d]	1.51	2.16
Group II (Asp)	12	88	4.58±0.15[a]	3.67	16.81
Group III (LA)	3	97	1.72±0.15[d]	1.30	2.23
Group IV (Spir)	1.5	98.5	1.36±0.11[d]	1.46	1.99
Group V (Asp+LA)	11	89	4.07±0.12[a]	3.51	15.69
Group VI (Asp+Spir)	8	92	3.26±0.20[b]	2.39	7.78
Group VII (Asp+LA+Spir)	6.5	93.5	2.35±0.20[c]	2.69	6.32

Different superscript letters in the same column of tail length showed significance difference at P< 0.05.

administration of *Spirulina* alone (GIII; Spir) or in combination with LA (GII; LA) with lowest damage in combined treated group (GIIIV; Asp+LA+Spir). However, no significant difference was noticed between GIV (Asp+L.A) and GV (Asp**)** or among the three normal control group (GI; control group, GII; LA and GIII; Spir).

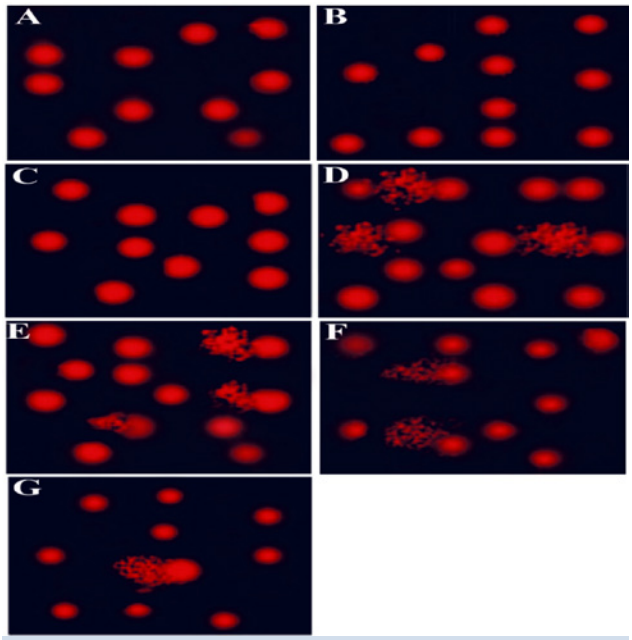

Figure 4. Photomicrographs representation of DNA damage in brain tissues using comet assay in Group I (Cont.) (A), Group II (Asp) (B), Group III (LA) (C), Group IV (Spir) (D), Group V (Asp+LA) (E), Group VI (Asp+Spir) (F) and Group VII (Asp+LA+Spir) (G)

DISCUSSION

Aspartame (E 951) is the most commonly used non-nutritive artificial sweeteners in over 100 countries in more than 6000 products pharmaceutical product and feed, drugs and including soft drinks, fruit juice, baked goods, chewing gum, candy, puddings, canned foods, ice cream, yogurt, table sweeteners and plenty of other foods and beverages (Magnuson et al., 2007).The present study was designed to investigate the possible harmful effects of aspartame induced DNA damage, inflammation, oxidative stress and molecular alterations on brain of rabbits. In the existing study oral aspartame administration to rabbits for 8 weeks resulted in significant increase in L-MDA concentration, decrease CAT activity and GSH concentration when compared with control normal group. These results are nearly similar with the recorded data of Mourad, (2011) who stated that, oral administration of ASP (40 mg/ kg b. wt) led to a significant elevation in LPO level in the liver, kidney and brain tissue and also indicated an increase in MDA level which was accompanied by a decrease in the activities of antioxidant enzymes (SOD and CAT) and GSH concentration in the liver of rat leading to degrade H_2O_2, more H_2O_2 could be converted to toxic hydroxyl radicals that may contribute to oxidative stress due to methanol metabolite from ASP. Moreover, Ashok and Sheela Devi, (2014) shown that a significant decrease in GSH concentration and glutathione reductase activity was reported in the brain of rats treated with aspartame at 100 and 500 mg/kg doses.Glutathione is decreased after repeated aspartame administration suggesting consumption of this important antioxidant defense mechanism by increased free radicals production due to aspartame administration which theoretically can further increase the vulnerability of the brain tissue to other oxidative insults (Tilsonha et al, 1991).Likewise, Feijó et al., (2013) exhibited that, oral administration of rats with aspartame (250 mg/kg b wt.) significantly increased brain tissue L-malondialdehyde concentration, LPO levels and markedly decreased GSH concentration. LPO is a free-radical-mediated process. In the entire rat brain regions after aspartame consumption, a marked increase in LPO was noted, which also supports the generation of free radicals. Generally, when the generation of reactive free radicals overwhelms the antioxidant defense, LPO of the cell membrane occurs. A marked increase in the LPO in the entire brain regions indicated this

result and possible loss of membrane integrity (Sohal et al., 2002).Meanwhile, treatment with *Spirulina* and alpha-lipoic acid to aspartame administered rabbits revealed a significant decrease in serum L-MDA level, decrease of CAT activity and GSH concentration when compared to aspartame group. Concerning to alpha lipoic acid, Li et al., (2013) exhibited that alpha-lipoic acid has been known to have positive effects on a wide variety of clinical conditions, which is completely consistent with its effect in decrease of the oxidative stress. Alpha-Lipoic Acid protects against oxidative stress both in peripheral tissues and central nervous system (Winiarska et al., 2008). Ying et al., (2004) demonstrated that alpha-lipoic acid can eliminate superoxide anions and that this process depends on both the concentration of alpha-lipoic acid and pH. Moreover, lipoic acid is a thiol containing nucleophile, reacts with endogenous electrophiles including free radicals or reactive drug metabolites and heavy metals. On the other hand, Konickova et al., (2014) reported that, *Spirulina* not only had anti-proliferative effects, but also inhibited the production of mitochondrial ROS and affected glutathione redox status. Supplementation with 1 or 5 % *Spirulina* induced GSH concentration; CAT activity and decrease L-MDA level the liver in intoxicated rats.Moreover, Abdel-Daim et al., (2013) described that the treatment with *Spirulina* significantly reduced oxidative stress and L-malondialdehyde in aspartame treated rats. Reactive oxygen species (ROS) attack and damage molecules in biological systems, leading to oxidative stress and various disorders and diseases occur. The antioxidant potential of *Spirulina* species and protective effects are mediated by phycocyanins, β-carotene, and other vitamins and minerals contained within *Spirulina*. Moreover, Phycocyanin appears to inhibit the generation of hydroxyl and peroxyl radicals, as well as lipid peroxidation. The higher lipid peroxidation observed could be due to a lower antioxidant capacity of the cells, and oxidative stress occurs in a cell or tissue when the concentration of reactive oxygen species (ROS) generated exceeds the antioxidant capability of that cell (Bermejo et al., 2008).Also, *Spirulina* is rich in β-carotene and the bioavailability is as good as the pure β-carotene, vitamin E and vitamin C and selenium, and *Spirulina* extracts could be effective against free radical induced lipid peroxidation which in turn may lead to cellular transformation (Pal et al., 2010). Furthermore, Sharoud, (2015) indicated that the protective effect of *Spirulina platensis* against paracetamol induced oxidative stress could be either direct by inhibiting lipid peroxidation and scavenging free radicals or indirect through the enhancement of the activity superoxide dismutase and the enzymatic free radicals scavengers in the cells. These properties could be attributed to the high levels of antioxidants such as c-phycocyanin, carotenoids, vitamins, minerals, lipids, proteins and carbohydrates. Lebda et al., (2017) who showed that aspartame and soft drinks induced upregulation of the relative mRNA expression levels of BAX and Casp3 and down-regulation of Bcl-2 genes in brain tissue of rats, suggesting an activation of cellular apoptosis. At the cellular level, aspartame can generate excessive reactive oxygen species (ROS) with a marked decrease in B-cell lymphoma 2 (Bcl-2), and increase in Bcl-2 like protein 4 (BAX) in rats' brain (Ashok and Sheeladevi 2014).

A significant up-regulation of BAX and AP-1 gene expression level were observed in brain of Asp-treated rabbits as compared to the normal control group. Similarly, Lebda et al., (2017) who showed that aspartame and soft drinks induced upregulation of the relative mRNA expression levels of BAX and Casp3 and down-regulation of Bcl-2 genes in brain tissue of rats suggesting an activation of cellular apoptosis. Similar results were reported earlier by El Haliem and Mohamed 2011 and Ardalan et al., 2017 who revealed that Aspartame administration at a dose of 250 mg/kg b.wt and 75 mg/kg b.wt, respectively, in rats caused significant up-regulation of the relative expression of TGF-β1, NF-κβ genes with significant down regulationin (IL-10) gene levels. ROS can trigger signal transduction pathways, primarily through nuclear factor-κB (NF-κB), promoting the production of TNF-α and increasing the production of anti- inflammatory cytokines, IL-10 (Park et al., 2008).Also Humphries and Pretorius (2008) who reported that administration of aspartame at a dose 50 mg/kg body weight for long time cause carcinogenic effect, also lead to up-regulation of AP-1 signaling and induced NF-κB signaling. Dowlati et al., (2010) reported that long term of oral administration of aspartame 75mg/kg b.wt significantly up regulated the expression of caspase-3activity and TNF-α in brain tissue of aspartame treated rat. Also, Soffritti et al., (2006) reported that the sweetener (aspartame) could increase brain TNF-α potent pro-inflammatory cytokine that is produced by glial cells involved in various physio-pathological conditions in the CNS of rats. Moreover, Mbazima et al., (2008) reported that, free radicals play a role in the initiation of apoptotic processes. The changes recognized in the expressions of genes decreasing in

Bcl-2expression and increasing of Bax and caspase-3 expression occurred. In neurons simultaneously, they give rise to apoptosis (Thomas et al., 2000). Furthermore, increase in the expression of the pro-apoptotic Bax gene lead to mitochondrial release of cytochrome c, which also triggers apoptosis. Caspases are closely associated with apoptosis. The caspase-cascade system played vital role in the induction, transduction and amplification of intracellular apoptotic signals. A depletion of intracellular GSH has been reported to occur with the onset of apoptosis) Mbazima, etal., 2008).

Additionally, both aspartate and glutamate act as neurotransmitters in the brain, carrying information from neuron to neuron. When there is an excess of neurotransmitter, certain neurons are killed by allowing too much calcium into the cells. This influx causes excessive numbers of free radicals to build up which kill the cells. The neural cell damage that is caused by excessive aspartate and glutamate is the reason they are referred to as excitotoxins. The excitotoxins are substances, usually acidic amino acids that react withspecialized receptors in the brain in such a way as to lead to destruction of certain types of neurons (Blaylock, 2002; Ho et al., 2003).In Asp administered rabbits and treated with LA and/ or Spir exhibited a significant down-regulation of caspase 3 and TNF-α gene expression as compared to Asp group. Vitamin C and E are the best antioxidants vitamins, both of which have been shown to be slightly effective in different models of neurodegeneration (Davoli et al., 1986). *Spirulina* strongly induces antioxidant enzyme activity, helps to prevent lipid peroxidation and DNA damage, and scavenges free radicals (Abdelkhalek et al., 2015).Similarly, Sun et al., (2011) reported that the expression of TNF-α, NF-κβ and IL-1b gene were significantly down-regulated following treatment by *Spirulina*. Also, *Spirulina* protected against neurotoxicity, hepatonephrotoxicity and colitis in animals by reducing oxidative stress (Abdel-Daim et al., 2015). Moreover, Juarez-Oropeza et al., (2009) reported that, *Spirulina* had both antioxidant and anti-inflammatory activities and down regulated the pro-inflammatory cytokines, which in turn might inhibit the neurodegeneration and oxidative stress thereby aids in maintaining proper brain and body health. Additionally, Ranney et al., (1976) recorded that, alpha-lipoic acid and *Spirulina* extract also extensively inhibit the inflammatory cascade by effectual modulation of inflammatory cytokines (IL-10 and IL-1), thus decrease the further exacerbation of aspartame brain injury mediated by inflammatory cytokines.

In the existing study a significant increase in DNA damage that was indicated by increase in tail length, tail DNA% and tail moment was observed in rabbits administrated with aspartame as compared to the control group. This increased DNA damage was significantly reduced after administration of *Spirulina* alone (GIII; Spir) or in combination with LA (GII; LA) with lowest damage in combined treated group (GVIIAsp+LA+Spir). Similarly, Findikli and Turkoglu (2014) showed that administration of aspartame 250 and 125mg/kg b.wt was indicated by an increasing of the tail length and tail DNA%. Consistent ingestion of food additives has been reported to induce toxic, genotoxic, and carcinogenic effects (Saad et al., 2014). The DNA damage induced by food additives depends on their transport across cellular/nuclear membranes, the activation and deactivation of intracellular enzymatic processes, the levels of radical scavengers, and the repair mechanisms in the target cell population. The comet assay has been used to determine the effects of these cellular processes on the amount of DNA damage induced. This assay is a powerful tool for determining genotoxicity, because it is simple and highly sensitive, has a short response time, and requires a relatively small number of cells and test substances (Čabarkapa et al., (2014).Aspartame caused DNA damage. Commonly used food sweeteners may be toxic at high concentrations in the long term. Lin et al., (2007) reported that DNA is a major drug target and can be damaged by harmful chemicals. The DNA damage caused by sweeteners may be associated with the generation of free radicals (reactive oxygen species), which cause DNA strand breaks and irreversible damage to proteins involved in DNA replication, repair, recombination, and transcription. Moreover, several mutagenic and genotoxic lipid peroxidation products, in particular malondialdehyde and 4-hydroxy-2-nonenal, have been shown to bind to DNA and to damage it (Eder et al., 2006). In the current study treatment with *Spirulina* platensis or and lipoic acid significantly reduced DNA damage that was indicated by comet assay in aspartame administered rabbits.These results are nearly similar to those recorded by Shirpoor et al., (2008) who reported that, alpha- lipoic acid partially alleviated the ethanol-induced DNA damage in developing hippocampus and cerebellum of rats. Hassan et al., (2012) stated that, supplementation with *Spirulina* succeed to inhibit DNA damage as indicated by the down-regulation of Fas (Fatty acid synthase) gene expression,

and decreased the percentage of DNA fragmentation and micro nucleated erythrocytes in aflatoxin intoxicated rats. Also, Saber et al., (2015) recorded that, co-treated rats with aluminum chloride and *Spirulina platensis* showed a significant decrease in all parameters of DNA damage in kidney (tail percentage, tail length, DNA tail percentage, and tail moment) when compared to the aluminium chloride -treated group. The existing results were confirmed by Ismail et al., (2009) who indicated that, polysaccharides of *Spirulina* enhanced cell nucleus enzyme activity, the process of DNA repair and the unscheduled DNA synthesis (Kajiet al., 2002).The anti-genotoxic effect of *Spirulina* may be related to its contents of phycocyanin and phycocyanobilin which also have strong anti-cyclooxygenase-2, antioxidant activity to scavenger peroxidinitrite and reduce peroxynitrite (OONO-) induced oxidative damage to DNA (Bhat and Madyastha, 2001).

CONCLUSION

In conclusion, the oral aspartame administration for 8 weeks resulted in significant increase in L-MDA concentration, decrease CAT activity and GSH concentration when compared with control normal group. The results of the present work revealed that *Spirulina* and alpha-lipoic acid alleviates the harmful effects of aspartame on brain tissue of rabbits. Also, *Spirulina* and alpha-lipoic acid has a protective antioxidant role in restoration of oxidative stress, a strong anti-inflammatory and anti-apoptotic effects on aspartame-induced brain damage. In this regard, the current study has brought a convincing indication preferring the usage of natural antioxidants like spirulina and alpha lipoic acid as a protective strategy against toxicity induced by aspartame.

CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Relationships between glucose-6-phosphate dehydrogenase, glutathione peroxidase, reduced nicotinamide adenine dinucleotide phosphate, total protein, malondialdehyde, total glutathione and vitamin C parameters in goat milkcells

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ABSTRACT: In this study, to reveal the antioxidant potential of goat milk cells, the activities of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPx) and the levels of reduced nicotinamide adenine dinucleotide phosphate (NADPH), total glutathione (tGSH), malondialdehyde (MDA), vitamin C (Vit C) and total protein (TP) in goat milk cells were determined and correlations between these parameters were evaluated. Milk samples were collected from 19 clinically healthy goats from a private goat farm. Briefly, milk cells were collected from milk by centrifugation and than they were sonicated. Supernatant G6PD, GPx activities and NADPH, tGSH, MDA, Vit C and total protein levels were determined by spectrophotometric methods. As regards correlations: milk cell MDA levels were positively correlated with milk cell tGSH (r=0, 725, p<0, 01), milk cell Vit C (r=0.622, P<0.01) and milk cell NADPH (r=0.763, P<0.01) levels. There was a positive correlation between milk cell GPx activity and milk cell NADPH levels (r=0.659, P<0.01). Milk cell tGSH levels were positively correlated with milk cell Vit C (r=0.615, P<0.05) and milk cell NADPH (r=0.846, P<0.01) levels. Milk cell NADPH levels was positively correlated with milk cell Vit C levels (r=0.791, P<0.01). As a conclusion, the antioxidant potential of goat milk cells were evaluated and discusses.

Keywords: Goat milk cell, GPx, G6PD, NADPH, tGSH

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INTRODUCTION

Though goat milk meets about 2% of the world's total annual milk supply, its contribution to human nutrition and economic well-being is extremely important in many parts of the world, particularly in the Mediterranean countries and the Middle East (Park 1994a, Park 1994b, Park and Haenlein 2007). Aleppo (Damascus) (Barıtçı and Adıgüzel, 2017) and Kilis goats (Keskin and Tüney, 2015) are two breeds in the Mediterranean and Southeastern Anatolia regions. Aleppo goat is a native breed of Syria and other Near East countries. It has been improved for over 40 years through genetic selection for milk and meat production and it has been given a high priority by the Food and Agriculture Organization (Mavrogenis et al., 2006). Kilis goat possesses crutial advantages in terms of high milk yield and reproductive traits with good toleration for warm environments and drought under extensive or semiintensive management conditions in arid and hot climates (Keskin et al., 2017). There is also an increasing demand for goat milk and its products in developed countries because people demand healthy food and also people who are allergic or have intolerance to cow milk turn towards goat milk and its products (Park, 1990; Park, 1994a; Park, 1994b; Chandan et al., 1992; Tziboula-Clarke, 2003). Although the general composition of goat milk is similar to cow milk, it is differentiated from cow milk with some features; goat milk contains on average 3.8% fat, 3.5% protein, 4.1% lactose and 0.8% ash; that is, more fat, protein and ash and less lactose than cow milk (Haenlein and Caccese, 1984). As goat milk contains a higher percentage of small-diameter fat globules than cow milk, and because of the qualitative and quantitative differences in milk proteins, especially in α-1 casein, it is easier to digest goat milk. Goat milk is rich in short and medium- chain mono and polyunsaturated fatty acids (caproic, caprylic, capric) (Djordjevic et al., 2019).

Healthy goat's milk has a higher number of somatic cells (270-2000×10^3 cells/ml, Souza et al 2012) than cow's milk-does (<200 ±10^3 cells/ml, Dang et al., 2008).The content of the somatic cells also vary between goat and cow's milk. While 40% to 87% of somatic cells in goat's milk are formed by neutrophils from PMNs (Souza et al., 2012; Shah et al., 2017), the rate of neutrophils in cow milk cells varies between 5% and 20% (Sarıkaya, 2006; Souza et al., 2012) and macrophages are the predominant cell type in healthy cow milk (Osstensson 1993).

Goats are reported to be more resistant to infectious conditions such as mastitis, than other species, presumebly as they have a higher number of neutrophils (Tian et al., 2005; Souza et al., 2012). Besides, antioxidants such as reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (rGSH), glucose-6-dehydrogenase (G6PD), glutathione peroxidase (GPx) and vitamin C (Vit C), have important roles in a cell's defence mechanism (Ralat et al., 2006; Stincone et al., 2015; Carole et al., 2007; Ighodaro and Akinloye, 2018).In a previous study (Akalın et al., 2019), we revealed the antioxidant potential of Holstein-Friesian cow milk cells by determining the levels of NADPH, GSH, GPx and G6PD and the relation of these parameters with mastitis, for the first time.Since it is thought that the antioxidant potential of the cells will change as the somatic cell count and somatic cell component change, we thought that revealing the antioxidant potential of goat milk cells will offer an insight to understand the goat milk cell defence mechanism.

Therefore, in order to investigate the antioxidant potential of goat milk cells, in the current study, it wasaimed to determine G6PD and GPx activities and NADPH, tGSH, MDA, Vit C and total proteinlevels in the cells of milks obtained from clinically healthy goats' udders and to investigate therelationships between the related parameters.

MATERIALS AND METHODS

Milk samples were collected from 19 healthy goats (10 Aleppo, 9 Kilis goats) aged between 1-4 and were fed in the same care and nutritional conditions in a private goat farm in April, inHatay region (36° 11' 56" North, 36° 9' 38" East). Milk was obtained while the owner was milking the goats returned from thepasture in the morning. Milk was taken from the right lobe of clinically healthy udders. During milking, the first 2-3 squeezes of milk were thrown away after the teat was wiped with 70%alcohol cotton, and milk samples were tested by California Mastitis Test (CMT) and CMT negative samples were included in the study. Collected milk samples were brought to the biochemistry laboratory in the cold chain and stored at -20°C until the treatment. Milk samples (14 ml each) were thawed and centrifuged at 600×g for 10 min at 4°C. After centrifugation, the supernatant was eliminated by removing the upper layer of fat with a cotton pad; the remaining cell pellet was washed twice with cold phosphate-buffered saline (PBS) and centrifuged at600×g for 10 min at +4°C. Finally, the supernatant was removed, and the remaining pellet wascompleted to 2 ml with PBS and sonicated (Bandelin Sonopuls HD 2070, Germany) (Akalın etal., 2016) for 5 repeti-

tions of 10 sec each, with a 30 sec cooling period (on ice) between each repetition. By this process, milk cells in 14 ml of milk was concentrated in 2 ml PBS. After sonication, the homogenates were centrifuged at 13, 000×g for 15 min at +4°C. Milk cell supernatant was used for the further analysis.

Determination of G6PD activity

Milk cell G6PD activity was determined by using the method developed by Beutler (1971) and calculated by the spectrophotometric measurement of the absorbance difference in optical density caused by the conversion of NADP+ to NADPH at 340 nm (UV 2100 UV-VIS Recording Spectrophotometer Shimadzu, Japan). Results are presented as IU/g protein.

Determination of GPx activity

Determination of GPx activity in milk cells was done according to the method described by Beutler (1975). According to this method, GPx catalyzes the conversion of rGSH to oxidizedglutathione (GSSG) in the presence of hydrogen peroxide (H_2O_2). Oxidized glutathione, formed by GPx in anenvironment where H_2O_2 is present, is converted back to GSH with the help of glutathionereductase and NADPH. The activity was calculated by the spectrophotometric measurement of the absorbance difference in optical density caused by the conversion of NADPH to NADP+ at340 nm. Results are presented as IU/mg protein.

Determination of NADPH Levels

NADPH levels in cell supernatants were determined spectrophotometrically using a commercial kit (Sigma MAK038). The reaction principle is based on the spectrophotometric (with ELISA reader, BioTek Instruments, µQuant, U.S.A) analysis of the reduction of formazan dye by NADPH at 565 nm, which is synthesized enzymatically in the pentose phosphate pathway. Results are presented as nmol/mg protein.

Determination of tGSH Levels

Total glutathione (GSSG+rGSH) levels in cell supernatants and milk serum were calculated using a commercial kit (Sigma CS0260). It is a kinetic method based on the principle of thereduction of 5.5'-dithiobis (2-nitrobenzoic) acid to trinitrobenzoat (TNB) by glutathione. When oxidized glutathione is regenerated by glutathione reductase and NADPH, TNB absorbance at412 nm can be measured by spectrophotometry. Results are presented as nmol/mg protein.

Determination of MDA Levels

Tissue MDA levels were determined spectrophotometrically according to the method proposed by Ohkawa et al. (1979). Principle: Tissue MDA determination; it is based on spectrophotometric measurement at 532 nm of the pink complex formed by MDA with TBA, which is the secondary product of lipid peroxidation, as a result of incubation of tissue homogenate in a boiling water bath for one hour under aerobic conditions and at pH:3.5. Results are presented as nmol/mg protein.

Determination of Vit C Levels

Milk vitamin C levels were calculated according to the manual spectrophotometric method of Haag (1985). Principle: Ascorbic acid (Vitamin C) is converted to dehydro ascorbic acid with mild oxidizing agents, dehydro ascorbic acid slowly converts to diketogulonic acid in mild acid solutions. Dehydro-ascorbic acid and diketogulonic acid react with 2.4-dinitrophenylhydrazine (DNPH) to form bis 2.4-dinitrophenylhydrazone. The results are given as mg/milk cells of 1 ml milk.

Determination of Total Protein Levels

Total protein levels in milk cell supernatants were determined by the Bradford (1976) method (Coomassie Brilliant Blue G, Sigma 27815-100 G). Protein concentration was determined spectrophotometrically by determining the absorbance at 595 nm. Bovine serum albumin (Merck 112018) was used as a standard. The results are given as mg/milk cells of 1 ml milk.

Statistics

The values obtained were evaluated by SPSS 22.0 program and descriptives (Mean ± Standard Error) were evaluated. Pearson correlation was performed for correlation analysis and $P<0.05$ indicated as statistically significant.

RESULTS

As shown in Table 1, goat milk cell G6PD and GPx activities were determined to be 1.81±0.48IU/g protein and 0.38±0.02 IU/mg protein, respectively. NADPH levels were 1.18±0.23 nmol/mg protein, tGSH levels were 101.73±25.50 nmol/mg protein, MDA levels were 1.43±0.35 nmol/mg protein, Vit C levels were 0.45±0.09 µg/milk cell of 1 ml milk and TP levels were 0.09±0.001 mg/milk cell of 1 ml milk.

Table 1. Some Biochemical Parameters in Goat Milk Cells

	Mean	SE	n
G6PD (IU/g protein)	1.81	0.48	19
GPx (IU/mg protein)	0.38	0.02	19
NADPH (nmol/mg protein)	1.18	0.23	17
tGSH (nmol/mg protein)	101.73	25.50	14
MDA (nmol/mg protein)	1.43	0.35	19
Vit C (µg/ milk cell of 1 ml milk)	0.45	0.09	19
TP (mg/ milk cell of 1 ml milk)	0.09	0.001	19

G6PD:Glucose-6-phosphate dehydrogenase, GPx: Glutathione peroxidase, NADPH:Reduced nicotinamideadenin dinucleotide, tGSH: Total Glutathione, VitC: Vitamin C, TP: Total Protein

Table 2. Correlations between the parameters (Spearman's correlation test)

	TP mg/ml	GPx U/ml	tGSH nmol/ml	Vit C µg/ml	NADPH pmol/ml	G6PD U/ml
MDA nmol/ml	0,454	0,408	0,725**	0,622**	0,763**	0,226
TP mg/ml		0,392	-0,150	0,063	0,377	-0,228
GPx U/ml			0,412	0,426	0,659**	-0,344
tGSH nmol/ml				0,615*	0,846**	0,266
Vit C µg/ml					0,791**	0,065
NADPH pmol/ml						-0,032

G6PD: Glucose-6-phosphate dehydrogenase, GPx: Glutathione peroxidase, NADPH: Reduced nicotinamideadenin dinucleotide, tGSH: Total Glutathione, VitC: Vitamin C, TP: Total Protein *P<0.05,**P<0.01.

As shown in Table 2, milk cell MDA levels were positively correlated with milk cell tGSH (r=0, 725, p<0, 01), milk cell Vit C (r=0.622, P<0.01) and milk cell NADPH (r=0.763, P<0.01) levels. There was a positive correlation between milk cell GPx activity and milk cell NADPH levels (r=0.659, P<0.01). Milk cell tGSH levels were positively correlated with milk cell Vit C (r=0.615, P<0.05) and milk cell NADPH (r=0.846, P<0.01) levels. Milk cell NADPH levels were positively correlated with milk cell Vit C levels (r=0.791, P<0.01).

DISCUSSION

Milk cells are composed of epithelial cells interfused to the milk from the breast tissue and leukocytes (neutrophils, lymphocytes and macrophages) that pass from the blood to the mammary gland and then into the milk. The content and the number of somatic cells in healthy goat milk differ from that in cow milk (Sarıkaya, 2006; Dang et al., 2008; Souza et al., 2012; Shah et al., 2017). Studies determining the protein levels of milk cells are quite limited. In the study conducted on cow milk somatic cells (Akalın et al., 2019), the total protein levels were determined as 0.374mg/1 ml cell supernatant in the cell pellet obtained by taking 50 ml of cow milk and concentrating it into 2 ml PBS. When the total protein levels in the present study were calculated with dilution coefficients (14 ml milk concentrated with 2 ml PBS: 0.09 mg/1 ml cellsupernatant), they were found to be slightly lower than the levels in the cow milk (approximately 0.321 mg/1 ml cell supernatant). No study investigating total protein levels of goat milk somaticcells has been found. The major component of goat milk somatic cell, unlike cow's milk is PMN. While 40%to 87% of somatic cells in goat milk (Souza et al., 2012; Shah et al., 2017) are formed by neutrophils from PMNs, the rate of neutrophils in cow milk cells varies between 5% and 20% (Sarıkaya, 2006; Souza et al., 2012). Moreover, 15% to 41% of somatic cells in healthy udder consist of macrophages whereas 9% to 20% of them consist of lymphocytes and 1% to 6% of them consist of epithelial cells (Paape et al., 2001). Determination of low total protein levels in goat milk cell compared to cow milk may be related to the difference in milk cell components.

The main goal of the pentose phosphate pathway is to produce NADPH, which has reducing power, and ribose-5-phosphate, which is the building block of DNA and RNA. NADPH is produced in the pentose phosphate pathway by glucose-6-phosphogluconate dehydrogenase (6PGD) and G6PD, from

NADP+ (oxidized NADPH) (Reuter et al., 1990; Stincone et al., 2015). While NADPH levels in cow milk cell were 4.24 nmol/mg protein (Akalın et al., 2019) and 5.99 nmol/mg protein (Akın et al. 2019), in the current study, the levels in goat milk (1.18 nmol / mg protein) were found to be lower than in the cow milk. As reported by Akın et al. (2019) and Akalın et al. (2019), a significant positive correlation was determined between cow milk cell NADPH levels and G6PD activity. However, in the current study no correlation was observed between these parameters in goat's milk. It can be speculated that NADPH is not controlled only by G6PD but also by 6PGD in the pentose phosphate pathway in the goat milk cell. It is suggested to evaluate 6PDG levels in goat milk cells to reveal the NADPH synthesis.

While positive correlations of milk leukocyte NADP+reductase activity (Ritter et al., 1977) and milk somatic cell G6PD activity (Akalın et al., 2019) with milk cell total protein levels in cows were reported, no correlation was found between total protein levels and G6PD activity in the current study. Glutathione protects cells against free radicals, reactive oxygen species, endogenous and exogenous toxic compounds. During the reduction, oxidized glutathione (GSSG) is formed and this molecule is reduced by GR enzyme which uses NADPH.While GPx provides detoxification of H_2O_2 in the cells, it allows conversion ofrGSH to GSSG (Ighodaro and Akinloye, 2018). In the current study, tGSH levels (101.73±25.5 nmol/mg protein) were found to be slightly lower than the levels reported for cow milk cells (122.88±13.08 and 142.16±37.06 nmol/mg protein) (Akalın et al., 2019; Akın et al., 2019, respectively). Also, goat milk cell NADPH levels were positively correlated with milk cell tGSH levels and GPx activity. While Akın et al. (2019) reported no significant correlation of NADPH with rGSH and GPx activity in cowmilk cells, Akalın et al. (2019) reported only a weak positive correlation between NADPH levels and GPx activity, and they thought that GSH levels and GPx activity in cow milk cells were also related to other mechanisms, and these parameters were not directly dependent on NADPH and G6PD. In goat milk cell, it can be concluded that NADPH is related to tGSH and GPx, unlike cow milk cell. While in their study, Akalın et al. (2019) determined GPx activity as 4.44±0.36 IU/mg protein incow milk cells, GPx activity was measured as 0.38±0.02 IU/mg protein in the current study conducted with goat milk cells. Just as in the cow milk cells (Akalın et al., 2019; Akın et al., 2019), no correlation was reported between total protein and GPx activity in the goat milk cells.

Vitamin C (ascorbic acid, ascorbate) is a water-soluble vitamin that causes the reduction of compounds such as molecular oxygen, nitrate, cytochrome a and c and is capable of reacting with free radicals in the aqueous environment. It reacts with superoxide and hydroxyl radicals and forms the first antioxidant defence against oxidant agents (Carole et al., 2007). In the current study, a significant positive correlation was determined between Vit C levels and tGSH levels. To our knowledge, no study was reported regarding correlation of milk cell Vit C and GSH levels. In a study conducted on lymphocytes, which were isolated from human blood, a significant and high level of positive correlation was found between tGSH and ascorbate (Lenton et al., 2000). Reduced glutathione is used to reduce oxidized Vit C (dehydroascorbic acid) to reduced form (ascorbic acid, ascorbate) (Winkler et al., 1994; May et al., 1997). The direct correlation between Vit C and GSH suggests that these two antioxidant agents may work synergistically with each other in the cell (Lenton et al., 2000). In the current study, a significant and high level of correlation was determined between Vit C and NADPH levels. There was no literature on the correlation between Vit C and NADPH levels in milk cells. On the other hand, ex vivo in human neutrophils, it has been reported that the activity of NADPH-oxidase enzyme, which uses NADPH as a substrate and enables the formation of superoxide radicals from molecular oxygen (Lassegue et al., 2012), is inhibited by the lipophilic derivative of ascorbic acid, thereby reducing the formation of superoxide radicals (Schmid et al., 1994). In the current study, the existence of a positive correlation between Vit C and NADPH in the absence of a correlation between Vit C and G6PD activity suggests that this might be due to NADPH-oxidase. It has been reported that thioredoxin reductase enzyme, which enables the conversion of dehydroascorbic acid to ascorbat in rat liver, uses NADPH as a reducing agent (May et al., 1997). Also, the positive correlation between Vit C and NADPH may be different in healthy and inflammative environment. Malondialdehyde is the final peroxidation product of fatty acids with multiple double bonds found in cell and organelle membranes. Increasing peroxidation of lipids by free radicals inmembranes causes an increase in MDA level. Malondialdehyde and other lipidperoxides can react with DNA or proteins and disrupt their structure (Gawel et al., 2004).The level MDA is served as a re-

liable biomarker of lipid peroxidation (LPO) and usually served as a marker of LPO (Acaroz et al., 2018). Nostudy on MDA levels in milk cells was found in the literature review. Goat milk cell MDA levels were determined as 1.43±0.35 nmol/mg protein. In addition, positive correlations of goat milk cell MDA levels with reducing agents such as tGSH, Vit C and NADPH were revealed. As MDA levels increase in goat milk cells, the increase seen in antioxidant molecules may indicate that these molecules play a role in the defence mechanism against oxidation and against the formation ofMDA.

CONCLUSION

In conclusion, positive correlations of milk cell MDA levels with milk cell tGSH, Vit C and NADPH levels, positive correlations of milk cell NADPH levels with milk cell tGSH, GPx and Vit C levels, positive correlation between milk cell Vit C and milk cell tGSH levels suggest that goat milk cells have an efficient antioxidant mechanism in healthy conditions which is some different from cow milk cells.

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CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Antibacterial activity of cinnamon oil against multidrug-resistant *Salmonella* serotypes and anti-quorum sensing potential

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ABSTRACT: The antimicrobial resistance of *Salmonella* and other foodborne pathogenic bacteria, witnessed in recent years, has become a significant health concern. Bacteria use chemical signals to communicate each other and regulate their behavior including virulence. Due to increasing antibiotic resistance, new drug development strategies are being investigated and the use of active ingredients of various medicinal and aromatic plants as alternatives to antibiotics is tested. This study aimed to determine the anti-quorum sensing (QS) activity of cinnamon oil (CO) on *Chromobacterium violaceum* and to evaluate antimicrobial activity of CO against multidrug-resistant (MDR) *Salmonella enterica* serotypes. Anti-QS activity was tested using biosensor strain and antibacterial activity was determined by a microdilution method according to EUCAST standards. CO was found effective on QS system of *Chromobacterium violaceum*. Nineteen foodborne pathogens isolated from different poultry/cow sourced foods and serotyped as *Salmonella enterica* subsp. *Enterica* serotype Infantis (15), Kentucky (1), Newport (1), Telaviv (1), and Typhimurium (1). *Salmonella* Infantis strains were found resistant to three or more antibiotic classes (with resistance to at least one antibiotic from each class) and categorized as MDR. The results concluded that CO has strong antibacterial activity against all *Salmonella enterica* serotypes with MIC between 0.125 μg/ml and 1.0 μg/ml. This research demonstrates that CO is a potential candidate for developing new antimicrobial agents, antiseptic solutions or natural food preservatives against MDR *Salmonella* serotypes while is also a potential anti-QS agent.

***Keywords*:** antibacterial activity; anti-quorum sensing activity; cinnamon oil; multidrug-resistant; *Salmonella enterica*

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INTRODUCTION

Quorum sensing (QS) is a communication system, includes the secretion of extracellular chemical signals that regulates the gene expression of bacterial virulence like biofilm formation, movement, pigment and enzyme production, depending on bacterial population density (Papenfort and Bassler 2016). Bacterial infections have become an alarming health problem, especially due to the increasing number of multiple antimicrobial drug-resistant bacteria and raise concerns about success in therapy due to resistance. And QS is one of the most important barriers to overcome in the fight against bacteria, as a mechanism responsible for the generation of virulence factors that play an important role in the pathogenicity and resistance (Zhao et al. 2020). Due to the increased antibiotic resistance, researchers are in search of new drug development strategies and also looking for different solutions as alternative treatment methods like the usability of active ingredients of various medicinal and aromatic plants (essential oils, herbs, spices), bacteriophages, probiotic microorganisms and their metabolites alongside synthesizing new chemicals (Kiymaci et al. 2018; Waters and Smyth 2015). Aromatic and medicinal plants are natural sources of various industries like pharmaceutical, food, chemical, cosmetics, and cinnamon, a tropical spice, is one of the most investigated sources of these plants (Akthar 2014; Arumugam et al. 2016; Swamy 2012; Swamy and Sinniah 2015). Cinnamon (extracts, essential oils) has some primary and secondary metabolites that show antibacterial activity including compounds like cinnamic acid, trans-cinnamaldehyde, cinnamate, etc. against some different pathogens (Kaskatepe et al. 2016; Vasconcelos et al. 2018).

Diarrheal illnesses are the most common foodborne diseases that affect millions of people worldwide. A World Health Organization (WHO) report claims these to be the leading cause of death in children under five years of age. According to the WHO report 2018 (WHO 2018), *Salmonella* is one of the four key global causative agents of gastroenteritis. *Salmonella* infection or salmonellosis, caused by consumption of foods contaminated with non-typhoidal *Salmonella enterica* (NTS) serotypes, is one of the major foodborne diseases worldwide and considered an important public health problem. The infection is self-limiting in the majority of cases, with no requirement for antibiotics. However, antibiotic medication is recommended to control infection in severe cases. Quinolones possess a broad range of antimicrobial activity against enteric pathogens but are not recommended for use in children. Instead, third generation cephalosporins are prescribed for treating critical infections in children. However, recent studies have reported increased resistance to antibiotics, particularly those used to treat infections by multi-drug resistant (MDR) NTS strains. These strains are associated with high mortality and morbidity and are therefore considered a major public health concern (Medalla et al. 2017).

In light of the recent surge in the development of MDR strains of enteric pathogens, past years have seen the use of natural products, particularly essential oils (EOs) with potent antimicrobial activity, for treating these infections. For instance, cinnamon oil (CO) is one such product that is used as a spice, condiment, and flavoring agent. Moreover, it has been a component of traditional medicine to treat anaphrodisia, vaginitis, shortness of breath, eye inflammation, rheumatism, neuralgia, leucorrhea, wounds, and sore teeth. Additionally, it has antioxidant and hypoglycemic properties and has been used against various pathogenic microorganisms. There is no literature investigating the efficacy of CO against *Salmonella* Infantis, *Salmonella* Kentucky, and *Salmonella* Telaviv.

In the present study, our first aim was to investigate the anti-QS activity of CO on *Chromobacterium violaceum (C. violaceum).* In Gram-negatives, the QS mechanism occurs via acyl-homoserine lactone (AHL) signal molecules. While *C. violaceum* (wild type) is a strain capable of producing short-chain AHLs and purple colored violacein pigment; mutant *C. violaceum* CV026 is a biosensor strain that does not produce AHL and pigments but can produce violacein pigment by recognizing short-chain AHL molecules (AHL molecules with chain length varying from C4 to C8) (McClean et al. 1997). With the addition of N-hexanoyl-L-homoserine lactone signal molecule to the medium, the mutant CV026 strain produces violacein pigment due to the QS mechanism.

Additionally, our aim was to determine the antibacterial activity of CO against *Salmonella enterica* subsp. *enterica* serotypes including MDR Infantis, Kentucky, Newport, Telaviv, and Typhimurium, isolated as foodborne pathogens from different poultry-cow sourced foods. To the best of our knowledge this is the first study that examines the inhibitory activity of CO against MDR *Salmonella* Infantis isolates.

MATERIALS AND METHODS

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) analysis of cinnamon oil

CO was purchased from a herbalist (Ministry of Food, Agriculture and Livestock of Turkey registration number TR-34-K-000495) and in our previous study the composition of compounds was analyzed by GC and GC-MS on an Agilent 6890N Network GC system and GC/MS analysis was performed on Agilent 5973 Network Mass Selective Detector integrated with the GC system, using an HP Innowax Capillary column with dimensions 60.0 m × 0.25 mm × 0.25 mm and helium as carrier gas (1.2 ml/min). The oven temperature was set to 60° for 10 min after injection, then increased to 220° with 4°/min heating ramp for 10 min and increased to 240° with 1°/min heating ramp without hold. Both injector and detector (FID) temperatures were 250°; split ratio was adjusted to 50:1. Injection volume was 2.0 µl. MS conditions were as follows: ionization energy, 70 eV; ion source temperature, 280°; interface temperature, 250°; mass range, 34-450 atomic mass units and the cinnamaldehyde content of CO was determined to be equal to 99.54% (Kaskatepe et al. 2016).

Anti-quorum sensing activity of cinnamon oil

A modified method was used, the fresh culture of *C. violaceum* CV026 at 30°C for 18 hours was taken and adjusted to Mc Farland 0.5 density (10^8cfu/ml). A hundred microliters *C. violaceum* CV026, and 50 µl N-hexanoyl-L-homoserine lactone was added to 10 ml soft Luria Bertani agar (0.9%) medium and poured into Petri plates after vortexing. CO (15 µl) was dropped on agar plates and incubated 30°C for 48 hours. Tests were carried out in duplicate (Erdonmez et al. 2018).

Bacteria and their characteristics (serotypes and antibacterial phenotypic and genotypic resistance profiles)

S. enterica strains (n =56), isolated from chicken meat, and cow ground meat and offal, were obtained from the Department of Food Engineering of the Middle East Technical University (METU, TURKEY) and in their previous study molecular confirmation of these strains was conducted by determining *invA* gene (*F:5'-GAACCCTCAGTTTTTCAACGTTTC-3', R:5'-TAGCCGTAACAACCAATACAAATG-3'*) by PCR (Kim et al. 2007) and serotyping of strains was performed using the White-Kauffmann-Le Minor scheme (Grimont and Weil 2007) at the laboratory of Public Health Agency of Turkey in Ankara. Antimicrobial resistance of fifty-six *S. enterica* isolates have been phenotypically determined by agar disk diffusion (CLSI 2016) test using eighteen antibiotics, including amikacin (AK) 30 µg, ampicillin (AMP) 10 µg, amoxicillin-clavulanic acid (AMC) 20/10 µg, cefoxitin (FOX) 30 µg, cephalothin (KF) 30 µg, ceftiofur (EFT) 30 µg, ceftriaxone (CRO) 30 µg, chloramphenicol (C) 10 µg, ciprofloxacin (CIP) 5 µg, ertapenem (ETP) 10 µg, gentamicin (GN) 10 µg, imipenem (IPM) 10 µg, kanamycin (K) 30 µg, nalidixic acid (N) 30 µg, streptomycin (S) 10 µg, sulfisoxazole (SF) 10 µg, sulfamethoxazole-trimethoprim (SXT) 10 µg, and tetracycline (T) 30 µg discs and also the antimicrobial gene resistance profiles of these strains have been investigated by screening 21 antimicrobial resistance genes shown in Table 1, encoding resistance against Class A beta-lactamase, ceftiofur, ceftriaxone, beta-lactamases, chloramphenicol, streptomycin, gentamicin, kanamycin, trimethoprim, sulfisoxazole, and tetracycline (Acar et al. 2017, Durul et al. 2015). The isolates that show resistance to three or more antibiotic classes (with resistance to at least one antibiotic from each class) were considered to be MDR.

Table 1. The antimicrobial resistance genes that were screened in the isolates (Soyer et al. 2013)

Antimicrobial gene	Antimicrobial
*bla*TEM-1	Class A beta-lactamase
*bla*PS13E-1	Class A beta-lactamase
*bla*CMY-2	Ceftiofur, Ceftriaxone
ampC	Beta-lactamases
cat1	Chloramphenicol
cat2	Chloramphenicol
Flo	Chloramphenicol
cmlA	Chloramphenicol
aadA1	Streptomycin
aadA2	Streptomycin
strA	Streptomycin
strB	Streptomycin
aacC2	Gentamicin, Kanamycin
aphA1-Iab	Kanamycin
dhfrI	Trimethoprim
dhfrXII	Trimethoprim
sulI	Sulfisoxazole
sulII	Sulfisoxazole
tetA	Tetracycline
tetB	Tetracycline
tetG	Tetracycline

Antibacterial activity of cinnamon oil

The minimal inhibitory concentration (MIC) of

CO was determined using the broth microdilution test, based on the recommendation of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards (EUCAST 2021). For this purpose, 100 µl cation adjusted Mueller-Hinton Broth (MHB) was added to 96-well micro-titer plates. Then, 100 µl CO was added into the first well and then was two-fold serially diluted. From a fresh culture of the bacterium, a suspension was prepared in phosphate-buffered saline; its turbidity was adjusted to an optical density equivalent to McFarland 0.5 using a nephelometer. The suspension was diluted to a ratio of 1:100 in MHB to obtain a final density of 5 × 10^5 cfu/ml. Finally, 100 µl of this bacterial suspension was added to each well. Micro-titer plates were incubated at 37 °C for 16 to 20 h. After incubation period, the MIC value was determined as the lowest CO concentration that inhibited bacterial growth. All tests were performed in duplicate.

RESULTS

In this study, anti-QS activity of the CO was evaluated as an inhibition of violacein pigment although signal molecule and CV026 strain together in the environment. As a result, it was determined that CO has inhibitory activity on N-hexanoyl-L-homoserine lactone molecule, in other words decreasing production of violacein pigment depending on the quorum sensing bacterial communication system.

For the antibacterial activity part of the study, nineteen of fifty-six *Salmonella enterica* isolates from chicken meat, and cow ground meat and offal, representing serotypes Infantis (15), Kentucky (1), Newport (1), Telaviv (1), and Typhimurium (1), were determined as resistant or MDR. All Infantis isolates were obtained from chicken meat. All foodborne *Salmonella* isolates in our study were resistant to at least one antibiotic. *Salmonella* Infantis strains were found resistant to three or more antibiotic classes (with resistance to at least one antibiotic from each class) and categorized as MDR. All *Salmonella* Infantis isolates (15) were resistant to N, and eight of these were resistant to K, S, SF, T, and N antibiotics. The results of the antibiotic resistance of these isolates are shown in Table 2.

Cinnamaldehyde (99.54%) was determined as the major component of the tested CO by GC and GC-MS. The antibacterial activity of CO was studied using the broth microdilution test, and the results were expressed as MIC value, as shown in Table 3. The lowest MIC value was determined as 0.125 (µg/ml) against some *Salmonella* Infantis and Kentucky serotypes.

Table 2. Antibiotic resistance of *Salmonella* isolates (Acar et al., 2017)

*METU ID	Isolates	The list of resistant antibiotics	The number of resistant antibiotics
MET S1-056	*S. enterica* Infantis	K, S, T, AMP, KF, SF, SXT, C, N	9
MET S1-050	*S. enterica* Infantis	K, S, T, AMP, SF, N	6
MET S1-674	*S. enterica* Infantis	K, S, T, SF, N	5
MET S1-669	*S. enterica* Infantis	S, AMP, KF, N	4
MET S1-788	*S. enterica* Infantis	SF, SXT, C, S, CIP, N, T	7
MET S1-785	*S. enterica* Infantis	SF, SXT, C, S, N, T	6
MET S1-750	*S. enterica* Infantis	SF, SXT, K, N, T	5
MET S1-782	*S. enterica* Infantis	SF, SXT, K, S, N, T	6
MET S1-759	*S. enterica* Infantis	SF, SXT, N, T	4
MET S1-777	*S. enterica* Infantis	SF, SXT, S, CIP, N, T	6
MET S1-792	*S. enterica* Infantis	SF, SXT, S, N, T	5
MET S1-668	*S. enterica* Infantis	S, SF, N	3
MET S1-492	*S. enterica* Infantis	S, T, N	3
MET S1-606	*S. enterica* Infantis	S, T, SF, N	4
MET S1-673	*S. enterica* Infantis	S, T, SF, N	4
MET S1-313	*S. enterica* Kentucky	T, N	2
MET S1-670	*S. enterica* Newport	SF	1
MET S1-063	*S. enterica* Telaviv	N	1
MET S1-625	*S. enterica* Typhimurium	S	1

*Middle East Technical University, Food Engineering Department Laboratory ID
AMP: Ampicillin, KF: Cephalothin, C: Chloramphenicol, CIP: Ciprofloxacin, N: Nalidixic acid, S: Streptomycin, SF: Sulfisoxazole, SXT: Sulfamethoxazole-trimethoprim, T: Tetracycline.

Table 3. Antibacterial activity of cinnamon oil

*METU ID	Isolates	MIC (µg/ml)
MET S1-056	*S. enterica* Infantis	0.125
MET S1-050	*S. enterica* Infantis	0.125
MET S1-674	*S. enterica* Infantis	0.50
MET S1-669	*S. enterica* Infantis	0.50
MET S1-788	*S. enterica* Infantis	1
MET S1-785	*S. enterica* Infantis	0.25
MET S1-750	*S. enterica* Infantis	0.25
MET S1-782	*S. enterica* Infantis	0.25
MET S1-759	*S. enterica* Infantis	0.50
MET S1-777	*S. enterica* Infantis	0.50
MET S1-792	*S. enterica* Infantis	1
MET S1-668	*S. enterica* Infantis	0.125
MET S1-492	*S. enterica* Infantis	0.125
MET S1-606	*S. enterica* Infantis	0.125
MET S1-673	*S. enterica* Infantis	0.50
MET S1-313	*S. enterica* Kentucky	0.125
MET S1-670	*S. enterica* Newport	0.50
MET S1-063	*S. enterica* Telaviv	0.50
MET S1-625	*S. enterica* Typhimurium	0.25

*Middle East Technical University, Food Engineering Department Laboratory ID

DISCUSSION

In the studies, it was reported that if the bacterial communication system is disrupted for any reason, the bacteria are not able to act in a coordinated manner and cannot create a successful infection process (Rasko and Sperandio 2010). Today, the methods preferred to combat bacterial infections are classical methods such as killing bacteria or stopping reproduction. However, the continuous use of these methods (such as protein synthesis inhibition, prevention of DNA replication, and effect on cell wall synthesis) results in the formation of resistant bacterial populations and ineffective treatment methods as well as economic losses (Aminov 2010). In the present study, we aimed to examine the inhibitory effect of CO against bacterial communication mechanism as a violacein inhibition like an initial level alternative treatment strategy for the attenuation of bacteria and their virulence and found CO effective.There are a limited number of studies on this subject. Similar to our study, Domínguez-Borbor et al. (2020) found that CO has QS inhibitory activity as anti-virulence therapy for vibriosis control, Alibi et al. (2020) specified the anti QS activity of *Cinnamomum* essential oil. Kavyani et al. (2019) underlined that cinnamon has inhibitory activity on the QS gene expression against *Pseudomonas aeruginosa* PAO1. Kalia et al. (2015) found that CO caused a decrease in biofilm-related DNA content and exopolysaccharide production of *Pseudomonas aeruginosa* in addition to inhibiting QS.

Salmonella genus is one of the primary causative agents of human and animal foodborne illnesses. Apart from being a public health concern, it adds to the economic costs in both developing and developed countries (Majowicz et al. 2010). *Salmonella* spp. commonly reside in poultry, beef, eggs, and some other fruits and vegetables. The symptoms of salmonellosis include diarrhea, fever, and abdominal cramps that develop between 12 and 72 h after infection in most people. Diarrhea is regarded as a major symptomatic factor as it can turn into a complication in infants, older people, and individuals with a compromised immune system. According to the CDC data, active food-related *Salmonella* outbreaks observed in 2018 were associated with *Salmonella* Enteritidis, Infantis, and Newport. The CDC report 2018 considered *Salmonella* serovars, particularly Typhimurium, to be the most frequently isolated agents from foods.

Recent years have witnessed a surge in both the number and variety of foodborne diseases. In particular, the increasing severity of salmonellosis, manifested as septicemia, is attributed to the development of MDR *Salmonella* strains that mainly affect children than adults (Ranjbar et al. 2012). The increasing use and misuse of antibiotics in human and veterinary

medicine has introduced antimicrobial resistance among *Salmonella* isolates in recent years (Foley and Lynne 2008). Its spread to the blood-stream and other body sites in hospitalized patients may turn fatal unless treated with antibiotics. Therefore, MDR *Salmonella* serotypes are regarded as an important health concern while treating *Salmonella* infections (CDC 2018).

To overcome the problem of multidrug resistance, recent studies have adopted treatment approaches based on antibiotic-free procedures to fight bacterial infections, for example, the use of EOs with antimicrobial properties. These natural products have widespread applications in the food and have been tested against different microorganisms as inhibitory agents in the form of food preservatives (Anwar et al. 2009; Burt 2004). Spices and their EOs, such as cinnamon, in addition to their use as a flavoring agent, have been the components of traditional medicine. These have been used for treating anaphrodisia, vaginitis, shortness of breath, eye inflammation, leucorrhea, rheumatism, neuralgia, wounds, and sore tooth. Moreover, some studies have reported cinnamon to possess hypoglycemic properties that are beneficial in lowering cholesterol levels (Bandara et al. 2012). It also has wound healing properties and acts as an anti-inflammatory compound (Gunawardena et al. 2014; Haddi et al. 2017; Tung et al. 2008). The major component of cinnamon EO that is responsible for its antimicrobial activity is cinnamaldehyde. This is compatible with our results that indicate high cinnamaldehyde content.

However, to our knowledge, no data related to anti-inhibitory effects of CO on MDR *Salmonella* serotypes exist. Therefore, the present study evaluated the inhibitory activity of CO against MDR *Salmonella* Infantis and some other serotypes (Kentucky, Newport, Typhimurium, and Telaviv). We believe this study to be the first of its kind, and therefore the results cannot be compared with other studies. *Salmonella* Infantis is known as the most frequent host-unspecific serovar to be associated with infections and outbreaks. It is also the fourth most common cause of human salmonellosis in Europe, with 1, 846 cases reported by the EU/EEA countries in 2014 (European Food Safety Authority [EFSA] and European Centre for Disease Prevention) (Almeida et al. 2013; ECDC 2016; McEwen and Fedorka-Cray 2002). The CO tested in the present study exhibited a high antibacterial activity between 0.125 µg/ml and 1.0 µg/ml against these isolates. Although some studies exist in the literature reporting significant antibacterial activity against *Salmonella enterica,* only few of these have specified the serotypes. For example, Yostawonkula et al. (2021) exhibited antibacterial activity of nano/microstructured hybrid composite particles containing CO against *Salmonella enterica*, Bagheri (2020) evaluated an antibacterial activity of CO against *Salmonella enterica* strains, Li et al. (2019) tested antimicrobial properties of hydroxypropyl methylcellulose-cinnamon essential oil emulsions and found effective against *Salmonella* Typhi, Paudel (2019) determined antimicrobial activity of cinnamon oil nanoemulsions on *Salmonella sp*., Chuesiang (2019) found effective cinnamon oil in oil-in-water nanoemulsions against *Salmonella* Typhimurium. Chen et al. (2013), in their study, determined the effect of CO on *Salmonella* Typhimurium proliferation; they reported a 1.3-log reduction (1.3 log) in *Salmonella* Typhimurium count in ground pork after treatment with CO. The minimum concentration required to achieve more than 1-log reduction in *Salmonella* population was 0.8% CO. On the same lines, Brnawi et al. (2018) tested the effects of cinnamon leaf and bark oil on *Salmonella* Typhimurium in strawberry shakes and observed a significant difference ($p< 0.05$) in log reduction of bacterial growth. Upadhyaya et al. (2015) indicated trans-cinnamaldehyde to reduce *Salmonella* Enteritidis colonization on eggshell and in yolk, whereas Todd et al. (2013) found a considerable decrease in bacterial population after CO treatment of infected romaine, iceberg, and spinach with *Salmonella* Newport at high concentrations and long treatment durations. Piovezan et al. (2014) found CO to effectively inhibit *Salmonella* Saintpaul growth at a concentration of 312 µg/ml.

Cinnamon has been found to have a strong antibacterial activity, consistent with other studies. In fact, in the present study, it was determined that multi-drug resistant isolates are sensitive to cinnamon oil and can also be effective as virulence inhibitors. However, to determine the usability of cinnamon in terms of public health and its ability to be added to existing treatment plans, in vivo studies and additional toxicity studies are required, and this situation appears as factors that limit the present study.

CONCLUSION

The results of the present study demonstrate that CO has strong antimicrobial activity against all tested foodborne *Salmonella enterica* isolates and anti-quorum sensing potential. Considering its inhibitory activity, especially against MDR *Salmonella* Infantis isolates, we believe CO could act as a natural antimi-

crobial ingredient in foods, thereby protecting from foodborne pathogens. Additionally, it is a potential candidate for developing new antimicrobial agents against MDR *Salmonella* isolates and also as a quorum sensing inhibitory agent. *Salmonella enterica* serotypes produce autoinducer 2 (AI-2) via the lux S synthase gene to coordinate virulence lux S gene expression with the QS mechanism, depending on the population density. Based on this, it is aimed to study the effectiveness of CO against AL-2 synthesis and QS gene expression of *Salmonella* serotypes in future studies.

CONFLICT OF INTEREST

The authors have no declaration of competing interests.

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Research article
Ερευνητικό άρθρο

Age-and sex-related changes in selected hematological parameters, lipid peroxidation and erythrocytes osmotic fragility of Turkish Angora cats

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ABSTRACT: This study was conducted to investigate the changes in selected hematological parameters, lipid peroxidation and osmotic fragility of erythrocytes in Angora cats depending on age and gender. For this purpose, the blood samples were collected from *vena saphena medialis* of 9 young and 14 adult cats which were also classified as male (n=12) and female (n=11). Following hematological analysis, samples were washed with PBS by centrifugation and 10% hematocrit suspension was prepared from the erythrocytes pellet for the osmotic fragility test. The concentration of malondialdehyde (MDA) was also measured from lysed erythrocytes to determine lipid peroxidation level. Red blood cells (RBCs), hemoglobin, and hematocrit were significantly ($P<0.001$) high in adults while the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were significantly ($P<0.05$) high in young cats. Erythrocyte MDA level was also higher statistically ($P<0.05$) in adult cats than in young cats. There was no significance ($P>0.05$) in these parameters between male and female cats. Findings of fragility tests showed that erythrocytes of young and male cats were statistically more susceptible to hypotonic NaCl solutions than those of adult and female cats, respectively ($P<0.01$, $P<0.05$). It was concluded that erythrocytes related parameters in Angora cats changed depending on age rather than gender except for stability of RBCs.

***Keywords*:** Angora cat, age, erythrocyte membrane stability, gender, osmotic fragility, oxidative stress

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INTRODUCTION

Angora cat is one of the kind cat breeds mainly originated from Ankara, Turkey. They have gold, blue or gold-blue colored eyes and generally white hairs. Researching Angora cats is vital as they are now an endangered species. Analysis of hematological parameters is the most common procedure in human and animal clinics for the diagnosis of many diseases. However, species-specific reference blood values can be affected by different factors such as age, breed, sex, environment and toxicants, and should be available to interpret the findings accurately (Spada et al., 2015; Şimşek et al., 2015; Turgut, 2000; Yiğit and Kabakçı, 2018). Therefore, it is very critical to investigate normal blood parameters of Angora cats from many perspectives.

The erythrocyte osmotic fragility (OF) is a commonly used test to measure the membrane resistance of RBCs for the assessment of blood-related disorders such as anemia. Although the OF test is not routinely used as a diagnostic hematological parameter by clinicians, it is known that erythrocyte is affected in some disease by increasing or decreasing fragility (Slappendel, 1998). Erythrocyte OF also provides information about the surface/volume ratio of RBCs, which may change in many pathological conditions (Beutler, 1990). This technique is based on the colorimetric measurement of the hemoglobin (Hb) content released from the RBCs, exposed to the progressively hypotonic NaCl solution. The level of hemolysis is expressed as a percentage by comparing RBCs completely lysed in distilled water (Perk et al., 1964). It was previously well reviewed that OF could be affected by some extrinsic (temperature, pH, oxygenation, and drugs) and intrinsic (age, gender, breed, species, genotype and phenotype) factors (Igbokwe, 2018). It was shown that OF decreased aging in sheep (Asri et al., 2006) and cattle (Basarab et al., 1980). It was determined that the osmotic fragility of aged erythrocytes in circulation decreased in elderly humans and bovine (Mosior and Gomułkiewicz, 1988). On the contrary, Rifkind et al. (1983) reported that OF increased in aged-erythrocytes of human. It was also found that osmotic fragility of erythrocytes was higher in male than female in cattle (Olayemi, 2007), fowls (Durotoye and Oyewale, 1988) and sheep (Durotoye, 1987), while it was lower in male than female in dogs (Ogunyemi and Olayemi, 2016), goats (Habibu et al., 2014), turkeys (Azeez et al., 2011) and humans (Olorunshola et al., 2012).

The membranes of RBCs consist of polyunsaturated fatty acids, phospholipids and cholesterol which relate to membrane stability and functions (Sako et al., 1989). Also, erythrocytes are constantly exposed to oxidative stress by free radicals produced by hemoglobin oxidation (Akila et al., 2007). Disruption of the redox activities of erythrocytes may result in membrane damage (Ojo et al., 2006). Aging might be a potential process for irreversible alterations related to the bioaccumulation of such oxidative disturbance in the cell (Akila et al., 2007). Lipid peroxidation is a series reaction producing free radicals in cell membranes and serves as an indicator of oxidative stress. It could be easily determined by the measurement of malondialdehyde (MDA), a product of lipid peroxidation, for determining oxidative disturbance (Halliwell, 1994; van Ginkel and Sevanian, 1994).

In Turkey, there are only few cat houses where Angora cats are saved and maintenance, except individual Angora cats keeper. It is aimed to create reference information about these cats while ensuring the protection and reproduction of these endangered animals. There are several pieces of research (Atmaca et al., 2014; Erat and Arikan, 2012; Simsek et al., 2015a; Şimşek et al., 2015) about Angora cats related to their physiological values. However, to the best of our knowledge, variations of the erythrocyte membrane resistance in Angora cats have not been defined depending on the age and gender previously. Therefore, the purpose of the present study was to determine the age-and sex-related changes in selected hematological parameters, lipid peroxidation and erythrocytes osmotic fragility of Turkish Angora cats.

MATERIAL AND METHODS

This study was carried out with nine young (less than 1 year old, 6.3±1.9 months) and fourteen adult (1-6 year-old, 44.6±18.6 months) Turkish Angora cats all healthy, vaccinated, fed with standard commercial dry cat food, and housed in Kirikkale University in the same conditions throughout the investigation. We only used the cats in our center for this study in case of changes in blood parameters depending on housing and feeding conditions. They were also grouped as male (n=12) and female (n=11) to investigate the effects of gender. The study design was approved by the Local Ethical Committee of Kirikkale University (2019/3-21).

Blood was collected into the heparinized test tubes by puncturing of *vena saphena medialis* for hematological analysis and obtaining hemolysate.The blood samples were immediately transported to the labora-

tory in the next building and analyzed for red blood cells (RBC), hemoglobin (Hb), hematocrit (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) by using an automatic blood analyzer (Abacus Junior Vet 5, Austria).

The rest of the blood samples were centrifuged at 1000 g for 10 min at 4 °C in the laboratory. The plasma and buffy coat were removed, and erythrocytes were washed 3 times with phosphate buffered sodium chloride solution (PBS, 154 mM NaCl, 10 mM sodium phosphate, pH 7.4). After the last centrifugation, a little part of the erythrocyte pellet was resuspended in PBS-NaCl to prepare 10% hematocrit to use the osmotic fragility test. The rest of erythrocyte pellet was hemolyzed with cold-deionized distilled water and stored at -80 °C for further MDA analysis. The MDA levels of erythrocytes were analyzed as a classical method previously described by Buege and Aust (1978).The hemoglobin content of erythrocytes was determined by the cyanmethemoglobin method (Drabkin and Austin, 1932).

The osmotic fragility of erythrocytes was evaluated in 2.5 mL of PBS containing gradually increased concentrations of NaCl from 0 to 0.85 in 13 tubes as previously defined by Tritschler et al. (2016) with some modification. Ten microliters of 10% hematocrit suspension were mixed with 2.5 mL of each PBS-NaCl dilutions and distilled water in different glass tubes and incubated for 30 min at laboratory temperature. Afterwards, all samples were centrifuged at 1000 g for 10 min, and the optical density of the supernatants was read with a spectrophotometer (Multiskan Go, Thermo Scientific, Finland) at 540 nm. The percentage of hemolysis in each tube containing different concentrations of NaCl was calculated by comparison with that of distilled water which was assumed 100 % hemolysis by using formula; % hemolysis = (absorbance of test tubes/absorbance of 0% NaCl dilution tube) x 100. The results were expressed as % hemolysis.

The threshold of P-value is considered 0.3 instead of 0.05 to obtain more accurate results of sample distribution tests for determining of reference interval (RI) in veterinary species that have a smaller population, according to guideline developed by the American Society of Veterinary Clinical Pathology (ASVCP) (Friedrichs et al., 2012).The Gaussian distribution of samples was determined by the Shapiro-Wilks test and the RI calculation of Gaussian and non-Gaussian samples were performed with parametric and robust methods using MedCalc Software Version 20 (Ostend, Belgium), respectively. Further statistical analysis of the data obtained from two groups (young and adult or male and female) was performed with SPSS 18.0 package program by using Student's T-test and Mann Whitney U test for parametric and non-parametric data, respectively. $P<0.05$ was considered statistically significant. It is also recommended when the number of between 20 and 40 reference samples are available, a table of ascending values along with a histogram and mean or median values should be reported (Friedrichs et al., 2012).

RESULTS

The histograms in Figure 1 show the population distributions of selected blood parameters while the histograms in Figure 2 represent the population distributions of erythrocyte MDA levels and hemolysis degrees of RBCs in different % NaCl concentrations.

Selected hematological parameters of young, adult, male, and female Angora cats, and reference ranges of them for domestic cats were shown in Table 1.There were significant differences between erythrocyte related values of young and adult cats while there were n o significant differences between the same parameters of male and female cats. According to these findings, RBC, Hb, and PCV were higher in adults, while MCV, MCH, and MCHC were lower than in young cats.

Table 2 represents the effects of age and gender on the MDA level of cats' erythrocytes. Malondialdehyde levels of adult cats were significantly ($P<0.05$) higher than that of young cats. However, the erythrocyte MDA levels of males or females were not different statistically ($P>0.05$).

Table 2 also shows the % NaCl concentrations of 10% and 90% hemolysis which are accepted initial and complete hemolysis, of erythrocytes in young, adult, male, and female cats. The erythrocytes OF curve of young and adult cats, and male and female cats represented in Figure 3 and Figure 4, respectively, show % hemolysis of RBCs in different dilutions of NaCl. Paired comparisons were made only at the NaCl concentrations range from initial hemolysis to complete hemolysis of RBCs. While erythrocytes of young cats were significantly more fragile than the adults at 0.50, 0.55, and 0.60 % of NaCl concentrations ($P< 0.01$, $P< 0.001$, $P< 0.001$ respectively), according to Figure 1, the erythrocytes fragility of male cats was significantly higher than that of females at 0.55 % ($P<0.01$) and 0.60 % ($P<0.05$) of NaCl concentrations (Figure 2).

Table 1. Changes in erythrocytes related values of Angora Cats depending on the age and gender

Parameters	Reference ranges*	Groups	n	Mean	SD	Median	Min	Max	Reference Interval	CI 90% of LRL	CI 90% of URL	P value
RBC (×10⁶/µL)	5 - 10	Young	9	6.69	1.24	6.56	5.18	8.95	4.65-8.73	3.55-5.75	7.63-9.83	<0.001
		Adult	14	9.72	1.29	9.62	8.36	12.97	7.10-11.87	5.91-8.45	10.56-13.18	
		Male	12	8.62	1.73	8.78	5.18	10.45	5.83-12.29	3.97-7.51	10.80-13.42	NS
		Female	11	7.89	2.24	7.17	5.52	12.97	3.03-11.92	1.12-5.05	9.10-14.10	
Hb (g/dL)	8 - 15	Young	9	11.30	0.92	11.40	10.10	12.80	9.78-12.81	8.97-10.60	11.99-13.62	<0.001
		Adult	14	13.01	0.69	13.01	11.30	13.90	11.88-14.42	11.21-12.51	1.68-14.92	
		Male	12	12.33	1.23	12.85	10.10	13.70	10.39-15.32	9.04-11.69	14.00-15.88	NS
		Female	11	12.05	1.16	12.10	10.60	13.90	10.14-13.94	9.12-11.17	12.92-14.97	
PCV (%)	24 - 45	Young	9	30.54	4.02	29.21	24.67	37.10	23.93-37.14	20.36-27.44	33.58-40.71	<0.001
		Adult	14	42.21	3.82	43.06	34.21	48.76	35.92-48.49	32.68-39.16	45.26-51.73	
		Male	12	38.35	7.17	41.04	24.67	45.45	25.93-54.66	19.52-34.53	48.17-60.63	NS
		Female	11	34.75	6.81	34.21	27.55	48.76	21.27-47.37	13.28-26.25	39.15-53.57	
MCV (fL)	39 - 45	Young	9	46.00	3.22	46.00	41.00	51.00	40.69-51.30	37.83-43.55	48.44-54.16	<0.05
		Adult	14	42.58	4.12	42.50	36.00	50.00	35.80-49.36	32.31-39.29	45.87-52.85	
		Male	12	44.50	2.88	44.50	41.00	50.00	38.93-49.83	35.44-40.62	46.88-51.91	NS
		Female	11	43.91	5.15	45.00	36.00	51.00	35.44-52.37	30.87-40.00	47.80-56.94	
MCH (pg)	12.5 - 17.5	Young	9	17.19	1.99	16.88	14.30	20.65	13.92-20.46	12.16-15.68	18.70-22.22	<0.001
		Adult	14	13.55	1.30	13.51	10.70	15.38	11.40-15.69	10.30-12.50	14.59-16.80	
		Male	12	14.66	2.11	14.00	12.46	19.50	11.20-18.17	9.41-12.98	16.34-19.91	NS
		Female	11	15.98	2.75	16.46	10.70	20.65	11.45-20.50	9.01-13.89	1806-22.04	
MCHC (g/dL)	30 - 36	Young	9	37.24	2.28	36.97	34.40	40.94	33.48-40.99	31.45-35.50	38.98-43.02	<0.001
		Adult	14	30.99	2.71	30.07	28.40	37.10	24.66-35.03	23.04-28.20	31.60-38.12	
		Male	12	32.79	4.05	30.47	28.56	40.94	22.08-39.62	20.14-27.08	34.90-43.38	NS
		Female	11	35.27	3.75	35.86	28.40	40.76	29.10-41.44	25.78-32.43	38.12-44.78	

SD: Standard deviation, CI: Confidence interval, LRL: Lower reference limits, URL: Upper reference limits, NS: Non-significant.
* Reference ranges of selected blood parameters for domestic cats (Blood and Studdert, 1988; Schalm et al., 1975).

Table 2. Changes in lipid peroxidation level and hemolytic range of erythrocytes of Angora cats depending on the age and gender

Parameters	Groups	n	Mean	SD	Median	Min	Max	Reference Interval	CI 90% of LRL	CI 90% of URL	P value
MDA nmol/mL	Young	9	29.31	9.69	32.69	16.67	43.59	11.51-50.63	4.72-21.73	37.42-56.09	<0.05
	Adult	14	38.97	10.69	39.10	21.79	57.69	21.39-56.55	13.34-29.44	48.50-64.60	
	Male	12	34.01	8.28	34.61	19.23	53.20	20.39-47.63	14.36-26.42	41.60-53.66	NS
	Female	11	36.28	15.17	38.46	16.67	57.69	6.48-66.32	5.80-21.90	52.64-72.74	
Initial Hemolysis (%NaCl)	Young	9	0.65	0.05	0.65	0.60	0.75	0.55-0.73	0.51-0.57	0.70-0.78	NS
	Adult	14	0.65	0.05	0.65	0.60	0.75	0.56-0.76	0.54-0.59	0.73-0.79	
	Male	12	0.65	0.04	0.65	0.60	0.75	0.57-0.74	0.55-0.60	0.71-0.77	NS
	Female	11	0.65	0.05	0.60	0.60	0.75	0.55-0.73	0.50-0.60	0.67-0.78	
Complete Hemolysis (%NaCl)	Young	9	0.50	0.06	0.50	0.40	0.55	0.35-0.57	0.31-0.40	0.53-0.61	NS
	Adult	14	0.45	0.04	0.40	0.40	0.50	0.38-0.49	0.34-0.40	0.46-0.52	
	Male	12	0.45	0.05	0.40	0.40	0.55	0.35-0.53	0.31-0.39	0.49-0.57	NS
	Female	11	0.45	0.04	0.45	0.40	0.50	0.39-0.50	0.36-0.43	0.47-0.54	

SD: Standard deviation, CI: Confidence interval, LRL: Lower reference limits, URL: Upper reference limits, NS: Non-significant.

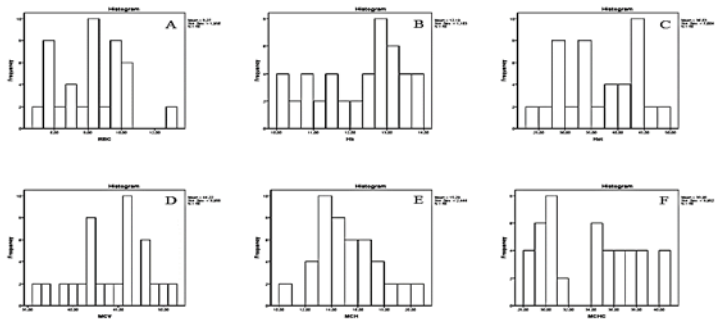

Figure 1. Histograms showing the 6 tested analyte population distributions: (A) RBC: Red blood cells, (B) Hb: Hemoglobin, (C) PCV: Package cell volume, (D) MCV: Mean corpuscular volume, (E) MCH: Mean corpuscular hemoglobin, (F) MCHC: Mean corpuscular hemoglobin concentration

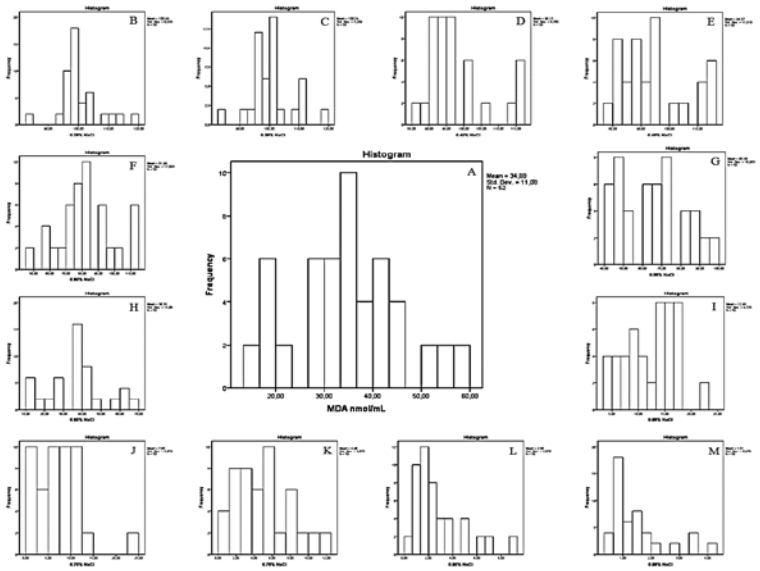

Figure 2. Histograms showing the 13 tested analyte population distributions: (A) MDA levels of erythrocytes, (B-M), hemolysis degrees of erythrocytes in different NaCl dilutions

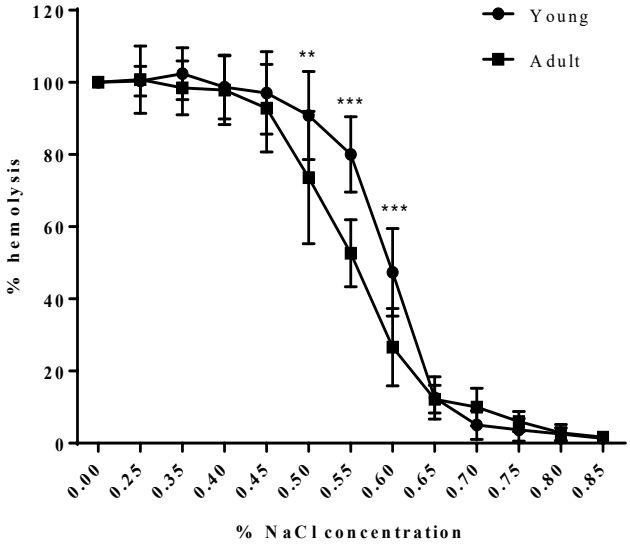

Figure 3. Erythrocyte osmotic fragility curve of young and adult Angora cats. The data expressed as mean plus standard error mean. Asterisk represents the statistically significance, **:P<0.01, ***:P<0.001

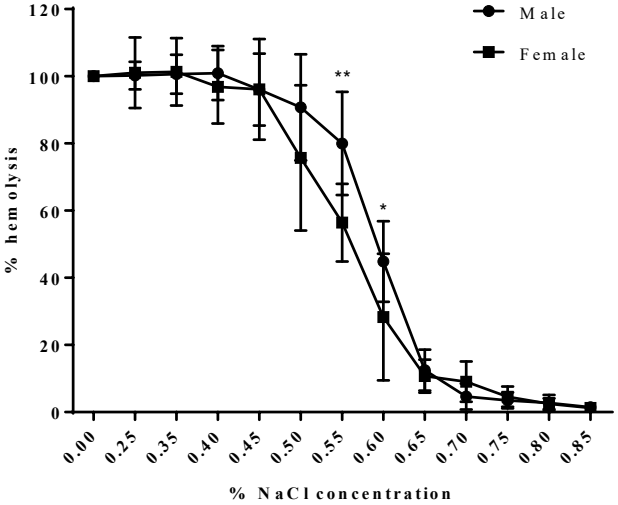

Figure 4. Erythrocyte osmotic fragility curve of male and female Angora cats. The data expressed as mean plus standard error mean. Asterisk represents the statistically significance, *:P<0.05, **:P<0.01

DISCUSSION

The blood parameters are determined not only for the diagnosis of diseases but also for evaluating the health status of living organisms since they are supplied much specific knowledge on the metabolic events in the body (Theml et al., 2004). In this study, we investigated some hematological parameters, lipid peroxidation level, and osmotic resistance of RBCs in healthy Angora cats in terms of age and gender. This study could be criticized for using a small sample size. However, we should note that Angora cat is an important and special cat breed belonging to Turkey, and well-known throughout the world. Unfortunately, it is an endangered breed (Erat and Arikan, 2012). Although, in guidelines the Clinical and Laboratory Standards Institute (CLSI), it is recommended minimum 120 samples for the preparation of reference limits (CLSI, 2010), it is difficult to find enough number of cats that are available, because there are only two centers which tries to protect and maintenance of Angora cats in our country. One of these centers is in our Faculty at Kırıkkale University where 25 cats are housed. The other Angora Cat House belongs to Ankara Pursaklar Municipality, where 40 cats have been saved and maintain here since 2017 (İnal, 2019). We did not want to include them, because their housing, feeding, and drinking conditions were different. In addition, two cats in our faciality were excluded from the study because they are too older (13- and 14-years-old). Therefore, the present study was carried out total of 23 cats, and both classification and grouping of them were performed as previously described by Olayemi et al. (2009).

