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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
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Review article
Ανασκόπηση

Injection site sarcomas in other species than the domestic cat

J. Stans*

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ABSTRACT: Injection site sarcomas (ISS) are tumours of tissues of mesenchymal origin that occur at an injection site. These tumours have been mainly described in cats as Feline injection-site sarcoma (FISS), but suspected cases have also been described in other species such as dogs. In other species than the domestic cat, these tumours are however much rarer. As a result, the body of literature is limited. This review aims to summarize the knowledge regarding ISS in species other than the domestic cat. In general, it seems that ISS can occur in a wide range of animals and that similar treatment strategies are employed as in cats. Like in cats, it seems that the benefit of the reasons for injection (such as vaccination and microchip implantation) could outweigh the risk of ISS development in other species.

Keywords: Injection site, sarcoma, oncology, cancer, animals

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INTRODUCTION

Injection site sarcomas (ISS) are tumours of tissues of mesenchymal origin that occur at an injection site (Zabielska-Koczywąs, Wojtalewicz, et al., 2017). These tumours have been mainly described in cats as Feline injection-site sarcomas (FISS), where they occur in about 1-10 of every 10,000 vaccinations (Zabielska-Koczywąs, Wojtalewicz, et al., 2017). A chronic inflammatory response at the injection site has been described as the hypothesis for the neoplastic disease (Woodward, 2011; Hartmann, Day, et al., 2015). Several studies have been conducted to investigate FISS. This includes studies regarding, among others, treatment strategies (Cohen, Wright, et al., 2001), risk factors (Kass, Spangler, et al., 2003) and immunohistochemistry (Carneiro, de Queiroz, et al., 2018). The current state of knowledge was summarized in several reviews (Saba, 2017; Zabielska-Koczywąs, Wojtalewicz, et al., 2017).

In addition to cats, suspected cases of these tumours have also been described in other feline species such as a lion (Kinne and Tarello, 2007) and in non-feline species such as dogs (Jacobs, Poehlmann, et al., 2017).This type of cancer is however very rare in other species than the domestic cat. As a result, the available literature about the condition in these species is sparse.

Due to the role of dogs as companion animals and the frequency of injections (e.g. for vaccinations and microchip implantations) performed, it is useful to also investigate ISS in these animals. Furthermore, investigating ISS in more exotic animals could provide more information on the condition in general and assess what factors (e.g. risk factors and treatment outcomes) are species-specific and which factors can be generalized.

The current review aims to briefly summarise the limited evidence about this condition in other species than the domestic cat.

SEARCH STRATEGY

To identify relevant literature regarding injection site sarcoma the Pubmed, Pubmed Central, Google Scholar and Web of Science databases were searched. Database-specific variants of the "injection site sarcoma", "injection site tumour","injection site cancer","-vaccine-associated sarcoma", "vaccine-associated tumour" and "vaccine-associated cancer", "foreign body sarcoma", "foreign body tumour", "foreign body cancer", "foreign body tumorigenesis", "microchip sarcoma", "microchip tumour" and "microchip cancer" search strings were used. Subsequently, the references of the selected publications were searched for further relevant literature.

IDENTIFIED LITERATURE

The relevant literature identified consisted mainly of case reports and case series describing a limited number of animals. The species for which literature was identified were: dogs (Jacobs, Poehlmann, et al., 2017), ferrets (Munday, Stedman, et al., 2003), a lion (Kinne and Tarello, 2007), a rabbit (Petterino, Modesto, et al., 2009) and a horse (Kannegieter, Schaaf, et al., 2010).

The dog is the most frequent species apart from cats for which suspected cases of ISS have been described. In 2003, Vascellari and colleagues described 15 cases of fibrosarcoma excised from where injections were thought to have been performed (Vascellari, Melchiotti, et al., 2003).In a later publication in 2006, Vascellari and colleagues described a case report of a dog with a fibrosarcoma in the back of the neck. The dog received several previous injections at this site, both for vaccinations and the placement of a microchip (Vascellari, Melchiotti, et al., 2006). In 2016, a case of extraskeletal osteosarcoma in the interscapular region in a dog was described (Selmic, Griffin, et al., 2016). The animal had received several injections at this site before. Jacobs et al. described a case of possible canine injection site sarcoma in 2017 (Jacobs, Poehlmann, et al., 2017).The dog developed tumours in the dorsocervical area after receiving injections at this site 3 weeks before.

In 2003, a retrospective case series of fibrosarcomas in ferrets was published (Munday, Stedman, et al., 2003). Seven of these fibrosarcomas occurred at a site regularly used for vaccinations.

In 2009, a case of interscapular fibrosarcoma in a rabbit was published (Petterino, Modesto, et al., 2009). The tumour developed at a site where several injections were performed. The authors stated that the histology of the tumour resembled that of a FISS.

A single case report of ISS in a horse was published in 2010 (Kannegieter, Schaaf, et al., 2010). This animal developed a fibrosarcoma 2 weeks after vaccination for equine influenza.

Finally, FISS has also been described in other fe-

line species than the domestic cat. In 2007, a case report of potential injection site sarcoma was described in a lion (Kinne and Tarello, 2007). The animal developed fibrosarcoma around 2 months after being vaccinated for feline leukaemia virus, feline rhinovirus and rabies. These authors also refer to a conference proceeding reporting a case of FISS in a tiger in 1998 (Kinne and Tarello, 2007). This publication was unfortunately not able to be sourced.

It is clear that the body of literature regarding ISS apart from the domestic cat is very limited as compared to literature about FISS. This supports the hypothesis that the condition is rare outside of the domestic cat. Other factors such as less reports and injection frequency in other species could also contribute to the smaller number of publications. It should also be noted that in most cases no assessment was performed to establish a causal relationship between an injection and the development of neoplastic disease. Additionally, almost all literature consists of case reports and case series describing a limited number of animals. Afinal observation is that a substantial amount of the identified literature regarding ISS apart from the domestic cat has been published more than 10 years ago.New research could provide important insights regarding potential changes in the incidence or characteristics of this condition.

CASE CHARACTERISTICS AND RISK FACTORS

The average age of cats affected by FISS was between 8 and 9 years in several studies (Hendrick, Shofer, et al., 1994; Doddy, Glickman, et al., 1996; Vascellari, Melchiotti, et al., 2003).The majority of FISS cases have been reportedto occur between 4 months and 3 years after injection (Hartmann, Day, et al., 2015). Fibrosarcomas are the most frequent kind of FISS(Doddy, Glickman, et al., 1996). In cats, it has been found that the risk of developing ISS does not vary between vaccine brands or manufacturers (Kass, Spangler, et al., 2003). The identified literature for ISS except that concerning the domestic cat was screened for several factors, including vaccine brand, age and injection site.

The dogs affected by potential ISS in the case series were on average 6.2 years old, but the age ranged from 7 months to 11 years (Vascellari, Melchiotti, et al., 2003). The animals in the case report were respectively 9, 6 and 11 years old (Vascellari, Melchiotti, et al., 2006; Selmic, Griffin, et al., 2016; Jacobs, Poehlmann, et al., 2017). In almost all of these cases, the presumed ISS was a fibrosarcoma, except for the 2016 case report (Selmic, Griffin, et al., 2016), where an osteosarcoma was diagnosed. Several breeds of dogs have been described that developed presumed ISS. In the case series (Vascellari, Melchiotti, et al., 2003), mixed breeds (6/15), Collie (1/15), German Shepherd (1/15), Schnauzer (1/15), Chow-Chow (1/15), Golden Retriever (1/15), American Pit Bull (1/15) Siberian Husky (1/15), Drahthaar (1/15) and Irish Setter (1/15) were reported. The case reports mention a French Bulldog (Vascellari, Melchiotti, et al., 2006), a Labrador (Selmic, Griffin, et al., 2016) and a Labrador Retriever(Jacobs, Poehlmann, et al., 2017). Based on these limited data, it is difficult to assess whether certain breeds have a predisposition to develop ISS. It is possible that this distribution is a normal variation or simply a reflection of the breeds that are usually kept. It is however apparent that the condition can develop in a wide variety of breeds. FISS has been estimated to occur in 1 - 10 of every 10,000 vaccinations in cats (Zabielska-Koczywąs, Wojtalewicz, et al., 2017). Because of the scarcity of the literature and the lack of epidemiological studies, it is difficult to assess the incidence of ISS in dogs. However, due to the widespread vaccination in dogs and the relative rarity of case reports in dogs as compared to cats, it can be hypothesized that the incidence in dogs is less than the incidence mentioned for FISS.

The age of the ferrets with sarcomas at an injection site in the study of Munday (Munday, Stedman, et al., 2003) ranged from 1 to 9 years. This study focused solely on fibrosarcomas. However, no literature regarding other sarcoma types at injection sites was identified for this species.

The dwarf rabbit in the 2009 case report was 1 year old when it received a vaccination (Petterino, Modesto, et al., 2009). However, when it developed a fibrosarcoma, it was already 8 years old.

The horse described in the case report of Kannegieter was 12 years old and a Quarterhorse x Arabian (Kannegieter, Schaaf, et al., 2010). It developed a fibrosarcoma.

The lion developing a fibrosarcoma was only 8 months old (Kinne and Tarello, 2007).

TREATMENT AND OUTCOME

FISS in the domestic cat is usually treated by surgical removal of the tumour, with wide margins.

If indicated, adjuvant treatment strategies such as radio- and chemotherapy can also be performed(Zabielska-Koczywąs, Wojtalewicz, et al., 2017). Despite treatment, frequent local recurrence has been described (Hartmann, Day, et al., 2015). Metastasis has also been described in several cases (Saba, 2017).

The samples of the 15 fibrosarcomas described by the group of Vascarelli (Vascellari, Melchiotti, et al., 2003) were obtained from dogs that underwent surgical removal of their tumour. It is not clear whether these animals received any additional therapy such as chemotherapy or radiation. The survival outcomes or the presence of metastatic disease were not reported. In the case report published in 2006, the tumour was surgically removed taking into account margins of 2 cm (Vascellari, Melchiotti, et al., 2006).The authors state that the dog was doing well and that there has been no sign of recurrence. The canine osteosarcoma described in 2016(Selmic, Griffin, et al., 2016) was initially surgically removed with wide margins. No metastasis was revealed at this time. A subsequent surgical site infection was treated with amikacin, followed by cefpodoxime proxetil and finally chloramphenicol after subsequent cultures. A carboplatin treatment was initiated to control microscopic metastases. During chemotherapy treatment, no metastases were identified. However, 1 month after the final cycle of chemotherapy, a suspected metastasis was identified in the right lung. Afterwards, a toceranib phosphate and cyclophosphamide treatment was initiated. Eighteen months after diagnosis, the animal presented with bone metastatic disease. Toceranib phosphate and cyclophosphamide treatment were discontinued. Afterwards, the animal was treated with fractioned radiotherapy. The animal was euthanised 20.5 months after diagnosis. The dog in the more recent case report (Jacobs, Poehlmann, et al., 2017) had a surgical removal of the tumour with margins of 3 cm. After 10 weeks, a recurrence of the tumour was noted. Lung metastasis was not present. A new surgical excision with 3 cm margins was carried out. Additionally, a three-days a week schedule of toceranib was started. No metastatic disease was noticed during this therapy. Fifty weeks after the first surgical removal, the same procedure had to be carried out for a third time due to a new recurrence, together with a continuation of the tyrosine kinase inhibitor therapy. The authors reported that the animal was still in remission almost 2 years after the diagnosis.

A multi-institutional retrospective study combining different treatment strategies in dogs could prove to be clinically useful if a large number of institutions participate and the inclusion criteria are not too strict, for example by allowing all FISS tumour types and treatment types.This way, a large number of cases can be assessed especially concerning relations between various treatment management strategies, quality of life after treatment and possible complications. A disadvantage of this kind of approach is that it is difficult to connect particular treatment strategies with survival outcome

The initial treatments for the ferrets in the case series of Munday and colleagues (Munday, Stedman, et al., 2003) were not detailed. It can however be assumed that the tumours were partially or completely surgically removed, since samples were available. Information about other therapies were not available. The authors report follow-up data for 3 of the animals that had fibrosarcoma at an injection site. One animal had a tumour recurrence 3 months after surgery and was euthanised. Two other animals did not have recurrence 8 and 12 months after surgery.

The fibrosarcoma in the rabbit in the case report of Petterino and colleagues (Petterino, Modesto, et al., 2009) was surgically removed with wide margins. After 2 months, the tumour recurred at the same site. The animal was subsequently euthanised.

The fibrosarcoma in the horse of the case report of Kinnegieter and colleagues (Kannegieter, Schaaf, et al., 2010) was surgically removed without radiotherapy or chemotherapy. No metastasis to lymph nodes or distant organs was identified. After 4 months, there was no recurrence of the tumour found.

In the case report by Kinneand Tarello (Kinne and Tarello, 2007), the lion was treated by surgical removal of the tumour. Despite radiotherapy being indicated, it was not performed due to practical considerations. The animal was later euthanised due to the poor prognosis. The time between diagnosis or treatment and death was not reported.

Due to the rarity of this disease in species outside of the domestic cat, it is unlikely that a clinical trial assessing treatment strategies will be feasible.
However, there is currently consensus that FISS should be treated with surgical removal. Based on the literature mentioned above, it seems that this treatment strategy is also the treatment of choice for ISS

in other species.

CONCLUSION

The literature regarding ISS outside of domesticated cats is very sparse. Despite this limited body of evidence, ISS has been described in dogs, ferrets, a rabbit, a horse and a lion. However, in most of these cases no assessment regarding a causal link between injections and the neoplasm was made. In general, treatment strategies of FISS and ISS in other species are similar. Based on the current literature, it seems that the benefits of the reasons for injection could outweigh the risk of developing ISS in all species. It should be noted, however, that this conclusion is based on a very limited amount of evidence. Further research should be performed to better understand the epidemiology, causes and risk factors of ISS in other species than the domestic cat and to discover optimal treatment strategies.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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Review article
Ανασκόπηση

Neuroacting drugs and its pharmacological response in relation to different stress status: A review

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ABSTRACT: This article intended to review many methods and types of stressors in the previous works of literature that describe the role of these stressors to induce modifications and alterations in the pharmacological response of the drugs acting on the nervous system (neuroacting drugs) in human and animal models. The current review focus on the different methods for inducing stress status which categorized as chemical, physical and miscellaneous stressors that affect on the well-known pharmacological response of the neuroacting drugs and by which mechanism can the stressor induce a modification in the drug target response with mentioning the findings related to changes in the pharmcologiacal response of the neuroacting drugs in previous literature. In conclusion, most studies suggest an alteration of the pharmacological response of neuroacting drugs, commonly by potentiating their efficacy and subsequent toxicity, due to different stressful methods, which may be obligated to the direct and indirect receptor modification (pharmacodynamic interaction) in addition to the direct pharmacokinetic influence on the essential parameters of absorption, distribution, metabolism, and excretion of the neuroacting drugs.

Keywords: animal model, interaction, neuroacting drugs, pharmacological response, stress

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INTRODUCTION

Stress means an increase in the free radical formation inside an organism's cells due to exposure to different stressful methods like chemicals, physical and other miscellaneous stressful agents (Lee and Jeong, 2007; Srivastava and Kumar, 2015). Different types of stressful methods causing an alteration in pharmacological response, especially for drugs that act on the nervous system. Stress may occur physiologically at the age of progressing and leads to an imbalance in the functions of the enzymes in the mitochondria responsible for energy production due to the accumulative effects of free radicals causing neurodegeneration (Navarro et al., 2002; Liguori et al., 2018). The goal of this review article was to focus on many methods and types of stressors in the previous works of literature that describe the role of these stressors with their mechanism for inducing modifications and alterations in the pharmacological response of the drugs acting on the nervous system in human and animal models because of the importance of the pharmacological response in the determining the actual benefits of using the drugs especially in clinical pharmacology.

BIOMARKERS USED FOR STRESS DETECTION

The state of oxidative stress (OS) is inferred using biochemical tests, the most important of which is the measurement of the glutathione and malondialdehyde concentrations, as well as the measurement of the total antioxidant status (TAS) (Dalle-Donne et al., 2006; Marrocco et al., 2017), which are among the essential vital signs indicating the occurrence of OS:

Glutathione: which consists of three peptide chains linked to the sulfur group; widely distributed in the organism's body and have an important and crucial part to metabolic as well as the defensive cell function by removing the free radical's toxicity that formed because of metabolic processes within the cell (Pastore et al., 2003; Dalle-Donne et al., 2006). Since the state of OS leads to a disruption of the antioxidant cellular biological defenses such as glutathione within the cells of the body of the organism, the state of the OS is inferred by measuring the glutathione concentration in the biological samples (e.g. Plasma and tissue) of the organism as its concentration decreases in the case of OS (Abdel Rahman, 1995; Patockova et al., 2003; Pastore et al., 2003; Dalle-Donne et al., 2006).

Malondialdehyde: The OS state occurrence leads to the cell membrane destruction of the body that contains unsaturated fats and this increases the level of the concentration of malondialdehyde compound, which is the final result of the lipid peroxidation process in the fat of the body cell membranes (peroxidation of unsaturated fatty acids, especially arachidonic acid). For this reason, the state of the OS is detected by measuring the malondialdehyde concentration (Patockova et al., 2003; Achuba et al., 2005; Del Rio et al., 2005; Dalle-Donne et al., 2006; Mendes et al., 2009), as the concentration of malondialdehyde rises, which is a sign of the stress. It is a toxic compound as it correlates with DNA and cellular proteins, causing genetic mutations, dysfunction of the cell, and a change in drug response (Marnett, 1999; Del Rio et al., 2005).

SELECTING AND CONSIDERING THE CENTRAL NERVOUS SYSTEM AS A TARGET FOR STRESS MODIFYING DRUGS

The central nervous system (CNS) differs from the rest of the body's systems by being more susceptible to stress (e.g., OS), due to its continued constant need for oxygen- significantly. It has a low concentration of antioxidants as well as has a high amount of polyunsaturated fats, and the fact that its large cellular compounds such as fats, carbohydrates, proteins, and nuclear acids that considered more susceptible to oxidation damage (Storz and Imlay, 1999; Patockova et al., 2003; Achuba et al., 2005; Sayre et al., 2008). The nervous tissue is more susceptible to the OS because of the production of high amount of free radicals. The reason is that the brain uses up to 20 % of the whole body oxygen, the CNS has a much amount of unsaturated fatty acids and the brain contains a high percentage of iron that stimulates metabolic processes. It has weak effectiveness of anti-oxidant enzymes, and these factors make the brain more susceptible to OS and thus have changed the effectiveness and pharmacological response to drugs that work on the nervous system (Pastore et al., 2003; Sayre et al., 2008).

TYPES OF STRESSORS

CHEMICAL STRESSORS

Hydrogen peroxide (H_2O_2): is one of the most common oxidizing compounds that stimulate free radical formation, the most important of which is the hydroxyl radical by Fenton Reaction. The hydroxyl radical is the leading cause of an OS that breaks down cell components, stimulates lipid peroxidation, and breaks down proteins, including protein receptors. It

stimulates regular or programmed death of the cell and is reliant on the concentration through direct oxidation of proteins and nucleic acids (Navarro et al., 2002; Patockova et al., 2003; Sayre et al., 2008). H_2O_2 reduces the effectiveness of dehydrogenases in the Krebs cycle, energy production, stimulates the growth factor and the receptor of the Aspartate neurotransmitter, which leads to an elevation in the calcium influx into the nerve cell and has a vital role for the serotonin receptor of rat brain, leading to poor behavior (Patockova et al., 2003; Sayre et al., 2008). Experimentally, H_2O_2 causes OS in chickens when given at 0.5% in water along with fourteen days and causes a neurobehavioral change in the open field activity (Mousa, 2012; 2014, Mousa and Mohammad, 2012a;b, Mousa, 2021; Mousa et al., 2021), besides modifying the pharmacokinetics (Mousa and Mohammad, 2012c).

Tertbutyl-hydroxyl peroxide: works by damaging the nerve cells in the brain by lowering body temperature and binding to the central and peripheral binding sites of the $GABA_A$ receptor on the outer surface of the mitochondrial membrane inside the neuron (Sarnowska et al., 2009).

Ethanol and nicotine: They cause OS by producing free radicals and depleting glutathione in the liver, kidney, lungs, and testes of rats, which is essential in the process of free radical disposal (Navasumrit et al., 2000; Husain et al., 2001).

Neuropeptide S: It induced OS, was found to alter the behavior of mice by causing a powerful OS that increases motor activity (Castro et al., 2009).

PHYSICAL STRESSORS

Mechanical immobilization: It has been observed that immobilization-induced stress by increasing the neurotransmitter dopamine level in the brain, and diazepam reduces the effects of this stress in rats (Hegarty and Vogel, 1995; Uehara et al., 2003). Restricting the movement of rats leads to stress. It alters activity measures used in the open field, as well as a change in behavior and a reduction of the glutathione of the nervous system (Nade and Yadav, 2010) while repeating restricting the movement, can induce stress in the rats and increases the neuron's sensitivity in the brain to diazepam which leads to an increased in its pharmacological response (Kalman et al., 1997).

Immersion: It was found that stress triggered by immersion by immersing chicken chicks in the water increased the number of places where the central benzodiazepine drugs were bound to the $GABA_A$ receptor, making these drugs closely bound to this receptor (Garcia et al., 2002).

Swimming: Which causes stress of rats (Motohashi et al., 1993) besides chickens (Marin and Arce, 1996) and increases the number of the benzodiazepine binding sites (central and peripheral) on the $GABA_A$ receptor of the nervous tissue without increasing number related to $GABA_A$ receptors, indicating an increased brain sensitivity to diazepam leading to an increase in its effect and pharmacological response to stress (Miller et al., 1987; Motohashi et al., 1993; Marin and Arce, 1996; Kalman et al., 1997).

Defeat Stress: It also increases the number of binding sites on the $GABA_A$ receptor in the brain without elevating the brain's number of $GABA_A$ receptors (Miller et al., 1987; Jie et al., 2018).

MISCELLANEOUS STRESSORS

Apomorphine: A drug that works on the CNS and is used as emetics causes OS in the rat brain and interferes with drugs that work on the nervous system and altering their pharmacological response (Moreira et al., 2003).

Xylazine: It was found that its administration with zolazepam and tiletamine in deer resulted in an OS in increasing the malondialdehyde concentration in the serum with rising glucose level (Yaralioglu-Gurgoze et al., 2005).

Chlorpyrifos: This is an insecticide that was found to cause OS in rats with an elevation in the process of lipid peroxidation of the red blood cells, indicating that it could interfere with the drugs administered with it (Mansour and Mossa, 2009).

Minerals (Cadmium, lead, mercury, and arsenic): They were found that exposure to these minerals causes OS by depleting antioxidants levels, which leads to increased active oxygen with rising of radicals such as the hydroxyl root besides high oxide root leading to the breakdown of proteins, fats, DNA, and the toxicity mechanism of these minerals may be attributed to their ability to cause OS (Storz and Imlay, 1999; Ercal et al., 2001; Jemai et al., 2007).

Sodium fluoride: causes OS, as this was inferred by the increase in the malondialdehyde concentration in the plasma of mice (Altintas et al., 2010).

MECHANISM OF STRESS INDUCTION

Only, the stressful agents are causing an elevation in the hydroxyl group (OH^-) (Fenton reaction), causing free radicals to be formed called Reactive Oxygen Species (ROS), which interact and destruct the cellular components like proteins (e.g., Receptors), carbohydrates, lipids, and nuclear acids (Figure 1) (Kar and Choudhury, 2016).

STRESS INDUCES A MODIFICATION IN THE BLOOD-BRAIN BARRIER

Many stressful methods destroy the blood-brain barrier (BBB), which may lead to more passage of the drugs acting on the nervous system. OS plays a role in increasing the permeability of the substance of the blood (e.g., Drugs) to the CNS through the BBB, which has an essential function in the balance of the CNS, as it was found that the OS works to change the location of the occludin (a protein responsible for the vital link between tight junction between the barrier cells) which increases the barrier's influence over substances and drug infiltration between the blood and the CNS (Lochhead et al., 2010; Daneman and Prat, 2015).

STRESS VERSUS NEUROTRANSMITTERS OF THE NERVOUS SYSTEM

Stressful agents that formed free radicals interact with the synthesis and release of the neurotransmitters in the presynaptic neuron in addition to its modification of the neurotransmitters' affinity and efficacy on their receptors on the postsynaptic neurons (Figure 2) (Kar and Choudhury, 2016). OS plays a significant part in the pathogenicity of multiple sclerosis, that destroys the myelin and axonal parts of neuron as well as free radical elevation, a decrease in concentrations of antioxidants in the blood and cerebrospinal fluid, and an increase in the neurotransmitter glutamate during the disease occurrence (Sayre et al., 2008). OS is causing degenerative diseases of neurons, affecting their susceptibility to neurotransmitters' secretion (Sayre et al., 2008). The OS destroys the neurons that produce Catecholamines such as adrenaline, noradrenaline, and dopamine in the brain, thereby leading to neurological diseases, including Parkinson's disease (Sayre et al., 2008). The H_2O_2 that causes OS destroys nerve cells in the brain of rats that producing neurotransmitters such as dopamine (Hussain et al., 1995) and it causes oxidation in the neurotransmitter dopamine to neurotoxic compound (Sayre et al., 2008). It was found that H_2O_2 increases the secretion of dopamine and noradrenaline neurotransmitters from the neurons of the brain of rats and increases their effect on their receptors by inhibiting the reuptake of these neurotransmitters into the neuron (Langeveld et al., 1995). H_2O_2 is used to induce OS and study neuropathological effects in the brain because it stimulates glutamate receptors by increasing secretion, increasing Nitric Oxide production, increasing the percentage of programmed neuronal cell death (Apoptosis) (Fatokun et al., 2007). The OS is the causative agent of diseases, neuropathy, and the cause of epilepsy cases, as it was found that stimulating the receptor of glutamate leads to the occurrence of these cases (Coyle and Puttfarcken, 1993).

EFFECT OF DIFFERENT STRESS METHODS ON THE PHARMACOLOGICAL RESPONSE OF SOME DRUGS ACTING ON THE NERVOUS SYSTEM

It was found that OS destroys the cell membrane by the lipid peroxidation process and leads to a change in the cell membrane's biological properties, including fluid entering the cell and disrupting or losing the receptor function in the cell membrane (Dalle-Donne et al., 2006; Donne et al., 2006). Stress factors involved in modulating the pharmacological responses of some neuroacting drugs were illustrated in Table 1.

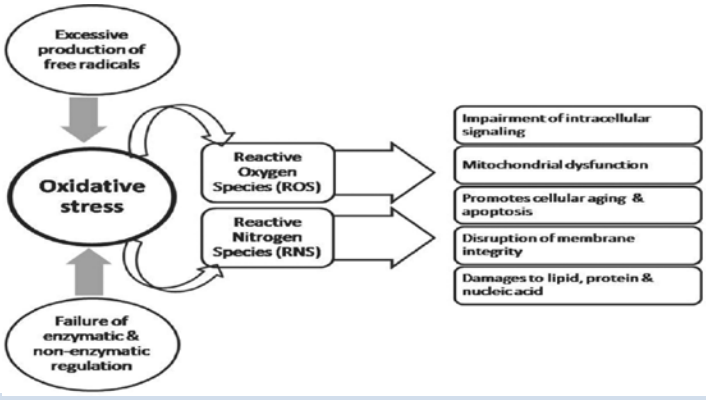

Figure 1. Flow chart of the impact of the OS on the cellular components (Kar and Choudhury, 2016).

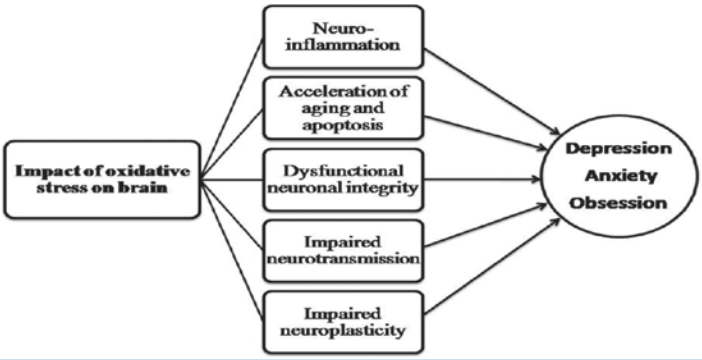

Figure 2. Flow chart of the OS involved in neurotransmitters and neuronal dysfunction (Kar and Choudhury, 2016).

Table 1. Summary of the stress factors involved in the modulation of the pharmacological responses of some neuroacting drugs

Stress factor	Neuroacting drug	Model	Drug response	Theory of interaction	Reference
H_2O_2	Diazepam	Chickens	+	+ affinity; -metabolism and excretion; K channels opening; modify pharmacokinetic parameters	Mousa and Mohammad, 2012a; 2012c; Zhang et al., 2002
	Xylazine	Chickens	+	+ affinity; - metabolism and excretion	Mousa and Mohammad, 2012b
	Ketamine	Chickens	+	+ affinity; - metabolism and excretion	Mousa, 2014
	Propofol	Chickens	+	+ affinity; - metabolism	Ahmed, 2010
	Detomidine-ketamine	Rabbits	+	Down regulation; - metabolism	Wohaieb et al., 1994
	Pentobarbital	Rats	+	+ affinity; - metabolism	Mohammad et al., 1999
	Neuroleptics	Rats	-	Activating Ca^{+2} channels; - NT release	Akaishi et al., 2004
	Benzoquinone	Rats	-	- affinity and metabolism	Baigi et al., 2008
	Chlorpyrifos	Rats	+	+ free radical formation	Mehta et al., 2009
	Paraquat	Rats	+	+ lipid peroxidation	Weidauer et al., 2004
	Antiepileptic drugs	Humans	-	+ lipid peroxidation; - antioxidant defense mechanism	Lopez et al., 2007
Cadmium	Detomidine-ketamine	Mice	+	+ oxidative damage in the CNS	Mohammad, 1994
	xylazine	Mice	+	+ inhibition of the CNS	Mohammad et al., 2000
Doxorubicin	Diazepam	Humans	+	+ inhibition of the CNS	Abdel Baky and Ali, 2009
Ethanol	Neuroacting steroids	Rats and Humans	+	+ basal levels	Porcu and Morrow, 2014
Nicotine	Propofol	Rats	+	Changes in brain metabolism	Khokhar and Tyndale, 2011
Immersion	Benzodiazepines	Chickens	+	+ affinity	Garcia et al., 2002
Restraint	Endotoxin	Mice	-	+ Glucocorticoid release	Kasahara et al., 2015
Foot shock	Neuroacting steroids	Rats	+	+ $GABA_A$ receptor function	Barbaccia et al., 1996

+: Increase; -: Decrease; NT: Neurotransmitter; CNS: Central nervous system

STRESS-INDUCING PHARMACODYNAMIC INTERACTION

Stress can induce a modification in the pharmacological response of the neuroacting drugs in one or more ways through increasing the binding sites at the receptors, increasing the receptors' susceptibility, and decreasing the numbers of the receptors (down-regulation). The stress can reduce the RNA production, which inhibits the development and production of protein substances in the cell, including protein receptors within the cell and those on the cell membrane, causing a reduction in the receptors' number (Crawford et al., 1997; Gunn et al., 2015).

STRESS-INDUCING PHARMACOKINETIC INTERACTION

Stress modifies drug disposition and availability to the target receptors by increasing the absorption of the drug from the site of treatment and alters their distribution by the destruction of the protein binding while decreasing the metabolism by affecting the cytochrome P_{450} enzymes responsible for drug elimination and effect termination and later decrease drug excretion (Mohammad et al., 1999; Mousa, 2012; Mousa and Mohammad, 2012c).

CONCLUSIONS

In conclusion, there are many methods of stressors used for induction of stress in animal models like chemical, physical and miscellaneous stressors and the majority of them practices the chemical method by using H_2O_2. Most studies suggest an alteration of the pharmacological response of neuroacting drugs, commonly by potentiating their efficacy and subsequent toxicity, due to different stress methods, which may be obligated to the direct and indirect receptor modification (pharmacodynamic interaction) in addition to the direct pharmacokinetic influence on the essential parameters of absorption, distribution, metabolism, and excretion of the neuroacting drugs**.**

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CONFLICT OF INTEREST

None declared.

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Research article
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Characterization of Faecal Enterococci from Wild Birds in Turkey and Its Importance in Antimicrobial Resistance

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SUMMARY: This research aimed to investigate the diversity of faecal enterococci isolated from wild birds, to detect their antibiotic resistance patterns and to determine their distribution of genes related to vancomycin resistance. Additionally, to investigate their virulence factors that are important in the development of the disease. One hundred seven cloacal/rectal samples were inoculated onto Enterococcus Agar, and presumptive colonies were identified and confirmed by PCR. Multiplex PCR assays were used to screen *vanA, vanB, vanC1 and vanC2/3*. The virulence-related genes; *ace, gelE, efa and agg* were determined by PCR. Among the 103 enterococci, 62 *E.faecalis*, 23 *E.faecium* 3 *E.gallinarum*, 2 *E.durans*, 1 *E.casseliflavus* and 12 *Enterococcus* spp. were identified. Of the 103 enterococci, 26 were found to be resistant against to three or more antibiotics. The highest percentages were detected for chloramphenicol (52%), tetracycline (33%) and erythromycin (30%). Two *E.gallinarum* isolates were harboring three virulence factors, and one isolate was carrying a single virulence factor. There is no virulence factor in the *E.casseliflavus* isolate. Also, *vanA and vanB* genes were not found. Forty-two of 103 enterococci were harboring virulence factors, more frequently in *E.faecalis*. Forty-two enterococci carried *efa A*, 31 isolates carried *gel* E, and *ace* was found in 18 isolates. Virulence gene *agg* was not detected. When the results of the study were evaluated in general, multiple drug resistance was described as 25%. Considering the risk of polluting the water resources of wild animals, it is suggested that the continuity of this type of epidemiological study in wildlife animals is necessary. In conclusion, the wild birds may act as substantial reservoirs carrying antimicrobial resistance among enterococci and estimate the potential risk for man, pets and farm animals.

Keywords: Wild birds, enterococci, antibiotic resistance, vancomycin resistance, virulence factors

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INTRODUCTION

The wildlife can act an essential role in antimicrobial resistance (AMR) dynamics and many wild animal species harbor multidrug-resistant (MDR) bacteria in a wide variety of natural environments (Vittecoq et al., 2016). Even though wild birds have only rare contact with antibiotics, they can acquire and disseminate resistant bacteria (Oravcova et al.,2018).Water contact, livestock, food chain and the soil appears are the main routes to the transmission of resistant bacteria originated from human or veterinary (Radhouani et al.,2012).

Enterococcus spp. is an intestinal flora bacteria of mammals, birds, invertebrates and some reptiles (Aarestrup, 2005) and is also found in various environments such as water, soil and sewage (Werner, 2012). Some species are important human pathogens and they have appeared as the fundamental causes of nosocomial infections worldwide (Poeta et al.,2005). Enterococci is an indicator of faecal contamination of environmental pollution. It is often used to detect AMR in both human and animal populations (Radhouani et al.,2012; Radimersky et al., 2010). In the world, *Enterococcus faecalis,* together with *Enterococcus faecium* are ranked as 3rd and 4thamong the nosocomial pathogens (Werner,2012).

Vancomycin is an antimicrobial agent used for Gram-positive bacterial infections and can moreover apply for preventive treatments in humans. Two types of vancomycin resistance have been defined in enterococci. The first is acquired type (*vanA, vanB, vanD, vanE, vanG, vanL*), which is observed mostly in *E.faecalis* and *E. faecium*species, is inducible resistance. The second one is the intrinsic type (*vanC*) which is related to *Enterococcus casseliflavus/flavescens* and *Enterococcus gallinarum* species, is a low-level resistance to vancomycin (Bagcigil et al.,2016; Silva et al.,2011). The emergence of vancomycin-resistant enterococci (VRE) with acquired mechanisms, exclusively those carrying *vanA, vanB or vanC* genes have been described worldwide in humans and animals with different incidences. Therefore, the significant increasing prevalence in VRE is seen as a worldwide health problem (Yahia et al.,2018). Various studies have described the incidence of VRE in wild birds, besides in other wild animals in many states (Poeta et al.,2005; Radhouani et al., 2014; Silva et al., 2010).

Severe infections due to enterococci are caused by the adhesion and secretory virulence genes. Virulence factors such as aggregation substance (*agg*), gelatinase (*gel*E), collagen-binding cell wall protein (*ace*), enterococcal surface protein (*esp*), hyaluronidase (*hyl*), endocarditis antigen (*efaA*) and cytolysin (*Cyl*) increase the severity of the infection and contribute to the pathogenesis of their infections by adhering to host tissue, colonisation, increasing invasion and modulating the host immune system (Celik et al., 2017).These genes have been demonstrated in wild birds and have been reported to pose a hazard to man (Klibi et al., 2015; Poeta et al.,2005).

The purpose of there search was to examine the diversity of faecal enterococci in wild birds, their antibiotic resistance patterns, their distribution of genes related to vancomycin resistance, and the occurrence of virulence factors.

MATERIALS AND METHODS

Samples

From one hundred and seven wild birds, cloacal swabs were recovered from November 2017 to February 2018 in Istanbul regions. The distribution of samples was as follows: 1- Passeriformes: crow (n=2), starling (n=1); 2- Non-Passeriformes: pigeon (n=26), collared dove (n=11), owl (n=1), little owl (n=2), tawny owl (n=1), long-eared owl (n=1), woodcock(n=2); 3- Waterfowl: white stork (n=1), grey heron (n=1), little gull (n=6), seagull (n=35), bittern (n=1), cormorant (n=2); 4- Birds of prey: kestrel (n=1), common buzzard (n=3), sparrow hawk (n=6), Short-toed snake eagle (n=1), honey buzzard (n=3). The samples were taken from the animals brought to the Wildlife Rehabilitation Centre of Faculty of Veterinary Medicine, Istanbul University Cerrahpasa, before the treatment.

Bacterial isolation

Swabs were inoculated into Enterococ Broth and incubated at 37°C for 24 hours. Cultures with colour change were subcultured onto Enterococ Agar (EA) when the black pinpoint colonies were selected as presumptive enterococci. Catalase negative, growth in 6.5% NaCl positive and esculin hydrolysis positive colonies estimated as presumptive enterococci and established by PCR (Ke et al., 1999). They were then identified by API ID20 Strep system (BioMérieux, France). PCR primers were presented in Table 1.

Antimicrobial susceptibility test

Isolates were tested by standard disk diffusion procedures to eight antimicrobials from 6 different antimicrobial classes (CLSI,2016). The antimicro-

bial agents tested were as follows: ciprofloxacin (5 μg), gentamicin (10 μg), streptomycin (30 μg), chloramphenicol (30 μg),tetracycline (30 μg), ampicillin (10 μg), erythromycin (10 μg), vancomycin (30 μg). MDR was considered as resistant at least three different antimicrobial classes.

Vancomycin Resistance Genes

Multiplex PCR assays were used with specific primers for *vanA*, *vanB, vanC1*and *vanC2/C3* (Kariyama et al.,2000). The multiplex PCR assay was made *E. faecalis* V583 (*vanB*), *E. faecium* BM4147 (*vanA*), *E. casseliflavus* DSMZ 20680 (v*anC2/C3*), *E. gallinarum* BM4174 (*vanC1*) and *E. faecium* CCUG542(vancomycin-susceptible) were used as reference strains (Table 1).

Virulence Genes

The occurrence of genes was established by PCR as described (Mannu et al.,2003). The tested genes were: aggregation substance (*agg*), gelatinase (*gel*E), endocarditis antigen (*efaA*), and collagen-binding cell wall protein (*ace*). *E. faecalis* ATCC 29212, *E. faecium* ATCC 6057 and *E. faecalis* NCDO 581 were used as reference strains for positive and negative controls.

RESULTS

Diversity of isolates

Enterococci were isolated in 96 out of 107 faecal samples (89.7%). One hundred three *Enterococcus* species recovered from 96 wild birds.The predominant species were *E.faecalis* (n=62), followed by *E. faecium* (n=23), *E.gallinarum* (n=3), *E.durans* (n=2) and *E.casseliflavus* (n=1). Twelve isolates could not be recognised to the species level. They were evaluated a s*Enterococcus* spp.

Antibiotic susceptibility testing

MDR was determined in 26 of 103 strains. The highest antibiotic resistance was found against chloramphenicol (53,4%), followed by tetracycline (32%), erythromycin (30%), streptomycin (21.3%), ampicillin (9.7%), ciprofloxacin (6.8%), gentamicin (5.8%).Vancomycin-resistance was not discovered in the isolates. MDR was determined 48% in *E.faecium* and 18% in *E.fecalis* isolates. Antibiotic resistance results of the isolates were presented in Table 2.

Table 1: PCR primers and their products

Gene and primers	Sequence	Product size (bp)	Reference
***Enterococcus* spp.**	5′-TACTGACAAACCATTCATGATG-3′ 5′-AACTTCGTCACCAACGCGAAC-3′	112	Ke et al., 1999
vanA	5’-CATGAATAGAATAAAAGTTGCAATA -3’ 5’-CCCCTTTAACGCTAATACGATCAA -3’	1.030	Devriese and Pot 1995
vanB	5’-AAGCTATGCAAGAAGCCATG -3’ 5’-CCGACAATCAAATCATCCTC -3’	536	Lopez et al. 2011
vanCl	5’-GGTATCAAGGAAACCTC -3’ 5’-CTTCCGCCATCATAGCT -3’	822	Devriese and Pot 1995
vanC2/C3	5’-CGGGGAAGATGGCAGTAT -3’ 5’-CGCAGGGACGGTGATTTT -3’	484	
***rrs* (16S rRNA)**	5’-GGATTAGATACCCTGGTAGTCC -3’ 5’-TCGTTGCGGGACTTAACCCAAC -3’	320	
ace	5’-AAAGTAGAATTAGATCCACAC-3’ 5’-TCTATCACATTCGGTTGCG-3’	320	Mannu et al. 2003
***gel*E**	5’-AGTTCATGTCTATTTTCTTCAC-3’ 5’-CTTCATTATTTACACGTTTG-3’	402	
efaA	5’-CGTGAGAAAGAAATGGAGGA-3’ 5’-CTACTAACACGTCACGAATG-3’	499	
AS	5’-CCAGTAATCAGTCCAGAAACAACC-3’ 5’-TAGCTTTTTTCATTCTTGTGTTTGTT-3’	406	

Table 2: Antibiotic resistance results of the isolates

	E.faecalis (n=62)	*E.faecium* (n=23)	*E.gallinarum* (n=3)	*E.casseliflavus* (n=2)	*E.durans* (n=1)	*Enterococcus spp* (n=12)
Erythromycin	15	11	-	-	2	3
Ciprofloxacin	2	3	-	1	-	1
Tetracycline	21	8	1	-	1	2
Gentamicin	3	2	-	-	-	1
Streptomycin	10	8	-	-	1	3
Ampicillin	2	6	-	-	-	2
Chloramphenicol	24	19	1	1	2	8
Vancomycin	-	-	-	-	-	-

Table 3: Patterns of antibiotic resistance of the isolates

	E.faecalis	*E.faecium*	*E.gallinarum*	*E.casseliflavus*	*E.durans*	*Enterococcus spp*
E+T+Ch	3	-	-	-	-	-
E+T+S	1	-	-	-	-	-
E+S+ Ch	-	1	-	-	-	1
E+A+ Ch	-	2	-	-	-	-
T+S+ Ch	-	2	-	-	-	-
C+A+ Ch	-	1	-	-	-	-
E+T+S+ Ch	3	3	-	-	1	1
E+C+T+S+ Ch	1	-	-	-	-	-
E+T+G+S+ Ch	2	1	-	-	-	-
C+G+S+A+ Ch	-	-	-	-	-	1
E+T+G+S+A+ Ch	1	-	-	-	-	-
E+C+T+S+A+ Ch	-	1	-	-	-	-
E+C+T+G+S+A+ Ch	-	1	-	-	-	-

C: ciprofloxacin G: gentamicin S: streptomycin T: tetracycline Ch: chloramphenicol A: ampicillin E: erythromycin

Thirteen different resistant patterns were observed among 103 enterococci isolates. Only one isolate (*E. faecium*) was resistant to all antibiotics used. Two of the isolates were resistant to 6 different antibiotic groups and 5 of the strains were resistant to 5 different antibiotic groups. MDR was described in 11 (17.7%) *E. faecalis* isolates and 11 (47.8%) *E. faecium* isolates. The AMR patterns of isolates are shown in Table 3.