The results of the hematologic parameters were within the reference ranges and highly similar to suggested standard values for cats (Blood and Studdert, 1988; Schalm et al., 1975). While erythrocyte related values were significantly different between young and adult cats, they did not differ between male and female cats. According to our study, RBC, Hb, and PCV values were higher in adults than in young cats whereas MCV, MCH, and MCHC were higher in young than in adults. Similarly, Simsek et al. (2015a) reported that RBC, PCV, and Hb values in adult Angora cats were higher than in 1.5-3 month-old kittens. Authors also showed that MCHC was higher in adults than in 1.5-3 month-old kittens, while MCV and MCH values were statistically the same in both groups. These findings were also in agreement with the reports on dogs (Olayemi et al., 2009) and goats (Elitok, 2012), in which RBC, Hb and PCV were significantly higher in adults than in the young. Whereas Yiğit et al. (2002) showed the same parameters were higher in young sheep compared to adults. Elitok (2012) showed that high level of MCV, MCH, MCHC in young goats significantly decreased with age which was parallel with our results. We suggest the differences between young and adult cats could be related to the life span or higher oxygen transport capacity of RBCs.

Our findings on RBC, PCV, Hb, MCV, MCH, and MCHC in female cats shown in Table 1 were fairly compatible with a previous study in which reported erythrocytes-related hematological parameters in 1-3-year-old female Angora cats (Şimşek et al., 2015). Olayemi et al. (2009) also did not find significant differences in RBC, PCV, Hb, MCV, MCH and MCHC in male and female Nigerian indigenous dogs. Furthermore, in a study carried out on Van cats, the PCV and Hb values did not differ according to age and gender (Sönmez and Ağaoğlu, 2010). However, Özkan et al. (2016) reported that PCV and Hb values of Van cats were significantly increased with the age, and in male cats. These different reports may be explained by the effects of other factors such as season or breed on blood parameters.

In this present study, lipid peroxidation level in erythrocytes was measured by MDA concentration and was significantly increased by age but there was no difference between male and female cats. Similarly, Simsek et al. (2015b) showed that healthy adult Angora goats had higher MDA levels in erythrocytes than that of young goats. Our results also support the findings of previous studies carried out on dogs (Gaál et al., 1996), mares (Aydilek and Şimşek, 2006), sheep (Salar-Amoli and Baghbanzadeh, 2010) and humans (Akila et al., 2007) which reported MDA concentration was affected by aging, but not gender. Denaturation of lipids, proteins and nucleic acids, and production of free radicals are normal process of cellular metabolism and are balanced by antioxidant mechanisms. Inadequate neutralization of free radicals emerging with ageing, leads to oxidation of cellular lipids, proteins, and carbohydrates (Matsubara and Machado, 1991). High MDA levels in adult cats may be associated with cellular membrane damage because of aging.

According to the findings of the present study, the concentration of NaCl lead to initial (10%) hemolysis was 0.65 % in all groups, and NaCl dilution lead to complete (90%) hemolysis was 0.50 % and 0.45 % in young cats and the others, respectively. These results

were in accordance with standard values of domestic cats as described by Schalm et al. (1975), in which minimum hemolysis was shown at 75% NaCl solution while maximum hemolysis was shown at lower 50% NaCl solution. Kohn et al. (2000) also reported that the initial and complete hemolysis of erythrocytes in non-anemic healthy (Abyssinian and Somali) cats were seen at 0.55 % and 0.45 % NaCl dilutions, respectively. Erythrocytes of young and male cats in the current study were more susceptible to hypotonic NaCl solutions than those of adult and female cats. In some previous reports, the erythrocytes OF in younger sheep (Asri et al., 2006), cattle (Basarab et al., 1980), and turkey (Azeez et al., 2011) were higher than that of older animals, which were compatible with the findings of the present study. This may be related to the short life span of RBCs in young as well as high MCV since it is considered to increase macrocytes (immature erythrocytes) count in blood. Macrocytes may have been easily broken in capillaries during circulation, which resulted in increased fragility when comparing in young and adult animals. Moreover, lower erythrocyte OF in adult cats may be associated with increased stability of cell membrane by aging as previously described by Grinna (1977). According to his report, cholesterol, phospholipids, and saturated fatty acid concentrations of erythrocyte's membrane increased during aging. However, this situation may lead to increased oxidative stress markers such as MDA, GSH, CAT in elderly/geriatric people (Akila et al., 2007). This agrees with the findings of the current study on MDA level (lipid peroxidation) in adult cats which was a higher concentration than that of young cats. It is well known that erythrocytes are more vulnerable to rupture because of the increased oxidative stress by the age, especially in old animals (Tyan, 1982). Contrary to expectations, higher MDA levels in adults did not cause lower OF in erythrocytes compared to young cats. Therefore, it is suggested that increased MDA level in adult Angora cats is not yet enough to increase OF in erythrocytes because they were not senior cats.

Erythrocytes OF in male Angora cats were higher than those in females especially at 0.55 and 0.60 % concentrations of NaCl solution. There are many incompatible reports on variations of erythrocyte OF according to gender in the literature. As compatible with the current study, RBCs of male cattle (Olayemi, 2007), sheep (Durotoye, 1987), and fowls (Durotoye and Oyewale, 1988) were more fragile to hypotonic NaCl solution compared to females. The authors have suggested that this may be related to the stabilizing effect of estrogen. However, erythrocytes OF in female Nigerian Indigenous dogs were higher than that of males (Olayemi et al., 2009) while there were no significant differences in erythrocytes OF between male and female horses (Andriichuk et al., 2014) and German Shepherd dogs (Ogunyemi and Olayemi, 2016). Unfortunately, the effects of gender on erythrocytes OF are still unclear.

CONCLUSION

In conclusion, the results of this study showed that erythrocytes related values of Angora cats may have variations depending on age and gender. Total RBCs, Hb, and PCV values were high in adults while MCV, MCH, and MCHC were high in young cats. These parameters were not altered according to gender. Malondialdehyde level, an indicator of lipid peroxidation was also higher in adults compared to young cats, but statistically the same in males and females. Osmotic fragility of erythrocytes decreased by age and male Angora cats have higher OF than females. It is suggested that the life span of RBCs may play a role in these age-related differences. However, no difference between genders in these values may be due to low levels of sex steroids in females during long anestrus periods. It was concluded that not only the age and gender difference affect the RBC related values, but also breed, season, and hormonal fluctuation might be responsible for these variations. In addition, although this is a small-scale qualitative study related to some blood parameters of Angora cats, these data may be useful for veterinarians, and seen as an opportunity to generate further hypotheses for researchers.

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CONFLICT OF INTEREST

It is declared that there is no conflict of interest between the authors.

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Research article
Ερευνητικό άρθρο

Investigation of Seroprevalence of Toxoplasmosis in Horses and Donkeys in Muş Province of Turkey*

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ABSTRACT: The purpose of this study was to determine the seroprevalence of Toxoplasmosis in equidae in the province of Muş of Turkey. The study material consisted of 210 equidae including 159 horses and 51 donkeys in Muş province. In serum samples, *anti-Toxoplasma gondii* antibodies and titers were detected using Sabin Feldman Dye Test (SFDT). Seropositivity was found in 115 (54.76 %) of the 210 equidae tested in the study. The rate of seropositivity in donkeys (92.16%) was higher than the rate in horses (42.77%), and statistical significance was observed (P<0.001). *T. gondii* antibody was detected in 68 (42.77%) of the horse sera. When *T. gondii* seropositivity was evaluated according to gender, it was found to be 47.92% in females and 32.92%in males. No statistical difference was observed between the gender groups (P> 0.05). When *T. gondii* seropositivity was evaluated according to age, seropositivity rate in those older than 10 years was found to be higher as 46.67%, but no statistical significance was observed among the age groups. *T. gondii* antibody was detected in 47 (92.16%) of donkey sera. When *T. gondii* seropositivity was evaluated according to gender, the rate of seropositivity was found to be 89.47% in females and 93.75% in males. No statistical significance was observed between the gender groups (P>0.05). When *T. gondii* seropositivity was evaluated according to age, the seropositivity rate in those older than 10 years was found to be higher as 96.30%, but no statistical significance was observed among the age groups (P>0.05). As a result of scanning the equidea in Muş province by SFDT, seropositivity rate was found as 42.77% in horses and 92.16% in donkeys.

Keywords: Donkey, Horse, SFDT, *Toxoplasma gondii*, Toxoplasmosis

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INTRODUCTION

Toxoplasmosis is a zoonotic, protozoal disease caused by the *Toxoplasma gondii (T. gondii)* agent (Dubey and Beattie, 1988). *T. gondii* has a wide variety of intermediate hosts from warm-blooded animals to humans. The agent is zoonotic in character and is an obligate intracellular protozoal parasite of feline (Garcia-Bocanegra et al., 2012).*T. gondii* is estimated to infect the population with a worldwide distribution, and to the extent that if it infects human, it is of great threatto animal health as well owing to its wide range of transmission (Kim and Weiss, 2008). Although *T. gondii* infection is subclinical in equidae, it may show symptoms such as fever, ataxia, retinal degeneration, encephalomyelitis, and may also lead to abortion or stillbirth (Güçlü et al., 2007; Miao et al., 2013). The disease is more severe in young and immunosuppressed animals (Güçlü et al., 2007; Kar and Güven, 2016).

To diagnose toxoplasmosis, serological tests such as Sabin-Feldman Dye Test (SFDT), Modified Plate Agglutination Test (MAT), Indirect Fluorescent Antibody Test (IFAT), Indirect Hemagglutination Test (IHAT), Enzyme-linked Immunosorbent Assay (ELISA), Immunosorbent Agglutination Assay (ISAGA), Lam Aglutination Test (LAT), Piezoelectric Immunoagglutination Assay (PIA), Western Blot (WB), Immunochromatographic Assay (ICT) and Avidity Test are applied (Liu et al., 2015).

Studies on investigation of the seroprevalence of toxoplasmosis in equidae (horses and donkeys) have been conducted in various region of Turkey. However, no study has been carried out in Muş province of Turkey. Therefore, in this study was aimed to determine the presence of *anti-Toxoplasma* antibodies and the seroprevalence of *T. gondii* by using SFDT in equidae from this province.

MATERIALS AND METHODS

Animals and sample collection

This research was carried out in accordance with the Sub-Committee Decision No: 2017/13 of Ataturk University, Faculty of Veterinary Medicine. The present study was conducted on 210 equidae (159 horses and 51 donkeys) which were randomly selected regardless of age and gender in Muş Province of Turkey (Table 1 and Table 2).The blood samples collected from the *vena jugularis* of the horses and donkeys were centrifuged at 3000 rpm, the sera were separated and stored at -80^{0}C until analyzed by the serological tests.

Serological examination

Serological tests of sera were performed by SFDT at the Parasitology Laboratory of Refik Saydam Hygiene Institute (Ankara, Turkey) (Sabin and Feldman, 1948). For the production of live antigen, *T. gondii* seronegative *Mus musculus* albino white mice ranging in age from 3-6 weeks were tested. For the continuation of live antigen production and the use of live antigen in tests, 2 ml of diluted liquid was produced by diluting with 0.9% Sodium Chloride (NaCl) solution to contain approximately 15-16 *T. gondii* tachyzoites in field controls performed *T. gondii* tachyzoites in field controls performed under the light microscope (10×40). Each mouse

Table 1. Foci, species and gender distribution of the examined group

Study region	Horse	Female horse	Male horse	Donkey	Female donkey	Male donkey	Total
Muş (Center)	73	45	28	25	4	21	98
Bulanık	18	11	7	19	8	11	37
Haskoy	35	21	14	1	1	0	36
Korkut	3	3	0	1	1	0	4
Malazgirt	20	10	10	5	5	0	25
Varto	10	6	4	0	0	0	10
Total	159	96	63	51	19	32	210

Table 2. Distribution of horses and donkeys in the study by age

Age/Species	Horse	Donkey	Total
0-5	28	9	37
6-10	56	15	71
>10	75	27	102
Total	159	51	210

was intraperitoneally administered 0.2 ml of diluted liquid containing tachyzoites. The obtained exudate was diluted with NaCl and homogenized, and when examined under a light microscope by adding activator serum, it was passaged to contain 25-30 tachyzoites. The obtained sera were then diluted in 1/16, 1/64, 1/128 ratios and the same amount of antigen (mixed with activator serum) was added and incubated for 50 minutes in a 37^0C water bath. After incubation, 0.025 ml of alkali methylene blue was added and mixed. 0.020 ml of it was taken and placed on the slide; applying a coverslip, it was examined at magnification under light microscope (10×40).The evaluation was made according to the staining pattern of the tachyzoites under the light microscope; if a tachyzoite was dyed more than 50%, the test was considered to be negative. If there was a tachyzoite that wasn't dyed more than 50%, the test was considered to be positive.

Statistical analysis

Statistical analysis of the data was made by SPSS 20.0 (SPSS Inc., Chicago, IL, USA) program to determine the seropositivity and seronegativity significance levels between the species, gender and age groups using the *Chi-square test* (X^2). Statistical significance in this study was defined as $P<0.05$.

RESULTS

Serological findings of horses and donkeys

While seropositivity were detected in 68 (42.77%) of 159 horses, 91 (57.23%) of them were seronegative. At the same time, while seropositivity were determined in 47 (92.16%) of 51 donkeys, seronegative were 4 (7.84%) of them. As a result, 115 (54.76%) equidae were found to be seropositive in total, and 95 (45.23%) found to be seronegative (Table 3).

Distribution of *T. gondii* seropositivity and antibody titers detected by SFDT in horses by gender and age

T. gondii antibodies were determined in 68 (42.77%) of the horse sera examined by SFDT. Of the seropositive sera; 66 (41.50%) yielded positivity at a titer of 1/16and 2 (1.25%) at 1/64. *T. gondii* seropositivity was determined as 47.92% (46/96) in females and 32.92% (22/63) in males. In addition, *T. gondii* seropositivity was detected as 46.67% (35/75) in those older than 10 years, as 42.86% (24/56) in those aged 5-10 years, and 32.14% (9/28) in 0-5 age group. However, there was no statistically significant difference between both gender and age groups (P>0.05) (Table 4).

Table 3. Serological findings of horses and donkeys

Species	Number of sera	Number and rate of positivity (%)	Number and rate of negativity (%)	*P value*	Antibody titer	
					1/16	1/64
Horse	159	68 (42.77%)	91 (57.23%)	0.001	66	2
Donkey	51	47 (92.16%)	4 (7.84%)		38	9
Total	210	115 (54.76%)	95 (11.58%)		104	11

Table 4. Distribution of *T. gondii* seropositivity and antibody titers detected by SFDT in horses by gender and age

Factor		Number of sera	Number and rate of positivity (%)	Number and rate of negativity (%)	*P value*	Antibody titer	
						1/16	1/64
Gender	**Female**	96	46 (47.92%)	50 (52.08%)	0.105	45	1
	Male	63	22 (% 32.92)	41 (65.08%)		21	1
Age	**0-5**	28	9 (32.14%)	19 (67.86%)	0.897	7	2
	5-10	56	24 (42.86%)	32 (57.14%)		24	0
	>10	75	35 (46.67%)	40 (53.33%)		35	0
	Total	159	68 (42.77%)	91 (57.23%)		66	2

Table 5. Distribution of *T. gondii* seropositivity and antibody titers detected by SFDT in donkeys by gender and age

Factor		Number of sera	Number and rate of positivity (%)	Number and rate of negativity (%)	P value	Antibody titer		
						1/16	1/64	1/128
Gender	**Female**	19	17 (% 89.47)	2 (% 10.53)	0.547	13	4	0
	Male	32	30 (% 93.75)	2 (% 6.25)		25	4	1
Age	**0-5**	9	7 (% 77.78)	2 (% 22.22)	0.198	5	2	0
	5-10	15	14 (% 93.33)	1 (% 66.67)		11	2	1
	>10	27	26 (% 96.30)	1 (% 3.70)		22	4	0
	Total	51	47 (% 92.16)	4 (% 7.84)		38	8	1

Distribution of *T. gondii* seropositivity and antibody titers detected by SFDT in donkeys by gender and age

T. gondii antibodies were determined in 47 (92.16%) of donkey sera examined by SFDT. Of the seropositive sera, 38 (74.51%) yielded positivity at a titer of 1/16, 8 (15.69%) at 1/64, and 1 (1.97%) at 1/128.*T. gondii* seropositivity was determined as 89.47% (17/19) in females and 93.75% (30/32) in males. In addition, *T. gondii* seropositivity was detected as 96.30% (26/27) in those older than 10 years, 93.33% (14/15) in those aged 5-10 years, and 77.78% (7/9) in 0-5 age group. However, there was no statistically significant difference between both gender and age groups (P>0.05) (Table 5).

DISCUSSION

The first study with horses in Turkey was conducted on 154 horses by SFDT about 50 years ago and seropositivity at a rate of 14.3% was detected (Dubey, 1998).It is reported that, under natural conditions, the prevalence of toxoplasmosis in horses ranges from 0% to 90% worldwide (Akkan et al., 2001).In addition, it is stated that the seroprevalence of *T.gondii* in donkeys worldwide varies between 11% and 62% (Machacova et al., 2014).Many factors are held responsible for this wide range of seropositivity, such as sensitivity and specificity of the serological test used, age of animals, climate, breeding and care standards, hygiene of shelters, and the number of samples taken (Pomares et al., 2011; Machacova et al., 2014).In the present study, the areas where horses and donkeys live, shelter conditions, shelter hygiene and contact with stray animals in Muş province support these suggestion.

Toxoplasma gondii causes subclinical infections in equidae (horses and donkeys). Therefore, the diagnosis of the infection is performed using various serological tests to detect *T. gondii* antibodies. A number of serological tests to detect antibodies to *T. gondii* have been thoroughly studied in various hosts (Dubey and Beattie, 1988).Although the requirement for the use of live parasites means that the SFDT is not commonly used, it remains the gold standard in many hosts.We therefore selected it for our study.Using the test, we found that the overall seroprevalence of toxoplasmosis 42.77% (68/159) in horses and % 92.16 (47/51) in donkeys in the province of Muş. Also, a statistical difference was observed between species (P<0.001) (Table 3).

When studies on Toxoplasmosis seroprevalence in horses are reviewed worldwide, varying levels of seropositivity have been reported. By different serological methods (ELISA, IFAT and MAT), seropositivities have been determined between 11.59%-22.7% in Brazil (Ribeiro et al., 2016; Almeida et al., 2017; Magalhães et al., 2017) %37.8-39% in Romania (Paştiu et al., 2015), 26% in Algeria (Mohamed-Cherif et al., 2015), 17.7% in Tunisia (Boughattas et al., 2011) and 14% in Iran (Razmi et al., 2016). The presented study, seropositivity (42.77%) detected in horses by was found to be higher than the seropositivity rates obtained by the study conducted in many countries (Table 3). The differences in the results from various studies worldwide on *T. gondii* seroprevalence in horses can be attributed to the factors such as types of serological tests employed, age of horses, location of the studies, the intended use of the animals, the number of final cat hosts and the level of contact thatthe horses have with these cats (Miller et al., 1972; Machacova et al., 2014; Paştiuet al., 2015).In our study, the high seropositivity in horses was attributed to related reasons.

When studies on toxoplasmosis seroprevalence in donkeys are reviewed worldwide, various levels of seropositivity have been reported. By different serological methods (ELISA, IFAT, LAT, MAT and PCR), seropositivities have been determined between 45% and 65% in Egypt (El-Ghaysh et al., 1998, Haridy et al., 2010), 5-8% in Italy (Machacova et al., 2014), 25.6% in USA (Dubey et al., 2014), 34% in Spain (García-Bocanegra et al., 2012), and 6.29% in China (Zhang et al., 2017). Also, two separate studies conducted in Egypt and China has linked the high rate of seropositivity to free rearing style of donkeys and to greater contact with cats (El-Ghaysh et al., 1998; Zhang et al., 2017). In addition, Machacova et al. (2014) have attributed the differences in seroprevalence rates to the number of donkeys subjected to test.In the present study, the seropositivity rate was determined as 92.16%, which was above the world average (Table 3). In addition, it was observed that high seropositivity was compatible with the related studies. That is, the free breeding of the donkeys tested explains the high rate of seropositivity.

When studies related to toxoplasmosis seroprevalence in horses in Turkeyare reviewed, various levels of seropositivity have been reported with different serological methods. By different serological methods (ELISA, IHAT, and SFDT), seropositivities have been determined between 2%-63.09% in Ankara (Babur et al., 1997; Babur et al., 1998; Güçlü et al., 2007; Gazyağcı et al., 2011), 10.44% in Kayseri (İnci et al., 2002), 7.2% in Niğde (Karatepe et al., 2010), 6.4% in Malatya (Aktaş et al., 1999), 1.80 % and 20.6%

in Kars (Aslantaş et al., 2001; Akça et al., 2004), % 1.74 in Van (Akkan et al., 2001), 13.5% and 28.4% in Hakkari (Göz et al., 2007), 6.35% in various provinces of Southeastern Anatolia (Diyarbakır, Gaziantep and Şanlıurfa) (Özkan et al., 2002) and 46.3% in the samples collected from various provinces of Turkey (Adana, Bursa, Gaziantep, İstanbul, İzmir and Konya) (Zhou et al., 2016). In the presented study, it is observed that the seropositivity rate is parallel to the seropositivity rates determined in other studies (Table 3).

When studies on toxoplasmosis seroprevalence in donkeys are reviewed in Turkey, various levels of seropositivity have been determined. İnci et al. (2002) have reported that seropositivity in 14 of the 33 donkeys (% 42.42) in Kayseri, and Balkaya et al. (2011) determined seropositivity in 57 of the 92 (62%) by SFDT in Erzurum.In the presented study, it was noted that the seropositivity was higher than the seropositivity rates determined in other studies (Table 3).The number of animals sampled can be considered as the reason for this difference.

Considering the relationship between toxoplasmosis and age in horses, Boughattas et al. (2011) have achieved a seropositivity rate of 21.27% in those over the age of 10; whereas Villa et al. (2018) reported a high rate of seropositivity in horses over 15 years old. Klun et al. (2017) have indicated that age was not statistically significant and they attributed the low rate of seropositivity to the young study population. In Turkey, Göz et al. (2007) stated that seroprevalence is higher in horses within the age range of 0-2 in Hakkari province. Karatepe et al. (2010) have reported that there was no statistical difference between the groups, although they detected seropositivity at a rate of 7.40% in 1-10 age group and 6.81% in 11-20 age group, in Niğde. In the present study, high levels of seropositivity were detected in horses over 10 years of age. However, as in other studies, no statistically significant difference was found between age groups (Table 4).

Looking at the relationship between toxoplasmosis and age in donkeys, Dubey et al. (2014) detected seropositivity in donkeys older than 30 months, while they did not detect seropositivity in those younger than 30 months. Machacova et al. (2014) stated that the positivity was higher in the animals in the elderly group, in their study by the LAT and IFAT. Balkaya et al. (2011), despite detecting seropositivity at rates of 38.6% in the age group 0-3, 50.9% at 4-6, and 19.3% at over the age of 7 in Erzurum Province, have not reported a statistical difference between age groups. In the presented study, high levels of seropositivity were detected in donkeys over 10 years of age. However, as in other studies, no statistically significant difference was found between age groups (Table 5).

When *T. gondii* seroprevalence in horses were evaluated with regards to gender groups, Haridy et al. (2009) have determined in Egypt, higher seropositivity in females (50%) than in males (22.2%), and inferred that females were more sensitive to the agent.Göz et al. (2007) reported a higher rate of seropositivity in females in Hakkari, while Güçlü et al. (2007) revealed a higher rate of seropositivity in males in Ankara. However, no statistical difference was found between the genders in both studies. In the present study, higher seropositivity was determined in females. However, as in other studies, no statistically significant difference was found between gender groups (Table 4).

When *T. gondii* seroprevalence in donkeys were evaluated with regards to gender groups, Haridy et al. (2010) in Egypt, Dubey et al. (2014) in USA and Machacova et al. (2014) in Italy have reported higher seropositivity rates in female donkeys.In addition, in Erzurum province of Turkey, a higher seropositivity rate has indicated in female donkeys, but no statistical difference was observed (Balkaya et al., 2011).On the other hand, in the present study, a higher seropositivity was found in male donkeys. However, as in other studies, no statistically significant difference was found between gender groups (Table 5).

In conclusion, Toxoplasmosis seroprevalence in horses and donkeys in Muş region of Turkey was determined as 42.77% in horses and as 92.16% in donkeys. The infection with zoonotic character has been ascertained to be critical for the public health and therefore should be taken in consideration. Also, it is necessary to conduct extensive research on the role of livestock in the epidemiology and transmission of Toxoplasmosis in Turkey.

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CONFLICT OF INTEREST

None reported.

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Research article
Ερευνητικό άρθρο

Effects of 25-hydroxycholecalciferol supplementation on breast meat quality and histomorphometric characteristics in broiler chickens

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ABSTRACT: This experiment was carried out to elucidate the influences of 25-hydroxycholecalciferol (25-OH-D_3) status in the diet and the sex of chickens on the sensory and histomorphometric characteristics of the breast muscle (pectoralis major) of broilers. We used a randomized design consisting of a 2×2 factorial arrangement with 25-OH-D_3 (3000 and 5000 IU kg/diet) and two sexes. Breast weight was not affected by either dietary 25-OH-D_3 status or bird sex. Cooking loss was decreased in female chickens but not in male chickens with additional 25-OH-D_3 supplementation. However, increasing 25-OH-D_3 levels in the diet reduced drip loss in male chickens but no change was observed in female chickens. A significant increase in breast meat pH_{24} was observed with increasing dietary levels of 25-OH-D_3; however, the squeezable water ratio and lightness (L^*), redness (a^*) and yellowness (b^*) values were comparable among the groups. The histomorphometric characteristics of the chicken breast meat throughout the growth period, except for the cross sectional area and number of fibres, were interactively influenced by 25-OH-D_3 and the sex of the bird. In conclusion, an improvement in 25-OH-D_3 status could be a practical tool to increase chicken breast meat quality with beneficial effects on its water holding capacity.

Keywords: Broiler, *Pectoralis major* Muscle Histology, Sensory Properties, Sex, Vitamin D.

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INTRODUCTION

Studies over the last decade have shown that increasing the level of 25-OH-D_3 in the diets of broiler chickens has beneficial implications for the yield and quality of breast meat. Enhancing the broiler chicken vitamin D status by feeding additional 25-OH-D_3 resulted in positive changes in the water holding capacity (WHC), pH and colour of the breast meat (Hutton et al., 2014; Starkey, 2014; Vignale et al., 2015; Bozkurt et al., 2017). However, almost all of these observations were obtained from male broiler chickens, and there are few studies using female chickens (Santiago et al., 2016). A recent exceptional one by Bozkurt et al. (2017) indicated significant sex and 25-OH-D_3 interactions for some meat quality traits. This finding stressed that the magnitude of the response to muscle development could differ between male and female chickens when the 25-OH-D_3 status of the diet changed. In addition, to the best of our knowledge, there are rare studies that have reported the effect of 25-OH-D_3 status on the histomorphometric characteristics of the pectoralis major muscle in broiler chickens (Chou et al., 2020). There are significant growth rate differences between fast-growing male and female broiler hybrids in association with different breast muscle development potentials (Ross, 2014; Cobb, 2018). Although there is clear evidence that 25-OH-D_3 modulates the structural characteristics and the development of the breast meat, the most valuable carcass cut-up part contributing over 30% of the overall broiler carcass weight, how male and female chickens differ in response to changes in dietary 25-OH-D_3 levels remains elusive.

There are reports that demonstrate a relationship between meat sensory characteristics including drip loss, cooking loss and pH, that determines WHC in pigs (Kim et al., 2007), cattle (Holmes and Ashmore, 1972) and sheep (Kadim et al., 1993) with different observations from male and female subjects. The effectiveness of adipose tissue in the activation of 25-OH-D_3 to 1α-25 (OH) $_2$-D_3, the most active form of Vitamin D_3, has been reported in studies with human subjects (Wamberg et al., 2012; Ryynänen et al., 2014). It is obvious that male chickens with more muscle and less adipose tissue than females will have a higher vitamin D requirement. Therefore, it is assumed that male broilers would have more responsive to increases in vitamin D levels in the feed than those the females in terms of lean muscle mass production with implications on sensory qualities of breast meat (Ceglia 2009; Ding et al., 2012). However, in broiler chickens, there is a dearth of studies using both male and female subjects in a comparative model.

We hypothesized that male chickens with a greater breast meat yield than females would benefit more from an addition of 25-OH-D_3 to their diet. Thus, our primary objective was to determine whether the improvement of 25-OH-D_3 status with the addition of 25-OH-D_3 would further impact the sensory attributes and histomorphometric characteristics of breast meat in male chickens compared with female chickens grown up to 42 days of age.

MATERIALS AND METHODS

Ethical approval

All procedures involving animals were approved by the Intuitional Animal Care and Use Committee of Aydın Adnan Menderes University, Aydın, Turkey (Ethical Committee approval no:64583101/2018/40).

Management

This experiment included a total of 720 one-day-old sexed chicks. Infectious bursal disease virus and Newcastle disease vaccines were implemented at 12 and 16 days of age, respectively. The environmentally controlled broiler test house was equipped with automatically adjustable heating, cooling, ventilation and lighting systems. Twenty-four wire-net pens were prepared, each having a floor space of 2.2 m^2 (125 x 177 cm) with five nipple drinkers and a tube-type feeder. The stocking density was 14 birds per m^2 floor space. The pens had concrete floor bedding and wood shavings litter with a thickness of approximately 6 cm.

The room temperature was maintained at 33 ºC from day 1 to 3 and thereafter gradually reduced until reaching 22 ºC on d 21 and then remained constant until the end of the trial. The light was provided by fluorescent bulbs for a period of 23 h/d from day 0 to 3, and then diminished 1 h every day until day 8, giving 18 h light per day during the remaining growth stage (i.e., from days 9 to 42). The experiment was terminated when the birds were 42 days of age.

Birds and experimental design

The experiment had a 2×2 factorial arrangement of treatments with 2 dietary regimens (diets with 3000 or 5000 IU 25-OH-D_3/kg) and 2 gender (male and female). Equal numbers of male (360) and female (360) 1-day-old broiler chicks (Ross 308) with an initial body weight of 43.6±2.9 g and 43.2±3.1 g, respec-

tively, were allocated into 4 equal groups, 180 chicks per group, with six replicates of 30 birds. Each pen was an experimental unit. The experiment was divided into two phases: a starter phase (1 to 28 days), and a finisher phase (29 to 42 days).

Experimental diets

The basal diet was a typical corn-soybean diet. The ingredient composition and nutrient content of the basal starter and grower diets are presented in Table 1. These diets contained no performance enhancer feed additives and were formulated to be nutritionally adequate in all essential nutrients. The diets were arranged as follows: (1) Control diet: The starter (from day 1 to 28) and finisher (from days 29 to 42) diets were formulated according to recommendations by the breeder (Ross, 2014). The concentrations of all macro and micronutrients, except for vitamin D_3, were chosen to represent the limitations published in the rearing guidelines for the strain (Nutrition Specifications of Ross 308 Broilers; Ross, 2014). Control diets contained 3000 IU/kg (75 μg/kg) cholecalciferol (vitamin D_3) to represent recent commercial applications in the field which coincides with the cholecalciferol content of vitamin premixes used in the feed industry for broiler chicken feed. (2) The control diet was further supplemented with 2000 IU/kg (50 μg/kg) 25-OH-D_3 to assess the effect of dietary vitamin D_3 intake by broilers over traditional levels of use in the field. All diets were mashed and were freshly prepared every two weeks. The diets and drinking water were available for ad libitum consumption throughout the experiment. The experimental diets were analysed three times to guarantee identical chemical compositions. The chemical compositions were determined according to protocols as outlined by the AOAC (1990). All feed samples were analysed for dry matter (934.01), ash (942.05), nitrogen (Kjeldahl procedure: 988.05), ether extract (920.39), and crude fibre (962.09).

Table 1. Composition of the experimental diets (as-fed basis)

Ingredient g/kg	Starter diet (d 1-28)	Finisher diet (d 29-42)
Corn, yellow, ground	367.31	431.15
Wheat	200.00	200.00
Soybean meal (48% crude protein)	357.00	286.00
Soy oil	36.50	49.00
Dicalcium phosphate	17.50	15.50
Limestone	11.00	8.50
Sodium chloride	2.40	2.64
L-Lysine HCL	1.00	0.20
DL-Methionine	2.05	2.21
L-Threonine	0.64	0.30
Vitamin-premix[a]	2.50	2.50
Mineral- premix[b]	1.00	1.00
Sodium bicarbonate	0.60	0.50
Anticoccidial[c]	0.50	0.50
Contents by analysis, %		
Dry matter	89.08	89.82
Crude protein	22.83	19.92
Ether extract	5.83	7.56
Crude fiber	3.23	3.14
Crude ash	6.52	5.99
Ca	1.18	0.99
P (total)	0.72	0.63
Calculated Contents %		
P (Available)	0.46	0.42
Lysine	1.29	1.17
Methionine	0.55	0.51
Methionine + Cysteine	0.96	0.94
Threonine	0.89	0.73
Linoleic acid	2.85	3.78
ME (kcal/kg)	3044	3234

[a]Provides per kg of diet: trans-retinol (vitamin A) 3.6 mg; vitamin D_3 (cholecalciferol) 75 mg; α-tocopherol acetate (vitamin E) 80 mg; menadione (vitamin K_3) 3.2 mg; thiamine (vitamin B_1) 3.2 mg; riboflavin (vitamin B_2) 8.6 mg; pyridoxine (vitamin B_6) 4.3 mg; cyanocobalamin (vitamin B_{12}) 0.02 mg; nicotinic acid 65 mg; Ca-D-pantothenate20 mg; folic acid 2.2 mg; D-biotin 0.22 mg.[b]Provides per kg of diet: Mn (MnO) 120 mg; Fe ($FeSO_4$) 20 mg; Zn (ZnO) 110 mg; Cu ($CuSO_4$) 16 mg; I ($CaIO_3$) 2 1.25 mg; Se (Na_2SeO_3) 0.30 mg; antioxidant (butyl hydroxy toluene) 125 mg.[c]Provides 100 mg monensin sodium per kg diet.

Dietary supplementation with 25-OH-D_3 was performed as follows. The feed grade 25-OH-D_3 used in this study (Hy-D; DSM Nutritional Products, Ltd., 4002 Basel, Switzerland) contained 69 µg/kg 25-OH-D_3, calculated to be equal to 2760 IU/kg provided by cholecalciferol (vitamin D_3) based on the conversion of 0.025 µg of cholecalciferol to 1 IU (NRC, 1994). The vitamin premix used in this study supplies 3000 IU (75.0 µg) vitamin D_3 per kg diet. Thus, a 725 g 25-OH-D_3 preparation was included per ton of basal diet to supply 50.0 µg 25-OH-D_3 per kg in the starter and finisher diets. The preparation of 25-OH-D_3 (i.e., 725 g) was balanced to 1 kg with finely ground soybean meal and homogenized using a laboratory type mixer for 30 seconds. Then, the premixture was supplemented with one ton of basal diet. Briefly, the control starter and finisher diets contained similar levels (i.e., 3000 IU/kg; 75 µg/kg) of cholecalciferol (vitamin D_3), while the die for the treatment group contained 5000 IU/kg (125 µg/kg) cholecalciferol (vitamin D_3).

Data collection

At days 14, 28 and 42, after 10 h feed withdrawal, two birds from each replicate representing average weight of the group (± 4%) were selected at random and slaughtered. Chickens were electrically stunned and exsanguinated via severing the jugular vein, bled for 45 second, scalded, mechanically defeathered and eviscerated. After weighing the absolute breast with bone and skin, the average 50 g of right half of breast muscle from each carcass at each age period were collected for histomorphometric analysis. At day 42, the breast muscles were placed in plastic bags and transported to the laboratory on ice to perform sensory characteristics of breast meat.

Meat quality analyses

pH_{24} and colour: All measurements were performed on the middle of the cranial section of the breast muscle at 24 h postmortem. For the determination of the pH of the meat samples, a pH meter (Hanna Instruments, HI 2211, Woonsocket, 02895 RI) was used. Colour measurements (lightness (L^*), redness (a^*) and yellowness (b^*)) were evaluated using a Minolta (Konica Minolta CR-400, Sensing INC. Sakai Osaka, 590-8551 Japan) at 24 h postmortem (Bozkurt et al., 2017).

Water holding capacity (WHC) : Drip loss, squeezable water and cooking loss were the main characteristics that determined the water holding capacity in this study. At 24 h postmortem, meat samples weighing between 5 and 8 g were placed between two filter papers and pressed at 10 kg for 5 minutes. The samples were reweighed, and the squeezed water weight (%) was calculated as follows: [(initial sample weight-final weight) /initial sample weight × 100]. Meat samples, approximately 4 cm in diameter, from each breast muscle were weighed, suspended on a hook inside a plastic bags and stored at 4 °C for 3 days. Each sample was reweighed, and the drip loss was calculated: [(initial sample weight-final weight) /initial weight × 100]. Fillets of approximately 60 g were weighed and cooked to an end-point temperature of 75 °C, cooled to room temperature and reweighed to determine the cooking loss: [(initial sample weight-final weight) / initial weight × 100] (Bozkurt et al., 2017).

Histomorphometry

For histological analysis, samples of the pectoralis major muscle were collected from 48 birds, 12 males and 12 females, from each group of chickens slaughtered at 42 days of age. The samples were *s*elected at 3 h post mortem from chickens having an average weight of 2.7 kg. Pectoralis major muscle samples were the "superficial layer" defined from approximately 0.2 cm to 1.2 cm below the breast muscle surface. The samples were immediately fixed in 10% buffered neutral formalin solution for 24 h, dehydrated in alcohol, cleared in xylene, infiltrated and finally embedded in paraffin (Khoshoii et al., 2013). The sections were cut at 5 µm thickness and stained with haematoxylin and eosin for general histological study (Bruck, 1975). Stained cross sections were viewed and photographed with a light microscope (Olympus BX53; Olympus, Tokyo, Japan) with a 10× objective lens and a 10× eyepiece (Tuma et al., 1962). Three photographs were taken from different cross-sections of each muscle. The samples were determined by using labSens software (Olympus Soft Imaging Solutions, Hamburg, Germany). A total of 144 preparations of pectoralis major muscle were used to determine the microstructure. Cross sectional area (CSA, μm^2) ; total fibres (TF) ; total number of fibres (NF; 1 μm^2) ; fibre diameter (FD; µm) ; FA: fibre area (μm^2) ; perimysium thickness (PT; µm) and endomysium thickness (ET; µm) were the criteria used for determining the microstructural characteristics of the pectoralis major muscle.

Statistical analysis

All data were subjected to two-way ANOVA using the JMP Statistical Package (SAS, 2018). Pen was

considered as an experimental unit for all measurements. The main effects of diet, sex and their interaction were assessed for all variables. Significant main effects were interpreted by comparing means using Student's t-tests. Means were considered significantly different when P<0.05, while P<0.10 was considered tendency.

RESULTS

The effects of 25-OH-D_3 status of the diet and the sex of the chickens on sensory characteristics of the breast meat (pectoralis major) are shown in Table 2. There was a significant 25-OH-D_3 by sex interaction for cooking loss in breast meat (P<0.05). Increasing the dietary 25-OH-D_3 levels from 3000 to 5000 IU decreased the cooking loss in the breast meat of female chickens, whereas no such effect was observed in males. The drip loss from breast muscle was slightly lower (P=0.067) in male chickens fed 5000 IU 25-OH-D_3 than in males fed 3000 IU 25-OH-D_3. However, no significant difference was found between females with different 25-OH-D_3 statuses, suggesting a 25-OH-D_3 by sex interaction for drip loss. The squeezable water ratio from the breast meat was not affected by the 25-OH-D_3 status or the sex of the bird. Dietary 25-OH-D_3 status and the sex of the chicken did not affect the lightness (L^*), redness (a^*) or yellowness (b^*) of the breast meat. An increase of 2000 IU in 25-OH-D_3 concentration in the diet increased the breast meat pH_{24} from 5.80 to 5.94 (P<0.05). No significant sex by 25-OH-D_3 interaction was found for any of the variables studied except for cooking loss. The absolute breast meat weight, CSA, TF and NF in the breast muscle were not affected by dietary 25-OH-D_3 status or the bird sex at any time point (i.e., 14, 28 and 42 days of age).

However, with regard to FD and FA, and PT and ET, no consistent tendencies were detected between dietary levels of 25-OH-D_3 and bird sex through the age periods of 14, 28 and 42. However, at d 42, in terms of FD, male birds benefited more from the increase in 25-OH-D_3 levels than females, indicating a significant 25-OH-D_3 by sex interaction (P=0.035). ET and PT were interactively affected by 25-OH-D_3 and bird sex at all age periods (P<0.01). While ET was similar between male and female chickens when they were fed a diet with 5000 IU 25-OH-D_3, female chickens had a higher ET than males treated with 3000 IU 25-OH-D_3 in the diet (P<0.01). However, with respect to PT, birds exhibited variable responses to alterations in 25-OH-D_3 status and bird sex all stages of the growth period.

Table 2. The effects of 25-OH-D_3 (IU/kg diet) status of the diet and gender of the chicken on sensory characteristics of the breast meat (Pectoralis major)

25-OH-D_3	Gender	Cooking loss %	Squeezable %	Drip loss %	pH_{24}	L^*	a^*	b^*
3000	Male	16.34[a]	9.90	3.27	5.80	58.77	1.58	7.87
	Female	17.24[a]	10.82	3.04	5.81	57.86	1.88	8.42
5000	Male	16.38[a]	9.66	2.63	5.90	58.28	1.57	8.43
	Female	14.96[b]	10.10	3.19	5.98	57.61	1.34	7.79
Pooled SEM[1]		0.265	0.560	0.106	0.031	0.320	0.120	0.150
	25-OH-D_3							
	3000	16.79	10.36	3.15	5.80[b]	38.31	1.73	8.15
	5000	15.67	9.88	2.91	5.94[a]	37.95	1.46	8.11
	Gender							
	Male	16.36	9.78	2.95	5.85	38.52	1.57	8.15
	Female	16.10	10.46	3.11	5.89	37.74	1.61	8.11
Source of variation[2]				Probability				
25-OH-D_3		0.041	0.646	0.256	0.037	0.410	0.257	0.911
Gender		0.628	0.740	0.437	0.455	0.081	0.880	0.887
25-OH-D_3 x Gender		0.035	0.275	0.067	0.535	0.785	0.275	0.053

[1]SEM: pooled standard error mean.
[2]Means in the same column within a treatment with no common superscript differ significantly (P<0.05).

Table 3. Effects of supplementation diet with 25-OH-D_3 (IU/kg diet) on muscle histomorphometric characteristics of Pectoralis major in broilers at 14 days age

25-OH-D_3	Gender	BW[3]	CSA	TF	NF	FD	FA	PT	ET
3000	Male	110	133	167	94	21.52[c]	859[b]	17.22[c]	6.86[c]
	Female	89	145	156	90	24.63[a]	914[a]	23.94[b]	8.19[a]
5000	Male	103	157	184	94	23.33[a]	885[b]	27.77[a]	7.80[b]
	Female	94	147	167	92	22.43[b]	913[a]	22.44[b]	7.49[b]
Pooled SEM[1]		2.451	6.100	6.503	2.450	0.108	6.652	0.519	0.081
	25-OH-D_3								
	3000	100	139	161	92	23.07	887	20.58	7.52
	5000	99	152	176	93	22.88	899	25.10	7.64
	Gender								
	Male	107	145	175	94	22.42	872	22.49	7.33
	Female	92	146	162	91	23.53	914	23.19	7.84
Source of variation[2]					Probability				
25-OH-D_3		0.902	0.314	0.285	0.879	0.368	0.001	0.000	0.002
Gender		0.158	0.930	0.306	0.497	0.000	0.348	0.646	0.456
25-OH-D_3 x Gender		0.461	0.372	0.823	0.878	0.000	0.037	0.000	0.000

[1]SEM: pooled standard error mean.
[2]Means in the same column within a treatment with no common superscript differ significantly ($P<0.05$).
[3]BW: Breast weight (g) ; CSA: Cross sectional area (μm^2) ; TF: Total number of fibres; NF: Number of fibres (1 μm^2) ; FD: Fibre diameter (μm) ; FA: Fibre area (μm^2) ;
PT: Perimysium thickness (μm) ; ET: Endomysium thickness (μm).

Table 4. Effects of supplementation diet with 25-OH-D_3 (IU/kg diet) on muscle histomorphometric characteristics of Pectoralis major in broilers at 28 days age

25-OH-D_3	Gender	BW[3]	CSA	TF	NF	FD	FA	PT	ET
3000	Male	429	217	87	43	37.88	1977	23.94[b]	8.23[c]
	Female	379	219	93	46	35.40	2049	30.60[a]	11.86[a]
5000	Male	419	209	95	42	37.08	2023	24.79[b]	9.53[b]
	Female	382	224	96	49	33.57	2251	21.49[c]	9.86[b]
Pooled SEM[1]		8.832	14.716	4.098	1.411	0.229	16.965	0.586	0.113
	25-OH-D_3								
	3000	404	218	90	44	36.64[a]	2014[b]	27.27	10.04
	5000	401	217	96	45	35.33[b]	2136[a]	23.14	9.70
	Gender								
	Male	424	213	92	42	37.48[a]	2001[b]	24.37	8.88
	Female	381	222	96	47	34.49[b]	2149[a]	26.04	10.86
Source of variation					Probability				
25-OH-D_3		0.962	0.957	0.161	0.899	0.004	0.000	0.152	0.125
Gender		0.078	0.762	0.703	0.145	0.000	0.000	0.000	0.000
25-OH-D_3 x Gender		0.844	0.831	0.315	0.363	0.268	0.453	0.000	0.000

[1]SEM: pooled standard error mean
[2]Means in the same column within a treatment with no common superscript differ significantly ($P<0.05$).
[3]BW: Breast weight (g) ; CSA: Cross sectional area (μm^2) ; TF: Total number of fibres; NF: Number of fibres (1 μm^2) ; FD: Fibre diameter (μm) ; FA: Fibre area (μm^2) ;
PT: Perimysium thickness (μm) ; ET: Endomysium thickness (μm).

Table 5. Effects of supplementation diet with 25-OH-D_3 (IU/kg diet) on muscle histomorphometric characteristics of Pectoralis major in broilers at 42 days age

25-OH-D_3	Gender	BW[3]	CSA	TF	NF	FD	FA	PT	ET
3000	Male	741	280	70	31	36.11[c]	3216	21.06	8.29[c]
	Female	701	295	58	28	45.30[b]	3629	22.11	11.39[ab]
5000	Male	737	279	71	31	47.45[b]	3423	24.81	12.34[a]
	Female	714	301	68	25	53.35[a]	3774	27.80	10.62[b]
Pooled SEM[1]		13.953	9.623	2.593	1.481	0.389	45.970	0.462	0.148
	25-OH-D_3								
	3000	721	287	64	30	40.71	3423	21.58[b]	9.84
	5000	726	290	69	28	50.40	3598	26.31[a]	11.48
	Gender								
	Male	739	279	70	31	41.78	3319[b]	22.93	10.32
	Female	708	297	63	27	49.33	3701[a]	24.96	11.00
Source of variation[2]						Probability			
25-OH-D_3		0.862	0.992	0.302	0.427	0.000	0.539	0.029	0.021
Gender		0.416	0.220	0.174	0.064	0.000	0.007	0.168	0.821
25-OH-D_3 x Gender		0.388	0.792	0.435	0.460	0.035	0.664	0.294	0.000

[1]SEM: pooled standard error mean.
[2]Means in the same column within a treatment with no common superscript differ significantly ($P<0.05$).
[3]BW: Breast weight (g) ; CSA: Cross sectional area (μm^2) ; TF: Total number of fibres; NF: Number of fibres (1 μm^2) ; FD: Fibre diameter (μm) ; FA: Fibre area (μm^2) ;
PT: Perimysium thickness (μm) ; ET: Endomysium thickness (μm).

DISCUSSION

Findings related to the sensory characteristics of chicken breast meat indicated that male and female chickens showed significant responses to dietary 25-OH-D_3 status in terms of cooking loss and drip loss, respectively. Cooking loss, a reliable indicator of WHC, was reduced in the carcasses of female birds with increasing 25-OH-D_3 supplementation, while no such effect was observed in males, suggesting a 25-OH-D_3 by sex interaction (P=0.035). With regard to cooking loss from breast meat, there has been no previous study that revealed the mechanism by which the sex of the bird affected their response to 25-OH-D_3. The data from the present experiment clearly indicate that higher levels of 25-OH-D_3 supplementation than the recommendations by the breeder (i.e., 4000 IU for the finisher period; Ross, 2014) and experimentally derived estimates, (i.e., 3000 IU) are beneficial to improve the WHC of chicken breast meat by decreasing cooking loss. Likewise, Bozkurt et al. (2017) indicated that slightly higher 25-OH-D_3 supplementation over the recommendations for the breed (i.e., 10%) markedly decreased squeezable water and cooking loss in male and female chickens at 38 days of age. Indeed, there are no specific nutrient recommendations chickens specific to sex regarding the ideal supplemental 25-OH-D_3 levels, only recommendations for both sexes (Ross, 2014; Cobb, 2018). Reduced cooking loss with increased 25-OH-D_3 status could be associated with increases in FD and FA, which are both indicative of an increase in myofibril area. This indicates a reduction in the size of the connective tissue, which facilitates the migration of intracellular water in the muscle (Hussein et al., 2019). The reduced drip loss and cooking loss in males and females, respectively, fed 5000 IU 25-OH-D_3 may be associated with the beneficial effects of 25-OH-D_3 on muscle fibre radial growth (Starkey, 2014; Świątkiewicz et al., 2017) since myofibrillar proteins are considered to be mainly responsible for the WHC of the meat (Ooizumi and Xiong, 2004). Therefore, it is reasonable that increasing the supplemental 25-OH-D_3 indirectly enhances the WHC in chicken breasts. Another plausible explanation for the decreased drip loss and cooking loss, reliable indicators that determine the WHC and consequently the raw meat quality with increased 25-OH-D_3 intake are the observed increases in FD and FA. As the CSA of the myofibres expands, the area occupied by the adipose and connective tissue would be limited, suggesting that the ability to retain liquid during refrigerated storage and cooking would be enhanced (Mazzoni et al., 2015) due to the increased diameter of the myofibrils. This is in parallel with the observations obtained from the current experiment that supplementation of the diet with 25-OH-D_3 at the level of 5000 IU increased the

CSA, FD and FA in the breast muscles of both sexes without increasing the TF and NF.

An increasing number of studies have shown that 25-OH-D_3 supplementation in the diet had positive effects on the bone (Zhang et al. 2020) and muscle development of broiler chickens (Hutton et al., 2014; Vignale et al., 2015; Bozkurt et al., 2017; Alves et al., 2018). However, until now, there have been no studies assessing the sex-specific characteristics of breast muscle for broiler chickens fed diets with different 25-OH-D_3 concentrations. In a recent example, Bozkurt et al. (2017) investigated the effects of superdosing 25-OH-D_3 (i.e., 5500 IU/kg diet) on the meat quality characteristics of broiler chickens. Moreover, to the authors' knowledge, no other study has investigated the interactions between sex and 25-OH-D_3 levels with regard to the structural characteristics of muscle meat in broiler chickens. Considering these facts mentioned above, it is challenging to elucidate the mechanism of sex based differences in breast muscle microstructure as a response to 25-OH-D_3 supplementation levels. A similar effect is expected for the sensory characteristics of breast meat because it was demonstrated that WHC in breast and thigh meat differently interact with muscle structural characteristics in male and female broilers, presumably due to the differences in their growth rate (Fanatico et al., 2005; Steczny and Kokoszynski 2019).

Muscle structural characteristics, including CSA, TF and NF, were not significantly affected by 25-OH-D_3 supplementation or the sex of the bird at any age period. In the present study, FD and FA in female chickens measured at day 42 were significantly higher than those in male chickens. However, despite the significant increases in FD in female chickens, the CSA was similar between male and female chickens in association with higher TF and NF in males than in females. This is not surprising and concurs with the findings by Scheuermann et al. (2003) that male chickens show higher myofibre density in the pectoralis major muscle than female chickens. In the present study, FD was increased with additional 25-OH-D_3 supplementation (3000 vs. 5000 IU/kg diet) ; however, the male birds benefited more from the 25-OH-D_3 supplementation. Regardless of sex, this is in agreement with earlier studies reporting the benefits of 25-OH-D_3 on muscle size (Starkey, 2014; Vignale et al., 2015). The beneficial effects of 25-OH-D_3 supplementation on FD and to a lesser extent on FA and CSA appear to be reflected in an improved WHC with significant decreases in cooking loss in female chickens. This suggests that it is possible to improve the WHC in female chicken breasts with additional 25-OH-D_3 supplementation, as already demonstrated by Bozkurt et al. (2017).

It was previously indicated that acidic meats, which are characterized by ultimate pH values lower than 5.7, have negative implications on WHC (Berri et al., 2005; Duclas et al., 2007). However, in the current experiment, squeezable water and drip loss, highly useful measurements to estimate WHC, were not influenced sex or dietary 25-OH-D_3 status. In this study, the pH of the breast meat was approximately 5.8, a value that is regarded as optimal at 24 h post-mortem. One peculiar finding from this experiment is that higher 25-OH-D_3 supplementation resulted in 0.14 increments in pH_{24} (i.e., 5.80 vs. 5.94).

Nevertheless, cooking loss, another indicator of WHC in edible meat, was significantly decreased with 25-OH-D_3 supplementation in the breast muscle of female chickens. The mechanism by which 25-OH-D_3 supplementation (3000 vs. 5000 IU) causes such a dramatic decrease in cooking loss is thought to be due to an increase in myofibriller protein accretion with a concomitant decrease in connective tissues and fat accumulation.

CSA, TF and NF were unaffected by either sex or 25-OH-D_3 status or their interactions at any time point. On the other hand, in regard to FD, FA, PT and ET measurements, no consistent responses to sex or 25-OH-D_3 status were found at the initial growth periods (i.e., 14 and 28 days of age). One possible explanation for this is that the beneficial effect of 25-OH-D_3 on muscle size in broilers is more prevalent from 5 weeks onwards when hypertrophy of the muscles speeds up to achieve a daily weight gain over 100 g (Scheuermann et al., 2003; Hutton et al., 2014; Vasquez et al., 2018). Muscle fibres with large diameters are dark, although the meat has a higher pH (Choi and Kim, 2009). In addition, Allen et al. (1997) found contrasting relationships between breast muscle pH and lightness (L^*) and yellowness (b^*) in broilers, whereas a positive correlation with redness (a^*) was noted. However, in the present experiment, despite greater FD and FA with additive 25-OH-D_3 supplementation, the colour of the breast meat was not influenced by the 25-OH-D_3 status of the diet regardless of the sex of the bird. This is in accordance with the findings of Lopez et al. (2011), who noticed that sex does not influence breast meat colour values (L^*, a^* and b^*)

in broilers. However, Hussein et al. (2019) reported higher a^* but lower b^* values in female chickens than in male chickens in terms of breast muscle colour. A contradictory pattern to that reported by Hüssein et al. (2019) was observed by Bozkurt et al. (2017) when evaluating the breast muscle colour response to supplemental 25-OH-D_3 in male and female Ross 308 broiler chickens.

CONCLUSIONS

In brief, some relationships between muscle structural characteristics and dietary 25-OH-D_3 status do exist in male and female broiler chickens at any time point, but the effects are much more pronounced at slaughter age (i.e., 42 days of age) than at earlier time points. The lack of significant 25-OH-D_3 by sex interactions for muscle structural characteristics shows that both sexes are quite similar in their response to 25-OH-D_3 levels in the diet, suggesting that no more than the typical nutritional 25-OH-D_3 specifications are needed for either male or female broiler chickens. Breast meat pH_{24} was significantly responsive to alterations in 25-OH-D_3 status but the meat colour was unchanged. The beneficial effects of increased 25-OH-D_3 supplementation on cooking loss are conclusive.

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CONFLICT OF INTEREST

The author declares that there are no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Flaxseed and sunflower oil affect egg production and quality in hens exposed to stress

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ABSTRACT: In the study, effects of dietary supplementations with flaxseed oil and sunflower oil on production performance, egg quality, and the eggs' sensory attributes in laying hens exposed to high stocking density stress were investigated. A total of one hundred and forty-four 38-week-old "Atak-S" breed laying hens were used. The hens were divided into two main groups as stress group and non-stress group, which both were further divided into three subgroups: basal diet, 2% flaxseed oil diet, and 2% sunflower oil diet groups. High stocking density stress was induced with a space allowance of 357 cm^2 per hen. All hens were weighed initially and just before the study has been completed, and the body weight gain was calculated. Egg production per hen was daily recorded, and production performance, mean egg weight, and egg mass were estimated. Moreover, eggshell weight, thickness, strength, albumen height, and egg yolk color were measured. Trained panelists evaluated egg samples collected from each subgroup regarding sensory attributes such as taste, flavor, color, and texture. Flaxseed oil decreased egg production and egg mass in the non-stress group while increasing the stress group's same parameters. Sunflower oil increased average egg weight in all hens and paled the egg yolk's yellow color in the stressed hens. Moreover, the non-stress group's eggs were more appealing in taste than those of the stress group. Flaxseed-supplemented diet enhanced the sensory attributes in the eggs of both stress and non-stress groups. Furthermore, neither of the oil supplementations generated a strange or repulsive odor in the eggs. In conclusion, dietary flaxseed oil supplementation might be recommended to improve egg production and egg sensory attributes in stress-exposed laying hens. Sunflower oil supplementation might be offered for increasing egg production and some sensory parameters in both stressed and unstressed hens.

Keywords: hens, flaxseed oil, sunflower oil, high stocking density, stress, egg quality

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INTRODUCTION

Stress is a crucial environmental factor that adversely affects production performance, immunity, and welfare, increases the mortality rate, creating severe economic damage in poultry. Chickens used in commercial production can be exposed to many stressors, such as chick placement, beak trimming, transfer of chickens to breeder houses, heat-cold, high humidity, poor ventilation, insufficient lighting, vaccination, and microbial or viral challenges (Surai et al., 2019). High stocking density is one of the major stress factors in extensive farming, yet the breeders tend to house more hens per pen due to economic concerns (Carey et al., 1995), which reduces the space allowance per hen, causes rivalry due to inadequacy of drinkers and feeders, consequently leading to stress (Puron et al., 1995).

High stocking density was shown to have reduced feed intake, egg production, egg mass (Jahanian and Mirfendereski, 2015), and increased floor and broken egg ratios in floor-type pens (Kang et al., 2016). Roberts, (2004) thoroughly documented that several stress factors reduced eggshell quality. On the other hand, high stocking density effects were investigated only in a few studies, creating conflicting results. Jahanian and Mirfendereski (2015) indicated that eggshell thickness was increased in hens exposed to high stocking density, whereas Kang et al. (2018) pointed out a reduction in eggshell strength. Egg quality declined in Japanese quails (El-Tarabany et al., 2016) and layer ducks (Xiong et al., 2020) due to high stocking density. On the other hand, egg quality was reported to have remained unchanged in some other studies (Geng et al., 2020).

It is unlikely to avoid stress factors in poultry farming, and thus, several studies have been conducted regarding the potential solutions, including dietary supplements to alleviate stress response in poultry exposed to various stressors (Clavijo and Flórez, 2018). In the presented study, flaxseed and sunflower oils were administered as dietary supplements to minimize the potential adverse effects of stress in hens.

Flax (*Linum usitatissimum* L.) is a plant grown since ancient times for its seeds and fibers (Carraro et al., 2012). Flaxseed contains a high amount of nutrients, such as proteins, vitamins, minerals, and antioxidants, and provides a rich energy source. It contains 35-40% vegetable oil, which is composed of 70% polyunsaturated (PUFA) (mainly alpha-linolenic acid, ALA), 20% monounsaturated (mainly oleic acid), and approximately 10% saturated fatty acids (palmitic and stearic acids) (Martinchik et al., 2012). It is also a good plant source of omega-3 fatty acid. It is used in poultry to enhance omega-3 levels in the carcass meat and eggs (Ahmad, 2017). However, it was reported that when the diet's flaxseed ratio exceeded 8%, cyanogenic glycoside and phytic acid contained in the flaxseed adversely affected feed digestibility resulting in reduced egg production (Beheshti Moghadam and Cherian, 2017). On the other hand, it is not the matter in question in terms of flaxseed oil, safely used at high concentrations in poultry diets. Flaxseed oil promoted growth in chicks, increased omega-3 level and oxidative stability of carcass, and improved carcass quality (Abbasi et al., 2019). Egg production was unchanged with flaxseed oil in some studies (Costa et al., 2008; Petrovic et al., 2012; Herkel et al., 2014). Nevertheless, studies demonstrated both an increase (Celebi- and Utlu, 2006) and a decrease (Jia et al., 2008) in egg production. Likewise, an increase (Yi et al. 2014), a decrease (Raes et al., 2002) were also reported concerning egg weight, while some studies demonstrated no difference (Steinhilber, 2005). The studies investigating the effects of dietary flaxseed oil supplementation on egg quality also revealed conflicting results. Although several authors showed no difference in extrinsic egg quality parameters (Mazalli et al., 2004; Costa et al., 2008; Raes et al., 2002), eggshell weight and thickness were reported to have decreased (Grobas et al., 2001), and albumen weight was increased (Schumann et al. 2000). Moreover, the fatty acids such as ALA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) contained in the flaxseed oil were transferred into the egg at high concentrations (Ehr et al., 2017), prolonging the freshness of eggs (Lee et al., 2016).

Sunflower (*Helianthus annuus* L.) is an oilseed plant widely grown in the world. The seeds contain high amounts of vegetable oil, protein, non-digestible crude fiber, vitamins, and minerals (Le Clef and Kemper, 2015). Its oil content was elevated to 60% by genetic improvement studies (Vilvert, 2018). It also contains various fatty acids, including linoleic acid (55-75%), oleic acid (15-25%), palmitic acid (6%), and stearic acid (2%) (Lacombe and Berville 2001). Sunflower meal (SFM) is used in poultry feeding as a source of energy and protein. However, its high fiber yet low lysine content restrains the diet's digestibility (Baghban-Kanani et al., 2018). Therefore, various enzymes, essentially lysine, are added to the diet that includes SFM; hence, it was reported that SFM

could be used in the diet of both broilers and laying hens at a 30% concentration, provided that the diet also contained enzymes (Seidavi et al., 2018). Once the diet included SFM, egg production, and quality were adversely affected, while -in contrast- concurrent niacin administration enhanced the relevant parameters (Baghban-Kanani et al., 2019). On the other hand, dietary sunflower oil supplementation showed no negative impact on egg production (Sangkaew et al., 2017), egg weight (Ceylan et al., 2011), and egg mass (Karunajeewa et al., 1989). Likewise, the intrinsic and extrinsic egg quality parameters were not negatively affected (Küçükersan et al., 2010; Dong et al., 2018).

The fatty acids in vegetable oils are accumulated in the egg yolk, causing lipid oxidation, hydrolysis, and polymerization (Wang et al., 2017), which -as a result- affected sensory attributes such as taste, flavor, color, and texture (Brelaz et al., 2019). On the other hand, stress further induced lipid oxidation (Eid et al., 2008), which might also alter sensory features. However, to the best of our knowledge, no data is available indicating the adverse effects of stocking density stress on the egg's sensory attributes.

The contradictory results concerning the effects of dietary flaxseed oil and sunflower oil supplementations despite the studies indicating their potential benefits in egg production and quality necessitates further studies. Furthermore, the potential positive effects of oil supplements on the relevant parameters and eggs' sensory attributes were not determined in stress-exposed hens.

The aim of the study was to investigate the effects of dietary flaxseed oil and sunflower oil supplementations on production performance, egg quality, and sensory characteristics in laying hens.

MATERIALS AND METHODS

Hens, study groups, and housing conditions

All experimental procedures were approved by the Animal Experiment Ethics Committee of the Istanbul University (Approval number 191-2018). A total of one hundred and forty-four 38-week-old Atak-S laying hens were included in the study. The hens were randomly divided into two main groups as the stress (n: 84) and the non-stress (n: 60) groups, which were further allocated into three subgroups: Basal diet, flaxseed oil diet, and sunflower oil diet groups, each containing 20 hens. Each sub-group was replicated four times. The stress group cages contained seven hens with a space allowance of 357cm$^{2/}$per hen, whilefive-hens were housed in each cage of the non-stress groupwith a space allowance of 500 cm^2 per hen. All hens were housed in stainless steel battery-type cages (width = 50 cm, length = 50 cm, height = 60 cm) which were equipped with linear feeders and nipple drinkers. The temperature, humidity, and ventilation were maintained by an automatic system according to the manufacturer's instructions in a 16/8 light-dark cycle.

Composition of the diets

All hens received the same basal diet. The feed was provided by a commercial company. The supplementation groups further received flaxseed oil and sunflower oils at 2%. The amount of feed per day was estimated according to the manufacturer's instructions. All hens were fed for 15 weeks. Water was provided *ad libitum.* The diets' nutrient contents were analyzed in a commercial company's laboratory (BanvitAş, Bandırma, Balıkesir). The diet's formulation and chemical composition were shown in Table 1 and Table 2, respectively.

Table 1. Formulation of the basal diet

Ingredients	Basal diet (%)
Soybean meal	33, 00
Sunflower meal	27, 00
Full-fat soybeans	11, 00
Maize	15, 00
Soybean oil	0, 90
DCP	2, 00
DL-Metiyonin	0, 10
Limestone	9, 70
Vitamin + mineral premix[1]	1, 00
Salt	0, 30

[1]Supplied per kg of diet: vitamin A (retinyl palmitate), 15, 000 IU; vitamin D3, 2, 500 IU; vitamin E (DL-α-tocopheryl acetate), 20 mg; vitamin B1, 3 mg; vitamin B2, 7, 5 mg; D-pantothenic acid, 25 mg; vitamin B6, 5 mg; vitamin B12, 0.002 mg; biotin, 0.5 mg; niacin, 25 mg; vitamin K3, 1.25 mg; folic acid, 1.5 mg; choline chloride, 750 mg; cobalt, 1.2 mg; copper, 8.8 mg; zinc, 84 mg; manganese, 106 mg; iron, 44 mg; iodine, 1.2 mg; and selenium, 0.15 mg.

Oil supplements

Flaxseed and sunflower oils were purchased from a commercial company (AyhanSezer Co Ltd., Bandırma, Balıkesir) and stored at +4°C during the study. The composition of fatty acids in oils was presented in Table 3.

Table 2. Chemical composition (calculated) and energy levels of the experimental diets (calculated)

Faty acid name	Faty acid number	Flaxseed oil (ppm)	Sunflower oil (ppm)
Caprylic acid	C8:0	Nd	1.047
Lauric acid	C12:0	Nd	1.231
Myristic acid	C14:0	1.509	1.99
Palmitic acid	C16:0	2.027	40.13
Palmitoleic acid	C16:1	Nd	1.874
Ginkgolic acid	C17:1	1.49	1.646
Stearic acid	C18:0	2.239	17.504
Oleic acid	C18:1 (n-9)	4.535	197.074
Linoleic acid	C18:2 (n-6)	3.885	303.261
Arachidic acid	C20:0	1.828	2.878
Alpha-linolenic acid	C18:3 (n-3)	6.625	1.811
Eicosenoic acid	C21:1 (n-9)	Nd	2.17
Heneicosanoic acid	C21:0	Nd	4.581
Behenic acid	C22:0	Nd	4.705
Tricosylic acid	C23:0	Nd	1.662
Nervonic acid	24:1 (n-9)	Nd	2.521

Nd = Not determined

Table 3. Fat acid comosition of flaxseed oil and sunflower oil

	Basal diet	Flaxseed oil	Sunflower oil
Dry matter (%)	89, 37	89, 42	89, 22
Crude protein, %	17, 29	16, 8	16, 69
Crude fat, %	3, 85	5, 44	5, 43
Crude fibre, %	2, 7	2, 48	2, 52
Ash, %	15, 15	16, 62	16, 59
Nitr-free core matter, %*	50, 38	48, 08	47, 99
Starch, %	39, 02	38, 58	38, 72
Sugar, %	3, 41	3, 4	3, 41
Calcium, %	3, 69	3, 71	3, 65
Utilizable phophorus, %	0, 43	0, 41	0, 39
ME, MJ/kg feed**	10, 96	11, 35	11, 35

Nitrogen-free core matter*, % = Dry matter, % - (crude protein, % + crude fat, % + crude fiber, % + ash, %). **ME, MJ/kg food = (0, 03431 x crude fat, g/kg) + (0, 01551 x crude protein, g/kg) + (0, 01669 x starch, g/kg) + (0, 01301 x sugar, g/kg).

Fatty acid analysis

An oil sample of 100µl was dissolved in 10 ml hexane. 2N KOH dissolved in 100 ml methanol was added and centrifuged for 30 sec. The methyl ester layer formed on the top by centrifuging was gently removed and used for the analysis (David et al., 2003). The fatty acid analysis of methyl esters was carried out using a gas chromatograph (Shimadzu GC-MS QP 2010 ULTRA, Kyoto, Japan) equipped with a fused silica capillary column (RTX-2330, 60 m length × 0.25 mm i.d. with a 0.25 µm film thickness). The column temperature, injection temperature, interface temperature, and ion source temperature were 100, 250, 250, and 200 °C, respectively. The fatty acids were identified from their peak retention times compared to the standards. Helium was used as the carrier gas at 2 µL/ min (Omri et al., 2019).

Performance parameters

Eggs were collected daily at the same hour, weighed on a precision scale with 0.1g accuracy, and the measurements were recorded. Egg production, mean egg weight, and egg mass were calculated (Egg production = the number of produced egg/number of hens x 100. Egg mass = egg production (%) x mean egg weight (g) (Araújo et al., 2015).

Quality parameters

The sensory attributes were evaluated in eggs collected at the last week of the study. Twenty eggs were

randomly selected from each subgroup. (A total of 60 eggs from the stressed group 3x20 and 60 eggs from the unstressed group 3x20). External quality parameters such as eggshell weight, thickness, and strength and internal quality parameters such as Haugh unit, albumen height, and yolk color were estimated. Eggshell weight was measured by a precision scale with 0.01g accuracy (Precisa XT 6200C, Dietikon, Switzerland). Other quality parameters were analyzed by a digital egg tester (DET6000, Nabel Co., Ltd, Kyoto, Japan), and eggshell thickness was measured with the equipment's accessory digital caliper rule.

Sensory attributes of eggs

Seven trained female panelists performed the sensory analysis. All panelists were professionals at the research/development laboratory of a commercial poultry company. A total of 72 eggs (12 eggs from each subgroup) collected on the same day were used to evaluate sensory attributes. The eggs were boiled for 9 min, cooled under tap water for 10 min, peeled, and then cut into four pieces along the longitudinal axis. The pieces were labeled according to the subgroup they belonged to and then offered to the panelists. Cold drinking water was provided to clear the mouth between tastings. The panelists scored the eggs on a scale of 1-5 concerning the parameters such as taste, flavor, color, and texture. The sum of the scores was obtained to rank the groups. The panelists were also asked to evaluate the eggs' odor, whether they smelled strange such as fish oil.

Statistical analysis

SPSS software (SPSS, Inc., Chicago, USA) was used for the statistical analysis. Initially, the Shapiro-Wilk test was applied to determine the distribution of the data. The normally distributed data were analyzed by a factorial ANOVA in a 2x3 factorial design. Stress and feed were held as constant factors, whereas the measured parameters were considered the dependent variables. The presence and absence of stress or three different diet types (basal, flaxseed oil-supplemented, and sunflower oil-supplemented) served as factors. Therefore, the main effects of stress and diet and stress-diet interaction were analyzed. When the interactive effect or the main effect of diet was significant, the source of difference was determined by the Post hoc tests, before which Levene's test was applied to check whether the data were homogeneously distributed. The Tukey test was performed for the Post hoc analysis of the homogenously distributed data (significant at P>0.005 level). The Games-Howel test was applied for the non-homogenous data (significant at P<0.005 level). Mean values and standard errors of the mean were used in tables and graphics. Results were determined significant if the P-value was 0.05 or lower.

RESULTS

The fatty acid contents of the flaxseed oil and sunflower oil were shown in Table 3. The flaxseed oil contained eight different fatty acids, the majority of which was composed of alpha-linolenic acid (n:3; omega-3) with a 27.45% concentration. The n:6/n:3 ratio was calculated as 0.58. Sunflower oil was rich in fatty acids with sixteen different components. Linoleic acid (n:6, omega-6) was the highest fatty acid content with a 51.74% concentration. The n:6/n:3 for the sunflower oil was calculated as 167.

Table 4. Effects of dietary flaxseed oil and sunflower oil supplementation and on body weight gain stressed and unstressed in laying hens

		Initial body weight (gr)	Final Body weight (gr)	Body weight gain (gr)
Stressed hens	Basal diet	1759.2 ± 17.1	1920.5 ± 31	161.2 ± 16.8
	Flaxseed oil	1763.3 ± 18.2	2032.3 ± 38	268.9 ± 40.3
	Sunflower oil	1773 ± 17.8	2048.6 ± 47	289.6 ± 52.3
Unstressed hens	Basal diet	1794.5 ± 20.8	2037.5 ± 44	264.5 ± 39.9
	Flaxseed oil	1804.2 ± 20.7	2055.2 ± 49	260.7 ± 49.5
	Sunflower oil	1773.1 ± 21.8	2046.3 ± 52	242.5 ± 46.8
		P= 0.180	*P* values	
		Stress	0.895	0.193
		Diet	0.132	0.648
		Stress x Diet	0.734	0.368

Body weights of the hens were presented in Table 4. The induced stress (P = 0.265) or dietary flaxseed oil and sunflower oil supplements (P = 0.227) had no significant impact on body weights. Besides, stress-diet interaction was also found insignificant (P = 0.288).

The effects on egg production, egg weight, and egg mass were shown in Figure 1. Based on the data, the stress-diet interaction affected egg production (P = 0.001). Flaxseed oil increased egg production in the stress-exposed hens, whereas -in contrast- egg production was reduced in the non-stress group. Sunflower oil showed no impact on egg production.

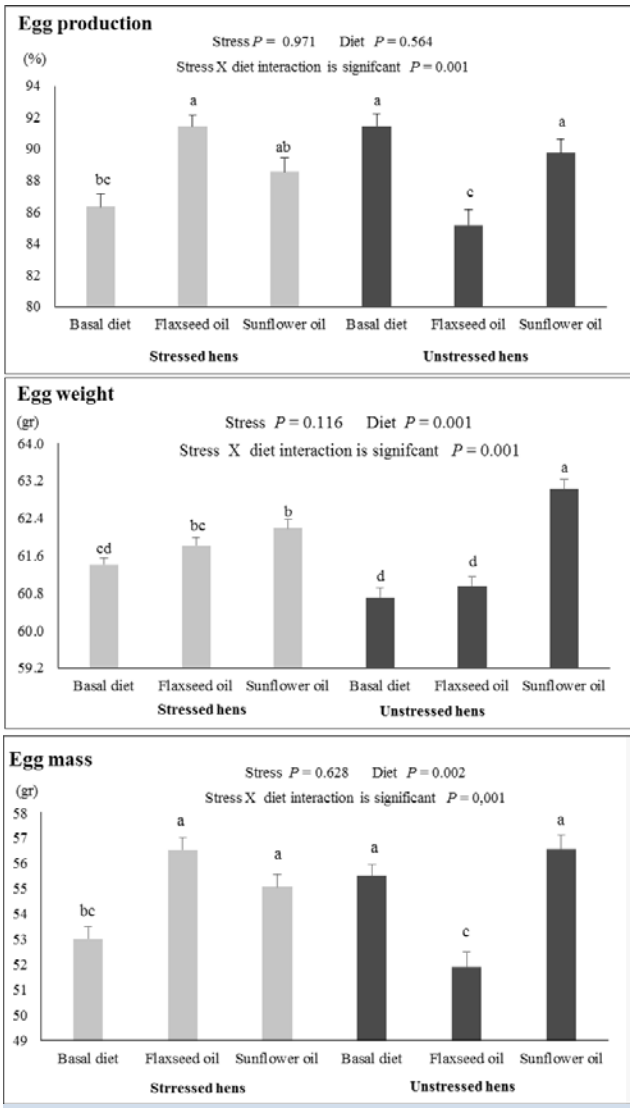

Figure 1. The effect of flaxseed oil and Sunflower oil on production performance in stressed and unstressed laying hens

Stress-diet interaction significantly affected egg weight (P = 0.001). Flaxseed oil generated a limited-level increase in egg weight in the stressed hens. No significant difference was noted in the non-stress group. Sunflower oil significantly increased egg weight regardless of absence or presence of stress.

The data revealed a significant stress-diet interaction effect on egg mass (P = 0.001). Flaxseed oil significantly increased egg mass in high stocking density stress-exposed hens while decreasing the unstressed birds' relevant parameter. In contrast, sunflower oil showed no significant impact on egg mass.

The data regarding eggs' external quality parameters were shown in Figure 2. The results revealed the noninfluence of stress, diet, and stress-diet interaction on eggshell weight (P = 0.491, P = 0.256, and P = 0.488, respectively), eggshell thickness (P = 0.720, P = 0.301, and P = 0.999, respectively), and eggshell strength (P = 0.232, P = 0.078, and P = 0.953, respectively).

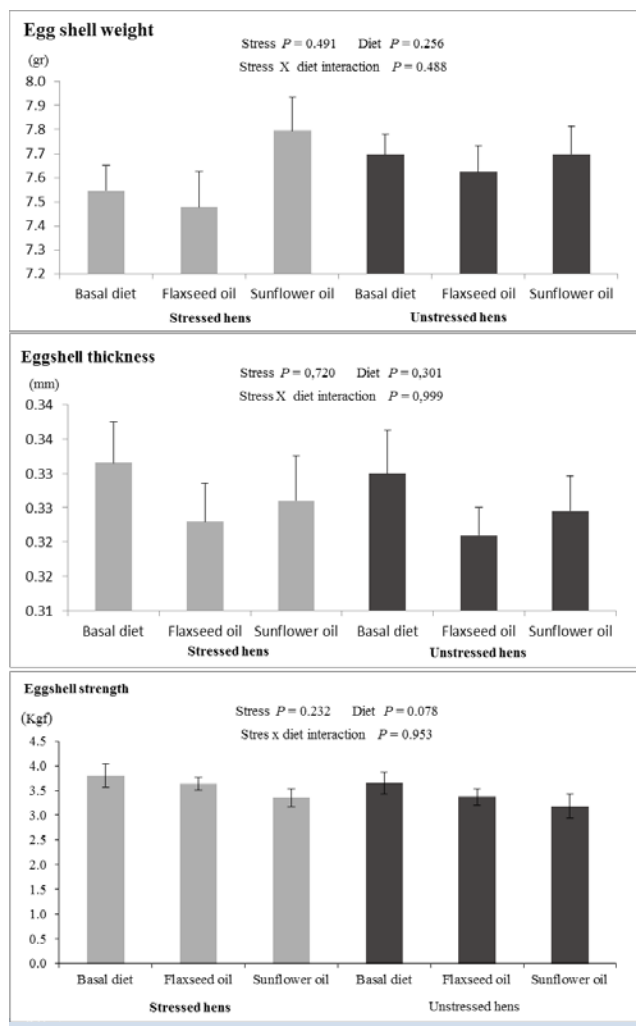

Figure 2. The effect of flaxseed oil and Sunflower oil on external egg quality parameters in stressed and unstressed laying hens

The data regarding eggs' internal quality parameters were shown in Figure 3. Based on the data, stress, diet, and stress-diet interaction did not affect Haugh unit (P = 0.736, P = 0.749, and P = 0.687, respectively) and albumen height (P = 0.493, P = 0.889, and P = 0.859, respectively). Nevertheless, the stress-diet interaction has a significant effect on egg yolk color (P = 0.029). Dietary sunflower oil supplement significantly reduced egg yolk yellow color intensity in the stressed hens while exerting no significant impact in the unstressed birds. Flaxseed oil did not generate a significant difference in hens' yolk color regardless of absence or presence of stress.

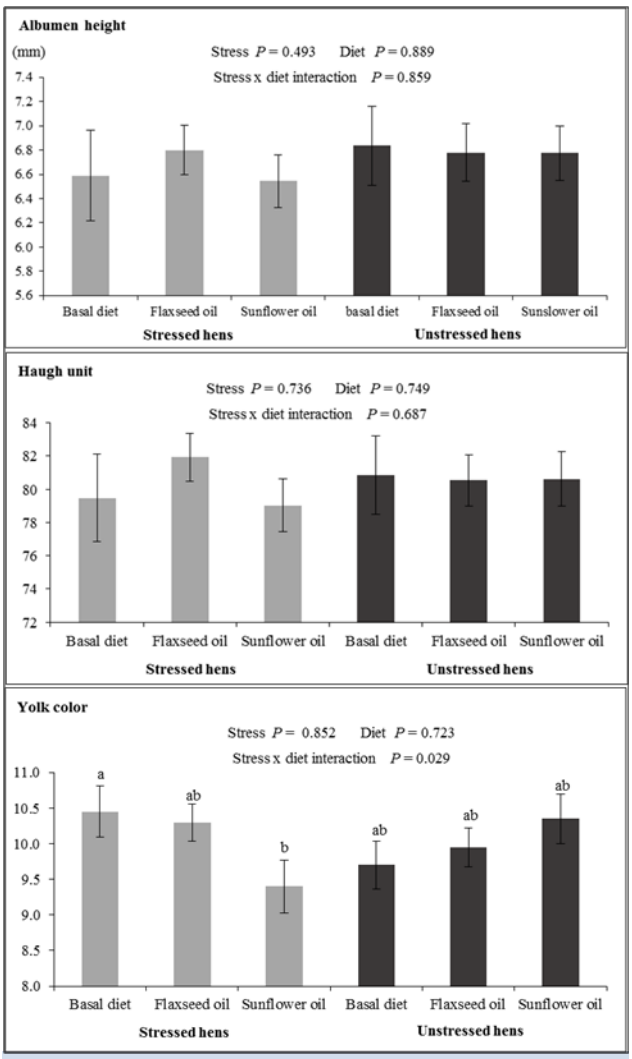

Figure 3. The effect of flaxseed oil and Sunflower oil on internal egg quality parameters in stressed and unstressed laying hens

Sensory quality tests revealed no repulsive odor in eggs. The unstressed hens' eggs undoubtedly tasted much better with a more desirable flavor than those of the stressed hens. When the diet groups of the stressed hens were compared, flaxseed oil supplementation elicited a more appealing taste in eggs than in other subgroups. No significant difference was noted in terms of flavor, yolk color, and texture. Likewise, in the non-stress group, eggs of hens fed with flaxseed oil-supplemented diet ranked first in terms of taste, followed by basal diet and sunflower oil received hens, respectively. Oil supplementations also improved the eggs' texture of the unstressed hens.

DISCUSSION

Fatty acid composition of dietary vegetable oil supplements -in particular- is of great importance while evaluating their potential benefits on production performance; hence, studies revealed divergent results concerning the effects of oil-supplemented diets in chickens (Ahmad, 2017). In the presented study, flaxseed oil contained approximately 37.6% saturated fatty acids (SFA) (myristic, palmitic, stearic, ginkgolic, and arachidic acid) and 62.3% unsaturated fatty acids (UFA) (alpha-linolenic acid, linoleic acid, and oleic acid) with an SFA/UFA ratio of 0.60. The fatty acid content of vegetable oils differs depending on environmental factors, plant variety (cultivar), and genotype (Wang et al., 2017). For instance, a study comparatively investigating the fatty acids compositions of four different flaxseeds revealed divergent results than the presented study, indicating that the SFA, UFA, and SFA/UFA ratios were 10-16%, 84-89%, and 0.12-0.19, respectively (Qiu et al., 2020). The SFA/UFA or n-6/n-3 PUFA ratios play a critical role in the manifestation of oils' biological effects in hens. Therefore, the fatty acids composition of vegetable oils should be analyzed before being administered as dietary supplements.

In the study, neither the stress model induced by high stocking density nor the dietary oil supplements impacted bodyweights. Bodyweight is known to have changed due to the type, duration, and severity of stressors. Altan et al. (2000) reported that body weight was decreased in heat stress-exposed broiler chicks, whereas Plavnik and Yahav (1998) obtained the exact opposite results. On the other hand, Nelson et al. (2018) showed no influence of stress on body weight, which is compatible with our findings. The presented study revealed consistent findings with previous studies concerning dietary oil supplementations' effects in terms of body weight. Likewise, flaxseed oil and sunflower oil supplements were reported not to have exerted a significant difference in body weights of laying hens (Eseceli and Kahraman 2003). Similarly,

sunflower oil-supplemented diet did not affect body weight in broiler chicks (Fébel et al., 2008). Constant body weight is desirable in laying hens after maturity, which reveals that energy balance is maintained. When body weight increases, feed intake should also be increased to compensate for the metabolic rate (Du Plessis and Erasmus, 1972). On the contrary, when body weight is reduced, dietary energy will be targeted to restore weight loss instead of enhancing production capacity. Economic loss is bound to be an inevitable outcome in either case. Therefore, the fact that the bodyweight remained unchanged either by stress or dietary applications was considered a positive output of the study.

The effect of dietary flaxseed oil on egg production differed depending on the absence and presence of stress. While it increased egg production and egg mass in the stressed hens, it generated an exact opposite effect in the unstressed birds. Flaxseed oil supplement was previously reported to have decreased egg production and egg mass. Petrovic et al. (2012) and Schumann et al. (2000) showed that diets containing 2% and 4% of flaxseed oil, respectively, reduced egg production in laying eggs. Shahid et al. (2020), consistently with our results, pointed out a decrease in egg mass in hens fed with a long-term (12 weeks) flaxseed oil-containing diet. It was previously stated that flaxseed diets adversely affected egg production by impairing the feed digestibility due to the anti-nutritional factors in flaxseed (Ahmad, 2017). However, why egg production was decreased by flaxseed oil supplementation decreased has not been clearly understood. Previous studies demonstrated that high energy level diets caused a decline in feed intake, leading to decreased egg production (Huang et al., 1989). In contrast, despite that sunflower oil is a high energy source, no adverse effect of sunflower oil-supplemented diet was noted on egg production in the study, which was thereby associated with an alternative underlying mechanism. When considering these oils' fatty acid compositions -unlike sunflower oil- flaxseed oil contains higher amounts of long-chain unsaturated fatty acids such as myristic acid, stearic acid, arachidic acid (Tablo 3), which were reported to have accelerated intestinal motility (Zhao et al., 2018). Furthermore, dietary oil supplementations were stated to have altered the transit of feed through the gastrointestinal tract and its absorption (Brelaz et al., 2019). Hence, flaxseed oil was shown to have increased intestinal motility due to its cholinergic and histaminergic properties (Parkman et al., 1999; Fabisiak et al., 2017). Therefore, it can be deduced that the decrease in egg production and egg mass in the unstressed hens that received flaxseed oil-supplemented diet was associated with increased intestinal motility and, accordingly, impaired absorption due to saturated fatty acids contained in flaxseed oil.

The fact that flaxseed oil increased egg production in the stressed hens was an intriguing finding. The egg-laying process is governed by the hypothalamic-pituitary-gonadal (HPG) axis in the hens, and the HPG axis is activated by gonadotropin-releasing hormone (GnRH) (Mishra et al., 2019). The high omega-3 content of flaxseed oil stimulates GnRH secretion (Tran et al., 2016), and one of the key stimulants of GnRH is catecholamines (Ottinger et al., 1995). Catecholamine release from the adrenal glands is elevated during stress response (Sabban, 2007). It can be deduced that GnRH production was stimulated both by catecholamine release, emerging as a stress response, and omega-3 contained in flaxseed oil in the stress-exposed hens fed with the flaxseed oil-supplemented diet. Therefore, it was considered that increased GnRH secretion might have resulted in an increase in egg production in the stressed hens that received flaxseed oil in their diet. Besides, corticotrophin-releasing factor (CRF) produced by the hypothalamus during stress is known to slow down intestinal motility, leading to prolonged transition of feed (Mertz, 2003), allowing improved feed absorption, which was considered to be associated with an increase in egg production and egg mass in the study.

Egg weight is a production parameter of great concern for breeders since consumers tend not to purchase small-sized eggs, which reduces their market value (Grobas et al., 1999). Physiological (hens' being at the initial phase of laying) and nutritional factors (a low-energy diet or low amino acid diet) influence the egg's size. Some stress factors are also known to reduce egg weight (Lara et al., 2013). Nevertheless, high stocking density did not affect egg weight in the presented study. Furthermore, flaxseed oil did not exert an adverse effect on egg weight. On the contrary, a slight increase was noted in egg weight in the stressed hens. On the other hand, sunflower oil substantially increased egg weight attributed to the sunflower oil's high linoleic acid (approximately 51.74%) content. It is considered that less effectiveness of flaxseed oil might have been associated with its lower linoleic acid (approximately 16.1%) content. Linoleic acid and other long-chain fatty acids within

lipoprotein complexes provide the egg's development and play a significant role in egg weight gain (March and MacMillan, 1990). Hence, oil supplementations rich in linoleic acid were reported to have increased egg weight (Ribeiro et al., 2007). Moreover, their positive influence on egg weight was documented to have been more prominent in hens with insufficient linoleic acid storage. (Grobas et al., 1999). Therefore, the positive effect of sunflower oil on egg weight was considered explicit by the increase achieved in the stressed hens in the presented study, unlike previous reports.

Egg quality is defined by the egg's intrinsic and extrinsic properties. In the study, eggshell weight, eggshell thickness, and eggshell strength were analyzed as extrinsic quality parameters. Corticosterone hormone released during stress response in poultry was indicated to have played a significant role in eggshell formation (Klingensmith et al., 1984). It adversely affects the laying period and reduces calbindin and osteopontin expressions effective in eggshell formation (Kim et al., 2015), impairing the shell quality such as weight, thickness, and strength. Concordantly, it was previously reported that specific stressors reduced eggshell weight (Mack et al., 2013), eggshell thickness (Mahmoud et al., 1996), and eggshell strength (Lin et al., 2004). In the presented study, the induced stress model (high stocking density stress) had no adverse effect on eggs' external quality parameters, which was considered to be associated with long-term stress exposure, allowing stress coping mechanisms to elicit adaptive responses to in-time reduced corticosterone release. Likewise, chronic stocking density stress was reported to have no impact on eggshell strength in hens (Garcia-Rebollar et al., 2008). The available data revealed the inefficaciousness of oil supplements on eggshell quality. It was previously reported that dietary sunflower oil supplementation did not affect eggshell weight (Ceylan and Çufadar, 2018), thickness (Cachaldora et al., 2005), and strength (Dong et al., 2018) (Promila et al., 2017). The conflicting results were indicated to have been linked with the dietary oils' fatty acid contents (Ding et al., 2017). High eggshell quality emerges as a crucial parameter through all aspects of the egg industry from production to consumption to avoid significant economic losses. The fact that eggshell quality was not affected with oil-supplemented diets was considered a favorable outcome.

Haugh unit and albumen height represent the eggs' protein content and freshness. High levels of these parameters are an indicator of good egg quality. Neither stress nor dietary oil supplementations affected Haugh unit and albumen height in the study, which is compatible with previous reports. It was shown that Haugh unit (Al-Saffar and Rose, 2002) and albumen height (El-Tarabany et al., 2016) were not influenced by stress. Likewise, dietary flaxseed oil and sunflower oil supplementations were reported to have no impact on the Haugh unit (Cherian et al., 2007; Midilli et al., 2009) and albumen height (Cedro et al., 2009). Egg yolk color is a crucial intrinsic egg quality parameter that determines consumer preferences. In the study, yolk color significantly faded in the stressed hens fed with a sunflower oil-supplemented diet while generating no significant impact in the unstressed birds (Figure 3). β-carotene is one of the main constituents of yolk coloration, and dietary intake of β-carotene is benefitted in metabolic regulation processes against stress exposure due to its good antioxidant properties. However, sunflower oil (Xixuan et al., 2000) and its high omega-6 content (Hollander et al., 1978) were shown to have decreased β-carotene absorption in the body. When the stress-exposed hens have received a sunflower oil-supplemented diet, β-carotene absorption is inevitably bound to have declined while a great deal of it is concurrently used up to compensate to the demands of the oxidative processes. Therefore, paling of yolk's yellow color in the stressed hens on sunflower oil diet was considered to be associated with reduced β-carotene levels. There is an existing false notion that an intense yellow color is an indicator of eggs' high nutritional value (Moreno et al., 2020) and thus affected consumer demand increasing the market value. However, no correlation was established between yolk color's intensity and the egg's nutritional value (Mızrak et al., 2012). On the other hand, yolk color should be taken into account while selecting eggs for their breeding merits since carotenoids facilitate antioxidant defense mechanisms in tissue responses to oxidative damage during the incubation period. (Blount et al., 2000). Even though this does not pose a problem concerning the eggs' nutritional value, a faded yellow yolk color in the stressed hens that received sunflower oil was considered an unfavorable result regarding both consumer demand and breeders concerning the selection of eggs to be kept for incubation.

Consumer demands are mostly directed by sensory properties of foods since the urge for purchase is essentially based on the color, odor, taste, and flavor of the food products (Ertaş and Doğruer, 2010), which

also applies to consumers preferences for eggs. Various vegetable oils and fatty acids have been widely used in recent years to enhance eggs' nutritional quality (Keten, 2019). On the other hand, dietary oil supplements were also reported to have altered eggs' sensory characteristics (Rokka et al., 2002). In the presented study, oil supplements did not adversely affect the eggs' sensory attributes. The absence of a strange odor in eggs was associated with the optimum concentrations of oils in the diet since it was previously shown that higher concentrations of vegetable oils deteriorated the taste and flavor of the eggs, generating a repulsive odor. (Hayat et al., 2010). The collected data revealed that dietary flaxseed oil supplementation improved the eggs' taste both in the stressed and unstressed hens. It was also previously reported in similar studies that flaxseed adversely affected the eggs' taste (Leskanich and Noble 1997; Subhani et al., 2020), which was indicated to have varied depending on the preparation method of oils, concentrations of their volatile components, or trimethylamine content (Parpinello et al., 2001). In the study, stress adversely affected the eggs' taste. Poor palatability was previously associated with the substantial role of oxidative processes of lipids contained in eggs in the stress-exposed hens (Imran et al., 2015). Likewise, oxidative stress was reported to have induced lipid oxidation in the egg as in tissues. (Bölükbaşı et al., 2007). The improved taste in the stressed hens' eggs that received a flaxseed oil-supplemented diet was considered to have resulted from flaxseed oil's antioxidant properties (Hashim et al., 2019).

CONCLUSION

Flaxseed oil increased production performance in the hens exposed to high stocking density stress, improved eggs' taste and texture in both stressed and unstressed birds with no impact on intrinsic or extrinsic egg quality parameters. Therefore, it was deduced that a flaxseed oil-supplemented diet might have generated beneficial effects in the stress-exposed hens. However, its potential adverse effect on egg production and egg mass should be carefully assessed in the unstressed hens. Dietary sunflower oil supplementation proved efficient in improving production performance and egg texture in both stressed and unstressed birds without exerting a negative impact on eggs' internal and external quality parameters except for egg yolk's yellow color. Therefore, it can be concluded that sunflower oil is recommended to be included in both stressed and unstressed hens' diets due to its benefits.

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CONFLICT OF INTEREST

There is no conflict of interest in the present publication.

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Research article
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Honey bee drone (*Apis mellifera*) sperm cryopreservation with rainbow trout seminal plasma supplemented extenders

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ABSTRACT: The current study aimed to investigate the effects of rainbow trout seminal plasma (RTSP) on frozen-thawed honey bee drone spermatozoa. Semen samples were collected from sexually mature drones and pooled. Then pooled semen was diluted with different concentrations of RTSP (2.5%/5% / 10%) and without RTSP (control) supplemented extenders. Motility, plasma membrane functionality, acrosome integrity, and mitochondrial function were negatively affected by the cryopreservation process but DNA integrity was not affected. Membrane lipid peroxidation status was also analyzed using the malondialdehyde (MDA) concentration at frozen-thawed. RTSP10 groups had a positive effect on sperm motility (64.00 ± 3.38%), plasma membrane functionality (72.07 ± 2.12%), acrosome integrity (86.20 ± 2.11%), and mitochondrial function (69.33 ± 2.94%) compared to the control group ($P< 0.05$). The study shows that RTSP supplemented extenders have beneficial effects on at frozen-thawed drone sperm parameters. The results of the present study demonstrated the advantage of using a 10% RTSP supplemented extender for drone sperm motility, plasma membrane functionality, and mitochondrial function.

Keywords: Drone semen, Rainbow trout, Seminal Plasma, Cryopreservation

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INTRODUCTION

The spermatozoon is one of the first living cells to be successfully cryopreserved (Benson et al., 2012). Cryopreservation of sperm without losing its fertilization feature contributes to the selection and protection of genelines with superior genetic characteristics (Nur et al., 2010). Achieving acceptable fertility results by freezing spermatozoa is under the influence of some important factors. These include freezing techniques, appropriate extenders, freezing and thawing process, species-specific spermatozoon physiology, and characteristics (Curry, 2000). Also, the freezing of sperm cells belonging to different species is privileged due to the different size, shape, and lipid components of the cells (Benson et al., 2012, Ustuner et al., 2016).Dronesemen has been successfully cryopreserved in recent years (Taylor et al., 2009, Hopkinset al., 2010, Wegener et al., 2014, Alcayet al., 2019b, Alcayet al., 2019a). However, the cryopreservation process may cause structural, biochemical, and functional damage in spermatozoa, leading to premature activation of spermatozoa against physiological stimulation in the female genital tract, and a decrease in sperm motility, vitality, plasma membrane integrity, acrosomal integrity, and fertility. These undesirable effects may cause an irreversible decrease in sperm parameters (Nur et al., 2010). Therefore, the success of the sperm cryopreservation process depends on the composition of the extenders which is a crucial factor to protect spermatozoon (Ustuner et al., 2016, Alcay et al., 2020).

Seminal plasma which contains biochemicals components regulates sperm function (Ciereszko et al., 2000, Glogowski et al., 2000). The seminal plasma has been used in the semen extenders for sperm cryopreservation of different species (Gunay et al., 2006, Ustuner et al., 2016, Alcay et al., 2020). Rainbow trout seminal plasma (RTSP) includes proteins (2.1 ± 0.3 mg mL), monosaccharides, aminoacids, free fatty acids, phospholipids, vitamins, and neutral lipids (Shaliutina-Kolešová et al., 2016, Ciereszko et al., 2000). Besides, RTSP supplemented extenders have been successfully used for ram and goat semen cryopreservation (Ustuner et al., 2016, Alcay et al., 2020). However, its effect on drone sperm cryopreservation has not been evaluated until now.

The cryopreservation of drone spermatozoa without losing its ability of fertilization contributes to the conservation of genelines. We hypothesized that RTSP supplementation in the semen extender could improve the frozen-thawed drone sperm viability and its longevity. Hence, the current study was designed to compare various concentrations of RTSP supplemented extenders for the cryopreservation of drone sperm using quality tests.

MATERIALS AND METHODS

This study was planned to research the efficiency of RTS supplementary extenders for cryopreservation of drone semen.To this end, extenders supplemented with various RTS concentrations (non-RTS, RTS 2.5%, RTSP 5%, and RTSP10%) were used to evaluate the frozen-thawed quality of drone spermatozoa.

Chemicals: The chemicals used in the study were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Extender preparation

According to the experimental design, various RTSP concentrations were added in certain proportions to each extender group.We prepared the experimental groups such as RTSP2, 5 (with 2.5% RTS), RTSP5 (with 5% RTSP), RTSP10 (with 10% RTSP), and control (non-RTS). Extenders contained Na Citrate (82.21 mmol), Catalase (1.59 mmol), KCI (5.34 mmol), NaHCO3 (24.87 mmol), Amoxicillin (0.82 mmol), and DMSO (10%). The pH value of the diluents prepared was scaled to be 8.1.

RTSP were obtained by the previously described method of Glogowski et al. (2000) and stored at −20 ºC for up to one monthbefore using for drone sperm cryopreservation.

Collection and dilution of semen

Healthy and strong honey bee colonies in Bursa Uludag University, Beekeeping Development-Application and Research Center were used for semen collection.

Sexually mature drones (16 days and older) were selected for semen collection and at least five colonies were used for this research. The pressure was applied to the thorax to induce ejaculation, and then the abdominal area was gently squeezed. Approximately 1 µl semen was collected from per drone using the Schley syringe under a stereomicroscope. Besides, to eliminate individual differences all semen was pooled. The volume of each pooled semen was portioned into four equal volumes, and a total of five pooled semen was used in the study. Each group of the extender was

individually diluted with control or RTS supplemented extenders to a final concentration of about $150x10^6$ (spermatozoa / mL).

Semen freezing and thawing

The method of cryopreservation and thawing was based on (Alcay et al., 2019b).According to this method, equilibrated drone sperm was filled into 0.25 mL straws.After the filling process, straws were frozen at 3°C/min from +5°C to -8°C and at 15°C/min from -8°C to -120°C in liquid nitrogen vapor using the Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France).Then the sperm-filled straws were immersed in liquid nitrogen and then stored in a liquid nitrogen container.

Semen evaluation

In the evaluation of frozen-thawed semen, plasma membrane integrity, acrosome integrity, sperm motility, and DNA integrity parameters were examined. A hypoosmotic swelling test (HOST) was used for plasma membrane functionality. FITC-Pisum sativum agglutinin (PSA-FITC) was used for acrosome integrity. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) wasused to assess DNA integrity. Evaluations were made by the same person during the study.

Motility

Drone semen motility assessment was performed using a phase-contrast microscope at 400x magnification (Olympus BX51-TF - OlympusOptical Co., Ltd., Japan) with the slide heated to 37 ° C.

Membrane functionality

For the assessment of the plasma membrane integrity, the hypoosmotic swelling test method was performed according to Alcayet al. (2019b). Following this method, the membrane integrity of the drone sperm was evaluated by observing the frizzled tails.

Acrosome Integrity

For this evaluation, a sample of 10 µl spermatozoa was added in 100 mL of PBS and centrifuged for 5 minutes. The sperm pellet obtained after centrifugation was resuspended with 100 mL PBS. Afterwards, smeared-slides were prepared and dried. After the drying process is over, the smears were left in acetone fixation at 4 °C for 15 minutes in a glass chalet (vertical, Hellendahl type).After fixation, smears were stained with FITC PSA solution for 1 hour at 37 ° C in a light-proof sample kit. After the staining process was completed, at least 200 drone spermatozoa emitting fluorescent light were evaluated under a fluorescent attachment microscope (Alcay et al., 2019a).

Mitochondrial Activity

Double fluorescent stains, PI, and Rhodamine (R123), was used to examine the mitochondrial integrity. For this analysis, Fraseret al. (2002) method was used. The results are expressed as a percentage (%).

DNA fragmentation

DNA fragmentation was evaluated by the TUNEL technique using In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with slight modifications (Wegener et al., 2014).

Malondialdehyde (MDA) concentrations

To assess the MDA concentrations the method of Sharafi et al. (2015) was performed. Briefly, 0.25 ml of diluted semen sample was treated with 0.25 ml of cold 20% (w/v) trichloroaceticacid to precipitate the protein. During the centrifugation, the precipitated protein was pelleted and the supernatant was incubated with (w/v) thiobarbituric acid for 10 minutes in a 100° C boiling water bath. After the incubation in the hot water bath, the sample was allowed to cool. Absorbance was determined using the Spectrophotometer (Mannheim Boehringer Photometer 4010). MDA concentrations were expressed as nmol/ml.

Statistical analysis

All of the data obtained in the study were analyzed using SPSS (SPSS 23.0 for Windows; SPSS, Chicago, IL, USA) and the results were represented as mean ± standard deviation. ShapiroWilk test was used as a normality test. Semen parameters were analyzed using one-way ANOVA followed by Tukey.

RESULTS

In the study, the percentages of motility, plasma membrane functional integrity, acrosome integrity, mitochondrial function, and DNA fragmentation rates of pooled semensamples were 87.00±4.47, 91.40±1.14, 94.00±1.58, 92.20±2.28, and 0.20±0.45 respectively. Spermatozoa quality was negatively affected by the cryopreservation process compared with the fresh pooled semen ($P < 0.05$). The table 1 shows the effects of different concentrations of RTSP on drone sperm parameters at frozen-thawed.

Table 1. The mean of studied sperm post-thawing parameters on different extender groups

Variable	RTSP Concentrations (%)			
	0	2.5	5	10
Motility (%)	52.00±3.68[a]	53.3±2.44[a]	59.67±4.42[b]	64.00±3.38[c]
HOST (%)	62.93±3.06[a]	65.73±3.55[b]	68.53±1.92[c]	72.07±2.12[d]
Acrosome integrity (%)	82.73±2.43[a]	83.00±3.25[a]	84.60±2.85[ab]	86.20±2.11[b]
Mitochondrial function (%)	59.73±2.71[a]	64.53±2.70[b]	66.27±2.63[b]	69.33±2.94[c]
DNA fragmentation (%)	0.71±0.99	0.53±0.74	0.60±0.74	0.53±0.74
MDA level (nmol/ml)	2.78±0.83	2.44±0.73	2.22±0.66	2.33±0.50

Data is presented in Mean± S.D.
Different letters within the same rows show significant differences among the groups (p<0.05).

The sperm motility was higher in RTSP5 and RTSP10, compared to control group (P <0.05). Also, the highest percentage of motility rates were obtained from the RTSP10 group (P < 0.05).The percentages of plasma membrane functional integrity were higher in RTSP compared to the control group (p<0.05). Besides, the best membrane integrity rate was obtained in the RTSP10 group (P < 0.05).

The better acrosome integrity was obtained in the RTSP10 group compared to the control and RTSP2.5 groups (P<0.05). The percentage of acrosome integrity wasnot found significantly different between RTSP5 and RTSP10 groups (P > 0.05).A better mitochondrial function rate was obtained in the RTSP groups compared with the control group (p<0.05). No significant differences were observed in sperm DNA damage between all groups (p>0.05). As shown in Table 1, it was found that the MDA levels were not significantly different between all groups (P>0.05).

DISCUSSION

Sperm freezing ensures that genetic material is preserved for a long time. However, it is known that the freezing-thawing process has negative effects on the fertilization ability of spermatozoa. These undesirable effects decrease motility, viability, plasma membrane, and acrosome integrities of spermatozoa (Nur et al., 2010, Alcay et al., 2019).In the study, we evaluated the effect of exogenous addition of RTSP in extenders on drone sperm quality at frozen-thawed.

Motility is one of the main sperm evaluation parameters and has an important effect on oocyte penetration (Yániz et al., 2020). In this study, we have shown that the presence of RTSP concentrations in cryopreservation media increased drone sperm motility compared to the control group at frozen-thawed (P<0.05). The motility values of drone spermatozoa cryopreserved with various semen extenders ranged between 25% - 62% (Alcay et al., 2015, Alcay et al., 2019b, Alcay et al., 2019a, Wegener et al; 2014, Wegener et al., 2012). Our study shows that frozen-thawed sperm motility rates in high dose RTSP groups (RTSP5 and RTSP10) were in good agreement with the findings of these studies. In our study, although RTSP supplementation caused a clear increase in motility, the RTSP2.5 group had not sufficient effect to make a statistical difference compared with the control group. When the RTSP doses were compared among each other, increasing doses of RTSP caused a gradual increase in motility.

The functional integrity of the plasma membrane that is essential for spermatozoon metabolism plays a crucial role in the oocyte fusion of spermatozoon (Yániz et al., 2013). However, cold shock and lipid peroxidation have negatively affect membrane permeability and integrity during cryopreservation (El-Kon et al., 2011). Therefore, it is crucial to keep integrity during the cryopreservation process to avoid cellular damage. HOST is the optimized test for detecting the subtle changes of spermatozoon membrane functionality (Maxwell et al., 1993, Nur et al., 2010). In the study, the plasma membrane functional integrity values in the RTSP10 group were higher than in the other groups (P < 0.05). The HOST values are in agreement with the earlier researches (Alcay et al., 2015, Alcay et al., 2019a, Alcay et al., 2019b).

The defected acrosome is one of the adverse effects of the sperm cryopreservation process (Ustuner et al., 2016, Nur et al., 2010). During oviposition, the queen releases few spermatozoa from the spermatheca, and then the acrosome reaction releases lytic enzymes that aid in the penetration of the vitelline membrane to fertilize the egg. Therefore, acrosome integrity is crucial for the fertility of frozen-thawed spermatozoa. In the study, there was no statistical difference among RTSP groups (RTSP2.5 and RTSP5) and the control group

for acrosome integrity rates. RTSP10 group preserved acrosome integrity better thanRTSP2.5 and control groups. These results are in agreement with the previous research (Alcay et al., 2019a, Alcayet al., 2019b, Nur et al., 2020).

Sperm needs the energy to carry out its functions and it can mostly obtain ATP through the glycolytic and oxidative phosphorylation pathways (Storey et al., 2008, Moscatelle et al., 2017). Mitochondria play an essential role in regulating sperm function (Yániz et al., 2020). Therefore, it is important to evaluate the mitochondrial function for sperm quality. In the study, mitochondrial function was better preserved in RTSP groups compared to the control group at frozen-thawed (P<0.05). Also, high dose RTSP preserved mitochondrial function compared to the other groups. Similar results were obtained in previous research (Ciereszko et al., 2017)

Protecting the integrity of DNA during freezing also has great importance not to disrupt the early development of the embryo (Nur et al., 2010). In this study, it was observed that drone semen was resistant to freezing-thawing process. No significant differences of DNA fragmentation were noticed between all groups (P>0.05). Similar result was obtained in previous research (Wegener et al., 2014). Oxidative damage may be evaluated by MDA levels which is a key product of polyunsaturatedfatty acid's peroxidation in thecells. In our study, no statistical differences were obtained for MDA levels in all groups (P>0.05). Catalase which was used in the main extenders for all groups might have been a positive effect against oxidative damage.

Considering all sperm parameters; the RTSP10 group was the optimum for drone semen preservation. Future studies might be focusing on RTSP supplementation to improve the cryopreservation values and to evaluate reproductive success (viable offspring) when used to fertilize the queens.

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CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Effects of milk yield and quality at post-calving period on Algerians cows' reproductive performances

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ABSTRACT: This study was carried out to determine the association between milk production characteristics at the beginning of lactation (the first three month of lactation) and the performance of reproduction within dairy cows of Algeria. Data were collected from Holstein cows (n=920) that calved between October 2017 and October 2019. Reproductive parameters included in the study were: calving to first-service interval (CFSI, days), calving to conception interval (CCI, days), services per conception (SPC), and conception rate at first-service (CRFS %). The milk parameters retained in the present study were: peak milk yield, cumulative milk yield in the first 30 days (Milk30), 60 days (Milk60) and 100 days (Milk100) postpartum of the cows. The milk composition traits analyzed were milk fat concentration (MF %) and milk protein concentration (MP %). These variables were used to calculate fat to protein ratio (FPR) for each stage of lactation (stage 1: 15-30 days, stage 2: 45-60 days and stage 3: 75-90 days postpartum). Pearson's correlation analysis was used to determine the correlation between reproductive parameters and milk production variables.

The obtained results showed that peak milk yield, cumulative milk yield in the first 60 days (Milk60) and 100 days (Milk100) were significantly and positively ($P<0.05$) correlated with all the measured fertility parameters. Furthermore, fat to protein ratio (FPR) and milk fat content correlated significantly and positively ($P<0.05$) with CFSI, CCI and SPC in the first two stages. The correlation between milk protein content with CFSI, CCI and SPC were negative and significant in the all three stages. Further research is required to identify the cause of this association.

***Keywords*:** Algeria, cows, milk quality, performance, reproduction.

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INTRODUCTION

Milk production and reproductive performance are two major determinants of dairy cows' profitability. While, genetic selection for milk yield has occurred, a reduction in fertility was observed. The question of possible antagonism between high milk production and reproductive performance was raised among the scientific researchers since long time. The relationship between fertility and milk production is often perceived, as a relatively current concern. Indeed, high reproductive efficiency is crucially important and has a high priority to maintain profitability in the dairy industry. The economic benefit in livestock is mainly associated with the reproduction of animals (Pryce et al., 2004). An efficient dairy production system should maximize the productivity of the individual by achieving the goal of one calve per cow per year. Effective reproduction results in optimal calving intervals, which, in turn, result in an optimal, milk production and calves per unit of time. In addition, low reproductive efficiency decreases herd gain by extending the calving interval, which results in less milk produced per cow and increasing replacement costs due to reproductive failure and veterinary charges. Therefore, well managing cows' fertility still a key element in ensuring the profitability and sustainability of the livestock.

After parturition, dairy cows in early postpartum period present a rapid increase in milk yield, nutritional requirements and a slow rise in dry matter intake. Most of them are unable to consume adequate quantity of feed needed for their milk production. As a result, cows enter the stage of negative energy balance (NEB) (Walsh et al., 2011). The severity and duration of the negative energy balance (NEB) may be influenced by genetic merit for milk yield and energy density or quantity of the feed offered (Buckley et al., 2003). According to Rossi et al. (2008), the extent of NEB is the main factor influencing the decline of reproductive efficiency in high-yielding dairy cows. The endocrine and metabolic signals associated with negative energy balance (NEB) signal to the reproductive tract, resulting in less regular estrous cycles and reduced embryo survival rates. Therefore, traits related to the extent and the duration of the postpartum negative NEB are of great interest as indicator traits to enhance improvement of reproductive performance. These important facts have to be elucidated in Algerian dairy herd in order to reduce the economic losses associated with non-professional farm management.

To estimate EB in early postpartum period, several traits have been associated with EB state and reproduction. Some authors put special attention to the rise in some metabolites and endocrine blood traits such as non-esterified fatty acids (NEFA) or beta hydroxyl butyrate (BHB) (Vanholder et al., 2005) ; body condition score (BCS) and milk composition (Friggens et al., 2007). In contrast to blood metabolites, which are invasive, difficult, and expensive to measure, milk composition data are easily available.

Recent research has demonstrated the potential of mid-infrared (MIR) analysis of milk to predict new phenotypes of interest such as milk composition; aptitude for transformation of milk and health status of cows. Because of its use by regular milk recording and milk payment systems to quantify the major milk components, MIR spectrometry is a rapid and cost-effective tool for recording phenotypes at the population level (De Marchi et al., 2014). Hence, MIR analysis of milk appears as a method of choice to provide indicators of fertility and EB that are collected routinely and that readily available. Furthermore, recent research has showed the importance of milk composition analysis for determining energy balance status and fertility in dairy cows (Buttchereit et al., 2010). These studies were conducted on Holstein dairy cows only. Despite, the well-known relationship between milk composition measures with fertility and EB Holstein cows, the expected beneficial effect of using these relationships as a management tool for evaluating the EB status and fertility of Holstein cows in Algeria is still unknown. Therefore, the objectives of this current study were to assess the reproductive performances of dairy cows in Algeria and their relationship with milk yield and milk composition in the first three months of lactation.

MATERIALS AND METHODS

Animals and management

The current study was performed from October 2017 to October 2019and included data from 920 purebred Holstein-Friesian dairy cows. Cows belong to eight commercial dairy herds located in the western of Algeria. An average lactation of the herd was 7600 kg of milk per 305 days and average parity of cows, included in the study was 2.8 (min.1-max. 4). The cows were kept in a free stall barn system and were feed with a mixed ration. Basic ration was composed of maize silage, grass and hay; lactating cows were supplemented with protein concentrate. All cows

could access to food, water and salt lick *ad-libitum* during the whole year in the stall. The cows were examined during estrus period by visual observation using standing to be mounted as a sign of estrus, 30 min at least three times daily (in the morning, at noon, and in the late afternoon). Cows were inseminated artificially after detection of estrus by an experienced veterinarian, in accordance with the international am-pm rule following detection of estrus. After 60-70 days post artificial insemination, if cows did not return to estrus, an experienced veterinarian using rectal palpation performed a pregnancy diagnosis.

Measurement of fertility traits

Bi-monthly surveys were conducted in each farm to collect data related to milk production and reproductive events (calving, insemination, pregnancy diagnosis) of dairy cows. These latter, were recorded by the farm managers according to cow's identification number, dates of calving and inseminations. The collected records were used to calculate the reproductive parameters. Their productive performance was characterized by referring to the following intervals: calving to first-service interval (**CFSI**) which is the number of days between parturition and the subsequent first-artificial insemination; calving to conception interval (**CCI**), represented the number of days between parturition and the artificial insemination that resulted in a pregnancy; services per conception (**SPC**), which means the number of artificial in semination that a cow required to conceive during the current lactation and conception rate at first-service (**CRFS, %),** (number of cows pregnant after the first AI divided by number of cows inseminated ×100).

Milk yield, sample collection and analysis

Cows were milked twice daily (morning and evening) by milking machines and the quantity of milk yield was measured by classical methods and was recorded daily at each milking for the first 90 days postpartum for individual cows. The daily recorded quantity of milk yield was used to calculate the peak milk yield, cumulative milk yield in the first 30 days (Milk30), 60 days (Milk60) and 100 days (Milk100) postpartum of the cows.

To determine milk composition variables, 2760 milk samples were collected during the first three month of lactation. In the post-partum period, each cow was sampled three times respectively at the 1st stage [1: 15-30 days]; 2nd stage [45-60 days] and 3rd stage [75-90 days].The milk samples were transported immediately after collection to the laboratory and stored overnight at 4°C for further analysis.

The milk composition analysis was carried by near infrared spectrophotometer Milko-Scan (Foss Electric, Denmark). The milk composition traits analyzed were milk fat concentration (MF %) and milk protein concentration (MP %). These variables were used to calculate fat to protein ratio (FPR) for each stage of lactation.

Statistical analysis

Statistical analyses were performed with SPSS software (Version 20.0). Pearson's correlation analysis was used to determine the correlation between reproductive parameters and the milk production variables. The results were considered significant when $P<0.05$.

RESULTS

Data related to the parameters of reproduction and milk yield recorded in 30, 60 and 100 days of lactation in the dairy herds are summarized in Table 1.

Table 1. Reproduction parameters and milk yield at 30, 60 and 100 days of lactation in Holstein-Friesiandairy herds of Algeria

Parameters	Mean±SD
Peak milk yield	24.3 ±2.12
Milk yield 30 days (kg)	660.02 ±35.1
Milk yield 60 days (kg)	1150.2 ±71.5
Milk yield 100 days (kg)	2030.2 ±131.5
CFSI (days)	132.4±54.4
CCI (days)	159.1± 49.5
SPC	1.85±1.17
CRFS (%)	59.1

CFSI: Calving to first service interval**; CCI:** Calving to conception interval; **SPC:** Services per conception; **CRFS**: Conception rate at first service

Data showing variation of milk composition for dairy herds according to the stages of postpartum period are summarized in Table 2.

Associations Between milk data records and reproductive outcomes

Correlation between reproduction parameters and milk data records are presented in Table 3.

Table 2. Milk composition characteristics at the post-partum period in Holstein's -Friesian's dairy farms of Algeria

Stage of lactation	Milk composition		
	Fat contents (%)	Protein contents (%)	Fat to protein ratio
1st [15-30 days]	4.68± 0.72	3.37 ± 0.39	1.41 ±0.36
2nd [45-60 days]	4.42±0.52	3.19± 0.28	1.39 ±0.33
3rd [75-90 days]	3.97 ± 0.41	3.31± 0.25	1.29± 0.27

Table 3. Correlation coefficients between reproduction parameters and milk composition records

Milk characteristics	Stages	Reproduction parameters			
		CFSI	CCI	SPC	CRFS
Protein content	1st [15-30 days]	-0.30**	-0.43**	-0.28*	0.35**
	2nd [45-60 days]	-0.49**	-0.48**	-0.34*	0.52*
	3rd [75-90 days]	-0.37*	-0.30*	-0.24*	0.44**
Fat content	1st [15-30 days]	0.33*	0.42*	0.31*	-0, 45*
	2nd [45-60 days]	0.27*	0.35*	0.26	-0.31*
	3rd [75-90 days]	0.23	0.28	0.10	0.15
Fat to protein ratio	1st [15-30 days]	0.39*	0.51**	0.11	- 0.43*
	2nd [45-60 days]	0.37*	0.36*	0.19	-0.29*
	3rd [75-90 days]	0.15	0.21	0.12	0.17
Milk yield	At 30 days [M30]	0.12	0.21	0.10	-0.15
	At 60 days [M60]	0.23*	0.29*	0.18	-0.27*
	At 100 days [M100]	0.35*	0.37*	0.17	-0.52**
	Peak of yield	0.45*	0.39*	0.29*	-0.28*

CFSI: Calving to first service interval**; CCI:** Calving to conception interval; **SPC:** Services per conception; **CRFS**: Conception rate at first service; ***= P<0.01, *=P<0.05,***

Milk yield and reproductive performance

Peak milk yield, cumulative milk yield in the first 60 days (Milk60) and 100 days (Milk100) were significantly (P < 0.05) correlated with all the measured fertility parameters. Conversely, cumulative milk yield in the first 30 days (Milk30) was not associated with fertility parameters.

The correlation coefficients between cumulative milk yield in the first 60 days (Milk60) and fertility parameters were 0.23, 0.29 and -0.27 for CFS, CCI and CRFS, respectively, while the correlation coefficients between cumulative milk yield in the first 100 days (Milk100) and fertility parameters for CFS, CCI and CRFS were 0.35, 0.37 and -52, respectively. Furthermore, the correlation coefficients between peak milk yield and CFS, CCI, CRFS were 0.45, 0.39 and -0.28, respectively.

Milk protein and reproductive performance

This study identified significant associations between early-lactation milk composition and subsequent fertility performance. Correlation coefficients between fertility traits and milk protein content was low in the first stage [15-30 days postpartum] and the third stage[75-90 days postpartum]. However, in the second stage[45-60 days postpartum], milk protein content was strongly and significantly, (P<0.05-0.01) correlated with all the fertility traits measured; calving to first service interval (CFSI), calving to conception interval (CCI), services per conception (SPC) and conception rate at first service (CRFS).

The correlation coefficients between milk protein concentration and calving first service interval were -0.30, 0.49 and -0.37 for first, second and third stages of lactation, respectively, while the correlation coefficients between milk protein concentration and CCI in first, second and third stage of lactation were -0.43, -0.48and -0.30, respectively. The strong relationship between the number of SPC and conception rate at first service with milkprotein concentration observed in stage 2 whose correlation coefficient was respectively -0.34 and 0.52.

Milk fat and reproductive performance

The strong relationship between calving to first service interval (CFSI), calving to conception interval (CCI), services per conception (SPC) and conception rate at first service (CRFS) with milk fat content was found in first stage. The correlation coefficients were respectively 0.33, 0.42, 0.31 and -0.45.

Fat to protein ratio and reproductive performance

The correlation coefficients magnitude between fertility parameters and fat to protein ratio was strong in first and second stage; except for service per conception (SPC) which was not significantly correlated with fat to protein ratio. Furthermore, in the third stage, fat to protein ratio was not significantly correlated with all fertility parameters.

DISCUSSION

This study was carried out to determine the association between milk production characteristics in the first three month of lactation and the performance of reproduction within dairy cows of Algeria. A negative correlation between the amounts of milk produced and the fertility of cows was observed in our survey. Similar results were reported by Walsh et al. (2011). This result might be related to the fact that higher milk production increased the difference between feed intake and dairy potential in early lactation stage, making cows genetically predisposed to a higher risk of negative energy balance (Patton et *al*. 2006).Dairy cows may present an energy deficiency situation at the post-partum period; as their low ingestion capacity does not cover the high energy requirements for milk production (Wathes et al., 2012). The deficit of energy could contribute to a decrease in fertility. This latter has particular consequences on the expression and duration of heat. Other studies have also shown that energy deficiency induced a decrease in the frequency of LH (luteinizing hormone) pulses and the development of ovarian insensitivity to LH (Walsh et al., 2011). Butler (2001) reported that high milk production in the first weeks of cows' lactation (between 20 and 180 days according to the studies) may decrease the ovulation frequency of the first dominant follicle, the frequency of LH peaks and thus delay the first ovulation. It also increases the predisposition for developing ovarian cysts and associated dysfunctions (Vanholder et al., 2006). Disenhauset al. (2002) observed a higher rate of abnormal cyclicity profiles within large dairy production in the first three weeks postpartum.

The most important findings from this study were that, early lactation milk protein concentration and reproductive performance was negatively associated. Our results were similar to other studies in which a positive association between milk protein concentrations and conception rate at first service were reported in early lactation (Alphonsus et al., 2014; Morton et al., 2016; Morton et al., 2017; Morton et al., 2018; Carty et al., 2020).The highest association ($P<0.01$) between protein concentration and the conception rate at first service was observed in the 3rd stage [75-90 days]. Catherine et al. (2020) showed a high probability of pregnancy in cows with high protein concentration in milk from 61 to 90 DIM. Cook and Green (2016) showed a positive relationship between probability of conception by 100 and 150 DIM and milk protein concentration at both first [0-30 days] and second [31-60] test days.

Douglas et al. (2016) showed that cows with high milk protein concentration have plasma profiles of selected metabolites and hormones that indicate greater partitioning of nutrients to physiological processes other than milk synthesis at the benefit of body condition. Morton et al. (2018) attributed the positive phenotypic associations between milk protein concentration and reproductive performance traits to factors affecting both milk protein concentration and reproductive performance. This relationship has been attributed to energy balance during early lactation (Madouasse et al., 2010; Alphonsus et al., 2014). In our study, the tested samples of milk showed content in proteins varying from 3.19 to 3.37. Negative energy balance has been associated with reduced reproductive performance, and milk protein concentration in early lactation is higher in cows with less negative energy balance (Patton et al., 2007). Buckley et al. (2003) and Patton et al. (2007) concluded that milk protein percentage in early lactation is a good indicator of a cow's energy balance and reproductive performance in dairy cows during this period. It is influenced by the level of energy supply to cows (Eicher, 2004). The energy balance of cows is only very weakly related to protein intakes. Only the essential amino acids (mainly methionine and lysine) digestible in the intestine (protected from digestion in the rumen) can have a positive effect on milk protein concentration. This latter is higher in the first week and decreases to its lowest level at the peak lactation. Further studies are needed by determining the content of methionine and lysine in milk of caws at the post-partum.

Most severe and prolonged NEB were observed in cows producing milk with lowest protein content (2.89%) (Fulkerson et al., 2001). Furthermore, milk protein content was positively associated with submission rate, pregnancy rate to first service, and pregnancy rate after 21 days breeding in a large field study (Morton, 2016). In line with findings, Madouasse et al. (2010) stated that cows with low milk protein content in the second month of lactation were less likely to be pregnant within 145 days of calving.

The milk fat of the first milk control is crucial because it allows quantifying somewhat the negative energy balance, which prevails at the very beginning of the lactation. In the present study, milk fat was decreased from 4.68 at the first stage to 3.97 at the third stage. The milk fat of the first milk control reflects the intensity of themobilization of reserve fats at the beginning of lactation. Kristula et al. (1995) demonstrated that Holstein cows with a milk fat > 45 g/L at the first dairy control subsequently had a significantly lower initial insemination rate than cows with lower milk fat. Milk fat concentration was positively associated with hazard of pregnancy in stage 1 (0-30 DIM). Other studies have identified higher fat concentrations in early lactation (<30 DIM) as negatively associated with subsequent fertility (Madouasse et al., 2010; Cook and Green, 2016). Cook and Green (2016) identified a negative association between milk fat percentage in the first 30 days in milk (DIM) with the probability of pregnancy at 100 DIM but not at 150 DIM. It suggests that the use of fat concentration to predict reproductive performance may be limited and that early lactation changes in fat concentration may be indicative of energy balance troubles. The higher mobilization of fat at the beginning of lactation may induce ketosis, which could be suspected indirectly through milk/protein ratio.

Our results, showed a significant relationship between the FPR ration in milk samples and CFSI, CCI and CRFS in the 60 days post-partum. In line with our findings, Podpecan et al. (2008), Löf et al. (2014) showed significant correlations between FPR and fertility. The fat to protein ratio is a calculated indicator of the nutritional status of dairy cows. It can be used to evaluate the energy balance and the level of the mobilization of reserve fats, especially at the beginning of lactation, a negative correlation between the fat to protein ratio and the energy balance. This relationship is stronger at the beginning than at mid-lactation (Buttchereit et al., 2010; Toni et al., 2011).

In the present study, fat to protein ratio decreased from 1.41 at the first stage to 1.29 at the third stage. In the case of an energy deficiency, the fat to protein ratio increased significantly. The maximum threshold value is set at 1.5 (Buttchereit et al., 2010; Toni et al., 2011). High fat to protein ratios in early lactation were associated with decreased reproductive performance, either by decreasing fertility or an extension of postpartum anestrus due to excessive loss of an increase in the rate of reform for infertility (Podpecan et al., 2008; Löf et al., 2014).

No relationship between FPR and fertility parameters was observed at the third stage [75-90 days] of lactation in post-partum. Dubois et al. (2006) indicated that cows with a fat to protein ratio >1.5 at the first postpartum control have a high incidence of cyclicity abnormalities (39.4%), compared to cows with <1.5 (25%), an increased risk of ovarian cyst, a lower conception rate at the first artificial insemination, delayed calving to conception interval and induce multiple services per conception.

CONCLUSION

Our study identified several associations between reproduction performance and milk protein concentration, milk fat and milk yield. In fact, high milk production decreases reproductive performance and higher milk protein concentration is associated with shorter calving to first service and calving to conception intervals. Furthermore, milk high fat concentration increased the number of days for conception. These results suggest that milk composition at early stage of lactation is likely to predict reproductive performance.It seems appropriate to carry outsimilar studies in other provinces of Algeriato better understand the association between reproductive performance and milk quality.

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CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Pharmacological effect of Lidan Tang against adjuvant-induced rheumatoid arthritis in rats

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ABSTRACT: Rheumatoid arthritis (RA) is a great concern across the globe. It is characterized as an autoimmune disease, where the body's immune system mistakenly attacks bone joints causing them to erode which leads to deformity in structure. Thus, the present study was conducted to elucidate the effect of Lidan Tang (LDT) decoction, a traditional Chinese medicine against rheumatoid arthritis. The rheumatoid arthritis was induced in Sprague-Dawley albino rats by intradermal injection of bovine collagen II type at the tail. Results suggested that LDT reduces paw swelling and arthritic scores in rats. The protective effect of LDT against RA was further substantiated by histopathological analysis of synovial tissue of rats, where LDT improves the architecture of the tissues as compared to CIA rats. It also reduces oxidative stress and inflammation in CIA rats as compared to the disease model group. The serum level of anti-collagen II-specific immunoglobulins (IgG_1 and IgG_{2a}) was reduced significantly in LDT treated group. In a western blot analysis, LDT treated group showed a significant reduction in the expression of NF-κB and COX-2 as compared to CIA rats. Collectively, our study for the first time demonstrated the protective effect of Lidan Tang decoction against rheumatoid arthritis.

Keywords: TCM, CIA, rheumatoid arthritis, NF-κB, COX-2.

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INTRODUCTION

The dramatic change in lifestyle in past decades has predisposed humans to many chronic diseases that affect their overall survivability (Carrera-Bastos et al. 2011). Among such diseases, Rheumatoid arthritis (RA) is a great concern for many health care workers across the globe. It is characterized as an autoimmune disease, where the body's immune system mistakenly attacks bone joints causing them to erode which leads to deformity in structure. It is also associated with inflammation, redness, swelling at the site due to the destruction of ligaments and tendons (Firestein and McInnes 2017; Smolen et al. 2018). The current clinical options to treat RA have been greatly dependent on the use of disease-modifying anti-rheumatic drugs (DMARDs) in combination with physical exercise or even surgery (Aletaha and Smolen 2018). However, these options are not sufficient to manage the disease; thus, it is an urgent need to discover newer agents that can provide relief against RA.

Various studies have shown a close connection between inflammation and oxidative stress (Sies 1986; Reuter et al. 2010; Fernández-Sánchez et al. 2011; Mittal et al. 2014; Hussain et al. 2016). It has been found that the inflammatory condition of RA has been greatly provoked by the generation of reactive oxygen species (ROS). It undermines the oxidative defense mechanism of the body by inducing lipid peroxidation, mitochondrial and DNA damage (Birben et al. 2012; Schieber and Chandel 2014). Moreover, accumulating shreds of evidence suggested that inflammation in RA provoked the release of various cytokines (IL-1β, IL-6, IL-10, IL-17, and TNF-α) from the immune cells (Feldmann 2002; McInnes and Schett 2007; Feldmann and Maini 2008). Studies have also shown that RA patients have a serum level of IgG in comparison to the normal population (Nandakumar et al. 2007; Khosroshahi et al. 2010). Thus, agents that inhibit inflammation and oxidative stress have shown a beneficial effect against RA.

Traditional Chinese medicine (TCM) has been used for centuries in China for treating various diseases and ailments. It's growing interest across the world as an alternative therapy to the modern-day conventional therapies (Yuan et al. 2016). Lidan Tang (LDT) decoction is a highly prescribed medication by the Beijing Integrated traditional Chinese and Western Medicine Hospital, China for the treatment of cholelithiasis. Recently, it was reported to exhibit a protective effect against high-fat-diet-induced gallstone in mice via reducing hepatic oxidative stress by promoting Keap1/Nrf2 pathway (Gao et al. 2020). However, till now, no study has reported the effect of LDT against RA. Thus, in the present manuscript, we intend to study the effect of LDT in adjuvant-induced arthritis in rats.

MATERIALS AND METHODS

Animals

Healthy male Sprague-Dawley albino rats were obtained from the institutional animal house and kept in a strict hygienic environment. They had free access to food and water in controlled humidity. The animal study was approved by the Xi'an Jiaotong University, China.

Establishment of the disease model

The chicken type II collagen (CII) after mixing with acetic acid (0.05 M) was suspended in complete Freund's adjuvant (CFA) (heat killed *Mycobacterium tuberculosis* H37Ra). The CFA mixture was admisntered into the dermis oftail of rats on the day 0. It was further admistered with CII and IFA on day 16. The control rats received no immunization.

Animal groups

Total five groups of rats were formed containg 12 rats in each group. The LDT decoction for administration to rats was prepared as per the given procedure elsewhere. (Gao et al. 2020) Th group treatment are as follows. The whole experiment period is 28 days and LDT decoction was admistered intra-peritonellly to the rats.

Group 1: Control (Saline for 28 days)
Group 2: CIA rats (Arthritis disease group)
Group 3: CIA + LDT (25 mg/kg)
Group 4: CIA + LDT (50 mg/kg)
Group 5: CIA + LDT (75 mg/kg)

The paw swelling of rats was examined on days 0, 14, and 28 of the experimental period using plethysmometer, . Moreover, the arthritic score of RA rats was considered as per the earlier reported method (Zhang et al. 2009)

Sample preparation

At the end of the experiment, i.e. 29th day, the rats were sacrificed, and serum was isolated from the blood of the rats for further biochemical estimation. The synovial joint tissue was cut out from the rats and

set in 10% formalin for histological assessment. On the other hand, an additional set of joint tissue was homogenized in phosphate buffer saline (10%) for further biochemical and molecular estimation.

Histopathological evaluation

The rat's synovial joint tissue was fixed for 24 hr after soaking in 10% buffered formalin. The tissues were processed and implanted in paraffin. A 5 µm thickness sections were cut, deparaffinized, dehydrated, and stained with hematoxylin and eosin (H&E) for the histological assessment estimation under a light microscope. Swelling and erythema of the paws (hind and fore) were graded using a 5-point scale: 0, no sign of swelling or erythema; 1, signs of swelling or erythema in the ankle or wrist; 2, signs of swelling or erythema in the ankle and tarsal or wrist and carpal; 3, signs of swelling or erythema extending to the metatarsals or metacarpals; and 4, signs of swelling or erythema involving the entire hind or fore paw. Hence, the maximum score was 8 (4 × 2 hind/fore paws).

Antioxidant enzymes and lipid peroxidation products

The joint-tissue homogenates were used to estimate the level of Glutathione (GSH) content, catalase (CAT) malondialdehyde (MDA) and superoxide dismutase (SOD) activities using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Estimation of Anti-collagen (C) II Ig and pro-inflammatory cytokines

Anti-collagen (C) II Ig and inflammatory marker levels Serum anti-CII IgG1 and G2a, IL-1β, IL-6, IL-10, IL-17, and TNF-α levels were determined using ELISA kits (Thermo Fisher Scientific).

Western blot analysis of NF-κB and COX-2

The isolated protein from the synovial joint tissues was probed to 10% SDS-PAGE for 50 min and transferred to PVDF membrane. The membrane was blocked with 5% defatted milk in tris-buffered saline with 0.1% Tween 20 for 1 h at 4°C and then incubated with rabbit anti-mouse NF-κB, COX-2 and β-actin primary antibodies at 4°C overnight. Afterward, the membrane was incubated with anti-mouse IgG HRP-conjugated secondary antibodies. The protein expression was perceived with an ECL detection kit.

Statistical analysis

Results are shown as means±standard deviation (SD). Statistical analysis was performed using ANOVA using Graphpad Prism (version 5). $P<0.05$ was taken as statistically significant.

RESULTS

LDT reduces paw swelling and arthritic score

Initially, the effect of LDT was investigated on swelling of the paw and arthritic score after induction

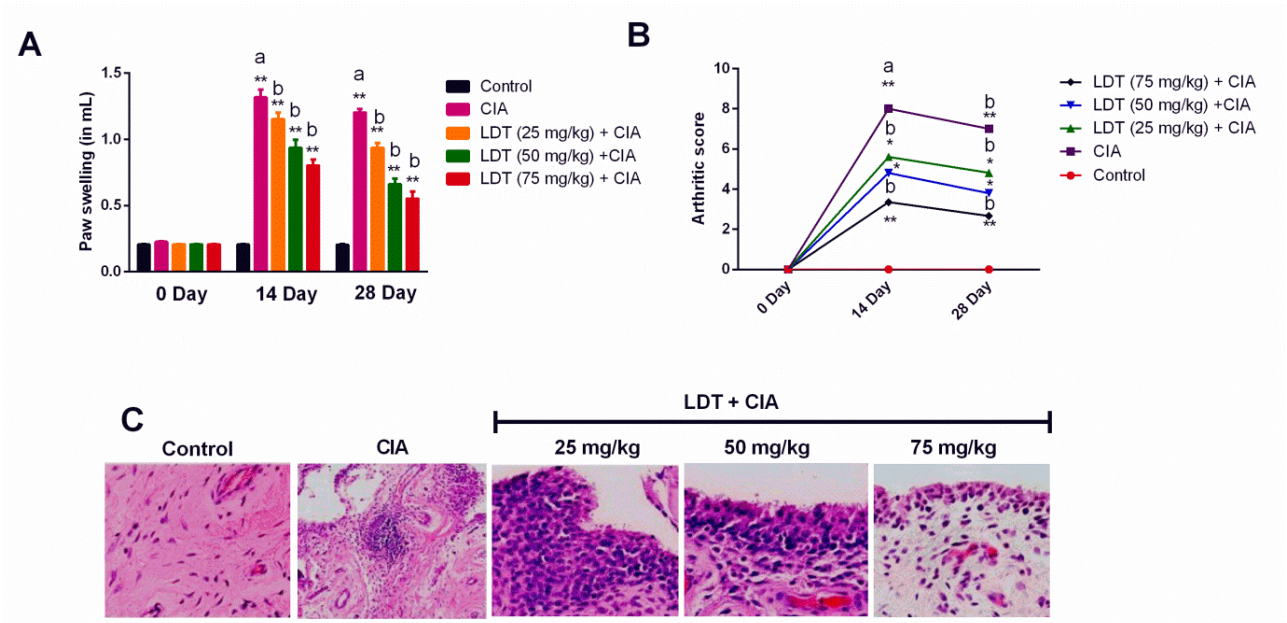

Figure 1. Effect of LDT on A) paw swelling, B) arthritic score and C) histopathological analysis of synovial joints of CIA and control rats. Data are represented as means ± SD. ** $p<0.01$, * $p<0.05$. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

of arthritis by CFA in rats. The rats from the CIA disease model group showed significantly increased swelling along and arthritic score as compared to control. However, upon administration of LDT, the studied indices were found significantly reduced as compared to the CIA model group in the treatment period, Fig. 1. The benefits of LDT were confirmed by the histological analysis of joint tissues, where it prevents tissue erosion and decreased permeability inflammatory infiltrates.

LDT reduces oxidative stress

The effect of LDT was further investigated on various indices of oxidative stress, such as MDA, GSH, CAT, and SOD in the joint tissues of rats. The level of GSH, SOD, and CAT was found significantly reduced in the CIA disease model together with an increase in MDA as compared to control. On the contrary, the levels of these biomarkers were found significantly improved in LDT treated group as compared to control ($p<0.05$), Fig. 2.

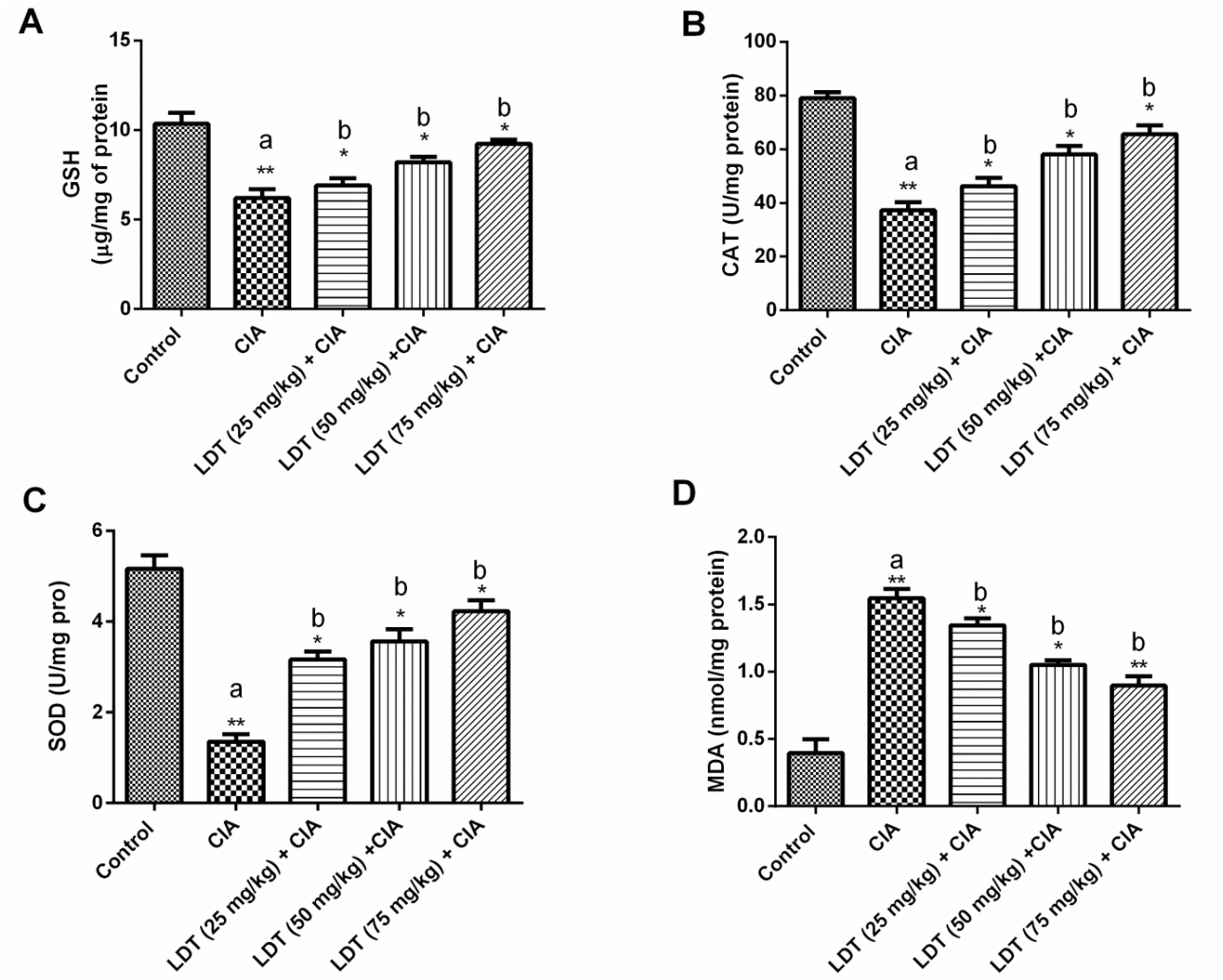

Figure 2. Effect of LDT on various indices of oxidative stress in CIA rats and control. A) GSH, (B) CAT, (C) SOD and (D) MDA. Data are represented as means ± SD. ** $p<0.01$, * $p<0.05$. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

LDT reduces serum Immunoglobins

The effect of LDT was investigated on the serum anti-CII IgG1 and G2a levels using ELISA analysis. The serum level of these tested immunoglobulins was found significantly elevated in the CIA disease model as compared to the control. However, in LDT treated rats, the level of serum anti-CII IgG1 and G2a was reduced significantly in a dose-dependent manner, Fig. 3 ($p<0.01$).

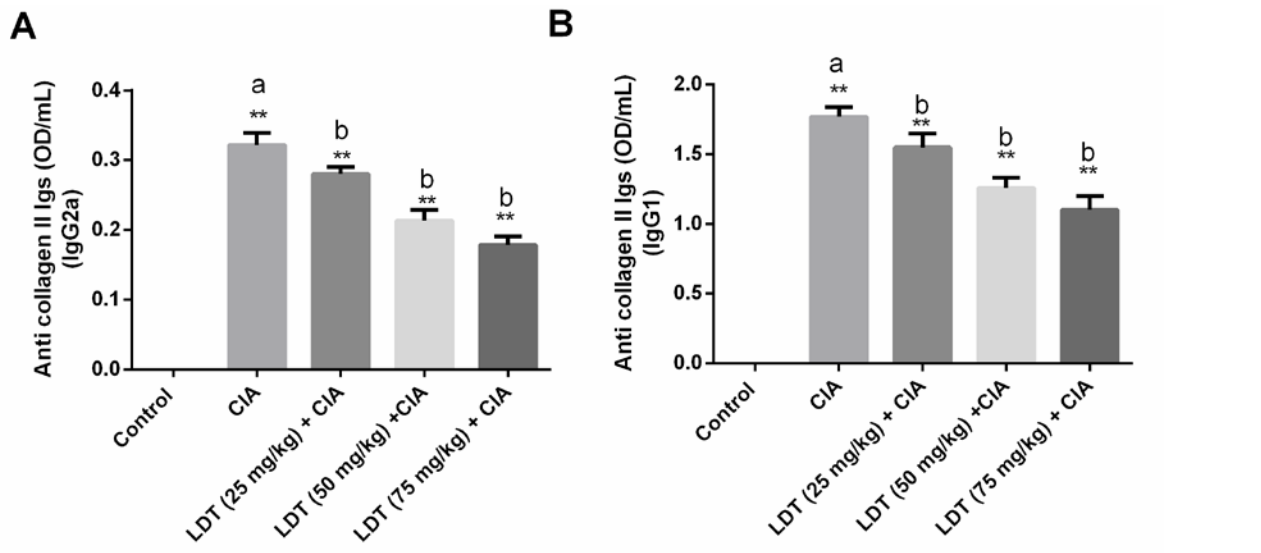

Figure 3. Effect of LDT on various serum anti-collagen II Ig levels in CIA rats and control. Data are represented as means ± SD. ** p<0.01, * p<0.05. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

LDT reduces serum pro-inflammatory cytokines

Standard ELISA was used to determine the effect of LDT on the serum level of various pro-inflammatory cytokines. As shown in Fig. 4, in comparison to control, the serum level of IL-1β, IL-6, IL-17, MCP-1, TNF-α was found significantly increased with low-level IL-10 in the CIA disease model group. On the contrary, LDT restored the level of these cytokines in comparison with the CIA disease group.

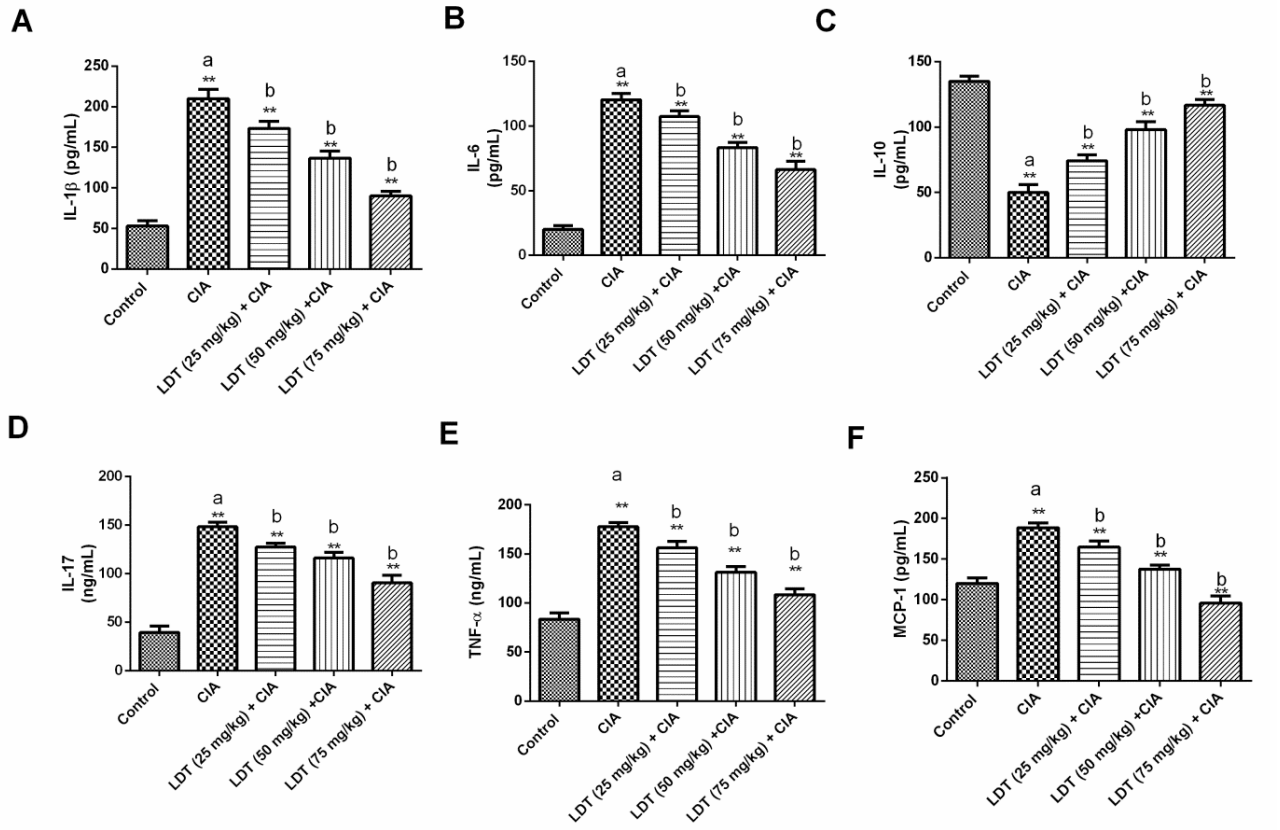

Figure 4. Effect of LDT on serum level of various pro-inflammatory cytokines in CIA and control rats. (A) IL-1β, (B) IL-6, (C) IL-10, (D) IL-17, (E) TNF-α and (F) MCP-1. Data are represented as means ± SD. ** p<0.01, * p<0.05. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

LDT reduces COX-2 and NF-κB in western blot analysis

Western blot analysis was conducted to determine the effect of LDT on the expression of COX-2 and NF-κB, an important inflammatory bio-marker. As shown in Fig. 5, LDT causes a significant reduction in the expression of COX-2 and NF-κB in rats as compared with the CIA disease model group.

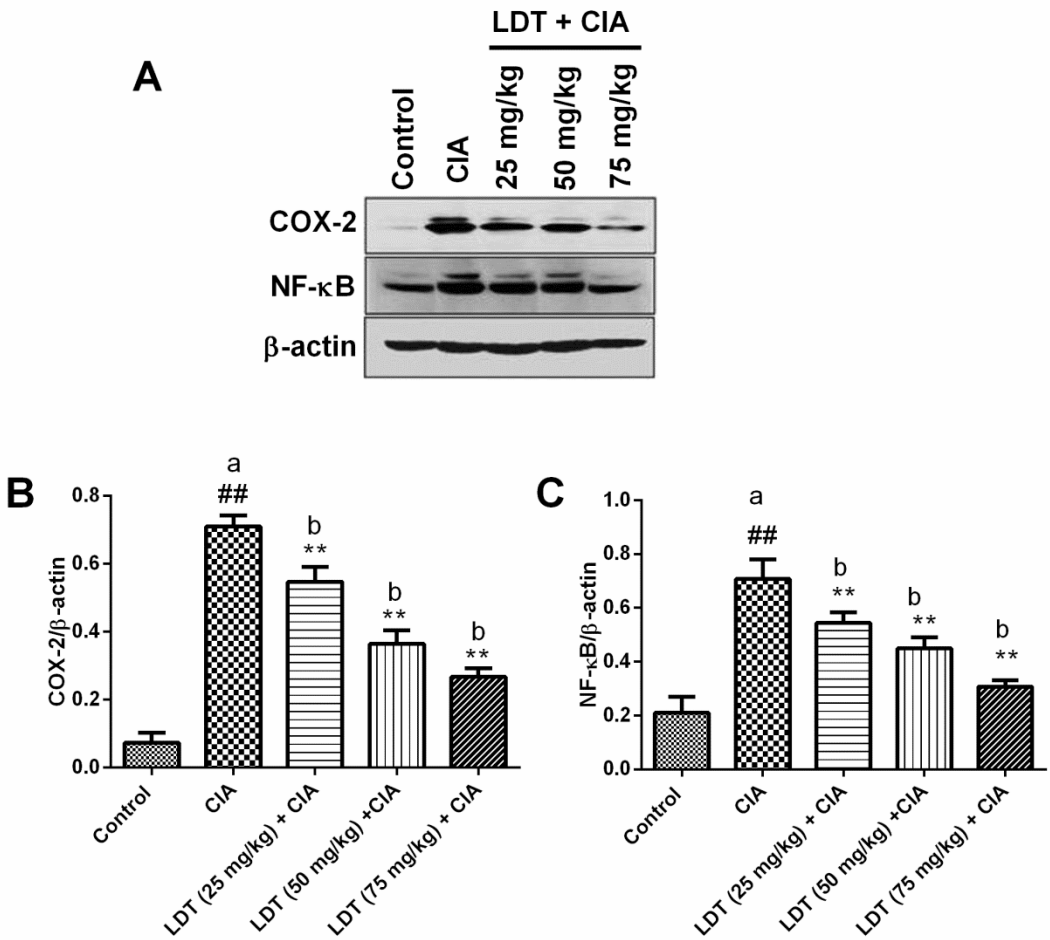

Figure 5. Effect of LDT on the (A) expression of COX-2 and NF-κB in western blot analysis, and representative bar-graph of (B) COX-2 and (D) NF-κB. Data are represented as means ± SD. ** $p<0.01$, * $p<0.05$. [a] CIA *vs*. control group, [b] LDT treatment group *vs*. CIA group

DISCUSSION

Since immemorial time, plants and their products have been used by mankind for treating their ailments and disease. These products have found significance in various traditional forms of medicines across the world, such as traditional Chinese medicine (TCM), Ayurveda, traditional Korean medicine (TKM), and other forms (Yuan et al. 2016). The holistic approach by these systems has garnered attention and as a result they have now blossomed into regulated systems of medicine (Efferth et al. 2007; Xu et al. 2013). In the past few decades, we have witnessed a significant rise in the research activities on the component of these medicines, which provides valid scientific evidence (Xue et al. 2013). Particularly, the medications used in TCM are often derived from hydro-alcoholic extracts of plants or combined extracts of the plant. Lidan Tang (LDT) decoction is a frequently used medical preparation used in TCM for the treatment of cholelithiasis (Gao et al. 2020). In the present manuscript, we have successfully shown the beneficial effect of LDT against adjuvant-induced (CIA) RA in rats. It has been found that LDT improved the pathological state of RA by reducing inflammation and oxidative stress in rats. Initially, the effect of LDT was investigated on the macroscopic features of RA in rats, such as paw swelling. Swelling is the common symptom of RA due to inflammation of the synovi-

al membrane and subsequent fluid build-up causing erosion and degradation of joint (Khurana and Berney 2005; Khanna et al. 2017). It has been found that LDT causes significant dose-dependent inhibition of paw swelling and associated arthritic score. This observation was further supported by histopathological examination of synovial joint tissues. Various studies have shown a close connection between inflammation and oxidative stress (Sies 1986; Reuter et al. 2010; Fernández-Sánchez et al. 2011; Mittal et al. 2014; Hussain et al. 2016). It has been found that the inflammatory condition of RA has been greatly provoked by the generation of reactive oxygen species (ROS). It undermines the oxidative defense mechanism of the body by inducing lipid peroxidation, mitochondrial and DNA damage (Birben et al. 2012; Schieber and Chandel 2014). Thus, agents reducing oxidative stress have shown a beneficial effect against RA. The effect of LDT was further investigated on various indices of enzymatic and non-enzymatic biomarkers of oxidative stress. It has been found that LDT reduces oxidative stress by the significant restoration of examined biomarkers near to normal. It has been suggested that reduction in oxidative stress by LDT prevents ligament damage and tissue erosion. The effect of LDT was further investigated on various pro-inflammatory cytokines. Accumulating shreds of evidence suggested that inflammation in RA provoked the release of various cytokines (IL-1β, IL-6, IL-10, IL-17, and TNF-α) from the immune cells (Feldmann 2002; McInnes and Schett 2007; Feldmann and Maini 2008). Therefore, inhibition of these overexpressed cytokines provides symptomatic relief against RA. In the present study, LDT significantly inhibited the release of tested cytokines in a dose-dependent manner. Moreover, LDT also causes a significant reduction of the serum level of anti-CII IgG, e.g. IgG1 and IgG2A, which is deemed as a characteristic hallmark of RA. Studies have shown that RA patients have a serum level of IgG in comparison to the normal population (Nandakumar et al. 2007; Khosroshahi et al. 2010). To understand the detailed mechanism of LDT, its effect was further quantified against pivotal mediators of inflammation cascade, NF-κB, and COX-2. Accumulating pieces of evidence suggested that NF-κB and COX-2 both mediates inflammation, hyperplasia, and tissue destruction in RA. It has been found that NF-κB activation promotes development of T helper 1 responses, activation, abnormal apoptosis and proliferation of RA fibroblast-like synovial cells, and differentiation and activation of bone resorbing activity of osteoclasts (van Loo and Beyaert 2011; Giridharan and Srinivasan 2018). Activated NF-κB has been detected in human synovial tissue on the early stage of joint inflammation, as well as in specimens obtained at the late stages of the disease. Analyses of nuclear extracts from synovial explants revealed the presence of increased NF-κB DNA binding activity in RA patients, but not in osteoarthritis patients. Immunohistochemical studies detected nuclear RelA (p65) and NF-κB1 (p50) mostly in RA endothelium and synovial lining, particularly in CD14-positive cells, and no staining in the normal synovium (Blom et al. 2014a, a, b; Ahmed et al. 2018). It is well established that NF-κB is involved in the regulation of multiple pro-inflammatory mechanisms. Activation of NF-κB is necessary and sufficient for the transcriptional activation of IL-1β, IL-6, IL-17, MCP-1, and TNF-α receptor activator of NF-κB ligand (RANKL), and cyclooxygenase 2 (COX-2), all of which are required for the initiation, amplification, and maintenance of chronic inflammations, including that seen in RA (Ghosh and Hayden 2008; Skaug et al. 2009; Sun 2012; Napetschnig and Wu 2013). Especially, TNF-α is critical in the pathogenesis of RA. In an inflammatory disorder, TNF-α is believed to play the role of early and crucial cytokine, which triggers various positive and negative feedback loops leading to exacerbated inflammation (Feldmann 2002; Palladino et al. 2003; Popa et al. 2007). Therefore, inhibition of NF-κB, cytokines and TNF-α expression is believed to inhibit RA. The activation of NF-κB also promotes upstream activation of COX-2 and responsible for controlling the transcription of this gene (Liang et al. 1999; Lee et al. 2004; Nishikori 2005; Ke et al. 2007; Ulivi et al. 2008). The results of the present study suggest that LDT causes significant inhibition of NF-κB and COX-2 and exhibit an ameliorative effect on inflammation in RA affected rats.