Vancomycin Resistance Genes

Four of the 103 isolates had an intrinsic resistance gene. Three *isolates* were harbored the *vanC1* gene. One isolate carried *vanC2* gene. No strains were harbored the *vanA* and the *vanB* genes.

Virulence Genes

Forty-three (41.7%) of 103 enterococci had virulence genes. Forty-two (40.7%) enterococci harbored *efaA*. Besides, the *gel*E gene was discovered in 31 (30.1%) isolates, and the *ace* gene was detected in 18 (17.4%) isolates. None of the isolates was harbored *agg* gene. The multiple virulence factors were determined in 31 enterococci. Seventeen isolates carried three virulence genes. 13 of 14 isolates carrying two genes contained *gel*E and *efaA* genes, while 1 of them carried *ace* and *efaA*genes.The multiple virulence factors were described in 3 of 3 *E. gallinarum*, followed by 27/33 *E.faecalis* isolates.While 60 isolates did not contain any virulence factor.Virulence genes patterns were presented in Table 4.

A total 26 multiple antibiotic-resistant isolates were harbored the *efa A* gene with 38.4%, the *gel*E gene with 26.9 % and the *ace* gene with 19.2 % prevalence.

Two *E.gallinarum* isolates were harboring three virulence factors and one isolate was carrying a single virulence factor. There is no virulence factor in the *E.casseliflavus* isolate.

The results of the diversity of wild birds, isolates, vancomycin resistance genes, virulence genes, and the level of AMR were summarised in Table 5.

Table 4: Virulence genes patterns

	E.faecalis	*E.faecium*	*E.gallinarum*	*E.casseliflavus*	*E.durans*	*Enterococcus spp*
Efa A	5	1	-	-	1	3
***gel*E**	1	-	-	-	-	-
ace	-	-	-	-	-	-
agg	-	-	-	-	-	-
gel*E + *efa A	11	1	1	-	-	-
ace* + *efaA	1	-	-	-	-	-
gel*E+*efa A* +*ace	14	1	2	-	-	-

Table 5: The results of the diversity of wild birds, isolates, vancomycin resistance genes, virulence genes and the level of AMR

Diversity of wild birds	Diversity of isolates						Vancomycin resistance genes				Virulence genes				
	E. spp	*E.f.*	*E.fc.*	*E.g.*	*E.c.*	*E.d.*	*van A*	*van B*	*van* C_1	*van* C_2/C_3	*ace*	*gel*E	*efa A*	*agg*	Level of AMR
Passeriformes															
crow (n=2)	-	2	-	-	-	-	-	-	-	-	1	1	1	-	-
starling (n=1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Non-Passeriformes															
pigeon (n=26)	5	8	13	-	-	2	-	-	-	-	1	5	7	-	26,9% (7/26)
collared dove (n=11)	-	5	2	1	1	-	-	-	C_1	C_2	2	4	4	-	18,2% (2/11)
little owl (n=2)	-	1	1	-	-	-	-	-	-	-	-	-	-	-	-
owl (n=1)	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
tawny owl (n=1)	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
long-eared owl (n=1)	-	-	1	-	-	-	-	-	-	-	-	-	-	-	100% (1/1)
woodcock (n=2)	-	1	-	1	-	-	-	-	C_1	-	-	2	2	-	-
Waterfowl															
white stork (n=1)	-	1	-	-	-	-	-	-	-	-	-	-	1	-	-
grey heron (n=1)	-	1	-	-	-	-	-	-	-	-	-	-	-	-	100% (1/1)
little gull (n=6)	1	5	-	-	-	-	-	-	-	-	1	1	3	-	33,3% (2/6)
seagull (n=35)	2	26	5	-	-	-	-	-	-	-	10	13	17	-	31,4% (11/35)
bittern (n=1)	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
cormorant (n=2)	1	-	-	1	-	-	-	-	C_1	-	1	1	1	-	50% (1/2)
Birds of prey															
honey buzzard (n=3)	1	2	-	-	-	-	-	-	-	-	-	1	1	-	-
Short-toed snake eagle (n=1)	-	1	-	-	-	-	-	-	-	-	1	1	1	-	-
sparrow hawk (n=6)	2	2	1	-	-	-	-	-	-	-	-	-	1	-	-
common buzzard (n=3)	-	3	-	-	-	-	-	-	-	-	1	2	2	-	-
kestrel (n=1)	-	1	-	-	-	-	-	-	-	-	-	-	1	-	-

E spp: Enterococcus spp E.f : E.faecalis E.fc.: E.faecium E.g.: E.gallinarum E.c.: E.casseliflavus E.d.: E.durans

DISCUSSION

Because of their variety in ecological niches and their ease in obtaining to man and environmental bacteria, wild birds can act as a pre-definitive role for the bacterial load. Although antibiotic treatment is not applied to wild animals, antibiotic-resistant bacteria were reported in them. It is still unclear how the MDR and related genes have spread from man and animals to wildlife. Many researchers emphasised several possible transmission routes, including direct contact with infected individuals, their faeces, food chain, water and soil (Han et al.,2011;Vittecoq et al., 2016; Yahia et al.,2018). Moreover, they underlined that wild mammals and birds could serve as reservoirs of resistance determinants, can spread and facilitate their transfer over large areas through the migration period or seasonal variations (Lozano et al., 2016; Oravcova et al.,2014; Splichalova et al.,2015). Then, wild birds can be regarded as an excellent indicator of the distribution of AMR in wildlife and could cause

a severe public health problem. (Lozano et al., 2016; Radimersky et al. 2010).

The authors reported the isolation of *Enterococcus* spp. from the wild bird's faeces worldwide. Blanco et al. (2006) isolated *E. faecium* and *E. durans* with a prevalence of up to 64 % from the Egyptian Vulture. *E. faecalis* and *E. gallinarum* were detached with 80 % prevalence from raptors and owls (Marrow et al., 2009). Radhouani et al. (2012) identified enterococci with a prevalence of 74 % from the Common Buzzard. Radimersky et al. (2010) determined the prevalence of enterococci as 58 % from feral domestic pigeons and identified the isolates as *E. faecalis, E. faecium, E. gallinarum, E. casseliflavus, E. hirae, E. durans* and *E. mundtii.* Splichalova et al. (2015) detected six different species from Coraciiform birds and determined the prevalence as 74 %. Yahia et al. (2018)recovered enterococci from wild birds with 52% prevalence. In Turkey, Akgul et al. (2016) determined the prevalence of enterococci as 23.8% from the gulls. In our study, the rate of enterococci from wild birds was 89.7%, which is higher than the other reports. The difference between rates could be based on multiple criteria, including geographical differences, sampling techniques and detection methods. Although a limited number of wild bird species were investigated in other studies, a lot of different species studied in this study can cause this difference.

Many authors emphasised that *E. faecalis* were the predominant enterococci with 25-67% prevalence (Poeta et al., 2005; Radimersky et al., 2010; Splichalova et al., 2015; Yahia et al., 2018). Conversely, some authors described that *E.faecium* was the main species with 48-82%. In the current research, *E.faecalis* prevalence is 60%, and *E.faecium* is 22%. Since these two species are considered as an emerging human pathogen, the high isolation rate of these species should not be ignored.

Even though infections due to the other enterococci species were much less frequent, the clinical cases have been reported (Cetinkaya et al., 2000; Janoskova and Kmet, 2004). Splichalova et al. (2015) indicated that the prevalence of *E.casseliflavus* was 32% in the coraciiform birds. Radimersky et al. (2010) showed that they identified *E.casseliflavus* and *E.gallinarum* from wild birds with a prevalence of 8% and *E.durans* with 13%. Klibi et al. (2015) reported 4% prevalence for *E.gallinarum, E.casseliflavus,* and 1% for *E.durans*. In the current study, although the isolation rate was low, *E.gallinarum*, *E.durans,* and *E.casseliflavus* were described with 3%, 2% and 1% rate, respectively.

In the list of "Critically Important Antimicrobials for Human Medicine" of the World Health Organization (WHO), chloramphenicol and tetracycline were stated as "highly important antimicrobials". Erythromycin, streptomycin, ciprofloxacin, gentamycin, and ampicillin were considered as "critically important antimicrobials" (WHO). According to the"List of Antimicrobials of Veterinary Importance" of the World Organisation for Animal Health (OIE), tetracycline and ampicillin were considered as "essential" agents and erythromycin and streptomycin were regarded as extremely important (OIE). In studies investigating antibiotic resistance profiles in different wild birds, mostly the highest resistance was seen to tetracycline (Han et al.,2011; Radhouani et al.,2012; Radimersky et al.,2010; Yahia et al., 2018). Further, erythromycin, ciprofloxacin, gentamicin, and lincomycin resistance were established in the range of highest to a moderate level (Klibi et al.,2015; Oravcova et al.,2014; Radhouani et al.,2012; Radimersky et al.,2010; Splichalova et al.,2015; Yahia et al.,2018). The prevalence of antimicrobial-resistant enterococci in wild birds investigated in this research was high. The primary resistance was found against chloramphenicol (53.4%). Since chloramphenicol is considered to be critically essential antimicrobials of the WHO, the high resistance detected in our region requires special attention for human health.

High resistance was also shown against tetracycline 32%, and erythromycin 30%. In recent years, interest in wildlife has started to increase. In many cities in our country, there is a veterinary faculty hospital and Wildlife Rehabilitation Center, which is connected to universities or ministries. As there are positive aspects, the release of animals recovered after antibiotic treatment to nature suggests that the increase of AMR in these wild animals may lead to adverse effects. The higher resistance to antibiotics can be attributed to this and also, as noted in the other publications, this may be due to the relatively higher use of these antibiotics in the medicine, in the agriculture and as a growth factor. Depending on these factors, it is possible for bacteria to gain resistance against antibiotics as a result of the pollution of nature and water resources. It is thought that Non-Passeriformes and Waterfowl birds such as pigeons, seagulls, and doves can be more affected by this contamination because they live more closely with the public. In this study, the results of the MDR (33.3% in Waterfowl birds, 25.6% in Non-Passeriformes birds, no resistance in Passeriformes birds and Birds of prey) (data not shown) support this hypothesis.

In enterococci isolated from animals and humans, MDR is widespread because of their natural (intrinsic) resistance mechanisms. They can adapt to the environment and can gain other antimicrobial resistance determinants (Lozano et al., 2016). Consequently, there is a minimal treatment option. In this research, the antibiotic resistance patterns were determined using seven antibiotics from 6 different antibiotic groups. MDR was shown in 26 isolates (25.2%), and our results were similar to the others (Radimersky et al.,2010; Santos et al., 2013). Resistance to three or more antibiotics was revealed 17.7% in *E.faecalis* and 47.8% in *E. faecium*. These results were parallel with Radhouani et al. (2014) indicated that AMR of *E.faecium* was highest than *E.faecalis* isolates.

Enterococci exhibit a natural resistance to many antimicrobial agents as well as various acquired AMR genes transmittable to other bacteria (Vittecoq et al.,2016). One of them vancomycin, which has recognised as essential healthcare causes Mondial, is two resistance mechanisms. The first is low-level intrinsic type, which is related with *E.gallinarum* (*vanC*1), *E. casseliflavus* (*vanC*2) and *E.flavescens* (*vanC*3) species. The second one is acquired type (*vanA/B/D/E/G/L/M/N*) and is frequently described in *E.faecium* and *E.faecalis.* (Bagcigil et al.,2016; Silva et al.,2011) The *vanA*-type resistance is highest common resistance factor than *vanB*-type (Werner,2012). The emergence of *vanA*, *van B* or *vanC* genes has been informed worldwide in wild birds with different incidences (1-10.5%) (Oravcova et al.,2014; Poeta et al., 2005; Radhouani et al.,2014; Silva et al., 2011; Yahia et al.,2018)

Acquired-Vancomycin resistance did not appear in this research. While 3.8% of isolates presented intrinsic vancomycin resistance. *E.gallinarum* contained *vanC1*, and one *E.casseliflavus* isolate harbored *vanC2*. Klibi et al. (2015) determined that intrinsic vancomycin resistance was recovered from 5.4% of faecal samples. Some researchers proposed that enterococcal species with low-level vancomycin resistance might frequently be existing in the microbiota of some birds (Lozano et al., 2016; Sellin et al., 2000).

The studies conducted on enterococci' virulence factors focused on wild animals are insufficient (Poeta et al., 2005; Radhouani et al.,2010; Silva et al.,2011). One of these limited studies was underlined *E. faecalis* has at least one virulence genes, and 38% of these isolates were harboured three virulence genes (Poeta et al., 2005). Radhouani et al. (2014) indicated that *E. faecalis* carry more virulence factors than *E.faecium*. In this research, the multiple virulence genes were detected more commonly in *E. faecalis* followed by *E. faecium* with 66.6 % prevalence. Fifty-three point two percent of *E. faecalis* was harbored at least one of the virulence genes, and most of them (81.8 %) carried the multiple virulence factors. In this research, also, the numerous virulence factors in *E. gallinarum* were detected with 100%; however, this high prevalence may be associated with a low number of isolates.

Endocarditis antigen was more frequently detected virulence treat genes coded by *efaA* gene (40.7%). Even though the biological role of *efaA* gene was relatively unknown, our results were similar with the studies which indicated the majority of genes that encode *efaA* in all type of samples (Creti et al.,2004; Eaton and Gasson, 2001).

Gelatinase, encoded by *gel*E gene, detection rate increased endocarditis cases in animals such as 75.3 % (Poeta et al.,2005). Contrarily, Klibi et al. (2015) reported the low prevalence of *gel*E with 12.3 % and Silva et al. (2011) described the absence of virulence factors in wild birds. In this research, the prevalence of the *gel*E was 30.1% and 43.5 % of *E.faecalis* strains had been found positive for gel production.

The *ace* gene which is an accessory colonisation factor encodes a putative protein with similar characteristics to mediate the adherence to collagen. Silva et al. (2011) detected *ace* virulence genes in 4 VRE isolates. Klibi et al. (2015) and Poeta et al. (2005) recognised the gene in nearly 10% of *E. faecalis* isolates. In our research, the prevalence of *ace* gene founded as 17.4%. On the contrary, *E.faecalis* prevalence was 24.1 %. These differences may occur from regional variation.

The *agg* gene, which is an aggregative pheromone, stimulated adherence to extra matrix protein. They have been previously found in enterococci species in a study (Semedo et al., 2003). Silva et al. (2011) reported the absence of *agg* in wild birds and this result supports these findings.

In the present research, the *efa* gene was the frequently carried gene in 26 AMR isolates with 38.4 % prevalence. The increasing virulence discovered in *E. faecalis* may probably conduct the extended of MDR in the environment (Yahia et al., 2018).

CONCLUSION

In our country, to our knowledge, this is a first report from the different species of the wild bird with

a significantly high prevalence. When the results of the study were evaluated in general, MDR was highlighted in a quarter of the isolates. Considering the hazard of polluting the water resources of wild birds, it is suggested that this type of epidemiological study should be maintained in wildlife animals.

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CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Association of the caprine calpastatin *Msp*I polymorphism with growth and reproduction traits in Saanen goats

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ABSTRACT: This study was designed to evaluate the effects of calpastatin (*CAST*) *Msp*I polymorphism on some growth and reproduction traits, including birth weight, first breeding weight, litter size, and average daily weight gain in Saanen goats. In this sense, blood samples obtained from 73 purebred female Saanen goats were used for genotyping. Genomic DNA was isolated by the phenol-chloroform method and used to determine *CAST* genotypes, including MM, MN, and NN, by means of the PCR-RFLP method. The population genetic parameters were estimated based on allelic distribution and the data were statistically analysed using analysis of variance (ANOVA) using a general linear model (GLM). Results revealed that N allele frequency was remarkably high (0.64) and the MM genotype was not present. The frequency of the heterozygous genotype was 59.62%. Concerning ANOVA results, significant differences were found between genotypes of the *CAST* locus concerning birth weight ($P<0.05$). In this respect, animals with the NN genotype were associated with higher birth weight means (2.85±0.29 kg) compared to heterozygous animals (2.53±0.24 kg). There was no significant association between the *CAST* marker and any of the remaining phenotypic traits evaluated. The present results suggest that focusing on this genomic region may be particularly useful in improving birth weight in goats which can be considered as an early indicator of post-weaning animal growth and survival.

Keywords: Saanen, birth weight, *CAST*, single nucleotide polymorphism

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INTRODUCTION

Goat breeding is preferred worldwide for dairy production, meat, leather, and hair, and moreover, selection for increased levels of reproductive performance and disease resistance is also included in breeding objectives (Korkmaz Ağaoğlu et al., 2012; Rupp et al., 2016).

Growth and reproduction, along with profitability in production, are significant functional traits in sustainable goat breeding programs. Even with goats reared primarily for milk, such as the Saanen breed, desired reproduction parameters make an important contribution by influencing the herd size and providing a profitable dairy goat management concerning decreased culling rates (Shelton, 1978). Growth traits are important indicators in evaluating the sustainability and profitability of goat production systems because they contribute to current and future production through influencing post-weaning animal growth (Hanford et al., 2006; Menezes et al., 2016).

From a biological standpoint, the genetic basis of growth and reproduction traits is a complex one. Numerous genes contribute to the expression of these functional traits, directly or indirectly. In this context, the discovery of new mutations/polymorphisms in functional genes or identification of novel associations between previously reported genotypic variations and growth/reproduction traits are not surprising circumstances. The calpastatin (*CAST*) gene, which encodes an endogenous inhibitor of the calpains (m- and μ calpain), has been reported to be widely expressed in reproductive tissues or organs and it regulates the calpain activity of cells (Chung and Davis, 2012; Garcia et al., 2006). As a Ca^{2+}-dependent cytosolic cysteine protease, calpain play pivotal roles in proteolytic modulation of Ca^{2+} mediated intracellular mechanisms, such as cell cycle and differentiation, signal transduction, and apoptosis (Hata et al., 2001). Calpain proteinase system, involving m-calpain, μ calpain, and calpastatin, has a significant role in normal postnatal skeletal muscle growth (Goll et al., 1998). Increased calpastatin activity is associated with decreased rates of muscle protein turnover, and hence, this negative correlation results in increased levels of skeletal muscle growth (Chung and Davis, 2012; Goll et al., 1998; Parr et al., 1992; Pringle et al., 1993). These investigations impel researchers to focus on the genotypic structure of the *CAST* gene for growth and reproductive traits in livestock. Caprine *CAST* gene is located on chromosome 7 (14,437,312-14,567,828 reverse strand) (Ensembl Genome Browser, 2021). Evaluating this genomic region may provide useful information about variations of growth in goat breeding based on the calcium-binding domain which is the major regulator of the calpastatin activity.

CAST gene is mainly associated with muscle development to the formation of the fibers and thus it may influence the growth performance of mammals (Goll et al., 1998).Polymorphisms in particular genes that affect complex quantitative traits may affect multiple traits. This pleiotropy is the main cause of the genetic correlations between corresponding traits (Bolormaa et al., 2014). For instance, polymorphisms associated with increased milk yield may also increase the weight or age at puberty (Collis et al., 2011).The potential relationship between *CAST* and reproduction traits was studied on the bovine genome (Collis et al., 2011; Bolormaa et al., 2014; Ortega et al., 2017). However, the current knowledge on the associations of caprine *CAST* variations with growth and reproduction traits is quite limited. A comprehensive evaluation of this genomic region for not only muscle development and growth but also essential reproduction traits may provide adequate genotypic consideration of dairy goat reproduction performance. Taken altogether, this study was, therefore, performed to evaluate the effects of *CAST Msp*I polymorphism on birth weight, first breeding weight, litter size, and average daily weight gain in Saanen goats.