CONCLUSION

Collectively, our present study successfully demonstrated the anti-RA effect of LDT against CIA induced RA in rats. LDT causes a significant reduction in oxidative stress, inflammation which results in a reduction of paw volume and arthritic score and improves the pathological state of RA rats.

CONFLICT OF INTEREST

None

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Research article
Ερευνητικό άρθρο

The influence of single lavender essential oil in honey bee prevention of American foulbrood

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ABSTRACT: Recently, there has been an increasing demand for natural, healthy, and safe products without residual antibiotics for human consumption, particularly bee products. Beekeepers have been struggling with this problem many years, having in mind often occurrence of American foulbrood (AFB), which is one of the most severe honey bee brood diseases, and in the past have been successfully eradicated with heavy usage of antibiotics. Such controlled, or mostly uncontrolled usage of antibiotics in fighting against American foulbrood lead to a residual quantity of antibiotic in honey. To overcome this problem, this research aimed to investigate the influence of single essential oil (*Lavandula angustifolia*) in protecting bees against AFB compared to the antibiotic oxytetracycline. Totally three treatments were formed artificially infected with *P. larvae* spore suspension, at concentration 2×10^9 spore/ml. The course of the disease was regularly monitored. Treatment one (T1) did not receive antibiotic therapy. Treatment two (T2) was given lavender essential oil at a concentration of 0.1% of sugar syrup. The treatment was applied for 30 days, at 48h intervals. Treatment three (T3) received antibiotics in the sugar syrup at a concentration of 0.1%, respectively. Clinical and laboratory examinations were performed on days 10, 20, 40 and 60, respectively. Besides, *L. angustifolia* essential oil rich in Ethanol, 2- (2-ethoxyethoxy) - (13.05%), linalool (10.71%), α-Terpinyl acetate (10.93%) and linalool acetate (9.60%), showed its positive effects against antibiotics in combat of American foulbrood, further research with a specifically designed qualitative and quantitative mixture of essential oils are more than necessary because single essential oil is not enough and didn't show expected results.

Keywords: Essential oils, bees, honey, nutrition, medicinal plants, lavender, welfare

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INTRODUCTION

Honey, bee bread, bee venom, bee pollen, propolis, and royal jelly are the products produced by honey bee colonies which prolonging, sustaining, and retaining the health of their consumers (Easton-Calabria et al., 2019). Nowadays, it is seen an increased interest in bee products, both traditional and contemporary ones (Durand & Fournier, 2017; Ignjatijević et al., 2019; Jenkins, 2016; Živanović et al., 2019). At the moment, modern science performs investigations which have the aim to analyze and discover the exact mode of action and directed health benefits and pharmacological properties of bee products (López-Romero et al., 2018; Srivastava et al., 2019). Today, honey and other honey bee products are conceptualized as a functional food with the ability to promote better physiological or psychological health compared to traditional remediated and nutritional food (Ignjatijević et al., 2019; Kevan & Menzel, 2012; Prodanović et al., 2019).

The main obstacle in the increased production of healthy honey bee products with functional and beneficial properties for humans is diseases of honey bee colonies and usage of antibiotics, which lead to final products with a high concentration of residual antibiotics (de Graaf et al., 2013).

The most often disease which makes serious healthy and economic problems to bees and producers is American Foulbrood (Hansen & Brødsgaard, 1999). Even after more than a century of American Foulbrood research, this fatal brood infection is still among the most harmful bee diseases (Genersch, 2008). Its etiological agent is the Gram-positive, spore-forming bacterium *Paenibacillus larvae* (Genersch, 2007). Enormous progress has been made, especially in the last thirty years, in the understanding of the disease and of the underlying host-pathogen interactions (Yoshiyama & Kimura, 2009). As a severe disease that affects the larval stage of honey bees, antibiotics have been wildly used to control this disease (Antúnez et al., 2011). Twenty years ago, the only drugs utilized for this purpose were sodium sulfathiazole and oxytetracycline hydrochloride, the latter also employed for the treatment of European foulbrood (Forsgren, 2010; Thompson et al., 2005).

Honey is a complex product that has always been considered as natural and healthy food (Eteraf-Oskouei & Najafi, 2013). Substances that could be present in non-negligible quantities in honey may be authorized or prohibited veterinary substances, such as various drugs and environmental pollutants such as pesticides and heavy metals (Al-Waili et al., 2012; Bogdanov, 2006). Because of all this reason modern science is trying to find an ecologically safe way to treat diseases of food animals (Copping & Duke, 2007; Puvača et al., 2020), as well as honey bees with natural substances (Fuselli et al., 2006; Kuzyšinová et al., 2016) without any adverse effects on bees themselves as well to their products intended for human consumption, especially to children (Sinha, 1997; Tagboto & Townson, 2001).

In that way, naturally, existing plants such as *Achillea millefolium*, *Thymus vulgaris*, *Ocimum basilicum, Taraxacum officinale,* and commercial phytotherapeutic product such as Protofil® have been reported as a possible solution in combat of honey bee diseases (Cristina et al., 2020).

Worldwide, in the last decade, the allopathic drugs against various honey bees' diseases were restricted to a few active substances and, finally, to only a single synthesis fumidil, an antibiotic obtained from *Aspergillus fumigatus* and is permitted only in third countries and in Canada as well (Cristina et al., 2020; van den Heever et al., 2014). Unfortunately, although an efficient product, due to the risk of residues, EMA has excluded this product from use in Europe in February 2016 (Özkök & Akyol, 2017; Puvača et al., 2013; van den Heever et al., 2014). In the given circumstances in the disease's treatment the ecologic phytotherapy, with the usage of whole medicinal plants or their parts and essential oils rich in bioactive substances, with recognized antiprotozoal activity currently being viewed as a great opportunity (Carson & Riley, 1995; Puvača et al., 2019; Puvača et al., 2020).

Recent findings show that the use of essential oils such as lavender and cinnamon can improve the health of bee colonies (Nicoleta & Silvia, 2020). Essential oils include a series of antimicrobial, antibacterial, antifungal, antiparasitic compounds from plants that play a role in reducing bacterial resistance. Natural antibiotics based on essential oils can be alternatives to chemically synthesized antibiotics, as they do not contaminate the bee products (Nicoleta & Silvia, 2020). Research performed by several researchers (Sammataro et al., 2009), using dietary essential oils, in honey bee colonies feeding, recorded changes in the size of the bee population, improvement of the queen fertility. Essential oils used *in vitro* and *in vivo* have shown to be very useful in the protection of honey bee colonies from pathogenic bacteria (Ebert et al.,

2007). Corresponding to the experiments carried out by (Roussenova, 2011), essential oils derived from different plants can play an unconventional role in controlling honey bee diseases, without any drug residues in bee products (Nicoleta & Silvia, 2020).

Roussenova (2011) aimed to determine the *in vitro* activity of various essential oils to field strains (isolated from apiaries in Bulgaria), and a reference *Paenibacillus larvae* strain concerning their utilization as alternative means for prevention and control of American foulbrood without antibiotics. Essential oils from thyme, cloves, cinnamon, marjoram, tea tree, sage, peppermint, oregano, grapefruit, lemongrass, and mandarin, but no lavender essential oils were used in this research. Obtained results from investigation have shown that essential oils exhibited a strong inhibitory effect against all tested *P. larvae* strains (Roussenova, 2011). The usage of essential oils for control of bacterial, fungal and parasitic honey bee colonies diseases takes more than a few benefits over standard methods. In the literature, resistance of bacteria to essential oils has not been recorded in the last forty years from the early beginnings (Hitokoto et al., 1980). Besides, ecological ingredients in bee products decompose quickly, their amount in honey is low, and they do not have a harmful impact on the health of honey purchasers (Nozal et al., 2002; Tasić, 2018; Vapa-Tankosić et al., 2020). It has been proven when essential oil of lavender is used in honey bee nutrition during the summer or winter period, it also expresses analgesic, mood stabilizers, anticonvulsant, curative, neuroprotective, and carminative properties (Koulivand et al., 2013).

Giving the *in vitro* investigation on the antibacterial potential of lavender essential oil, it has been shown that it inhibits the growth of infected areas between 8 and 30 mm in size, with amounts of lavender oil between 1 and 20 µL (Roller et al., 2009).

Having in mind, this research aimed to investigate the influence of lavender (*Lavandula angustifolia*) essential oil in honey bee production protecting bees against AFB compared to the antibiotic oxytetracycline (OTC).

MATERIALS AND METHODS

Biological experiment with honey bees was performed following the EU legislation and principle of the Three Rs within Directive 2010/63/EU.

The experiment was performed in field conditions, in the hives outside, in the northern part of Serbia, the Autonomous Province of Vojvodina. The study included 15 honey bee colonies distributed in three treatments. Each honey bee colony consisted of about 1500 young worker bees and a young breeder queen. All treatments in the experiment were infected with *P. larvae* spore suspension, at concentration 2×10^9 spore/ml. The course of the disease was regularly monitored. Treatment one (T1) did not receive oxytetracycline therapy. Treatment two (T2) was given lavender essential oil at a concentration of 0.1% of sugar syrup. The therapy was applied for 30 days, at 24h intervals. Treatment three (T3) received oxytetracycline in the sugar syrup at a concentration of 0.1%, respectively. Clinical and laboratory examinations were performed on days 10, 20, 40, and 60. Experimental design with honey bees is presented in Table 1.

In this experiment honey bee colonies free from American foulbrood were chosen. Examination of the honey from the investigated hives revealed the absence of *P. larvae* spores. In purpose of establishing a high hygiene instinct in bees "Pin-test" was used (Facchini et al., 2019; Spivak & Downey, 1998). Artificial infection was performed using referent strains of *P. larvae* (5875 CAMP, Czech Republic). A particular nutritive culture medium J-agar was used, consisting of 20 g agar, 5 g tryptone, 15 g yeast extract, 3 g K_2HPO_4, 2 g glucose, and one liter of demineralized water. Media was used as a part of the suspension for bees.

Table 1. Experimental design with the application of essential oil and oxytetracycline in artificially infected honey bees with American Foulbrood

Parameter	Treatments		
	T1	T2	T3
Hives	5	5	5
Honey bee colony	1500 bees Young breeder queen	1500 bees Young breeder queen	1500 bees Young breeder queen
Sugar syrup and supplements	10 mL/day syrup	10 mL/day syrup 0.1% Lavender essential oil	10 mL/day syrup 0.1% Oxytetracycline

The microscopic examination method was used to observe the morphology of *P. larvae* to distinguish the American Foulbrood from other diseases. A preliminary check to the inspected diseased larvae in a direct way by using suitable spores stain such as 0.2% carbon fuchsin. About two drops of water were mixed with the larvae. The suspension was then transferred by loop and smeared on a glass slide as a thin film. The slide was then stained. After washing and drying the slide, it was examined under the microscope for *P. larvae* spores. A smear of suspected larvae colony was taken on a slide, Gram stained, the slide was flooded with crystal violet for 1 min, washed then flooded again with iodine solution for the same time, the decolorizing agent was used as ethanol for 5 sec, the final steps involved applying safranin, after each step the slide was rinsed with water for 5 sec. The prepared slide was then examined under the microscope. The bacterium was identified as *P. larvae* with gram positive rods 0.5-0.6 μm wide and 1.5-6 μm long (Nizar et al., 2015).

A bacteriological method such as bacterial cultivation and isolation to obtain pure cultures. Several media for cultivating *P. larvae* were examined previously to choose the appropriate one. The media used, such as sheep blood was approved by OIE (World Organisation for Animal Health, 2008) and described by other investigators as a good quality nurturing media for *P. larvae*. Ram blood agar with 8% defibrinated blood, nutritive medium with 2% NaCl, and nutritive medium with 5% NaCl, nutritive broth adjusted at 6.8 pH, was used in our research, respectively.

Antibiotic containing 55 mg oxytetracycline / 1 g of powder for oral application purchased from local veterinary medical supply was used in artificial honey bee infection.

Lavender (*Lavandula angustifolia*) essential oil analysis was performed by Gas chromatography (GC) and gas chromatography-mass spectrometric (GC-MS) on used thyme essential oil using an Agilent 7890A GC equipped with an inert 5975C XL EI/CI mass spectrometer detector (MSD) and flame ionization detector (FID) connected by capillary flow technology 2-way splitter with make-up. An HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was used. The GC oven temperature was programmed from 60 to 300 °C at a rate of 3 °C min^{-1} and held for 15 min. Helium was used as the carrier gas at 16.255 psi (constant pressure mode). An auto-injection system (Agilent 7683B Series Injector) was employed to inject 1 μL of the sample. The sample was analyzed in the splitless mode. The injector temperature was 300 °C and the detector temperature 300 °C. MS data were acquired in the EI mode with scan range 30-550 m/z, source temperature 230 °C, and quadrupole temperature 150 °C; the solvent delay was 3 min (Puvača et al., 2020).

RESULTS AND DISCUSSION

On the tenth day, microscopic examination showed the presence of vegetative rods of *P. larvae* in two colonies of the treatment T1. Clinical testing on day 20 confirmed symptoms of American foulbrood in the same treatment, with *patchy* comb appearance (Figure 2), and dark colorcappings (Figure 1) with brown colored larvae, elastic and sticky (Figure 3). Bacteriological examination revealed vegetative rods of *P. larvae* (Figure 4).

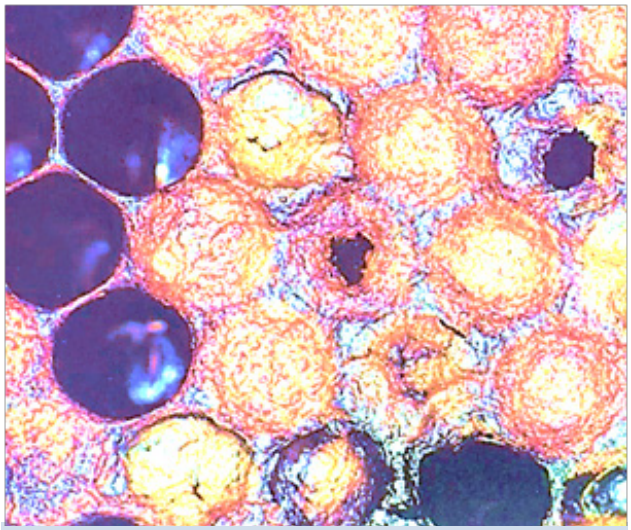

Figure 1. The characteristic appearance of the capping

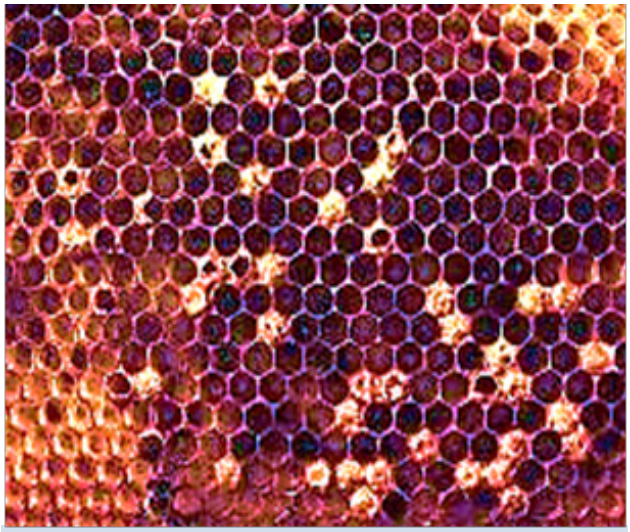

Figure 2. Patchy comb

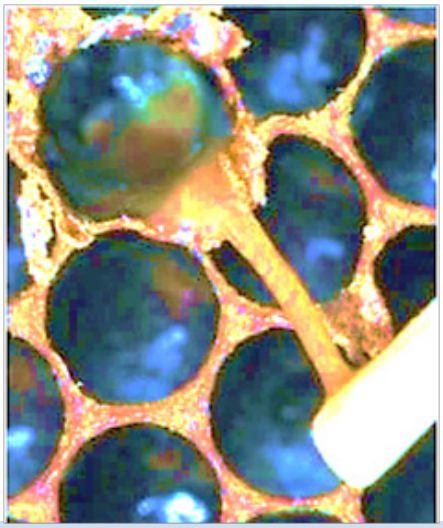

Figure 3. Rotten dead larva

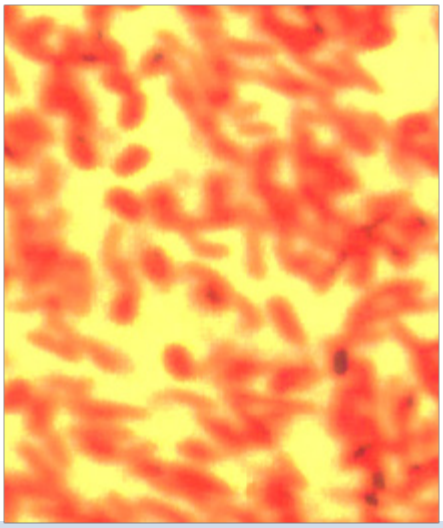

Figure 4. *P. larvae* (spores and rods)

In treatments T2 and T3 clinical signs of the disease were not expected; however, bacteriological analyses showed the presence of bacterial rods. Clinical and bacteriological analyses on day 40 showed the American foulbrood in all treatments. In the treatments, T1 and T2 two and three honey bee colonies were alive, respectively. In the treatment T3 with applications of oxytetracycline, all honey bee colonies were alive. On day 60, there were not left any alive honey bee colony in treatment T1, while only one alive colony was recorded in treatment T2 with the application of lavender essential oil, but with pronounced clinical signs of the disease. In treatment, T3 with the addition of oxytetracycline two alive colonies was seen with significant symptoms of the American Foulbrood and positive bacteriological findings (Table 2).

American foulbrood is a lethal disease of the honey bee brood and the infection often appears in closed broods. The susceptible larvae are essential for the infection, but also the development of the disease is dictated by the number of *P. larvae* spores. The number of spores varies depending on different factors. Sturtevant and Revell (Sturtevant & Revell, 1953) in the middle fifties induced artificial infection with American foulbrood with 5×10^7 spores of *P. larvae*/g honey, while forty years later was observed that feeding bee colonies with honey containing 2×10^9 spore/g necessarily caused the outbreak of the disease. Frequent feeding of larvae in the first weeks of life may introduce a large number of*P. larvae* spores into the larvae itself and cause an outbreak of disease. Some investigations have shown that only ten spores are enough for one-day-old larva, and millions of them are needed to induce the infection in 4 to 5-days-old larvae. Research of Plavša et al. (2011) indicated that in the artificial infection with *P. larvae* showed first clinical symptoms of American foulbrood and the first spores were detected after 25 days post-infection, while the honey bees treated with oxytetracycline in sugar syrup early signs of the disease have shown on 47 days post-infection. Khan et al. (2019) have highlighted that communicable diseases are not only the past but also the present problem in developing as well as developed countries.

The medicinal plants and nano-silver have been used against the pathogenic microbes. Herbal medicines are generally used for healthcare because they have a low price and a rich source of antimicrobial properties. Like medicinal plants, silver nanoparticles also have new applications in biomedical fields due to their inherent therapeutic performance. As in other species, as well in honey bees' medicinal plants and silver nanoparticles show their antiviral, bactericidal, and fungicidal properties. The applications of medicinal plants against honey bee pathogen such as fungi (*Ascosphaera apis*), mites (*Varroa* spp. and *Tropilaelaps* sp.), bacteria (*Melissococcus plutonius Paenibacillus larvae*), and microsporidia (*Nosema apis* and *Nosema ceranae*), could be a possible natural solution against synthetic drugs which have been used over the years disputes their harmful effect on bees and decrease quality and safety of honey. Wiese et al. (2018) have tested six major plant terpenes and their corresponding acetates, characterizing six natural *Thymus vulgaris* chemotypes, for their antimicrobial activity on bacteria associated with European

Table 2. Clinical signs of honey bee colonies with the application of essential oil and oxytetracycline artificially infected with American Foulbroo

Control periods	Treatments		
	T1	T2	T3
Day 10			
Descriptive control	Comb appearance without the changes	Comb appearance without the changes	Comb appearance without the changes
Bacteriological control	Two colonies positive on bacterial rods Three colonies positive on *P. larvae* spores	Negative	Negative
Day 20			
Descriptive control	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color	Compact and convex cappings, one colony cappings are in a dark color	Compact and convex cappings
Bacteriological control	Two colonies positive on bacterial rods Three colonies positive on *P. larvae* spores	Two colonies positive on bacterial rods One colony positive on *P. larvae* spores	Negative
Day 40			
Descriptive control	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color in three colonies
Bacteriological control	Two colonies positive on *P. larvae* spores	One colony positive on bacterial rods Three colonies positive on *P. larvae* spores	Two colonies positive on bacterial rods Three colonies positive on *P. larvae* spores
Day 60			
Descriptive control	-	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color	*Patchy* comb, seals in a dark color, larvae are elastic and in brown color in two colonies
Bacteriological control	-	One colony positive on *P. larvae* spores	Two colonies positive on *P. larvae* spores

foulbrood. The same group of authors (Wiese et al., 2018) has concluded that bee-forageable thyme product terpenes (mainly from pollen) yield effective oxytetracycline activity by reducing the growth of bee disease-associated bacteria and can be detected with different response levels by the honey bees' antennae. Kuzyšinová et al. (2016) have given insights into the use of antibiotic therapy in countries that permit this therapy is disputable regarding its low effectiveness, development of resistant bacterial strains, and residues in honey bee products. Because of all the harmful effects of antibiotics, alternative methods of prevention or therapy of American foulbrood have been considered. They are based mostly on substances of natural origin that neither adversely affect the honey bee products nor put some load on the environment. Such substances include for example probiotics, prebiotics, fatty acids, plant essential oils, and other plant materials. These substances are commonly used in the prevention or treatment of a whole range of diseases of farm and pet animals and have also recently been used in beekeeping. This "green" and healthy approach is especially important having in mind an interest in substances of natural origin which increasing constantly for many years and recently discovered are of great interest to the researchers. This interest also applies to bee products because of their extensive nutritional and therapeutic properties (Kieliszek et al., 2018).

The results are presented in Table 3, which reveals the most dominant subgroup of the phenolic compound of the investigated essential oil. The conducted analyses showed that *L. angustifolia* essential oil is the richest in Ethanol, 2- (2-ethoxyethoxy) - (13.05%). The results of our research emphasized that the lavender essential oil was also rich in linalool (10.71%), α-Terpinyl acetate (10.93%) and linalool acetate (9.60%). Ethanol, 2- (2-ethoxyethoxy) - (CH-

$_3CH_2OCH_2CH_2OCH_2CH_2OH$) is known as a solvent for dyes, nitrocellulose, paints, inks, and resins. It is a component of wood stains for wood, for setting the twist and conditioning yarns and cloth, in textile printing, textile soaps, lacquers, penetration enhancer in cosmetics, drying varnishes, and enamels, and brake fluids (Da Porto et al., 2009). It used to determine the saponification values of oils and as a neutral solvent for mineral oil-soap and mineral oil-sulfated oil mixtures. Linalool is a monoterpenoid that is octa-1, 6-diene substituted by methyl groups at positions 3 and 7 and a hydroxy group at position 3. It has been isolated from plants like *Ocimum canum*. It has a role as a plant metabolite, a volatile oil component, an antimicrobial agent, and a fragrance (González-Rivera et al., 2016). It is tertiary alcohol and a monoterpenoid. While α-Terpinyl acetate is found in cardamom and it is often used as a flavoring agent.

Several findings were introduced, testing the ability of different essential oils to inhibit the growth of *P. larvae* (Kuzyšinová et al., 2016). The highest activity against *P. larvae* in essential oils of lemongrass, thyme, and chamomile were noticed. Like our study with lavender essential oil, peppermint and Andean thyme showed inhibitory activity against the causative agent of American foulbrood (Fuselli et al., 2006), respectively.

Table 3. Identified phenolic compounds of the Lavender (***Lavandula angustifolia***) essential oil, %

Compound	RT [2]	RI [3]	*L.angustifolia* Composition ± SD
α-Thujene	5.632	923	0.05±0.00
α-Pinene	5.816	930	0.72±0.01
Camphene	6.229	944	0.25±0.00
Sabinene	6.928	970	0.12±0.01
β-Pinene	7.033	974	0.60±0.02
Myrcene	7.428	988	0.56±0.01
Ethanol, 2- (2-ethoxyethoxy) -	7.863	1003	13.05±0.04
Hexyl acetate	8.146	1011	0.13±0.00
α-Terpinene	8.297	1015	0.41±0.01
p-Cymene	8.570	1022	0.87±0.00
Limonene	8.713	1026	2.23±0.06
1, 8-Cineole	8.805	1029	5.55±0.03
β- (Z) -Ocimene	9.033	1035	0.06±0.00
γ-Terpinene	9.828	1056	0.05±0.00
Terpinolene	10.985	1088	0.04±0.01
Linalool	11.405	1110	10.71±0.01
Camphor	13.267	1143	3.72±0.00
Isoborneol	13.787	1154	1.04±0.02
Borneol	14.173	1163	0.46±0.02
Isononyl acetate	14.530	1171	3.45±0.00
Terpinen-4-ol	14.696	1175	0.90±0.01
α-Terpineol	15.577	1188	2.00±0.03
Citronellol	16.923	1226	2.50±0.04
Geraniol	18.110	1254	1.28±0.01
Linalool acetate	18.194	1255	9.60±0.02
Bornyl acetate	19.562	1285	0.21±0.00
α-Terpinyl acetate	22.374	1349	10.93±0.04
Neryl acetate	23.038	1364	0.44±0.00
Geranyl acetate	23.898	1364	0.80±0.00
Caryophyllene (E-)	25.443	1420	1.80±0.01
NI [1]			25.10
Total peak area			98030240

[1] Not identified; [2] Retention time; [3] Experimental retention indices based on n-alkane series under identical experimental conditions and comparison was done with the mass spectra library search NIST; SD—standard deviation calculated for n (n = 3) gas chromatography-mass spectrometric (GC-MS) analysis.

In most cases usually, the essential oils from oregano, thyme, and clove with strong inhibition of *P. larvae*, or essential oils from chamomile, rosemary and fennel with weak antibacterial activity were investigated while the investigation with essential oil of lavender in inhibition of *P. larvae*, performed just a few (Laird & Phillips, 2012).

It was recorded that among the very useful extracts of medicinal plants belongs those of Indian lilac (*Melia azadirachta*) with MIC equal to 10-800 µg/ml, Ceylon cinnamon with MIC 25-100 µg/ml and lemon grass with MIC 50-100 µg/ml (Ács et al., 2018).

Even when investigating the inhibitory activity of essential oils against pathogens, one must not forget to consider their potential toxic effect on honey bees. Many investigations have shown that essential oils can be used in honey bees because their toxicity to them is none or minimal. Large number of essential oils inhibit the growth of *P. larvae* and also presents LD50 values of the respective extracts for honey bees. Still, for example, peppermint oil as entirely nontoxic and LD50 values of thymol reached 100 mg/Kg, of cinnamon oil 50 mg/Kg and clove oil 200 mg/Kg (Albo et al., 2003).

CONCLUSIONS

Based on the obtained results, lavender essential oil rich in Ethanol, 2- (2-ethoxyethoxy) -, linalool, α-Terpinyl acetate, and linalool acetate, showed its positive effects in this experiment, indicating that lavender essential oil can be useful for the prevention or slow down the course of the disease (comparing with control group T1). Still, it is not enough for the healing process of American foulbrood. Further research with a specially designed qualitative and quantitative mixture of essential oils is more than necessary because single essential oil is not enough and didn't show expected results. Consumers search for the highest quality products, preferably with health benefits, rich in vitamins, valuable bio elements, and nutrients. Therefore, honey that is rich in beneficial ingredients has proved to fulfill these expectations, but further research in fighting the bee's diseases without any adverse effect on honey bees' products is essential very soon.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Ketamine or propofol anesthesia in dogs: how do they affect cytokines, antioxidants and neutrophil functions?

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ABSTRACT: The objective of the study is to investigate the effects of ketamine and propofol on cytokines, antioxidant defense system, and neutrophil functions in dogs. A total of 24 dogs were used. Dogs were divided into two groups as ketamine and propofol. The ketamine group received ketamine (5 mg/kg) intravenously while the propofol group received propofol (4 mg/kg) intravenously.Blood samples were collected before sedation and 30 minutes after induction of anesthesia.Serum antioxidant and cytokine levels were analyzed and neutrophil functions were determined. Respiration rate, serum malondialdehyde, IL-4, IL-6 levels, and phagocytic and chemotaxic activity of neutrophils were decreased (P=0.001, P=0.010, P=0.014, P=0.039, P=0.008, and P=0.037, respectively), oxygen saturation were increased (P=0.025) in the ketamine group. Serum IL-6 and IFN-γ level were decreased (P=0.015 and P=0.032 respectively), chemotactic activity of neutrophils were increased (P=0.049) in propofol group. The administration of ketamine was found to have a positive effect both on the antioxidant system and the neutrophil. On the other hand, positive and negative effects of propofol on different parts of the immune system were observed. Therefore, the results should be taken into account when designing an anesthesia protocol for dogs to predict possible defense system reactions during the postoperative period.

***Keywords*:** Dog, anesthesia, cytokine, antioxidant, neutrophil activation

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INTRODUCTION

Today, it is not enough for anesthetic drugs to provide only an effective anesthesia. In addition, evaluation of the potential effects on the immune system, defense mechanisms and cytokines is important to minimize possible postoperative complications (Soneja et al., 2005). Ketamine, which is frequently used in veterinary practice, is a dissociative anesthetic and is preferred due to its non-suppressive effects on the cardiorespiratory system in clinical doses (Muir, 2010). Propofol is commonly used for induction and maintenance of anesthesia (Jungheinrich et al., 2002).

Antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) play a protective role against oxidative stress (Szczubial et al., 2015). Ketamine exhibits antioxidant features in experimental studies in animals (Wang et al., 2019). However, the reported cases are quite limited in dogs (Camkerten et al., 2016). Propofol, which is structurally similar to antioxidants, is reported to be protective against oxidative stress by increasing SOD, CAT and GSH-Px activities in dogs (DeLa Cruzet al., 1999; Lee and Kim, 2012).

Cytokines are small proteins involved in generation of an immune response and inflammatory reactions. Anesthetics might have had an impact on cytokine production and proinflammatory-antiinflammatory cytokine balance (Sheeran and Hall, 1997). Human research revealed the suppressive effects of ketamine (Lisowska et al., 2013) and propofol (Chen et al., 2005) on proinflammatory cytokines. However, studies in dogs are very limited. Therefore, the possible effects of anesthetics on cytokines are considered worthy of investigation.

Neutrophils, phagocytic cells, are the first line of cellular defence against pathogens. It is not sufficient to merely estimate the count of neutrophils in the peripheral circulation to evaluate their effectiveness. Therefore, their phagocytic and chemotactic activities and as well as oxidative burst should also be explored because the involved mechanisms play an important role in generating an immune response by sending signals to other cells of the defence system (Dinauer, 2007; Van Kessel et al., 2014). Anesthetics agents are known to affect the circulatory neutrophil count and chemotaxis (Morisaki et al., 1998) but their effects on neutrophils are not limited to the relevant events. Propofol and ketamine were indicated to suppress phagocytic activity, oxidative burst, and chemotactic activity in humans (Nishinaet al., 1998; Cruz et al., 2017). Particularly, propofol inhibited superoxide production by neutrophils in dogs (Sato et al., 2016), while inhibition of phagocytic response by polymorphonuclear leukocytes for ketamine was shown in vitro animal studies (Son et al., 2009), but no clinical study was referred.

Ketamine and propofol affect the body's defence and antioxidant systems. Although cytokines play a substantial role in the relevant interactions, this mechanism has not been thoroughly investigated, considering species-related differences in animals. The aim of this study is to investigate the effects of ketamine and propofol on cytokines, antioxidants and neutrophil functions just 30 minutes after their administration for anesthesia induction in dogs premedicate with alfa 2- adrenergic agonists. It was hypothesized that ketamine or propofol, used as anesthesia agents in dogs, will affect inflammatory response, antioxidant defence mechanism and neutrophils in a different way. This was thought to have important effects on the patient's recovery from anesthesia period.

MATERIALS AND METHODS

The study was approved by the Istanbul University Experimental Animals Local Ethics Committee (no. 35980450-050.01.04) with informed owner consent.

Animals

A total of 24 healthy, male dogs aged 1to6 years old, ASA I and II, were enrolled in the study (Table 1). The mean body weight (± standard deviation) was 22.17 ± 13.02 kg. Physical examination, complete blood count, and biochemical parameters were applied on each animal. Food and water intake were withheld for 8 hours and 1 hour, respectively, before anesthesia.

Experimental design

The article was prepared according to the guidelines proposed by the CONSORT (Moheret al., 2010) (Figure 1). The dogs were randomly allocated into two groups (n = 12) "ketamine" or "propofol" group. A catheter was inserted into the cephalic vein for intravenous injections. For premedication, xylazine (ROMPUN, BAYER) at 0.5 mg/kg was administered intravenously (IV) to all dogs. Five minutes after premedication, dogs in the ketamine group received ketamine (ALFAMINE, EGE-VET) at a dose of 5 mg/kg IV, while the animals in propofol group received 4 mg/kg propofol (PROPOFOL, FRESENIUS) IV. Endotracheal intubation was performed a cuffed tube

Table 1. The breed and age distribution of the ketamine and propofol groups and the reasons for the anesthesia of the cases

	Ketamine (n = 12)	Propofol (n = 12)
Breed		
Golden Retriever	2	1
English Cocker Spaniel	1	0
Cross Breed	3	2
Anatolian Sheepdog	1	1
English Setter	1	2
French Bulldog	1	1
German Shepherd Dog	1	0
Chow Chow	1	1
Rottweiler	1	0
Terrier	0	2
Pug	0	1
Jack Russell Terrier	0	1
Age (years)		
1	4	4
2	1	0
3	3	2
4	3	3
5	0	0
6	1	3
Reasons for anesthesia		
Osteosynthesis	7	8
Soft tissue surgery	5	4

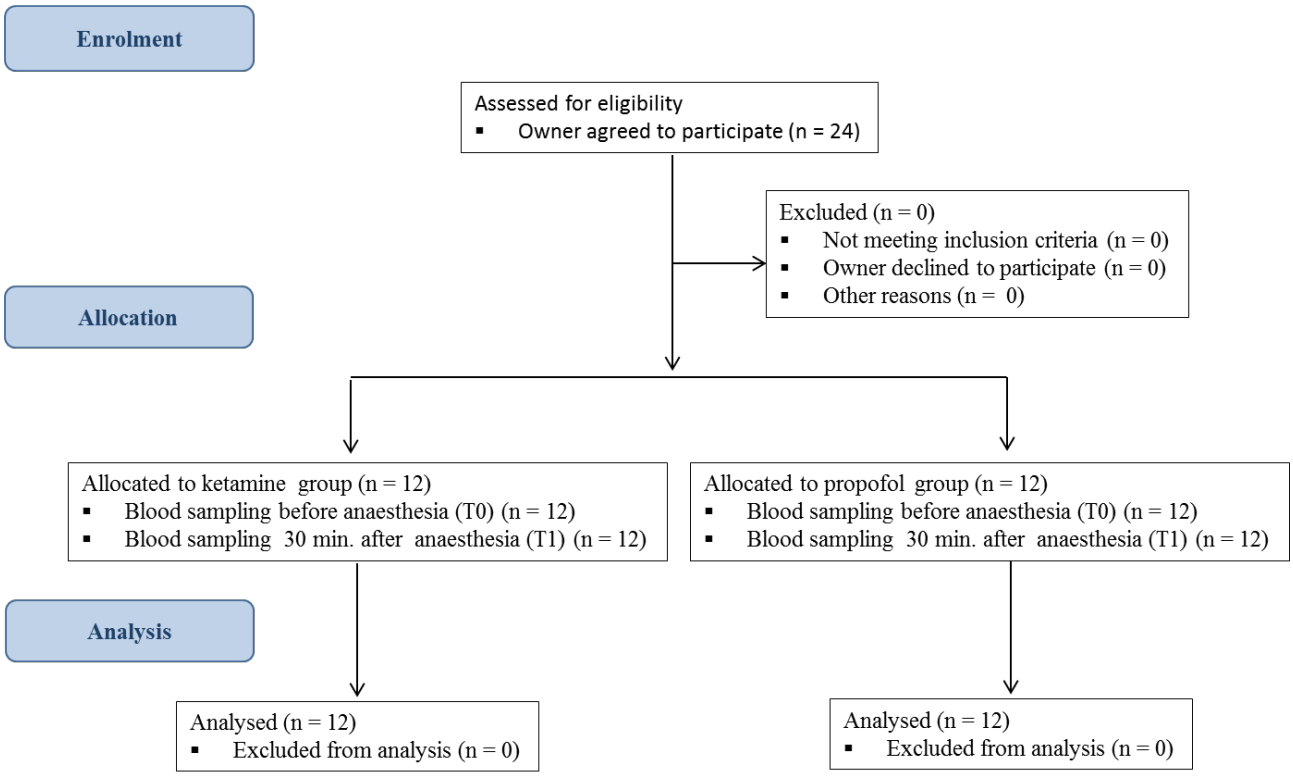

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) flow diagram

(WILLY RÜSCH) and anesthesia was maintained with isoflurane at a concentration of 2% (vaporizer setting) in 100% oxygen in spontaneous breathing. All dogs received Lactate Ringer's solution (10 ml/kg/hr, IV) during anesthesia.

Monitoring of cardiorespiratory parameters

Heart rate (HR), respiration rate (RR), end-tidal carbon dioxide ($ETCO_2$), hemoglobin oxygen saturation (SpO_2) and rectal temperatures (RT) of all cases were monitored throughout the anesthesia by a multifunctional ECG monitor (Advisor V9212 AR, SURGIVET). Data were recorded every 5 minutes, before sedation (T0) and at 30 minutes after anesthesia induction (T1) and before the surgical incision were assessed.

Blood sampling

Blood samples, which were taken from jugular vein at times T0 and T1, were collected into both serum and heparinized tubes, each containing 3 ml of blood. Heparin-containing whole blood samples were used to assess neutrophil functions while anticoagulant free blood specimens were centrifuged at 3000 g to obtain serum samples. The samples were stored at -80 ºC for further analysis.

Analysis of oxidative stress parameters

Serum MDA level was measured in all cases to assess oxidative stress that ketamine and propofol anesthesia might have induced. Serum SOD, CAT, and GSH-Px levels were measured to determine the efficacy of the antioxidant defence system. Analyses of MDA and antioxidant enzymes were performed by the ELISA method using dog-specific commercial kits (ABBKINE, ABBKINE SCIENTIFIC) and following the manufacturer's instructions. Serum samples were used to detect changes in the antioxidant defence system.

Plasma thiobarbituric acid reactive substances (TBARS) were estimated according to the method of Yoshoiko et al. (1979). The assay was based on the reaction of two molecules of thiobarbituric acid with one molecule malondialdehyde. This formed a coloured complex with a maximum absorbance at 532 nm. Plasma Cu-Zn superoxide dismutase SOD activity was determined according to method of Sun et al. (1988) by inhibition of nitroblue tetrazolium (NBT) reduction with xanthine-xanthine oxidase used as a superoxide generator. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. CAT activity was determined by modified method described by Yasmineh et al. (1995). The assay was based on the decomposition of H_2O_2 in buffer by catalase enzyme in the plasma. GSH-Px activity was measured using spectrophotometrickits in accordance with the manufacturer's instructions. This method is based on that of Paglia and Valentine (1967).Glutathione Peroxidase (GPX) catalyses the oxidation of Glutathione (GSH) by Cumene Hydroperoxide. In the presence of Glutathione Reductase (GR) and NADPH the oxidised Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. The decrease in absorbance at 340 nm is measured.

Assessment of cytokine level

Interlukin-2, IL-6, IL-8, IFN-γ levels, and IL-4, IL-10 ve TGF-β levels were measured to assess the possible effects of anesthesia on pro-inflammatory and anti-inflammatory cytokines, respectively, by the ELISA method using dog-specific commercial kits (ABBKINE, ABBKINE SCIENTIFIC) according to the manufacturer's instructions. Serum samples were used to detect cytokine levels.

Isolation of neutrophils and assessment of neutrophil functions

Three millilitres heparinized blood was layered carefully onto same amount of the Histopaque density gradient 1.5 ml Histopaque-1077 was layered onto 1.5 ml Histopaque-1119, (HISTOPAQUE, SIGMA-ALDRICH). The samples were then centrifuged at 340 g for 30 minutes at room temperature. After centrifugation the supernatant was discarded, and neutrophil containing Histopaque-1119 band was collected carefully by Pasteur pipet and transferred to a conical tube. Obtained cells were washed with 6 ml Hank's balanced salt solution (HBSS) three times (10 minutes at room temperature, 300 g) (Strasseret al., 1998). The cell pellets were re-suspended in 1 ml PBS and investigated for cell viability and measurement of neutrophil function. Cell numbers were adjusted to 1×10^6 cell/ml. Cell viability was determined by flow cytometer using rhodamine-123 (Robinsonet al., 1997).

To assess phagocytic activity, oxidative burst, and chemotactic activity, 20 µl of cell suspension was pipetted into 3 tubes that were added 1 ml PBS, 10 µldihydrorhodamine-123 (DHR-123, SIGMA-ALDRICH) and incubated in a 37 ºC water bath for 5 minutes. After incubation, 20 µl of E. coli solution (5

x 10^9 E. coli/ml), 10 µl (20 mg/ml DMSO) phorbol 12-myristate 13-acetate (PMA, SIGMA-ALDRICH), and 10 µl (4 mg/ml DMSO) N-Formylmethionyl-leucyl-phenylalanine (fMLP, SIGMA-ALDRICH) were added to the first, second and the third tube, respectively, to provoke phagocytic activity, oxidative burst, and chemotactic activity. After the stimulants were added (minute 0), the cell suspension was analyzed in flow cytometry. Mean fluorescence intensity was measured using the FL1 detector. After the first measurement, the cell suspension was incubated for 20 minutes in the water bath (37 ºC) and afterward, the mean fluorescence intensity was measured again. Phagocytic activity, oxidative burst, and chemotactic activity was calculated as the ratio of MFI value at 20 minutes. MFI-1 value at 0. minute (Bilgic et al., 2008). Flow cytometric analyses were performed on a flow cytometry equipped with cell quest software (FACS calibur, BD BIOSCIENCES).

Statistics

All statistical analysis were performed with IBM SPSS Statistics Version 21 program (SPSS Inc., CHICAGO, ILLINOIS). First, Shapiro-Wilk test was used to check whether the data obtained was normally distributed. The presence of a difference between the data obtained at T0 and T1 was analysed using paired samples t-test for normally distributed data. Non-parametric Wilcoxon signed rank test was used when the normality assumption was violated. The results are expressed as means ± standard error of the mean (SEM) for parametric variables and median with interquartile range (IQR) for nonparametric variables. Significance was established at $p < 0.05$ level.

RESULTS

The effects of ketamine or propofol on certain physiological parameters are shown in Table 2. No significant change was recorded in the ketamine group concerning HR and RT (p=0.352 and p=0.711) compared to the values before anesthesia. However, RR was significantly decreased (p=0.001) while $SpO_2$was increased (p=0.025) at T1. No significant change was noted in the dogs that received propofol regarding HR, SpO_2, and RT (p=0.069, p=0.243, and p=0206, respectively) while RR was significantly decreased (p=0.012) at T1 compared to before anesthesia. Mean values of $ETCO_2$ levels that were measured after anaesthesia, were43.1 ± 7.02 and 41.3 ± 9.7 in ketamine and propofol groups, respectively.

The effects of ketamine or propofol on the antioxidant defense system are presented in Table 3.It was found that serum MDA level decreased significantly 30 minutes after ketamine administration, but there was no significant change in serum CAT, SOD and GSH-Px levels (p=0.540, p=0.689, and p=0.656, respectively). On the other hand, there was no significant change in serum MDA (p=0.273) or SOD, CAT, and GSH-Px levels (p=0.699, p=0.278, and p=0.255) in dogs anesthetized with propofol.

Table 2. Heart rate (HR, beats per min), respiratory rate (RR, breaths per min), heamoglobin oxygen saturation (% SpO2) and rectal temperature (RT, °C) measured before sedation (T0) and 30 minute after anesthesia induction (T1) in dogs anesthetized with ketamine or propofol

		HR (bpm)			RR (bpm)			SpO_2 (%)		RT (°C)	
		Mean	±	SEM	Mean	±	SEM	Median	(IQR)	Median	(IQR)
Ketamine	T0	107.0	±	8.55	81.71	±	7.13	90	(89-92)	38.7	(38.3- 39.1)
	T1	89.8	±	12.93	10.21	±	2.89	94	(93-95)	39.0	(38.1-39.5)
	P values	0.352			0.001			0.025		0.711	
		Median		(IQR)	Median		(IQR)	Mean	± SEM	Mean	± SEM
Propofol	T0	115.0		(100-128)	55		(24-120)	92.5	± 0.98	38.9	± 0.11
	T1	85.5		(72-129)	12		(10-16)	94.1	± 0.82	38.7	± 0.17
	P values	0.069			0.012			0.243		0.206	

T0 = before sedation, T1 = 30 minutes after anesthesia induction. SEM= Standard error of mean, IQR = Interquartile range. Normally distributed data were presented as mean ± SEM, non-normally distributed data were presented as median and interquartile range (IQR). n = 12 in ketamine and propofol groups.

Table 3. Serum malondialdehide (MDA), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels measured before sedation (T0) and 30 minute after anesthesia induction (T1) in dogs anesthetized with ketamine or propofol

		MDA (mmol/ml)			SOD (ng/ml)		CAT (ng/ml)			GSH-Px (ng/ml)		
		Mean		SEM	Median	(IQR)	Mean		SEM	Mean		SEM
Ketamine	T0	6.06	±	0.61	5.66	(5.2-6.3)	53.7	±	3.93	49.15	±	4.44
	T1	5.35	±	0.41	491	(4.4-6.1)	52.4	±	3.32	46.19	±	5.76
	P values	0.010			0.540		0.689			0.656		
		Mean		SEM	Median	(IQR)	Median		(IQR)	Mean		SEM
Propofol	T0	5.02	±	0.33	6.8	(5.5- 7.2)	59.23		(55.5-104.3)	52.46	±	5.65
	T1	5.60	±	0.38	6.3	(5.3-10.8)	65.29		(59.0-92.8)	48.37	±	4.45
	P values	0.273			0.699		0.278			0.225		

MDA = malondialdehide, CAT = catalase, SOD = superoxide dismutase, and GSH-Px = glutathione peroxidase, T0 = before sedation, T1 = 30 minutes after anesthesia induction. Normally distributed data were presented as mean ± SEM, non-normally distributed data were presented as median and interquartile range (IQR). n = 12 in ketamine and propofol groups.

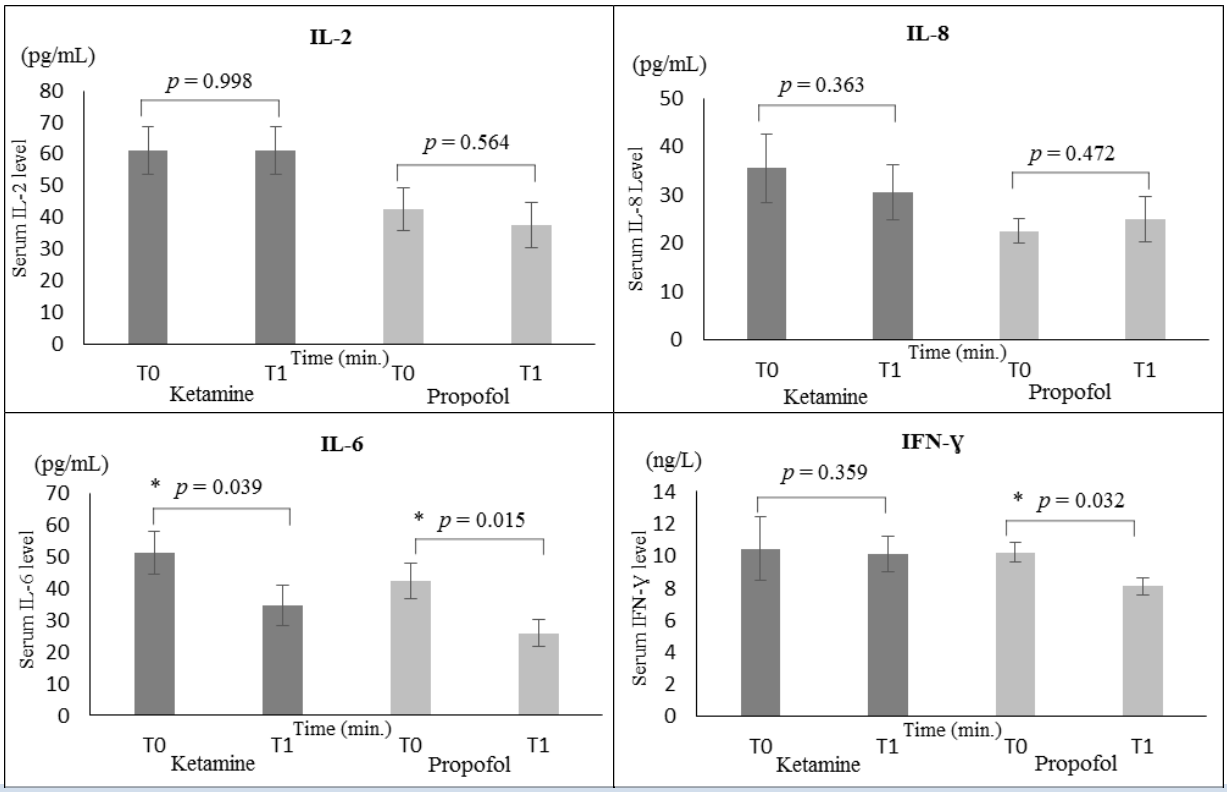

Figure 2. Mean values of interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8) and interferon gamma (IFN-γ) of dogs in ketamine and propofol groups; before sedation (T0) and 30 minutes after anesthesia induction (T1). The error bars show the standard error of mean (SEM). * Indicates that the difference between T0 and T1 is significant. n = 12 in ketamine and propofol groups

Findings regarding pro-inflammatory cytokines are given in Figure 2. Thirty minutes after ketamine administration, serum IL-6 levels were significantly decreased compared to the value before anesthesia (p=0.039), but serum IL-2, IL-8 and IFN-γ levels did not change (p=0.988, p=0.363, and p=0.359, respectively). Propofol had no significant effect on serum IL-2 and IL-8 levels (p=0.564 and p=0.472, respectively) while IL-6 and IFN-γ levels were decreased (p=0.015 and p=0.032, respectively) compared to them before anesthesia.

Data regarding anti-inflammatory cytokines are shown in Figure 3. Serum IL-4 level in the dogs that received ketamine was significantly decreased (p=0.014) However, IL-10 and TGF-β remained un-

changed compared to the values before anesthesia (p=0.833 and p=0.688, respectively). In the propofol group, serum IL-4, IL-10, and TGF-β levels were not significantly affected compared to the values before anesthesia (p=0.432, p=0.470, and p=0.358, respectively).

The effects of ketamine or propofol on neutrophil functions were presented in Figure 4. It was determined that the phagocytic and chemotaxic activity of neutrophils decreased 30 minutes after ketamine administration (p=0.008 and p=0.037, respectively), however, the oxidative burst was not affected (p=0.419) at T1. Propofol did not alter the phagocytic activity and the oxidative burst (p =0.676 and p=0.904, respectively), but the chemotactic activity was increased (p=0.049) at T1.

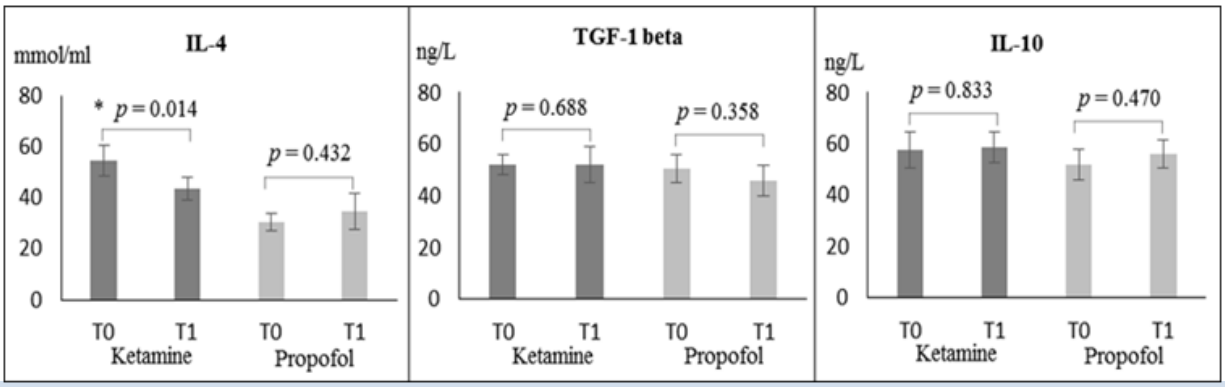

Figure 3. Mean values of interleukin-4 (IL-4), transforming growth factor-1 beta (TGF-1 beta) and interleukin-10 (IL-10) of dogs in ketamine and propofol groups; before sedation (T0) and 30 minutes after anesthesia induction (T1). The error bars show the standard error of mean (SEM). * Indicates that the difference between T0 and T1 is significant. n = 12 in ketamine and propofol groups

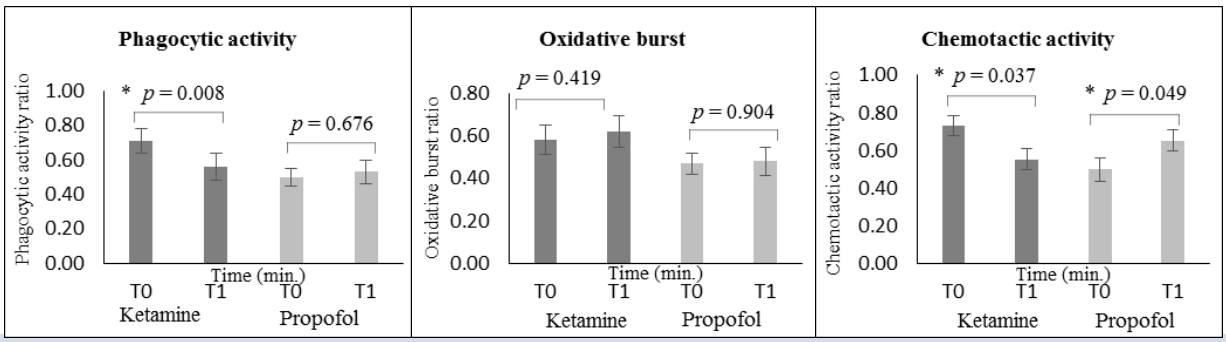

Figure 4. Mean values of phagocytic activity, oxidative burst, and chemotaxic activity of dogs in ketamine and propofol groups; before sedation (T0) and 30 minutes after anesthesia induction (T1). The error bars show the standard error of mean (SEM). * Indicates that the difference between T0 and T1 is significant. n = 12 in ketamine and propofol groups

DISCUSSION

The majority of anesthetics suppress the cardiorespiratory system by causing oxidative stress (Cruz et al., 2017). In this study, it was observed that anesthetics did not significantly affect HR but decreased RR (Table 2). Even though no apnoea occurred due to the initiation of anesthesia either with ketamine or propofol, it was considered that RR decreased with the effect of ongoing anesthesia. It is clear that respiratory suppression and a decrease in SpO_2 following administration of xylazine in premedication (Guzel et al., 2018). Therefore, changes in RR and SpO_2 following xylazine were not taken into consideration in this study. In the ketamine group, RR decreased but SpO_2 increased following 30 minutes after ketamine administration. This increase was that due to the dogs being ventilated with 100% oxygen after intubation. Despite the quantitative increase in SpO_2 with propofol at T1, this increasewas not found to be statistically significant, which is compatible with the references (McDonelland Kerr, 2007) indicating that propofol suppresses respiration as much as barbiturates whereas ketamine does not exert the same effect. In the study, $ETCO_2$value was found to be remained within the acceptable levels (McDonelland Kerr, 2007) in both groups. Hypothermia was not observed since the parameters were evaluated for a short duration (Guzel et al., 2018).

The most important defensive tasks are held by antioxidant enzymes such as SOD, CAT, and GSH-Px against the free radical injury in the body (Szczubialet al., 2015). Lupp et al. (1998) stated that ketamine suppresses the oxidative process by reducing lipid peroxidation, while Reinkeet al. (1998) reported that ketamine increased free radicals in serum. Kamiloglu et al. (2009) indicated that ketamine-xylazine anesthesia suppressed the free radical production and thus supported the antioxidant mechanisms. In this study, it was determined that MDA levels, which are the determinants of oxidative stress, decreased in the ketamine group, while SOD, CAT and GSH-Px levels did not change. This result showed that ketamine anesthesia reduced free radical formation and did not trigger oxidative stress. It was indicated that propofol increased GSH-Px level versus a decrease in MDA level (Akin et al., 2015).On the contrary, Tomsıč et al. (2018) stated that anesthesia with either propofol or sevoflurane did not affect the level of MDA, SOD and GSH-Px activities. In the study, propofol did not alter the levels of oxidative stress parameters such as SOD, CAT, GSH-Px, and MDA, which is compatible with the results of Tomsıč et al. (2018). It was evaluated that MDA levels in dogs receiving ketamine were found to be lower than those given propofol, and that ketamine anesthesia was more protective against oxidative stress.

Our data on cytokines shows that ketamine does not affect other proinflammatory cytokines studied except IL-6 (Figure 2). Previous studies have shown that IL-6 production, which is increased by using various stimuli (Yamaguchi et al., 2017) or as a result of surgical intervention (Welters et al., 2011), is suppressed by ketamine. It is possible to observe a similar effect in propofol. Thus, it is reported that propofol inhibits increased IL-6 biosynthesis as a result of lipopolysaccharide stimulation (Chen et al., 2005) or due to surgical intervention (Sayed et al., 2015). In this study, both ketamine and propofol evidentially decreased the IL-6 levels irrespective of either any cellular stimulation or the existence of surgical intervention (Figure 2). Normally, IL-6 is a pleiotropic cytokine that affects hepatocytes, megakaryocytes, neutrophils, T and B cells as well as synovial and dermal fibroblasts (Tanaka et al., 2014), which occurs as a result of the long-term exposure to IL-6 stimulation. In this study, it was considered more pertinent to assess the acute effects of IL-6 since it is a one-time application. In the early phases, IL-6 initiates the inflammatory reactions by increasing the expression of acute-phase proteins such as fibrinogen, c- reactive protein, and serum amyloid A or the adhesion molecules (ICAM-1, VCAM-1) (Barnes et al., 2011). Moreover, IL-6 causes apoptosis of neutrophils (McLoughlin et al., 2003). The suppression of IL-6 production by the anesthetics used in the study was found significant in terms of inhibition of possible inflammatory reactions and neutrophil apoptosis.

In this study, IFN- γ levels were significantly decreased in the propofol group unlike the ketamine group (Figure 2). It is highly unlikely to pronounce the precise cause of the relevant decrease in IFN- γ by propofol merely based on the available data. Nevertheless, T cells and NK cells were shown to be the major sources of IFN-γ (Ye et al., 1995). Supportively, it was reported that propofol inhibited T cell proliferation and activity by the aid of lymphocyte function-associated antigen-1 (LFA-1) (Yukiet al., 2011) and hence it was assumed that the decrease in IFN-γ might have been associated with the inhibition of T cells. However, it should be noted that the decrease in serum IFN-γ level might have been linked with the receptor status of IFN-γ since it was previously reported that propofol increased the expression of IFN-γ receptors on LPS stimulated whole blood cultures (Brand et al., 2001). An increase in IFN- γ receptors leads to the rapid elimination of IFN-γ from the circulation (Farrarand Schreiber, 1993). The decrease in serum IFN-γ after the induction of propofol occurred due to the depletion in secretion may be considered the downside of the protocol because IFN-γ undertakes critically important tasks such as the activation of macrophages, antigen presentation, upregulation of the non-specific immune response, and lymphocyte-endothelial cell interactions in the acute phases (Billiau, 1996). In the study, it was shown that propofol did not affect anti-inflammatory cytokines in dogs while ketamine decreased merely IL-4 levels (Figure 3). IL-4 is secreted from mast cells, Th2 cells, eosinophils, and basophils (Gadani et al., 2012). It was also reported that IL-4 adversely affected macrophages and dendritic cells (Hershey et al., 1997) and concordantly a decrease in IL-4 level with ketamine may be considered an affirmative effect because macrophages and dendritic cells are the most important antigen-presenting cells in the body. These cells are the link between the specific and the non-specific defence systems. One of the important properties of IL-4 is its capability to reduce Th1 cells (Hershey et al., 1997). Therefore, the decrease in IL-4 in the ketamine group was deduced to be a positive outcome since the de-

creased IL-4 secretion with ketamine did not exert a negative impact on Th1 cells in dogs, which was supported by the fact that ketamine did not decrease the levels of IFN-γ that is primarily produced by Th1 cells (Yang et al., 1999) and hence the IFN-γ levels were decreased in the propofol group unlike ketamine (Figure 2).

In vitro studies in humans (Helleret al., 1998) and dogs (Son et al., 2009) showedthat ketamine inhibited phagocytic and chemotactic activities of neutrophils. The inhibitory effect of ketamine on neutrophil functions has also been shown in vivo in the presented study, which raises the question of why ketamine reduces phagocytic and chemotactic activity. It is well known that mitochondrial ATP is required as the source of energy to maintain phagocytic and chemotactic activities (Son et al., 2009) and hence ketamine reduced the chemotactic activity by reducing mitochondrial membrane potential and thus ATP synthesis in previous in vitro studies (Chang et al., 2005). Therefore, it was considered that the reduction in phagocytic and chemotactic activities in the dogs that received ketamine in the study might have resulted from the suppression of the mitochondrial ATP production. Moreover, ketamine is known to inhibit pseudopod formation and elastase secretion in activated neutrophils (Craciun et al., 2013). Since pseudopod formation and elastase secretion play an important role in phagocytosis, the relevant concept was assumed to be one of the causes of the reduction in phagocytic activity of neutrophils in the study. Unlike ketamine, no reduction in neutrophil functions was detected in the dogs that received propofol. On the contrary, the chemotactic activity was increased (Figure 4). In vitro studies in humans demonstrated that propofol inhibited chemotactic activity and the other neutrophil functions through formyl peptide receptor-1 (FPR-1) (Yang et al., 2013). It was indicated that the relevant receptors are absent or are expressed in quite low quantities in dogs and some other animals (Linnekin et al., 1990). Therefore, it is little wonder that propofol did not inhibit chemotactic activity and vice versa in the dog, which was considered to be associated with a species-related diversity. However, there is no sufficient data available to precisely elucidate the underlying mechanisms.

CONCLUSIONS

In this clinical study, the anesthetic agents that were used had no effect on the cardiorespiratory system other than what was predicted.Thirty minutes after induction of anesthesia, ketamine prevented oxidative stress whereas propofol had no impact on the antioxidant defense system. The inhibition of IL-4 production by ketamine was considered to be a favorable outcome in regard to the defense system; however, the decreases in IL-6 levels, as well as the reduction in phagocytic and chemotactic capabilities of neutrophils were the adverse effects of ketamine. At the same time the suppression of IL-6 and IFN-γ production was an unfavorable effect of propofol on the defense system while the increase in the chemotactic activity of neutrophils was considered a positive aspect. In conclusion, it can be deduced that overall data obtained from the study should be noted while preparing anesthesia protocols particularly in terms of predicting possible postoperative reactions in the defense system. More research is needed in regards of the duration of the aforementioned results.

CONFLICT OF INTEREST STATEMENT

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Research article
Ερευνητικό άρθρο

The effect of the challenge with Newcastle disease virus on the gastrointestinal bacterial population in Japanese quail (*Coturnix japonica*)

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ABSTRACT: Newcastle disease (ND), caused by virulent strains of Newcastle disease virus (NDV), is a devastating disease of poultry worldwide. The effect of a challenge with NDV on bacterial population in quail is poorly documented, so for this purpose, a total of 100 day-old Japanese quail were purchased and divided into 2 equal groups randomly. Each group was divided into 2 subgroups. The birds in group A challenged with a velogenic chicken isolate of NDV. The birds in group B did not challenge with NDV as the control group. For the determination of *lactobacillus* counts in the intestine and crop of Japanese quail, at the end of the period, 10 birds of each subgroup were chosen randomly. One gram of the crop and ileocecal content were taken and cultured on MRS for determination of *lactobacillus* counts. The colony-forming units of *Escherichia coli* in digesta of ileocecal on Mac Conkey agar were investigated. The results of this study showed that the challenge with a velogenic chicken isolate of NDV could increase colony-forming units of *Escherichia coli* in group A compared to the control group. Also, it reduced lactobacillus counts of intestine and crop compared to the control group. So it concluded that velogenic chicken isolate of NDV influences microflora of intestine and crop of Japanese quail.

Keywords: birds, *Escherichia coli*, gut, *lactobacillus*, velogenic NDV.

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INTRODUCTION

The microflora in the gastrointestinal tract of birds plays an important role in growth performance, nutrition, detoxification of certain compounds, and protection against pathogenic bacteria (Roto et al., 2016; Varmuzova et al., 2016; Sikandar et al., 2020). There is an active and complex microbial community in the animal's gastrointestinal tract, which plays key roles in the immune system, nutrition absorption, pathogenesis, and further in the health and physiological functions of the host; thus, the use of intestinal microflora as probiotic has become one of the hot topics in the international research, especially the use of lactobacillus (Yadav and Jha, 2019). Some studies suggest that various strains of lactobacilli have a stimulating effect on antibody-mediated response in chicken and such effect is dependent on the strain of *Lactobacillus* used and the type (layer- or meat-type) and age of the chicken (Brisbin et al., 2011).However, it remains to be elucidated how probiotics enhance antibody-mediated immune response. It is speculated that probiotics can stimulate the production of Th2 cytokines (e.g., IL-4 and IL-10), which may subsequently enhance the immune response mediated by antibodies (Haghighi et al., 2005). Lactobacillus is one of the predominant bacteria in the animal's gastrointestinal tract (Li et al., 2020).Lactobacillus can produce abundant lactic and acetic acids to lower the pH value in the gastrointestinal tract (Zhao et al., 2021), or compete for the nutrients and epithelial adhesion sites with pathogens to inhibit the growth of pathogens or have the excellent properties of acid and bile salt tolerance as well as the power capacity of colonization and adhesion, and so on (Monteagudo-Mera et al., 2019). Lactobacilli used as probiotics are non-pathogenicgram-positive bacteria that live in the animal intestine. In the chicken, as well as the ability to limit food-borne pathogens and to improve production parameters, the administration of various members of the Lactobacillus species could stimulate multiple aspects of the immune response. These activities include improving systemic antibody response, increasing the number of intestinal epithelial lymphocytes (IELs) expressing CD3, CD4, CD8, and T cell receptors (TCR), modulation of chicken chemokine and cytokine gene expression, and improving the function of T cells (Talazadehet al., 2016).Several lactobacilli strains have been shown to decrease the population of *Salmonella*, *Campylobacter,* and some other non-beneficial bacterial groups in the chicken gut (Nakphaichit et al., 2011).Researchers also demonstrated that multispecies probiotics containing *L. salivarius*, and *L. reuteri* significantly reduced cecal colonization by *C. jejuni*, indicating that probiotic products can also be used to improve food safety by reducing the population of human pathogens, such as *C. jejuni*, in chicken (Ghareeb et al., 2012).Despite the interest in the administration of probiotics in commercial poultry production, to date, there is little information about the mechanisms of stimulation of chicken immune response by probiotic bacteria (Talazadeh et al., 2016). The diagnosis of lactobacilli is carried out by culture-dependent techniques (isolation and culture, microscopic examination, physiological and biochemical events) (Karami et al., 2017). Controlling pathogenic microorganisms and enhancing beneficial microorganisms in the digestive content of the gut is important. The intestinal microflora is relatively stable under common circumstances but is easily influenced by various diseases. Since bacteria may play a role in the disease process due to the modification of intestinal innate immunity by dysbiosis and translocation of bacteria, changes of normal microflora in the digestive tract are important sites in the susceptibility of birds to bacterial infection (Cui et al., 2018). ND is a lethal viral disease of poultry and is caused by specified viruses of the avian Paramyxovirus serotype 1 (PMV-1), the serotype of the genus *Rubulavirus*, belonging to the family *Paramyxoviridae.* There are nine serotypes of avian paramyxoviruses designated APMV-1 to APMV-9 (Talazadeh et al., 2016). There is a wide variation in the infectivity of the disease produced by NDV in birds. The pathogenicity of various NDV strains varies from apathogenic strains to velogenic highly virulent strains (Cattoli et al., 2011). The virulence of NDV strains varies greatly with the host, but breed or genetic stock does not appear to have a significant effect on the susceptibility of chickens to the disease (Talazadeh and Mayahi, 2013). NDV has also been categorized into five pathotypes based on clinical signs in infected chickens, including a) viscerotropic velogenic, b) neurotropic velogenic, c) mesogenic, d) lentogenic or respiratory and e) asymptomatic (Getabalew et al., 2019).Viscerotropic velogenic ND (VVND) has been reported in Brazil in ducks, pigeons, quail *(Coturnix),* turkeys, teal, and guan.Although Japanese quails are more resistant to NDV than chickens, the severity of the disease may increase under stress conditions (Mazlan et al., 2017). Today, quail is extensively reared in several countries of the world for human consumption. In the poultry world, quail meat production is negligible compared

to broilers, but occupies a relevant place in poultry breeding and contributes to the variety in poultry meat production (Jeke et al., 2018). Quails are bred for egg and meat production, and the relative importance of their two products varies between countries (Jeke et al., 2018). In recent years, commercial production of quail has increased in some regions of Iran, and a part of the protein demands of Iranian people is provided with the meat of this bird. As the quail industry has developed in the world and also in Iran, therefore it is necessary to study more about the effect of a challenge with NDV in this bird.In the present survey, we intend to determine the effects of a challenge with a velogenic chicken isolate of NDV on the *lactobacillus* numbers (as beneficial microflora) in intestine and crop, and also *Escherichia coli* numbers (as important opportunistic pathogen) in the intestine of Japanese quail.

MATERIAL AND METHODS

Ethics statement

I declare all ethical standards have been respected in the preparation of this article. Ethical permission was granted by the Shahid Chamran University of Ahvaz Ethical Commission for Animal Experiments (22 May 2020) under verification number EE /99.3.02.47185/scu.ac.ir

Virus

Based on the nucleotide sequence, the velogenic NDV used in this experiment was previously characterized as genotype VII (subgenotype VIId) and assigned an accession number of NDa: KP347437. Initially, the virus was propagated twice in 9-day-old embryonated chicken eggs through inoculation into the chorioallantoic sac. The 50% embryo infective dose (EID50) was calculated for the second passage according to the method of Reed and Muench, and the harvested allantoic fluid was used as inoculum as specified in the experimental design.

Vaccination program: At 20 days of age, the chicks of each group were vaccinated with Newcastle B_1 strain (commercial vaccines Avishield® ND B1 was provided by Genera Inc. (Croatia)) via eye-drop.

Animal husbandry, experimental design, and diets

A total of 100 day-old Japanese quail (average body weight 9.53g), were divided into two equal groups randomly.Each group was divided into two subgroups of 25 quails. The birds in group A challenged with a velogenic chicken isolate of NDV. The birds in group B did not challenge with NDV as the control group. Chicks were reared in standard conditions (temperature, ventilation, and light) for 56 days and throughout the trial, birds had free access to water and feed. A standard basal diet in pellet form was provided to the birds. There was no mortality in any of the groups during the study period.They were housed in cages separately in the animal research unit of Shahid Chamran University of Ahvaz (Iran) and received feed and water ad libitum during the experiment. At 34 days of age, when the sera were negative for maternal antibodies in conventional hemagglutination- inhibition (HI) test, the birds in groups A were inoculated with 100 µL (50 µL/eye) of NDV-infected allantoic fluid containing 10^5 EID50 of viral inoculum, whereas the birds in groups B received distilled water by the same route.

Determination of *lactobacillus* counts in intestine and crop

For the determination of *Lactobacillus* counts, 10 and 20 days postinoculation, 10 birds of each subgroup (20 birds of each treatment) were chosen randomly. The contents of the distal part of the small intestine (10 cm anterior to the junction with caecum and rectum) and crop were separately collected, and used for microbial assays. The populations of lactobacillus were estimated as CFU g^{-1}. Sterilized phosphate-buffered saline (PBS) (9 mL) was added to 1 g of fresh materials (1:10), and then subsequent dilutions were prepared. 50 microliters of each dilution were cultured on MRS at 37°C for 48 hours, under microaerophilic conditions, and the presence of bacteria was then determined (Talazadeh et al., 2016).

Escherichia coli counts in the intestinal contents

10 and 20 days postinoculation, for determination of populations of *Escherichia coli* in intestinal digesta of birds, 10 birds of each subgroup (20 birds of each treatment) were chosen randomly. The contents of the distal part of the small intestine (10 cm anterior to the junction with caecum and rectum) of birds were collected and used for microbial assays. The populations of *E. coli* were estimated as CFU g^{-1}. Sterilized phosphate-buffered saline (PBS) (99 mL) was added to 1 g of fresh material (1:100), and then subsequent dilutions were prepared. Samples were cultured on Mac Conkey agar (Merck, Germany), at 37 °C for 24 hours, and the presence of *E. coli* then determined. The original data for *Escherichia coli* counts were

transformed to log10 CFU g^{-1} of intestinal content for statistical analysis (Talazadeh et al., 2016).

Statistical analysis

The data were submitted to analysis of variance using the Statistical Package for Social Sciences (SPSS) version 18.0. Mean differences among treatments were evaluated through the One Way ANOVA LSD Test at P< 0.05.

RESULTS

Table 1. The effect of velogenic NDV on lactobacillus counts in ileocecal contents of quails in MRS Agar

Medium / groups	10 days postinoculation	20 days postinoculation
A (challenge) (10^5)	25 ± 3.9^b	38 ± 3^b
B (control) (10^5)	48 ± 4.1^a	57 ± 5.5^a

*CFU/g± standard deviation of means
Columns with different superscripts (a and b) are significantly different (P < 0.05).

Table 2. The effect of velogenic NDV on lactobacillus counts in the crop of quails in MRS Agar

days / groups	10 days postinoculation	20 days postinoculation
A (challenge) (10^5)	16.5 ± 0.7^b	26 ± 3.5^b
B (control) (10^5)	44 ± 1.4^a	59 ± 5.2^a

*CFU/g± standard deviation of means
Columns with different superscripts (a and b) are significantly different (P < 0.05).

Table 3. The effect of velogenic NDV on *E.coli* numbers in ileocecal contents of quails on Mac Conkey agar

days / groups	10 days postinoculation	20 days postinoculation
A (challenge)	9 ± 1.3^b	20 ± 1.5^b
B (control)	6.5 ± 2.5^a	16 ± 3.2^a

log CFU g^{-1} ± standard deviation of means
Columns with different superscripts (a and b) are significantly different (P < 0.05).

According to these tables, the results of this study showed that challenge with velogenic NDV decreased significantly lactobacillus counts in crop and ileocecal contents of quails compared to the control group and increased significantly *E.coli* numbers in ileocecal contents of quails compared to the control group (Table 1-3).

DISCUSSION

Our results suggest that the challenge with a velogenic chicken isolate of NDV decreased the proliferation of beneficial bacteria and increased the presence of gram-negative bacteria. This study indicates that velogenic NDV infection interferes with the intestinal microbiome in quails. The intestinal microflora is relatively stable under common circumstances but is easily influenced by various diseases (Ma et al., 2017).NDV is the causative agent of ND, which is one of the most highly contagious diseases in chickens, and result in severe economic losses to the poultry industry worldwide (Turmagambetova et al., 2017). In Iran, NDV is endemic in different parts of the country, causing enormous losses due to high mortality, sub-optimal production, slaughterhouse condemnation of carcasses, and high prevention and treatment expenses. In recent years, outbreaks of ND have been occasionally observed in different avian species in Iran, including Japanese quail (Momayez et al., 2007), ostrich (Ghiamirad et al., 2010), exotic caged birds (Madadgar et al., 2013), and broiler chickens (Mehrabanpour et al., 2014). Cui et al. (2018) showed that the NDV infection may be associated with the dysbiosis of gut flora and NDV infection interferes with the formation of the intestinal microbiome in newly hatched chicks and loss of a subset of bacteria along with decreased richness and diversity were observed in the gastrointestinal tract of NDV infected newly hatched chicks (Cui et al., 2018). Similar phenomena are also found in many other important poultry diseases like *Eimeria tenella* (Zhou et al., 2017), Marek's disease virus (Perumbakkam et al., 2014), avian leukosis virus (Ma et al., 2017), etc. The normal microbiota of the gastrointestinal tract of chickens plays an important role in inhibiting the establishment of intestinal pathogens

(Güntheret al., 2016). Indeed, some important pathogens like *Rhodoplanes* were found to be enriched in both the duodenum and the ceca of the NDV infected chicks. It is resumed that *Rhodoplanes sp.* might be an emerging human pathogen involved in unknown febrile conditions and could cause local infection of any tissues or organs (Zhang et al., 2011). Besides, the duodenum showed relatively homogeneous flora among individuals, but NDV infection made a preference for implantation of known conditionedpathogens. Similarly, the ceca of normal chicks were dominated by *Paenibacillus* or *Enterococcus* at hatch. Several *Paenibacillus species* produce antimicrobial substances that affect a wide spectrum of microorganisms such as fungi, soil bacteria, plant pathogenic bacteria, and even important anaerobic pathogens such as *Clostridium botulinum* and *Paenibacillus pasadenensis* (Passera et al., 2017). In line with this, more *Epulopiscium* and *Clostridium* were established accompanied by the complete loss of *Paenibacillus* after the infection of NDV in newly hatched individuals. *Clostridium* contains around 100 species that include common free-living bacteria, as well as important pathogens. A previous study conferred that bacterial infections could also be enhanced by NDV in a mice model (Cuiet al., 2018). Therefore, NDV infection increased the chance of secondary infection since the intestinal gut might be a great source of conditionedpathogens. Bacterial dysbiosis has been linked to altered immune function and/or persistent inflammation. Understanding the roles of microbiota in intestinal mucosal immunity should offer novel insight into gastrointestinal disease pathophysiology and deliver new immunotherapy strategies (Chang and Lin, 2016).Microbial dysbiosis and translocation are associated with systemic immune activation in HIV and SIV infections, which in turn helps the increase of virus load in the host (Marchetti et al., 2011).It is increasingly observed that the NDV infection may be associated with the dysbiosis of gut flora. NDV infection is associated with mucosal damage in chickens. Thus, it is imperative to better understand the interplay between intestinal microbiota changes and the pathogenesis of NDV infection. The data will be useful for future studies related to the pathophysiology of NDV in birds and for experiments evaluating the interactions of NDV and bacteria, and other mixed infections in poultry. It has been reported that NDV infection of chicks induced disproportion of gastrointestinal tract microbial population (Cui et al., 2018). A similar phenomenon was also found in our study.Indeed, in the present study, the velogenic chicken isolate of NDV, induced disproportion of the gastrointestinal tract microbial population. So *Escherichia coli* as an important opportunistic pathogen was found to be enriched in the intestine of the NDV infected quails. Therefore, a velogenic chicken isolate of NDV increased the chance of secondary infection since the intestinal gut might be a great source of conditioned pathogens. Also, in the present study, a velogenic chicken isolate of NDV decreased lactobacillus counts of intestine and crop compared to the control group. This study showed that the challenge with a velogenic chicken isolate of NDV decreased the proliferation of beneficial microflora and increased the presence of gram-negative bacteria. This study indicates that velogenic NDV infection interferes with the intestinal microbiome in quails. The data will be useful for future studies related to the pathophysiology of NDV in quails and for experiments evaluating the interactions of NDV and bacteria, and other mixed infections in poultry.

CONCLUSIONS

In the present study, the velogenic chicken isolate of NDV, induced disproportion of the gastrointestinal tract microbial population. So *Escherichia coli* as an important opportunistic pathogen was found to be enriched in the intestine of the NDV infected quails. Therefore, a velogenic chicken isolate of NDV increased the chance of secondary infection since the intestinal gut might be a great source of conditioned pathogens. Also, in the present study, a velogenic chicken isolate of NDV decreased lactobacillus counts of the intestine and crop as beneficial microflora. So it concluded that NDV may have serious consequences in quail farms and according to these findings vaccination against NDV in quail farms of Iran is highly recommended. Controlling factors like isolation of the farms, applying bio-security, decreasing stress conditions, certification of quail movement, and particularly enforcement of vaccination programs, etc., must be considered to improve disease security and reduce danger of spreading of infection.

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CONFLICT OF INTEREST

None was declared by the authors.

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Research article
Ερευνητικό άρθρο

Effect of thyme essential oil and enzyme supplements on performance, carcass and blood metabolites in broilers

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ABSTRACT: This experiment was conducted to evaluate the effects of thyme essential oil and enzyme supplementation to corn/wheat based diets on performance, carcass traits and blood metabolites in broilers. This experiment was carried out with 432 Ross-308 broilers (54.3±0.18) as a factorial arrangement with 36 units (12 birds per replicate) in a completely randomized design from 1 up to 42 days. Results showed that the basic ration and meal type significantly affected the performance (P<0.05). The use of wheat increased feed intake (FI) and feed conversion ratio (FCR), while soybean increased FI and weight gain (WG) and improved FCR (P<0.05). Meat and blood parameters and blood parameters were not affected by the experimental treatments (P>0.05). Use of enzyme supplement or thyme essential oil in treatments containing wheat is recommended, because it can improve broilers performance parameters.

Keywords: Broiler, Blood metabolites, Enzyme, Performance and Thyme essential oil

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INTRODUCTION

Thyme (*Thymus vulgaris*) was originally planted in the Mediterranean and currently cultivated in gardens and fields as an aromatic and medicinal plant (Çiftçi et al., 2018). Thyme active substances can stimulate the secretion of pancreatic enzymes, thereby improving digestion efficiency and absorption of protein and other nutrients from the bird's gastrointestinal tract (Hashemipour et al., 2016). The willing for the elimination of growth promoting antibiotics to poultry diets led to a gradual increase in the use of medicinal plants and their derivatives as safer alternatives (Yadav et al., 2016; Naghi Shokri et al., 2017; Devi et al., 2018; Huang et al., 2018; Cimrin et al., 2020; Sevim et al., 2020). These substances have the potential to promote, along with growth, other positive aspects such as improving health (Attia et al., 2017; Mehdi et al., 2018; Yesilbag et al., 2020) and improving the gut microbial population (Adeyemo et al., 2016; Rahman and Yang, 2018; Abd El-Ghany and Eraky, 2019).

Reducing the cost combined with the saving of the ration can play a crucial role in the final price of the poultry products, which may increase their consumption. Alongside these advantages, the use of wheat instead of corn in poultry diets also has some limitations, including the presence of inhibitors such as non-starch polysaccharides and phytates in wheat grain (Yaghobfar and Kalantar, 2017).

There are various methods for reducing antinutrients in feeds, such as using enzyme supplements and essential oils of medicinal plants. These enzymes degrade antinutritional factors and break down their complex, thus increase protein digestibility and improve poultry performance.

Wade et al. (2018) reported that broilers which received 100 mg / kg of thyme had higher weight gain (WG). Cross et al. (2007) studied the effects of thyme essence on growth, digestibility, and intestinal microflora of broilers, stating that using thyme essence had positive effects on poultry performance. Cao et al. (2010) found that supplementation of thyme essential oil in the poultry diet significantly improved feed intake (FI) ($P<0.05$).

Pournazari et al. (2017) reported that the use of thyme extract increased live weight (LW) and FI. In the study by Çiftçi et al. (2018) thyme-containing treatments reduced serum glucose, triglyceride, total cholesterol, uric acid and total protein in Japanese quail *(Coturnix coturnix Japonica).* Hedayati and Manafi (2018) reported that probiotics and herbal composition (including thyme extract) increased daily WG and decreased feed conversion ratio (FCR).

Hosseini and Mimandipour (2018) reported that thyme extract increased daily WG and improved FCR. Witkowska et al. (2019) reported that thyme extract had no adverse effect on poultry health and increased the immune response of poultry. Sugout and Mohammed (2019) suggested that thyme powder could be used as a growth promoter in the poultry diet of 1% without adverse effects on poultry. In the study of Abo-Eid et al. (2019), thyme extract showed the best anticholesteryl activity and reduced crude protein in poultry. Therefore, the aim of this study was to determine the effects of thyme essential oil and enzyme supplementation on performance and blood metabolites.

MATERIALS AND METHODS

In this experiment, 432 Ross-308 broilers (54.3±0.18) were divided into three factorial trials with 36 units. The tested factors were the carbohydrate (corn and wheat), the meal type (soybean and rapeseed) and the additive type (thyme essential oil and enzyme). The experiment was conducted in a completely randomized design in two periods, including growing (11 to 24 days) and finisher (24 to 42 days). Diets conformed to the advised levels of nutrients, as established by the Ross-308 broiler nutrition specification, and using the UFFDA software program (Hamidi et al., 2021).

In the first three days, the saloon lighting was continuous, and from the fourth day it changed to 23 hours light and 1 hour dark. In the first day, the saloon temperature was 34 °C. From the first week onwards and every two weeks, the temperature was dropping by 2 degrees. In the sixth week, the temperature was set at 20 °C and remained until the end of the experiment.

Feed conversion ratio was calculated considering the wastage and specifying the age of chickens.

The chemical compounds of enzyme and thyme extract, used in the experiments, are listed below (Tables 1 and 2).

Table 1. Chemical composition of thyme essential oil (GC-MS analysis)

Chemical composition	amount
α - Pinene	1.47
Camphene	0.03
β - Pinene	0.04
Myrcene	0.05
ρ - Cymene	14.32
Limonene	0.24
Ocimene	0.09
Terpinene	22.46
3, 8 - ρ - Menthadine	0.05
Linalool Oxide	0.1
Fenchone	0.02
Linalool	4.96
4- Terpineol	1.39
Thymol	43.84
Cavacrol	10.94

Table 2. Chemical composition of COMBO enzyme Bland 10X

Chemical composition	amount
Cellulase	>750.000 CU Unite/Kg
Fungal Amylase	>300.000 SKB Unite/Kg
Fungal Protease	>10.000.000 HUT Unite/Kg
Nutral Protease	>1.000.000 PC Unite/Kg
Alkaline Protease	>12 Anion Unite/Kg
Xylanase	>200.000 XU Unite/Kg
Beta Glucanase	>200.000 BG Unite/Kg
Hemicellulase	>200.000 HCU Unite/Kg
Lipaze	>750.000 FIP Unite/Kg
Pectinase	Activities Present
Mananase	Activities Present

In this study, the body weight (BW) of the broilers and the daily feed intake (FI) were recorded. Health status and mortality rates were recorded daily during the experimental period. The resulting data was used to calculate the feed conversion ratio (FCR).

At the end of the experimental period, on the 42nd day, two birds per replicate (one male and one female) were selected and were slaughtered to determine carcass traits. The percentage of the carcass was determined comparing heart weight, abdominal fat, gizzard, liver, breast, and thigh weights to the corresponding carcass weights. At the day 42, blood samples from 3 birds per treatment were randomly taken via the wing vein. The samples were centrifuged (3000 rpm × 10 min) after clotting (2h) to obtain serum and stored at -20ºC before analysis. Glucose, cholesterol, triglyceride, albumin and high-density lipoprotein-cholesterol (HDL) were measured using commercial kits (Pars Azmoon, Tehran, Iran) (Sigolo et al., 2019).

Data were subjected to statistical analysis according to a completely randomized design as a factorial arrangement of 2 × 3 using the general linear model procedure of SAS 9.4 (2018). Means were compared using Tukey's tests at 5% probability, according to the following model:

$$Y_{ijkl}=\mu+A_i+B_j+C_k+AB_{ij}+AC_{ik}+BC_{jk}+ABC_{ijk}+\varepsilon_{ijkl}$$

Where Y_{ijkl} = dependent variable, μ = overall mean, A_i = the effect of Factor A, Bj = the effect of Factor B, C_k = the effect of Factor C, ABij = Interaction of Factors A×B, AC_{ik} = Interaction of Factors A×C, BC_{jk} = Interactivity of Factors B×C ABC_{ijk} = Interaction of Factors A×B×C and ε_{ijkl} = the random error.

RESULTS

Feed Intake

As can be seen in Table 3, the main effects of the basal diet, meal, and the interactions between the basal diet × type of meal, the basal diet × additive and the type of meal and additive had a significant effect on FI in the grower period (P <0.05). In the finisher periods, the main effects of the basal diet, and the interaction effects of the basal diet and the type of the meal had a significant effect on FI (P <0.05). However, other experimental treatments were not statistically significant (P >0.05).

Weight Gain

The main effects of meal type as well as the interaction effects of dietary meal × meal type, basal diet × additive and meal type × additive had a significant effect on weight gain (P <0.05). In the finisher periods, the main effects of the basal diet (corn or wheat) as well as the interactions of the basal diet × meal type had a significant effect on weight gain (P <0.05).

Feed Conversion Ratio

As shown in the Table 3, the main effects of the basal diet, meal, and the interactions between the basal diet × type of meal, the base diet × additive and the type of meal and additive had a significant effect on the FCR (P <0.05).

Table 3. Effect of supplementation thyme essential oil and enzyme on daily weight gain (WG; g/chick/day), feed intake (FI, g/chick/duration), and feed conversion ratio (FCR)

	Growing period (12-21 day)			Finisher period (22-42 day)		
Treatments	FI	WG	FCR	FI	WG	FCR
Ration type						
Corn	54.36b	40.34	1.35b	159.12b	77.23b	2.06
Wheat	56.97a	39.42	1.44a	162.2a	80.01a	2.02
SEM	0.083	1.031	0.003	0.032	0.284	0.19
Meal type						
Soybean	58.74a	40.03a	1.47b	157.02	79.84	1.97
Rapeseed	55.12b	35.27b	1.56a	156.94	78.03	2.01
SEM	0.083	1.031	0.003	0.032	0.284	0.19
Supplementation type						
Essential oil	55.93	36.05	1.55	156.89	76.25	2.05
Enzyme	56.01	36.42	1.54	157.01	76.94	2.04
SEM	0.083	1.031	0.003	0.032	0.284	0.19
Ration × Meal						
Corn × Soybean	59.4a	40.19a	1.48d	159.35ab	78.04b	2.04
Corn × Rapeseed	57.39ab	37.95b	1.51c	158.79b	77.43b	2.05
Wheat × Soybean	56.24bc	36.49c	1.54b	160.35ab	79.32a	2.02
Wheat × Rapeseed	54.73c	34.78d	1.57a	161.01a	79.95a	2.01
SEM	0.842	0.019	0.002	0.812	0.472	0.009
Ration × Supplementation						
Corn × Essential oil	59.12a	40.53a	1.46b	159.72	79.54	2.01
Corn × Enzyme	59.33a	40.64a	1.46b	159.91	79.65	2.01
Wheat × Essential oil	55.18b	36.12b	1.53a	160.01	79.78	2.00
Wheat × Enzyme	55.39b	36.25b	1.53a	161.20	80.01	2.01
SEM	1.032	1.074	0.005	0.947	0.663	0.004
Meal × Supplementation						
Soybean × Essential oil	57.83a	38.72a	1.49a	157.32	77.01	2.04
Soybean × Enzyme	58.02a	39.37a	1.47b	158.12	77.13	2.05
Rapeseed × Essential oil	54.94b	35.29b	1.56b	155.73	76.63	2.03
Rapeseed × Enzyme	54.98b	35.78b	1.54c	156.49	76.95	2.03
SEM	0.642	0.532	0.004	1.735	0.812	0.012
Ration × Meal × Supplementation						
Corn × Soybean × Essential oil	59.85	40.89	1.46	158.25	78.32	2.02
Corn × Soybean × Enzyme	59.97	40.93	1.46	159.12	79.17	2.01
Corn × Rapeseed × Essential oil	57.72	39.76	1.45	157.02	77.64	2.02
Corn × Rapeseed × Enzyme	58.03	40.12	1.45	157.34	77.83	2.02
Wheat × Soybean × Essential oil	56.89	39.00	1.46	159.26	79.97	1.99
Wheat × Soybean × Enzyme	56.95	39.12	1.46	160.24	80.12	2.00
Wheat × Rapeseed × Essential oil	55.78	38.74	1.44	159.87	79.22	2.02
Wheat × Rapeseed × Enzyme	56.01	38.92	1.44	160.32	79.49	2.02
SEM	1.825	1.027	0.009	2.021	1.324	0.027

Carcass traits

The effect of using different feeds on carcass traits of broilers at the end of the experimental period is shown in Table 4. Experimental treatments had no significant effect on carcass traits (p >0.05).

Table 4. Effect of supplementation thyme essential oil and enzyme on carcass traits (%)

Treatments	Carcass	Breast	Tights	Abdominal fat	Gizzard	Liver	Heart
Ration type							
Corn	71.22	34.19	25.14	2.53	2.35	3.67	0.743
Wheat	70.37	34.58	25.76	2.45	2.42	3.45	0.735
SEM	0.714	0.526	0.326	0.166	0.078	0.088	0.029
Meal type							
Soybean	70.79	34.26	25.59	2.08	2.42	3.60	0.778
Rapeseed	70.71	34.02	25.02	2.84	2.60	3.65	0.685
SEM	0.714	0.526	0.326	0.166	0.078	0.088	0.029
Supplementation type							
Essential oil	72.16	34.29	26.52	2.66	2.60	2.76	0.765
Enzyme	70.88	34.38	25.33	2.60	2.15	3.43	0.687
SEM	0.714	0.526	0.326	0.166	0.078	0.088	0.029
Ration × Meal							
Corn × Soybean	71.11	34.65	25.33	2.76	2.51	3.42	0.733
Corn × Rapeseed	69.13	34.74	25.42	1.98	2.46	3.49	0.775
Wheat × Soybean	70.31	34.53	25.59	2.22	2.44	3.53	0.784
Wheat × Rapeseed	70.38	34.34	25.08	2.86	2.63	3.48	0.755
SEM	0.683	0.702	0.249	0.178	0.095	0.101	0.048
Ration × Supplementation							
Corn × Essential oil	69.66	34.63	24.94	1.98	2.57	3.46	0.836
Corn × Enzyme	69.95	34.52	24.39	2.26	2.52	3.49	0.827
Wheat × Essential oil	70.15	34.22	25.18	2.53	2.60	3.55	0.779
Wheat × Enzyme	71.71	34.55	25.38	2.61	2.49	3.77	0.758
SEM	0.683	0.702	0.249	0.178	0.095	0.101	0.048
Meal × Supplementation							
Soybean × Essential oil	69.36	33.74	24.10	2.03	2.55	3.44	0.875
Soybean × Enzyme	71.21	33.79	24.86	2.75	2.62	3.78	0.706
Rapeseed × Essential oil	72.18	34.41	24.97	2.73	2.71	3.81	0.663
Rapeseed × Enzyme	73.28	34.71	25.22	2.79	2.59	3.65	0.753
SEM	0.683	0.702	0.249	0.178	0.095	0.101	0.048
Ration × Meal × Supplementation							
Corn × Soybean × Essential oil	71.92	33.90	26.23	2.19	2.64	3.74	0.720
Corn × Soybean × Enzyme	70.21	34.31	24.82	2.92	2.58	3.52	0.663
Corn × Rapeseed × Essential oil	71.04	34.79	25.54	2.60	2.50	3.71	0.866
Corn × Rapeseed × Enzyme	72.75	35.77	25.11	2.55	2.08	3.23	0.723
Wheat × Soybean × Essential oil	70.76	34.38	24.76	2.67	2.55	3.34	0.740
Wheat × Soybean × Enzyme	66.69	35.19	26.23	2.11	2.42	3.63	0.736
Wheat × Rapeseed × Essential oil	69.01	32.98	27.93	2.65	2.22	3.63	0.650
Wheat × Rapeseed × Enzyme	71.46	34.91	25.91	2.85	2.47	3.51	0.726
SEM	1.278	0.996	0.647	0.374	0.187	0.155	0.052

Blood Parameters

The effect of the different treatments on serum metabolites is presented in Table 5. Experimental treatments had no significant effect on serum metabolites (p >0.05).

Table 5. Effect of supplementation thyme essential oil and enzyme on blood biochemical parameters (mg/dL)

Treatments	Glucose	Cholesterol	Triglyceride	Abumin	HDL
Ration type					
Corn	117.49	149.23	32.95	1.61	88.96
Wheat	159.73	172.58	40.90	1.71	96.32
SEM	29	32	12	0.165	5.57
Meal type					
Soybean	140.92	135.02	47.72	1.94	78.40
Rapeseed	176.23	130.96	39.77	1.92	71.36
SEM	29	32	12	0.165	5.57
Supplementation type					
Essential oil	131.12	100.50	64.77	1.40	61.76
Enzyme	144.22	95.43	82.95	1.42	62.05
SEM	29	32	12	0.165	5.57
Ration × Meal					
Corn × Soybean	153.46	144.16	61.37	1.63	89.60
Corn × Rapeseed	145.54	158.37	64.24	1.68	91.52
Wheat × Soybean	141.58	153.29	64.77	1.74	95.04
Wheat × Rapeseed	150.16	157.92	65.28	1.81	91.84
SEM	23	25	14	0.213	4.39
Ration × Supplementation					
Corn × Essential oil	154.13	95.43	81.81	1.74	71.36
Corn × Enzyme	154.45	124.87	87.50	1.76	74.24
Wheat × Essential oil	157.09	108.62	88.11	1.71	88.96
Wheat × Enzyme	156.76	145.17	115.90	1.79	89.60
SEM	23	25	18	0.501	4.87
Meal × Supplementation					
Soybean × Essential oil	160.72	121.82	37.17	1.79	58.24
Soybean × Enzyme	169.96	125.13	34.10	1.68	57.14
Rapeseed × Essential oil	168.12	130.95	40.95	1.11	61.06
Rapeseed × Enzyme	170.87	132.66	44.32	1.50	62.19
SEM	23	25	15	0.612	5.03
Ration × Meal × Supplementation					
Corn × Soybean × Essential oil	157.57	153.29	45.45	1.88	95.07
Corn × Soybean × Enzyme	160.72	166.49	87.60	1.93	96.32
Corn × Rapeseed × Essential oil	169.96	157.36	32.95	1.97	100.01
Corn × Rapeseed × Enzyme	118.48	150.32	34.09	2.05	99.53
Wheat × Soybean × Essential oil	172.60	174.49	64.27	1.89	104.00
Wheat × Soybean × Enzyme	185.80	168.22	59.09	2.21	102.54
Wheat × Rapeseed × Essential oil	177.23	169.37	62.35	1.92	99.98
Wheat × Rapeseed × Enzyme	184.00	176.08	61.08	2.64	89.33
SEM	32	27	17	0.812	5.76

DISCUSSION

Feed Intake

On the one hand, the present data showed that wheat diets contained more than the double concentration of soluble NSP relative to corn diets, which may be implicated in the higher jejunal digestion viscosity observed in birds fed wheat diets, and this may have increased their feed intake due to the lack of energy supply. On the other hand, the relative increase in FI in experimental groups containing wheat can be due to various causes, including fiber and stimulation of the secretory and digestive tracts in the stomach, intestine, liver, pancreas and bile. Cross et al. (2007) stated that the use of thyme oil improves the FCR in broilers but has no significant effect on WG and FI. Shakouri and Kermanshahi (2007) reported that using enzyme leads to a significant increase in daily FI of broilers and also Zakeri and Kafashi (2011) reported that using enzyme had a significant effect on daily FI of broilers. Non-starch polysaccharides such as pectin, cellulose and amyloid in canola in the intestine absorb a large amount of water, increasing the concentration and viscosity of the digestive contents. Increased viscosity decreases the speed of digestion and reduces FI.

Weight Gain (WG)

It was predictable that non-viscous feed ingredients such as corn result in better weight gain than viscous feed ingredients such as wheat which have high levels of soluble and viscous NSP, but it turned out quite differently. However, the interactions of enzyme and grain type clearly showed the role of enzyme addition in this matter. On the other hand, probably due to the high levels of NSP in this feed, it has led to an increased viscosity in the poultry intestine. Viscosity increases the length of the small intestine by increasing absorption, and consequently, it increases weight gain. Another possibility is that using wheat may have led to increased WG due to the digestive system's palatability or stimulation (due to high fiber), leading to increased WG, which is advisable at this stage of the report. Rapeseed meal could be used as an economically viable alternative to soybean meal in poultry diets. The significant reduction in performance parameters of broiler chicks during the growing period could be the result of the high glucosinolate content of the experimental diets. The tolerance to glucosinolates in younger birds is less, which impairs thyroid functions. As the birds grow, the thyroid develops, and mature birds can tolerate a relatively high amount of glucosinolates (Mnisi and Mlambo, 2018). Nobakht et al. (2010) reported that using different levels of nettle, mackerel, and cactus herbs in early and growing periods had significant effects on broiler performance. The results of this experiment were in agreement with the results of Nasiri et al. (2011). Shakouri and Kermanshahi (2007) reported that the use of the enzyme resulted in a significant increase in the DWG of broilers that did not match the results obtained in this study.

Feed Conversion Ratio (FCR)

Starch is the dominant energy source in corn. The significant improvement in ileal starch digestibility could have contributed to the increase in available energy content of the corn diet, and higher energy efficiency with less feed intake also leads to improved FCR. The higher WG and reduced FCR in canola treatments could be attributed to humic acid and enzymes, which may have improved the feed efficiency given the reduced feed intake (Disetlhe et al., 2018). Similarly, to our results, Al-Harthi (2017) reported that supplementation of poultry diet with phytase enzyme improves FI, BW and FCR of broilers. Improvement of digestion and absorption of nutrients in poultry intestine, by medicinal plants, is related to the increased secretion of digestive enzymes such as lipase, amylase and protease, theincreased digestibility, and the increased intestinal length and depth and number of villi. Nutrient retention in the gut is higher, which gives more opportunities for them to be absorbed. (Yadav and Jha, 2019).

Carcass traits

Due to the increasing poultry age, the digestive system's development led to improving the secretion of enzymes required for digestion. It resulted that experimental diets did not significantly affect carcass traits (Bautil et al., 2019; David et al., 2020). Improving digestion and absorption of nutrients in poultry intestines during the use of medicinal plants is related to the effect of medicinal plants on increasing the secretion of digestive enzymes, such as lipase, amylase and protease and increasing digestive function, increasing intestinal length and depth and number of villi (Thapa et al., 2019; Sharma et al., 2019). Since the use of essential oils of medicinal plants reduced the harmful microbial population of the gastrointestinal tract, the rate of proteins and amino acids degradation was decreased, and more of them were absorbed and stored in the body.

Blood Parameters

Experimental treatments had no effect on serum glucose, cholesterol, triglyceride, albumin and high-density lipoprotein-cholesterol (HDL) concentrations. Blood biochemical parameters are closely associated with health status and play their role as important indicators of health conditions in birds. Together with other serum metabolites, albumin concentration indicates the adequacy of protein in the diet and the efficiency with which the broilers are utilizing it; therefore, both protein and energy sources have been able to meet the needs of poultry equally (Yang et al., 2018; Hatab et al., 2020). Saki et al. (2014) reported that dietary inclusion of phytogenic feed additives did not change the serum cholesterol and triglyceride levels in laying hens. Soltan et al. (2008) demonstrated that aniseed supplementation in the broiler diet did not affect the broiler serum cholesterol levels. Our results were in accordance with Saki et al. (2014) and Soltan et al. (2008) reports.

CONCLUSION

Even though increased viscosity, wheat in the diets led to an increase in the length of the gastrointestinal tract and improved performance parameters. This study recommends using enzymes or essential oils to reduce antinutrients' negative aspects and increase performance.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationship that could influence the work reported in this paper.

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Research article
Ερευνητικό άρθρο

The seroepidemiology of pestivirus infection in sheep in Afyonkarahisar province of Turkey and the analysis of associated risk factors

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ABSTRACT: Border disease virus (BDV) is a pestivirus that causes considerable economic losses in the sheep industry due to its effect on breeding and health. This study's goal was to determine the seroprevalence of pestivirus infection, the ratio of persistently infected (PI) animals, and risk factors associated with the disease in sheep flocks between 2019-2020. To this end, 460 blood serum samples collected from eight sheep flocks were examined using commercial Enzyme-Linked Immuno-Sorbent Assay (ELISA) test kits to determine the presence of antibodies (Ab) and antigens (Ag) against pestiviruses (bovine viral diarrhea virus, border disease virus). Risk factors associated with pestivirus infection were statistically evaluated in terms of significance in the logistic regression model according to epidemiological data and information obtained from flock owners. Individual seropositivity was analyzed by Generalized Estimating Equations (GEEs) for associated responses. The overall apparent animal level seroprevalence was estimated to be 24.57% (95% CI: 20.85-28.7). The overall true seroprevalence was calculated to be 25.51% (95% CI: 21.65-29.60). The rate of positive sheep in each flock varied between 8.33-57.14%. The ratio of PI sheep among the 460 animals tested was found to be 0.43%. The relationship between the age groups was statistically significant (p-value:0.0002<0.05; $\chi 2$:13.15). Management type, age, the presence of cattle in the farm, landscape and the status of other clinical diseases were identified as important risk factors associated with individual pestivirus seropositivity. The results of this study indicate that it will contribute to the creation of national control eradication and monitoring plans and the development of strategies and that the potential risk of sheep as a pestivirus reservoir, especially for cattle that use common pastures, should be considered in future studies.

***Keywords*:** pestivirus, sheep, BDV, risk factors, epidemiology, Turkey

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INTRODUCTION

Pestiviruses infect small and large ruminant species, causing significant economic losses worldwide due to their effect on breeding and health (Krametter-Froetscher et al., 2007). Bovine viral diarrhea virus 1 (BVDV1), bovine viral diarrhea virus 2 (BVDV2), classical swine fever virus (CSFV), and border disease virus (BDV) are in the Pestivirus genus in the Flaviviridae family. Since 2017, the International Committee on Virus Taxonomy (ICTV) has renamed the aforementioned four species as Pestivirus A, B, C and D, respectively, and in addition to these, Pestivirus E (pronghorn pestivirus), Pestivirus F (Bungowannah virus), Pestivirus G (giraffe pestivirus), Pestivirus H (HoBi-like pestivirus), Pestivirus I (Aydin-like pestivirus), Pestivirus J (rat pestivirus), and Pestivirus K (atypical porcine pestivirus) were added, and a total of 11 species were identified (Simmonds et al., 2017). BDV is in genetic and antigenic affinity with CSFV and BVDV (Marco et al. 2007; Feknous et al., 2018).

BDV infection is commonly observed in sheep, and it has been reported that it can also cause disease in cattle, goat and pig species (Paton et al., 1995; Braun et al., 2014; Schweizer and Peterhans, 2014; Feknous et al., 2018). BDV is the primary cause of congenital infections in sheep, and it can cause acute, foetal, and persistent infections. The main route of transmission of pestiviruses is horizontal transmission through transiently infected and PI animals. Furthermore, BVDV-1, BVDV-2, BDV, CSFV, and HoBi-like pestiviruses can be transmitted vertically. The disease usually causes abortions in pregnant animals, low birth weight and dog-hair appearance in lambs (Van Campen and Frolich 2001; Monies et al., 2004; Kittelberger and Pigott, 2008; Şevik 2018).

Prevalence studies constitute a prerequisite for control and eradication programs, and pestiviruses are among the main causes of reproductive problems and immune system effects in cattle and sheep in most countries (Radostits et al., 2007). Understanding the relationship between seroprevalence and PI animals can be used to assess the immunization potential of sheep not exposed to disease prior to breeding (Nettleton, 2000; Berriatua et al., 2004).An important aspect of pestivirus control and eradication programs implemented in various continents and countries of the world is the detection and eliminating of PI animals (Lindberg and Alenius, 1999; Berriatua et al., 2006). PI animals are infected during early embryonic and fetal development, and unlike individuals who become infected in the late period of pregnancy, they are seronegative and shed the virus throughout their lives. As a result, detecting PI animals is epidemiologically important since PI animals are the most significant source of infection for other animals (Houe, 1999).ELISA kits are preferred in pestivirus studies due to their advantages such as fast screening of many samples, sensitivity and being economic (Gonzalez et al., 2014; Hanon et al., 2018). The presence of viral antigens in PI animals can be detected by the ELISA method, which is a fast technique that is most commonly used in blood samples (Sandvik, 2005; Avci and Yavru, 2014).

In Turkey, sheep breeding is carried out in the form of large, medium, small flocks or a traditional family business within technical and economic opportunities according to the climate and natural conditions. There is no pestivirus vaccination program for small ruminants in Turkey. Although there are studies on the seroepidemiology of pestiviruses in sheep flocks in Turkey, there are almost no studies on the determination and analysis of risk factors associated with the disease. In the present study, it was aimed to predict the seroepidemiology and spread of pestivirus in sheep flocks in Afyonkarahisar province of Turkey and determine associated risk factors. It is expected that such epidemiological studies will contribute to the reduction of the prevalence of pestivirus infection, the creation of control eradication and monitoring plans for the country, and the development of strategies.

MATERIAL AND METHODS

Study area and description

Afyonkarahisar province is located between 37° 45 and 39°17 north latitude and 29° 40 and 31°43 east longitude. In the Master Plan of the Ministry of Agriculture and Forestry, the province is divided into four agro-ecological sub-regions, considering the agricultural diversity and climate data (I. Sub-Region, II. Sub-Region, III. Sub-Region, IV. Sub-Region) (Fig. 1). Although Afyonkarahisar province is located in the Aegean Region, it is under the influence of the continental climate, and winters are heavily snowy, and summers are hot and dry. The region of the province ranks first in the Aegean region with the number of small ruminants of 1 045 000 and has an important position in terms of animal husbandry potential (TUIK, 2020).

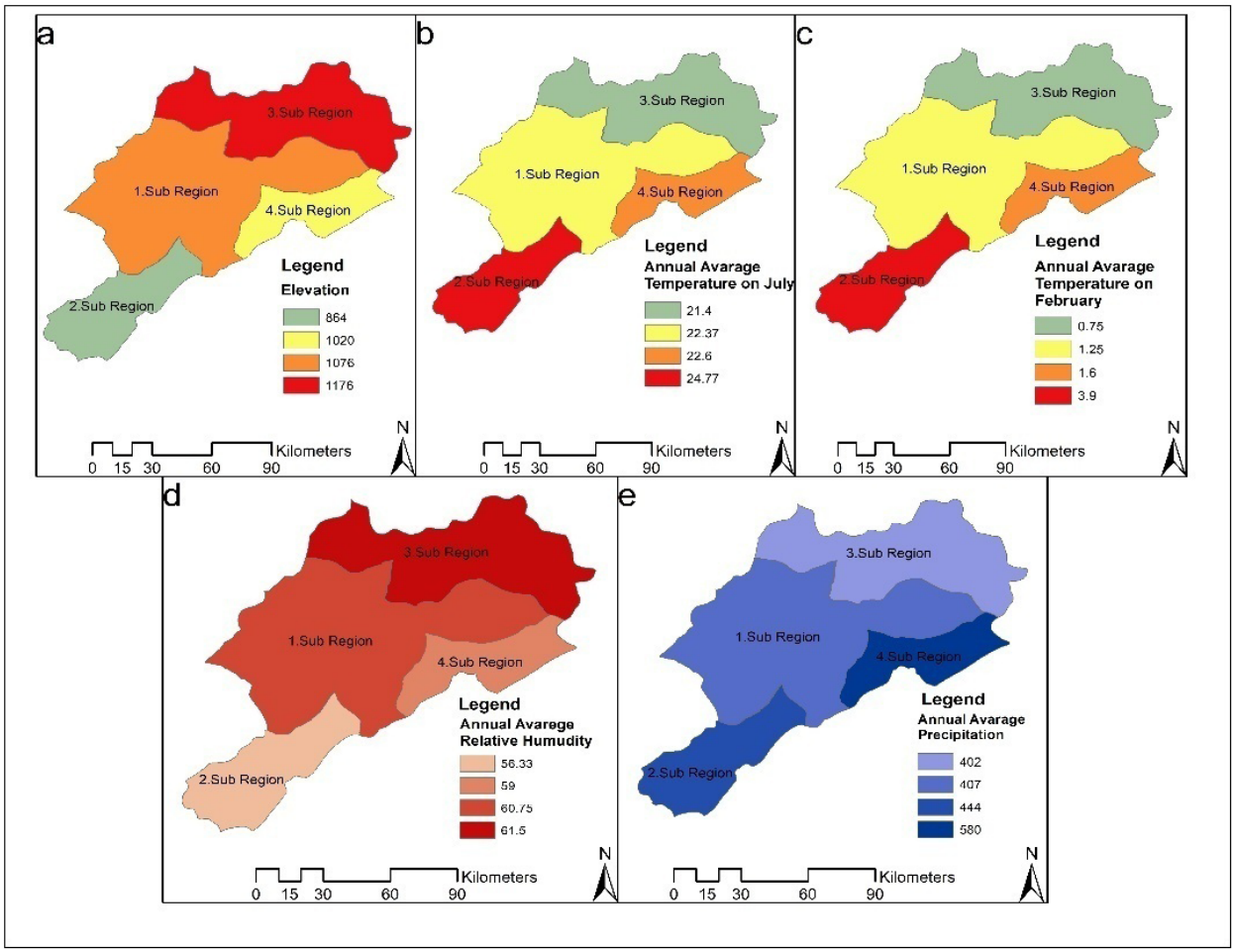

Figure 1. The study area

Samples, data management and analysis

In this study, the random sampling method was chosen for 460 animals from eight sheep flocks that were not vaccinated against pestivirus in the region of Afyonkarahisar province between 2019-2020. The required minimum within-flock sample size was computed on the basis of the epidemiologic research (Thrusfield, 2005; Dohoo et al., 2010). Four hundred sixty blood samples were drawn from the *vena jugularis* of the sheep selected, 10 ml were taken into blood tubes and centrifuged at 3000 rpm for 20 min. The serum obtained after this procedure was transferred to 1.5 ml sterile Eppendorf tubes. It was stored at -20 ^{0}C until being tested. Approval for the study protocol was obtained from Pamukkale University Animal Experiments Ethics Committee (Report No: PAUHADYEK-2019/36). Sheep owners were interviewed directly during the same visit with drawing the blood sample to obtain information on epidemiological data. Flock size, composition, animal age, animal movements, the presence of goats and cows in the enterprise, farming management (nomadic-sedentary), health status (abortion, stillbirth, joint disorders, nervous symptoms, other diseases, etc.) data were saved in Microsoft Excel spreadsheets.

Antibody ELISA

The serum samples collected were examined by indirect ELISA (IDEXX BVDV/MD/BDV p80 protein Ab IDEXX Laboratories, Westbrook, Maine, USA) in terms of the presence of antibodies against pestivirus. The antibody ELISA utilized to test serum samples is an ELISA based on the highly conserved p80 (non-structural pestivirus polypeptide) protein. The antibody ELISA test was carried out following the procedure reported by the manufacturer. The Optical Density (OD) values of the test results were read in an ELISA reader at 450 nm wavelength. The percentage inhibition (PI) values of the test sera were calculated according to the formula presented below by comparing them with the negative control.

S/N%= (OD_{S450}/ ($OD_{N450controlmean}$) ×100 where OD_{450} is the optical density at 450 nm of the sample (S) and negative control mean (N). The samples with the values of 0.50 and above were evaluated as negative, between 0.40 and 0.50 as suspicious, and below 0.40 as positive.

Antigen ELISA

The commercial BVDV antigen ELISA (IDEXX BVDV Ag/Serum Plus IDEXX Laboratories, Westbrook, Maine, USA) kit was utilized to detect the presence of pestivirus antigens. The test, based on the principle of detecting viral antigens in study samples, was performed according to the procedure reported by the manufacturer. Ag ELISA results were calculated for each sample.

$$S\text{-}N = OD_{S450} - OD_{N450controlmean}$$

where OD_{450} refers to the optical density at 450 nm of the sample (S) and negative control mean (N). The samples with the values of 0.30 and below were evaluated as negative and above 0.30 as positive. ELISA-antigen positive animals were sampled again two weeks later to verify whether these animals corresponded to PI status or suffered a transient infection.

Statistical analysis

Data processing was carried out in the R program (R Core Team, 2018). Descriptive statistics were performed to determine the ratio of flocks with respect to the corresponding ratios of seropositive animals. GEE was performed by utilizing the R "geepack" package (R Package geepack for Generalized Estimating Equations) to estimate the overall within-flock seroprevalence. Potential risk factor parameters associated with pestivirus infection were compared in terms of seroprevalence. Logistic regression analysis was conducted to determine the impact shares of risk factors thought to be effective on pestivirus infection. The disease was defined as positive (1) or negative (0) according to the serological test results of 460 samples included in the application in the study. According to the serological test results, it was defined as a categorical dependent variable (Y) that can take a value with two results. The interpretation of the model was carried out using odds ratios (OR).Each of the risk factors was examined with seropositivity using contingency tables. Fisher's exact test and chi-square test were used to examine the relationships between response variables and explanatory variables. The value of $p<0.05$ was accepted as statistically significant. Factors associated to BDV seropositivity with a $p < 0.10$ were included in the multiple model. Multiple logistic regression was used to assess the associations of potential risk factors with BDV seropositivity. The final model fit was assessed with Hosmer-Lemeshow goodness-of-fit test (Dohoo et al., 2010)

RESULTS

Of the 460 sera tested by antibody ELISA, 113 samples were detected as seropositive. The apparent overall within-flock seroprevalence was estimated to be 24.57% (95% CI: 20.85-28.7) based on the GEE model. The rate of positive sheep in each flock varied between 8.33-57.14%. The overall true seroprevalence was calculated to be 25.51% (95% CI: 21.65-29.60), considering the sensitivity (96.3%) and specificity (100%) of the Ab-ELISA test kit. The relationship between the animals' age groups (1-3 years old; >3 years old) was found to be statistically significant (p-value:0.0003<0.05; χ^2:13.15). Detailed results are presented in Table 1.

Table 1. Prevalence of antibodies to BDV with 95% CI according to GEE model

Flock	No of sampled animals	positive	prevalance%	95% CI
I.	48	4	8.33	3.29-19.55
II.	56	32	57.14	44.14-69.23
III.	60	12	20.00	11.83-31.78
IV.	58	10	17.24	9.64-28.91
V.	54	17	31.48	20.68-44.74
VI.	58	8	13.79	7.16-24.93
VII.	64	14	21.88	13.5-33.43
VIII.	62	16	25.81	16.55-37.88
	460	113	24.57	20.85-28.7

Of the 460 serum samples examined by antigen ELISA, 9 (1.96%) were determined to be positive for the presence of antigen, they were negative for the presence of BDV-specific antibodies. The said animals were resampled 14 days later. Of the 9 animals which were positive for the BDV antigen in the first sampling, 2 were positive in the second sampling, and 2 samples were negative for BDV-specific antibodies. It was determined that these PI animals had moderate to high seropositivity, and there were more animals aged > 3 years. Two sheep (0.43%) determined to be PI were removed from the enterprises they belonged to.

In the study, the differences in the ratio of seropositive animals were analyzed in terms of the animal purchase, abortion histories, sub-region conditions, flock management, flock composition, the presence of other clinical diseases in the flock (pneumonia, gastrointestinal, respiratory problems, etc.) variables. The detected differences between the variants in question were statistically assessed following epidemiologic principles. The odds ratio was calculated to be 1.56 times higher in terms of flock management (the nomadic status compared to the sedentary status), the odds ratio was calculated to be 0.45 times higher in terms of the age range (animals aged above 3 years compared to animals in the 1-3 age range), the odds ratio was calculated to be 1.57 times higher in terms of the presence of cows in the flock (yes/no), the odds ratio was calculated to be 1.54 times higher in terms of the presence of other clinical diseases in the flock (yes/no).Detailed results involving odds ratios for these risk factors and the analysis of risk factors associated with seropositive BD are presented in Table 2.The multiple regression logistic model determined that age, flock effect, the presence of cattle in the farm, and the status of other clinical diseases are risk factors for BDV infection (Table 3).

Table 2. Risk factors related to seropositive for Border Disease

Factors	Categories	positive	negative	*p*-value	OR	95% CI
Agro-ecological sub region	I.	24	75	0.01	0.50	0.28-0.88
	II.	55	86	0.06	2.09	0.95-4.58
	III.	11	72	0.15	1.58	0.83-3.01
	IV.	23	114			
Other clinical diseases	yes	58	141	0.04	1.54	1.00-2.36
	no	55	206			
Age	1-3 years	46	209	0.0003	0.45	0.29- 0.69
	>3 years	67	138			
Flock status	sheep with cattle	57	136	0.03	1.57	1.03-2.42
	no cattle	56	211			
	sheep with goat	36	138	0.13*	0.70	0.45-1.11
	no goat	77	209			
	mixed (goat or cattle)	61	143	0.01	1.67	1.09-2.56
	sheep only	52	204			

Table 3. Factors included in the multiple logistic regression analysis

Variable	Categories	b	SE	OR	95% CI	p-value
Flock effect	nomadic*					
	sedentary	-0.53	0.23	0.58	0.37-0.92	0.02
Age	1-3 years*					
	>3 years	0.83	0.26	0.61	1.46-3.54	< 0.001
Other clinical diseases	Yes*					
	No	0.46	0.23	1.58	1.02-2.45	0.03
Flock status	sheep with cattle*					
	no cattle	-0.560	0.24	0.57	0.36-0.89	0.01

*Reference category
b: regression coefficient
SE: Standard Error
OR:Odss Ratio

DISCUSSION

Seroprevalence studies are essential in determining the exposure to pestiviruses in a flock, detecting active infection in a flock, and in the preparation of control eradication programs and the creation of implementation strategies (Houe, 1999). Different methods are used for the diagnosis of pestivirus infection, and blocking and indirect ELISA methods are commonly used serological tests (Sandvik, 2005). The detection of antibodies by ELISA in ruminants after pestivirus infection provides reliable results in terms of seroconversion (Feknous et al., 2018). ELISA-based tests have been used in studies to determine the seroepidemiology of pestivirus infection in small ruminants in various countries and continents of the world (Krametter-Froetscher et al., 2007; Mishra et al., 2009; Avci and Yavru, 2014; Feknous et al., 2018).

Seroprevalence rates in sheep vary between 0-50% on a country basis (Nettleton et al., 1998). In this study, the apparent overall within-flock prevalence was determined to be 24.57% (95% CI 20.85-28.7). The overall true seroprevalence was calculated to be 25.51% (95% CI 21.65-29.60). The study results are similar to the individual animal seroprevalence findings in the studies conducted in Austria (29.4%), India (23.4%), Spain (17.9%), Iran (21.20%), and the inland and coastal zones of Turkey (18.94%) (Mainar-Jaime et al., 1999; Okur-Gumuşova et al., 2006; Mishra et al., 2009; Krametter-Froetscher et al., 2010; Muhammadi et al., 2011). However, a various seroprevalence rate of 51-90.9% was observed at the individual level in some studies in other countries (Berriatua et al. 2004; Gaffuri et al., 2006; Schiefer et al., 2006; Valdazo-Gonzalez et al., 2006). These variable seroprevalence rates can be attributed to many factors such as the management system specific to the country in general, the biosecurity measures at the entry of new animals into farms, poor housing conditions, insufficient information about the disease, and the lack of screening tests at regular intervals.

The prevalence of PI sheep or viremic sheep worldwide is between 0.3% and 20%. The prevalence of PI is 0.32% in Austria and varies between 0.24-0.6% in Spain (Valdazo-González et al., 2008; Martin et al., 2015). The PI sheep rate in this study was determined to be 0.43%. The reason why this rate is lower compared to the rates in some other studies can be explained by the fact that there are animals slaughtered for economic purposes and this is a factor that reduces the probability of detecting PI animals during sampling (Valdazo-González et al., 2006). Another reason may be due to the time the infection has occurred in the flock. Furthermore, the circulation of the infection in the field is provided by acutely and persistently infected animals. Although the number of PI animals is usually low, it ensures the continuity of the virus in the flock. Therefore, it is very important to check all animals in the flock periodically from virological aspects and determine whether newborns are persistently infected.

According to the correlation test between seropositivity and seronegativity in the distribution of the age groups in the study, it was detected that positivity increased with increasing age. A higher seroprevalence rate was detected in sheep aged > 3 years old (32.68%) compared to sheep aged between 1-3 years (18%), which is explained by their long-term exposure to pestiviruses. The high seroprevalence rates observed in the study in animals aged > 3 years can be explained by the increased chance of exposure to the virus in older animals compared to younger animals (Berriatua et al., 2004; Mishra et al., 2008). The fact that the sheep sampled in the study are older than one year, the decrease in maternal antibodies until this age, and the presence of the antibodies detected since sheep in Turkey are not vaccinated against pestiviruses depend on the direct exposure to pestiviruses.

BDV prevalence and its association with various risk factors were analyzed. An investigation of risk factors associated with the disease has become significant in taking control measures for infection. In the current research, the rates of seroprevalence varying between the sub-regions were also observed. Seroprevalence (P) was lower in sub-region III (P=13.25%; OR:2.09; 95% CI: 0.95-4.58) compared to sub-region II (39%). The low seroprevalence (13.25%) observed in sub-region III can be explained by the fact that most of the flocks in that region are immobile. It can also be associated with the presence of mountainous areas in this sub-region. Such conditions may be related to the absence of contact with infected flocks and the fact that transmission also depends on the degree of contact between animals (Nettleton et al., 1992; Feknous et al., 2018).

A lower prevalence was detected in sheep flocks in the sedentary system (P= 20.18%; OR: 1.56; 95% CI: 1.01-2.41) compared to transhumant flocks (P=28.14%). The high prevalence observed in transhumant flocks can be explained by the fact that flocks are on the move to reach more grazing land and there

may be contact with other flocks. It has also been reported in previous studies that transhumance has a significant effect as a risk factor at the individual level (Tabbaa et al., 1995; Krametter-Froetscher et al., 2007; Martin et al., 2015).

In some of the pestivirus seropositive sheep flocks, a history of clinical symptoms including pneumonia, weakness, enteritis, a history of reproductive failure, abortion, stillbirth and birth of small and weak lambs is based on breeders' statements. According to the statements of flock owners, it has been observed that seroprevalence is higher in flocks with other clinical diseases (diarrhea, respiratory problems, weak lambs) in the flock history in comparison with flocks without clinical symptoms and the odds ratio increases in direct proportion (P=29.14%; OR:1.54; 95% CI: 1.00-2.36). Seropositivity was determined to be statistically significant (p-value :0.04<0.05; χ^2:3.97). In this context, pestivirus infections in sheep should not be ignored because they may cause the suppression of the immune system against common pathogens that cause economic losses in farming.

Furthermore, the presence of cattle on the farm in terms of flock status was calculated to increase the odds ratio for BDV by 1.57 times compared to the absence of cattle (p-value:0.03<0.05; OR:1.57; 95% CI:1.03-2.42). Likewise, the presence of cattle and goats on the farm was calculated to increase the odds ratio for BDV by 1.67 times compared to sheep farms alone (p-value:0.01<0.05; OR:1.67; 95% CI: 1.09-2.56). This can be explained by the presence of pestivirus transmission cases from cattle to sheep or from sheep to cattle, as has been reported in previous studies (McCullough et al., 1987; Paton et al., 1995; Lindberg et al., 1999; Krametter-Froetscher et al., 2007; Rosamilia et al., 2013; Kaiser et al., 2017; Şevik, 2020).

CONCLUSIONS

As a result, the prevalence of BDV in sheep may be an important risk factor for pestiviral infection of other species in the region, cattle that share common pastures with sheep. When seropositivity and PI rate of BDV infection are evaluated together, the removal of PI animals from the flock, taking and implementing biosecurity measures, ensuring the protection and control of the disease will be important for the livestock sector. Further studies aimed at isolating the BDV strains circulating in Turkey will help to understand the molecular epidemiology and dynamics of the pestivirus infections in the country better.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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Use of Multivariate Adaptive Regression Splines, Classification Trees and ROC Curve in Diagnosis of Subclinical Mastitis in Dairy Cattle

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ABSTRACT: Subclinical mastitis is one of the most significant diseases that cause economic losses in dairy cattle farming. This research was conducted on 112 heads of Holstein Friesian dairy cattle to reveal the relationship between subclinical mastitis and milk composition and milk quality.In the study, CMT (California Mastitis Test) and CSCC (Classified Somatic Cell Count) used in the diagnosis of subclinical mastitis were used as a binary response variable i.e. healthy and unhealthy. Potential predictors included here were lactation number, days in milk (DIM), darkness-lightness ranges between 0=black and 100=white (L*), green-red ranges between - a*=-60 and a*=+60 (a*), blue-yellow ranges between -b*=-60 and b*=+60 (b*), redness-yellowness (Hue°), vividness-dullness (Chroma), milk fat, milk protein, lactose, milk freezing point, solid non-fat SNF, density, solids, pH, and electrical conductivity. Classification and Regression Tree (CART), Chi-Squared Automatic Interaction Detection (CHAID), Exhaustive Chi-Squared Automatic Interaction Detection (Ex-CHAID), Quick, Unbiased, Efficient, Statistical Tree (QUEST), and multivariate adaptive regression splines (MARS) were used as data mining algorithms that help to make an accurate decision about detecting influential factors increasing the risk of subclinical mastitis.

In conclusion, better classification performances of CART and MARS data mining algorithms were determined compared with those of the remaining algorithms to correctly discriminate between healthy and unhealthy cows.

***Keywords*:** CMT, CSCC, Classification trees, MARS algorithm, Subclinical Mastitis, Milk quality.

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INTRODUCTION

A major part of total milk production in developed countries is produced by dairy cattle that are more suitable to intensive production compared to small ruminants i.e. sheep and goat.Dairy milk is a significant nutrient-rich food for growing healthy human generations and a vital animal product for progressing the country's economy. The quality of milk that is indispensable for food safety is closely associated with the udder health of cows. Mastitis is one of the important diseases for dairy cattle in the world, and it occurs as a result of inflammation of the udder tissues due to infection.It is an animal welfare problem that negatively affects milk quality and causes economic losses in milk yield per cow (Green et al., 2002; Sharma et al., 2011; Walsh et al., 2011). Variability of mastitis disease was ascribed to genetic (breed, herd) and environmental factors i.e. lactation number, lactation stage, parity, calving month, age, calving year, feeding, and managerial conditions (Aytekin et al., 2018; Sinha et al., 2021). Akdag et al. (2017) reported that udder-related traits were necessary to be considered in dairy cattle breeding strategies. Ural (2013) evaluated the relationship between subclinical mastitis and udder traits in Holstein-Friesian dairy cattle reared in the Aydın province of Turkey and emphasized that it was necessary to ascertain udder traits decreasing SCC amount of the cow's milk for selection strategies. Subclinical mastitis is a significant determinant that adversely affects profitability in a dairy farm, depending on the health status of cows that can change based on managerial conditions.

Detection of subclinical mastitis in dairy cattle is made by California Mastitis Test (CMT), White Side Test (WST), Surf Field Mastitis Test, Sodium Lauryl Sulphate Test (SLST), Somatic Cell Count (SCC), Electrical Conductivity (EC), milk colour-related sensor device, biochemical analyses, and the occurrence of pathogens in milk (Eyduran et al., 2005; Islam et al., 2011; Gáspárdy et al., 2012; Aytekin and Boztepe, 2014; Hoque et al., 2015; Mpatswenumugabo et al., 2017). El-Sayed et al., (2015) studied the relationship of bacteria counts with SCC, EC, and chemical composition of the cow's milk as part of subclinical mastitis diagnosis. Da Costa Ribeiro et al., (2016) mentioned that chlorine and lactose amounts of milk can be used in the diagnosis of subclinical mastitis.

EC, CMT, and SCC diagnosis techniques were appraised relatively as part of the detection of subclinical mastitis (Špakauskas et al., 2006; Kandiwa et al., 2017). It was reported that high SCC amounts in the cow milk decreased casein, lactose, and fat amounts of milk, which shortened milk shelf-life and obstructed conversion of the milk to other milk products (Eyduran et al., 2005). Ribeiro et al, . (2016) reported that changes in lactose, protein, and fat could be with enhancing SCC amount. El-Sayed et al., (2015) reported that EC in the milk of a healthy animal had the range of 4.4 and 5.5 (mS/cm).

Relationships between udder characteristics, milk composition, lactation milk yield and milk quality and composition etc. of subclinical mastitis (SCC or CMT) in dairy cattle were investigated (Sharma et al., 2011; Kaşıkçı et al., 2012; Akdag et al., 2017; De Oliveira Mouraet al., 2017).From the point of improving new breeding strategies, it is imperative to determine the relationship between subclinical mastitis and the amount, quality, composition of the cow's milk together with environmental factors mentioned above. However, there are few previous studies in dairy cattle. Moura et al., (2017) evaluated the correlation between subclinical mastitis (EC and SCC) and the Physico-chemical composition of the milk in Zebu cows. Tiwari et al., (2017) utilized ROC (Receiver Operator Characteristics) analysis to estimate threshold values regarding EC, pH, and SCC that help to diagnose subclinical mastitis of crossbreed cows raised under subtropical conditions. Aytekin et al., (2018) comprehensively investigated the relationship of subclinical mastitis (CMT) with calving month, electrical conductivity, colour, composition, and quality traits of milk in cattle breeds i.e. Brown Swiss and Holstein-Friesian with the methodology of tree-structured CART) data mining algorithm. To make a better decision about the reliable diagnosis of the subclinical mastitis with EC, SCC, and CMT in dairy cattle, application of powerful statistical techniques i.e. Logistic Regression (Altay et al., 2019; Kılıç and Keskin, 2019), CART (Aytekin et al., 2018), support vector machines (SVM, Mammadova and Keskin 2015), artificial neural network (ANN, Mikail and Keskin, 2015, Fuzzy Logic (Coskun and Zulkadir, 2018) and adaptive neuro-fuzzy inference system (ANFIS, Mikail and Keskin, 2015) may be a noteworthy opportunity for animal breeders. However, the applicability of the MARS data mining algorithm, which is a non-parametric regression method that discloses the high dimensional relationship between sets of dependent and explanatory variables without necessitating distributional and functional assumptions of the variables, has not yet been recognized in subclinical

mastitis diagnosis with the aid of EC, SCC and CMT together with quality, composition, and colour traits of the cow's milk in dairy cattle. Therefore, the main aims of this investigation were to find milk quality, composition, and colour traits affecting subclinical mastitis based on CMT and CSCC as binary variables i.e. healthy and unhealthy through CART, CHAID, Exhaustive CHAID, QUEST, and MARS classification algorithms, and to obtain evidence to select the best subclinical mastitis diagnosis method between CMT and CSCC, depending upon classification performances of the data mining algorithms.

MATERIALS AND METHODS

Materials

In this study, 112 head of Holstein Friesian cattle used were obtained from a private farm in Konya, Turkey. Milk samples were collected in the morning milkingduringthe summer of 2019.Milk sampling was performed with primiparous dairy cows averaging 152.30±14.95 (Mean±SE) days in milk (DIM) and was taken from cows without a clinical history of mastitis in herd. All cows fed ad libitum with a mixture of concentrated feed and forage such as straw, alfalfa, fescue grass, and corn silage.

Milking, Milk samples, and Milk analysis

Holstein Friesian cows were housed in a free-stall barn and fed ad libitum with a mixture of concentrated feed and forage as total mixed ration (TMR). Dairy cows milked three times daily in a 2 x 15 parallel milking parlor ENGS, EcoHerd, Version 1.01). Since there is no classification tree-based power analysis, a power analysis based on logistic regression, which is the simplest classification, has been performed. The probability of mastitis = 0.3235, odds ratio = 4.3731, and the number of animals with 90% power calculated by reference to the study (Altay et al., 2019) is 97.In order to have the power of the test in the study over 90% and to provide a homogeneous data structure, the number of animals was determined as 112.The data of 112 cows with first lactation number were obtained from the herd management system. Milk samples were obtained from 112 dairy cattle that had been milked with two milkers by using sampling equipment during milking time to represent homogeneous of all milk. After the morning milking, analyses were immediately conducted. Fat (%), protein (%), lactose (%), freezing point (^{o}C), SNF (%), density (kg/m^3), total solids (%), pH, and conductivity (uS/cm) traits, which were examined as milk components, were immediately analyzed two times with the help of an ultrasonic milk analyser (LACTOSCAN MMC30, Milkotronic Ltd, Bulgaria). Somatic Cell Count (SCC) in milk was analyzed by an electronic counter (Nucleocounter SCC-100, Chemometec, Denmark). California mastitis test (CMT) scores of all samples were determined by using a same solution, equipment and expert. Milk samples were homogeneously taken from each cow at the milking by using milk sampler. Then, milk samples were placed in a plastic test paddle, divided into 4 separate wells, in order to determine mastitis status. CMT solution was added on the milk samples taken and after mixing same direction in an oval shape for about 20 seconds, it was diagnosed by the expert (Shitandi and Kihumbu, 2004). All milk samples were screened for subclinical mastitis by the CMT to determine the healthy and unhealthy status of cows. Also, colour characteristics of milk samples were measured for CIELAB system measuring parameters by the Minolta Chroma Meter CR-400 (Konica Minolta, Inc., Osaka, Japan) (CIELAB, 1976). By using The L*, a*, and b* colour values, Hue°and Chroma values were calculated using the formula Hue° =Tan^{-1} x (b*/a*) and Chroma =√ (a^ (*2) +) b^ (*2).

Statistical analysis

CSCC and CMT as a subclinical mastitis diagnosis test were binary dependent variables i.e. healthy and unhealthy. Animals whose CSCC amount is less than 200 000 in 1 cc milk were accepted as healthy; otherwise, they were considered unhealthy. Classification performances of CART (Breiman et al., 1984), CHAID (Kass, 1980), Exhaustive CHAID (Biggs et al., 1991), QUEST (Loh and Shih, 1997), and MARS (Friedman, 1991) algorithms were evaluated comparatively based on accuracy, sensitivity, specificity and area under ROC curve. CART, CHAID, Exhaustive CHAID, and QUEST produces a tree structure to yield the highest accuracy rates as soon as possible. CART (Kovalchuk et al., 2017; Kovalchuk et al., 2018) and QUEST work according to binary node splitting rule, but CHAID and Exhaustive CHAID algorithms run based on multiway node splitting rule (Akin et al., 2018). As a modified form of the CART algorithm, MARS is used to find predictors with hinges function for a better solution of binary logistic regression. Maximum tree depth was used for CART (5), QUEST (5), and both CHAID algorithms (3) by default. In the 5-fold cross-validation, the whole data set (112 records) was randomly separated into 10 approx. equal parts of 21 or 22 records, from which nine were

used to train a given type of prediction model and one served as an independent test set. This process was repeated 5 times. The minimum number of parent and child nodes was 10 and 5 for decision trees i.e. CART, CHAID, Exhaustive CHAID, and QUEST. Optimal trees of the decision tree algorithms were produced after their resubstition costs were very close to corresponding cross-validation costs (Tyasi et al., 2021). Accuracy is an algorithm's proportion of correctly classifying healthy and unhealthy animals. Sensitivity is the algorithm's proportion of correctly classifying unhealthy animals. Specificity is the algorithm's proportion of correctly classifying healthy animals (Grzesiak and Zaborski, 2012). The confusionmatrix for the classifier algorithms is given in Table 1.

Table 1. Confusion table for the classifier algorithms

		Predicted as	
		Unhealthy	**Healthy**
Observed	**Unhealthy**	A	B
	Healthy	C	D

The expressions A, D, B, and C gave in the following equation represent the numbers of true positive, true negative, false positive, and false negative, respectively. The formula developed by (Hanley and McNei, 1982) was used to determine AUC (AUCse).

Accuracy = (A+D) / (A+B+C+D)

Sensitivity = A / (A+B)

Specificity = D / (C+D)

Error proportion = 1 - Accuracy

$$^{se}AUC= \sqrt{\frac{AUC(1-AUC)+(n_A-1)(q1-AUC^2)+(n_B-1)(q2-AUC^2)}{n_A n_B}}$$

n_A=A+C and n_B=B+D

$$q1= \frac{AUC}{2-AUC} \quad \text{and} \quad q2= \frac{2AUC^2}{1+AUC}$$

Pairs of algorithms in the area under ROC curve were compared based onthe z test.

Statistical analyses associated with CART, CHAID, Exhaustive CHAID, and QUEST were IBM SPSS 23 (IBM Corp. Released, 2015). MARS analysis was performed using earth (v5.1.2; Milborrow, 2019) and caret (v6.0.86; Kuhn, 2020) packages of R software (R Core Team, 2020; Kuhn and Johnson, 2013; Eyduran et al., 2019; Akin et al., 2020). The trial version 19.5.1 of the MedCalc software was used to calculate the area under ROC curve and comparison (AUC) and to compare pairs of algorithms in the area. Also, logistic regression-based power analysis used to determine the sample size in the study was performed in G*Power package program version 3.1.7 (Faul et al., 2013).

RESULTS AND DISCUSSION

Table 2 presents descriptive statistics of milk quality, composition, and colour traits for each diagnostic test. Although the method averages are close to each other in terms of the traits considered in general (except for SSC), it was observed that there were differences between the average traits of healthy and unhealthy animals regardless of the method.

Table 2. Descriptive statistics of parameters of milk quality for each diagnosis test

Variables	Methods	Diagnosis	N	Minimum	Maximum	Mean±SE	StDev	CoefVar
DIM (day)	**CMT**	**Healthy**	63	6.00	499.00	123.20±13.20	105.00	85.16
		Unhealthy	49	11.00	424.00	181.40±16.70	116.80	64.39
	CSCC	**Healthy**	77	6.00	499.00	128.50±12.50	110.10	85.72
		Unhealthy	35	22.00	424.00	193.20±18.50	109.70	56.76
Morning Milk (kg)	**CMT**	**Healthy**	63	6.90	21.60	13.14±0.49	3.87	29.48
		Unhealthy	49	1.90	19.10	11.01±0.59	4.12	37.43
	CSCC	**Healthy**	77	5.60	21.60	12.98±0.43	3.80	29.27
		Unhealthy	35	1.90	18.80	10.52±0.73	4.30	40.86
L	**CMT**	**Healthy**	63	81.42	89.75	86.90±0.21	1.64	1.89
		Unhealthy	49	82.98	89.35	86.25±0.20	1.38	1.59
	CSCC	**Healthy**	77	81.42	89.75	86.86±0.18	1.61	1.86
		Unhealthy	35	82.98	88.56	86.07±0.22	1.28	1.49
a	**CMT**	**Healthy**	63	-3.55	4.11	-2.34±0.14	1.09	-46.33
		Unhealthy	49	-3.56	-1.71	-2.70±0.07	0.49	-17.98
	CSCC	**Healthy**	77	-3.55	4.11	-2.33±0.11	0.99	-42.39
		Unhealthy	35	-3.56	-1.95	-2.87±0.08	0.45	-15.55

b	**CMT**	**Healthy**	63	-0.45	6.95	2.65±0.19	1.50	56.56
		Unhealthy	49	-0.08	8.05	3.63±0.30	2.12	58.29
	CSCC	**Healthy**	77	-0.45	6.95	2.58±0.17	1.46	56.38
		Unhealthy	35	0.16	8.05	4.16±0.37	2.17	52.05
H	**CMT**	**Healthy**	63	-66.73	59.40	-38.65±3.05	24.21	-62.63
		Unhealthy	49	-66.14	1.83	-48.11±2.50	17.51	-36.40
	CSCC	**Healthy**	77	-66.73	59.40	-39.19±2.67	23.42	-59.75
		Unhealthy	35	-66.14	-3.86	-50.70±2.70	15.95	-31.47
C	**CMT**	**Healthy**	63	1.91	8.07	3.80±0.15	1.20	31.55
		Unhealthy	49	2.37	8.80	4.68±0.26	1.82	38.84
	CSCC	**Healthy**	77	1.91	8.07	3.73±0.13	1.14	30.57
		Unhealthy	35	2.38	8.80	5.18±0.32	1.87	36.05
Fat (%)	**CMT**	**Healthy**	63	1.97	4.98	3.53±0.09	0.70	19.68
		Unhealthy	49	2.22	4.96	3.85±0.09	0.60	15.70
	CSCC	**Healthy**	77	1.97	4.98	3.56±0.07	0.66	18.49
		Unhealthy	35	2.22	4.96	3.93±0.11	0.64	16.37
Protein (%)	**CMT**	**Healthy**	63	2.71	3.53	3.24±0.02	0.14	4.33
		Unhealthy	49	2.62	3.52	3.24±0.02	0.15	4.67
	CSCC	**Healthy**	77	2.62	3.53	3.22±0.02	0.16	4.83
		Unhealthy	35	3.06	3.52	3.27±0.02	0.11	3.40
Lactose (%)	**CMT**	**Healthy**	63	4.06	5.28	4.85±0.03	0.21	4.31
		Unhealthy	49	3.92	5.27	4.84±0.03	0.23	4.73
	CSCC	**Healthy**	77	3.92	5.28	4.83±0.03	0.23	4.82
		Unhealthy	35	4.57	5.27	4.89±0.03	0.17	3.54
Freezing Point (^{o}C)	**CMT**	**Healthy**	63	-0.62	-0.47	-0.56±0.01	0.03	-4.90
		Unhealthy	49	-0.63	-0.45	-0.57±0.01	0.03	-5.38
	CSCC	**Healthy**	77	-0.62	-0.45	-0.56±0.01	0.03	-5.39
		Unhealthy	35	-0.63	-0.53	-0.57±0.01	0.02	-4.21
SNF (%)	**CMT**	**Healthy**	63	7.39	9.62	8.83±0.05	0.38	4.30
		Unhealthy	49	7.13	9.59	8.81±0.06	0.42	4.73
	CSCC	**Healthy**	77	7.13	9.62	8.79±0.05	0.42	4.83
		Unhealthy	35	8.32	9.59	8.90±0.05	0.31	3.53
Density (kg/m^3)	**CMT**	**Healthy**	63	1023.97	1033.29	1030.59±0.19	1.52	4.98
		Unhealthy	49	1023.74	1032.43	1030.24±0.22	1.55	5.12
	CSCC	**Healthy**	77	1023.74	1033.29	1030.41±0.19	1.70	5.58
		Unhealthy	35	1027.49	1032.43	1030.49±0.19	1.12	3.68
Solids (%)	**CMT**	**Healthy**	63	0.61	0.79	0.73±0.01	0.03	4.27
		Unhealthy	49	0.59	0.79	0.72±0.01	0.03	4.69
	CSCC	**Healthy**	77	0.59	0.79	0.72±0.01	0.03	4.78
		Unhealthy	35	0.68	0.79	0.73±0.01	0.02	3.51
pH	**CMT**	**Healthy**	63	6.30	7.00	6.53±0.03	0.22	3.34
		Unhealthy	49	5.64	7.08	6.64±0.04	0.31	4.61
	CSCC	**Healthy**	77	6.26	7.00	6.52±0.02	0.2	3.24
		Unhealthy	35	5.64	7.08	6.71±0.05	0.32	4.77
Conductivity (µS/cm)	**CMT**	**Healthy**	63	3.35	6.22	5.05±0.09	0.74	14.58
		Unhealthy	49	3.36	7.31	4.91±0.13	0.87	17.76
	CSCC	**Healthy**	77	3.35	7.31	5.14±0.09	0.75	14.56
		Unhealthy	35	3.36	6.05	4.67±0.14	0.82	17.56

Table 3 presents the classification performances of the tested data mining algorithms for each subclinical mastitis diagnosis. The areas under ROC (AUC) were found to be significant in all algorithms used in the diagnosis of subclinical mastitis ($P < 0.05$).

The best classification performance for the CMT diagnosis test was recorded for MARS data mining algorithms with sensitivity (0.857), specificity (0.809), and accuracy (0.830) rates above 0.80. MARS algorithm correctly classified 85.7 (%) of unhealthy cows, 80.9 (%) of healthy cows, and 83.0 (%) of all the cows. Based on the CMT diagnosis test, MARS had the biggest area under ROC curve of 0.869 as the best agreement between sensitivity and specificity in the CMT diagnosis test. The best classifier algorithm for detecting subclinical mastitis using CMT was found as MARS, followed by CART. The present CART findings were in near agreement with those recorded by Aytekin et al. (2018) who found that 77.2 (%) of the Brown Swiss and Holstein-Friesian cows were unhealthy (sensitivity), 95.7 (%) of them were healthy in CMT diagnostic test.

Among classifier algorithms whose performances were tested for CSCC subclinical mastitis diagnosis test in the current work, CART was determined to be a promising algorithm that had a sensitivity (0.917), specificity (0.725), accuracy (0.848) rates, and the largest area of 0.890 under ROC curve. CART algorithm correctly classified 91.7 (%) of the cows detected as unhealthy by CSCC, correctly classified 84.8 (%) of healthy and unhealthy cows. Based on the CSCC subclinical diagnosis test, MARS had the biggest area under ROC curve of 0.890 as the best agreement between sensitivity and specificity in CSCC.

As a result of the CSCC diagnosis test, the best agreement between the sensitivity and specificity rates was recorded in the CART classifier according to the largest area under ROC curve constructed for the classifier and the highest sensitivity rate of 91.7 (%). Among the combinations of the algorithm and the diagnosis test, the best one was understood to be the combination of the CART algorithm and CSCC diagnosis test. It could be suggested that CSCC that correctly captured 91.7 (%) of unhealthy cows was the best subclinical diagnosis test in the CART classifier. However, the MARS algorithm correctly classified 93.5 (%) of healthy cows in the CSCC diagnosis test as a result of the highest specificity rate estimated for the algorithm. Higher accuracy estimates of the algorithms evaluated for the CSCC diagnosis test here were obtained (0.786 to 0.848) compared to those recorded by Mammadova and Keskin (2015) with an accuracy rate of 50 (%) for support vector machines and Mikail and Keskin (2015) with the accuracy rates of 36 and 65 (%) for ANN and ANFIS algorithms in SCC diagnosis test. Aytekin et al, . (2018) emphasized easy interpretation of the visual results obtained from CART in the CMT diagnosis test.

The flexible MARS model providing very high classification quality for CMT diagnosis test was produced by two milk colour traits i.e. “a” and “C”.

$GLM_{unhealthy}$ = -0.9504965 - 1.043345 * max (0, a + 2.97) + 1.154928 * max (0, C - 4.46). In this case, the probability of being unhealthy for any cow based on the CMT diagnosis test can be estimated with the help of $P_{UNHEALTHY} = \exp^{GLMunhealthy} / (1 + \exp^{GLMunhealthy})$ where exp the base of natural logarithm whose value is 2.718. For example, the probability of being unhealthy for a cow with a= -2.20 and C=3.29 based on the CMT diagnosis test can be calculated as follows:

$GLM_{unhealthy}$ = -0.9504965 - 1.043345 * max (0, -2.20 + 2.97) + 1.154928 * max (0, 3.29 - 4.46).

$GLM_{unhealthy}$ = -0.9504965 - 1.043345 * max (0, -2.20 + 2.97) + 1.154928 * max (0, 3.29 - 4.46).

Where, max (0, -2.20 + 2.97) = max (0, 0.77) = 0.77 max (0, 3.29 - 4.46) = max (0, -1.17) =0

$GLM_{unhealthy}$ = -0.9504965 - 1.043345 * 0.77 + 1.154928 *0

$GLM_{unhealthy}$ = -0.9504965 - 1.043345 * 0.77 = -1.75387215

$P_{UNHEALTHY} = \exp^{GLMunhealthy} / (1 + \exp^{GLMunhealthy})$

$P_{UNHEALTHY} = \exp^{(-1.75387215)} / (1 + \exp^{(-1.75387215)})$ = 0.1731024 / (1+0.1731024)

$P_{UNHEALTHY}$ = 0.1475595 is expressed as the probability of being unhealthy for a cow with a= -2.20 and C=3.29).

Table 3. Classification performances of the algorithms for each diagnosis test

Methods	Algorithm	Sensitivity	Specificity	AUC	Accuracy	Associated Criterion	P-value
CMT	CART	0.716	0.839	0.742	0.750	0.5788	0.000
	CHAID	0.644	0.773	0.634	0.670	0.5631	0.015
	Exhaustive CHAID	0.716	0.667	0.740	0.696	0.3717	0.000
	QUEST	0.644	0.720	0.628	0.661	0.5382	0.020
	MARS	0.857	0.809	0.869	0.830	0.3894	0.000
CSCC	CART	0.917	0.725	0.890	0.848	0.3394	0.000
	CHAID	0.800	0.773	0.710	0.795	0.4864	0.000
	Exhaustive CHAID	0.800	0.773	0.710	0.795	0.4864	0.000
	QUEST	0.791	0.762	0.696	0.786	0.4853	0.010
	MARS	0.514	0.935	0.776	0.804	0.4843	0.004

Comparison of the algorithms in the area under the ROC curve for both subclinical mastitis diagnostic tests is given in Table 4. The classification performance order for CMT diagnostic test was MARS > CART = Exhaustive CHAID > CHAID = QUEST in area under ROC curve. The classification performance order recorded for CSCC diagnostic test was CART > MARS > CHAID=Exhaustive CHAID=QUEST in terms of the area under ROC curve. CSCC was found superior to CMT in the area under ROC curve of the CART classifier. But, no significant difference was found between CSCC and CMT tests in the area under ROC curve for CHAID, QUEST, and MARS classifiers. A significant point is that an area of 0.869 under ROC curve was estimated as the second-largest value numerically (Table 4). The present ROC curve results indicated that most of the algorithms showed similar trends for CMT and CSCC in comparing subclinical mastitis tests within CHAID, Exhaustive CHAID, QUEST, and MARS algorithms, which was in agreement with those reported by some authors who reported the suitability of EC with CMT and SCC (Kaşıkçı et al., 2012; Aytekin et al., 2018). Mammadova and Keskin (2015) obtained 50% accuracy, 92% specificity, and 89% sensitivity using support vector machines algorithm as well as 43% accuracy, 79% specificity, and 75% sensitivity using binary logistic regression analysis based on SCC subclinical mastitis diagnosis test. Both accuracy rates reported by Mammadova and Keskin (2015) were lower than those of the tested algorithms employed for CSCC and CMT diagnosis tests here.

Table 4. Comparison of the algorithms in the area under ROC curve

Methods	CART	CHAID	Exhause-CHAID	QUEST	MARS
CMT	0.742 ± 0.050^{Bb}	0.634 ± 0.054^{Ca}	0.740 ± 0.047^{Ba}	0.628 ± 0.054^{Ca}	0.869 ± 0.036^{Aa}
CSCC	0.890 ± 0.035^{Aa}	0.710 ± 0.052^{Ba}	0.710 ± 0.058^{Ba}	0.696 ± 0.059^{Ba}	0.776 ± 0.058^{Ba}

$^{A, B}$The difference between the algorithms with the capital letter in CMT or CSCC row is significant (comparison of the algorithms)
$^{a, b}$The difference between diagnostic tests with the letter in any algorithm column is significant (comparison of the subclinical mastitis diagnostic tests)

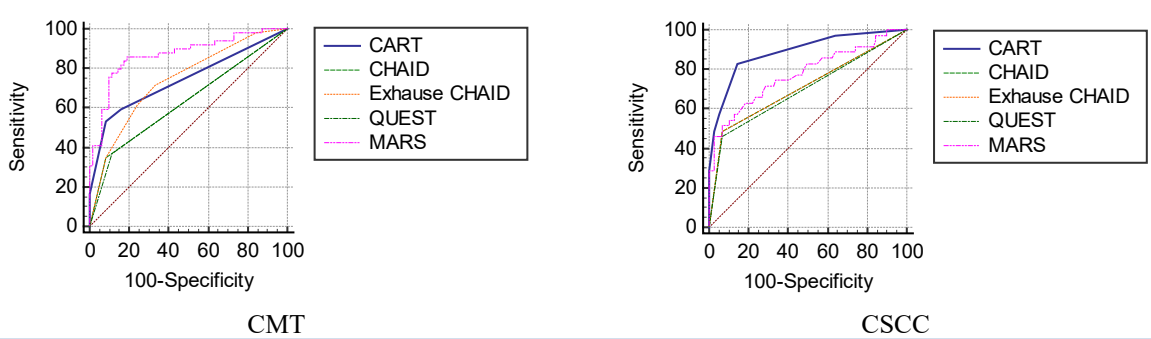

Figure 1. ROC curves of classifier algorithms for each diagnosis test

In the CSCC method used in the diagnosis of subclinical mastitis, Aytekin et al, . (2018) reported that milk color features a *, C *, fat and DIM compatible markers, while in CMT method, EC, “L” and “a”, fat, freezing point, and calving month properties. It has been identified as an important indicator.