MATERIALS AND METHODS

Animals, sampling, and DNA extraction

A total of 73 purebred female Saanen goats that were grown on the same farm, Bursa Uludag University, Faculty of Veterinary Medicine Practice and Research Farm, located in the South Marmara region of Turkey (40° 14′ N and 28° 52′ E) were used. All of the animals were raised with the same feeding and management conditions. Animals were housed indoor and were fed with a concentrate feed in pellet form which contains 18% crude protein, 12 MJ/kg metabolizable energy, and alfalfa as roughage. During the experiment, goats had free access to water and had 0.50 kg/per animal concentrate feed and *ad libitum* roughage. All animals were fed twice daily (at 09:00 and 16:00). Measurement of phenotypic traits was performed according to Rashidi et al. (2011). Litter size was the number of kids born per parturition. The birth weight was the weight of the kids and it was recorded within 12 hours of birth. Average daily weight

gain (ADWG) was calculated from birth (BW) to the time reaching the first breeding weight (FBW) and it was calculated as ADWG = (FBW−BW)/age of goat at first breeding. The first breeding weight was the weight of goats when reaching the breeding age. Ethical approval was received from Bursa Uludag University local Research Ethics Committee(App. No: 2020-02/09). Blood samples (approximately 4 mL) were taken from jugular veins. Genomic DNA was extracted using a phenol-chloroform method according to Green and Sambrook (2012). To evaluate DNA quantification (ng/μL) and purity (260/280) analysis, a NanoDrop spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA) was used.

Genotyping

In this study, genotyping of the SNP in the caprine *CAST* gene was performed by PCR-RFLP. A 622 bp fragment in the exon 1C/1D region of the *CAST* gene was amplified from the purified genomic DNA. PCR reactions were performed in a total volume of 25 μL, using 2.50 μL DNA sample (approximately 60 ng genomic DNA) as a template, 12.50 μL PCR master mix (OneTaq Quick-Load 2x MM with Standard Buffer, New England BioLabs Inc., Ipswich, Cat#M0486S, USA), 1 μL (0.5 μM) of each primer, and 8 μL of nuclease-free water (Thermo Scientific). Primer sequences were as follows:

Forward: 5'-TGGGGCCCAATGACGCCATC-GATG-3'

Reverse: 5'-GGTGGAGCAGCACTTCTGAT-CACC-3'

The primers were used based on the ovine sequences (Palmer et al., 1998) and were verified for caprine genome specificity by conducting BLAST searches of the NCBI Gen-Bank database. The PCR profile included an initial denaturation step at 95°C for 5 min, 30 cycles of 94°C (1 min), 60°C (1 min) and 72°C (2 min), and a final extension step of 8 min at 72°C. The PCR quality control was verified by electrophoresis (85-90 V for 45 min) using 10 μL of the amplified product in 2% (w/v) agarose gels (Sigma Aldrich, Steinheim, Germany) stained with ethidium bromide (Sigma Aldrich) with the concentration of 1 μg mL^{-1}. Afterward, 15 μL of PCR product (if verified) was digested with 10 U of *Msp*I restriction enzyme (New England BioLabs, Cat#R0106S), with 10x NEB buffer (New England BioLabs, Cat#B7004S) by incubating at 37 °C overnight. The digested products were then subjected to 3% (w/v) agarose gel electrophoresis. PCR-RFLP reactions were performed in a thermal cycler (Palm Cycler GC1-96, Corbett Research, Australia). The electrophoresis patterns were visualized by a gel imaging system (DNR-Minilumi, DNR Bio-Imaging Systems, Israel). Allele sizes were estimated by comparison to a 100-bp ladder (100-1500 bp, Biomatik Corporation, Ontario, Canada).

Statistical analysis

Estimation of allelic and genotypic frequencies and the Hardy-Weinberg equilibrium (HWE) testing (α=0.05) were performed by using Cervus v3.0 software. Indices of genetic diversity (effectiveness of allele incidence) including observed (experimental) heterozygosity (H_{exp}) / homozygosity (Ho) and the polymorphism information content (PIC) were estimated based on the formulas indicated by Nei and Roychoudhury (1974) and Botstein et al. (1980), respectively. The expected (theoretical) heterozygosity (H_{the}), the effective number of alleles (Ne), and the level of possible variability realization (LVPR-*V*%) were calculated as described by Crow and Kimura (1970). The fixation index (F_{IS}) was estimated from the values of theoretical (H_{the}) and experimental (H_{exp}) heterozygosities using the following formula:

$$F_{IS} = (H_{the} - H_{exp}) / H_{the}$$

The phenotypic traits were birth weight, first breeding weight, litter size, and average daily weight gain. Minitab (Minitab, Pennsylvania, USA, v17.1.0) was used as statistical software. The data were evaluated utilizing ANOVA using a general linear model (GLM) according to the following statistical models:

Model [1] was used to test the effects of *CAST* genotypes on the birth weight of kids:

$$Y_{ijklmn} = \mu + B_i + S_j + C_k + G_l + I_m + e_{ijklmn}$$

where: Y_{ijklmn} = the studied trait, μ = the overall mean, B_i = Birth year (i=2012-2018), S_j = Season (j=spring, summer, winter), C_k = Litter size (k=single, twin, triplet), G_l = *CAST* genotypes (l=MN, NN), I_m = two-way interactions, e_{ijklmn} = random error.

Model [2] was used to test the effects of *CAST* genotypes on first breeding weight, and average daily weight gain:

$$Y_{ijklmn} = \mu + B_i + S_j + G_k + \beta W_l + I_m + e_{ijklmn}$$

where: Y_{ijklmn} = the studied trait, μ = the overall

mean, B_i = Birth year (i=2012-2018), S_j = Season (j=spring, summer, winter), G_k = *CAST* genotypes (k=MN, NN), βW_l = regression effect of birth weight, I_m=two-way interactions, e_{ijklmn} = random error.

Model [3] was used to test the effects of *CAST* genotypes on litter size:

$$Y_{ijklm} = \mu + B_i + S_j + G_k + I_l + e_{ijklm}$$

where: Y_{ijklm} = the studied trait, μ = the overall mean, B_i = Birth year (i=2012-2018), S_j = Season (j=spring, summer, winter), G_k = *CAST* genotypes (k=MN, NN), I_l=two-way interactions, e_{ijklm} = random error.

A probability level of $P<0.05$ was considered statistically significant. Two-way interactions were not statistically significant ($P>0.05$) and they will not be discussed further.

RESULTS

PCR-RFLP patterns

The amplification of the gene encoding calpastatin using the appropriate primers yielded a 622bp amplicon (Figure 1). The cleavage of the PCR product with the *Msp*I nuclease resulted in three bands (622bp, 336bp, and 286bp) for heterozygous genotype (MN). The DNA amplified from NN animals remained undigested (622 bp) with the corresponding restriction enzyme (Figure 2). MM genotype (336bp and 286bp) was not observed in the present study.

Genetic variability

The frequency of allele N (0.64) of the caprine *CASTMsp*I polymorphism was much higher than M (0.36). The frequency of heterozygous genotype was quite high (59.62%) and the MM genotype was unseen, as shown in Table 1.

Table 1. Genotype and allele and frequencies of *CAST Msp*I polymorphism in caprine *CAST* gene, population genetic indices (H_{the}, H_{exp}, Ho, F_{IS}, LPVR, Ne, PIC) and compatibility with the Hardy-Weinberg equilibrium (HWE).

Locus	*CAST*		
Genotypes	NN	MN	MM
n	21	52	0
GF	40.38	59.62	0
EGF	30.30	33.50	9.30
Alleles	N		M
AF	0.64		0.36
H_{the}		0.4657	
H_{exp}		0.4608	
Ho		0.5392	
F_{IS}		0.0205	
LPVR (*V%*)		0.4499	
Ne		1.8546	
PIC		0.3546	
χ^2(HWE)		22.3395	
P		0.000*	

CAST - calpastatin. *n* - number of experimental goats. GF - genotype frequency. EGF - the expected genotype distribution according to HWE. AF - allele frequency. H_{the} - theoretical heterozygosity. H_{exp} - experimental heterozygosity Ho - homozygosity. F_{IS} - fixation index. LPVR - level of possible variability realization. Ne - number of effective alleles. PIC - polymorphism information content. χ^2(HWE) - Hardy-Weinberg equilibrium χ^2 value.
*not consistent with HWE.

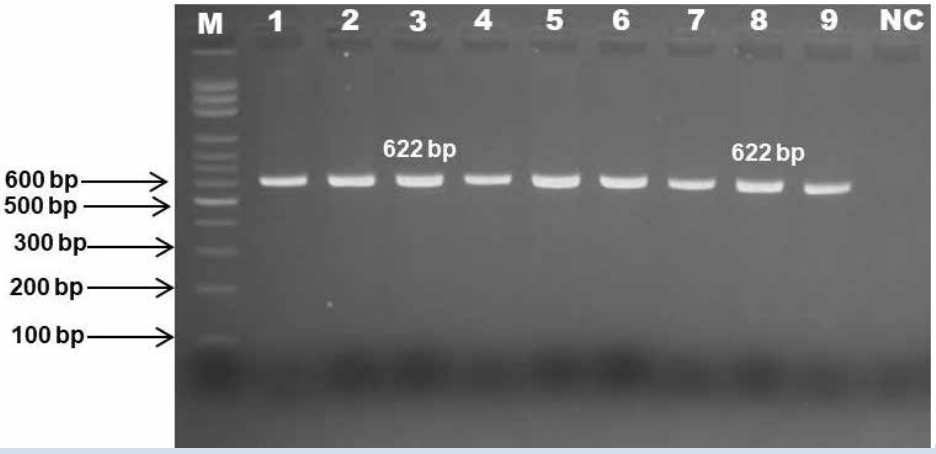

Figure 1. The electrophoresis pattern of PCR amplification (622 bp amplicon) for caprine *CAST* locus (M: Marker, 100-1500bp; NC: Negative control; bp: Base pair)

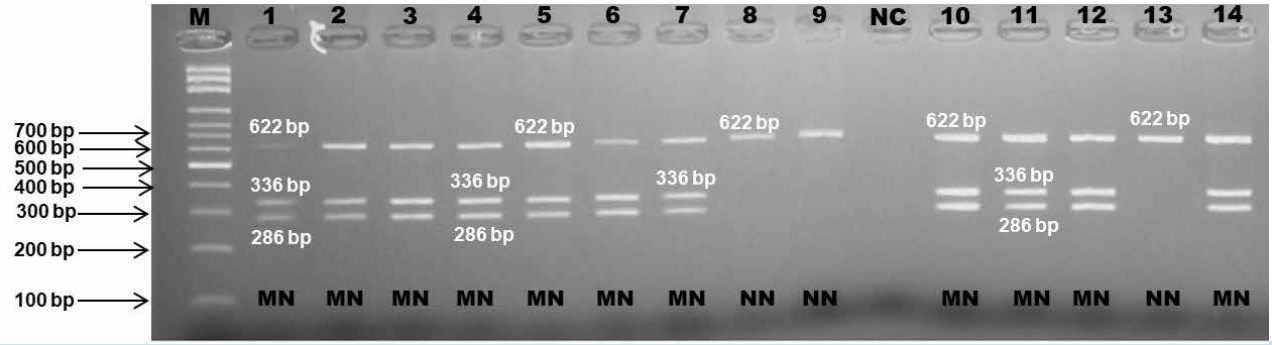

Figure 2. The electrophoresis pattern of restriction enzyme digestion of PCR product with *Msp*I for caprine *CAST* genotypes including NN and MN (M: Marker; NC: Negative control; bp: Base pair; Lines 8, 9, and 13: NN; Lines 1-7, 10-12, and 14: MN)

Table 2. The least-square means for the genotype effects of *CAST Msp*I polymorphism on studied traits (*n*=73)

Traits	NN Genotype		MN Genotype		Significance (*P*-value)
	Mean	Standard error	Mean	Standard error	
Birth weight (kg)	2.851	0.286	2.532	0.243	**0.032**
First breeding weight (kg)	40.191	4.322	42.460	3.160	0.490
Average daily weight gain (kg)*	0.050	0.005	0.053	0.003	0.723
Litter size	2.190	0.120	2.021	0.079	0.242

* Calculated based on the period between birth and reaching the first breeding weight.

HWE testing revealed that the population was determined not to be compatible with the equilibrium (P<0.001). The population genetic indices are presented in Table 1. Moderate levels of these indices were distinctly observable. The number of heterozygous genotype carriers was remarkably high (n=52) and the minor allele frequency was 0.36 (M allele). This resulted in the desirable genetic variability of Ne (1.85), although only two genotypes were observed (NN and MN).

Marker associations

Table 2 shows the least-squares means, standard errors, and the levels of significance concerning the effects of the *CAST* marker on birth weight, first breeding weight, litter size, and average daily weight gain. Significant differences were found between the genotypes of the *CAST* locus about birth weight (P<0.05). Animals with the NN genotype were associated with higher birth weight means (2.85±0.28 kg) compared to heterozygous animals (2.53±0.24 kg). There was no association between the marker and any of the remaining phenotypic traitsevaluated (first breeding weight, litter size, and average daily weight gain).

DISCUSSION

Genetic diversity

The present results indicated that there was a deviation from HWE for the *CAST* marker (P<0.001). Population characteristics in relation to selection process dynamics and inbreeding levels may cause this disequilibrium (Lacorte et al., 2006). Moreover, the typical structure of dairy herds which are under intense selection with a few sires should be considered when evaluating HWE. Population genetic parameters are very important indicators in the assessment of population structure for genetic variation. They also indicate the quality and suitability of genetic markers in a particular population (Ardicli et al., 2019). The low levels of H_{exp} indicate high inbreeding rates and it should be considered as a potential problem for the herd and should be accompanied by detailed pedigree information. On the other hand, the effectiveness of selected loci is determined by Ne values (Trakovická et al., 2013). In this study, the H_{exp} value was 0.4608, whereas, Ne value was 1.8546, and thus, the results indicated an admissible level of genetic variability in the analysed Saanen population at the considered locus. This interpretation was partially confirmed by a relatively low level of F_{IS} (0.0205) because this value can be considered as a good indicator for eventual heterozygosity and it displays the degree to which heterozygosity decreases (Duifhuis-Rivera et al., 2014). LVPR value, which is associated with homozygosity in the considered population (Miluchová et al., 2013), was determined to be 0.4499. Besides, present results

revealed that *CASTMsp*I polymorphism was moderately informative according to the classification of PIC values (high polymorphism if PIC>0.50, moderate polymorphism if 0.25<PIC<0.50, and low polymorphism if PIC<0.25) suggested by Botstein et al. (1980) (Table 1). Another important point is that the MM genotype was not present. This situation partially causes a negative impact on population impact on population genetic indices which is directly related to allele frequency distributions.

Marker effect on trait means

The relationship of caprine *CASTMsp*I polymorphism to birth weight, litter size, first breeding weight, and average daily weight gain was evaluated in this study. A member of the calpain-calpastatin system was chosen because it plays a crucial role in growth regulation. As Goll et al. (1998) indicated skeletal muscle growth is significantly associated with muscle protein synthesis and degradation and size/ number of skeletal muscle cells. There is fair evidence of a relationship between increased skeletal muscle growth and decreased muscle protein degradation (Chung and Davis, 2012; Goll et al., 1998). This is a cause of decreased levels of calpain activity which is regulated by calpastatin activity. Thus, the genetic mechanisms underlying the impacts of the calpain-calpastatin system on growth traits may partially explain the variations between individuals. Byun et al. (2008) suggested that *CAST* is an excellent candidate gene for controlling growth in livestock. The results of this study suggest that there is a significant association between the *CASTMsp*I polymorphism and the birth weight of kids in Saanen goats (P<0.05). Animals with the NN genotype had +0.319 kg heavier birth weight compared to the MN genotype. The birth weight of kids is one of the most important and reliable indicators of breed efficiency in the breeding plans for commercial goat production systems. This trait is known to be highly variable and is significantly affected both by genetic and environmental factors (Mioč et al., 2011). It is important to note that birth weight is associated with kid mortality which generally occurs at birth and from birth to weaning compared to mortality weaning to breeding age. Evaluating the effectiveness of preweaning management is imperative to have a desired kid survival rate (Awemu et al., 1999; Hailu et al., 2006). The present study indicates that selecting animals with the NN genotype of the *CASTMsp*I polymorphism induced higher means of birth weight and this application may contribute to well-handled preweaning management. However, there was no association of *CAST* marker with first breeding weight, average daily weight gain, and litter size. As in the present study, Byun et al. (2008) suggested that the *CAST* gene is an important regulator of birth weight, but has only a limited effect on growth rate to weaning in Romney lambs. Similar results were presented by Chung and Davis (2012) indicating a potential relationship between ovine *CAST* and birth weight. There is limited information about the relationship of the *CAST* marker to growth and reproduction parameters in goats. To the best of the author's knowledge, this is the first study suggesting a significant association between caprine *CASTMsp*I polymorphism and birth weight. The application area of genomic selection is quite limited in goat breeding compared to sheep and, especially, cattle. Thus, there is plenty of room for improvement in evaluating the genetic base of quantitative traits regarding the caprine genome.

Litter size or the number of kids born per parturition is an important indicator of productivity in goat breeding. In the present study, the litter size for the NN genotype was 2.19 whereas it was determined to be 2.02 in heterozygous animals. On the other hand, the heterozygous animals seemed to be heavier than the NN genotype concerning the first breeding weight of the goats (42.46 kg and 40.19 kg, respectively). However, this difference was not substantiated in association analysis (P>0.05). A similar implementation may be considered for the average daily weight gain from birth to first breeding. Growth and reproductive traits are known to show wide variations among breeds or populations and even different populations of the same breed in the same environmental conditions. It is important to note that considering different combinations of the polymorphisms based on the genotypic interactions through epistasis, genetic linkage, and pleiotropy may be worthy to provide a broad aspect in understanding the genetic basis of the quantitative traits. Recently, the trend of selection has gradually evolved from traditional procedures to marker-assisted selection or genomic selection in developed countries. These genomic methods allow breeders or researchers to use quantitative trait loci and candidate genes, which directly or indirectly affect the phenotype of individuals (Trakovická et al., 2013).

Improvement of production traits to increase economic gain is the preeminent aim of livestock. Never-

theless, the importance of functional traits including growth and reproduction cannot be underestimated to achieve a sustainable production system, especially in small ruminant breeding.

CONCLUSIONS

This paper points out the need for genetics research on the caprine genome regarding economically important quantitative traits and it also indicates a potential association between *CASTMsp*I polymorphism and birth weight in Saanen breed. The NN genotype may have a favourable influence on the birth weight of kids, and thus, we first suggested that caprine *CAST* could be regarded as a candidate molecular marker for birth weight. Further studies with larger populations are required to understand the complex biological nature of genetic basis on growth and reproduction traits in goats.

CONFLICT OF INTEREST STATEMENT

None of the authors of this article has any conflict of interest.

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Research article
Ερευνητικό άρθρο

Replacement of Beef Fat in Meatball with Oleogels (Black Cumin Seed Oil/ Sunflower Oil)

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ABSTRACT: Meat products contain fatty acids, especially saturated fatty acids, which cause adverse health effects. The effect of fats on meat products isnot only concerned with health, but also about the product's sensorial or textural properties. The research aimed to develop a new, healthier meatball formula in which the fat is substituted by oleogel gelled by carnauba wax and made with sunflower oil and black seed oil mixture. The effect of substituting animal fat with oleogel on color values, cooking parameters, lipid oxidation, and the textural properties of meatballs were determined. The effect of oleogel type on the TBA values of 50 and 75% substituted samples was statistically significant ($p < 0.05$), and TBA values of the samples with oleogel substituted were higher than others at the end of the storage. The effect of substitution rates on the texture profile of meatball samples was found to be statistically significant ($p < 0.05$). The oleogel (25%) added group scored significantly ($p < 0.05$)higher than the control group in appearance, flavor, texture, juiciness, oiliness, and overall acceptability, and was not found difference between the treatment groups.

Keywords: Black seed oil, Meatball,Oleogel, Sunflower oil, TBA

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INTRODUCTION

Meat and meat products contain saturated fatty acids, which may contribute to cardiovascular disease. In recent years, consumer demand for healthy products has been increased. So studies on the substitution of fats with healthier lipids in meat products (meatballs, pates, sausages, etc.) have increased rapidly (Kouzounis et al., 2017). The substitution of animal fats increases the concentration of unsaturated fatty acids while decreasing the concentration of saturated fatty acids in meat products. This is beneficial in terms of disease prevention (Dominguez et al., 2017). Animal fat has a technological, physicochemical, sensorial, and textural impact on the product in addition to its health benefits (Dominguez et al., 2017; Fagundes De Oliveira et al., 2017).

Black seed (*Nigella sativa*) and black seed oil have traditionally been used to treat various diseases. (Burits and Bucar, 2000). Black seed contains essential oils (Piras et al., 2013)as well as bioactive compounds such as phenolics, which have antioxidant properties(Burits and Bucar, 2000). Several studies have found that black seed has antioxidative properties. The majority of the studies substituted vegetable oils for fats, resulting in higher lipid oxidation levels due to the unsaturated fatty acid composition of the vegetable oils (Delgado-Pando et al., 2011). Thus, the combination of sunflower oil and black seed oil improves the antioxidant capacity of the oil, which would be used to produce oleogel.

New techniques for gelation of edible oils have been developed in order to form solid-like properties in the oil without altering its chemical structure. Oleogels have applications in the food, cosmetics, and pharmaceutical industries. (Co and Marangoni, 2012). Carnauba wax is extracted from the leaf and petiole of the *Copernicia cerifera* palm tree. During the production of carnauba wax, the leaves of the trees are cut, dried, and beaten until the wax is in powder form(Koonce and Brown, 1941).

The aim of the study was to determine the effect of substituting animal fat with an oleogel (sunflower/ black cumin seed oil mixture) structured by carnauba wax on color values, cooking parameters, lipid oxidation, textural and sensorial properties.