As part of CSCC as a diagnostic test for subclinical mastitis, CART was selected as the best classifier (Table 1). 68.8 (%) of the 112 cows in the CSCC diagnosis test were characterized as healthy, and 31.2 (%) of them were classified as unhealthy (Node 0).

The CART classification tree of the CSCC diagnosis test is presented in Figure 2. At the top of the CART classification tree structure, Node 0 was divided into two smaller subgroups i.e. Node 1 (a subgroup of the cows with C < 5.195) and Node 2 (a subgroup of the cows with C > 5.195) according to C milk colour trait at the first tree depth. 81.4 (%) of the cows with C < 5.195 were determined as healthy (Node 1) ; however, 73.1 (%) of the cows with C > 5.195 were characterized as unhealthy (Node 2).

At the second tree depth, Node 1 was branched into two smaller subgroups i.e. Node 3 and Node 4 according to the DIM trait. 96.6 (%) of the cows with C < 5.195 and DIM < 53.5 days were found as healthy (Node 3). 73.7 (%) of the cows with C < 5.195 and DIM > 53.5 days were described as healthy.

At the second tree depth, Node 2 was split into two subgroups i.e. Node 5 and Node 6 according to “a” milk colour trait.

All of the cows with C > 5.195 and a < - 3.195 were determined as unhealthy (Node 5), whereas 56.2 (%) of the cows with C > 5.195 and a > - 3.195 were identified as unhealthy (Node 6). Cut-off values of 5.195 C and - 3.195 a could provide some hints for breeders to detect subclinical mastitis.

At the third tree depth, Node 4 was partitioned into two smaller subgroups i.e. Node 7 and Node 8 according to the DIM trait. In the CART tree diagram, 77.8 (%) of the cows with C < 5.195 and 54.5 < DIM < 77.5 days were recorded as unhealthy (Node 7), but 83.3 (%) of the cows with C < 5.195 and DIM > 77.5 days were characterized as healthy (Node 8).

At the fourth tree depth of the CART algorithm, Node 8 was divided into two smaller subgroups i.e. Node 9 and Node 10 according to milk fat trait. 88.4 (%) of the cows with C < 5.195, DIM > 77.5 days, and milk fat < 4.250 (%) were ascertained as healthy. 60 (%) of the cows with C < 5.195, DIM > 77.5 days and milk fat > 4.250 (Node 10) were found as unhealthy.

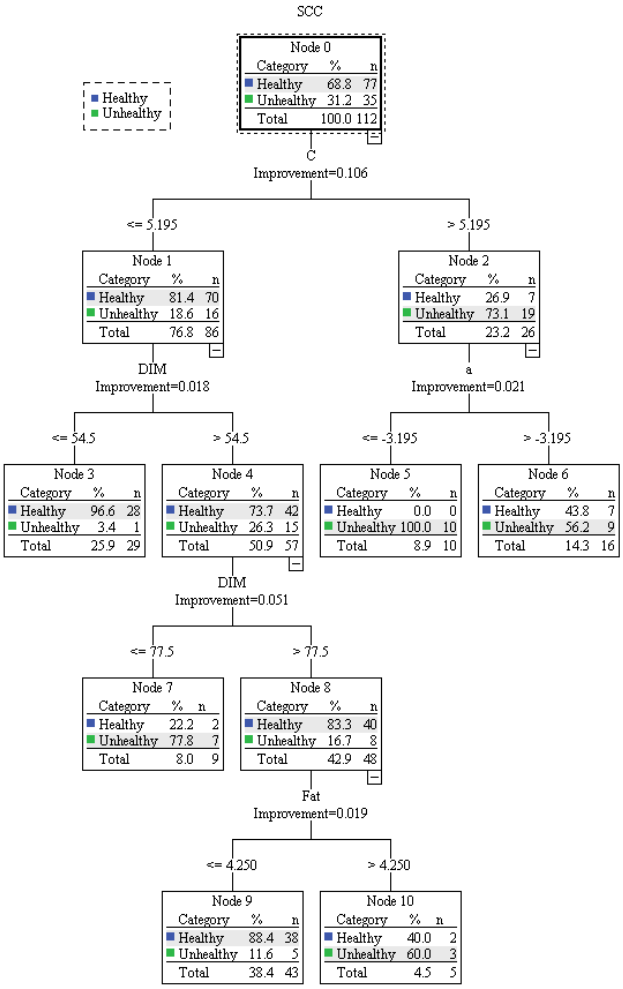

Figure2. CART classification tree of the CSCC diagnosis test.

Aytekin et al. (2018) reported that the CART classification tree created for the CMT subclinical mastitis diagnosis test consists of calving month, EC, milk fat, milk freezing point and milk color (L* and a*). Whereas, the MARS data mining algorithm gave the best classification performance for the CMT and produced an easier interpretable equation for more correctly classifying healthy and unhealthy cows when compared with the results of CART, ANN, ANFIS, SVM algorithms published elsewhere (Mammadova and Keskin 2015; Mikail and Keskin 2015). In this regard, the use of the MARS algorithm that helps to find cut-off values of significant milk traits discriminating unhealthy and healthy cows is advisable together with subclinical mastitis diagnosis tests.

CART analysis produced by Aytekin et al., (2018) reflected that subclinical mastitis risk of cows with

EC > 5.695 and fat > 3.160 (%) was 3.62 times more in comparison with that of those with EC > 5.695 and fat < 3.160. In the study, the risk of cows (60%) with milk fat > 4.250 (Node 10) was found to be 5.17 times more than the risk of cows with fat < 4. 250 (11.6%) (Node 9) when C < 5.195 and DIM > 77.5 days were considered. CSCC and CMT diagnosis tests produced almost similar tendenciesbased on QUEST, MARS, and both CHAID algorithms (Table 3). This finding was nearly in agreement with the previous statements of some authors (Kaşıkçı et al., 2012; Aytekin et al., 2018).

Wide variation in mastitis disease diagnosis was attributed to genetic (breed, herd) and environmental factors i.e. lactation number, lactation stage, lactation period, parity, udder traits, calving month, age, calving year, feeding and managerial conditions, especially statistical analysis techniques.

With the scope of subclinical mastitis detection, the usability of CHAID and especially MARS algorithms were scarce in the literature to reveal the relationship of the mastitis risk with milk quality, composition, colour traits. In this respect, further studies are still required for subclinical mastitis diagnosis with the support of data mining algorithms.

CONCLUSION

In herd management, it is desirable to identify unhealthy animals as soon as possible to obtain healthy products for animal health, and also human health. Also, the failure of farms to distinguish between healthy and unhealthy animals will cause economic losses because of management. With the development of data mining methods in recent years, it will benefit herd management by closing the gaps in diagnosing mastitis. In diagnosis, increasing the detection of mastitis in unhealthy animals and decreasing the margin of error will make a great contribution to herd management.

In the current work, the relationship of subclinical mastitis with milk quality, colour, and composition traits was investigated based on data mining algorithms i.e. CART, CHAID, Exhaustive CHAID, QUEST, and MARS. Among these algorithms, MARS gave the best performance by correctly classifying unhealthy and healthy animals in CMT diagnosis, while the CART algorithm was found to be the best classifier in CSCC (P <0.05). According to the results of CART analysis obtained for SCC, all cows with a < -3.195 and C > 5.195 in their milk could be said to be at risk of subclinical mastitis. The subclinical mastitis risk of cows with C > 5.195 was observed to be four times more than that of those with C < 5.195 in their milk. No significant differences of CSCC and CMT diagnosis tests in the area under ROC curve for other algorithms except for CART were obtained. For a generalization of the current results, the examination of much larger populations in different cattle breeds was recommendable.

In conclusion, the application of CART and MARS algorithms may a good choice for cattle breeders to find cut-off values of influential milk traits correctly discriminating healthy and unhealthy cows.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

The Effects of dietary cysteamine on performance, hatchability, plasma parameters and sex hormone levels in quails (*Coturnix coturnix japonica*)

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ABSTRACT: This study was designed to determine the effects of a single dose of dietary Cysteamine (Cys) on growth performance parameters, meat properties and laying performance, hatchability, plasma glucose, cholesterol, and sex hormone levels of quails. For this purpose, two separated animal experiments have designed as growth period Trial 1 (T1) and laying period Trial 2 (T2) and experiments have conducted one after another.

Trial 1 (T1) : A total of two hundred, 1- day old quail (*Coturnix coturnix japonica*) chicks were randomly allocated to 1 of two treatment groups with 5 replicates, either a control group (basal diet) or a treatment group that contained 90 mg Cys per kg basal diet. During the 5-weekexperimentalperiod, the performance data regarding the growth period were collected. At the end of T1, a total of 30 quail were slaughtered, and the color values, pH, and meat macronutrients were determined in breast meat.Data from T1 showed that the inclusion of 90 mg Cys in the diet affectedneither growth performance nor breast meat parameters of quail.

Trial 2 (T2) : At the end of T1, 40 from each treatment and a total of 80, six-week-old quail were selected randomly to be used in the laying period experiment and allocated to two dietary treatment groups. Forty quails from the control group of T1 were fed with a laying period basal diet. Forty quails from the Cys group of T1 were fed with a laying period diet that contained Cys (90 mg/kg basal diet). During the 10-week laying trial egg production and hatching performance data collected. At the end of the trial, a total of 20 quails were sacrificed and plasma glucose, cholesterol, and sex hormone levels were detected. Results of T2 displayed that 90 mg /kg feed dietary Cysincreased egg mass ($P< 0.05$), however, it was ineffective on other laying and hatching performance parameters. Whereas plasma glucose level was not altered, plasma cholesterol level significantly decreased with the presence of Cys in the diet ($P< 0.05$). Moreover, dietary Cys significantly increased plasma sex hormones levels in both sexes. In conclusion, 90 mg dietary Cys did not show any improvement or depressing effect on the growth, laying, or hatching performance of quails however, its cholesterol-lowering impact and boosting effects on plasma sex hormones are noteworthy.

***Keywords*:** meat, egg, cholesterol, estradiol, testosterone

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INTRODUCTION

Cysteamine (Cys) is an amino thiol and has defined as a natural bioactive substance that modulates the endocrine and metabolic status of animals (Rideau et al. 1990b; McLeod et al. 1995; Hu et al. 2008a). Cys occurs by decarboxylation of cysteine amino acid and, entering into the coenzyme-A structure, take the role in energy metabolism (Doğan et al. 2014). With the discovery that Cys is being part of the coenzyme A pathway, different formulas such as cysteamine hydrochloride, phosphocysteamine, and cysteamine bitartrate have used for various therapeutic strategies (Besouw et al. 2013). Furthermore, there are shreds of evidence that Cys rises the growth rate in mammals and poultry by increasing the growth hormone release (Hall et al. 1986; Yang et al. 2006).Recently reported that Cys has been shown to be effective in increasing growth and improving efficiency in a range of livestock species (Sun et al. 2017). On the other hand, Cys was reported to promote the maturation and fertilization of oocytes and to improve embryo developmental rates *in- vitro* (Urdaneta et al. 2004a; Hu et al. 2008a; De Matos and Furnus 2000a). Despite considerable knowledge about the metabolic functions of Cys, its action mechanism still unclear. Cys has rarely investigated as a dietary supplement in domestic animals including poultry. However, reported that a low dosage of Cys (generally ≤ 80 mg) administration may increase the synthesis of glutathione which is a powerful antioxidant. Contrarily, high dosage Cys administration (generally ≥ 100) may cause oxidative stress (Besouw et al. 2013).The quest for effective alternative feed additive to reduce the costs and optimize production in poultry production continues, and examination of bioactive substances such as Cys that may influence performance, and product quality has become more essential.Cys is an example of an additive studied in countries such as China and Thailand with the purpose of establishing beneficial effects on the performance of pigs and broilers (Nunes et al. 2012). The present study conducted in order to provide scientific data regarding usage of Cys as a feed supplement in poultry diets. Herewith, in this study quail was chosen as an experimental animal due to some advantages like early sexual maturity (6-7 weeks of age) and a high reproduction rate (Shim and Vohra 1984) has chosen. Therefore, the effects of dietary Cys (cysteamine hydrochloride) have investigated in quails' growth and laying periods.Thereby, two experiments that followed each other were designed to examine the effects of a single dose, 90 mg/kg feed dietary Cys on growth performance and meat macronutrients, laying performance, hatching parameters, and plasma cholesterol, glucose, estradiol, testosterone, and Follicle-stimulating hormone (FSH) hormone levels.

MATERIALS AND METHODS

The animal experiments were conducted under the Prof. Dr. Orhan Düzgüneş application and research farm facility of Selcuk University Agriculture Faculty. All animal experiments were carried out according to the local ethics committee directives of Selcuk University.

Experimental design

Trial 1 (T1) :A total of 200, one-day-old quail chicks without sex discrimination were used for a 5-week growth period and this experiment named T1. The quail chicks were randomly divided into two treatment groups, each treatment had 100 quails with five replicates; 20 quails each. The chicks were reared in 33 x 40 x 28 cm size cages under the semi-controlled environment terms (ventilation controlling system) as every compartment of cages had a water nipple, manger, and heater. During the trial, 23 - h light - 1 h dark lighting program were applied. Feed and water were provided *ad-libitum*.

Trial 2 (T2) : At the end of the growth period trial (T1), 10 male and 30 female quail chicks from each treatment group, i.e., a total of 80 quails were randomly selected by considering cage dimensions and gender discrimination. Selected birds were allocated in two treatment groups with five replicates for 10 week laying period, and each compartment contained two males and six females (a total of 8 quails). Birds from the T1 control group were placed in the control group of T2 and were fed with the laying period basal diet. Birds from the T1 Cys group were placed in the Cys group of T2 and fed with a laying period basal diet contained 90 mg Cys kg/feed.Feeding of quails with T2 experimental diets was started when quails were 6 week- old age, however, performance data regarding egg production have got to collect after quails have reached 8 week-old age.The performance data and egg samples were collected during the T2. Quails were reared in 28 x 30 x 40 cm size cages, under the semi-controlled periphery terms (ventilation controlling system) as every compartment of cages had a water nipple, manger, and heater. The lighting programme considered as 16 - h light - 8 h darkness. Feed and water provided as *ad-libitum*.

Experimental diets

Feed raw materials was provided by a local feed factory and nutrient contents of experimental diets were formulated as the National Research Council (NRC 1994). Cysteamine (100% purity, crystal form, Cysteaminium chloride) was provided from Merck® (Darmstadt, Germany).

T1: Growth period diets were contained 23.88% crude protein and 2901 kcal metabolizable energy/kg (Table 1). The control group diet of T1 was the basal growth period diet without Cys supplementation. Cys group diet of T1 was a growth period diet with 90 mg cysteamine supplementation per kg basal diet.

T2: Laying period diets were contained 19.16% crude protein and 2900 kcal metabolizable energy/kg (Table 1).The control group diet of T2 was the basal laying period diet without Cys supplementation. Cys group diet of T2 was a laying period diet with 90 mg cysteamine supplementation per kg basal diet.

Table 1. Nutritional compositions of experimental diets used at T1 and T2

Ingredients (g/kg)	Experimental Diets T1	T2
Barley	76.5	77.5
Corn	380	413.4
Soybean meal	409	295
Sunflower meal	50	71
Soybean oil	45	57
Limestone	11	60
DCP	18.5	15.5
Salt	4	4
Vitamin Premix [A]	2.5	2.5
Mineral Premix [B]	1	1
Lysine	1	1.6
Methionine	1.5	1.5
Nutrients (%)		
Crude Protein *	23.88	19.16
Moisture *	7.73	7.81
Ash*	8.04	12.25
Cellulose	7.51	7.48
Metabolizable energy (kcal/kg)	2901	2900
Calcium	0.96	2.85
Available phosphorus	0.47	0.40
DL-Methionine	0.54	0.50
Methionine + Cysteine	0.90	0.82
L-Lysine	1.35	1.14

T1:Trial 1,T2:Trial 2 ,[A]**:** Premix provided the following per kg of diet: Vitamin A, 15,000 IU; Vitamin D3 1500 IU; Vitamin K 5.0 mg; Vitamin B1 3 mg, Vitamin B2 6 mg; Vitamin B6 5 mg; Vitamin B12 0.03 mg; Niacin 30 mg; Biotin 0.1 mg; Calcium D-pantotenat 12.0 mg; Folic acid 1.0 mg; Choline chloride provides 400 mg [B]**:** Premix provided the following per kg of diet: Manganese 80 mg; Iron 35 mg; Zinc 50 mg; Copper 5,0mg: Iodine 2 mg, Cobalt 0.04mg. *Analyzed value.

Performance parameters

T1: The initial live-weights (LW) of quail were determined at the beginning of the experiment. Thereafter, the average LW was were measured weekly throughout 5 weeks. Before every LW measurement, the manger was cleaned and the average feed intake (FI) was calculated as the difference between the given and residual feed. The LW of each subgroup of quail were gauged in plastic containers, and thelive-weight gains (WG) of treatment groups were calculated on a weekly basis. At the end of the trial, the LW of quails were recorded as final body weight (FBW). During the experiment, daily mortality was recorded. The feed conversion ratio (FCR) of the groups was calculated using average WG and average FI values. At the end of T1, a total of 30 (15 from each treatment group and 3 from each subgroup) quail were randomly selected and slaughtered. Viscera and feet were removed and carcass weight (CW) and carcass yield (CY) were calculated.

T2: The egg production (EP) was recorded daily while the egg weight (EW) was recorded weekly for 10 weeks period. The egg mass (EM) was calculated as EM= (EP × EW) /100. The FI was calculated by the difference between the given and residual feed and recorded every week.The FCR was calculated as FCR = FI /EM.

Meat macronutrients, color values and pH

Breast muscles were split from carcasses from T1 treatment, and the samples were held until the analyses time (24 hours) in refrigerator +4 ℃. The proximate composition of quail meat was determined according to the AOAC method (A.O.A.C. 2000). The moisture value of breast meat was determined after drying in oven at 105 ± 3°C and the total ash content was analyzed with ash furnace at 550 ± 5°C. The crude protein content of meat samples was determined with Kjeldahl and fat content wasdetermined with soxhlet device. The pH value of breast meat was determined by portable pH meter (WTW 2A20-1012 Waterproof pH-Meter) (Horwitz and Latimer 2000) and color values (L*, a*, b*) by Minolta Chroma Meter CR 400 (Minolta Co., Osaka, Japan) (Hunt et al. 1991). The L*, a* and b* parameters correspond to the lightness (-100/+100, dark/white), redness (-100/+100, green/red) and yellowness (-100/+100, blue/yellow), respectively.

Hatching parameters

At the last three weeks of T2, 50 eggs from each

group (total of 100 eggs) were collected for determination of hatchability parameters. Eggs were incubated at 37.5°C and 55-60% humidity for 14 days. Then the eggs were transferred to hatching machinesat 37.2°C and 75% humidity. After the completion of the outputs, embryo deaths and infertility were determined by macroscopic analysis of eggs for calculation of hatchability ratio (HR), fertility ratio (FR), and hatchability of fertile eggs (HOF) (Aygün et al. 2012).

Plasma biochemical analyses

At the end of T2, one male and one female quail from each subgroup; 10 quails from each treatment (total of 20 quails) were slaughtered. Euthanasia of the birds was realized decapitation method. Blood samples were collected from the jugular vein and transferred to heparin-coated glass tubes. The sample tubes were centrifuged at 3000rpm (rpm= round per minute) for 10 minutes and plasma was obtained. Plasma glucose and total cholesterol levels were detected with a Siemens-dimension RLA-MAX (Germany) device using the photometric method. Levels of estradiol, testosterone, and FSH hormones in blood plasma were determined automatically by Siemens-advia centaur XP (Germany) device using the chemiluminescence method (Erdinçler et al. 2004).

Statistical analysis

Statistical analysis was performed using Minitab (Minitab 2000). Growth, laying, and hatching performance and meat properties data differences were determined using by T-test. General linear model, 2x2 factorial design was applied to plasma biochemical and hormone data, and differences were determined by using Tukey test ($P< 0.05$).

RESULTS

Results of T1

In the present study, Cys was used as dietary supplements to investigate its effects on quail's growth performance parameters. Data obtained from T1 are summarized in Table 2. and results showed that 90 mg Cys supplementation in the diet did not affect any parameter on growth period of quails.

Effects of the feeding of quails with 90 mg additional dietary Cys during T1 on meat color, pH, and meat nutrients content are presented in Table 3. Data showed that at the end of 5 weeks T1, Cys supplementation did not change meat color and pH values and not affect the macronutrients composition of meat compared with the control group.

Table 2. Effects of dietary cysteamine on growth performance parameters in T1

	Control group	Cysteamine group	*P value*
FCR	3.06±0.051	2.95±0.028	*0.103*
FI (0-5 Week) (g)	472.30±13.0	459.87±1.80	0.410
AWG (0-5 Week) (g)	154.29±2.50	156.01±1.40	*0.569*
FBW (g)	173.8±4.50	181.72±2.90	*0.189*
CW (g)	123.47±2.4	130.43±2.2	*0.070*
CY (%)	71.1±0.58	71.89±2.00	*0.727*

T1: Trial 1, FCR: Feed conversion ratio, FC: Feed intake, AWG: Average weight gain, FBW: Final body weight, CW: Carcass weight, CY: Carcass yield

Table 3. Effects of dietary cysteamine on meat color, pH value and nutrient composition at the end of T1

		Control group	Cysteamine group	*P value*
Meat color values				
	L*	42.37±0.58	41.77±1.1	0.634
	a*	6.21±0.32	6.81±0.16	0.156
	b*	3.59±0.39	3.09±0.31	0.364
Meat pH		5.68±0.01	5.64±0.02	0.205
Meat macronutrients (%)				
	Dry matter	29.31±0.46	30.47±0.27	0.070
	Ash	1.35±0.05	1.36±0.03	0.829
	Fat	2.69±0.33	3.12±0.21	0.311
	Protein	23.78±0.39	24.67±0.28	0.105

T1: Trial 1

Results of T2

Data regards on impact of diet Cys on laying performance parameters of quails are demonstrated in Table 4. Outcomes of T2 displayed that long term Cys feeding of quails was not effective on FI, FCR, EW and EP in laying period however, with the inclusion of Cys in diet, EM increased significantly (P< 0.05).

Determined hatching parameters during T2 are summarized in Table 5. None of the hatching parameters did not affected by 90 mg dietary Cys significantly.

Alterations of plasma glucose, cholesterol, estradiol, FSH, and testosterone levels detected at the end of T2 are shown in Table 6. Plasma glucose levels of quails were similar between sexes and, Cys in diet did not affect plasma glucose levels of animals. On the other hand, both, sex, and treatment were effective on plasma cholesterol levels of quails and, it was higher in male quails than females and Cys inclusion in diet significantly decreased blood cholesterol in both sexes (P< 0.05). Plasma estradiol levels as expected was significantly higher in the female quails than male and Cys in diet increased plasma estradiol level (P< 0.05). Additionally, Cys caused higher plasma estradiol level in male whereas decreased in female quails (P< 0.05). Difference of plasma FSH levels between two sexes was insignificant but, dietary Cys raised up plasma FSH level according to control group and, the highest plasma FSH level detected in Male × Cys group (P< 0.05). Plasma testosterone levels of quails were dramatically different between two sexes and, testosterone levels of males quite higher than females (P< 0.05). Dietary Cys caused significantly high plasma testosterone level than control group, and whit the inclusion of 90 mg in diet the plasma testosterone was not change in female whereas significantly boost in male quails (P< 0.05). Results demonstrated that 90 mg dietary Cys per kg basal diet independently gender displayed cholesterol- lowering and boosting-sex hormones effects in quail as a bioactive component.

Table 4. Effects of dietary cysteamine on laying performance parameters in T2

	Control group	Cysteamine group	*P value*
FI (Weekly) (g)	238.4 ±14.0	231.50±8.80	0.698
FCR	3.03±0.28	2.70±0.24	0.401
EM (g)	10.26±0.15 ^{B}	11.35±0.38 ^{A}	0.045
EW (g)	12.44±0.25	12.71±0.18	0.417
EP (%)	82.67±2.80	89.15±2.00	0.102

$^{A, B, C}$ Means in the same row with a different superscript differ significantly (P<0.05). T2: Trial 2, FCR: Feed conversion ratio, FC: Feed intake, EM: Egg mass, EW: Egg weight, EP: Egg production

Table 5. Effects of dietary cysteamine on hatching performance parameters in T2

(%)	Control group	Cysteamine group	*P value*
HR	89,10±4,5	92,28±1,3	0,541
HOF	94,52±2,8	94,10±1,4	0,897
FR	94,48±2,2	98,11±0,78	0,200

HR: Hatchability ratio, HOF: hatchability of fertile eggs, FR: fertility ratio

Table 6. Effects of dietary cysteamine on plasma biochemical and sex hormone levels at the end of T2

		Glucose (mg/dL)	Cholesterol (mg/dL)	FSH (mIU/mL)	Estradiol (ng/dL)	Testosterone (ng/dL)
Sex	*Male*	271.4	276.5 ^{A}	0.129 ^{A}	2.925 ^{B}	375.93^{A}
	Female	268.9	163.6 ^{B}	0.111 ^{A}	7.690 ^{A}	26.60^{B}
Treatments	*Control*	271.4	226.5 ^{A}	0.069 ^{B}	5.221 ^{B}	94.69^{B}
	Cysteamine	268.9	213.6 ^{B}	0.171 ^{A}	5.393 ^{A}	307.84^{A}
Sex × Treatment	*Male×control*	271.8	284.0 ^{A}	0.044 ^{C}	2.393 ^{D}	164.50^{B}
	Female×control	271.0	169.0 ^{B}	0.094^{B}	8.049 ^{A}	24.87^{C}
	Male×Cysteamine	271.0	269.0 ^{A}	0.214^{A}	3.456 ^{C}	587.35^{A}
	Female×Cysteamine	266.8	158.2 ^{B}	0.128^{B}	7.330 ^{B}	28.34^{C}
	SEM	1.631	13.24	0.013	0.557	52.572

$^{A, B, C}$ Means in the same column with a different superscript differ significantly (P<0.05). SEM: Standard error meaning, T2: Trial 2

DISCUSSION

Growth performance and meat properties

Studies regarding the effects of Cys on growth have reported that the positive effects of Cys supplementation for broiler chickens (Yang et al. 2006; Hu et al. 2008b). Moreover, some studies declared that improving effects of dietary Cys depends on its dosage (Jie et al. 2006; Ma et al. 2009). Many studies reported similar positive effects of dietary Cys on growth (Zhengkang 1993; Nunes et al. 2012). On the other hand, despite evidence Cys presence induces better growth, our results revealed that 90 mg/kg dietary Cys was ineffective on the growth performance of quails. These results may be due to that Cys can decrease the activity of digestive enzymes and can negatively affect the growth (Yang et al. 2005; Yang et al. 2006). Additionally, Jeitner and Lawrence (2001) reported that Cys may cause formation of hydrogen peroxide molecules and oxidative stress, and also decreases the activity of glutathione peroxidase. Clearly, in the present study, the dosage of dietary Cys for growing quails was not at the toxic levels that may negatively affect the growth performance. Nevertheless, 90 mg/kg diet Cys did not display the expected improving impact on the growth of quails.

Meat color is being the main factor that governs consumers' prefers decisions (Bai et al. 2018) and meat pH value is an indicator for the shelf life as it may influence the water holding capacity (Allen et al. 1998). It has been reported that dietary Cys supplementation could improve antioxidant status and delay meat discoloration by improving glutathione levels and antioxidant activity (Bai et al. 2018). Researches related to dietary Cys and its' effect on pH value and meat color have conducted in pigs and Zhu et al. (2018) reported that dietary Cys was ineffective on meat pH. Another study stated that Cys presence in piglet diets was not changed color values of meat (Tao et al. 2020). However, Bai et al. (2018) noticed that whereas dietary Cys reduces color discoloration depend on the administered dose, ineffective on meat pH value. To our knowledge, there are no studies related to the effects of dietary Cys on meat macronutrients that may contribute to this section. However, some researchers reported that Cys could increase the carcass lean percentage (Tao et al. 2020). Although little is known about the role of Cys on protein and lipid metabolism. In this study, 90 mg Cys inclusion to diet did not change dry matter, protein, fat, or ash contents of quail meat.

Laying and hatching performance

Few researches were conducted regarding the effects of dietary Cys on the laying poultry performance. However, some studies showed that Cys in the diet may improve the laying performance. For example, Hu et al. (2008a) reported that Cys at level of 400 mg / kg feed of breeder broiler ration significantly increased the average laying rate by 2.24% but egg weight was not affected. Li and Liu (2005) reported that Cys inclusion to the diet significantly increased the egg production and egg weight were in laying hens in the late production period. The results of the current study showed that 90 mg Cys per kg basal diet increased the in egg production per bird and this parameter was found significantly higher than the control group ($P< 0.05$). However, unfortunately, there is no scientific clarification or sufficient confirming data to explain the relationship between the increasing in the EM and dietary Cys. *In-vitro* studies reported that Cys has regulatory effects on oocyte maturation and fertilization and also increased embryo growth rates (Urdaneta et al. 2004b; De Matos and Furnus 2000b). It has been thought that dietary Cys may improve the incubation parameters of poultry. Similar to our study, Hu et al. (2008b) reported that addition of Cys to diet has not affected on fertility and hatchability in breeder broiler. Clearly, administration methods (*in-ovo* or oral) and dosage may effective factors or the effect of Cys on hatchability parameters. This study proved that 90 mg Cys in the diet was not effective on hatching performance of quails.

Plasma metabolites and sex hormones

Birds maintain higher plasma glucose concentrations than other vertebrates and smaller bird species have higher blood glucose level (Braun and Sweazea 2008). In this study, both sexes of laying quails had similar plasma glucose levels, and among the plasma parameters, only glucose level not changed by the addition of Cys in the diet. Rideau et al. (1990a) demonstrated that inoculation of 300 mg Cys per kg body weight to day-old male chicks increased plasma glucose level. Apparently, the dosage and administration method may modulate the effects of Cys on the plasma glucose level of poultry. On the other hand, in this study, the changes in plasma cholesterol levels of laying quails were dramatic and the differences were significant in both sexes and treatments ($P< 0.05$). Our results revealed that the plasma cholesterol level of quails are dissimilar between sexes and coherently with the current study Marks and Washburn (1991)

reported that cholesterol levels in quail may show differences between sexes or lines. Compatible with our results, there is evidence that the addition of Cys in the diet decrease the blood cholesterol in poultry and Xiao-jie et al. (2004) reported that 100 mg Cys per kg body weight in the geese diet decreased the cholesterol level in plasma and influence the lipid metabolism. Wittwer et al. (1987) confirmed a significant reduction of plasma cholesterol after supplementing Cys in the diet of cholesterol-fed rabbits, while and Zhu et al. (2007) reported that Cys lowered plasma low-density lipoprotein) levels in rats. Moreover, a study suggested that oral supplementation of Cys at level of 340 mg per kg body weight inhibited cholesterol secretion into bile (Salam et al. 2005). Data of the present study supported the cholesterol-lowering effects of dietary Cys.

Former studies, regard on Cys administration in avian species generally have focused on blood growth hormone alterations. In this study growth hormone level of birds, could not be detected. But sex hormone levels did alter significantly ($P< 0.05$) (Table 6). Data of this study displayed that the effects of dietary Cys on the plasma FSH levels depended on the sex. Consequently, the alterations of FSH level affected testosterone and estradiol levels differently in the two sexes. The effect of FSH on male and female reproductive hormones in poultry can be explained as follows: Vanmontfort et al. (1995) reported that there is a positive correlation between FSH and testosterone hormones in male animals and Baird et al. (1981) stated that estradiol secretion is controlled by LH (Luteinizing hormone). It is not found any experimental or *in-vivo* study show the impact of dietary Cys on plasma sex hormone levels, however, obviously, this study proved 90 mg in the diet showed boosting effect on sex hormones levels of quails.

CONCLUSIONS

In a conclusion, according to data from the two experiments (T1 and T2), 90 mg Cys in quails' diet was ineffective on growth, laying, and hatching performance parameters as well as meat properties. However, the cholesterol-lowering effect and sex hormone-raising effects of dietary Cys were remarkable. In a nutshell, it looks that using Cys at the level of 90 mg per kg feed has not a growth promoter effect on the growth or laying performance of quails. Studies regarding cysteamine and its effects on poultry performance and metabolism are inadequate. The results of the current study may contribute to further research on this topic and clearly is needed more research on cysteamine usage in poultry diets.

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CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Impact of the protein content in pollen on honey bees (*Apis mellifera* L.) lifespan

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ABSTRACT: The objective of the research work was to determine the protein content in identified pollen grains and the lifespan of worker honey bees fed with pollen with different protein content. In this study the number of the identified floral species was nine. The percentage of the total protein content in the bee-collected pollen pellets ranged from 11.5% for *Chondrilla juncea* to 25.1% for that of *Brassica napus*, and the average value was 18.5%. The average protein content of entomophilous plants was 18.5%, whereas it was 18.4% in anemophilous plants. Protein is one of the main components in pollen, and vary among different floral species source. The lifespan of worker bees ranged from 25 days when they consumed pollen from *Brassica napus* (25.1% protein content) to 14.3 days when they consumed pollen from *Zea mays* (17% protein content). The present study was performed to draw a comparison between the lifespan of worker bees fed with pollen with different protein content and significant differences were found. The worker honey bees lived longer when they consumed pollen with higher protein content. There is prolonged lifespan of bees fed with pollen from *Cirsium* sp., *Helianthus annuus* and *Chondrilla juncea*, as compared to bees fed with pollen from *Zea mays*, despite the higher protein content in the latter's pollen.

Keywords: pollen nutrition; protein needs; honey bees

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INTRODUCTION

Pollen satisfies the dietary needs of bees for protein (Herbert, 1992). Pollen differs in its nutritional value to bees (Vivino and Palmer, 1944; Standifer, 1967; Haydak, 1970; Crailsheim, 1990). Protein content in pollen ranges from 3.8% to 40.8% (Stanley and Linskens, 1974; Herbert and Shimanuki, 1978; Loper and Berdel, 1980). According to Kleinschmidt and Kondos (1977) protein content in pollen ranges from 7% to 37%, and for Roulston et al. (2000) ranged from 2.5% for *Cupressus arizonica* to 61.7% for *Dodecatheon clevalandii*. Liolios et al. (2015) showed that the protein content of pollen ranged from 12.8% for pollen from *Smilax* sp. to 30.1% for that of *Fallopia* sp., with an average of 20.8%. According to Kleinschmidt and Kondos (1976) protein content in pollen less than 20% cannot satisfy colony requirements for optimum brood rearing.

According to Avetisyan (1983) are 20 amino acids in pollen, but for honey bee's body 10 amino acids-threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine, arginine and tryptophan are essential of their diet (De Groot, 1953).

High protein content in pollen contains necessary amino acids in high concentrations (Wille et al., 1985). On turn most pollen types contain all necessary amino acids (Herbert, 1992). Individual amino acids are important in bee development (Herbert et al., 1970). There is a close relation between the nutritional value of pollen and the development, reproduction, and productivity of the bee colonies (Radev et al., 2014).

The honey bee (*Apis mellifera* L.) is using for aging research (Rueppell et al., 2004a). The lifespan of honey bees is influenced of different factors (Maurizio, 1961; Brucner, 1980; Winston and Katz, 1982; Milne, 1982, 1985). High level of protein content in pollen gives a long-lived bees, while low protein content minimises the longevity of bees (Kleinschmidt and Kondos, 1978; Schmidt et al., 1987). According to Levchenko (Stashenko, 1988), the level of protein content in pollen significantly affects the bee biology. Low protein content in pollen reduces the resistance of honey bees to diseases (Matilla and Ottis, 2006). Deficiency of quality protein in the diet of the bee colony can be one of the reasons for the emergence of invasion and infectious diseases (Bilash, 1990). One of the best ways to fight against dangerous diseases is by feeding the bees with pollen (Lavrehin and Pankova, 1983).

The purpose of this study was to determine the lifespan of worker honey bees according to consuming identified pollen with different protein content. In the literature there is not much information about pollen protein content of identified floral species and lifespan of honey bees.

MATERIALS AND METHODS

Pollen collecting

Pollen bottom traps (Figure 1) were placed at five bee hives and the pollen pellets harvested every second day from April until September in area of Belozem (Bulgaria) (N 42.201860/E 25.049330). The pollen loads from each hive were analyzed carefully. Four hundred and ten samples of bee-collected pollen pellets were separated over white paper according to their colour, shape, and texture (Figure 2).

Figure 1. Inside pollen trap

Figure 2. Separated pollen for identifing

Sampling

The labeled pollen samples were stored in separate vials in a freezer at -18 °C. The plant species represented by each pollen sample was identified through a microscopic examination of pollen grains (Figure 3). Palynological analysis was carried out using a similar methodology as Louveaux et al. (1978). To identify the pollen, is referred to the database of the laboratory of Apiculture-Sericulture of the Agricultural school of Aristotle University in Thessaloniki, Greece.

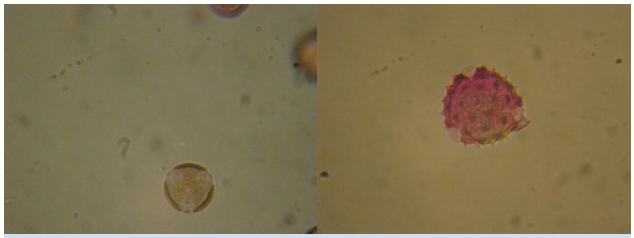

Figure 3. Microscopic pollen grains

Kjeldahl method

For nitrogen content determination, pollen was analyzed using the Kjeldahl method. The crude protein content was estimated using the factor 5.60 (Rabie et al., 1983) times the volume of HCL added to the pollen. In this case, the following formula applied: protein (%) = 0.791*V/m, where: V = the volume (mL) of HCl used for titration and m = the amount of pollen (g). Two replicates from each sample were analyzed, and the results were averaged.

Lifespan of worker honey bees

Nine groups with three cages 10*10*10 cm each were created (Figure 4). One hundred newly emerged worker honey bees were placed in each cage and fed with 50% sugar syrup (1:1 sugar/water). By pipet (Figure 5), a quantity of 20 ml sugar syrup containing two grams pollen from each identified floral species for each cage group were added. The mixed syrup was replaced every day with new one. All cages were placed under the same conditions, covered for darkness in a room at temperature 28 °C. In pre study fed only with sugar syrup, honey bees lived longer than 10 days at this temperature. The alive worker honey bees were checked every day and the last remaining found dead in each cage was reported for lifespan.

Figure 4. Cage with wax comb and honey bees

Figure 5. Pipet with sugar syrup and pollen

Statistical analysis

The results were statistically processed by used a Student's T-test and "Anova".

RESULTS

In the present study, identified pollen grains from nine floral species were used. They represent plants which are favored by honey bees. The largest number of taxa come from Asteraceae - 5, and 4 families by one representative (Figure 6). The results underline the fact that pollen from different plant species has a greatly varying protein content. The percentage of the total protein content in bee-collected pollen grains ranged from: 11.5% for *Chondrilla juncea* to 25.1% for *Brassica napus*, and the average value was 18.5%.

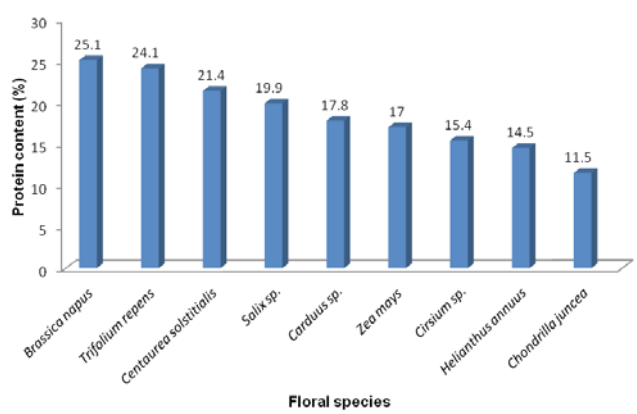

Figure 6. Protein content (dry matter %) of 9 different pollen species

Significant difference in the protein content in pollen of entomophilous plants - 18.5% compared to protein content in pollen of anemophilous plants - 18.4% was not found ($P < 0.05$, Student's t-test). Salix sp. was presented as an anemophilous plant in this study. The protein content values of Salix sp. and the other anemophilous plant *Zea mays* were pretty high. The result obtained for *Zea mays* could be attributed to hybrid variety.

Table1. Average lifespan (in days) of worker bees fed with sugar syrup and pollen of different plant species from Bulgaria

	Lifespan of bees I cage	Lifespan of bees II cage	Lifespan of bees III cage	Average lifespan of bees
Worker bees fed with sugar syrup and pollen from *Brassica napus*	26	24	25	25 a
Worker bees fed with sugar syrup and pollen from *Trifolim repens*	24	23	25	24 a
Worker bees fed with sugar syrup and pollen from *Centaurea solstitislis*	23	23	24	23.3 a
Worker bees fed with sugar syrup and pollen from *Salix* sp.	23	24	23	23.3 a
Worker bees fed with sugar syrup and pollen from *Carduus* sp.	23	24	22	23 a
Worker bees fed with sugar syrup and pollen from *Zea mays*	15	14	14	14.3 b
Worker bees fed with sugar syrup and pollen from *Cirsium* sp.	18	20	22	20 ac
Worker bees fed with sugar syrup and pollen from *Helianthus annuus*	17	19	16	17.3 c
Worker bees fed with sugar syrup and pollen from *Chondrilla juncea*	17	16	18	17 c

The data in Table 1 shows a slight reduction of the lifespan of worker bees when they consumed pollen with lower protein content. The longest lifespan was observed in the bees which consumed pollen from *Brassica napus* (25 days), while the shortest - in bees which consumed pollen from anemophilous plant species *Zea mays* (14.3 days). The present study was performed to make a comparison between the lifespan of worker bees fed with pollen from: *Brassica napus* (25.1% protein content), *Trifolim repens* (24.1% protein content), *Centaurea solstitialis* (21.4% protein content), *Salix* sp. (19.9% protein content), *Carduus* sp. (17.8% protein content), *Zea mays* (17% protein content), *Cirsium* sp. (15.4% protein content), *Helianthus annuus* (14.5% protein content) and *Chondrilla juncea* (11.5% protein content).

When comparing the results (Anova: Single factor), significant differences were found between the lifespan of worker bees fed with pollen from *Brassica napus* (25 days), *Trifolim repens* (24 days), *Centaurea solstitialis* (23.3 days), *Salix* sp. (23.3 days) and *Carduus* sp. (23 days), as compared to bees fed with pollen from *Zea mays* (14.3 days), *Helianthus annuus* (17.3 days) and *Chondrilla juncea* (17 days) (F > F crit). Significant differences were also found between the lifespan of worker bees fed with pollen from *Brassica napus* (25 days) and *Trifolim repens* (24 days), compared to bees fed with pollen from *Cirsium* sp. (20 days) (F > F crit).

The statistical analysis showed no significant differences (F ≤ F crit) in the lifespan of bees fed with pollen from *Cirsium* sp. (20 days) compared to bees fed with pollen of: 1) *Centaurea solstitialis* (23.3 days), *Salix* sp. (23.3 days) and *Carduus* sp. (23 days) ; 2) *Helianthus annuus* (17.3 days) and *Chondrilla juncea* (17 days).

It is noted, there was a prolonged lifespan for bees fed with pollen from *Cirsium* sp., *Helianthus annuus* and *Chondrilla juncea*, in comparison to bees fed with pollen from *Zea mays*, despite the higher protein content in the latter's pollen.

DISCUSSION

In this study, the results for pollen protein content come from some of worker bees' favorite plant species. Protein content in pollen depends on the plant visited by bees. The knowledge of pollen protein content of floral species in the area of beekeeping is the main aspect of utmost importance related to honey bees vitality and development.

The results presented in this study provided additional insight into the impact of protein content in pollen to honey bees lifespan. This would be useful information for beekeepers and would help them in bee colonies development. There are not many studies about the lifespan of bees fed with identified pollen with known protein content. The lifespan studies are

for bees which are a part of honey bee colonies.

According to: Taranov (Azimov, 1969), Tyunin (Azimov, 1969) and Azimov (1969) the lifespan of bees is about 30 days; El-Dib (Azimov, 1969) the lifespan from the Caucasian breed of bees is 27-29 days; Omholt and Amdam (2004) the life of bees varies from 3 to 4 weeks in the summer to over 6 months in the winter. According to Radev (2013) in a period of intensive rearing of brood the lifespan of honey bees is over 47 days on average.

It was found that the protein content in pollen of *Zea mays* is higher in comparison to that of *Cirsium* sp., *Helianthus annuus* and *Chondrilla juncea*, but the lifespan of the worker bees fed with that pollen is shorter than that of the bees fed with pollen from the other three floral species. This difference could be attributed to hybrid variety of the cultivated plant or other components in the pollen and needs further research. *Zea mays* is a cultivated plant and could be contaminated by a pesticide. Further research must be provided for components in pollen from *Zea mays* to establish why honey bees which consume pollen from it have the shortest lifespan.

According to Höcherl et al. (2012), extensive cultivation of *Zea mays* could force bees to collect mainly of it pollen instead of other kinds, and these honey bees fed maize pollen had shorter lifespan.

This research was a prerequisite for studying the impact of protein content in pollen on honey bees' lifespan. Analysis provided us with exclusive information about the nutrition of honey bees and could be used for further research. Every pollen type contains components such as: vitamins, minerals, enzymes, lipids, sugars and etc. in different amounts and bees are better off consuming mixed pollen rather than only one pollen type.

CONCLUSIONS

Protein is a main component of pollen and its content vary among different floral species. The lifespan of worker honey bees was longer when they consumed pollen with higher protein content.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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Research article
Ερευνητικό άρθρο

Effect of zinc supplementation during cryopreservation on post-thaw chicken semen parameters and fertility

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ABSTRACT: The present study evaluated supplementation of zinc from different sources during chicken semen cryopreservation on post-thaw semen quality and fertility. Adult White Leghorn chicken semen was cryopreserved using 4% dimethyl sulfoxide (DMSO) in Sasaki diluent (SD).In the semen cryomixture zinc oxide (6.25 and 12.5 μM) and zinc sulphate (100 and 200 μM) were added before freezing process. The plastic semen straws were thawed at 5°C for 100 sec and samples were evaluated for sperm motility, live, abnormal and acrosome intact sperm as well as lipid peroxidation levels in the seminal plasma. The semen cryopreservation and evaluation were repeated on six occasions. Fertility of the zinc supplemented semen was evaluated by inseminating into White Leghorn hens. Zinc supplementation did not affect any of the post-thaw sperm parameters. The lipid peroxidation and fertility were similar between the treatments. In conclusion, supplementation of zinc as zinc oxide or zinc sulphate during chicken semen cryopreservation does not affect post-thaw semen parameters or fertility.

***Keywords*:** Chicken, Fertility, Semen cryopreservation, Zinc oxide, Zinc sulphate

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INTRODUCTION

Conservation of chicken through semen cryopreservation is a management tool. The semen cryopreservation protocol is almost well standardized in cattle, however, similar consistent fertility results could not be achieved in chicken because of reasons such as line or breed variability and uniqueness of the chicken sperm (Long, 2006). Different studies have evaluated compounds added during the cryopreservation process to improve the fertility outcome from post-thaw chicken semen (Zhandi et al., 2017; Pranay Kumar et al., 2019).

Zinc is an important mineral having role in testicular development and steroidogenesis (Hamdi et al., 1997). In sperm zinc acts as a second messenger having role in motility and fertility (Chu, 2018). Zinc is an important component of metalloproteins and has role in sperm homeostasis and fertilizing ability (Kerns et al., 2018).The human sperm chromatin got stabilized and DNA damage prevented when zinc is added prior to cryopreservation (Kotdawala et al., 2012). Zinc inhibited the in vitro generated superoxide anion in human semen (Gavella and Lipovac, 1998). Zinc supplementation in bull semen cryopreservation mixture improved sperm motility, viability and total antioxidant capacity (Dorostkar et al., 2014). Recently, zinc oxide supplementation in chicken semen during freezing has been shown to improve post-thaw in vitro tested semen parameters (Zhandi et al., 2020). However, this study has not reported on the effect on fertility. Furthermore, the effect of zinc sulphate supplementation during chicken semen cryopreservation is not known. Thus, the present study evaluated the effect of zinc in cryopreservation media on post-thaw semen quality and fertility in chicken.

MATERIALS AND METHODS

Semen from White Leghorn (IWK line) roosters aged 45 weeks was collected by dorso-abdominal massage method (Burrows and Quinn, 1937), pooled and cryopreserved with 4% DMSO. The birds were maintained in individual cages in an open-sided house at the institute poultry farm. The experimental protocol was approved by the Institutional Animal Ethics Committee. The pooled semen sample was equilibrated for 30 min at 5°C and then diluted using Sasaki diluent (D (+) -glucose- 0.2 g, D (+) - trehalose dehydrate- 3.8 g, L-glutamic acid, monosodium salt- 1.2 g, Potassium acetate- 0.3 g, Magnesium acetate tetrahydrate- 0.08 g, Potassium citrate monohydrate- 0.05 g, BES- 0.4 g, Bis-Tris- 0.4 g in 100 ml distilled water, final pH 6.8; Sasaki et al., 2010). The effect of zinc during semen cryopreservation was evaluated using zinc oxide at 6.25 and 12.5 µM, and zinc sulphate at 100 and 200µM. The zinc levels were selected based on the earlier published reports (Ghallab et al. 2017; Zhandi et al. 2020).Samples with only cryoprotectant served as control. After mixing diluent containing different concentration of zinc the samples were maintained at 5°C for 30 min. The semen cryomixture was then loaded into 0.5 ml French straws with the final sperm concentration 2000 x 10^6/ml and placed 4.5 cm over liquid nitrogen for 30 min after which they were plunged and stored in liquid nitrogen. The procedure of cryopreservation and post-thaw evaluation was repeated six times. The straws were stored for a minimum of a week and thawed at 5°C for 100 sec for evaluation and insemination. The thawed samples were assessed for progressive sperm motility, live sperm, abnormal sperm, and intact sperm acrosome. Insemination in 47 weeks old White Leghorn hens (8 hens/treatment) with thawed semen (200 million sperm/0.1 ml semen) was done three times at four days interval. The eggs collected were incubated under standard incubation conditions. The fertility was determined by candling the eggs on 18th day of incubation and expressed as percent fertility (Total number of fertile eggs/Total number of eggs incubated) x 100.

The progressively motile sperm in each sample was scored subjectively by evaluating a drop of semen on a Makler chamber under 20x magnification.

The live and abnormal sperm were assessed using Eosin-Nigrosin stain (Campbell et al., 1953). A semen smear was prepared after mixing a drop of semen and a drop of stain, air dried and examined under high (1000x) magnification. The live membrane intact sperm that were clear in appearance were counted and percent live sperm calculated. A total of 200 sperm were counted in each slide. The abnormal sperm percent assessed based on morphological abnormalities were also estimated in the same slides.

The intact sperm acrosome was evaluated as per Pope et al. (1991).Semen (10 µl) was mixed with equal volume of stain [1% (wt/vol) rose Bengal, 1% (wt/vol) fast green FCF and 40% ethanol in citric acid (0.1 M) disodium phosphate (0.2 M) buffer (Mc Ilvaine's, pH 7.2-7.3)] and left for 70 sec. A smear of the mixture was made, dried and evaluated at high magnification (1000x). The sperm having intact acrosome was identified by the blue stained acrosomal

caps while no stained cap could be observed in the acrosome reacted sperm. The acrosome intact sperm percent was calculated by counting a minimum of 200 sperm in each sample.

The lipid peroxidation was evaluated by the thiobarbituric acid method (Hsieh et al., 2006) and expressed as the malondialdehyde (MDA) concentration in seminal plasma. The seminal plasma was separated by centrifuging semen samples at 3000 x g for 5 min and stored until analysis. The assay was performed by mixing 0.9 ml of distilled water and 0.1 ml seminal plasma in a glass tube followed by addition of 0.5 ml of thiobarbituric acid reagent. The tubes were kept in a boiling water bath for an hour. The cooled content of the tubes was measured for absorbance against blank at 540 nm in a colorimeter.

The data is presented as mean ± SE. Data were analyzed in SAS 9.2 and $P<0.05$ was considered significant. The different zinc treatments were compared by one-way ANOVA with Tukey's post hoc test. The data were arcsine transformed where appropriate before analysis.

RESULTS

The sperm motility, live and abnormal sperm and acrosome intact sperm values were similar between the treatments (Table 1). There was no difference in lipid peroxidation levels between the treatment groups. The fertility from zinc supplemented treatments was similar to that of the control cryopreservation treatment.

DISCUSSION

Semen cryopreservation is a stressful event for the sperm where it undergoes structural as well as functional damages. Few reports have indicated beneficial effects of zinc addition during semen cryopreservation. In the present study addition of zinc during semen cryopreservation was evaluated. Two salts of zinc, zinc oxide and zinc sulphate, were supplemented at concentrations reported elsewhere and post-thaw semen parameters and fertility studied.

In the present study zinc oxide addition in the cryopreservation extender had no effect on post-thaw semen parameters. This is in contrast to other reports in chicken (Zhandi et al., 2020) and human (Kotdawala et al., 2012) where addition of zinc prior to freezing improved the post-thaw sperm motility. The chicken sperm motility and mitochondrial membrane potential were improved only in the 1µg/ml treatment and higher level had no effect (Zhandi et al., 2020). However, the beneficial effects of addition of zinc on fertility was not reported. In the present study zinc oxide inclusion did not improve the fertility from cryopreserved semen. The zinc oxide concentration used in the present study was similar to Zhandi et al. (2020), however, no positive changes on semen parameters were observed. These differing results may be due to the different chicken breeds used in the studies. Furthermore, the differing results may also be due to the different freezing medium and straw size used in the studies. In vitro addition of zinc to human semen has been shown to reduce lipid peroxidation level (Gavella and Lipovac 1998).Zinc is a component in antioxidant enzymes that reduce the superoxide anions generated during oxidative stress which in turn results in lower lipid peroxidation level. In the present study the post-thaw seminal plasma MDA concentration was unaffected by supplementation of zinc salts. There are no reports of zinc inclusion during chicken semen cryopreservation on post-thaw semen lipid peroxidation for comparison of the results of the present study.

Table 1. Effect of zinc supplementation in cryopreservation medium on post-thaw semen parameters and fertility

Parameters	4% DMSO	Zinc oxide (6.25µM)	Zinc oxide (12.5µM)	Zinc sulphate (100 µM)	Zinc sulphate (200 µM)
Progressive sperm motility (%)	15.0 ± 1.29	13.33 ± 1.67	20.0 ± 2.24	17.5 ± 1.71	15.83 ± 1.54
Live sperm (%)	25.8 ± 0.57	26.78 ± 1.28	28.72 ± 1.63	30.57 ± 0.84	28.63 ± 1.71
Abnormal sperm (%)	2.82 ± 0.18	2.8 ± 0.25	2.6 ± 0.23	2.65 ± 0.16	3.12 ± 0.23
Acrosome intact sperm (%)	89.67 ± 0.42	91.0 ± 1.21	89.0 ± 2.0	83.83 ± 2.43	85.83 ± 2.21
Seminal plasma Lipid peroxidation (nM MDA/ml)	0.88 ± 0.08	1.03 ± 0.08	0.89 ± 0.03	0.93 ± 0.02	0.91 ± 0.05
Fertility (%)	5.61 ± 2.95	7.12 ± 3.79	3.33 ± 2.25	1.81 ± 1.81	0
Number of eggs incubated	52	60	65	54	66

Inclusion of zinc sulphate at 200µM during stallion semen cryopreservation has been shown to improve motility, viability, plasma membrane integrity and acrosome status (Ghallab et al., 2017). Supplementing 0.288 mg/L zinc sulphate in bull semen extender during cryopreservation improved the sperm motility and total antioxidant capacity, however, higher levels were found to be deleterious (Dorostkar et al., 2014). In the present study zinc sulphate was used at 100 and 200 µM concentrations. In the earlier studies in other species similar or higher concentrations have produced beneficial effects. This difference in result may be due to the species difference.

In conclusion, addition of zinc as zinc oxide or zinc sulphate during chicken semen cryopreservation does not affect the post-thaw semen parameters or fertility.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
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Microbiological characterization of bee pollen from the Aegean region of Turkey

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ABSTRACT: This study aims to characterize the microbiological safety of Turkish bee pollen for the first time. Twenty-one bee pollen samples were purchased from local beekeepers between September 2020 and October 2020 in Muğla, İzmir, Kütahya and Afyon provinces in the Aegean region of Turkey. The samples were analyzed for total aerobic bacteria, total coliform bacteria, total psychotropic microorganisms, total lactic acid bacteria, *Staphylococcus aureus*, sulfite-reducing clostridia spores, yeast and mold. Total ochratoxin and aflatoxin levels were measured using a lateral flow kit in samples contaminated with mycotoxigenic molds. The counts of the microorganisms ranged from 3.70 to 5.42 log CFU g^{-1} for the aerobic mesophilic, 3.0 to 5.40 log CFU g^{-1} for psychrotrophs, 3.85 to 5.78 log CFU g^{-1} for lactic acid bacteria (LAB), 3.0 to 5.45 log CFU g^{-1} for yeasts-molds, and <10 to 5.0 log CFU g^{-1} for total coliforms in the samples. Besides, *S. aureus* and sulfite-reducing clostridia were not detected among the samples. The predominant mold genera in samples were *Aspergillus* sp. *Penicillium* sp. and *Alternaria* sp. Five samples contaminated with mycotoxigenic molds had aflatoxins in the range of 2.96-9.71 μg/kg. According to the study results, comprehensive prevention, control and surveillance strategies need to be established to reduce bacteria and mycotoxin contaminations in bee pollen. Legal regulation of bee pollen as a food supplement and legal limit of mycotoxins in bee pollen should be defined.

***Keywords*:** Bacteria; Bee pollen; Mold; Mycotoxins; Yeast.

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INTRODUCTION

Bee pollen is a pellet of pollen collected by worker honeybees. The bees store the pollen in the broods to be used as the primary food source for the development of brood and young bees (Carroll et al., 2017). It has wide biological activities known and used since antiquity due to having bioactive contents including amino acids, lipids, carbohydrates, mineral salts, vitamins, phenolic compounds, and flavonoids (Komosinska-Vassev et al., 2015). There has been a growing interest in better understanding its biological properties, and numerous studies reported that the pollen exhibits a variety of beneficial therapeutic properties including antioxidant (Kocot et al., 2018; Tutun et al., 2021), antimicrobial (Erkmen and Ozcan, 2008), antiinflammatory (Eteraf-Oskouei et al., 2020) and wound healing (Olczyk et al., 2016).

Although most of the studies conducted on bee pollen have focused on its chemical composition, chemical residues, botanical origin, and other biological activities, there have been limited studies on its safety risk related to microbiological hazard (Komosinska-Vassev et al., 2015; Mauriello et al., 2017; Puvača, 2018). Its water content and nutritionally important components make pollen an ideal environment for the growth of various bacteria, mold, and yeast (Estevinho et al., 2012). Bee pollen may contain some pathogens including viruses, parasites and bacteria. Also, manipulations by a beekeeper in bee pollen production and storage can lead to a significant increase in the contamination levels of bee pollen by pathogenic microorganisms (Mauriello et al., 2017; de Sousa Pereira et al., 2019; Lika et al., 2021). Toxigenic molds in the pollens can produce one or more mycotoxins that are toxic to vertebrates and other animal groups in low concentrations (Kostic et al., 2019; Tutun and Kahraman, 2020). Thus, the presence of microorganisms in bee pollen can both favor the spoilage of the bee pollen and cause diseases in humans. Its microbial quality in terms of the health and safety of consumers is important and should be monitored properly. This study aimed to investigate the microbiological characterization of honeybee pollens from the Aegean region of Turkey.

MATERIAL AND METHODS

Sampling of bee pollen

Twenty-one pollen samples were purchased from local beekeepers and warehouses linked to these apiaries between September 2020 and October 2020 in four provinces (Muğla, Kütahya, Afyon and İzmir) belong to the Aegean Region of Turkey (Table 1). All the samples were sent to the laboratory in sterile glass vials and stored at 4°C, until testing. They were analyzed within 48 h of sampling in the Laboratory of the Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University.

Physicochemical analysis

The water activity (a_w) of the pollen samples was determined using a hygrometer (Testo 650, Germany) and the a_w value was recorded at 20±0.2 ºC. Moisture content in pollen samples was measured using a moisture analyzer (AND MX-500, AD Company). The pH value of each sample was measured using a pH meter (WTW Lab-pH Meter InoLab® pH 7110) at 25ºC ± 2ºC after 10 g of each sample was dissolved in 90 ml deionized water.

Microbial analysis

Microbiological determinations were evaluated as described previously by De-Melo et al. (2015). The samples were analyzed for total aerobic bacteria, total coliform bacteria, total psychotropic microorganism, total lactic acid bacteria, *Staphylococcus aureus*, sulfite-reducing clostridia spores, yeast and mold.

Table1. Sampling regions

Sample code	Number of samples	Province	District	Number of Apiary
1-5	5	Muğla	Döğüşbelen	A-E
6-7	2	Muğla	Seydikemer	F-G
8-11	4	Muğla	Dalaman	H-K
12-13	2	Muğla	Menteşe	L-M
14-15	2	Muğla	Datça	N-O
16	1	Muğla	Milas	P
17	1	İzmir	Seferihisar	Q
18, 19	2	Kütahya	Kadınhanı	R, S
20, 21	2	Afyon	İscehisar	T, U

Sample preparation

A 10 g sample from each bee pollen was placed aseptically into sterile stomacher bags (VWR, Belgium), diluted in 90 mL of sterile saline peptone water (0.85%±0.1%, w/v) and homogenized in a stomacher (Stomacher 400, Seward, London, UK) for 2 min at room temperature. Then, serial dilutions were made in 9 mL of sterile saline peptone water (1:10, v/v). The appropriate dilutions were plated by spread plate technique (0.1 mL) in duplicate and counted after incubation. The colonies were expressed as log colony forming units g^{-1} (log CFU g^{-1}).

Microbial enumeration

Count of total aerobic microflora was performed within 24-36 h after the spread plate inoculation of 0.1 mL of each dilution on plate count agar (PCA, Merck, 5463) and incubation at 30 °C. To count the total psychotropic microorganism, 0.1 mL of each dilution were inoculated on PCA and incubated at 7±0.5 °C for 10 days. To determine the total lactic acid bacteria (LAB) count, 0.1 mL of each aliquot was inoculated onto de Man, Rogosa and Sharpe agar (MRS, Merck 110660) and incubated under anaerobic conditions using Anaerocult® A (Merck 113829) with anaerobe jar at 30°C for 48-72 h. Enumeration of total coliforms was carried out by spreading on plates of violet red bile glucose agar (VRBG, Merck 110275). Plates were overlaid with VRBG agar. Then, solidified plates were incubated at 37°C for 24-48 h. For the determination of *S. aureus*, 0.1 ml aliquots of the dilutions were transferred to Baird-Parker agar (BP, Merck, 105406) enriched with egg yolk and incubated for 24 h at 37±2°C. The occurrence of sulfite-reducing clostridia spores was determined according the procedure as defined by ISO 15213:2003 (2003). The dilutions (10, 5 and 1 mL) were placed in a water bath at 80°C for 10 min, inoculated in petri dishes containing sulfite iron agar (SIA, Merck, 110864) and incubated at 37ºC for 48 h under anaerobic conditions. The serial dilutions of pollen homogenates were inoculated into dichloran rose Bengal chloramphenicol agar (DRBC, Merck 1.00466) and incubated at 25±1 °C for 5-7 days. At the end of the incubation period, yeast and mold colonies were counted.

Isolation and identification of yeast and mold

Single colonies of different yeast grown on DRBC agar were sub-cultured on Sabouraud's dextrose agar (SDA, Merck, 105438) supplemented with chloramphenicol (Oxoid, SR0078E) and incubated in aerobic conditions at 37 °C for 24-48 h. Isolated yeasts were defined at the genus level by colony morphology, Gram staining, germ tube test and urease tests (Seeliger, 1956; Quinn et al., 2011; Khadka et al., 2016). In the germ test, *Candida albicans* ATCC 90028 was used as positive control and *C. tropicalis* ATCC 13803 was used as a negative control. The yeast isolates were sub-cultured to CHROMagar Candida (CHROMagar, Paris, France) and incubated at 37°C for 24-48 h under aerobic conditions. At the end of the period, the growth ability and colony colors of the yeast isolates on CHROMagar Candida were evaluated according to the manufacturer's instructions. *C. albicans*, *C. tropicalis*, *C. krusei*, *C. kefyr* or *C. glabrata* and *Candida* sp. appear as green, metallic blue, purple fuzzy, mauve-brown and white to mauve colored colonies, respectively.

Single colonies of different mold grown on DRBC agar were duplicate sub-cultured on SDA supplemented with chloramphenicol and incubated at 25°C and 37°C for 4-7 days. Slides were prepared by the sticky tape method and stained with lactophenol cotton blue solution for microscopic examination of mold colonies (Quinn et al., 1999). The morphological characteristics of isolated colonies were identified based on macroscopic (colony appearance) and microscopic (hyphae, conidia, conidiophores and arrangement of spores) examination at genera level (Quinn et al., 1999; Pitt and Hocking 2009).

Mycotoxin analyses

Total aflatoxin and ochratoxin levels were analyzed in the bee pollen samples contaminated with *Aspergillus* sp. and/or *Penicillium* sp. The concentrations of total aflatoxin and ochratoxin in the samples (n=5) contaminated with *Aspergillus* sp. and/or *Penicillium* sp. were quantified by Symmetric Total Green 0-30 Lateral Flow kit (ProGnosis Biotech, Larissa, Greece) and Symmetric Ochratoxin Lateral Flow kit (ProGnosis Biotech, Larissa, Greece) according to the manufacturer's instructions, respectively.

Statistical analysis

Analyses were performed in duplicate. The Pearson correlation coefficient was calculated with SPSS 21 (SPSS Inc., IBM Corporation, Armonk, New York, USA). A p-value less than 0.05 is statistically significant.

RESULTS

The values of moisture, a_w and pH of bee pollens

ranged from 12.92% to 20.99%, 0.59 to 0.78 and 3.78 to 4.71, respectively (Table 2). While the samples from Menteşe had lower water content and the a_w values, the samples from Kütahya province had higher than the others.

The microbial communities of bee pollens are shown in Table 2. The counts of microorganisms ranged from 3.70 to 5.42 log CFU g^{-1} for the aerobic mesophilic bacteria, 3.0 to 5.40 log CFU g^{-1} for psychrotrophs, 3.85 to 5.78 log CFU g^{-1} for LAB, 3.0 to 5.45 log CFU g^{-1} for yeasts and molds, and <10 to 5.0 log CFU g^{-1} for total coliforms. *S. aureus* and sulfite-reducing clostridia were not detected among the samples. No correlation was shown between counts of bacteria including aerobic mesophilic bacteria, psychrotrophs and total coliform and the a_w and %RH values for bee pollen (Table 3). A positive correlation was observed between LAB counts and a_w ($r=0.507$; $p<0.05$) and %RH values ($r=0.551$; $p<0.01$) for bee pollens. Also, there is a significant positive correlation between yeast-mold communities and a_w ($r=0.464$; $p<0.05$) and %RH values ($r=0.529$; $p<0.05$) of bee pollen. These results indicate that a_w and %RH had a positive effect on the yeast-mold and LAB growth.

Totally 21 fungal isolates representing 4 fungal species were detected in all bee pollen samples (Table 4). Fourteen yeasts were isolated in pure form on SDA. White-cream color, smooth, pure growth yeast colonies were observed on SDA. Gram-positive, 3-4 µm in diameter, oval-round, budding yeast cells were seen in these colonies by microscopic examination. All yeast isolates were urease negative and no germ tube formation was detected in any of the isolates. Colonies of yeast isolates in CHROMagar Candida were white to mauve in color and the isolates were interpreted as *Candida* sp. The *Candida* sp. was the most frequently found with 14 isolates. *Penicillium* sp. (5 isolates from Seydikemer, Dalaman and Kütahya regions) was the second most prevalent fungal genera. *Aspergillus* sp. and *Alternaria* sp.were the least fungal contaminant found in bee pollen.

Total aflatoxin and ochratoxin production were analyzed in 5 samples contaminated with *Aspergillus* sp. and *Penicillium* sp. There was a good accuracy with recovery 95% and 96%, and the detection limit were 1.20 and 1.50 µg $kg^{-1}$for aflatoxin and ochratoxin, respectively.

Total aflatoxin levels ranged from 2.96 to 9.71 µg kg^{-1} (Table 5). The samples from the Dalaman region had the highest total aflatoxin level (9.71 µg kg^{-1}). Ochratoxin was not detected in the samples.

Table 2. Microbial and physicochemical evaluation of 21 bee pollen samples

Regions	n	Aerobic mesophilic	Psychotrophs	LAB	Yeast -mold	Total coliforms	*S. aureus*	Sulfite-reducing Clostridia	a_w	pH	%RH
Döğüşbelen	5	4.20 -5.42	4.08-4.71	5.18-5.46	3.00-4.04	3.48-3.78	<10	<10	0.70-0.72	4.18-4.36	18.05-18.72
Seydikemer	2	4.30-4.88	4.11-4.46	4.60-5.04	3.48-4.65	3.50-3.80	<10	<10	0.66-0.68	4.27-4.36	16.26-16.48
Dalaman	4	3.95-4.65	3.00-4.60	3.85-5.78	3.00-5.45	3.48-4.65	<10	<10	0.67-0.75	4.03-4.44	16.49-20.99
Menteşe	2	3.95-3.98	3.00-4.30	4.00	3.00	<10	<10	<10	0.59-0.60	4.57-4.61	12.92-13.50
Datça	2	4.30-4.90	5.23-5.40	5.00-5.20	3.00	3.00-4.60	<10	<10	0.69-0.72	4.09-4.45	17.20-19.03
Milas	1	4.30	3.30	5.48	5.48	<10	<10	<10	0.69	4.28	20.90
Seferihisar	1	3.78	3.48	4.00	5.00	<10	<10	<10	0.76	3.78	20.55
Kütahya	2	3.81-4.30	4.00-4.60	4.00-5.30	4.40-4.48	<10	<10	<10	0.68-0.78	3.93-4.45	16.94-24.74
Afyon	2	3.70-3.72	3.00-4.70	5.00	3.00-5.00	3.40-5.00	<10	<10	0.71-0.72	4.24-4.71	17.66-19.95

Note: microbial communities express as log CFU g^{-1}

Table 3. Correlation between the microbial counts, moisture content and a_w in bee pollen

	a_w	%RH
Aerobic mesophilic	0.119	0.136
Psychotrophs	0.212	0.137
LAB	0.507*	0.551**
Yeast-mold	0.464*	0.529*
Total coliforms	-0.99	-0.710
a_w	1.000	0.916**
%RH	0.916**	1.000

* $p<0.05$; ** $p<0.01$

Table 4. Fungal species isolated from bee pollen

Areas	Sample No	*Candida* sp.	*Aspergillus* sp.	*Penicillium* sp.	*Alternaria* sp.
Döğüşbelen	1-5	+	-	-	-
Seydikemer	6	+	-	+	-
Seydikemer	7	+	-	-	-
Dalaman	8	+	-	+	-
Dalaman	9	+	-	+	+
Dalaman	10	+	+	+	-
Dalaman	11	-	-	-	-
Menteşe	12-13	-	-	-	-
Datça	14-15	-	-	-	-
Milas	16	-	-	-	-
Seferihisar	17	-	-	-	-
Kütahya	18	+	-	-	-
Kütahya	19	+	-	+	-
Afyon	20-21	+	-	-	-

Table 5. Total aflatoxin and ochratoxin levels of the pollen samples

Sample Region	**Total Aflatoxin (µg/kg)**	**Ochratoxin (µg/kg)**
Seydikemer	2.96	*
Dalaman	8.04	*
Dalaman	9.71	*
Dalaman	5.03	*
Kütahya	4.92	*

*: Not detected.

DISCUSSION

Since bee pollen moisture value plays an important role in organoleptic properties and extension of their shelf life, it is one of the important parameters for assessing quality control of this product (Anjos et al., 2019). The higher a_w values give the opportunity to the proliferation of microorganisms including pathogenic bacteria grown at a_w>0.85 and fungi grown well at a_w>0.6 (Mathlouthi, 2001; Rahman, 2010). In the current study, the average a_w, pH and moisture values of the bee pollen were 0.69, 4.30, and 18.05%, respectively. The $a_w$values of all samples were greater than 0.6, which enables microorganisms to grow in stored bee pollen (Rahman, 2010). The pH average and water content of the samples in the current study were similar to those of bee pollens reported in the literature (Estevinho et al., 2012; Petrovic et al., 2014; Arslan and Durmaz, 2019; Beev et al., 2020; Dinkov, 2020) and the Brazilian legislation (pH, 4.0-6.0). In the current study, although the a_w and %RH values of the bee pollens had a positive effect on the growth of LAB and yeast-mold, they did not show a positive effect on the growth of the bacteria including aerobic mesophilic bacteria, psychrotrophs and total coliform. The reason for LAB and yeast-mold growth may be high a_w and %RH in pollen samples. Many literatures also confirm these results (Gonzalez et al., 2005; Hani et al., 2012; Nuvoloni et al., 2021)

The studies on the microbiological safety of fresh bee pollen samples showed a vast majority of microbial diversity according to inadequate hygienic manipulations during harvesting, processing and storage conditions (Nogueira et al., 2012; De-melo et al., 2015; Beev et al., 2020). In the present survey, some samples had not perfect microbial quality characteristics and similar observations were reported by other researchers (Belhadj et al., 2014; Shevtsova et al., 2014; Beev et al., 2020). There was a variation in the microbiological quality among those collected from a different apiary in the same province. In a sample collected in Döğüşbelen province the higher aerobic mesophilic count (5.42 log CFU g^{-1}) was observed, for psychrotrophs the highest value was observed in pollen sample from Datça (5.40 log CFU g^{-1}). The sample from Milas province had the highest counts of LAB and yeast-mold counts (5.48 and 5.48 log CFU g^{-1}, respectively). The highest value of total coliform was observed in one sample from Afyon province (5.0 log CFU g^{-1}). As a member of the *Enterobacteriaceae*

family, the presence of coliforms that cause various food-borne diseases in humans is linked to fecal contamination of food (Jay et al., 2005). It is well known that *S. aureus* can produce thermostable enterotoxin that causes food poisoning (Argudin et al., 2010) and *Clostridium botulinum* is responsible for serious neuroparalytic illness due to produce neurotoxin (Vasquez, 2009). All fresh bee pollen samples were negative for *S. aureus* and sulfite-reducing clostridia. This is a promising result considering the importance of these microorganisms are potentially pathogenic and have significant effects on public health.

In the present study, the most prevalent fungi genus in bee pollen samples was *Candida* sp., followed by *Penicillium* sp., *Aspergillus* sp. and *Alternaria* sp., which is similar to those of previous studies on bee pollen (Kostic et al., 2017; Sinkevičienė et al. 2019; Beev et al., 2020). In terms of the yeast-mold count, most of the samples (Except 3) are comply with the European Union regulations which allow for up to 50.000 CFU $g^{-1}$of yeast and mold in bee pollen samples. There is no regulation on microbiological quality standards of bee pollen in Turkey.

Mycotoxins belong to the category of toxic secondary metabolites produced by *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp. and cause health hazards to people and animals. The mycotoxin-producing fungi contamination of foods may result in the production of mycotoxins such as aflatoxins and ochratoxins (Gilberth et al., 2000; Jay et al., 2005). We analyzed the total aflatoxin and ochratoxin in the samples contaminated with *Aspergillus* sp. and/or *Penicillium* sp. and detected the only aflatoxin in the range of 2.96-9.71 µg kg^{-1} in all tested samples (average 6.13 µg kg^{-1}). According to Regulation of European Commission (No: 165/2010) and Turkish Food Codex (No: 26/2008), the maximum levels of total aflatoxin is from 2.0 µg kg^{-1} to 15.0 µg kg^{-1} depending on the type of foods, such as maize, wheat, and milk. However, there is no information about the maximum levels for mycotoxins in bee pollen in these regulations. Campos et al. (2008) proposed that maximum permissible concentration (MPC) in pollen should be set at 2.0 µg kg^{-1} for aflatoxin B1 and 4.2 µg kg^{-1} for total aflatoxins. On the other hand, since bee pollen is consumed less than foods with the tolerable limit for mycotoxins, these proposed MPCs may not be realistic. In addition, according to the coexistence of mycotoxins in bee pollen, the MPC should be reconsidered, as some combined mycotoxins have a more detrimental effect on human health (Smith et al., 2016; Kostic et al., 2019). Determining the MPC of mycotoxins especially aflatoxins and ochratoxins in bee pollens will be of great importance.

Bee pollen has ideal nutritional and chemical composition for bacterial colonization and growth. Improper movements while beekeepers collect and process bee pollen may result in microorganism contamination (Mauriello et al., 2017). Several factors, such as humidity, temperature, drying process, and storage conditions can affect the growth of different types of microorganisms in bee pollen (Kostic et al., 2017). In the current study, the reason for the high microorganism load in a few samples could be the results of contact of the bee pollen with microorganisms by beekeepers' manipulation mistakes. Aegean region, situated in the western part of Turkey, has a Mediterranean climate that provides a favorable condition for microbial growth especially fungi. Also, the high moisture and temperature in these regions could contribute to the microbial growth in the samples (Arruda et al., 2017; Tarazona et al., 2019).

Monitoring of the microbiological quality of raw bee pollen allows evaluating of processing and storage conditions (Rocha, 2013; De Melo et al., 2015). Microbiological contamination of bee pollen can happen during the steps of production, due to inadequate hygienic practices, the lack of proper handling at the harvesting, transportation and storage steps (Nogueira et al. 2012). It is known that each apiary has different collection and processing practices. Therefore, the variation in the counts of microorganisms observed in samples is acceptable considering different apiary manipulation.

CONCLUSION

This is the first study in Turkey to describe the microbiological quality of bee pollen from the Aegean region. The findings suggest that microbiological criteria should be established for bee pollen regulated by national authorities. Adoption of proper harvesting practice and post-harvest processing techniques can help to prevent possible microbial contamination of Turkish bee pollen by equipment or handling. Also, quality control and monitoring systems are necessary for maintaining the safety of bee pollen for humans.

CONFLICT OF INTEREST

None declared by the authors.

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Research article
Ερευνητικό άρθρο

Mycotoxins occurrence in food commodities, their associated hazards and control strategies

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ABSTRACT: Globally, the food is contaminated by various means, but microorganisms are predominant factor in contaminating the food and agriculture commodities. Among microorganisms, fungi are mainly involved in the spoilage of food due to their diversified nature and minimal requirement for growth. The toxigenic fungi associated with mycotoxins, can grow during any stage of food chain including harvesting, handling, distribution and storage. Mycotoxins are fungal secondary metabolites and their production is influenced by various factors such as environmental conditions, crop type and storageconditions. Mycotoxins in agriculture commodities expose serious health hazards. This review entails different types of mycotoxins involved in the spoilage of food and agriculture commodities, their potential health hazard, maximum allowable limits of mycotoxins in different food commodities and possible control strategies. In developing countries, regulatory authorities need to establish quality control strategies and limits of mycotoxins in food, in order to ensure the consumer safety.