MATERIALS AND METHODS

Sunflower oil and black cumin seed oil were purchased from a local market in Afyonkarahisar. Carnauba wax obtained was from a trader in İstanbul, The Brown Swiss bulls were slaughtered at a commercial slaughterhouse. Samples of the thin flank of the carcasses were obtained 24 h post mortem from a local butcher. The flank was dissected into muscle and fat. Muscle and fat were comminuted separately at 0°C through a 6 and 3 mm plate, respectively, (Mateka EPA 22T, İstanbul, Turkey) and transported in cold conditions to the Food Science and Technology laboratory of Afyonkarahisar University of Health Sciences Department of Nutrition and Dietetics in 10 min. They were held in a refrigerator until the preparation of the meatballs. Other chemicals,butylatedhydroxyanisole, hexane, isopropanol, tetra methoxy propane, and thiobarbituric acid was purchased from Sigma-Aldrich (St. Louis,MO, USA).

Oleogel preparation

Oleogel was prepared according tothe method of Lim et al., (2017) and Öğütcü and Yilmaz (2015) with slight differences. Three different oleogel were prepared with sunflower oil, the second one used 90% sunflower oil (OG) and 10% black cumin seed oil mixture (OG10),and the last one was 80% sunflower oil and 20% black cumin seed oil mixture (OG20). Oleogels were prepared by the addition of carnauba wax to the sunflower oil and sunflower black cumin seed oil mixture at a ratio of 7. 5% (w/v). This concentration was selected because of the appearance and texture of the oleogels (only sensorial evaluation was made by the researcher, below this percentage, the oleogel mixture was more fluid, and above this concentration the color of the oleogel was darker in color). After adding the oil and wax, the mixture heated to 82 °C and held at this temperature for 5 minutes after having a clear appearance. The oleogel mixture was allowed to cool to room temperature and stored at 4 °C at refrigerator overnight.

Meatball preparation

Beef fat added a ratio of 25% of meat**,** and salt was added to the mixture at 2 %. The minced meat and fat were kneaded by hand for 5 min. The meatball dough was cut into 25 gram pieces and rounded, then put onto plates covered with cling film and stored in the refrigerator (4±2 °C) for 6 days. After the preparation of the control group, other groups were prepared in the same manner, except for fat addition. The fat was substituted by oleogels (OG, OG10, OG20) at a ratio of 25, 50 and 75% of fat content in each group. Meatball samples were prepared in two replications.

Instrumental color determination

A colorimeter(X-Rite (Ci6X)) was used to determine the CIE color parameters (L*, a*, b*) of the meatball samples. Before determining the values, the colorimeter was calibrated with white and black plaque after calibration readings were taken from three different points of the samples.

Lipid oxidation

Thiobarbituricacid (TBA) Analysis

TBA values were determined according tothe method of (Pikul et al., 1989). 10g of meatball wasmixed with 35 ml of 4 % perchloric acid and 1 ml BHA added to the mixture. The mixture was homogenized at 13800 rpm for 1 minute. After homogenization, the slurry was filtered and washed with 5 ml distilled water. The filtrate was filled to 50 ml with perchloric acid. The filtrate (5ml) and 5 ml of TBA (0. 02 M) were mixed and heated at 80 °C for 1 hour. After heating, the mixture was cooled to room temperature for 10 min, and the absorbance of samples was read at 532 nm wavelength. The results were given from the calibration curve, which was prepared by the tetramethoxypropane (TMP). Results were given per mg malondialdehyde (MDA)/ kg meatball sample.

Conjugated dienes analysis

A 0. 5 g meatball sample was suspended and homogenized with 5 ml distilled water. 0. 5 ml aliquot was mixed with 5 ml of extraction solution (3:1, hexane: isopropanol) for 1 minute. After extraction, the solution was centrifuged at 2000*g* for 5 min. The supernatant of the samples was measured at 223 nm wavelength, and the result was given as micromole per mg meat sample(Juntachote et al., 2007).

Cooking parameters

Cooking yield (CY)

The cooking yield of the meatballs was determined by the ratio of the weight of cooked meatballs to the weight of raw meatballs. The results were expressed by (%) of the initial weight. (Murphy et al., 1975).

Moisture retention (MR)

Moisture retention was determined as the amount of moisture in the sample of 100g cooked meatballs. The equation of (El-Magoli et al., 1996) was used to determine this;

MR(%): (CY (%) * Moisture of Cooked meatball) / 100

Fat retention (FR)

Fat retention was determined by the equation of (Murphy et al., 1975).

FR (%): (Fat content of cooked meatball)*(Cooked meatball weight) / (Fat content of uncooked meatball)*(Uncooked meatball weight) *100

Diameter reduction (DR)

The diameter of the meatballs was calculated using a manual caliper before cooking and after cooking. Diameter reduction was calculated according to the equation of

DR: (Uncooked meatball diameter - Cooked meatball diameter) / Uncooked meatball diameter *100

(Kilincceker and Yilmaz, 2019)

Texture profile analysis

Texture profile analysis was conducted with an analyzer (TA-HD Plus, Stable Micro Systems, UK) with a 25 kg load cell (Bourne, 1978). The meatballs were put into a cylindrical container. The height of the samples was 2 cm, and the analysis was done at room temperature. The probe was 10 mm above the meatball, and the test speed was 5 mm/sec. The meatball was compressed twiceat 50% by the P/36R probe. Hardness, adhesiveness, springiness, cohesiveness, gumminess, chewiness, and resilience of the samples were determined by the software program of the instrument.

Sensorial analysis

Sensorial characteristics of the samples were assessed by 12- member semi-trained panel who are students at the Department of Nutrition and Dietetics in Afyonkarahisar Health Sciences University. The panelists have a mean age of 20 years who regularly consumed meatballs. Meatballs were grilled in an electric oven at 180 °C for 15 minutes. Between the samples, bread and water were provided to the panelists to cleanse the palate. 9- point scale was used for the evaluation of the samples which “9” indicates the highest acceptability and “1” is the lowest. Appearance, odor, flavor, texture, juiciness and overall acceptance were evaluated (Gokalp et al., 1999).

Statistical analysis

A software program was used to perform analysis of variance (ANOVA) on the obtained data (SPSS 20). The Shapiro-Wilk test was used to determine the normality of the data distribution (Shapiro and Wilk, 1965). The data is given in the form of a mean value

and a standard deviation. If the distribution was normal, the difference in means was determined using the Tukey Test, and if the distribution was not normal, the difference in means was determined using the Dunnett's T3 test. All analyses were performed in triplicate; color, TBA, and conjugated dienes analysis were performed on days 0, 2, 4, and 6, and cooking parameters and texture profile analysis of cooked meatballs were performed on day 0.

RESULTS

Instrumental determination of color

The storage time had no statistically significant impact on the L* and a* values of all samples (p>0. 05) (Table 1). Except for the 25% and 75% substituted OG 20 and control groups, the effect of the storage time was statistically significant (p0. 05) when the b* value results were examined. The effect of substitution rates on the L* values of OG meatball samples on days 0, 4, and 6 and OG10 samples on days 2, 4, and 6 was found to be statistically significant (p 0. 05), while the OG20 group samples showed no significant difference.

The effect of storage time, rate of substitution, and type of oleogel on the a* values of meatball samples was determined to be statistically insignificant. Red color is associated with meat/meat product quality in customer preferences. Since no dye or spice was applied to the meatball formula in this study, there has been no masking effect of the different lipid sources in meatball samples in the manner of an a* value.

Different findings were published by Kouzounis et al., (2017) in a similar study, and they concluded that the fat sources can affect the product's instrumental color parameters. By replacing the fats in frankfurters with organogels, Barbut et al. (2016)found that the lightness of the frankfurters was reduced. They also said that replacing fat with organogels had hardly effect on redness and yellowness values. According to Wolfer et al., (2018), the processing of frankfurter style sausages using oleogels instead of pork meat has a lower L* value than the control group, and the cross-section color of the sausages in the control group was redder.

Thiobarbituric acid (TBA) analysis

The oxidation of lipids in meat and meat products is an essential quality characteristic. The deterioration of lipids not only result in an unpleasant taste, but it also affects the texture, color, and shelf life of the product(Poyato et al., 2015).

There was an increase in TBA values, which is expected depending on time (Figure 1). However, in some groups, the increase was not linear, and there were occasional increases and decreases. This may be due to the instability of the oxidation product compounds.

On the first day, the differences in TBA values between the control group and the samples prepared using different substitution rates and oleogel added samples were statistically significant, except the OG10 oleogel samples (p<0. 05). The TBA values were changed to 0. 15-0. 20, 0. 13-0. 18, and 0. 23-0. 51 for the OG, OG10, and OG20 groups, respectively, while the control group was 0. 09 mg MDA/kg meat sample. When the effect of substitution rates on TBA values of the same oleogel added samples was evaluated at the end of storage, statistically significant differences (p0. 05) were found. TBA values of meatballs prepared with different substitution rates and with OG10 (0. 27-0. 31 mg MDA/kg meat) and OG20 (0. 28-0. 31 mg MDA/kg meat) type oleogels were found to be statistically lower on the last day of storage than the control (0. 60 mg MDA/kg meat) group, in which there was no statistically significant difference between the substitution rates. The effect of oleogel type on the TBA values of 50 and 75 % substituted samples was statistically significant, and the TBA values of OG substituted samples were higher at the end of storage than the others. This decrease may be related to the antioxidant compounds found in black cumin seed oil.

Malonaldehyde loss may be caused by interactions with other molecules (amino acids, proteins) or intermolecular reactions (polymerization) (Jamora and Rhee, 2002). Yilmazand Öğütcü (2015) concluded that the rate of oxidation in cookies could be influenced by the solid fat content, storage conditions, processing conditions, and ingredients with antioxidant activity. Delgado-Pando et al. (2011)concluded that formulation and storage time had an impact on the TBA values of healthy frankfurters with fat replacers. Gómez-Estaca et al., (2019) showed that ethylcellulose oleogel substituted products have the highest initial TBA values, which increases during storage. They suggested that the degree of lipid oxidation was related to the oleogels' process parameters. According to da da Silva et al. (2019), the TBA values of modified sausages were significantly lower as compared to the control groups. Poyato et al. (2015)concluded that replacing fat in burger patties with gelled carrageenan-containing emulsion results in substantially lower TBA values as compared to the control.

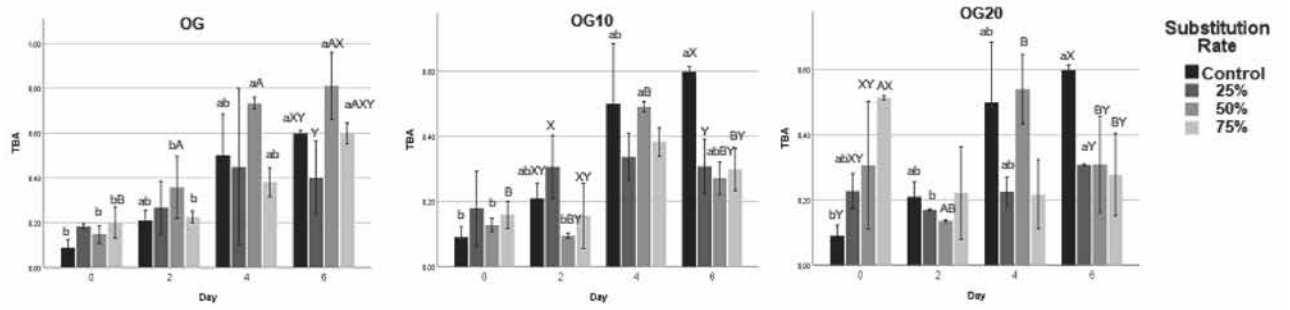

Figure 1. TBA values of meatball samples whose fats were substituted with oleogels.

[a-c]: Different superscript lowercase letters show differences between the storage days with respect to the same substitution rates and same oleogel types (p< 0. 05). [A-D]: Different superscript uppercase letters show differences between the oleogel type with respect to the same storage day and substitution rate (p < 0. 05). [X-Z]: Different superscript uppercase letters show differences between the substitution rates with respect to the same storage day and oleogel type (p < 0. 05). OG: Oleogel prepared by sunflower oil, OG 10: Oleogel prepared by mixing black cumin seed oil:sunflower oil (10:90), OG 20: Oleogel prepared by mixing black cumin seed oil:sunflower oil (20:80).

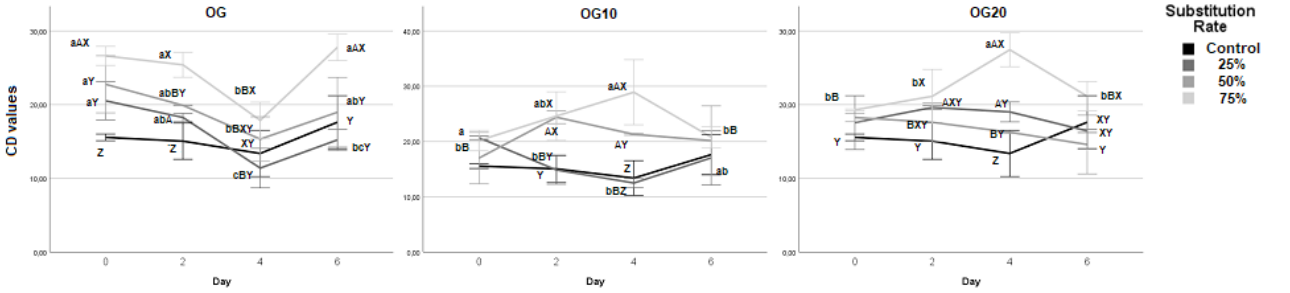

Figure 2. CD values of meatball samples whose fats were substituted with oleogels.

[a-c]: Different superscript lowercase letters show differences between the storage days with respect to the same substitution rates and same oleogel types (p< 0. 05). [A-D]: Different superscript uppercase letters show differences between the oleogel type with respect to the same storage day and substitution rate (p < 0. 05). [X-Z]: Different superscript uppercase letters show differences between the substitution rates with respect to the same storage day and oleogel type (p < 0. 05). OG: Oleogel prepared by sunflower oil, OG 10: Oleogel prepared by mixing black cumin seed oil:sunflower oil (10:90), OG 20: Oleogel prepared by mixing black cumin seed oil:sunflower oil (20:80).

Conjugated dienes analysis

Figure 2 shows the findings of the conjugated dienes analysis. According to the results, the CD values of meatballs were statistically (p<0. 05) different in meatball samples made with different substitution rates in the same oleogel type groups at the beginning of storage. At the beginning of storage, the OG and OG20 groups had the highest conjugated diene values with a substitution ratio of 75% and the OG10 group had a substitution ratio of 25%.

Conjugated diene measurement shows the oxidative stability of the fats or oils in the product which formed by the triplet oxygen or singlet oxygen (Akhtar et al., 2018). Juntachote et al. (2007)concluded that storage period reduced the conjugated diene value, but our result shows the opposite. Except for OG10 and OG20 added samples with a substitution rate of 75%, conjugated diene values decreased until the fourth day and increased on the sixth day in this study. Conjugated diene values in these two groups rise until the fourth day of storage, then rise again on the sixth day. This situation has arisen as a result of an increase in the amount of unsaturated fatty acids. According to Juntachote et al. (2007), the decomposition of the conjugated dienehydroperoxides increases the TBA values of the samples. The samples with the lowest TBA values had the highest conjugated diene values in the study. This can be explained by the fact that the compounds present in black cumin seed oil inhibit the formation of TBA substances.

Table 1. Color parameters of meatball samples whose fats were substituted with oleogels.

	Day	OG				OG10				OG20			
		Substitution Rate				Substitution Rate				Substitution Rate			
		Control	25%	50%	75%	Control	25%	50%	75%	Control	25%	50%	75%
L*	0	42. 30±3. 28^{XY}	45. 91±1. 26^{X}	44. 97±2. 56^{X}	37. 04±0. 19^{BY}	42. 30±3. 28	44. 88±3. 06	45. 47±6. 00	41. 31±4. 09^{abAB}	42. 30±3. 28^{X}	45. 20±0. 43^{XY}	46. 19±1. 28^{XY}	44. 06±2. 54^{AY}
	2	42. 25±0. 99	43. 97±5. 17	41. 66±1. 67	38. 94±2. 24^{B}	42. 25±0. 99^{XY}	44. 81±2. 67^{X}	40. 90±3. 12^{XY}	36. 90±0. 81b^{BY}	42. 25±0. 99	43. 84±3. 59	44. 92±4. 27	42. 80±1. 05^{A}
	4	46. 16±2. 49^{X}	47. 86±0. 62^{X}	40. 81±0. 91^{BY}	40. 78±2. 11^{Y}	46. 16±2. 49	45. 91±3. 40	46. 62±2. 50^{A}	39. 66±3. 59^{ab}	46. 16±2. 49	47. 82±1. 74	46. 20±1. 86^{A}	43. 92±2. 06
	6	46. 18±1. 74	46. 00±1. 14	42. 90±1. 42	40. 52±4. 03	46. 18±1. 74	42. 72±1. 96	41. 91±3. 04	41. 81±0. 47^{a}	46. 18±1. 74	44. 52±2. 79	42. 50±2. 24	42. 57±1. 54
a*	0	17. 44±1. 14^{aXY}	16. 81±1. 14^{aY}	19. 83±0. 70^{aAX}	16. 40±0. 56^{aAY}	17. 44±1. 14^{a}	14. 78±2. 46^{ab}	13. 38±1. 41^{abB}	14. 77±0. 70^{aAB}	17. 44±1. 14^{aX}	14. 89±0. 81^{aXY}	14. 67±1. 78^{aBXY}	13. 34±1. 33^{aBY}
	2	12. 75±0. 35^{bY}	13. 67±1. 84^{abXY}	15. 82±0. 99^{bAX}	13. 58±0. 69^{bXY}	12. 75±0. 35^{bY}	14. 50±0. 36^{aX}	12. 98±0. 31^{aBY}	13. 07±0. 81^{abY}	12. 75±0. 35^{b}	12. 96±1. 47^{ab}	11. 03±0. 74^{bB}	11. 37±1. 74^{ab}
	4	8. 15±0. 14^{cZ}	10. 61±0. 88^{bcY}	14. 45±0. 77^{bcAX}	8. 68±0. 86^{cZ}	8. 15±0. 14^{c}	10. 24±2. 03^{ab}	10. 74±0. 90^{abB}	9. 87±0. 96^{bc}	8. 15±0. 14^{cY}	10. 93±0. 42^{bX}	10. 27±1. 09^{bBXY}	9. 89±1. 37^{abXY}
	6	8. 44±0. 97^{c}	8. 25±0. 34^{bB}	9. 07±1. 60^{c}	7. 02±0. 87^{c}	8. 44±0. 97^{c}	10. 19±1. 03^{bA}	9. 64±0. 83^{b}	8. 27±0. 41^{c}	8. 44±0. 97^{cY}	10. 64±0. 35^{bAX}	8. 54±0. 33^{bY}	8. 37±0. 89^{bY}
b*	0	15. 98±1. 18^{X}	17. 06±0. 48^{aX}	17. 51±1. 39^{X}	13. 21±0. 10^{Y}	15. 98±1. 18	16. 08±0. 35	15. 22±1. 32	14. 30±1. 43	15. 98±1. 18	16. 14±0. 71	16. 29±0. 51^{a}	15. 34±1. 03
	2	14. 47±0. 76	15. 42±2. 09^{ab}	15. 66±1. 07	12. 73±0. 75	14. 47±0. 76^{XY}	16. 17±0. 51^{X}	14. 43±0. 66^{XY}	12. 97±0. 94^{Y}	14. 47±0. 76	14. 83±1. 62	14. 70±0. 46^{ab}	14. 78±1. 37
	4	14. 34±0. 80^{X}	15. 71±0. 37^{abX}	14. 09±1. 44^{X}	11. 40±0. 63^{Y}	14. 34±0. 80^{XY}	15. 01±0. 56^{X}	15. 75±1. 14^{X}	12. 07±1. 28^{Y}	14. 34±0. 80	16. 04±1. 20	15. 98±1. 16^{a}	13. 75±0. 92
	6	14. 54±0. 93^{X}	14. 79±0. 56^{bX}	13. 89±1. 06^{XY}	11. 70±0. 99^{BY}	14. 54±0. 93	14. 05±1. 07	12. 93±2. 01	12. 67±0. 43^{AB}	14. 54±0. 93	13. 94±0. 92	13. 24±1. 00^{b}	14. 08±0. 96^{A}

$^{a-c}$: Within each column different superscript lowercase letters show differences between the storage days with respect to the same substitution rates and same oleogel types (p< 0. 05). $^{A-B}$: Within each row different superscript uppercase letterss how differences between the oleogel type with respect to the same storage day and substitution rate (p < 0. 05). $^{X-Z}$: Within each row different superscript uppercase letters show differences between the substitution rates with respect to the same storage day and oleogel type (p < 0. 05). OG: Oleogel prepared by sunflower oil, OG 10: Oleogel prepared by mixing black cuminseed oil: sunflower oil (10:90), OG 20: Oleogel prepared by mixing black cuminseed oil: sunflower oil (20:80).

Table 2. Texture profile analyse results of meatball samples whose fats were substituted with oleogels.

	Hardness (N)				Adhesiveness			
Oleogel	Control	25%	50%	75%	Control	25%	50%	75%
OG	145. 75±18. 13^{a}	77. 32±26. 41^{b}	46. 12±8. 38^{bB}	67. 34±8. 94^{bA}	ND	-0. 67±0. 72	-0. 77±0. 29^{A}	-2. 02±2. 09
OG10	145. 75±18. 13^{a}	55. 89±15. 43^{b}	59. 74±5. 18^{bA}	38. 34±3. 86^{bB}	ND	-0. 86±0. 59^{a}	-0. 91±0. 17^{aA}	-4. 47±2. 28^{b}
OG20	145. 75±18. 13^{a}	48. 72±4. 10^{b}	45. 24±1. 89^{bB}	30. 38±7. 98^{bB}	ND	-1. 46±0. 48	-2. 10±0. 64^{B}	-6. 33±5. 92
	Springiness				Cohesiveness			
OG	0. 85±0. 02^{a}	0. 74±0. 07^{ab}	0. 70±0. 01^{bA}	0. 70±0. 09^{ab}	0. 62±0. 04^{a}	0. 42±0. 07^{b}	0. 40±0. 06^{b}	0. 40±0. 07^{b}
OG10	0. 85±0. 02^{a}	0. 70±0. 03^{b}	0. 66±0. 02^{bAB}	0. 62±0. 04^{b}	0. 62±0. 04^{a}	0. 42±0. 03^{b}	0. 34±0. 03^{bc}	0. 32±0. 09^{c}
OG20	0. 85±0. 02^{a}	0. 71±0. 01^{b}	0. 62±0. 04^{cB}	0. 61±0. 04^{c}	0. 62±0. 04^{a}	0. 50±0. 06^{ab}	0. 32±0. 05^{c}	0. 37±0. 06^{bc}
	Gumminess				Chewiness			
OG	9185. 81±945. 69^{a}	3178. 44±682. 56^{b}	1832. 36±164. 74^{bAB}	2697. 61±174. 87^{bA}	7830. 82±851. 40^{a}	2377. 93±687. 43^{b}	1271. 91±122. 16^{bAB}	1900. 80±321. 03^{bA}
OG10	9185. 81±945. 69^{a}	2407. 81±711. 75^{b}	2069. 74±363. 34^{bA}	1221. 16±284. 84^{bB}	7830. 82±851. 40^{a}	1694. 97±553. 08^{b}	1364. 57±216. 91^{bA}	757. 97±160. 24^{bB}
OG20	9185. 81±945. 69^{a}	2494. 91±505. 59^{b}	1473. 41±194. 57^{bB}	1112. 53±180. 98^{bB}	7830. 82±851. 40^{a}	1765. 06±348. 50^{b}	917. 38±184. 57b^{bB}	682. 55±146. 64^{bB}
	Resilience							
OG	0. 24±0. 03^{a}	0. 14±0. 05^{ab}	0. 14±0. 03^{b}	0. 14±0. 03^{b}				
OG10	0. 24±0. 03^{a}	0. 15±0. 02^{b}	0. 10±0. 01^{b}	0. 09±0. 03^{b}				
OG20	0. 24±0. 03^{a}	0. 18±0. 02^{ab}	0. 10±0. 02^{c}	0. 12±0. 04^{bc}				

a-c: Within each row different superscript lower case letters showdifferences between the substitution rates (p< 0. 05). A-D: Within each column different superscript uppercaseletters show differences between the oleogel types (p < 0. 05). OG: Oleogel prepared by sunflower oil, OG 10: Oleogel prepared by mixing black cuminseed oil: sunflower oil (10:90), OG 20: Oleogel prepared by mixing black cuminseed oil: sunflower oil (20:80).

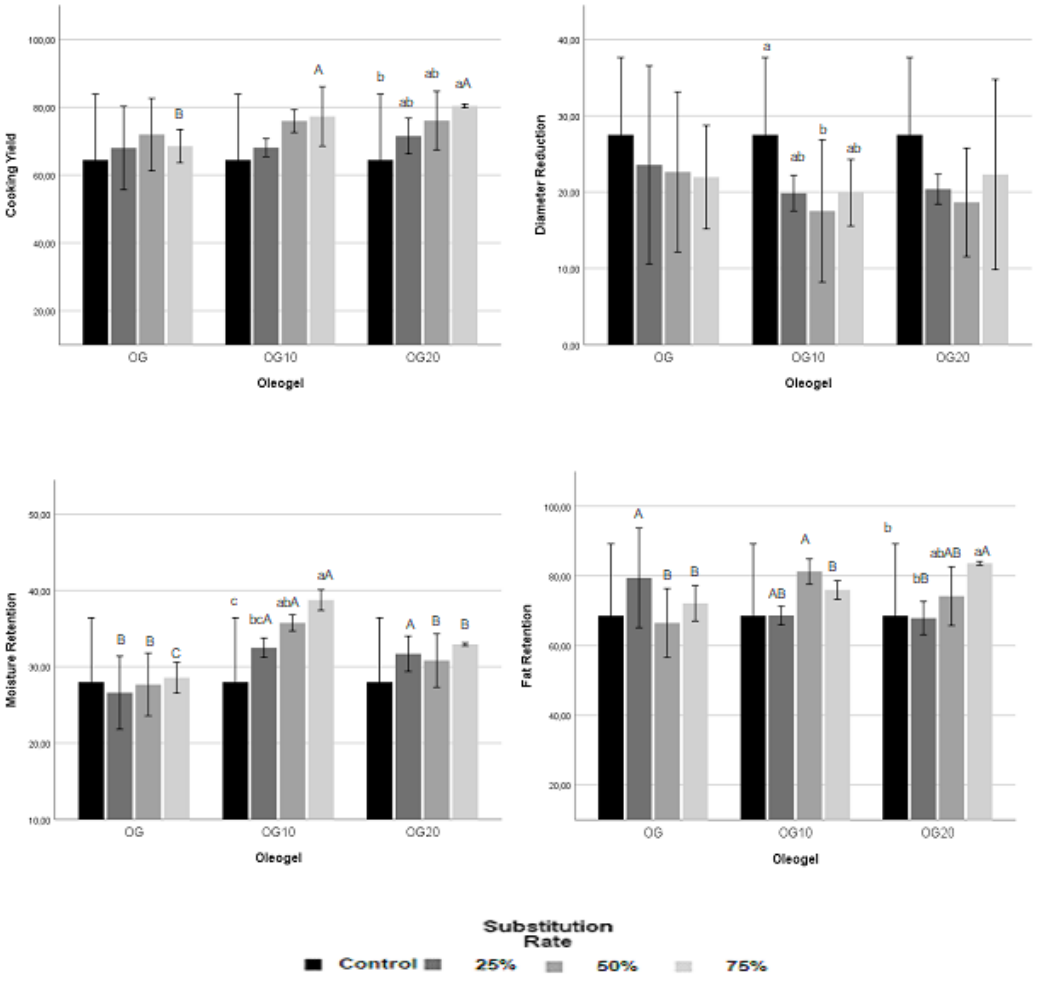

Figure 3. Cooking parameters of meatball samples whose fats were substituted with oleogels

a-c: Different superscript lowercase letters show differences between the substitution rates (p< 0. 05). A-D: Different superscript uppercase letters show differences between the oleogel types (p < 0. 05). OG: Oleogel prepared by sunflower oil, OG 10: Oleogel prepared by mixing black cumin seed oil:sunflower oil (10:90), OG 20: Oleogel prepared by mixing black cumin seed oil:sunflower oil (20:80).

Cooking parameters

Figure 3 shows the cooking parameters of meatball samples prepared with fat substitution by different oleogels at different substitution rates. Except for 75%, there was no significant difference observed between the oleogel types in terms of different substitution rates. Furthermore, no significant difference was observed between the control group and the fats substituted at a 25% ratio groups. However, meatball fats substituted at 50% and 75% had significantly (p<0. 05) higher cooking yields than the control and 25% substituted classes.

Figure 3 shows that the diameter reduction of the meatball samples ranged from 17. 51 to 23. 55%. The effect of substitution rate was significantly (p <0. 05) lower than the diameters of the meatballs only in the OG10 added samples.

By increasing the substitution rate in samples where fats were substituted with OG10, the moisture retention of the meatball samples was increased. In terms of moisture retention, there was a significant (p <0. 05) difference between the oleogel types with different substitution rates of samples. The oleogel type impacted fat retention in all substitution rates, and substitution rates impacted fat retention values in the OG10 and OG20 samples.

Barbut et al. (2016) reported that oleogels in meat products reduced cooking losses, and da Silva et al. (2019) reported that high pork back fat reduced cooking losses in sausages. This may be due to the presence of organogel in the matrix, which causes large fat globules in the matrix to help minimize liquid losses. Moghtadaei et al. (2018)showed that increasing the replacement amount of oleogels in burgers made

with different replacement ratios reduced cooking loss. According to Fagundes De Oliveira et al. (2017), reformulated burgers with pork skin and canola oil gels have lower diameter reduction and cooking loss values.

Texture profile analysis

The effect of substitution rates on the hardness, springiness, cohesiveness, gumminess, chewiness and resilience values of the meatball samples was found to be statistically significant ($p<0.05$) (Table 2). As compared to the control group, the hardness, springiness, cohesiveness, gumminess, chewiness, and resilience values of all fat substituted samples decreased. This is to be predicted because animal fats have an effect not only on health but also on the texture properties of the food. The effect of oleogel type on the cohesiveness and resilience values in all substitution rates was found to be statistically insignificant ($p>0.05$).

Kouzounis et al. (2017) found similar findings for the hardness, gumminess, and chewiness values of frankfurters made with pork lard or sunflower oil oleogel. They concluded that the hardness value of a lard-based frankfurter was higher than that of oleogel. They also concluded that any differences in these parameters could be attributed to the properties of oleogel as well as its interaction with the meat dough. Barbut et al. (2016) showed that substituting canola oil or organogel for pork fat results in significantly lower hardness scores. They reached the conclusion that the difference may be due to the scale of fat globules.

Sensory analysis results

Sensory analysis results (Figure 4) showed that the meatballs' color, taste, texture, juiciness, oiliness, and overall acceptability scores were significantly different.

They were similar in terms of appearance, taste, and oiliness ratings.

The OG25 group had the highest scores for appearance, flavor, texture, juiciness, oiliness, and overall acceptability and was significantly different from the control group, but there was no difference between the OG25 and the other groups. Although, the results showed that all values of the control group were the lowest except flavor. Fat content is important for both the textural and sensorial characteristics of the product, and also for human health. Because of the adverse affects of animal fats, healthy lipids may be substituted. As a consequence, this sensory analysis yields valuable results for developing new formulations and products for consumer acceptance.

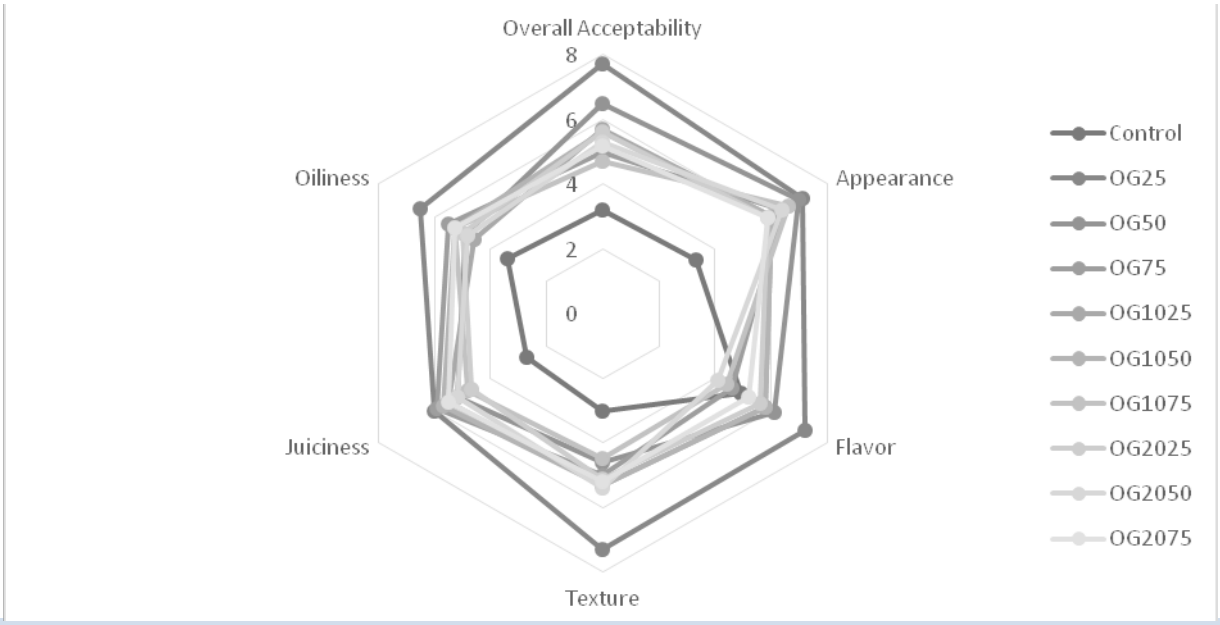

Figure 4. The sensory analysis on the average scores of the meatball samples

OG25: Meatball fat substituted with OG at 25% level, OG50: Meatball fat substituted with OG at 50% level, OG75: Meatball fat substituted with OG at 75% level, OG1025: Meatball fat substituted with OG10 at 25% level, OG1050: Meatball fat substituted with OG10 at 50% level, OG1075: Meatball fat substituted with OG10 at 75% level, OG2025: Meatball fat substituted with OG20 at 25% level, OG2050: Meatball fat substituted with OG20 at 50% level, OG2075: Meatball fat substituted with OG20 at 75% level.

CONCLUSIONS

TBA values of meatballs are reduced during storage when black seed oil is added to the oleogel mixture. These oleogel research must be conducted in greater depth and may be applicable to the development of new healthy goods. More research is required to determine the properties of oleogels made from sunflower oil and black seed oil. Oleogels can be made using a variety of gelling agents.

CONFLICT OF INTEREST STATEMENT

None declared.

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Research article
Ερευνητικό άρθρο

Complicated corneal ulceration in cats: diagnosis and treatment outcomes of 80 cases (2014-2018)

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ABSTRACT: The study describes the most common clinical and endoscopic findings associated with complicated corneal ulcers in cats and evaluates the short-term outcomes after surgical interventions. Eighty client-owned cats of different breeds with corneal ulcers were included. Cats were clinically evaluated to initially determine corneal abnormalities. Endoscopic examination of the corneas was performed to determine anterior and posterior segments' abnormalities. Non-healing superficial ulcer was treated by superficial keratectomy and deep stromal ulcers were treated using conjunctival flaps. Corneal sequestrum were treated by partial keratectomies and conjunctival flaps. Anterior synechiae were treated via peripheral iridectomy and separation of the adhesion between the iris and the inner cornea. Symblepharon were treated by removal of the adhered conjunctival membrane from the cornea. Unresponsive endophthalmitis was treated surgically by exenteration. Outcomes after surgical managements of selected corneal abnormalities were assessed clinically and endoscopically. Non-healing superficial ulcer, deep stromal ulcer with descemetocele, endophthalmitis, symblepharon, corneal sequestration and anterior synechiae with secondary glaucoma and corneal scarring were the recorded complications of corneal ulcer. FHV-1 was a common etiologic factor of corneal ulceration. Persistent corneal scars of varying shape and size developed in cats with deep stromal ulcer, anterior synechiae, and corneal sequestration. Domestic shorthaired and Persian cats were the most predisposed breeds to FHV-1 infection and subsequent corneal ulceration. Management of patients with corneal ulcer would prevent serious complications. No age or sex predisposition to complicated corneal ulceration in cats was noticed.

Keywords: ophthalmology, cat, ulcer, perforation, corneal surgery

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INTRODUCTION

Corneal ulceration is one of the common eye problems affecting humans (Whitcher et al., 2001), and a wide variety of animal species (Bentley, 2005; Michau et al., 2003).It can cause varying degrees of visual impairment or even blindness. Corneal ulcers are commonly seen in veterinary practice, and although uncomplicated ulcers would likely heal without veterinary attention, a complicated ulcer requires optimal management (Maggs, 2008). Corneal transparency and clarity are associated with its unique collagen and extracellular matrix organization (Mohan et al., 2005). Decreased corneal protection or increased corneal abrasion may result into ulceration. Uncomplicated stromal injuries tend to undergo avascular healing; however, infected or destructive lesions usually stimulate and require vascularized healing (Maggs, 2008). After injury, stromal keratocytes synthesize collagen, glycosaminoglycans, and mucoprotein of the ground substance and transform to fibroblasts that produce nontransparent collagen (Hankanson and Merideth, 1987). The nontransparent collagen causes different levels of visual impairment (Wilkie and Whittaker, 1997). Furthermore, collagen fibrils produced during repair of a stromal lesion are laid down in an irregular pattern and often produce a grey "wispy" opacity within the cornea that interferes with light transmission (Vanore et al., 2007). In the Persian, Siamese, and occasionally Domestic shorthaired (DSH) cats, slow-healing epithelial defects caused by a variety of etiologic factors may result in corneal nigrum or sequestrum (Featherstone and Sansom, 2004). This may produce a brownish-black plaque or deposit that is usually located centrally or para-centrally and stimulates vascularization (Laguna et al., 2015).

Ulcerative keratitis management often requires a combination of medical and surgical therapeutic strategies (Barros et al., 2005; Vanore et al., 2007). In deep corneal ulceration, several surgical procedures have been proposed including conjunctival and (Hankanson and Merideth, 1987) corneal grafts (Wichayacoop et al., 2009), corneo-conjunctival transposition(Vanore et al., 2007), porcine small intestinal submucosa graft (Goulle, 2012), amniotic membrane transplantation (Barachetti et al., 2010), and application of tissue adhesives(Hankanson and Merideth, 1987; Barros et al., 2005).

Feline herpesvirus-1 (FHV-1) is one of the most frequently encountered causes of conjunctivitis and keratitis in cats(Stiles et al., 1997) and commonly produces an epithelial ulcer that may be dendritic or geographic (Groth et al., 2014). In severe complicated cases, deep stromal ulceration, descemetocele, or even globe rupture may develop (Stiles, 2014). Diagnosis of herpesvirus ocular lesions is often difficult due to the carrier state and the ubiquitous nature of the virus (Stiles and Townsend, 2007). Nevertheless, it can be either cultured, or detected with PCR, from 10% of conjunctivae and 50% of corneas of clinically normal cats (Stiles and Townsend, 2007).

The purposes of the study were the determination of the most common etiologic factors and manifestations associated with complicated corneal ulcers in cats, clinical, endoscopic and laboratory findings of complicated corneal ulceration, and evaluation of the short-term treatment outcomes.

MATERIALS AND METHODS

Animals

The present study was carried out on 80 client-owned cats examined by the Ophthalmology Service at the Department of Small Animal Surgery, Faculty of Veterinary Medicine, Cairo University, Egypt from September 2014 to December 2018.Informed consent was obtained from the owners of the 80 cats enrolled in the present clinical study and no ethical approval was acquired. Cats that had non-healing superficial or deep stromal corneal ulceration and complicated corneal ulcer were included in the study, whereas those with superficial non-complicated or corneal erosion were excluded. Patients that had epithelial erosion or herpetic keratitis were also excluded from the study reported here. Data collected from the clients included signalment, history of traumatic eye injury, duration of clinical signs, and history of previous medications.

Clinical and endoscopic examination

Clinical evaluation was initially performed by a qualified ophthalmologist (KMA) via slit lamp examination (SL 14 handheld slit lamp, Kowa, Tokyo, Japan) for detection of the current corneal abnormalities. Commercially available fluorescein strips (Bio-Glo® Fluorescein sodium Strips 1 mg; HUB pharmaceuticals, LLC., Rancho Cucamonga, CA, USA) were used to detect the type of corneal ulcer. Cats suspected to have anterior or posterior segment abnormality were examined endoscopically (Abd-Elhamid et al., 2014), using an 8.5 mm probe (Eickemeyer video endoscope unit supplied with halogen light source 150

watt, Vetlux, Tuttlingen, Germany). The endoscope unit was connected through an adapter to a computer device. Cats were sedated using xylazine hydrochloride 2% (Xylaject®;ADWIA, Cairo, Egypt) in a dose of 1mg/kg *b.w. i.m*. Topical anesthesia (Douet et al., 2013) of the cornea was achieved via instillation of Benoxate hydrochloride 0.4% (Benox®; EIPICO, Cairo, Egypt). The endoscopic probe was then gently placed on the corneal surface and series of images and video loops were captured for each examined cat. Measurement of intraocular pressure (IOP) was done using Tonopen tonometer (Tonopen XL®, Reichert Technologies, NY, USA).

Laboratory examination

Isolation and detection of FHV-1 and Mycoplasma felis

Sample collection and handling

Corneal swabs were collected by rolling dry cotton-tipped applicators along the mucosal surface of the conjunctiva or region of corneal ulceration. Separate swab samples were taken from each cat for viral and mycoplasma culture. Each sample was placed into a sterile tube containing 1 m*l* of sterile PBS then stored at refrigerator temperature (2-5°C).

Isolation of Mycoplasma felis

The corneal swabs were tested for the presence *of M. felis* on PPLO agar (Difco Laboratories) supplemented with thallous acetate (0.5 g/*l*), ampicillin (0.4 g/*l*), inactivated horse serum (250 m*l*/*l*), D-glucose (10 g/*l*) and freshly prepared yeast extract (70 m*l*/*l*). Incubation was performed at 37°C for 2-5 days in an atmosphere of 10% CO_2. *Mycoplasma* isolates were sub-cultured on ox blood agar in order to estimate hemolysis. *M. felis* was identified by colony characteristics and biochemical reactions (Razin and Freundt, 1984; Kirchhoff et al., 1985).

Detection of FHV-1 using PCR

DNA was extracted from the swabs using a QIAamp DNA mini kit (Qiagen). The swab was placed in a 1.5 m*l* micro centrifuge tube containing 300 m*l* of saline and incubated at 37°C for 10 min on a shaker. Approximately 200 m*l* of saline was then removed and placed in a fresh 1.5 m*l* tube, and DNA was extracted according to the manufacturer's protocol. The reaction was performed with master mix, primers (Table 1), PCR water and 5 m*l* of template DNA. Amplifications consisted of initial incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec; 58°C for 30 sec, and 72°C for 30 sec; with final extension at 72°C for 5 min (Sandmeyer *et al.*, 2010).