***Keywords*:** mycotoxins, secondary metabolites, food spoilage, aflatoxins, maximum allowable limits

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INTRODUCTION

Food contamination is a problem since ages and fungal contamination has been identified as main cause of food spoilage (Ashiq, 2015). Since ancient times, spoilage of crops and other food commodities have shown the presence of fungi and molds (Umesha et al., 2017). There is a high chance of fungal contamination if agricultural commodities are not stored properly and are exposed to high moisture content. Once agricultural commodities are infected by fungi, the infection spreads through the different stages i.e. harvesting, processing, and storage due to favorable conditions for the fungal growth (da Rochaet al., 2014).

The population of the world is expected to reach 8.2 billion by 2030 and 842 million people were estimated undernourished during 2011-2013. In coming decades, the food supply chains will be associated with growing challenges related to urbanization, family size, population aging and consumer concerns for healthy and sustainable food production. These trends will have a significance influence on food supply which will need to be more efficient in order to meet the demands. As a result, the food supply will need to grow within the domain of available natural resources and technologies (FAO, Food and Agriculture Organization of the United Nations, 2014).

The word "mycotoxin" means a poisonous substance that is produced by fungi. Food contamination by mycotoxins is a major food safety threat and possess several health risks to the users depending on the specific type of mycotoxin consumption, exposure level and a person's health status (Reddyet al., 2010). Mycotoxins are natural contaminants and present a serious challenge due to their diversified nature in terms of chemical structure and symptoms in humans and animals (Zychowski et al., 2013). The predominant effects of mycotoxins include carcinogenicity and neurotoxicity (Kolpinet al., 2014). The presence of mycotoxins not only poses a threat to human and animal health but it also accounts for significant economic losses, these losses can arise at any step during the food supply chain from farm to fork level (Rodrigues et al., 2011). The prevention can be best solution to overcome the mycotoxin contamination due to diversified nature of mycotoxins to contaminate wide variety of food products along the supply chain and difficulties associated with its detection (Anater et al., 2016).

Generally, most of the mycotoxins are low molecular weight secondary metabolites that apparently have no function in the metabolism of fungi (da Rocha et al., 2014). Mycotoxin chemical structure ranges mostly from simple C4 compounds to complex compounds (Paterson and Lima, 2010).Many species of fungi produce mycotoxins and few mycotoxins have been reported to exhibit carcinogenic potential in humans and animals (Huffman et al., 2010). Among mycotoxin producing fungi, *Fusarium, Aspergillus, and Penicillium* species are main mycotoxin producers and are called field fungi due to their ability to contaminate various food commodities (Jajić et al., 2019; Ashiq, 2015). Mycotoxins including aflatoxins (AFs), fumonisins (FMN), ochratoxin A (OTA), trichothecenes (include deoxynivalenol (DON) and T-2 toxin), and zearalenone (ZEN) gained more awareness because of their high frequency of occurrence and adverse health effects to humans and animals (Bhat et al., 2010).The consumption of mycotoxin contaminated food can result in carcinogenic, immunosuppressive, and teratogenic effects (Binder et al., 2007). The mycotoxins mainly target kidneys, liver, immune and nervous systems and in humans' general manifestations of mycotoxicosis are diarrhea, gastrointestinal distress and vomiting (Bhat et al., 2010).

The ingestion, inhalation and absorption of mycotoxins can cause mortality in humans and animals (Bankole and Adebanjo, 2003). The term mycotoxicosisis associated with the ingestion of mycotoxin contaminated food by animals or humans (Binder et al., 2007). The mycotoxicosis can be experienced through indirect exposure to products of animals (meat or milk) which are contaminated with mycotoxin (Bankole and Adebanjo, 2003).

The foods contamination by mycotoxins can be avoided by maintaining the higher quality of food during the entire food supply chain. The high-income developed countries have less exposure to high mycotoxins level due their food safety standards and regulations (Ashiq, 2015). In hot and humid areas of the world, food spoilage thorough fungi are more common (Sabahat et al., 2010; Thompson and Henke, 2000). The developing countries with high temperature and relative humidity need to adopt the modern food safety standards and regulations in the entire food supply chain to minimize the fungal and mycotoxin food contamination. The present review summarizes different types of food contaminating mycotoxins, their influence on human and animal health and possible control strategies.

MYCOTOXINS

The word mycotoxin is derived from Greek words, i.e. mykes indicates fungi or molds and toxicum means "poison". In 1960s the word "mycotoxin" was first used to explain the toxin in animal feed related to contaminated peanuts and the death of turkeys in England called Turkey-X-disease (Bennett, 1987). Normally the word 'mycotoxin' is used for relatively low molecular weight toxic chemicals (Mw<500 Da) (da Rocha et al. 2014). Generally, the effects of mycotoxins on humans and animals vary with change in their molecular structures (Miller, 1995).

FACTORS AFFECTING MYCOTOXINS PRODUCTION

Fungi are dependent on oxygen due to their aerobic nature; therefore, fungi have to face the consequences of oxygenic conditions such as presence of reactive oxygen species. The reactive oxygen species are produced during metabolic processes and their production can be influenced by environmental stress (Halliwell and Gutteridge, 2007). The accumulation of reactive species can potentiate morphological and metabolic transitions in fungi which in turn can result in toxin synthesis (Reverberi et al., 2010).

Miller (2001) reported that secondary metabolites are produced from one of the primary metabolites due to limitation of one or more nutrient. Proline, asparagine and tryptophan can increase the biosynthesis of AFs in *A. parasiticus* (Reverberi et al., 2010) however, their presence can reduce the production of AFs in *A. flavus* (Wilkinson et al., 2007). Temperature, pH, water activity and various other environmental factors significantly affect the production of mycotoxins such as OTA and AFs (Chein et al., 2019a). The environment-based factors influence the mycotoxin synthesis at transcription level and even the exposure of suboptimal quantities of fungicides can potentiate the biosynthesis of mycotoxins (Schmidt-Heydt et al., 2007).

The nature and production quantity of mycotoxins are mainly influenced by synergies of various factors: available nutrition, temperature, types of substrate, moisture content conditions, humidity, colony maturity, co-occurrence of mycotoxins with other fungi, and competing with other microbes and stress factors (Rao, 2001). The major contributing factors in the production of mycotoxins and consequences of their consumption are summarized in Figure 1.

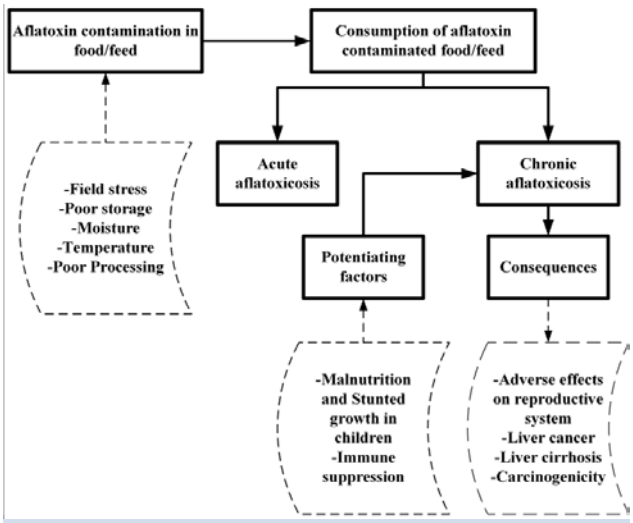

Figure 1. Contributing factors for mycotoxins contamination and consequences of their consumption (adopted from Bbosaet al. 2013)

MYCOTOXIGENIC FUNGI

Several fungal genera produce mycotoxins including *Aspergillus, Fusarium, Penicillium*, *Alternaria* and *Claviceps* spp. (Majeed et al., 2021; Reddy et al., 2010). These different mycotoxigenic fungi genera are most abundant and have a strong environmental relation with human food materials (Samsudin and Abdullah, 2013). *Fusarium* spp is reported as mycotoxigenic fungi in cereal crops as well as other food commodities. *Aspergillus* spp and *Penicillium* spp are most common pathogens for plants and food commodities during drying and storage periods (Mohale et al., 2013).

TYPES OF MYCOTOXINS

AFLATOXINS

Aflatoxins (AFs) are well-known mycotoxins and in 1960s, for the first time reported in the UK when turkey poults 100, 000 suffered and died (known Turkey X disease) after consuming AFs contaminated peanut meal (CAST, 1989).AFs are derivatives of difuranocoumarin produced by species of *A. flavus* and *A. parasiticus* via polyketide mechanism (Ellakany et al., 2018; Turner et al., 2009). Among the 18 AFs categories, (AFB1 and AFB2) B series, (AFG1 and AFG2) G series and (AFM1 and AFM2) M series have been identified as the most important AFs affecting humans and animals (Figure 2). B and G series of AFs are characterized on the basis of their fluorescence under ultraviolet light (B = blue, G = green) (da Rocha et al. 2014). M series is associated with B series hydroxylated derivatives, reported in dairy

cattle, milk, meat and various mammals which consumed contaminated food and feed with AFs (Acaroz et al., 2019a; Chen et al., 2005). AFB1 and AFG1 dihydroxy derivatives are AFB2 and AFG2, respectively (Chunet al., 2007). The main reason that AFB1 and AFG1 are generally more toxic and carcinogenic than AFB2 and AFG2 is the presence of a double bond in the form of vinyl ether in their terminal furan ring, which is the active site and intensifies their fluorescence. Thus, their active site can experience a reaction of reduction leading to a change in activity (Turner et al., 2009). AFs are stable at constant high temperatures with minimum loss through cooking or pasteurization. AFs are unstable with UV, intense pH (< 3 or >10) values and oxidizing agents in the presence of oxygen (Herzallah, 2009).

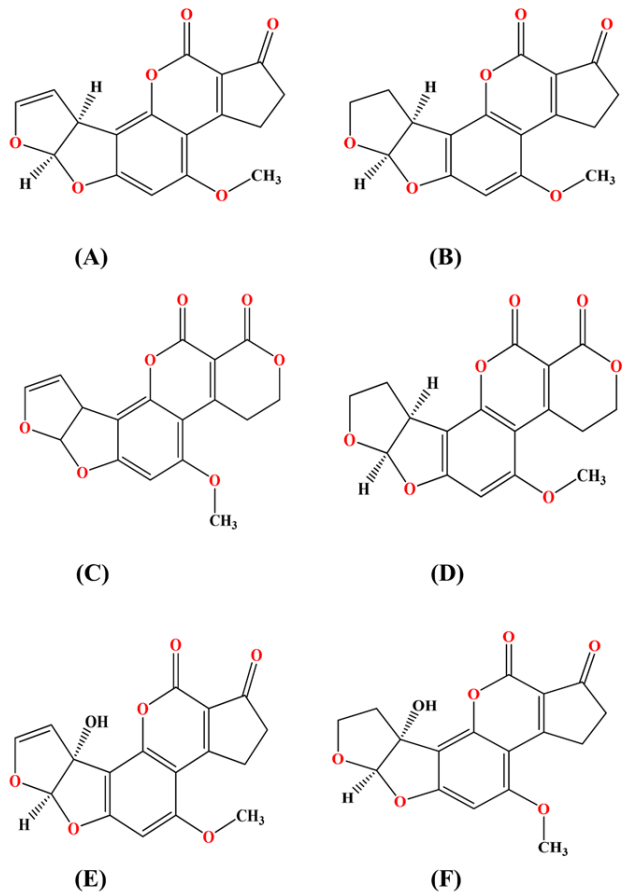

Figure 2. Chemical structure of (A) Aflatoxin B1 (B) Aflatoxin B2 (C) Aflatoxin G1 (D) Aflatoxin G2 (E) Aflatoxin M1 (F) Aflatoxin M2

A. flavus only produces B AFs (B1 and B2) and around 40% of *A. flavus* species produce AFs. *A. parasiticus* produces G AFs (G1 and G2) (Umesha et al., 2017). Morphologically *A. nomius* is like *A. flavus* but it produces both B and G AFs. *A. flavus* commonly occurs in crops in the world's warm temperature regions (Pepeljnjak et al., 2004).

AFs are mainly produced by *A. Parasiticus* and *A. flavus*, the optimum growth temperature rangesare25 to 35 °C and 28 to 30 °C, respectively (Bhat et al., 2010).AFs are of main concern in hot and humid areas, as the optimum temperature in warm areas of the world are favorable for fungal growth (Fernandez-Cruz et al., 2010).AFs are typically found in agriculture products like cereals (barley, sorghum, wheat, maize and rice), spices (ginger, turmeric, coriander, black pepper, and chili), tree nuts (nuts, walnuts, peanuts, pistachios and almonds) and oilseeds (cotton soybean, sunflower and sesame) (Acaroz et al., 2019b; Firdous et al., 2012). Whereas AFM1 and AFM2 are mainly present in milk, milk products and meat (da Rocha et al., 2014).

Health Hazards of Aflatoxin

In tropical and subtropical regions, including Africa and Asia, various studies have been reported that indicated the adverse effects of AFs food contamination (Acaroz et al., 2019a, 2019b).The ingestion of food heavily contaminated with AFs can cause death in various cases (Ashiq, 2015). A wide variety of animals are affected by AFs including rodents, cattle, poultry fish and swine. Though the AFs response depends on the level of exposure, exposure duration, nutrition status, health, age, and environmental factors (Wagacha and Muthomi, 2008).

AFs are carcinogenic, teratogenic, hepatotoxic, mutagens, immunosuppressants and can induce various other serious hazards in animals and humans (da Rocha et al., 2014; Eaton and Gallagher, 1994). AFs are categorized as group 1 carcinogens by International Agency for Research on Cancer (IARC, 1993). AFs interfere with the protein synthesis due to their DNA binding capacity, hence, effect various essential cellular metabolisms and immune system (da Rocha et al., 2014). Generally, aflatoxicoses is recognized as disease associated with the AFs consumption. The death can be encountered in acute aflatoxicoses, whereas the chronic conditions can induce cancer, immunosuppression and hepatotoxicity (Zain, 2011).

FUMONISINS (FMNS)

FMNs were first discovered and reported in 1988 (Bennett andKlich, 2003). FMNs are mainly produced by *Fusarium* genera (*Fusarium proliferatum, F. verticillioides, and F. nygamai*) and 28 different

types of FMNs have been isolated and classified in four groups (A, B, C and P).The different species of *Fusarium*genera produce FMNs, particularly *F. verticillioides* formerly *Fusarium moniliforme, F. proliferatum*, *F. anthophilum*, as well as *Alternaria alternata* (Omurtag 2008). *Aspergillus niger* has been reported to produce FMNs like B2 and B4, and a new B series of FMNs (FB6) was recognized from *A.niger* (Huffman et al., 2010).

The major types of FMNs (FB1, FB2, and FB3) are contaminants of natural cereals (Omurtag, 2008). Among FMNs, the most important and abundant mycotoxin family member is FB1 (Figure 3) (Reddy et al., 2010).FMNs are polyketide metabolites derived from repetitive condensation of acetate units or other short carboxylic acids by a similar mechanism of enzyme bound for fatty acid synthesis (Huffman et al., 2010).

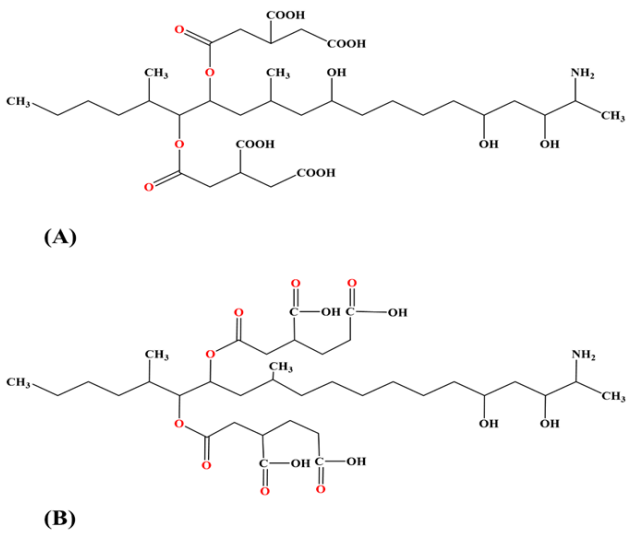

Figure 3. Chemical structure of (A) Fumonisin B1 (B) Fumonisin B2

Temperature and humidity are important factors for *Fusarium* contamination and synthesis of mycotoxins (Omurtag, 2008).The optimum temperature and water activity for the production of FMNs were reported in the range of 15-30 °C and 0.9-0.995, respectively (Sanchis and Magan, 2004).The presence of FMNs was found in agriculture and food commodities including bovine milk, dried figs, corn, products of corn, medicinal plants, and herbal tea (Omurtag et al., 2010).FMNs were observed as common contaminant of feeds and food in Philippines, South America, China, USA, Africa, France, Italy, Indonesia, and Thailand (Kumar et al., 2008).

Health Hazards of Fumonisins

Human intake of FMNs contaminated foods has been associated with esophageal cancer in Asia, South Africa, and Central America, (Alizadeh et al., 2012; Marasas et al., 2004).FMNs are categorized as a group 2B substance (human carcinogenic) by International Agency for Research on Cancer (IARC, 1993) and immunosuppressive by World Health Organization (WHO, 2002).The chronic FMNs effect on animals include impairment of basic immune function, nephrotoxicity, hepatotoxicity, respiratory disorders, and reduced milk production (Diaz et al., 2000). FMNs do not interact with DNA like AFs, however, due to similarity with sphingosine it might intervene with the biosynthesis of sphingolipids (Shier, 1992), which in turn influence the essential cellular activities as sphingolipids are essential for membranes, inter and intra cellular communication (Merrillet al., 1993).

OCHRATOXINS (OTS)

In South Africa, Ochratoxin A (OTA) was first reported in 1965 (Van der Merwe et al., 1965) and isolated from cornmeal contaminated by *Aspergillus ochraceus*. Later in 1969, ochratoxin (OT) was isolated and reported in the United States from corn (Shotwell et al., 1969).OTA was later identified as secondary metabolite associated with *Aspergillus* and *Penicillium* spp (Duarteet al., 2010). Historical reports revealed that OTA was found in Egyptian tombs and considered for the suspicious deaths of many architects (Pittet, 1998).

OTA contains 7-carboxy-5-chloro-8-hydroxy-3, 4 dihydro- (3R) -methylisocoumarin in a carboxyl group linked to L-ß-phenylalanine (Figure 4) (Fernandez-Cruz et al., 2010). OTA is a crystalline white powder that is stable in food processes but unstable in the presence of light.Acid hydrolysis of OTA changes it to phenylalanine and an optically active lactone acid named OTα (IARC, 1993).

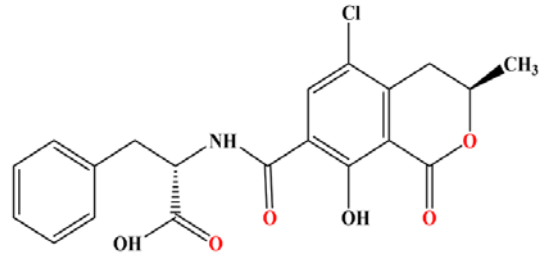

Figure 4. Chemical structure of Ochratoxin A

OTA is produced by various species of *Aspergillus* and *Penicillium* genera. Main producers are *A.*

carbonarius, and *A. ochraceus*, (Bachaet al., 2009). Some species of *Aspergillus (A. niger, A. carbonarius, A. ochraceus, P. verrucosum)* are responsible for OTA production (Bhat et al*.,* 2010). OTA is a natural contaminant in various foods, like cocoa, corn flour, cereals, dried fruits, maize, soya beans, peanuts, nuts, fish, milk, eggs, poultry, kidney beans, tea, and some herbs (Batista et al*.,* 2009). Hence, in tropical areas, it is however linked to moldy green coffee beans. It is also found in a coffee brew and roasted coffee beans (Sibanda et al., 2002). OTA contaminates spices, and dried fruit, whereas, grapes are commonly contaminated with OTA during storage (Bhat et al., 2010). Exposure of OTA to human generally occurs by the intake of poorly stored food products. The presence of OTA has been observed in the tissues and organs of animals and humans, including blood, breast milk, and meat (Kumar et al., 2008). The optimum temperature and water activity to produce OTA are 25-30 °C and 0.98, respectively (Milani, 2013).

Health Hazards of OTA

OTA has been associated with carcinogenic, nephrotoxic, teratogenic, and immunosuppressive effects in humans and animals (da Rocha et al., 2014).OTA was categorized as carcinogen to humans (group 2B) by the International Agency for Research on Cancer (IARC, 1993).OTA was linked with Balkan endemic nephropathy (BEN), a disease of kidney which was observed incertain areas of Balkan countries (Pfohl-Leszkowicz et al., 2002).The previous reports reported OTA as a potent teratogen, immunosuppressive, liver toxin, and carcinogen in animals (Pfohl-Leszkowicz and Manderville, 2007).

TRICHOTHECENES (TCT)

In 1949, Trichothecin from *Trichothecium roseum* was isolated and defined for the first time by Freeman and Morrison. Trichothecin discovery was associated with other TCT for example, T-2 toxin (T- 2), and deoxynivalenol (DON) (Omurtag, 2008).TCTs are chemically defined by a tetracyclic sesquiterpenoid 12, 13-epoxytrichothec-9-ene ring system (Zöllner and Mayer-Helm, 2006). TCTs were further categorized as macrocyclic, or non-macrocyclic, depending on macrocyclic presence of esteror an ester-ether bridge between C-4 and C-15 (Merhej et al., 2011).

TCT is a family of mycotoxins that includes more than200 compounds divided into four subclasses (Group A-D), based on their functional characteristic. The most toxic TCTs are Group A (Bhat et al., 2010). Generally, TCT are found as contaminants in cereals and their derivative (Foroud and Eudes, 2009). TCT found in food/feedstuffs are produced by *Fusarium graminearum* and *F.culmorum*. *F.pseudograminearum, F. graminearum*, and *F. culmorum* are accountable of producing deoxynivalenol (DON) toxins (Figure 5) which is a member of TCT (Ashiq, 2015; Glenn, 2007). TCTs are leading source of contamination in grains like oats, maize, barley and wheat (Zöllner and Mayer-Helm, 2006).TCT have also been observed in cereal products and milk (Spanjer et al., 2008). *F. culmorum, F. sporotrichioides* and *F. graminearum*, were reported to produce DON toxins, (Merhej et al., 2011).

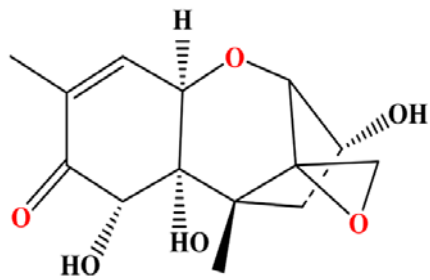

Figure 5. Chemical structure of Deoxynivalenol (DON)

Health Hazards of TCT

In humans and animals TCTs are associated with serious and chronic toxicosis. In eukaryotes contact of TCT results in slowed growth. Therefore, TCTs can affect wide range of invertebrates, mammals, fish, and plants (Wannemacher et al., 1997). TCTs can inhibit cell division, RNA and DNA synthesis, which might influence the structure and integrity of membranes, as well as function of mitochondria (Cundliffe and Davis, 1977), DON andT-2 toxins affect immunity through inhibition of cell and protein synthesis (Bhat et al.2010). DON lowers the body's level of antibodies and immunoglobulins (Richard, 2007).

ZEARALENONE (ZEN)

ZENs are estrogenic lactone resorcylic acid compounds mainly produced by *Fusarium* species (Diekman and Green, 1992). The production of ZEN is mainly associated with *Fusarium graminearum* and *F. culmorum* (Logriecoet al., 2002).ZEN is a non-steroidal mycotoxin and referred asF-2 toxin (Zinedine et al., 2006).ZEN is biosynthesized by various species of *Fusarium* via a polyketide pathway (Huffman et al., 2010). Chemically ZEN is 3, 4, 5, 6, 9, 10-hexahydro-14, 16-dihydroxy-3-methyl- 1*H*-2 benzoxacyclotetradecin 1, 7 (8*H*) - dione, is a macrocyclic ß-resor-

cylic acid lactone (Figure 6) (Cozzini and Dellafiora, 2012).

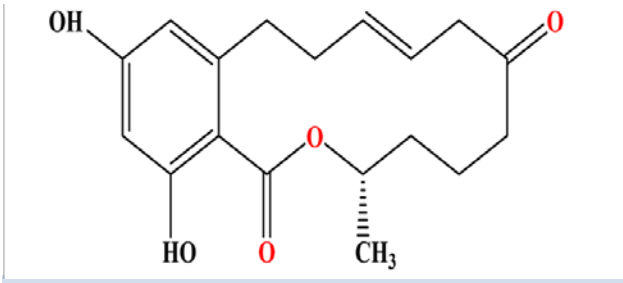

Figure 6. Chemical structure of Zearalenone

The crops that are mainly contaminated by ZENs are wheat, corn, oats, maize, barley, rice, millet, and sorghum (Zinedine et al., 2006). The presence of ZEN was reported in cereal by-products of corn and soya meal (Schollenberger et al., 2007), eggs (Sypecka et al., 2004) and milk (Seeling et al., 2005). ZENs are stable to heat, but under alkaline conditions the toxins can be degraded at a high temperature (>150 °C) (European Commission, 2000). Children are more affected by ZEN contaminated foods because of their high consuming rate of cereal based foods (Bhat et al., 2010).

Health Hazards of ZEN

ZENs may cause abortion, infertility, and problems with reproduction system (especially swine) and are linked with cervical cancer (El-Nezamiet al., 2002).ZEN contaminated feed ingestion leads to interference with exocrine and endocrine systems. Like other environmental estrogens, ZEN is able to interfere with the function of sex steroids (Bhat et al., 2010). Metabolites of ZEN bind to receptors of estrogen and activate transcription of genes (Fink-Gremmels and Malekinejad, 2007). Because of ZEN estrogenic activity, contaminated feed with ZEN showed changes in the reproductive tract, fertility reduction, and rise in number of fetal resorptions (Morgavi and Riley, 2007).Among animals, pigs have been the most sensitive to ZEN and poultry is least affected (Bhat et al., 2010).

MYCOTOXIN REGULATIONS

Food and Agriculture Organization declared mycotoxins as the major contaminants, accountable for 25% of food crops across the world. The mycotoxin contamination presents a serious threat to food security and economy (Aiko and Mehta, 2015; Alshannaq and Yu, 2017). Maximum allowable limits of different types of mycotoxins in food and feed have been specified by various national and international monitory organizations. The US Food and Drug Administration (FDA), World health Organization (WHO), European Union (EU), and several other countries have set the maximum limit of the mycotoxins in food, which are summarized in Table 1.

Table 1. Maximum allowable limits of mycotoxins in food commodities

Country/ Region	Mycotoxins	Products	Maximum limit (ppb)	References
European Union	Aflatoxins: B1 G1, B2, G2	Maize, wheat, rice, spices, almonds, oil seeds, dried fruits, cheese	0.1-8 4-15	EC (European Commission 2010).
	Aflatoxin M1	Milk, eggs, meat	0.5	CAC (Codex Alimentarius Commission 2015).
	Fumonisins (FB1 and FB2)	Maize-based breakfast cereals and maize-based snacks	800	(EC 2007).
		Raw maize grain	4000	(CAC 2015)
		Maize flour and maize meal	2000	(CAC 2015)
		Maize intended for direct human consumption	1000	(EC 2007).
	Ochratoxin A	Cereals, dry fruits, wine, spices, oat, raisins, coffee, cocoa, soybeans, meat	0.5-15	(EC 2006).
		Wheat, barley and rye	5	(CAC 2015).

	Deoxynivalenol (DON)	wheat, oats and maize	1250	(EC 2007).
		Flour, meal, semolina and flakes derived from wheat, maize or barley	1000	(CAC 2015).
		Cereal grains (wheat, maize and barley) destined for further processing.	2000	(CAC 2015).
	Zearalenone	Unprocessed cereals other than maize	100	(EC 2007).
		Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	100	(EC 2007).
		cereal flour, bran and germ for direct human consumption	75	(EC 2007).
USA	Aflatoxins B1, B2, G1, G2	Total aflatoxins in food for human consumption corn, peanut products, cottonseed meal, maize, wheat, rice, peanut, sorghum, pistachio, almond, ground nuts, tree nuts, figs, cottonseed, spices	20	USDA (United States Department of Agriculture 2015).
	Aflatoxin M1	Milk, milk products	0.5	FDA (Food and Drug Administration 2011)
	Total Fumonisins (FB1, FB2 and FB3)	Cereals	2000-4000	(Alshannaqand Yu 2017).
		Corn products and cleaned maize used for popcorn	2000-3000	
	Ochratoxin A	Cereals, wheat, barley, and rye and derived products.	5	(CAC 2015).
China	Aflatoxin B1	Corn, corn flour (grits, flake) and corn products, peanut and its products, peanut oil, corn oil	20	(Clever 2018)
		Paddy rice, brown rice, rice. Vegetable oil and fat	10	
		Wheat, barley, other grains,	5.0	
		Wheat flour, cereal, other husked grains and bean products, other cooked nuts and seeds. Soy sauce, vinegar, fermented paste		
	Aflatoxin M1	Foods intended for special dietary uses.	0.5	
		Milk and milk products	0.5	
	Ochratoxin A	Grains and grain products, beans and bean products, Baked coffee beans, ground coffee (roast coffee)	5.0	(Clever 2018).
		Instant coffee	10.0	
	Deoxynivalenol	Corn, corn flour (grits, flake). Barley, wheat, cereal, wheat flour	1000	(Clever 2018).
	Zearalenone	Wheat, wheat flour, corn, corn flour (grits, flake)	60	(Clever 2018).

South Korea	Aflatoxin B1	Grains, cereal products, dried fruits, Meju, and streamed rice	10	(Yoshizawa 2011; Chun 2011).
	Aflatoxin B1	Baby foods	0.1	(Chun 2011).
	Aflatoxin B2, G1, and G2	Grains, cereal products, dried fruits, Meju, streamed rice, and baby foods	15	
	Aflatoxin M1	Raw milks and milks prior to manufacturing processing	0.5	(Yoshizawa 2011; Chun 2011).
	Fumonisin B1, B2	Grain products, Cereals processed corn products for popcorn, confectionaries (contain>50% corns).	1000	(Chun 2011).
		Corn processed food, corn powder	2000	
		Corn	4000	
	Deoxynivalenol	Grain and their processed foods	1000	(Chun 2011).
		Corn and their processed foods	20000	
		Cereals	500	
	Ochratoxin A	Meju	20	(Chun 2011).
		Instant coffee and raisins	10	
		Red pepper powder	7	
		Grains and their processed food (grinding, cutting, etc). Coffee beans, and roasted coffee.	5	
		Baby foods for infants and young children	0.5	
	Zearalenone	Grains and processed grain foods	200	(Chun 2011).
		Confectionaries	50	
		Baby foods	20	
Indonesia	Total aflatoxins	Corn feed	50	(Suparmoet al. 2011)
	Total aflatoxins	All foods	35	
	Aflatoxin B1	All foods	20	
	Aflatoxin B1	Peanut, corn and their products	15-20	
	Aflatoxin M1	Dried milk and related products	5	
	Aflatoxin M1	Milk, drink milk products, fermented milk and rennin hydrolyzed milk products, concentrated milk, cream, cheese, pudding, yogurt, whey and their products	0.5	
	Fumonisin B1, B2	Corn (raw material)	2000	(Suparmoet al. 2011)
		Corn foods products, e.g., popcorn, corn chips	1000	
	Ochratoxin A	Spices	20	(Suparmoet al. 2011)
		Instant Coffee	10	
		Cereals (rice, corn, sorghum, wheat) and their products and coffee	5	
	Zearalenone	Maize	Not detectable	FAO (2003)
Japan	Total	All foods	10	(Kawamura 2011; Srianujata 2011)
	Aflatoxin B1	Rice	10	(Srianujata 2011; Yoshizawa 2011).
	Aflatoxin B1	Other grains	5	(Srianujata 2011).
	Deoxynivalenol	Wheat	1, 100	(FAO 2003).
	Zearalenone	Compound feeds	1000	(FAO 2003).

Brazil	Aflatoxins B1, G1, B2, G2	Oil seeds, nuts, dried fruits, cereals, spices	20	(Anfossiet al. 2016).
	Aflatoxin M1	Milk and infant formula	0.5-5	
	Fumonisins	Maize	2000-5000	(Anfossiet al. 2016).
	Ochratoxin A	Cereals, dried fruits, coffee, cocoa, wine, beer, grape juice, spices, liquorice, blood products	2-30	(Anfossiet al. 2016).
	Deoxynivalenol	Cereals, bakery products	750-3000	(Anfossiet al. 2016).
	Zearalenone	Cereals, bakery products, maize oil	200-1000	(Anfossiet al. 2016).
India	Aflatoxins	Oil seeds, nuts, dried fruits, cereals, spices	30	(Anfossiet al. 2016)
	Aflatoxin M1	Milk and infant formula	0.5	
	Ochratoxin A	Cereals, dried fruits, coffee, cocoa, wine, beer, grape juice, spices, liquorice, blood products	20	(Anfossiet al. 2016).
	Deoxynivalenol	Cereals, bakery products	1000	(Anfossiet al. 2016).
Russia	Aflatoxin B1	Maize	5	(Abdallah et al.
	Aflatoxin M1	Milk	0.5	2015).
	Ochratoxin A	Cereals, dried fruits, coffee, cocoa, wine, beer, grape juice, spices, liquorice, blood products	5	Anfossiet al. 2016).
	Deoxynivalenol	Cereals, bakery products	700-1000	Anfossiet al. 2016).
	Zearalenone	Cereals, bakery products, maize oil	1000	Zinedineet al. 2007).

CONTROL STRATEGIES FOR MYCOTOXIN CONTAMINATION

It is difficult to control contamination of mycotoxin in the field, during harvesting, storage and transportation of food and feed commodities (Umesha et al., 2017).The factors like soil moisture, invasion mostly with insects and mineral deficiencies contribute to mycotoxin contamination (Murphy et al., 2006). The way to protect and ensure consumers safety is to prevent fungal contamination of food/feed commodities which eventually leads to mycotoxin production (Figure 7). Plantation and pre-harvesting approaches can be improved by exercising preventing measures to reduce mycotoxin contamination. The control of mycotoxins in food produce is essential for public health and can be achieved by number of strategies such as, prevention of fungal contamination in food and feed, decontamination of mycotoxins and continuous surveillance. The fungal growth in food and feedstuff can be prevented by implementation of hygiene practices, drying and storage under appropriate conditions (Tola and Kebede, 2016). The high moisture content of food produce favors the growth of fungi and mycotoxin production. The drying of food produce can reduce the moisture content and hence prevents the growth of fungi and mycotoxins (Cheinet al., 2019a). The mold growth and accumulation of mycotoxins in food can be prevented by using natural preservatives such as acetic acid, lactic acid, benzoic acid and various essential oils (Chein et al., 2019b; Sriwattanachai et al., 2018).

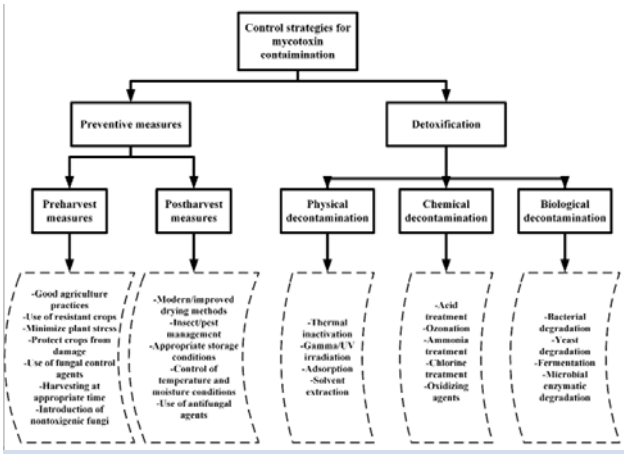

Figure 7. Aflatoxin prevention and control strategies in food and feed commodities. (Adopted from Ashiq, 2015)

Prevention of mycotoxin contamination may not always be possible, so decontamination process is important (Aiko and Mehta, 2015; Rustom, 1997). The mycotoxins can be decontaminated by physical and chemical treatments. Irradiation, cooking, boiling and extrusion are categorized as physical treatments

for decontamination of mycotoxins. During food processing, food undergoes heat treatment that might result in thermal inactivation of mycotoxins. However, most of the mycotoxins are heat stable and may not be easily inactivated by heat processing (Bullerman and Bianchini, 2007). The thermal deactivation of mycotoxins is influenced by certain essential factors such as temperature, water content and duration of exposure to heat. Mycotoxins can be decontaminated by chemical treatment however, the resultant degradation products might influence the food quality and safety (Aiko and Mehta, 2015).

BIODEGRADATION OF MYCOTOXINS

In comparison to other degradation approaches, biodegradation provides a better chance of deactivation of mycotoxin. Certain microbes and enzymes may reduce secondary metabolites toxicity such as AFs, FMNs, OTAs, TCTs, and ZENs can be converted into less toxic metabolites through changes in the structure of these mycotoxins (Pintonet al., 2010). Biodegradation has been widely used in many countries to detoxify mycotoxins. Cleavage of ring, acetylation, hydrolysis, glycosylation, deamination, and decarboxylation are the key procedures for biotransformation reactions (Guan et al., 2008). Biodegradation typically begins with the mycotoxin's identification through high performance liquid chromatography or enzyme linked immunosorbent test following by incubation media with specific microbes (Ding et al., 2015). In addition, many bacterial species can detoxify mycotoxins by biotransformation mechanism (Dalié et al., 2010; Tokai et al., 2005).The degradation of mycotoxins by different microorganisms are summarized in table 2. Lactic acid bacteria (LAB) were reported to inhibit the accumulation of mycotoxins and it was found that the inhibition of mycotoxin accumulation was not dependent on low pH rather it was associated with production of low molecular weight LAB metabolites (Dalié et al., 2010).

Earlier studies focused on mycotoxin toxicity and biodegradation mechanisms. The mechanism of microbial degradation of mycotoxins involves the use of microbial catabolic pathways, which results in fewer toxic effects or harmless end products. (Yang et al., 2014).

Table 2. Biodegradation of mycotoxins by microbes

Mycotoxin	Microbes	Biodegradable products	Mechanism	References
Aflatoxin	*Nocardia corynebacteroides, Corynebacterium rubrum, Pseudomonas putida, Rhodococcus spp, and Saccharomyces Cerevisiae*	Aflatoxicol; Aflatoxin M1; Aflatoxin B2a	Act on lactone ring	(Adeboet al. 2015; Du et al. 2017).
Fumonisin B1	*Exophialaspinifera, Sphingopyxisspp, Sphingomonas spp.*	Fumonisin hydrolyzed B1 (HFB1)	Removes tricarballylate groups with carboxylesterases in C-14 and C-15	(Du *et al.* 2017; Vanhoutte*et al.* 2016)
Ochratoxin A	*Bacillus licheniformis, Bacillus spp, Brevibacteriumiodinum, Acinetobacter calcoaceticus, Brevibacterium epidermidis, Lactobacillus acidophilus, Aspergillus versicolor, Aspergillus niger, Aspergillus ochraceus, Rhodoturulaspp, Saccharomyces spp.*	Phenylalanine; Ochratoxin A	Hydrolyze the amide bond	(Du *et al.* 2017; Vanhoutte*et al.* 2016)
Trichothecenes	*Blastobotrys capitulate, Trichomonascus, Aspergillus, Curtobacteriumspp, Anaerovibriolipolytica, Selenomonas and Saccharomyces*	3-acetyl T-2 toxin; T-2 toxin 3-glucoside; Neosolaniol	Acetylation deacetylation, deep oxidation, oxygenation, Epimerization, and glucosylation	(Du *et al.* 2017; Vanhoutte*et al.* 2016)
Zearalenone	*Mucor bainieri, Rhizopus spp, Cunninghamellabainieri, Alternaria alternate, Thamidium elegans, Aspergillus ochraceous, Rhodococcus spp, Streptomyces rimosus, Trichosporonmycotoxinivorans, Pseudomonas spp, Aspergillus niger and Acinetobacter spp*	(α-ZEL) and β-zearalenol (β-ZEL) ; ZOM-1; 2, 4- dimethoxyl zearalenone; zearalenone- 4-β-D-glucoside	Cleavage of the lactone ring and change of the hydroxyl groupC-4	(Du *et al.* 2017; Vanhoutte*et al.* 2016)

CONCLUSION

The fungal contamination and mycotoxin accumulation account for major food spoilage in the world and can greatly influence the world economy. The presence of mycotoxins in food or feedstuff not only accounts for food spoilage but poses a serious health hazard to both humans and animals. Therefore, the control of mycotoxins in food and feed is essential to ensure the food safety and food security. Mycotoxins accumulation in food can be controlled by implementation of good agriculture practices. The consumption of mycotoxin contaminated food can lead to serious health hazards. Mycotoxins can be eliminated from food or feedstuff by physical and chemical decontamination. The complete elimination of mycotoxins from food and feed commodities is a difficult task and requires a combined effort from policy makers, government agencies, farmers, processors and distributors. However, the developing countries should opt for the modern strategies for minimizing the mycotoxin contamination to control their level in food commodities within the maximum allowable limits. The maximum allowable limits for mycotoxins, their control strategies should be communicated with farmers and processors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Hemato-biochemical Profile of Ostriches (*Struthio Camelus*) based on Gender and Age of Birds

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ABSTRACT: The present study was conducted to determine the hematobiochemical analysis of ostrich (*Struthio camelus)* with respect to gender and progressive age. A total of 40 apparently healthy ostriches of either sex were spitted into young (subgroups; upto 1 year, 1 to 2 year, 2 to 3 year) and adult groups (subgroups; 3 to 4 years and > 4 years). Blood samples were collected from the wing vein of the ostriches maintained in one of the ostrich farms near the Gogra in Pakistan. For hematobiochemistry analysis, the serum samples were obtained by centrifugation of collected blood samples and kept in 1ml aliquots. The hematobiochemical parameters included total protein, glucose, urea, uric acid, creatinine, aspartate amino transferase, alanine aminotransferase, gamma Glutamyl transferase, lactic dehydrogenase, alkaline phosphatase, cholesterol, triglycerides, sodium, potassium, calcium, phosphorus and magnesium. The results showed a significant age-related difference in all serum biochemical values, however, the non-significant difference were observed in males and females within the same age group. The mean values of cholesterol, HDL-C, uric acid and creatinine were non-significant ($P>0.05$) between two age groups from young to adult age and between the sexes of the same age group. The statistical analysis explained that sodium (Na) and potassium (K) values were significantly ($P<0.05$) increased in young than the adult ostriches. Keeping in view these findings, we may conclude that this preliminary study embarked on establishing a set of reference values in serum biochemistry of ostrich in Pakistan.

***Keywords*:** kidney function test, liver function test, minerals, Serum biochemistry

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INTRODUCTION

Poultry production systems have been integrated with human livelihoods for thousands of years, enhancing diet, income, and food and nutrition security (Shoaib et al., 2020; Yasmin et al., 2020; Farooq et al., 2021).Ostrich (*Struthio camelus*) belongs to a family of flightless birds (Ratites) and lives in deserts and savannahs of Africa and the Arabian Peninsula. The ostrich is called a camel bird due to its similarities with the dromedaries. It was denominated by Linnaeus in 1758, like *Struthio camelus* (Deeming, 2001). An adult ostrich, the largest non-flying bird in the world, weighs about 150 kg and is mainly raised for meat production because of its low cholesterol and fat content (Mancevica and Mugurevics, 2013; Elhashmi et al., 2021). Ostrich farming is a developing industry all over the world. This expansion in the ostrich farming over the globe is observed more pronounced in the past two decades (Al-Sobyail et al., 2011; Zaima et al., 2021a). Current survey indicates that about 0.55 Million ostriches are slaughtered per annum globally. Initially, in Pakistan, ostrich farms started as a part of the agro-tourism industry, but since then, ostrich meat (volais) is gaining wide acceptance (Zaima et al., 2021b).

In ostrich farming, the birds are more susceptible to health issues that results from unappropriated management of the breeders that results in environmental and nutritional stress (Miranda et al., 2008). To assess the health status of birds, hematobiochemical and specific plasma metabolites provide valuable information that can be helpful for the disease evaluation. These parameters are not only influenced by nutrition, body scoring, gender, age, captivity and circadian rhythm but can also be taken as physiological indicator that act as relevant diagnostic tool in veterinary medicine (Durgun et al., 2005; de Almeida et al., 2018; Moryani et al., 2021). These blood standards have specific values for sex, age and species. Another advantage is that they reflect diet and management practices, which explains why they are essential in the evaluation of clinical and nutritional disorders that affect birds (Blue-McLendon and Green, 2010)

For successful ostrich farming, diagnosis and monitoring of ostrich health need to be done routinely. Thus, clinical hematology and serum biochemistry are widely used as a diagnostic tool. No serum biochemistry parameters have been published or compiled for an ostrich in the Pakistani environment. Hence, this preliminary study was embarked on to establish a set of reference values in serum biochemistry of ostrich in Pakistan.

MATERIALS AND METHODS

Ethical Concern

Ethical clearance for this research was granted by the Animal Care and Use Committee of University of Agriculture, Faisalabad-Pakistan (Ethical clearance number:962). No bird was harmed otherwise and no health hazard to the handlers of such type of slaughtering.

Experimental Groups

A total of 40 clinically healthy ostriches of either sex (20 males, 20 females) comprising five age groups of equal size (n=8) viz; young one (Up to 1 year, 1-2 year and 2-3 year) and adult (3-4 year and above 4 years) were used in this study (Table 1).

Table 1. Grouping of youngand adult birds.

Groups	Age	Male	Female	Total
Young Group	Up to 1 year	**4**	**4**	**8**
	1-2 year	**4**	**4**	**8**
	2-3 year	**4**	**4**	**8**
Adult Group	3-4 year	**4**	**4**	**8**
	Above 4 years	**4**	**4**	**8**
Total		**20**	**20**	**40**

Collection of Blood Samples

Blood samples were collected from the wing vein of the ostriches maintained in one of the ostrich farms near the Gogra in Pakistan. Two test tubes were used for the collection of blood samples from each ostrich. Serum was separated by centrifugation @ 500g for ten minutes of blood and collected in 1ml aliquots following by storage at -20°C. Then serum samples were used for respective analytical determinations.

Biochemical Analysis

Glucose

The level of glucose in serum was measured with the help of a commercially available diagnostic kit, which is known as Flutiest ® GLU- Analyticon diagnostic kit.

Serum Lipid Profile

Total serum cholesterol and triglycerides (mg/dL) were measured with the help of a commercial reagent kit byDia Sys Diagnostic Systems (Cat. No. 5760) USA while HDL-Cholesterol (mg/dL) in serum was

measured with the help of a commercially available reagent kit (Randox Laboratories Limited, UK). To calculate VLDL (Very low density lipids) and LDL (Low density lipids) were measured from following formulas

$$\text{VLDL}= \frac{\text{triglycerides}}{5}$$

LDL=HDL+total cholestrol-Triglycerides

Serum Proteins

The total proteins and albumin in serum were measured with the help of a commercially available Monoreagent Diagnostic Kit, K031, prepared by Bioclin®, laboratory, München, Germany.

Liver Function Markers

The concentration of ALT (Alanine transaminase) and AST (Aspartate aminotransferase) in serum were measured with the help of a commercially available diagnostic kit, which were prepared by Randox laboratories, UK and its reference number BT294QY.

Serum Enzymes

The concentration of ALP (Alkaline phosphatase) in serum was measured with the help of a commercially available diagnostic kit, which was prepared by ALP Fortress diagnostics, limited, Bioclin® München, Germany.The concentration of GGT (Gamma-glutamyl transferase) in serum was measured with the help of a commercially available diagnostic kit, which was prepared by Transferase GGT kinetic diagnostic kit. The concentration of LDH (Lactate dehydrogenase) in serum was measured with the help of a commercially available diagnostic kit, which was prepared by LDH Fortress diagnostics, limited, Bioclin®.

Renal function test

The concentration of creatinine in serum was measured with the help of a commercially available reagent kit prepared by the Creatinine Ecoline Merck diagnostic kit while Breuer and Breuer diagnostic kit was used for urea and uric aciddetermination in serum.

Mineral Profile

Some macro minerals like calcium (Ca), magnesium (Mg), phosphorus (P), sodium (Na) and potassium (K) were determined in serum. Ca, Mg and P concentration in serum was determined through the wet digestion process and its absorbance was determined by the atomic absorbance spectrophotometer. Sodium and potassium concentration in serum was measured through a flame photometer.

Statistical analysis

Factorial one-way analysis of variance (ANOVA) was used to compare the means of parameters. Tukey's test was used to compare the group's mean at a 5% level of significance

RESULTS

Serum Glucose

Analysis of variance of glucose in young and adult ostriches of both sexes are presented in Table 2. The values of the glucose showed increasing trend ($P < 0.05$) among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years) and maximum value was observed in above 4 years group (Fig 2). Statistical analysis described the mean values of glucose were significantly ($P<0.05$) different among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years) and adults group (3-4 year and above 4 years), but these values showed a non-significant ($P>0.05$) difference between the sexes of the same age group and between the adults group. A rapid increase in the parameters of serum glucose were observed in young ostriches, however which maintain a plateau after attaining adult age (Fig 2)

Serum Lipid Profile

Statistical results described that the mean values of cholesterol and HDL-C were non-significant ($P>0.05$) among the five age groups from young to adult age and between the sexes of the same age group. However, significant ($P<0.05$) increasing trend was seen in the mean values of triglycerides, LDL-C and VLDL-C from young to adult age (Fig 2). The maximum ($P<0.05$) value of these parameters was measured in the adult ostriches regardless of gender as shown in table 2 and Fig 2.

Serum Proteins

Analysis of variance of total serum proteins and albumin young's and adult ostriches of both sexes is presented in Table 2. The statistical analysis showed that the age is directly related to serum proteins values (Fig 2). The mean values of the total serum proteins and albumin increased ($P < 0.05$) among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years). Mean values of total serum protein and albumin were

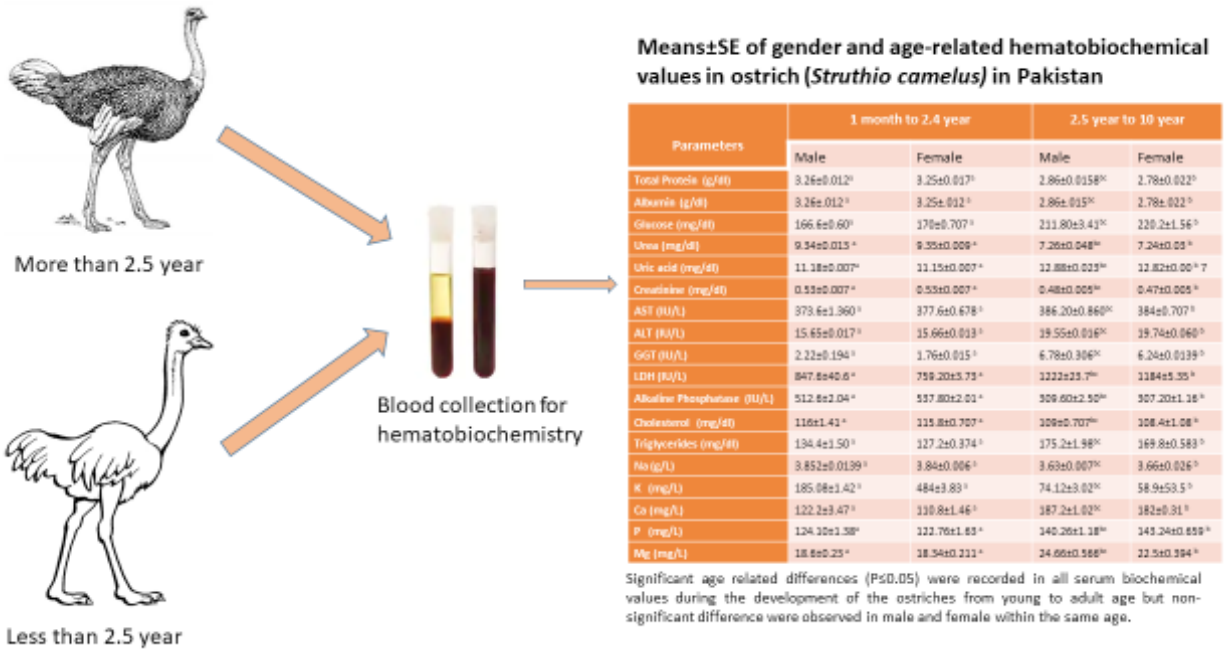

Figure 1. Schematic Representation of Hematobiochemistry

Table 2. Means ± SE values of different age-related hematobiochemical values in ostriches (*Struthio camelus).*

	Parameters	Means±SE (n=40)	Male (n=20)	Female (n=20)	Young Group			Mature Group	
					Upto 1 year (n=8)	1-2 year (n=8)	2-3 year (n=8)	3-4 year (n=8)	Above 4 year (n=8)
Serum Glucose	Glucose (mg/dl)	196.8±10.2	199.6±8.32^{a}	197.8±8.21^{a}	166.6±0.60^{a}	180±0.707^{b}	207±1.88^{c}	211.80±3.41^{d}	220.2±1.56^{d}
Serum Proteins	Total Protein (g/dl)	3.81±0.47	3.84±0.55^{a}	3.71±0.44^{a}	2.56±0.01^{a}	2.96±0.02^{b}	3.88±0.05^{c}	4.86±0.08^{d}	4.78±0.08^{d}
	Albumin (g/dl)	2.98±0.34	3.28±0.46^{a}	3.11±0.49^{a}	1.98±0.01^{a}	2.26±0.02^{b}	2.91±0.06^{c}	3.76±0.04^{d}	3.58±0.05^{d}
Serum Lipid Profile	Cholesterol (mg/dl)	110.38±0.91	113.53±1.12^{a}	111.25±1.01^{a}	110.1±0.21^{a}	111.8±0.71^{a}	113±1.41^{a}	109±0.71^{a}	108.4±1.08^{a}
	Triglycerides (mg/dl)	152.96±9.52	156.79±12.92^{a}	15.36±11.52^{a}	127.2±0.37^{a}	134.4±1.50^{b}	158.2±1.56^{c}	175.2±1.98^{d}	169.8±0.58^{d}
	HDL (mg/dl)	29.01±9.80	32.01±6.68^{a}	331.12±5.98^{a}	27.24±0.18^{a}	28.40±0.38^{a}	28.40±0.36^{a}	30.74±0.59^{a}	30.30±0.07^{a}
	LDL-C (mg/dl)	65.24±9.80	68.34±7.80^{a}	66.41±7.65^{a}	39.80±1.31^{a}	46.04±0.82^{a}	69.80±11.31^{a}	78.9±17.3^{b}	91.64±0.96^{b}
	VLDL (mg/dl)	31.22±1.86	34.22±2.61^{a}	33.21±2.53^{a}	25.44±0.07^{a}	29.88±0.31^{b}	30.24±0.09^{c}	35.04±0.39^{d}	35.52±1.62^{d}
Serum Enzymes	GGT (µkat/l)	4.37±1.02	4.92±2.10^{a}	4.97±2.02^{a}	1.76±0.02^{a}	2.22±0.19^{a}	4.86±0.04^{a}	6.78±0.31^{b}	6.24±0.01^{b}
	LDH (µkat/l)	1030.6±94.7	1030.6±89.62^{a}	1080.4±88.7^{a}	759.20±3.73^{a}	847.6±40.6^{b}	1140.10±15.56^{c}	1222±23.7^{d}	1184±5.35^{d}
	ALP (µkat/l)	431.1±50.7	470.1±57.87^{a}	467.1±56.45^{a}	488.7±1.16^{a}	512.6±2.04^{b}	537.80±2.01^{c}	309.60±2.50^{d}	307.20±1.16^{d}
Liver Function Test	AST (µkat/l)	391.02±5.67	397.23±6.77^{a}	395.12±6.71^{a}	373.6±1.360^{a}	381.6±1.21^{b}	397.6±0.74^{c}	402.20±0.86^{d}	400±0.71^{d}
	ALT (µkat/l)	16.92±1.44	17.72±2.54^{a}	17.62±2.54^{a}	11.98±0.01^{a}	15.65±0.02^{b}	17.66±0.04^{c}	19.55±0.06^{d}	19.74±0.06^{d}
Kidney Function Test	Urea (mg/dl)	8.53±0.57	9.45±0.68^{a}	9.43±0.67^{a}	8.66±0.05^{a}	9.34±0.03^{b}	10.13±0.05^{c}	7.26±0.05^{d}	7.24±0.05^{d}
	Uric acid (mg/dl)	11.64±0.53	12.76±0.65^{a}	12.56±0.69^{a}	10.18±0.008^{a}	11.18±0.01^{a}	11.15±0.01^{a}	12.88±0.02^{a}	12.82±0.01^{a}
	Creatinine (mg/dl)	0.49±0.02	0.48±0.03^{a}	0.47±0.03^{a}	0.44±0.006^{a}	0.53±0.01^{b}	0.53±0.01^{c}	0.48±0.01^{d}	0.47±0.01^{d}
Serum Mineral Profile	Na (g/L)	4.24±0.33	4.88±0.68^{a}	4.74±0.65^{a}	3.85±0.01^{a}	4.84±0.04^{b}	5.21±0.06^{c}	3.66±0.026^{d}	3.63±0.007^{d}
	K (mg/L)	130.2±5.29	133.2±6.72^{a}	131.2±6.29^{a}	150.08±1.42^{a}	164±2.83^{b}	184±3.83^{c}	78.9±3.53^{d}	74.12±3.02^{d}
	Ca (mg/L)	148.4±15.5	151.42±17.85^{a}	150.4±16.85^{a}	110.8±1.46^{a}	122.2±3.47^{b}	140±1.37^{c}	182±0.31^{d}	187.2±1.02^{d}
	P (mg/L)	130.82±5.29	133.82±7.29^{a}	131.61±6.82^{a}	115.10±1.38^{a}	122.76±1.63^{b}	132.76±1.88^{c}	143.24±0.65^{d}	140.26±1.18^{d}
	Mg (mg/L)	20.69±1.44	21.99±1.97^{a}	21.69±1.84^{a}	16.6±0.23^{a}	18.34±0.211^{b}	21.34±0.51^{c}	22.5±0.394^{d}	24.66±0.566^{d}

abcd: mean values having different alphabet differ significantly from one another (P≤0.05).

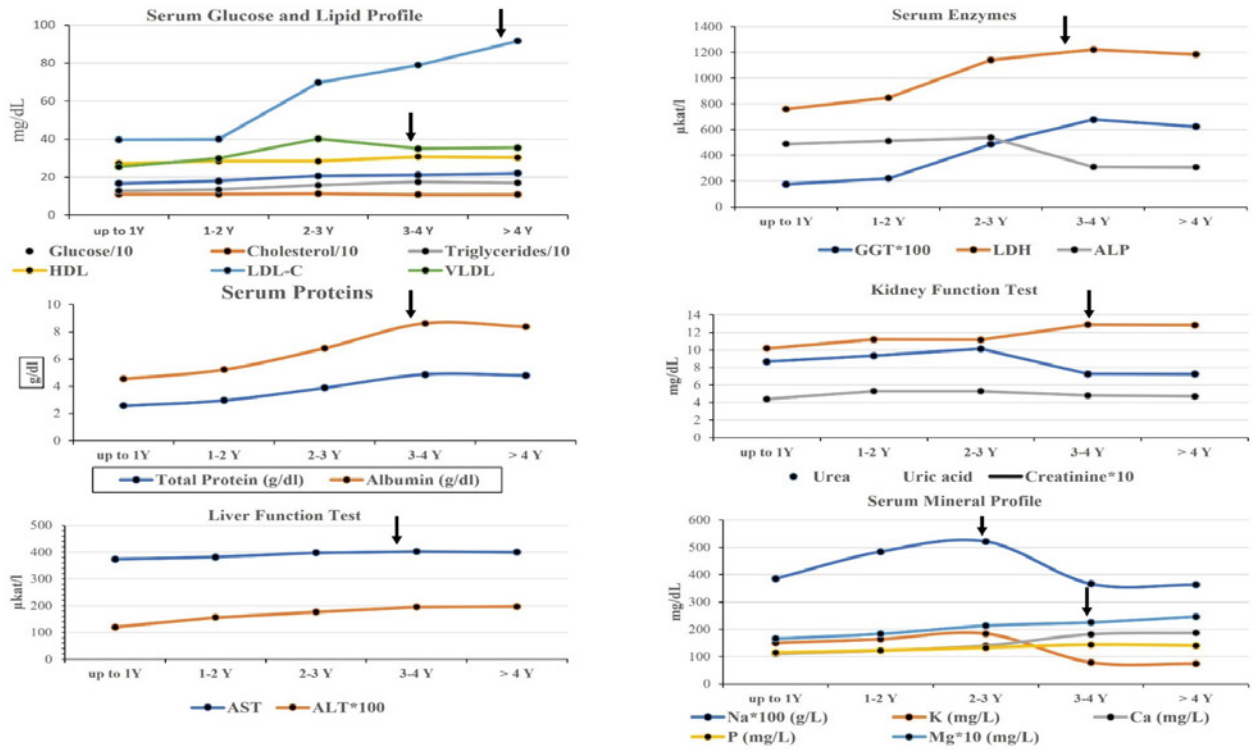

Figure 2. The pattern of different hematobiochemical values in the ostriches in relation with the progressive age. The black arrow showed the plateau of specific value at specific age where it maintained at particular point

significantly (P<0.05) greater in adults as compared to young groups. These values were statistically non-significant (P>0.05) among the sexes.

Liver Function Markers

The mean values of the liver function test, including serum Aspartate transaminase (AST) and serum Alanine transaminase (ALT) levels were presented in Table 2 and fig 3. The statistical analysis showed that the age is directly related to Liver Function values with the highest value in above 4 years birds. The mean values of AST and ALT increased ($P < 0.05$) among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years). Mean values of AST and ALT were significantly (P<0.05) greater in adults as compared to young groups. These values were statistically non-significant (P>0.05) among the sexes but significant (P<0.05) differences were observed among the mean values of the young and adult age groups between the adults group. (Table 2 and Fig 2). A rapid increase in the parameters of liver function markers were observed in young ostriches, however which maintain a plateau after attaining adult age (Fig 2)

Serum Enzymes

Analysis of variance of Gamma Glutamyl Transferase (GGT), Lactic Dehydrogenase (LDH) and Alkaline Phosphatase (ALP) in young and adult ostriches of both sexesare been presented in table 2.The statistical results of serum enzymes value, GGT and LDH were significantly (P<0.05) greater in the adult ostriches' group as compared with the young ostrich group but the mean values of ALP were significantly (P<0.05) greater in young's as compared adult ostriches (Fig 2).These values showed a non-significant (P>0.05) difference between the sexes of the same age group and between the adult age grouped. Gamma Glutamyl Transferase GGT (µkat/l) value was significantly (P<0.05) affected by the age of birds as greater values were observed in adult groups (Table 2).A rapid increase in the parameters of serum enzymes we observed in young ostriches, however which maintain a plateau after attaining adult age (Fig 2)

Renal function test

The mean values of urea (mg/dl) showed increasing trend ($P < 0.05$) among all young groups (i.e., up to 1 year, 1 to 2 years, 2 to 3 years) and were found significantly (P<0.05) greater in young's as compared to adult groups. Although, these values were statistically non-significant (P>0.05) between the sexes but significant (P<0.05) differences were observed among the mean values of the young and adult age groups (Fig 2). Statistical results described the mean

values of uric acid and creatinine were non-significant (P>0.05) between two age groups from young to adult age and also between the sexes of the same age group (Table 2).

Mineral Profile

The mean values of the mineral profile, including Sodium (Na; g/L), potassium (K; mg/L), calcium (Ca; mg/L), phosphorus (P; mg/L) and magnesium (Mg; mg/L) are summarized in Table 2.The statistical analysis explained that Na and K values were significantly (P<0.05) increased in the young group than the adult group of ostriches (Fig 2). However, the values of Mg, Ca and P were significantly (P<0.05) increase in the adult group of ostriches as compared to the young group of ostriches. The values of all mineral profiles followed non-significant changes in males and females within the same age, as shown in Table 2.

DISCUSSION

The poultry birds are extensively studied in Pakistan other than the ostriches. The ostrich farming is flourishing in Pakistan under the umbrella of Punjab Livestock and Dairy Department. The hematobiochemical parameters are considered the physiological indicators that helps to assess the health status and act as relevant diagnostic tool in the veterinary medicine. Therefore, this study was focused to observe the changes in hematobiochemical parameters of the ostriches in relation to progressive age and to establish a set of reference values of ostrich in Pakistan.

Generally, blood glucose level was greater in birds as compared to mammals (200 to 500 mg/dl). Contrary to mammals, glucose homeostasis in birds was controlled by glucagon because of the abundance of alpha cells in the pancreas (Thrall et al., 2004). In this study, the glucose level of young ostriches in males and females was 170 ± 0.71 mg/dl and 166.6 ± 0.60 mg/dl, respectively, which was in accord with the Khazraiinia et al. (2006) and Albokhadaim et al. (2012b).De Almeida et al., 2018 reported these values in emu which is lower than our findings of adult ostriches. Significantly (P<0.05), greater glucose in adult birds may be attributed to more consumption and type of diet. Moreover, oscillations in glucose levels may be associated with the management of animals, concerning aspects like stress.

Mean values of cholesterol level of different age groups were found no difference (P>0.05) and the same trend was described by Khazraiinia et al. (2006) and Samour et al. (2011). The same trend was observed in the emu (de Almeida et al., 2018). Serum cholesterol levels for most bird species range from 100 to 250 mg/dL (Lumeij 2008).

The mean values of triglycerides were significantly greater (P<0.05) in the adult group of ostriches as compared to the young and the same trend was described by Thrall et al. (2004) and Khazraiinia et al. (2006), however, Khaki et al. (2012) reports the lower limits than this study. Cholesterol values were recorded a significant change during the end of the egg-laying period of ostriches (Hrabcáková et al., 2014). Despite many published reports showing lower levels of cholesterol and fat in ostrich meat and lower levels of fat in broiler chicken meat compared with cattle and sheep meat, this study showed lower serum lipids in birds.

The mean values of total serum proteins and Albumin were significantly (P<0.05) different between two age groups from young to adult age (Table 2) there is paucity of literature describing the age relating changes in ostriches. But De Almeida et al. (2018) reported the comparable values of these parameters in emus. Young birds have a high rate of metabolism due to fast muscle and feather growth that required a high protein diet.

Significantly (P<0.05), increasing trend of ALP and AST was seen with the increasing age in ostriches that could be related to the greater metabolic rate, which was directly related to the turnover characteristics of cell and tissue in the growing phase (Bovera, 2007). The values of ALP found in this study were in line with Samour et al. (2011), who reported the value of ALP as 531±198 µkat/l in young ostriches. Greater levels of ALP indicate elevated osteoblastic activity, trauma and disease condition (Harr, 2006). The AST (µkat/l) value determined in this study were slightly greater than the values determined by Verstappen et al., (2002) in adult ostriches 321 ± 56µkat/l. AST was not necessarily sensitive to liver damage but also used in conjunction with Creatine Kinase (CK), a muscle-specific enzyme (Harr, 2006). The greater value of AST can be linked to the muscle stress and trauma associated with handling during blood sampling (Samour et al., 2011). Gamma Glutamyl Transferase GGT (µkat/l) value was significantly (P<0.05) affected by the age of birds as greater values were observed in group B (Table 2). Verstappen et al. (2002) reported the value of GGT in adult ostriches as 0.25± 0.47 µkat/l, which was not even close to the present study results. GGT value can be increased in inflammation, neoplasia and

biliary obstruction (Harr, 2006). This difference in the present study can be attributed to the different analytical methods and health status of the birds.

Lactic Dehydrogenase LDH values determined by (Samour et al., 2011) in one-years-old ostrich was 437.8 ± 38 µkat/l that was lower than the value of the present study. Age was directly related to the LDH value as in adult birds. These results were comparable with Verstappen et al. (2002), who reported 1107 ± 470 µkat/l level of LDH. These variations in the LDH value might be due to its close association with the skeleton muscles, handling and restraining of the ostrich. Moreover, discrepancies in LDH value may also be due to the difference in sampling time as its half-life is shorter than other enzymes.

The urea level was observed in the current study, falls under the normal ranges (0-10 mg/dl) in non-carnivores' birds but significantly ($P < 0.05$) changed with the progressing age. Urea was excreted by the renal corpuscles, which depends upon the hydration status of the birds and it was used diagnostic tool for the pre-renal azotemia in some birds (Thrall et al., 2004). The current study revealed the numeric value of uric acid showed a non-significant trend among different groups and gender. It was a nitrogenous waste. Its value in birds was generally greater than mammals (Khazraiinia et al., 2006; Bovera et al., 2007). In birds, uric acid is used as a health indicator because its sequential evaluation gives an idea about the progression and treatment of disease. The mean value of creatinine level in ostriches was similar to the previous studies as measured greater levels in this study (Thrall et al., 2004; Khazraiinia et al., 2006). The significantly greater levels of creatinine for group B are difficult to explain: the creatinine level is considered an index of muscle metabolism, and a creatinine increase normally occurs when the muscular tissue turnover accelerates. However, this result may be related to the greater levels of AST recorded between the sexes of the same age group. Same trend has been reported in eagles by Nazifi et al. (2008). In this study, the mean values of urea were non-significant ($P>0.05$)

Na and K values were significantly ($P<0.05$) increased in the young group than the adult group of ostriches. But the values of Mg, Ca and P were significantly ($P<0.05$) increase in the adult group of ostriches as compared to the young group of ostriches. The values of all mineral profiles were not so much changed in males and females within the same age, as shown in table 2. In this study, the serum mineral values of calcium, phosphorous, and magnesium were significantly greater in adult birds, but sodium and potassium were more elevated in young bird and were in agreement with Bovera et al., (2007). Calcium and phosphorous were essential for laying birds and bone mineralization. Deficiencies in these minerals may lead to different abnormal egg formation and bone. Osteoid matrix deficiencies were also associated with the other micro-minerals like copper, zinc magnesia, etc. Calcium and phosphorus values were recorded a significant change during the end of the egg-laying period of ostriches. Some variations were observed in the values obtained in the present study and the values mentioned in the relevant literature (Jaime et al., 2010). These differences in observations might be due to variations in management, environmental temperature, sample storage, and the time duration between processing and collection. The present study augments the existing diagnostic tools for health problems and routine monitoring of the health status of ostriches.

CONCLUSION

The information presented in this research paper provides the knowledge with important gender and age-related changes data that can be used for diagnosis of diseases and monitoring of ostrich health that needs to be done routinely. Close scrutiny of the data indicates that the serum biochemical parameters values is rapidly increased during the young age; however, it maintains a plateau with minor increments in the adult age. The sex of the birds had no significant effect on serum biochemical parameters in ostriches (*Struthio camelus*). These findings can be extremely helpful for diagnosis of pathological processes and routine monitoring of the health status of ostriches.

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CONFLICT OF INTERESTS

All the authors declared they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Aggressive behavior in cats exposed to trauma

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ABSTRACT: Aggressive behavior is an important behavioral problem in cats. This issue can occur as a reaction when there is disease or pain in a normal cat. The aetiology of the agressive behavior is beyond disease and pain.The aim of this study is to evaluate the behavior changes of cats exposed to trauma using behavior scoring system and demeanour scoring system. This study is consisted of 135 cats of different breeds, ages and genders with high rise syndrome and traffic accidents. These cats were given a detailed clinical and radiological examination. Demeanour scoring system, behavior tests, and visual analog scale were used to identify behavior changes and pain in cats. The findings from this study showed that cats exposed to trauma may experience behavioral changes or agression, and this may result from pain or stress from trauma.

Keywords: Aggressive behavior, Behavioral changes, Cats, Trauma

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INTRODUCTION

Aggressive behavior, which remains a current subject in cats, is a complex phenomenon that depends on genetics and many different environmental factors (Penar and Klocek, 2018).Among the factors that lead to aggressive behavior, biochemical and physiological processes, psychological factors including tendency and mood, emotional reactions, motor actions and vegetative reactions, and environmental factors such as false socialization, hostile environment or irresponsible animal owners are very important (Penar and Klocek, 2018). Aggressive behavior is defined as "attacks, attack attempts or attack threats" and this definition does not necessarily include the desire to harm aggressive behavior (O'Hearem 2009; Penar and Klocek, 2018).Behavioral issues of cats include drawing furniture, aggressive behavior, anxiety, overstimulation, and exaggerated vocalization. Both passive and active aggressive behavior is a more common problem (Strickler and Shull, 2014; Penar and Klocek, 2018).

Aggressive behavior can occur as a reaction when there is disease or pain in a normal cat (Camps et al., 2015). If a cat is suffering, the normal touch of its owner can be very disturbing, and the animal can instinctively respond with aggressive behavior (Camps et al., 2015). Aggressive behavior also disappears if the underlying cause is removed or the disease is cured (Penar and Klocek, 2018).On the contrary, in some cases, aggressive behavior or aggressive response of the animal is not always disappearing, cat not as tolerant any longer for manipulation due to bad experience.

Behavior scoring system has been developed for domestic cats to evaluate their responses to stress, as the Zeiler et al., (2014) aimed at detecting a change in behavior in healthy cats undergoing short-term hospitalization. Recent advances in pain scoring that Mills et al., (2020) investigated the correlation between behavior problems and painful processes, have been recorded in cats and dogs. However, the nature of the cat (the individual temperament or friendly, confident, shy, protective or wild) is not taken into account when describing the type of aggressive response. Any cat that is not friendly and does not feel confident could incorrectly be classified as suffering. Increased stress worsens the perception of pain, and recovery is delayed in healthy cats (Zeiler et al., 2014).Stress may not be the source of pain, but all stress factors can alter behavior. Therefore, it is important to determine an effective behavior scoring system, the individual temperament of the cat and changes in daily behavior in the home context or in hospitalization. It will be more sensitive to detect pain by adding individual temperament and behavior to pain scoring systems.

The visual analog scale (VAS) consists of a 100 mm line labeled "no pain in one end" and "extreme pain" in the other, and the pain indicating the line is scored by the blind author (Tan and Yayla, 2018; Yayla et al., 2019).

High Rise Syndrome (HRS) is a term used to describe cats falling from two or higher floors, resulting in injury or less often death (Vnuk et al., 2004).

The aim of this study is to evaluate the behavior changes of cats exposed to trauma using behavior scoring system and demeanour scoring system.

MATERIALS AND METHODS

The study was conducted on cats following approval by the Animal Research Local Ethics Committee of Dicle University (Dicle Universty-HADYEK, E--020-73418).

Study animals

Permission was obtained from the owners and clinical protocol was recorded before starting the study.

The study material consisted of 135 cats of different breeds, ages and genders with high rise syndrome and traffic accidents between 2019 and 2020 at Dicle University Faculty of Veterinary Medicine, Department of Surgery. These cats were given a detailed clinical and radiological examination. In addition to neurological and orthopedic evaluation, major trauma protocol was applied in all cases. In addition, all cats in the study were injected with Tranexamic acid (Transamin® 5% ampoule, Teva, IV) and Methylprednisolone (Prednol L® 20 mg, Mustafa Nevzat, IM) for a possible internal bleeding, spinal cord injury and shock picture that may develop. Fluid support was provided for (0.9% NS, 0.09% NaCl®, Baxter, IV).Then the evaluation of the cats was carried out as follows.

Usage and evaluation of scales

In previous studies, "Demeanour scoring system (DSS) " used by Zeiler et al. (2014) and "behavior test (Owner-assigned response scores) " used by Vaisanen et al. (2007) were used to identify behavior changes in cats included in the study. In addition, visual analog

scale (VAS) was used to evaluate the pain (Merola-andMills, 2015; Yayla et al., 2019).

DSS consisted of 8 multiple choice questions (5 hands-off and 3 hands-on questions) to describe the cat's movement. Each question was given a score of 0 to 5 depending on the option selected, and the total DSS score is the simple sum of the answers to all questions (Table 1). Owner-assigned response scoring, which is a behavior test, consisted of two parts as previously described (Vaisanen et al., 2007). The first part consisted of a list of 10 individual aspects of cat behavior. This included overall level of activity, time spent sleeping, playfulness, aggressive behavior (as table 1), closeness (ie, the cat's desire to be in close proximity to a person), willingness to stay on the lap, attention seeking, withdrawal or hiding, vocalization (other sounds other than purring or hissing), purring, and appetite. This scoring was as follows; significantly increased (+2), increased to some extent (+1), no change (0), decreased to some degree (-1) and clearly decreased (-2). The second part consists of 9 statements about the behavior that the owners are asked to indicate the degree of observation about the time it takes to be brought to the hospital after trauma. Cats move differently than normal, have a lower posture than normal, have a different attitude than normal, sleep in different positions than normal, sleep in different places than usual, restless than normal, scary than normal, vocalize differently than normal and respond differently than normal. The answers received were clearly yes (+2), to some extent yes (+1) and no (0).

The VAS consisted of a 100 mm line labeled "no pain at one end" and the other "extreme pain", and the perceived pain intensity indicating the line was marked by blind writer.

While scoring, one researcher person (first author) met with the patient owners (Table 1, DSS score) and each cat was evaluated by this person (first author) immediately after trauma.

For the cats included in the study, it was recorded that age, gender, breed, whether they had undergone surgery before. In addition, the cat's position in the family (0 = only one animal; 100 = full member of the family), and now anxiety or degree of distress (0 = not at all stressed; 100 = extremely stressed) were noted. Also, cats with aggressive behavior in their historytowards humans and veterinary clinics were not included in the study. All these evaluations were made by a single person (SY) immediately after the trauma. Subsequent follow-up continued for a week, either by phone or face to face.

Table 1. The total range of total scores obtainable (0 to 25) divided into the five different demeanour categories (from Zeiler et al., 2014).

Total Score	Category	Definition of category and note on possible link with pain scoring systems
0	Friendly and confident	Cat is "loveable" with attention seeking. Will approach handler for human-cat interaction. The pain score may be considered true as these cats are assumed to be able to reflect their true demeanour.
1-8	Friendly and shy	Cat is friendly and "loveable" when handled but will not seek interaction with human. These cats may mask pain and thus the score obtained in the pain score may be lower than the true pain experienced.
9-18	Withdrawn and protective	Cat remains ridged and appears "uneasy" when handled. Handler does not feel in danger of being attacked by cat during handling. These cats may mask pain very well. The pain score may be lower than the true pain experienced.
19-24	Withdrawn and aggressive	Cat is ridged or frozen and may demonstrate pawing, biting, or clawing when handled. May or may not be associated with growling. Handler feels in danger of being hurt when handling this cat. These cats may mask their pain very well. The pain score may be lower than the true pain experienced.
25	Overtly aggressive	Handler at risk of being injured when handling this cat. Cat makes aggressive gestures including hissing, growling, pawing, clawing and rapid aggressive movement towards handling attempts. These cats are a challenge to handle and evaluate correctly. This makes pain scoring a challenge or perhaps even impossible.