Treatment

The surgical procedure applied for each case based on whether the eye was potentially visual or not and on time to healing, recurrence rate, and availability of the technique at the time of presentation. Time to healing was defined as the number of days from surgery until removal of sutures and discontinuation of medication therapy. The surgical procedures for the reported complications were summarized in Table 2.

Table 1. The primers used for PCR of the samples suspected to have feline herpes virus-1

Primers	Amplicon
F 5′-CGG GAA AAT CCA GTA CGA GT-3′ R 5′-AGG AAG AGT TCG GCG GTA TT-3′	200 bp

Table 2. The treatment protocols for the 80 cats with complicated corneal ulcer

Corneal abnormalities	Number of cats	Surgical procedure
Deep stromal ulcer with descemetocele	**22**	Conjunctival flap
Nonhealing corneal ulcer	**16**	Superficial keratectomy
Unresponsive endophthalmitis	**14**	Exenteration
Symblepharon	**6**	Surgical removal of the adhered conjunctival tissues from the cornea
	5	Exenteration
Anterior synechia with secondary glaucoma	**8**	Peripheral iridectomy with removal of the adhered iris strands from the inner surface of the cornea
Chronic ulcer with granulation tissue	**5**	Superficial keratectomy and conjunctival flap
Corneal sequestration	**4**	Superficial keratectomy and third eye lid flap

Cats undergoing surgery were premedicated with atropine sulphate (0.04 mg/kg) and xylazine hydrochloride 2% (Xylaject®; ADWIA, Cairo, Egypt) in a dose of 1 mg/kg and anesthetized with ketamine hydrochloride 5% (Keiran; EIMC pharmaceuticals Co., Cairo, Egypt) in a dose of 20 mg/kg. Corneal anesthesia was established as previously described for endoscopic examination (Douet et al., 2013). The eye was prepared for aseptic surgery and draped routinely.

Cats with indolent (non-healing superficial) corneal ulcer were treated by superficial keratectomy under a binocular surgical microscope (12.5x; 66 VISION TECH CO., LTD. China) with the cat under general anesthesia. The technique utilized a microsurgical blade to excise the superficial stroma and epithelium under and around the indolent ulcer (Chavkin, 1990). Cats with deep stromal ulcer were treated by a conjunctival flap. The conjunctival flap was created in the bulbar conjunctiva using Steven's tenotomy scissors, with the base of the flap being attached to the limbus. The length of the flap was adjusted to cover the corneal defect without tension. The flap was then sutured to the borders of the corneal defect using 7-0 monofilament polypropylene (Prolene®; Ethicon, USA) in a simple interrupted. The sutures were placed deeply in the corneal stroma without penetrating the cornea completely (Gelatt and Brooks, 2011). Corneal sequestrum was managed surgically in 4 cats (5%) via partial keratectomy and conjunctival flap (Kirschner et al., 1991). A 64 Beaver blade was used to sharply remove the necrotic lesion from the cornea. The dissection was continued through the stromal thickness to a depth that allowed removal of the majority of the pigment associated with the sequestrum then a conjunctival flap was applied. Healed corneal ulcer (in 7 eyes; 8.75%) with granulation tissue were treated by use of conjunctival flap after surgical excision of the granulation tissue.

Cats diagnosed with anterior synechiae and secondary angle closure glaucoma (8 eyes; 10%) were treated surgically by peripheral iridectomy and separation of the adhesion between the iris and the inner cornea (Chavkin et al., 1990). Symblepharon were treated by removal of the adhered conjunctival membrane from the cornea in 6 out of 11 cats. In the remaining 5 cats the eye was presented by marked hypotony and phthisis bulbi, these eyes were not potential for vision and were treated by exenteration. Daily ophthalmic application of tobramycin (Tobrin®; Alcon, Cairo, Egypt) and dexapanthenol (Corneregel® ophthalmic gel; MINIPHARM, Cairo, Egypt) was performed 4-6 times a day after each surgical procedure. Medication dosages were decreased and then discontinued as healing occurred.

Cats diagnosed with corneal perforation followed by unresponsive endophthalmitis were treated by exenteration of the eye globe (Slatter, 2001).

RESULTS

Animals

Among the presented 80 cats, there were 46 (57.5 %) Domestic Shorthaired (DSH), 25 (31.25 %) Persian, 5 (6.25 %) Siamese, and 4 (5 %) Himalayan cats. Mean (±SD) age of the cats on initial evaluation was 21.2±1.6 months (range, 3months to 6 years). Fifty-two were females and twenty-eight were males. Seventy-four cats were sexually intact and six spayed. Thirty-seven (46.3%) cats had bilateral corneal abnormalities, 24 (30%) cats had right-sided corneal disease, and 19 (23.8%) cats had left-sided corneal disease. A history of short-term ophthalmic application of tobramycin and dexamethasone was reported in 15 cats.

Clinical findings

Clinical findings of complicated corneal ulcer of the 80 cats enrolled in this study included; deep stromal ulcers with descemetocele (22 cats; 27.5%), indolent corneal ulcer and superficial nonhealing ulcer due to FHV-1 infection (16 cats; 20%), endophthalmitis with corneal involvement (14 cats; 17.5%), symblepharon (11 cats; 13.75%), anterior synechiae with secondary glaucoma and corneal scarring (8 cats; 10%), chronic ulcers with granulation tissue protruding on the surface of the cornea (5 cats, 6.25%), corneal sequestrum (4 cats; 5%). Corneal abnormalities associated with each cat breed are demonstrated in Table 3.

The superficial nonhealing ulcers were presented after unsuccessful treatment for several weeks and were associated with corneal vascularization and detached epithelium at the edges of the ulcer (Figure 1a), these ulcers stained with fluorescein dye that migrated beyond the edges of the ulcer (Figure 1b), while in FHV-1 infected cats they were geographically distributed with no or minimal corneal vascularization (Figure 1c). Deep stromal ulcers were associated with corneal melting, descemetocele and variable degrees of corneal edema and vascularization (Figure 1d). In cats with chronic ulcers, corneal vascularization and

Table 3. The distribution of corneal abnormalities in the enrolled 80 cats with corneal ulcers.

Corneal abnormalities	Number (n)	Percentage (%)	Breed (n)
Deep stromal ulcer with descemetocele	22	27.5%	Persian (11)
			Domestic shorthaired (9)
			Himalayan (2)
Nonhealing superficial ulcer	16	20 %	Domestic shorthaired (9)
			Persian (5)
			Siamese (2)
Unresponsive endophthalmitis	14	17.5%	Domestic shorthaired (11)
			Persian (2)
			Siamese (1)
Symblepharon	11	13.75%	Domestic shorthaired (6)
			Persian (5)
Anterior synechia with secondary glaucoma	8	10%	Domestic shorthaired (6)
			Himalayan (2)
Old healed ulcer with granulation tissue	5	6.25%	Domestic shorthaired (5)
Corneal sequestration	4	5%	Persian (2)
			Siamese (2)

granulation tissue were noticed over the central and paracentral quadrants of the corneal surface (Figure 2 a and b). The corneal sequestrum appeared as well-defined medium to relatively large sized blackish area (Figure 2c and d) at the central and paracentral cornea and were associated with corneal vascularization. All cats with corneal sequestration had a history of long-term use of ophthalmic corticosteroids before presentation. One of the sequestra cases was a Persian cat showing associated diffuse corneal edema and neovascularization (Figure 3a). Keratitis with corneal ulceration and granulation tissue secondary to *FHV-1* and *Mycoplasma felis* infection showed reddish to pink granulation tissue covering most of the corneal surface (Figure 3b). Unresponsive endophthalmitis secondary to perforated corneal ulcers were associated with corneal melting and large fibrin clot protruding over most of the corneal surface (Figure 4a and b).

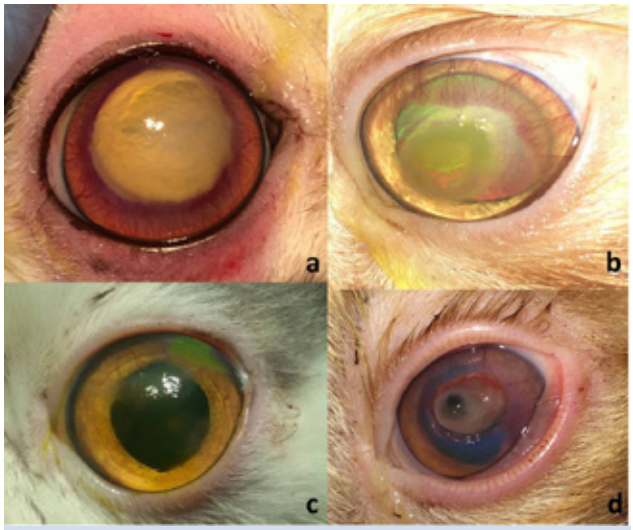

Figure 1. Photograph showing the clinical presentation of the superficial non- healing and deep stromal ulcer. (a) Superficial ulcer with corneal vascularization in a 14-month-old Himalayan cat. (b) Superficial corneal ulcer stained with fluorescein dye in a 2-year-old Persian cat with corneal vascularization; this ulcer was secondary to herpes virus infection. (c) Geographically distributed corneal ulcer in a 6-month-old Persian cat infected with herpes virus. (d) Deep stromal ulcer with descemetocele and corneal melting in a 4-year-old DSH cat.

Endoscopic findings

Endoscopic evaluation in patients presented with anterior segment abnormalities showed long iris strands arising from the pupil borders adhered to the inner cornea in 8 cats (Figure 5 a & b). One cat with perforated cornea showed immature cataract and anterior synechia (Figure5c). Endoscopy one month after surgical removal of the iris strands adhering to the inner cornea showed normal shaped pupil with normal optic nerve head (ONH) and clear retinal blood vessels (Figure 5 d).

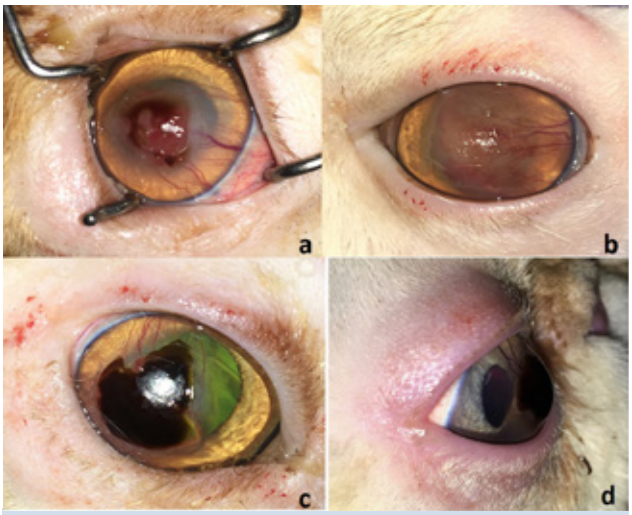

Figure 2. Representative clinical photographs complicated corneal ulcer with over granulation tissue and corneal vascularization in a 12-month-old DSH cat (a) and in a 2-year-old DSH (b). (c)A well-defined large sized corneal sequestrum secondary to FHV-1 infection in a 6-year-old Persian cat. (d) A well-defined medium sized corneal sequestrum secondary to herpes keratitis in a 6-year-old Persian cat.

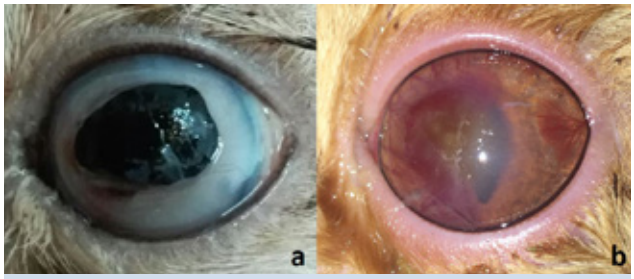

Figure 3. A relatively large sized corneal sequestrum with corneal edema and neovascularization in a 3-year-old Persian cat (a). Corneal ulceration with reddish to pink granulation tissue covering most of the corneal surface and corneal vascularization in a 6-month-old Domestic shorthaired kitten infected with FHV-1 and *Mycoplasma felis* (b).

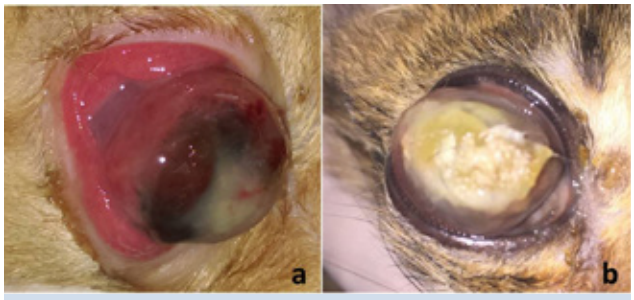

Figure 4. Clinical photograph showing perforated corneal ulcers with unresponsive endophthalmitis and large protruding fibrin clot in 3-year (a), 6-year (b), old DSH cat. FHV-1 was isolated from these2 cats.

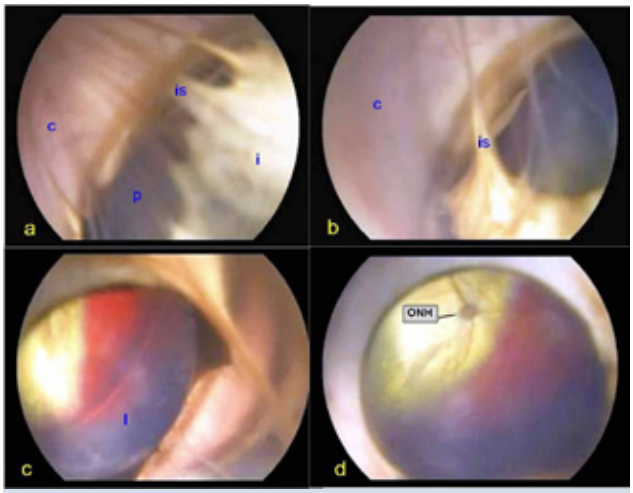

Figure 5. (a and b) Endoscopic images of cats with anterior segment abnormalities illustrating long iris strands *"is"* arising from the pupil *"p"* borders and adhering to the inner cornea *"c"*. (c) Endoscopic image of a cat with perforated cornea showing immature cataract *"l"* and anterior synechia. (d) Normal shaped pupil with normal optic nerve head (ONH) and clear retinal blood vessels

Laboratory findings

FHV-1 was isolated from 57 out of 80 (71.3%) cats, and *Mycoplasma felis* was isolated from 15 out of the 57 (26.3%) cats infected with FHV-1. Cats infected with FHV-1 included 18 (31.6%) with deep stromal ulcer; 12 (21.1%) with superficial ulcer; 9 (15.8%) with symblepharon; 7 (12.2%) with anterior synechiae with secondary glaucoma and corneal scarring; 5 (8.7%) with endophthalmitis with corneal melting; 4 (7%) with corneal perforation; one (1.8%) with corneal sequestration and one (1.8%) with corneal ulceration with granulation tissue. Among the 57 FHV-1 infected cats, there were 36 (63.1%) DSH cats, 19 (33.3%) Persian cats, one (1.8%) Himalayan and one (1.8%) Siamese cat. Among the 15 cats infected with *Mycoplasma felis,* 6 (40%) showed deep stromal ulcer, 4 (26.7%) showed superficial ulcer, 3 (20%) had symblepharon and 2 (13.3%) had endophthalmitis.

Treatment outcomes

Granulation tissue and corneal sequestration were removed successfully from the surface of the cornea (Figure 6 a,b and c). The conjunctival flap was stable and well-fixed throughout the treatment period (Figure 7 a and b).Successful healing of the corneal defect after removal of the corneal sequestrum or granulation tissue was noticed 3 weeks postoperatively (Figure 8a). All reported superficial corneal ulcers were healed successfully 3 weeks postoperatively

with relative elimination of the associated symptoms. Residues of granulation tissue and variable degrees of fibrosis that disappeared completely after 3 months postoperatively were reported in 6 (42.8 %) out of 14 cats with indolent ulcer (Figure 8b and c).

Three out of 4 (75%) cats with corneal sequestration showed successful healing of the corneal defect with variable degrees of corneal fibrosis with associated varying degrees of corneal opacity and vision impairment (Figure 8d). In one Persian cat, with long lasting sequestrum and diffuse corneal edema, the necrotic material was deep and involved all layers of the cornea. Removal of the necrotic material results in corneal perforation, subsequent ocular hypotony and the condition ended up with exenteration. In 22 cats with deep stromal ulcer, lysis of the corneal stroma was minimized, and the corneas were healed successfully 4 weeks post-surgery. However, variable degrees of granulation tissue and corneal fibrosis (Figure 8e) were developed in 16 out of 22 (72.7%) cats with deep stromal ulcer. The adhered conjunctival membrane was removed successfully in 6 out of 11 cats with symblepharon and the corneal lesion was healed with granulation tissue, fibrosis and / or melanosis (Figure 8f).

Cats with anterior synechiae and secondary glaucoma showed complete recovery (Figure 5d) and the intraocular pressure was reduced from 33 to 24 mmHg one week after surgery. Recurrence of anterior synechia was identified via endoscopic examination in one Himalayan cat, one month after surgery with associated corneal scarring.

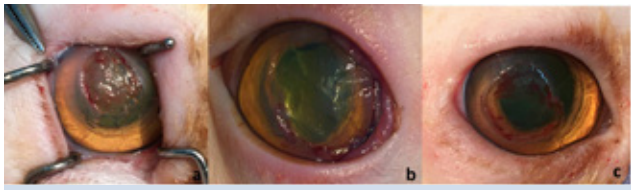

Figure 6. Intraoperative radiograph representing the successful removal of the over granulation tissue from the cat in figure 2a (a) and figure 2b (b) and the corneal sequestrum from the cat in figure 2c (c).

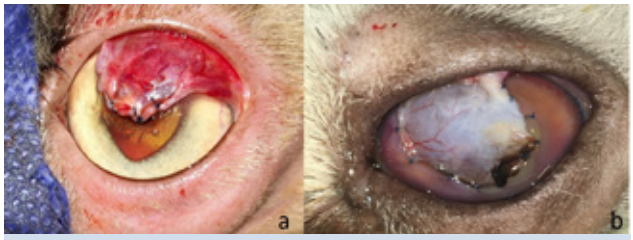

Figure 7 a and b. Photograph representing the conjunctival flaps used in this study

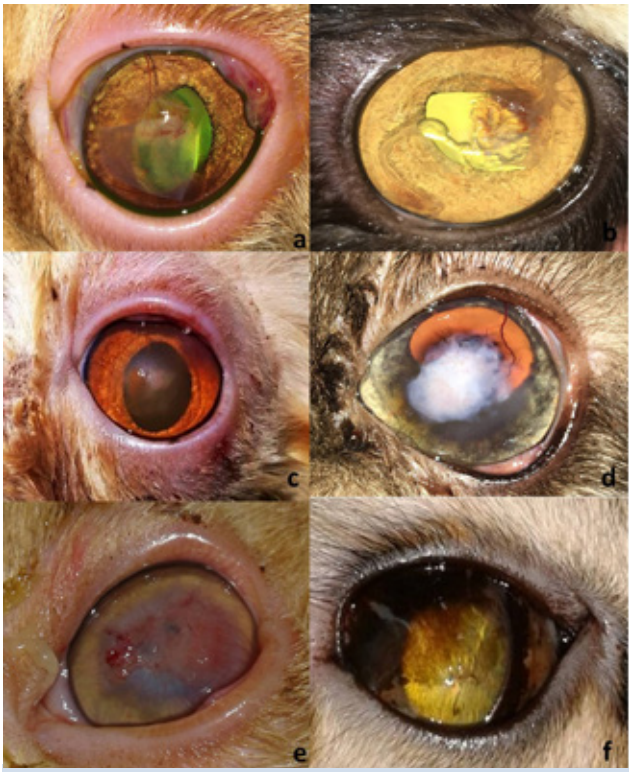

Figure 8. Post-operative radiograph showing the successful healing of the corneal defect after removal of the corneal sequestrum (a) and the healing of indolent ulcer and deep stromal ulcer with residues of granulation tissue (b) and fibrosis (c). (d) Representing severe degree of corneal fibrosis after removal of long lasting corneal sequestrum. (e) adhesion between the conjunctival tissue, granulation tissue and fibrosis was noticed in a cat with deep stromal ulcer after removal of the conjunctival flap. (f) Corneal pigmentation observed in a DSH cat one month after surgical treatment of the symblepharon

DISCUSSION

In the present study, corneal ulcer complications were evidenced in young and adult, male and female cats. The age range (3 months to 6 years) of the cats with complicated corneal ulcer differed from that reported for Boxers (2 to 10 years) (Gelatt and Samuelson, 1982). FHV-1 infection is expected to be the most common initial cause of ocular diseases in young growing cats. Infection with FHV-1 in the neonatal period, prior to eyelid opening, can lead to extensive corneal damage and globe rupture in severe cases (Nasisse et al., 1995). This is relatively in agreement with the study reported here, as there were 9 (15.8%) FHV-1 infected cats with corneal perforation and incurable endophthalmitis with extensive corneal damage. In our study, FHV-1 was isolated from approximately 71.3% of the presented cats with a higher incidence reported in Domestic shorthaired cats (63.1%) followed by Persian cats (33.3%). The high incidence documented in Domestic shorthaired

cats may be attributed to lack of vaccination in these cats. Bilateral corneal abnormalities were reported in approximately 46.3% cats. This relatively high percentage may be due to transmission of the disease to the contralateral eye (Gelatt and Samuelson, 1982), or co-infection with FHV-1.