Table 2. Distribution of patients evaluated and scoring scale

Cause of trauma		DSS					Total
		1 (0)	2 (1-8)	3 (9-18)	4 (19-24)	5 (25)	
High rise (n=110)	Medical treatment	12	8	6	3	3	32
	Surgery	35	21	14	7	1*	78
Traffic accident (n=25)	Medical treatment	2	2	1	1	1	7
	Surgery	7	5	3	1	2	18
Total		56	36	24	12	7*	135

*shows statistically different in each row, P < 0.05

Table 3. Scores consist of two part for the extent of change in various behaviors

Part I	clearly decreased (-2)	decreased to some extent (-1)	no change (0)	increased to some extent (1)	clearly increased (2)
Overall level of activity	41	32	21	5	1*
Time spent sleeping	4*	14	21	29	32
Playfulness	1*	6	14	35	44
Aggressive behavior	0*	0	92	3	5
Closeness	0*	5	18	35	42
Willingness to stay in the lap	2*	8	57	23	10
Attention seeking	10	14	31	25	20
Withdrawal or hiding	8	15	42	24	11
Purring	25	23	35	12	5*
Appetite	17	26	24	23	10

*shows statistically different in each row, P < 0.05

Part II	no change (0)	changed to some extent (1)	clearly changed (2)
Way of movement	15	35	55
Posture (lowered)	40	32	28
Demeanor	32	37	31
Sleep (position)	41	30	29
Restlessness (increased)	65	27	8*
Fearfulness (increased)	60	24	16
Vocalization	65	21	14
Response to touch	55	27	18

*shows statistically different in each row, P < 0.05

Treatment protocol

Operations such as orthopedic surgery and palatorrhaphy were performed under general anesthesia (2-3 mg/kg Xylazin (Rompun®, Bayer 20 mg/ml/im, 10-20 mg/kg dose Ketamine (Ketaso®l, Interhas, 100 mg/ml/im) in cats. Postoperative analgesia management (meloxicam (Metacam® 20mg / ml IV) was performed for 3 days. Also, other cats that did not undergo surgery received medical treatment for wound or soft tissue damage.

Data on the post-treatment period of all animals undergoing surgery and receiving medical treatment were not included in the study, since standardization cannot be achieved.

Behavioral tests were performed after trauma and operative situations were not included.

Statistical analysis

Statistical analysis of the data was performed using the Minitab-17 software package. All data from the study were considered nonparametric, and for calculations, the sign test of median was used with P<0.05 was accepted as significant.

RESULTS

It was determined that the cases included in the study were cats with 81.48% high rise syndrome and 18.52% affected by traffic accident. Sixty-three (46.66%) of the cases were male and 72 (53.34%)

were female. The cases were distributed between 1 and 8 years in terms of age. Body weights of the patients varied between 0.8 kg and 4.5 kg. There was no statistically significant difference in body weight, age and gender.

Distribution of patients evaluated and scoring scale (Table 2) and scores of change in various behaviors (Table 3) are summarized in tables.

According to DSS, aggressive behavior was observed in 7 of all casesat the time of arrivaltotheclinic and this was found statistically significant ($P<0.05$). In addition, 4 of these 7 affected cats did not have any orthopedic disorders (such as osteosynthesis, vertebral stabilization) or other conditions requiring surgical treatment (such as cleft palate, hernia, tissue loss wounds). It was learned that these cats returned to condition before trauma on the 3rd day was during hospitalization or after discharge (Table 2). However, a significant behavioral change was not observed in 56 cats who received medical treatment (14) and undergoing surgery (42) (Table 2).

In the behavioral change scale results, a statistically significant difference was observed, especially in terms of aggressivity, similar to DSS (Table 3).

There was no significant difference between high rise (mean 35, min 0- max 89) and traffic accidents (mean 42, min 0- max 95) in cats evaluated with VAS. There was a significant difference between those who undergoing surgery (mean 38, min 0- max 85) and those who received medical treatment (mean 41, min 0- max 90). In addition, the VAS score (mean 75, min 60- max 95) of 7 patients with 5th grade in DSS was higher than the others.

DISCUSSION

Behavioral scores have been defined to evaluate behavior changes in cats against various stressors (Zeiler et al., 2014). DSS and owner-assigned response scoring have been recently used by different authors, and their results have been reported (Zeiler et al., 2014; Vaisanen et al., 2007). In our study, we aimed to evaluate the behavior changes of cats exposed to trauma by using these two tests and VAS.

Recently, many scales related to pain in cats and dogs have been described, and many of them have been used especially in the assessment of postoperative pain (Tan and Yayla, 2018; Yayla et al., 2019). However, in these pain tests or scales, many situations related to cat's behavior are not considered. Some behaviors such as the habitus and friendly, confident, shy, protective and wild behaviors are important in cats. Increased stress aggravates pain and delays recovery. Stress factors may not cause pain, but they do cause a change in behavior. Therefore, an effective behavior scoring system should consider the cat's individual temperament and be able to monitor daily behavioral changes. If individual temperament and behavior are included in pain scoring systems, it may be more sensitive in detecting pain (Brondani et al., 2011; Gaynor and Muir, 2009; Zeiler et al., 2014). In our study, while VAS was used as pain score, DSS and owner-assigned response scoring were used together in terms of behavioral changes. Three different scales covering both animal behavior and pain issues were evaluated together.

Feline behavioral experts have shown the types of aggressive behavior as aggressive behavior caused by lack of socialization, play aggressive behavior, aggressive behavior associated with petting, redirected aggressive behavior, pain-associated aggressive behavior, predatory behavior and inter-cat aggressive behavior (Tuzio et al., 2004). It is also important to understand normal behavior in cats as stated in the guide. Besides, knowing normal social behavior and communication can prevent aggressive behavior (Tuzio et al., 2004).

The aggressive behavior of felines is a complex issue (Penar and Klocek, 2018). Aggressive behavior can occur in cats due to many different causes. Hunger, thirst, pain, all sorts of disappointment, as well as fear, are among the causes (Beata, 2001). The cause may not always be pain or a disease, and this cause is sometimes difficult to determine (Camps et al., 2015). In our study, trauma stress was considered as the cause of aggressive behavior and therefore this study was conducted on cats exposed to trauma. As a source of trauma, cats with traffic accident and high-rise syndrome were chosen. Aggressive behavior appeared in only 7 of 135 cats. Pain management and medical treatment were performed in 4 of 7 cats. Therefore, we do not think that aggressive behavior in cats exposed to trauma is related to the severity of trauma. In addition, the cat's aggressive behavior or predisposition to aggressive behavior or other individual characteristics may be more important than trauma stress.

Another important issue in controlling post-traumatic pain or aggressive behavior change is the patient's home care and the relationship with the owner

(Camps et al., 2015). The cat's attitude towards the owner at home or its relationship with other animals should be evaluated. Post-traumatic cats can be normal at home while very aggressive in the veterinary clinic. On the contrary, if conditions such as nutrition or wound care cannot be achieved when aggressive movements are observed at home, this should not be neglected. On comforting measures, the owner of the cat should be warned or recommended. Scoring should be done to evaluate pain and effective pain management should be applied. According to the results obtained in our study, it was seen that 5/7 of the cats who had aggressive behavior were calmer at home, only when they became aggressive in the clinic. The other two cats were observed to be aggressive towards their owners, but these cats returned to normal when their treatment was completed.

Understanding aggressive behavior in cats is a very different matter. Communication with the cat is important both for the examination of the cat without any damage and for the veterinarian to prevent damage and for the simple interventions. Cat owners also worry that their cats are suffering from pain. Therefore, it is necessary to deal with aggressive behavior in cats. Owners of cats as well as veterinarians should be aware of the types and causes of aggressive behavior in cats. It should be thoroughly evaluated in terms of aggressive behavior or behavior change.

CONCLUSION

As a result, it should be known that cats exposed to trauma may experience pain, even if there is no orthopedic disorder or surgical treatment, and that pain or trauma stress can cause behavioral changes. In addition, we think that the behavioral categories in Table 1 should be known and taken into account in the treatment process.

An interesting finding was that only 7 out of 135 cats displayed aggressive behavior according to the behavioral scores we used. It is also noteworthy that the remaining cats did not change despite their potential to exhibit aggressive behavior.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Evaluation of seroprevalence and associated risk factors of toxoplasmosis in sheep and goats in District Jhang-Pakistan

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ABSTRACT: Toxoplasmosis is a zoonotic infection caused by a pathogenic protozoan, *Toxoplasma gondii,* responsible for huge economic and health losses in developing countries.The current study was conducted to assess the seroprevalence of toxoplasmosis and associated risk factors in sheep and goats in District Jhang, Punjab, Pakistan.Blood samples (n=400) were collected from both genders of goats (n=219) and sheep (n=181) from four Tehsils of District Jhang along with a comprehensive questionnaire to evaluate the risk factors associated with the disease endemicity and spread. For assessing the seroprevalence, the samples were examined using Latex agglutination test. Additional data regarding hygienic conditions, water source, gender, breed, age of animal was also collected on a predesigned questionnaire.The overall seroprevalence of *Toxoplasma gondii* was found 34.25% (137/400) in District Jhang. Higher seroprevalence was recorded in goats {36.52% (80/219)}as compared to sheep {31.49% (57/181)}, however, it was non-significant (p>0.05). Gender-wise seroprevalence was found 32.59% (44/135) and 35.09% (93/265) in male and female animals, respectively (p>0.05). Further, the association of toxoplasmosis between different age groups was significantly higher in older animals having age >24 months 42.75% (62/145) than younger animals with age <12 months 26.60% (29/109) and 11-24 months 31.50% (46/146) (p<0.05). The seroprevalence was also higher 40.81% (80/196) in animals drinking water from outdoor water source than in animals drinking from indoor water source 27.94% (57/204) (p<0.05). Moreover, seroprevalence was significantly higher 43.11% (97/225) in animals kept in vicinity of cats than in absence of cats 22.85% (40/175) (p<0.05). However, reproductive status, breeds, flock size had non-significant impact on the prevalence of *T. gondii*. Thus, it is concluded that the presence of cats near animals, larger flock size, older age of animals, and poor hygienic conditions are main risk factors of toxoplasmosis in sheep and goats and these could be a potential threat of infection for livestock industry and public health.

***Keywords*:** *Toxoplasma gondii;* Goats; Sheep; Latex agglutination test; Cats; Pakistan.

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INTRODUCTION

Toxoplasmosis is a common zoonotic disease caused by a parasite *Toxoplasma gondii* with high prevalence in human population. This parasite is distributed worldwide and is considered as one of the most successful parasite. *T. gondii* infects a large number of species, including domestic animals. Toxoplasmosis is more prominent in small ruminants like goats and sheep and causes huge economic losses due to abortion and neonatal deaths in infected animals. Humans can also be infected with this disease using contaminated and undercooked meat and milk of infected animals (Dubey and Jones, 2008).

Due to zoonotic potential of toxoplasmosis globally, several serological studies show that antibodies against *T. gondii* are found in more than one third of human population. However, prevalence of *T. gondii* in human population varies greatly in different countries due to variations in cultures and customs; and even shows variations among different ethnic groups within one country (Sroka, 2001). Toxoplasmosis has been found 20-30% in USA, 25% in Japan, 60% in Netherlands, 60% in Italy, 35% in Finland and 50%-60% in Poland (Abu-Dalbouh et al., 2012).

T. gondii is an obligate intracellular pathogenic protozoan that belongs to family Sarcocystidiae (Sibley et al., 2009). *T. gondii* has a number of strains and are mainly divided into three classes i.e. Type I, Type II and Type III (Boothroyd and Grigg, 2002). Type I is recognized as an infectious agent that causes infection in rodents. Type II is a causative agent of toxoplasmosis in small ruminants like goats, sheep etc. However, it has been found that Type III is not an infectious strain of toxoplasmosis (Boothroyd, 2009).

T. gondii completes its sexual stage in cats because these are definitive hosts (Petersen and Schmidt, 2003). Cats spread oocysts through their feces. Humans, warm blooded animals and large number of other animals act as intermediate hosts of *T. gondii.* Cats or other members of felids shed oocysts after completion of sexual stage where sporozoites are harbored (Bisson et al., 2000). When oocysts contaminated food is ingested, sporozoites found in oocysts enter into the gastrointestinal tract of the secondary host (sheep, goat, buffalo, cattle, mice, birds, and humans) and completes its asexual period of life cycle.

In livestock, the main route of transmission of the parasite is through ingestion of sporulated oocysts. These oocysts are produced in wild and domestic cats during the sexual stage of their life cycle. These animals are major reservoir of the parasite. However, it has been noted that this infection does not exist or rarely found if cats are absent. So cats have a critical role in spreading this infection (Jones and Dubey, 2012). Sheep and goats are commonly infected with *T. gondii*. In sheep and goats, *T. gondii* infection causes significant losses due to abortion and because of zoonotic transmission through consumption of infected milk and meat (Afonso et al., 2013).*T. gondii* can cause early embryonic death, fetal death, resorption, stillbirth, abortion, mummification and neonatal death in sheep depending on the stage of gestation (Jones et al., 20001).

T. gondii is a zoonotic parasite which may transmit to humans through consuming raw or undercooked meat containing infectious tissue cysts, taking food or water infected with sporulated oocysts, using goat or sheep milk contaminated with *T. gondii* tachyzoites (Ahmad et al., 2015), and parasite may also be transmitted to humans through organ transplantation, blood transfusion and transplacentally transmitted from mother to fetus (Ramzan et al., 2009). In humans, infection does not show any symptoms, however, individuals suffering from AIDS and immunocompromised may show some complications. In humans, toxoplasmosis may be congenital or acquired (Tenter et al., 2000).*T. gondii* causes both acute and chronic infections. The major symptoms of the toxoplasmosis are flu-like mild illness having characteristics such as fatigue, fever and headache. Sometimes the disease exists without proper signs and symptoms. Immunocompromised people like HIV infected patients and pregnant women may suffer serious illness characterized by diarrhea, weight loss, liver diseases, pneumonia, and central nervous system infection. In case of severe infection, toxoplasmosis can even cause death.

Numerous studies have been conducted on the prevalence of *T. gondii* in different animal species in various parts of the world. Prevalence of *T. gondii* has been reported in goats and sheep in different parts of Punjab, Pakistan. Prevalence of toxoplasmosis was 19.9% in sheep of Southern Punjab (Dubey, 2004) and 14.3% and 18.2% in goats and sheep of Pothowar region, respectively (Ganter, 2008). In Rahim YarKhan, Pakistan seroprevalence was reported 25.4 % and 11.2% in goats and sheep, respectively (Spisak et al., 2010). In District Jhang, rearing of goats and sheep is carried out on large scale in rural areas where livelihood of a huge community of the farmers

depend upon raising of these animals. However, there is no comprehensive data available on seroprevalence of *T. gondii* infection in goats and sheep in District Jhang. Keeping in view the consequences of the disease and its impact, the current study was designed to determine the seroprevalence of *T. gondii* in goats and sheep and associated risk factors e.g., gender, age, breed, water source, presence of cats, flock size, hygienic conditions with the infection.

MATERIALS AND METHODS

Study design

The presentstudy was carried out in four Tehsils (Jhang, Shorkot, Athara Hazari, and Ahmadpur Sial) of District Jhang, Punjab, Pakistan. The study was conducted in accordance with the Ethical Principles in Animal Experimentation, and before starting the research project, necessary ethical approval was obtained from the ethical review committee, Government College University, Faisalabad, Pakistan.

Collection of blood samples

Blood samples were collected from jugular vein of sheep and goats. Blood samples (n=400) were collected from goats (n=219) and sheep (n=181) present in the study area during the period from February, 2018 to July, 2018.To make the scope of study wider, blood samples were collected from male and females of three breeds of goats (teddy, beetal and juttle) and three breeds of sheep (desi, thalli and kajli) present in the area of study. One hundred samples were collected from each of the Tehsil to carry out the study.

Serum separation

After collection of blood, samples were promptly transported to the Microbiology Laboratory, College of Veterinary and Animal Sciences, Jhang for separation of serum. Serum samples were separated through centrifugation at 3000 rpm for 15 minutes. All serum samples were properly labelled and stored at -20°C until further analysis.

Questionnaire surveys

A questionnaire was designed to obtain the information from the farmers about the sampled animals regarding their age, sex, breed, herd size, abortion history, biosecurity, management practices, source of dinkingwater, and presence of cats in the premesis. Based on collected information, animals were grouped into three classes based on their age (<12 months, 13 to 24 months and >24 months), hygienic conditions (low, moderate and high). Low referred to farms that were cleaned after ≤2 days, moderate means that farms were cleaned >2 days and high means that farms were cleaned daily. Sources of water were categorized into an indoor water source (inside farmhouse) and an outdoor water source (ponds). Based on flock size, animals were divided into three classes (flock having <10 animals, flocks with 11 - 30 animals and flocks >30 animals). Reproductive status of animals (pregnant, non-pregnant and lactation) was also recorded. The data were recorded in a pre-designed questionnaire.

Latex agglutination test

Antibodies specific for *T. gondii* were measured using latex agglutination test (LAT) using a commercially available toxoplasmosis latex kit manufactured by Antech Diagnostic, UK according to the manufacturer's instructions. Latex reagent contains a suspension of polystyrene particles coated with antigens of *T. gondii*. Agglutination appears when the parasite is present in the serum in positive control while negative control does not show such agglutination. Antigen-antibody reactions occur when serum containing antibodies against *T. gondii* were tested and reactions can be easily visualized due to agglutination.The serum and all reagents were brought to room temperature. A drop of diluted serum (40µL) was placed into each well of test slide followed by the addition of a drop of latex reagent and mixed well. The presence and absence of agglutination were observed within four minutes. The positive sera indicated clear agglutination while in negative sera, no agglutination was observed.

Statistical analysis

The data was analyzed using SPSS version 23.0 IBM Inc. USA. Odds ratio was calculated using SPSS by comparing healthy and diseased animals to determine *T. gondii* infections. To measure the association between various variables Pearson's Chi-Square test was used. The difference was considered statistically significant at $p \leq 0.05$ and non-significant at $p > 0.05$.

RESULTS

The present study is the first epidemiological study carried out to evaluate the seroprevalence of the *T. gondii* infection in goats and sheep from District Jhang, Punjab, Pakistan using latex agglutination test (LAT). Among 400 serum samples collected from goats and sheep, 137 samples were found pos-

itive. Hence, the overall seroprevalence of *T. gondii* in goats and sheep in district Jhang was 34.25% (137/400**)** (Figure 1). The seroprevalence of *T. gondii* in goats (36.52%) was higher than seroprevalence in sheep (31.28%). Out of 219 serum samples collected from goats, 80 were found positive, so seroprevalence of *T. gondii* was 36.52% (80/219). While 57 out of 181 blood samples collected from sheep were positive so, the seroprevalence was 31.49% (57/181).

Tehsil-wise seroprevalence of *T. gondii* in goats was 44.64%, 35.38%, 22.72%, and 40.74% in Tehsil Jhang, Tehsil Athara Hazari, Tehsil Shorkot and Tehsil Ahmadpur Sial, respectively. So, the highest seroprevalence (44.64%) was found in goats of Tehsil Jhang and lowest (22.72%) in goats of Tehsil Shorkot. In sheep, the seroprevalence was 38.63%, 25.71%, 30.35% and 30.43% in Tehsil Jhang, Tehsil Athara Hazari, Tehsil Shorkot and Tehsil Ahmadpur Sial, respectively. So, the highest prevalence in sheep was found in Tehsil Jhang (38.63%) and the lowest (25.17%) in Tehsil Athara Hazari.

There has been a close association reported between toxoplasmosis in goats and sheep and various risk factors such as gender, age, presence of cats, flock size, source of water, breed etc. The current data showed that seroprevalence of *T. gondii* in female goats was higher 37.57% (59/157) than male goats 33.87% (21/62), however, this difference was statistically non-significant. However, seroprevalence was little bit higher in male sheep 33.33% (21/63) than female sheep 30.50% (36/118).

It was also found that seroprevalence was higher in older age groups (>24 months) than younger animals having age <12 months and between 13-24 months. The prevalence of *T. gondii* in goats having age <12, 13-24 and >24 months was 30% (15/50), 39.71% (33/83) and 41.02% (32/78), respectively. Hence, in goats highest seroprevalence was found in animals having age >24 months. In sheep, seroprevalence was 29.72 % (11/37), 30% (18/60) and 37.83% (28/74) in above gropus, respectively.

Cats are definitive hosts of *T. gondii*. Current study demonstrated that seroprevalence was significantly higher in those animals living in the farms where cats were present than those farms where no cats were present. Prevalence of *T. gondii* in sheep and goats living in vicinity of cats was found 47.61% (60/126) and 38.53% (42/109), respectively. However, seroprevalence in absence of cats was 21.50% (20/93) and 20.83% (15/72) in goats and sheep, respectively.

The current data showed a positive correlation between flock size of goats and sheep and seroprevalence of *T. gondii*. Highest seroprevalence (43.66%) was found in those goats that live in flock size of >30 as compared to animals living in flock comprising of less than 10 (40%) and between 11 to 30 animals (29.59%).

The seroprevalence was also higher 40.81% (80/196) in animals drinking water from outdoor water source than in animals drinking water from indoor water source 27.94% (57/204) ($p<0.05$). While, based on different breeds of goats, seroprevalence of *T. gondii* in Teddy breed was 50% (23/46), Juttle 46.42% (13/28) and Beetal 30.34% (44/145). Whereas the seroprevalence based on different breeds of sheep was Thalli 44.11% (15/34), Kajli 41.93% (13/31), and Desi 25% (29/116) (Table 1).

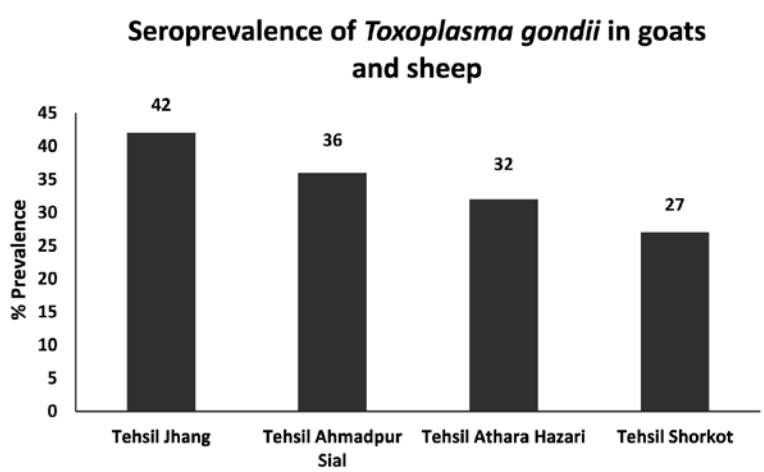

Figure 1. Seroprevalence of *T. gondii* in different Tehsils of District Jhang

Table 1. Demographic and risk factor association of *Toxoplama gondii* in goats and sheep

Risk Factor	Tested	Positive	Negative	Prevalence	Chi-Square	*P*-Value	Odds Ratio	95% Confidence interval	
								Lower	Upper
1- Animal Type									
Goat	219	80	139	36.52%					
Sheep	181	57	124	31.49%	2.398	0.122	1.392	0.915	2.118
2-Place									
Tehsil Jhang	100	42	58	42%					
Tehsil Athara Hazari	100	32	68	32%					
Tehsil Shorkot	100	27	73	27%					
Tehsil Ahmadpur Sial	100	36	64	36%	5.362	0.147	--	--	--
3-Breed									
Teddy	46	23	23	50%					
Beetal	145	44	101	30.34%					
Juttle	28	13	15	46.42%					
Desi	116	29	87	25%					
Thalli	34	15	19	44.11%					
Kajli	31	13	18	41.93%	9.700	0.084	--	--	--
4-Gender									
Male	135	44	91	32.59%					
Female	265	93	172	35.09%	0.249	0.618	0.894	0.576	1.388
5-Age (months)									
<12	105	26	79	24.76%					
13-24	143	51	92	35.66%					
>24	152	60	92	39.47%	7.978	0.019	--	--	--
6-Flock Size									
<10	97	27	70	27.83%					
11--30	135	40	85	29.62%					
>30	168	58	110	34.52%	0.010	0.995	--	--	--
7-Cats									
Yes	235	102	133	43.40%					
No	165	35	130	21.21%	17.932	0.000	2.558	1.646	3.974
8-Reproductive status (Female animals)									
Pregnant	91	34	57	37.36%					
Non-Pregnant	116	36	80	31.03%					
Lactation	68	25	43	36.76%	3.161	0.206	--	--	--
9-Water Source									
Outdoor	230	86	144	37.39%					
Indoor	170	51	119	30.00%	7.358	0.007	1.779	1.171	2.701
10- Hygienic Conditions									
Low	124	56	68	45.16%					
Moderate	191	61	130	31.93%					
High	85	20	65	23.52%	11.347	0.003	--	--	--

DISCUSSION

There exist great variations in prevalence of *T. gondii* across the world and rates of infection ranges from 0 to 100 percent in various countries (Villagra-Blanco et al., 2019). This difference in prevalence depends upon traditions, customs, weather conditions, husbandry practices, presence or absence of cats and difference in age (Atail et al., 2017). The current study has given an insight on toxoplasmosis and revealed the widespread occurrence of *T. gondii* among the domestic goats and sheep raised in District Jhang, Punjab. In the present study overall seroprevalence including both species was found 34.25%.Current prevalence rate i.e. 34.25% that is higher than those reported by (Andrade et al., 2013) in South Africa and (Romanelli et al., 2007) in North America as 4.3% and 11.2%, respectively. However, present prevalence rate is lower than reported earlier (42.8%) by (Ahmed et al., 2016) in Pakistan, 49.43% reported by (Sechi et al., 2013) in eastern Slovakia, 52% reported by (Gebremedhin et al., 2013) in Multan, Pakistan and 52% reported by Buxton et al., 2007 in El-Gadrif state. These variations in the seroprevalencerates with the above reports could be due to differences in their customs, traditions, lifestyles of the inhabitants, weather conditions, age of the animals and husbandrypractices (Gazzonis et al., 2015).

It has been reported in several studies describing various risk factors have significantassociation with toxoplasmosis in goats and sheep (Samra et al., 2007; Wang et al., 2011; Ahmad and Tasawar, 2016; Turcekova et al., 2013; Ramzan et al., 2009). These risk factors include source of water, age, flock size, presence of cats, hygienic conditions etc. Cats are definitive host of *T. gondii* and can pass oocysts of the parasite in their feces, hence one of major source of environmental contamination (Rashid et al., 2016; Onyiche and Ademola, 2015). Moreover, the prevalence rate may also be linked with the presence of cats that excrete oocysts, which after sporulation become infectious to man and animals (Zhao et al., 2015). In present study, the association of these risk factors was also checked.

It was found that seroprevalence was higher in older age groups (>24 months) than younger animals having age <12 months and between 13-24 months. The prevalence of *T. gondii* in animals having age <12, 13-24 and >24 months was 24.76% (26/105), 35.66% (51/143) and 39.43% (60/152) respectively. So, the present study revealed the fact that prevalence increases with an increase in the age of animals. Present results are supported by some previous reports describing that seroprevalence of *T. gondii* increases with an increase of age of animals (Roberts et al., 2001; Raeghi et al., 2011; Lopes et al., 2010; Fu et al., 2009). These results suggest that usually, animals acquire infection post-nataly. The increase in infection rates with the increase in age might be due to fact that old animals have a high opportunity of contact with various predisposing risk factors or ingestion of infective oocysts from the environment (Gottstein, 1995). Furthermore, older animals have low immunity as compared to younger ones and hence, less resistant to infection (Hove et al., 2005).

Cats are definitive hosts of *T. gondii*. So their presence or absence among sampled animals might affect the prevalence of parasite. Current study confirmed the presence of cats as an important factor in the epidemiology of the toxoplasmosis. It is found that cats are positively associated with the infection caused by *T. gondii*. Prevalence was significantly higher in those animals living in the farms where cats were present than those farms where no cats were present. Prevalence of *T. gondii* in sheep and goats living in vicinity of cats was found 43.40% (102/235), while seroprevalence in absence of cats was found 21.21% (35/165). The results are due to the reason that cats shed millions of oocysts in the environment which could be ingested by animals along with food and water thus causing infection. Other studies have also confirmed that a significant association is found between the prevalence of toxoplasmosis and the presence of cats in the vicinity (Ahmad and Qayyum, 2014) reported the results from a study conducted in Poland that the presence of wandering cats is an important risk factor that transmits the infection to sheep and goat. Current results are in agreement with the findings from previous studies reporting an increase in seroprevalence in goats and sheep due to presence of cats (Cenci-Goga et al., 2013; Deyo et al., 2009; Fotiric-Aksic, 2013).

In current study it was found that seroprevalence was high in those animals that live in poor and moderate hygienic conditions as compared to those living in a well hygienic conditions. Seroprevalence in animals was highest 45.16% (56/124) among those who live in poor hygienic conditions while it was 31.93% (61/191) and 23.52% (20/85) in animals living in moderate and hygienic conditions, respectively. These findings are in agreement with those reported by others (Montoya and Liesenfeld, 2004; Camossi

et al., 2011). The risk of contamination of food and water with oocysts is greatly reduced due to proper cleaning at farms, thus decreasing the risk of Toxoplasmosis. While poor cleaning conditions at farms increases the risk of contamination of water and food by oocysts and eventually increases the risk of toxoplasmosis.

The results of current study showed a positive correlation between flock size of goats and sheep and seroprevalence of *T. gondii.* The highest prevalence i.e. 34.52% (56/168) was found in those goats that live in flock size of more than 30 as compared to animals living in a flock comprising of less than 10 i.e.27.83% (27/97) and between 11 to 30 animals 29.62% (40/135). These results are in agreement with the results reported by (Sadek et al., 2015). They found that there exists a positive correlation (r=0.9275) between flock size and prevalence of toxoplasmosis. They suggested that chance of toxoplasmosis increases with large flock size as compared to small flock size. The reason is that in large flock size animals have a greater chance to come in contact with each other and infectious material such as cat's feces because floor space of the pen per animal is less (Robert-Gangneux et al., 2017). Besides, animals in large flock size might have received less care from managers as compared to smaller flock size where nutrition and care could be better (Vismarra et al., 2017). Similar correlation was also found by others (Tavassoli et al., 2013).

CONCLUSIONS

Present study witnessed the high prevalence of Toxoplasmosis in study area and it is further concluded that presence of cats near animals, large flock size, older age of animals, and poor hygienic conditions are the main risk factors of Toxoplasmosis in sheep and goats in District Jhang which are potential threat of infection for human population of study area. Moreover, further studies are needed for countrywide screening of the food animals thatmight create awareness in animal farmers and help policymakers to formulate suitable approaches to control this disease.

CONFLICT OF INTEREST

There is no potential conflict of interest among the authors listed in this manuscript.

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Research article
Ερευνητικό άρθρο

Molecular and histopathologic investigation of Pestivirus, *Chlamydophila abortus* and *Listeria monocytogenes* infections in aborted sheep foetuses

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ABSTRACT: The aims of this study was to investigate the presence of pestiviruses, *Chlamydophila abortus* (*C. abortus*) and *Listeria monocytogenes* (*L. monocytogenes*) and histopathological findings caused by these agents in aborted sheep foetuses. A total of 52 aborted sheep foetuses, aged between 1 to 5 months of gestation, were collected from Konya province in Turkey. Molecular techniques were used for the detection of pestivirus RNA, *C. abortus* and *L. monocytogenes* DNA in investigated samples. Pestivirus RNA was detected in 6 (11.5%) of the 52 aborted sheep foetuses whereas *C. abortus* DNA was determined in 8 (15.4%) foetuses. However, *L. monocytogenes* DNA was not detected in investigated samples. The significant histopathological findings were hypomyelination, degeneration and necrosis of neurons in the brain, interstitial pneumonia, mononuclear infiltrations in the liver, hyperemia and proximal tubule degeneration in the cortex of kidney in pestivirus positive samples and multifocal purulent-necrotic foci, diffuse neutrophils and mononuclear infiltrations in the liver and spleen, hyperaemia, bleeding, intramyelinic and perivascular oedema, gliosis, neuronophagia in the brain, hyperemia, proximal tubule degeneration and neutrophil granulocyte infiltration in the cortex of kidney were observed in *C. abortus* positive samples. The results of the study show that *C. abortus* and pestivirus infections play an important role in abortion in sheep.

Keywords: Border disease virus; *Chlamydophila abortus*; Ewe; *Listeria monocytogenes*; Pathology

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INTRODUCTION

Small ruminants significantly contribute to the national socio-economic development through their high meat or milk yield, their rapid generational turnover, and their high number, which exceeds 38 million according to 2018 data in Turkey (TÜİK, 2019). However, in livestock enterprises, the most important risk factor that threatens sustainability is abortion. Previous studies have shown that pestiviruses, *Chlamydophila abortus* (*C. abortus*) and *Listeria monocytogenes* (*L. monocytogenes*) are important infectious agents that cause abortion in small ruminants in Turkey (Bulut et al., 2018; Kalender et al., 2013; Karaca et al., 2007).

Border disease virus (BDV) and bovine viral diarrhoea virus (BVDV), which infect sheep and goats are members of the *Pestivirus* genus in the *Flaviviridae* family. These two pestivirus infections are globally distributed, and the presence of immunotolerant, persistently infected animals lead to significant economic losses and reproductive losses as well as to respiratory disease and diarrhoea in ruminants. Pestivirus infections have been more widely reported in sheep than in goats. Congenital infection of small ruminants can lead to abortion, malformations and birth of persistently infected offsprings (Bulut et al., 2018; Constable et al., 2017; Feknous et al., 2018; Sozzi et al., 2019). Histopathologically, pestivirus infections are characterized by central myelination deficiency, along with demyelination and acute necrotising and inflammatory lymphoproliferative lesions (Constable et al., 2017).

Chlamydophila abortus (previously named *Chlamydia psittaci*), an intracellular microorganism and zoonotic agent, causes abortion in sheep by infecting epithelial cells and macrophages. *C. abortus* can persist for several months in non-pregnant sheep without any clinical signs. The principal sources of this microorganism include vaginal discharges, foetuses and placentas (Livingstone et al., 2017). Histopathologically, necrotic and inflammatory foci can be found in the liver of infected foetuses, and small focal necrosis can be found in the lungs, spleen and rarely in the brain (Kalender et al., 2013).

Listeria monocytogenes, a facultatively anaerobic, gram-positive microorganism found in silage and soils, causes listeriosis, a serious and life-threatening disease affecting a wide range of animals (Wang et al., 2018). Listeriosis in ruminants is characterized by encephalitis, abortion and neonatal septicaemia with miliary visceral abscesses. In cases of utero foetal death with autolysis, no recognisable lesions can be seen. However, in some foetuses, multifocal hepatocellular necrosis or microabscesses are commonly numerous in the liver but occur less in the spleen (Constable et al., 2017). Similar lesions are present in the lung, kidney, spleen and brain and are usually only visible microscopically (Schlafer and Foster, 2016).

In Turkey, the seroprevalence of pestiviruses, *C. abortus* and *L. monocytogenes* in small ruminants varied between 0 - 90.27%, 1.6 - 29.3% and 34.69 - 58.39%, respectively (Gökçe et al., 2007; Kalender et al., 2013; Karaca et al., 2007; Ural and Erol, 2017). These infections have not received enough attention in abortion cases in Turkey. The epidemiological screen is one of the main appropriate control strategies to limit the spread of these infections. The objective of this study were to investigate presence of pestiviruses, *C. abortus* and *L. monocytogenes* in abortion cases of sheep and describe the histopathological lesions caused by these agents in sheep foetuses.

MATERIAL AND METHODS

Samples

The current study was performed during the lambing season of 2018. The Konya province, according to the data of Turkish Statistical Institute for the year 2018 ranked second with sheep populations of about 2 million. A total of 52 aborted sheep foetuses were collected from the Konya province in Turkey. Sheep foetuses aborted were aged between 1 to 5 months of gestation. At necropsy, tissue samples (lung, liver, spleen, kidney and brain) were collected from aborted sheep foetuses, and placed in PBS for molecular analyses and in 10% buffered formalin for histopathological examination. The study was approved by the Animal Ethical Committee of Hatay Mustafa Kemal University (Approval No. 2017/1-1).

Nucleic Acid Extraction for PCR

Foetal tissue samples were homogenized in PBS using mortar and pestle. DNA extraction was performed from the tissue homogenates using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the kit's instructions. RNA extraction was carried out from the homogenized foetal tissue samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Both DNA and RNA extracts were stored at -85 °C until required for PCR analyses. DNase/RNase-free distilled water

was used as negative control in detection of agents.

Detection of *C. abortus*

A PCR method described by Thiele et al., (1992) was used to detect 16S rRNA sequence of *C. abortus*. PCR was performed in a final volume of 25 µl of a mixture containing 2A: 5′-GCTTTTCTAATTTA-CACC-3′ and 2B: 5′-ATAGGGTTGAGACTATCCA-CAT-3′ primers. Amplification was performed using Techne Thermal Cycler (Bibby Scientific Limited, Staffordshire, UK) with the following conditions: 40 cycles of 94 °C for 2 minutes, 50 °C for 1 minute and 72 °C for 75 seconds, and final extension at 72 °C for 7 minutes. Amplified DNA products were electrophoresed at 90V for 1 hour in 1.5% agarose gel stained with ethidium bromide and examined under UV illumination. A 116 bp fragment was deemed positive for *C. abortus* (Thiele et al., 1992).

Detection of L. monocytogenes

Real time PCR was performed using *L. monocytogenes* prfA gene specific primers and probe described by Rossmanith et al. (2006) with a probe master mix kit (Roche, Germany). Real time PCR reactions were performed in Lightcycler 2.0 PCR machine (Roche, Indianapolis, USA) with the following conditions: an initial denaturing step at 95 °C for 10 minutes, followed by 45 cycles of 94 °C for 15 seconds, 64 °C for 1 minute. The samples that had a Ct value <35 were considered positive (Rossmanith et al., 2006).

Detection of Pestiviruses by RT-PCR

One-step RT-PCR was used for the detection of pestivirus RNA. The assay was carried out in a 25 µl of a mixture containing primers 324: 5′-ATGCCCT-TAGTAGGACTAGCA-3′ and 326: 5′-TCAACTC-CATGTGCCATGTAC-3′, which amplify 288 bp region of the 5’ UTR region (Vilcek et al., 1994), with using one step RT-PCR kit (Qiagen, Hilden, Germany). Amplification was performed using Techne Thermal Cycler (Bibby Scientific Limited, Staffordshire, UK) with the following conditions: reverse transcription step at 50 °C for of 30 minutes and initial PCR activation step at 95 °C for 15 minutes, followed by 35 cycles of 94 °C for 1 minute, 57 °C for 1 minute and 72°C for 1 minute, and final extension at 72 °C for 10 minutes. Amplified PCR products were analysed on 1.5% agarose gel stained with ethidium bromide after electrophoresis at 90 V for 60 min. A 288 bp fragment was deemed positive for pestiviruses (Vilcek et al., 1994).

Histopathological evaluation

The tissues (lung, liver, spleen, kidney and brain) were fixed 10% buffered formalin for histopathology. Tissues were routinely processed and paraffin-embedded. The sections in 5 µm thickness from paraffin blocks were prepared and stained with Haematoxylin and Eosin stain (HE). These samples were observed under the light microscope (Olympus BX50-F4, Tokyo, Japan). For significant cases, microscopic images were photographed and transferred into digital camera system (Olympus DP12-BSW, microscopic, Tokyo, Japan).

RESULTS

Detection of *C. abortus* and L. monocytogenes by PCR

Out of 52 aborted sheep foetuses, 8 were found positive for *C. abortus* by PCR, which was directly performed on tissue samples obtained from the aborted foetuses. The age of the *C. abortus* positive foetuses were 2.5 months old (n=2) and 4-5 months old (n=6). *L. monocytogenes* DNA was not detected in the investigated samples.

Detection of Pestivirus RNA by RT-PCR

Pestivirus RNA was detected in 6 (11.5%) out of the 52 aborted sheep foetuses. The age of the pestivirus positive foetuses were 1 month old (n=1), 2 months old (n=2), 3 months old (n=2) and 4 months old (n=1). In this study, dual infection was not detected in the investigated samples.

Histopathological Findings in Foetal Tissues

Histopathological examination of six pestivirus-infected foetuses aborted at 3-5 months of gestation revealed the presence of severe hyperaemia, areas of multifocal haemorrhage, oedema and hypomiyelination (Figs. 1A-C), degeneration and necrosis of neurons, neuronophagia (Fig. 1B), gliosis in brain, hyperaemia in the meninges and infiltrations of mononuclear cells (especially of lymphocytes in one abortion) (Fig. 1D). In the lungs, we observed severe hyperaemia, mononuclear cell infiltration in the interalveolar septum, and in one abortion, enlargement of the interlobular septum and oedema. In the liver, considerably intense mononuclear cell infiltration in the portal area as well as severe hyperaemia and hydropic degeneration in hepatocytes were detected only in one abortion (Fig. 1E). Renal hyperemia was detected in

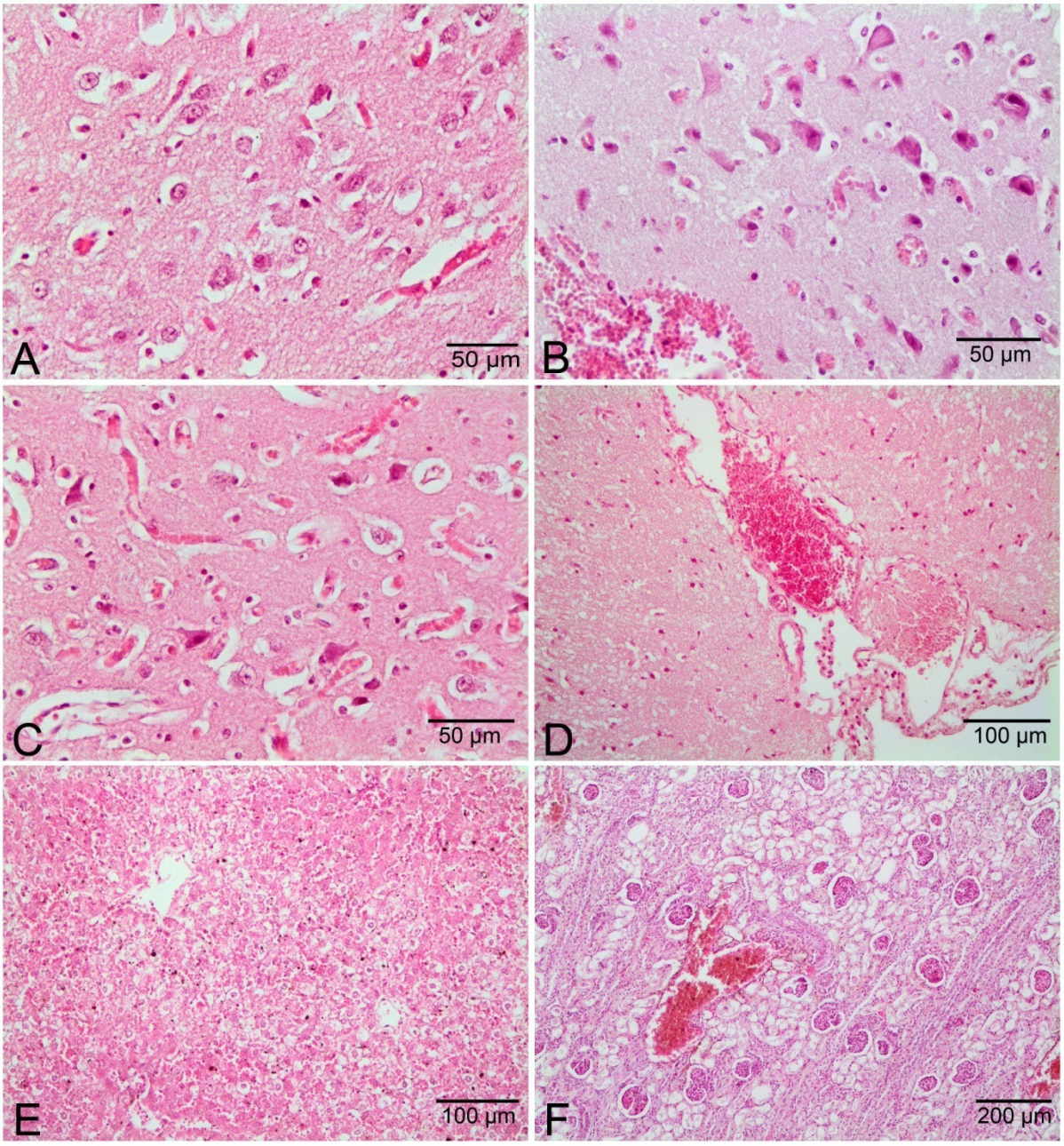

Figure 1. Hypomyelination (A), haemorrhage, degeneration and necrosis in neurons (B), hyperaemia, intramiyelinic oedema (C), hyperaemia and mononuclear cells in the meninges (D), hydropic degeneration in hepatocytes (E), tubule degenerations and metanephros or atrophy of some glomeruli (F). HE staining

all cases; in one abortion, haemorrhage and tubule degenerations were observed, whereas metanephros or atrophy of some glomeruli was observed in another abortion (Fig. 1F). Hyperaemia was also observed in the spleen of the aborted foetuses.

Histopathological examination of tissues obtained from eight *C. abortus*-infected foetuses aborted at 2.5-5 months of gestation revealed hyperaemia, degeneration and desquamation in the epithelium of bronchi and bronchioles as well as severe neutrophil granulocyte, lymphocyte, macrophage infiltration and mild neutrophil granulocyte infiltration in the alveol and interalveolar septum (Fig. 2A). In the liver, we observed hyperaemia, multifocal purulent-necrotic foci, diffuse neutrophil granulocyte and mononuclear cell infiltrations of limited number of lymphocytes and plasma cells and of macrophages and hepatocyte degenerations; in two abortions, degeneration and necrosis of hepatocytes were observed, whereas in

four abortions, intense neutrophil granulocyte infiltration was observed especially in the portal area and around the vena centralis (Figs. 2B-C). In the spleen, neutrophil granulocyte infiltration and multifocal purulent-necrotic foci were detected (Fig. 2D). In the central nervous system, hyperaemia in the brain and meninges, bleeding, intramyelinic and perivascular oedema, gliosis, neuronophagia and ischemic neuronal changes were observed; in one abortion, mononuclear cell infiltration was noticed in the brain (Fig. 2E). In the kidney, hyperaemia, proximal tubule degeneration and neutrophil granulocyte infiltration in the cortex and pelvis were observed in one abortion (Fig. 2F).

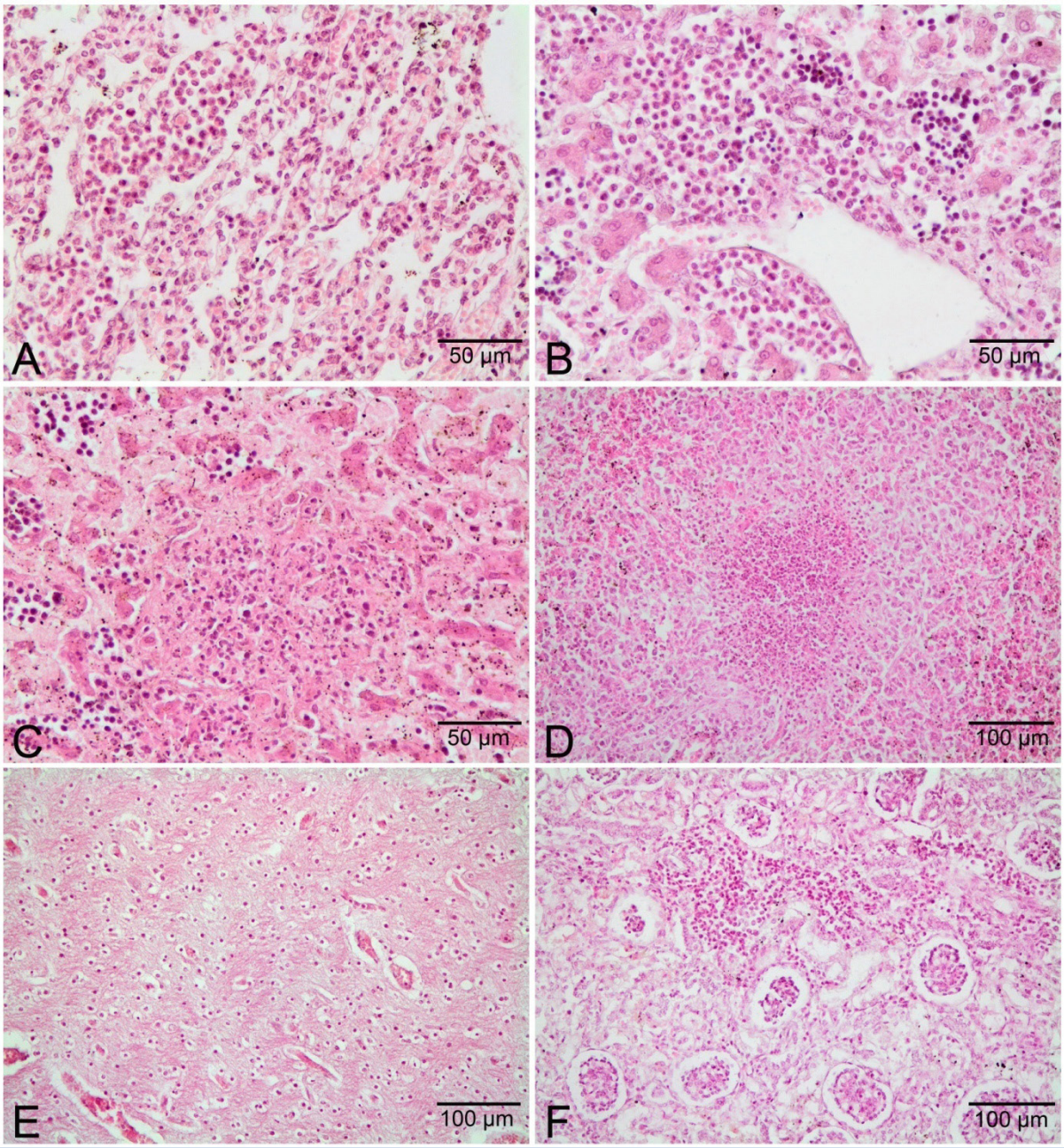

Figure 2. Neutrophil granulocyte infiltration in the alveol and interalveolar tissue (A), intense neutrophil infiltration around the vena centralis (B), multifocal purulent-necrotic foci in the liver (C) and in the spleen (D), hyperaemia and intramyelinic oedema in the brain (E), proximal tubule degeneration and neutrophil infiltration in the kidney (F). HE staining

DISCUSSION AND CONCLUSION

When *C. abortus*, which is the etiologic agent of enzootic abortion in sheep, is introduced into naive flocks, abortion rates can reach up to 25%-60% (Pugh et al., 2020). Serological studies conducted in different regions of Turkey have shown that the presence of *C. abortus*-specific antibodies among flocks that have history of abortion ranged from 1.6% to 46.6% (Gökçe et al., 2007). In this study, *C. abortus* DNA was detected in 8 (15.3%) of the 52 aborted sheep foetuses. This finding is consistent with a previous report (Arif et al., 2020; Heidari et al., 2018), although the current prevalence rate of *C. abortus* in aborted sheep foetuses is higher than the reported rates (3.49% and 6%) (Kalender et al., 2013). Possible explanations for this discrepancy include the number of sampled animals, time of sampling, individual differences and farm management.

Ewes infected with *C. abortus* are generally aborted within the last 2-3 weeks of gestation (Pugh et al., 2020). In the current study, six of the *C. abortus*-positive foetuses were aborted at 4-4.5 months of gestation. However, two *C. abortus*-positive foetuses were aborted at less than 3 months of gestation. Similar results were obtained by a previous study (Longbottom et al., 2013). Longbottom et al., reported that *C. abortus*-infected ewes are aborted at 74-138 days of gestation. This condition can be explained by latent infection with *C. abortus*, presence of secondary infections, individual differences or non-infectious factors. Microscopically, necrotic and inflammatory foci can be found in the liver, spleen and rarely in the brain of *C. abortus* positive sheep foetuses (Buxton et al., 2002; Kalender et al., 2013). Similarly, multifocal purulent-necrotic foci in the liver and spleen are observed in this study. There may be mononuclear cell infiltration in hepatic portal areas and multifocal areas of hepatitis (Buxton et al., 2002; Constable et al., 2017; Kalender et al., 2013; Longbottom et al., 2013; Navarro et al., 2004; Schlafer & Foster, 2016). However, in our study, intense neutrophil granulocyte infiltration was seen in the portal area and around the vena centralis in four of the foetuses infected with *C. abortus*. Thickening by mononuclear cell in interalveolar septum (Kalender et al., 2013; Longbottom et al., 2013; Navarro et al., 2004; Schlafer and Foster, 2016), infiltration of alveolar spaces by histiocytes and rare neutrophils in some cases in the lung (Miller et al., 1990) and in the brain, mild meningoencephalitis with vasculitis and hemorrhage (Kalender et al., 2013; Schlafer and Foster, 2016), small foci of leucomalacia, confined to the cerebral white matter cores, minimal focal microgliosis in the thalamus and midbrain (Buxton et al., 2002) have also been reported. In our study, mild neutrophil granulocyte infiltration in the interalveolar septum and changes in the epithelium of bronchi and bronchioles were observed. While mononuclear cell infiltration was noticed similarly in only one abortion in the brain, in other infected foetuses, bleeding, intramyelinic and perivascular oedema, gliosis and ischemic neuronal changes were observed in the brain.

Listeria monocytogenes can also cause abortion in sheep (Shoukat et al., 2014). The detection rates of *L. monocytogenes* in sheep abortions are 2.83% in Kashmir Region in India (Shoukat et al., 2014), 8.3% in Denmark (Agerholm et al., 2006) and 25% in Austria (Wagner et al., 2005). In Turkey, the presence of *L. monocytogenes* has been frequently identified serologically in cattle and small ruminants. The reported seroprevalence of *L. monocytogenes* in sheep is 25.8% in Bursa (Kennerman et al., 2000), whereas that in goats is 34.69%-60.81% in different provinces of Turkey (Karaca et al., 2007; K. Ural et al., 2009). We investigated the presence of *L. monocytogenes* in tissue samples of aborted sheep foetuses by using real-time PCR; however, *L. monocytogenes* DNA was not detected in investigated samples.

Both BDV and BVDV can infect small ruminants and cause abortion (Sozzi et al., 2019). Therefore, in this study only detection of pestiviruses was performed and genetic characterization of pestiviruses was not performed. In this study, the rate of pestivirus infections in sheep abortions was 11.5% (6/52). This finding is consistent with previous report (Şevik, 2018), although the current rate was higher than the reported rates (0.93%-3%) (Çokçalışkan, 2002; Oguzoglu et al., 2009). Possible explanations for this discrepancy include the detection method, the number of sampled animals and farm management. Ewes infected with pestiviruses generally abort at 60-85 days of gestation. Additionally, abortion may result if infection occurs beyond day 85 of gestation (Menzies, 2007). In the current study, three of the pestivirus-positive foetuses were aborted at 3-4 months of gestation. However, three pestivirus-positive foetuses were aborted at less than 3 months of gestation. This situation can be explained by strain of pestivirus, infection period and immune status of infected animals.

Chlamydophila abortus and *L. monocytogenes* DNA and pestivirus RNA were not detected in 38

aborted sheep foetuses. Abortion in these cases may be related with other infectious agents such as *Brucella sp.*, *Salmonella sp.*, akabane virus and bluetongue virus (Pugh et al., 2020) or may be related with non-infectious factors (nutritional and management factors).

In histopathological examination of pestivirus infected animals, hypomyelination, which usually occurs without signs of inflammation, is seen in all parts of the brain and spinal cord (Schlafer and Foster, 2016). It has been reported necrotizing and/or non-suppurative meningo-encephalomyelitis, often accompanied by hypomyelinogenesis in the brain. The most consistent finding are periventricular leukomalacia with perivascular mononuclear cell infiltration and gliosis (Krametter-Froetscher et al., 2010; Oguzoglu et al., 2009). In this study, severe hyperemia, areas of multifocal haemorrhage, intramiyelinic oedema and similarly, hypomiyelination, degeneration and necrosis in some neurons and gliosis were observed in the brain of the aborted foetuses. A moderate leucocytostasis of the sinusoids has been seen in the liver and kidneys showed the moderate pigment nephrosis (Krametter-Froetscher et al., 2010). In our study, considerably intense mononuclear infiltration in the portal area, severe hyperaemia in several cases and hydropic degeneration in hepatocytes were detected in the liver of one aborted foetus. While renal hyperemia was detected in all cases, haemorrhage, tubule degenerations and metanephros or atrophy of some glomeruli were observed in another abortion. Metanephrosis and atrophy could not be distinguished in this study. Interstitial pneumonia characterised by mononuclear cell infiltration was seen in the lung (Krametter-Froetscher et al., 2010; Oguzoglu et al., 2009). We observed severe hyperaemia, mononuclear infiltration in the interalveolar tissue and also, enlargement of the interlobular septum and oedema in one abortion in the lungs. Although our findings violently demonstrated the common association of pestiviruses and *C. abortus* in sheep abortions, this does not rule out the possible involvement of other pathogens as the cause of the abortions in sheep. However, further studies are needed to determine detailed epidemiologic screening for other pathogens associated with sheep abortions.

In conclusion, our study investigated the prevalence of *C. abortus*, *L. monocytogenes* and pestivirus infections in sheep abortions. The current findings are only specific for the surveyed province of Turkey and may not be representative for other regions of the country. Our results show that *C. abortus* and pestivirus infections play important role in abortion in sheep. Further studies on different infectious agents that can induce abortion in large number of small ruminants will provide a deeper understanding of the role of infectious agents in abortion cases.

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CONFLICT OF INTEREST

None declared by the authors.

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Case report
Κλινικό περιστατικό

Polypoid Cystitis in a dog - A case report

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ABSTRACT: Polypoid cystitis is a rare disease of younger male dogs. The aim of this article is to report a case of polypoid cystitis, first in Greece, which was observed in a dog. The 11-year-old dog had a 3-month history of hematuria. Upon surgical approach to the retroperitoneal area an irregularly thickened wall was revealed, and additionally six (6) cysts 1-6 mm in diameter protruded from the mucosal surface. Histological examination confirmed transitional cell hyperplasia covering a core of proliferating connective tissue, edema and a neutrophilic inflammatory cell infiltrate with areas of ulceration and hemorrhage. These lesions are consisted with polypoid cystitis which is is a rare-benign disease, histopathology is the means of definitive diagnosis and it is best treated with surgical cystotomy.

***Keywords*:** polypoid cystitis, urinary bladder

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CASE HISTORY

A 11-year-old intact male German shepherd dog (GSD) was presented with 3-month history of hematuria. Urine culture was not performed as the dog had previously undergone broad spectrum antibiotic therapy, however clinical signs returned after discontinuation. Ultrasound and X-ray imaging was not elucidative, therefore, exploratory surgery was scheduled.

Upon surgical approach to the retroperitoneal area, the bladder wall was revealed to be irregularly thickened. Six (6) cysts 1-6 mm in diameter protruded from the mucosal surface. Surgical biopsy samples from the bladder polypoid masses of dog were obtained. The other organs were normal.

Tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with Hematoxylin and Eosin (H-E stain).

Histological examination confirmed transitional cell hyperplasia covering a core of proliferating connective tissue and polypoid projections were detected into the lumen. In addition to these findings, there was edema and an inflammatory cell infiltrate (neutrophils) with areas of ulceration and hemorrhage. All the above, which compose the mentioned criteria by Martinez et al. (2003) for establishing a definitive diagnosis of PC, along with the fact that the basement membrane was intact, very rarely are seen all together in the same specimen, hence the rarity of our case, which is the first reported PC case in dog in Greece. The latter is attributed usually to the unacceptable condition of the sample as the proliferating tissue, obtained during cystoscopy is very delicate and tiny. After an 8 month period the dog was free of symptoms, confirming our diagnosis.

DISCUSSION

Polypoid cystitis (PC) is a rare disease of the urinary bladder in dogs and grossly, in tangential sections, or in case where only small surgical specimens are obtained for biopsy, may be incorrectly taken for urothelial carcinoma - transitional cell carcinoma (UC/TCC), which stands as the most commonly diagnosed tumor in the urinary bladder of domestic animals. The predominant patient group are male medium aged dogs (D.J. Meuten and T.L.K. Meuten, 2017).

Of all reported malignancies in the dog, urinary bladder cancer accounts for about 2%, with invasive urothelial carcinoma (UC) being the most common form. Differentiation of the non-UC conditions (such as PC) from UC is of major importance, since the treatment and prognosis vary in a great degree (Fulkerson and Knapp, 2020). The similar gross morphologic appearance of a great number of neoplasms is the reason why PC cannot be easily diagnosed macroscopically. The golden standard of definitive diagnosis is histopathology. The obtained biopsies during cystoscopy will confirm in most cases the diagnosis of polypoid cystitis (Martinez et al., 2003), while in other cases conflicting results may require rediagnosis (Butty et al., 2021). Confirmation of UC when dealing with cases that are not definitive can be accomplished with the aid of immunohistochemistry (IHC) (uroplakin III, GATA-3) (Fulkerson and Knapp, 2020).

UC is mostly located at the bladder neck or trigone region, although such lesions can be found in all anatomical parts of the canine bladder (Martinez et al., 2003; Fulkerson and Knapp, 2020). In a study of 17 dogs with polypoid cystitis, the most favorable location is the cranioventral bladder mucosa (apex) and the ventral mucosa (Martinez et al., 2003).

The causative agents of polyp formation in dogs is still unknown. It can be induced from a chronic mucosal irritation of the urinary bladder, which is due to UTI (Urinary Tract Infection) or cystic calculi. Chronic irritation can be caused by cystic calculi and, as a result a polyp formation is enhanced even without the presence of UTI. Up to this day remains unknown whether UTI is a cause or effect of polyp formation. Another fact that needs to be clarified in the future is the role of some bacteria, such as *Proteus spp.,* in the pathogenesis of polypoid cystitis. In human medicine, the use of indwelling urinary catheters and the resulted chronic bladder irritation is implicated in the presence of PC. One study even suggested that the main cause of all these might be the drainage conditions, implying as a key factor the associated negative pressure in the bladder (Martinez et al., 2003).

Macroscopically, the surface of the mucosal bladder is elevated by one or multiple nodular to polypoid lesions that protrude into the lumen with a ranging diameter, from 5 mm to 4 cm according to Martinez et al. (2003), or 2-3 cm as reported by D.J. Meuten and T.L.K. Meuten (2017). Also edema and inflammation may cause the thickening of the bladder wall. Microscopically, the polyp consists of urothelial cell hyperplasia (Figures 1-2) that covers a core of proliferating connective tissue. Hyperplasia induce the

formation of Brunn's nests (varying sized foci of urothelium-Figure 3). Mucoid degeneration and dysplastic or even metaplastic foci can be found at the mucus-secreting glands (cystitis glandularis-Figure 4) along with edema, congestion, ulceration, hemorrhage and inflammation (D.J. Meuten and T.L.K. Meuten, 2017). All the above findings were observed in our case simultaneously which is rather not very common.

The histological criteria to establish a definitive diagnosis of polypoid cystitis are (1) microscopically detectable polypoid projections of mucosa and stroma into the lumen, (2) evidence of epithelial proliferation (thickening, mitotic figures, or down-growth of epithelial cords into the stroma), (3) stromal edema, (4) inflammation, and (5) stromal hemorrhage and hemosiderin accumulation. Apart from these, intraepithelial lumina often filled with proteinaceous secretions, ulcerations, erosions, granulation tissue, or epithelial atypia, also constitute characteristic lesions. These criteria are mentioned by Martinez et al. (2003) and were confirmed in our study. In 2009, a Korean essay by Im et al. reported the same histological findings also.One additional criterion of major importance is that the basement membrane must be intact, without infiltrations along the entire length. In our case the basement membrane had not been penetrated and all lesions were confined to the mucosa of the bladder.

The most common clinical sign of PC is hematuria, as a result of hemorrhage from the polyp mucosa due to chronic irritation (Martinez et al., 2003).

PC shares the same clinical signs with other conditions such as neoplasia (other that UC), chronic cystitis, fibroepithelial polyps, granulomatous cystitis/urethritis, gossypiboma, calculi, inflammatory pseudotumor and UC (Fulkerson and Knapp, 2020). The biologic behavior of PC warrants further study as malignant transformation into TCC cannot be ruled out (Butty et al., 2021).

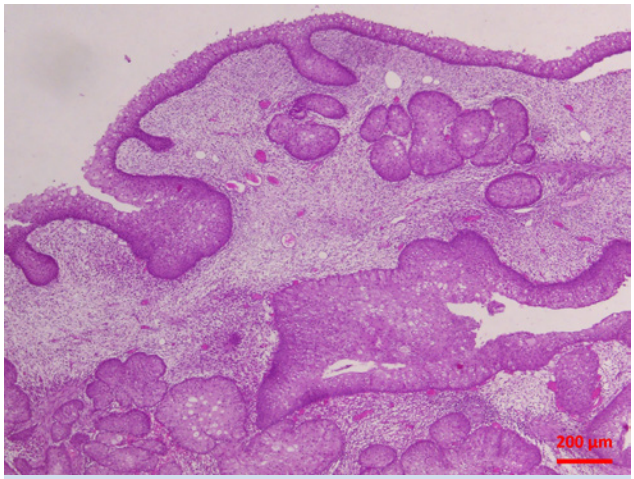
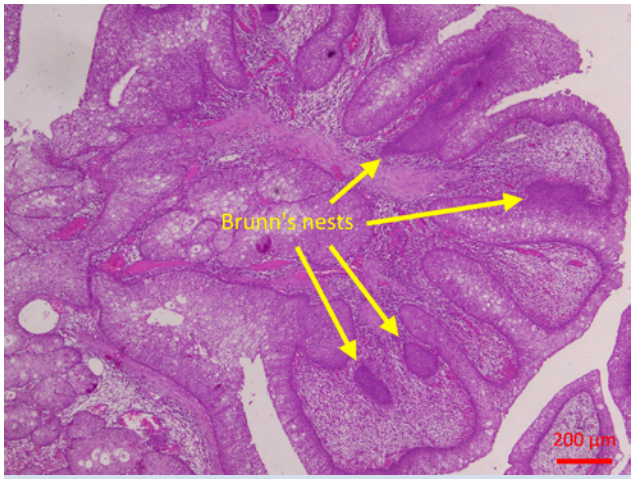


Figure 1.



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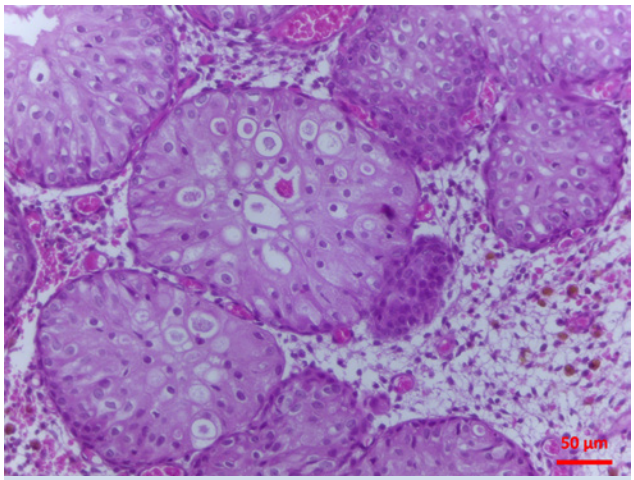

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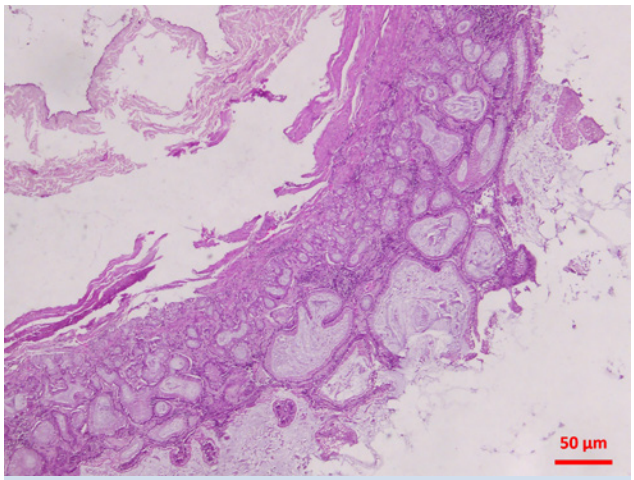

Figure 4.

The best way to evaluate the urinary bladder is surgical cystotomy. In case of cystic calculi, these are composed of struvite, apatite plus struvite and calcium oxalate. Antibiotics such as amoxicillin [alone or in combination with clavulanic acid (Augmentin®, GlaxoSmithKline)] and cephalexin (Ceporex®, Intervet/Schering-Plough Animal Health) are the most common primary selected. Aggressive medical management can even sometimes cause polyps complete regression and gives the chance to control clinical signs (Martinez et al., 2003).

In conclusion, PC is an uncommon disease, very similar with urothelial carcinoma (UC) concerning clinical and macroscopical findings. Histopathologic examination using certain criteria is the most accurate way to differentiate from UC and treat and prognose accordingly. In our best knowledge, this is the first report of PC in dog in Greece.

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Case report
Κλινικό περιστατικό

Clinical, radiographic and bronchoscopic findings of tracheal collapse in a calf

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ABSTRACT: A six-day-old calf was presented to the Fırat University Veterinary Teaching Hospital with a history of wheezing (noisy respiration) and dyspnea that worsens over exercise and drinking. The calf shown upper respiratory distress symptoms including extended head and neck, dilated nostrils, severe inspiratory stridor with characteristic goose-honk cough, and exophthalmos. Upon these findings, upper airway obstruction was suspected and cervical-thoracic radiographs and bronchoscopy were decided to be performed focusing to obtain detailed visualization of the upper respiratory tract. Thoracic radiograph, a significant tracheal lumenn arrowing was visible at the area of the thoracic inlet, beginning from the caudal cervical region (C5) and extending to the cranial thorax (T1). Also, severe dorsoventral narrowing was detected, approximately 15 cm after entering the tracheal lumen by the bronchoscope. A diagnosis of tracheal collapse was established based on clinical, radiographic, and bronchoscopic findings. It is important to remember that tracheal collapse should be considered in the differential diagnosis of calves that show symptoms from the upper respiratory tract such as extended head and neck, dilated nostrils, severe inspiratory stridor with characteristic goose-honk cough.

***Keywords*:** Calf; inspiratory dyspnea; honking sound cough; tracheal collapse

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CASE HISTORY

Tracheal collapse occurs due to tracheal lumen narrowing most often at a dorsoventral direction or sporadically to lateral-to-lateral direction and is characterized by symptoms compatible to upper respiratory tract disease (Woolums, 2015). Tracheal collapse is an uncommon disease of cattle, and affected animals usually show clinical signs within the first weeks of age (Jelinski and Vanderkop, 1990; Ashworth et al. 1992). Although the etiology is not known precisely, it may be related to cranial thoracic trauma, tracheostomies, roping, dystocia, and congenital defects (Woolums, 2015). This paper aims to present clinical, radiographic, and bronchoscopic findings of tracheal collapse in a calf.

A six-day-old male, Holstein calf, was admitted to the Fırat University Veterinary Teaching Hospital with a history of wheezing (noisy respiration), dyspnea that worsens over exercise and drinking and goose-honk cough. The owner reported that the calf was born with dystocia, and it has been started to show respiratory signs after birth that gradually exacerbated. Prior to admission to the Fırat University Veterinary Teaching Hospital treatment was initiated by another veterinarian containing of an antibiotic for three days. At presentation the calf showed upper respiratory tract symptoms including extended head and neck, dilated nostrils, severe inspiratory stridor with characteristic goose-honk cough, and exophthalmos. Heart rate was 144 beats/minute (reference interval: 100-140 beats/minute) respiratory rate was 88 breaths/minute (reference interval: 30-60 breaths/minute), and body temperature was 40 °C (reference interval: 38.5-39.5 °C). Hematocrit was 30% (reference interval: 22-33%) and total white blood cell count was 10.3 x 10^3/µL (reference interval: 4.9-12 x 10^3/µL). While plasma total protein concentration was decreased (5 g/dL, reference interval: 6.74-7.46 g/dL), plasma fibrinogen concentration (10 g/L, reference interval: 2-7 g/L) was increased. Upon these findings, upper airway obstruction was suspected. To obtain detailed visualization of the upper respiratory tract, radiography and bronchoscopy were scheduled.

Right and left lateral recumbent radiographs of the cervical and thoracal region were performed with digital radiography (CPI CMP 200 DR, Communications & Power Industries LLC., Palo Alto, CA; RAD-14, Varian Medical Systems, Salt Lake City, UT; Canon CXDI-50G, Canon Inc., Tokyo, Japan) selecting appropriate exposure setting (10mAh and 70 kV).On the thoracic radiograph, significant tracheal collapse was visible at the area of the thoracic inlet, beginning at the caudal cervical region (C5) and extending until the cranial thorax (T1) (Figure 1). No rib fractures or an extra-tracheal mass compressing the tracheal lumen were detected. After radiography, bronchoscopic examination was performed to confirm the diagnosis and to evaluate the degree of the tracheal collapse along with other possible intraluminal pathology. For this purpose, the calf was premedicated with an intramuscular injection of xylazine at a dose of 0.05 mg/kg and was restrained in sternal recumbency.A flexible fiberoptic bronchoscope (Karl Storz, Tuttlingen, Germany) was inserted through the right nostril, nasal cavity, pharynx, larynx, and trachea. There was no visible lesion in the nasal cavity, pharynx, and larynx (Figure 2a). However, severe dorsoventral narrowing of the tracheal lumen starting approximately 15 cm after entering the trachea was displayed (Figure 2b). The tracheal collapse extended until the tracheal bifurcation. No additional lesions of the tracheal lumen were detected before tracheal bifurcation.

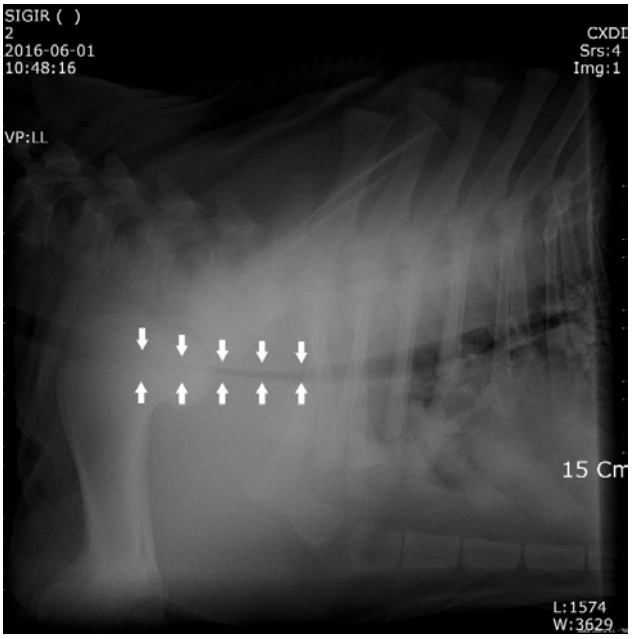

Figure 1. Right lateral thoracal radiography of calf. The severe tracheal collapse was evident between C5-T1 (arrows).

Although there is no grading system for tracheal collapse in calves, a classification system reported for dogs based on the loss of tracheal luminal diameter-has long been established (Tappin, 2016). According to this grading system, the tracheal collapse was graded from 1 to 4 point: 25% loss of the tracheal lumen diameter was described as grade 1, 50% loss of the lu-

men was described as grade 2, 75% loss of the lumen was described as grade 3, complete loss of the lumen was described as grade 4. In the present case, the loss of tracheal intraluminal diameter was approximately 75% and was classified as grade 3 tracheal collapse.

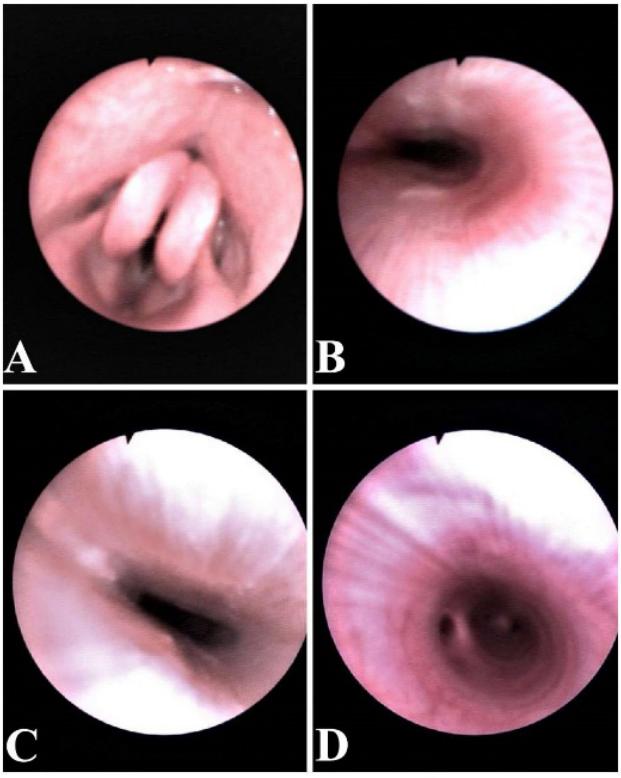

Figure 2. Normal appearance of the larynx (A).The starting point of the tracheal collapse (B) and the most severe collapsed segment of the trachea (C). Normal appearance of the tracheal lumen just before the bifurcation point (D).

DISCUSSION

Tracheal collapse is mostly diagnosed in dogs and can also encounter in cats, goats, and horses (Belli et al., 2003; Aleman et al., 2008; Mims et al., 2008; Johnson and Pollard, 2010). Also several cases have been reported in calves of different age (Fingland et al., 1990; Jelinski and Vanderkop, 1990; Ashworth et al., 1992; Faillace et al., 2018; Verdemal et al. 2019). The etiology is not yet completely understood, but it is speculated that thoracic trauma, dystocia, rib fracture, and congenital defects may cause tracheal collapse (Woolums, 2015). Also, Ashworth et al. (1992) stated that heritable chondrodysplasia must be considered as a cause of the tracheal collapse. In previous reports, the calves suffered from tracheal collapse were of various age ranging from 15 days to 4 months old, and a rib fracture that was evidenced of cranial thoracic trauma during delivery was most commonly diagnosed in those cases (Fingland et al., 1990; Jelinski and Vanderkop, 1990; Ashworth et al., 1992; Faillace et al., 2018; Verdemal et al. 2019). The time of onset of clinical signs was also considerably variable. Jelinski and Vanderkop (1990) reported that their case started to show clinical signs associated with tracheal collapseten days after birth. Another publication reported that the mean age of ten calves with tracheal collapse at the onset of clinical signs was 2.7 weeks and 9.4 weeks upon presentation (Fingland et al., 1990). In the present report, the age of the calf that was presented for examination was in accordance with previous reports.

Additionally, the clinical signs associated with the tracheal collapse in the present case started soon after birth, unlike previous reported cases. There may be two possible explanations for the earlier onset of the clinical findings in the present case compared to previous ones. The first explanation is that it may be associated with the dorsoventral compression of the cranial thorax during delivery. Secondly, it may have been caused by a congenital anomaly like a heritable chondrodysplasia, as mentioned by Ashworth et al. (1992). The latter was thought to be the reason in this case report.

In conclusion, the diagnosis of tracheal collapse can be easily made using radiographic and bronchoscopic imaging. Tracheal collapse should be considered in the differential diagnosis in calves showing symptoms compatible with upper respiratory tract disease such as extended head and neck, dilated nostrils, severe inspiratory stridor with characteristic goose-honk cough.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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