In the study reported here, cats with deep stromal ulcer showed different degrees of visual impairment according to the size of corneal ulcer (Ali and Hassan, 2020). In previous studies, symblepharon was reported to be a serious complication associated with local herpetic infection (Martin and Stiles, 1998; Slatter, 2001). In these studies, complete blindness was evidenced in FHV-1 infected cats secondary to symblepharon, as 5 out of 11 cats with symblepharon were presented with phthisis bulbi with the eyes not potential for vision and were treated by exenteration (Slatter, 2001). Corneal sequestrum is a serious complication of long-lasting corneal ulcer or after long-term treatment with topical corticosteroids (Nasisse et al., 1995). In the present study, corneal sequestrum was superficial, affecting the anterior third of the corneal stroma, and was easily removed in all cats without complications, except in a Persian cat where the lesion extended to the Descemet's membrane and caused corneal perforation. This case ended up with hypotony and phthisis bulbi.

In the current study, superficial keratectomy and conjunctival flap were convenient treating options in cats with superficial corneal ulcer and deep stromal ulcer. This may be due to the fact that conjunctival flap provides corneal support and fibrovascular tissue to fill corneal defects (Tandon et al., 2010 and Ali and Hassan, 2020)). Additionally, it brings blood supply and blood-associated immune components along with natural anti-collagenase to the lesion (Tandon et al., 2010). In this study, the use of conjunctival flap also allowed proper visualization and monitoring of the rest of the cornea, as well as the surrounding structures of each treated eye. Persistent corneal scars developed in cats with deep stromal ulcer and anterior synechiae caused varying degrees of visual impairment. This may be attributed to the lack of collagen fibrils produced during repair of the stromal lesion to be laid down in a regular lattice pattern. The previously described ocular endoscopic technique (Abd-Elhamid et al., 2014), was used in the present study to determine anterior chamber abnormalities in cats with corneal edema and corneal neovascularization. Moreover, this technique was useful for monitoring the outcome after surgical removal of the incarcerated iris strands that were adherent to the inner surface of the cornea. A confirmatory diagnostic method for detecting the underlying cause (FHV-1) of complicated corneal ulcers in cats was the use of PCR in this study. Future investigation on larger populations of different animal species with complicated corneal ulceration is still warranted.

In conclusion, the most common complications of corneal ulceration in cats included deep stromal ulcer with descemetocele, endophthalmitis with corneal involvement, symblepharon, corneal sequestration and anterior synechiae with secondary glaucoma and corneal scarring. FHV-1 is thought to be the initial etiologic factor or incriminated in the pathogenesis of corneal ulceration in cats. Persistent corneal scars of varying shape and size can frequently develop in cats with deep stromal ulcer, anterior synechiae, and corneal sequestration. Domestic shorthaired and Persian cats were the most predisposed breeds to FHV-1 infection and subsequent corneal ulceration. Immediate management of patients with corneal ulcer would prevent serious complications associated with this disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest and no competing financial interest related to this report.

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Research article
Ερευνητικό άρθρο

A New Outbreak in Sea Bass Farming in Turkey: *Aeromonas veronii*

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ABSTRACT: Turkey produces most of the European sea bassin Europe and bacterial diseases are the main cause of economic loss during the production cycle. This research presents the first extended study of the *Aeromonas veronii* infection in sea bass on the Turkish coast of the Aegean Sea. An outbreak was observed in three different sea bass farms and diseased fish with clinical symptoms were sampled. Abdominal distention, hemorrhages on the body and anus, enlarged spleen and liver, and hemorrhages in the internal organs were detected from clinical and pathological examinations. Biochemical and molecular identification confirmed the pathogen to be *A.veronii*.The histopathological observations demonstrated that the pathogen caused bacterial colonies in the fibrous connective tissue, granuloma, and vacuolar degeneration.The primary causes of the disease were proved through an infection experiment. 80% and 90% mortality were calculated in 0.85 x 10^8 CFU ml^{-1} and 1.28 x 10^8 CFU ml^{-1} experimentally infected groups with clinical and pathological signs of the disease respectively. Recently, the pathological symptoms of the disease had been confused with pasteurellosis infection in cage farms but the presence of *A.veronii* has been confirmed in the current study. A detailed study is needed to investigate the overall status of the disease in the Aegean Sea in order to design an appropriate preventive strategy.

Keywords: *Dicentrarchus labrax, Aeromonas veronii,* Aegean Sea, infectious fish disease

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INTRODUCTION

Turkey is a significant producer of farmed fish in Europe, and sea bass (*Dicentrarchus labrax*) is one of the main species produced. The increase in national aquaculture output in the last 30 years has led to various problems; among these is the prevalence of several bacterial fish pathogens in the Mediterranean area. The number of new pathogens identified has increased over time, and one of the most common causes of the emergence of infectious diseases in marine fish is the *Aeromonas* species.

Aeromonas species are Gram-negative, facultative anaerobic bacteria that have a ubiquitous presence, including in psychrophilic and mesophilic organisms and humans (Nerland, 1996; Austin and Austin, 2007; Janda and Abbott, 2010). These bacteria were previously reported as the causative agent of bacterial hemorrhagic septicemia (BHS), motile aeromonad septicemia (MAS), and epizootic ulcerative syndrome (EUS) in many marine and freshwater fish species (Austin and Austin, 2007; Martinez-Murcia et al., 2008; Liu et al., 2016). In addition, motile aeromonads have been reported with several clinical signs,such as ulceration, fin and tail rot, abdominal distention, and exophthalmia (Sreedharan et al., 2011). Because *Aeromonas* species affect many fish species, interest in understanding the role of the pathogen has been rising in fish farms (Guzman-Murillo et al., 2000). *Aeromonas veronii* is the most virulent of the *Aeromonas* species,which include *Aeromonas caviae*, *Aeromonas hydrophila* and *Aeromonas sobria* (Janda and Kokka, 1991; Sreedharan et al., 2011). It causes pneumonia, soft tissue and wound infection and gastroenteritis in humans (Janda and Abbott, 2010), and hemorrhagic septicemia and epizootic ulcerative syndrome in fish (Cai et al., 2012).

The diseases caused by *A.veronii* have been reported from several locations around the world and from several fish species: cultured channel catfish (*Ictalurus punctatus*) (Liu et al., 2016), cultured snakehead fish (*Ophiocephalus argus*) (Zheng et al., 2012), Chinese longsnout catfish (*Leiocassislongirostris*) (Cai et al., 2012), Nile tilapia (*Oreochromis niloticus*) (Hassan et al., 2017), carp (*Cyprinus carpio*) (Gong et al., 2010; Yu et al., 2010), Siberian sturgeon (*Acipenser baerii*) (Ma et al., 2009) and European sea bass (*D. labrax*) (Smyrli et al., 2017).

To our knowledge, this is the first extended study of infection by *A. veronii*in cultured sea bass in the Aegean coast of Turkey. Sea bass is one of the most produced commercial fish species in the Mediterranean region, and Turkey is the leading producer of sea bass in the Aegean Sea. This infection is thus a significant concern with high mortality and economic loss. The pathological symptoms were initially confused with another major bacterial disease, pasteurellosis; however,it was not previously possible to isolate the agent from chronic cases in fish farms. The study presented here is the first detailed investigation from the Aegean coast of Turkey of the *A. veronii* infection, which is a serious and contagious disease agent in sea bass cage culture, with pure isolation, the biochemical and molecular identification of the agent, and the determination of its histopathological effects and pathogenicity obtained via an experimental infection.

MATERIALS AND METHODS

The outbreak was observed in three different sea bass farms between 2017 and 2019 in the South Aegean region, Turkey. Diseased fish samples that approached commercial size (250-350 g) were sampledover a period of three years (a total of approximately 270 fish) from acute and chronic cases and transferred to the Fish Disease and Biotechnology Laboratory, Faculty of Fisheries, Izmir Katip Celebi University. Clinical, microbiological and pathological examinations were performed, and bacterial isolates from the anterior of the kidneys, spleen and liver were streaked on tryptic soy agar (TSA, Merck) supplemented with 2% NaCl and tryptic soy agar supplemented with sheep blood in order to isolate the causative pathogen. The TSA plates were incubated at 25°C for 24-48 h and colony morphology was observed. The pure colonies were streaked again on TSA for biochemical analyses. Motility, Gram staining was implemented, and catalase and oxidase activity were detected according to standard procedures (Austin and Austin, 2007). The isolates were identified using API 20E tests (BioMerieux S.A., France) by determining the biochemical characteristics of the bacteria.

Molecular identification of the bacteria was accomplished with 16SrRNA gene amplification. A EurXGeneMATRIX Tissue Bacteria DNA Isolation Kit (Poland) was used for DNA isolation, and the density and quality of the DNA were determined with a Thermo Scientific Nanodrop 2000 (USA). The PCR amplification reactions were performed by employing universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3').The amplification was carried out after initial denaturation at 95 °C for 5 minutes, fol-

lowed by 35 cycles at 95 °C for 45 seconds, 57 °C for 45 seconds and 72 °C for 1 minute, then 72 °C for 5 minutes as the final extension. Band screening of the PCR products was observed in the gel electrophoresis. Amplified products of template DNA were sent to the Macrogen direct sequencing service (Macrogen, Holland) for sequence determination. The sequenced DNA data were matched with the GenBank database using the BLASTN 2.6.1. algorithm and a phylogenetic tree was formed by the neighbor-joining method with MEGA7 software.

For histological examination, the liver, spleen, gill, and heart were sampled from diseased fish with clinical signs and preserved in 10% buffered formalin after necropsy. The tissues were then processed routinely and prepared into paraffin blocks. The blocks of tissues were cut to 5 µm thickness and stained with Hematoxylin and Eosin (H-E) and examined under a light microscope (Culling et al. 1985).

The pathogenicity of *A.veronii* was tested on European sea bass which had no infection history. Three duplicated experimental groups were constituted and 10 healthy fish (weighing about 350 g each) were placed in each of 300 L tanks in the recirculated aquaculture system in Faculty of Fisheries Research Center, Izmir Katip Celebi University.

The isolated and identified *A.veronii* strain was grown in Tryptic Soy Broth (TSB, Merck) supplemented with 0.5% NaCl for 20 h at 25°C. The number of colony-forming units (CFU) per ml was determined by plating seven-fold serial dilutions on TSA plates. 0.1 ml bacterial suspension of 0.85 x 10^8 CFU ml^{-1} and 1.28 x 10^8 CFU ml^{-1} *A.veronii* were injected by intraperitoneal injection (IP) into two of the groups, while 0.1 ml PBS were injected IP into the control groups under the same environmental conditions. Moribund and dead fish were observed and clinical and pathological examinations were conducted over a period of 10 days.

RESULTS

The disease caused massive mortality in sea bass farms between May (19 °C) and November (20 °C) in the same region, and cumulative losses were observed over three years from chronic cases. All the sea bass farms are located in the same area and the symptoms of the disease were observed in all of them. Infected fish were observed near the surface of the water and exhibited a loss of appetite. The clinical findings indicated abdominal distention, hemorrhages on the head and around the mouth, especially on the upper and lower jaw,operculum, and ulcerative lesions on the ventral side of the body and around the anus (Figure 1). Necropsy showed enlarged liver, spleen, heart, and kidney with whitish nodules, multiple granulomas, and focal necrosis (Figure 2).

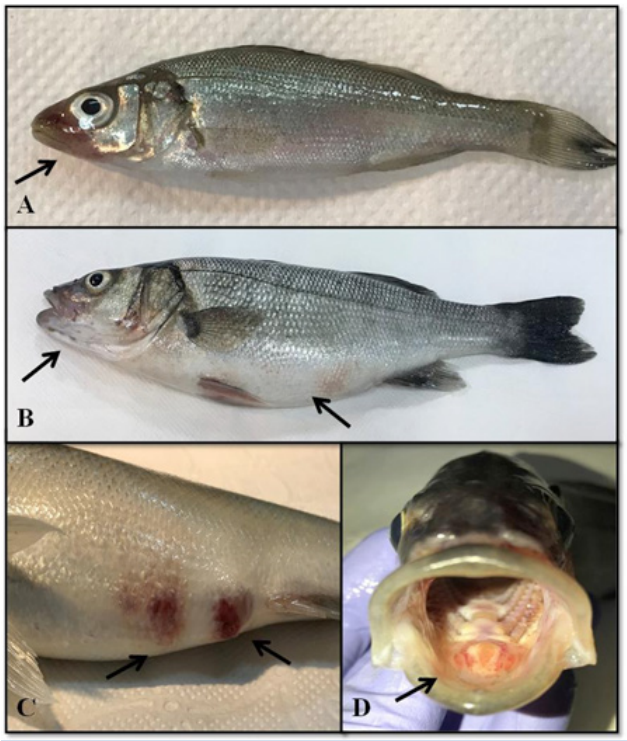

Figure 1. Clinical signs of the disease: A: hemorrhages on head and around mouth; B: hemorrhages around mouth and abdominal distention; C: ulcerative lesions on the ventral side of the body and around anus; D: hemorrhages on the upper and lower jaw

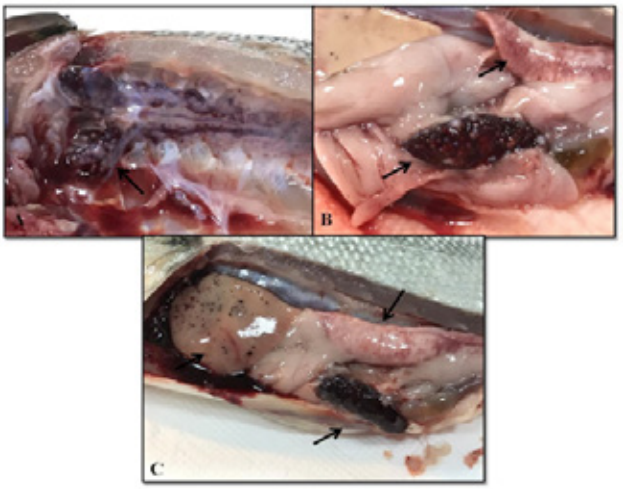

Figure 2. Pathological findings of the disease: A: multiple granulomas on kidney; B: hemorrhages on gonads and internal fat, whitish nodules on spleen; C: petechiae on liver with anemia, hemorrhages on gonads and internal fat, whitish nodules on spleen

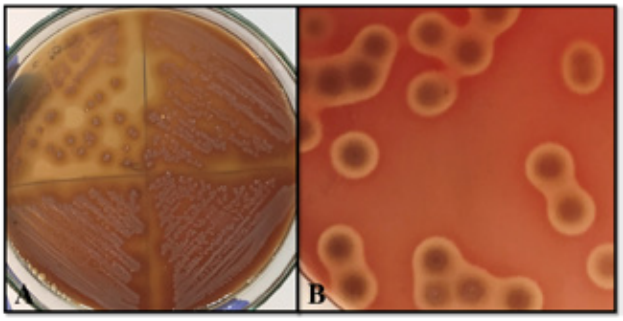

Figure 3. A: Pure *A. veroniic* olonies on TSA; B: Hemolysis of *A. veronii* on BTSA

Smooth and round colonies were detected on the TSA with brown pigmentation on the medium and these caused hemolysis on the BTSA after 48 h incubation (Figure 3). The isolates were found to be motile, Gram-negative, oxidase, and catalase positive rod-shaped bacteria. The morphologic and API 20E biochemical test results are presented in Table 1. ONPG (ortho-nitro-phenyl-galactoside), LDC (lysine decarboxylase), ODC (ornithine decarboxylase), citrate utilization, TDA (tryptophan deaminase), indole, VP (Voges Proskauer test), gelatin, glucose, mannitol and sucrose were found to be positive, while ADH (arginine dihydrolase), H_2S, urease, inositol, sorbitol, rhamnose, melibiose, amygdalin and arabinose were detected to be negative in all isolates (Table 1).

Table 1. Morphologic and biochemical test results of the isolated *Aeromonas veronii* strain

Aeromonas veronii			
Gram stain	-	TDA	+
Motility	+	Indole	+
Oxidase	+	VP	+
Catalase	+	Gelatin	+
O/129	-	Glucose	+
OF	+	Mannitol	+
ONPG	+	Inositol	-
ADH	-	Sorbitol	-
LDC	+	Rhamnose	-
ODC	+	Sucrose	+
Citrate utilization	+	Melibiose	-
H_2S	-	Amygdalin	-
Urease	-	Arabinose	-

The 16S rRNA sequence results of the isolated strains showed 99% sequence similarity with *A.veronii* in the BLASTN 2.6.1 database and were registered in NCBI GenBank with accession number MT126417. The phylogenetic tree of the isolated strain and other homologous sequences which were isolated from different fish species is presented in Figure 4.

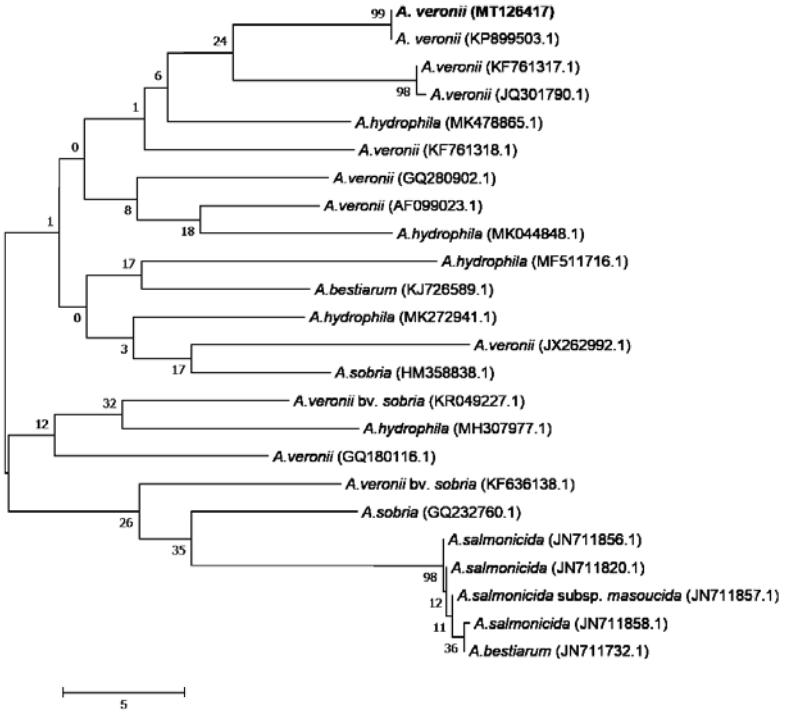

Figure 4. Phylogenetic tree of *Aeromonas veronii* MT126417 (in bold) and related matches isolated from different fish species in GenBank (NCBI) based on 16S rRNA sequences using MEGA7 with Maximum Composite Likelihood Method, 1000 bootstrap replicates. The bootstrap values are shown next to the branches and the scale bars present the distance values.

The histopathological results for the spleen, liver, gill and heart tissues are presented in Figure 5. Degeneration in cells such as lymphocytic cell infiltration was noticed in the spleen tissue. In the liver,granuloma, intravenous hyperemia, hemorrhages between cells, vacuolar degeneration, necrotic tissue were observed, as well asbacterial colonies in the fibrous connective tissue. Lamellar epithelial hypertrophy and hyperplasia with degenerative changes were found in the gill epithelium. In addition, degeneration and lymphocyte cell infiltration were determined, especially in subendocardial cells (Figure 5).

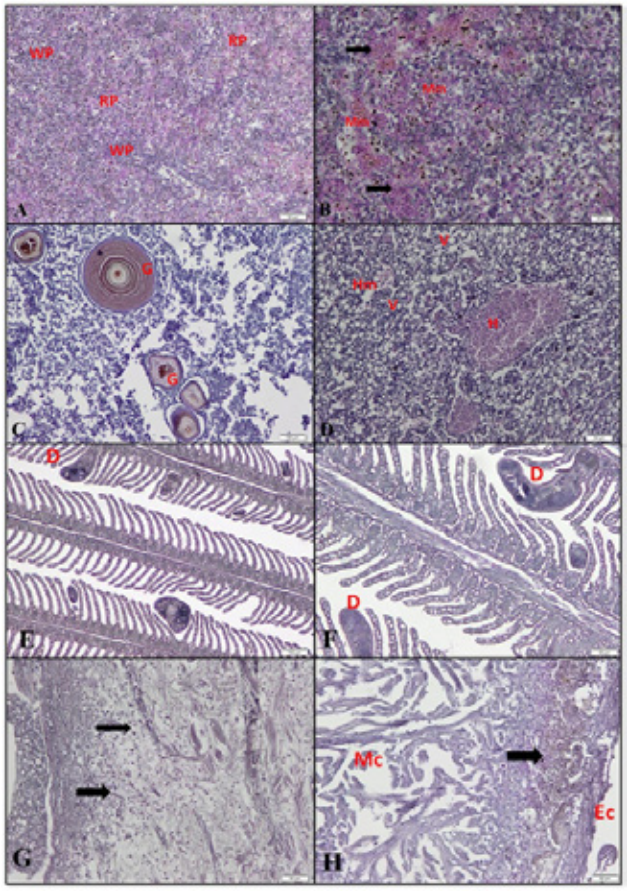

Figure 5. Hematoxylin and Eosin (H-E) stain of *Aeromonas veronii* infected tissues; A (X 200) WP: white pulp; RP: red pulp -B (X 400): spleen tissue with degeneration in cells;Mm: melanomacrophage and lymphocytic cell infiltration; C (X 100) G: granuloma - D (X 200): V: vacuolar degeneration;H: hyperemia;Hm: hemorrhages between cells and bacterial colonies in liver tissue, E (X 100) - F (X 200) D: degeneration - G (X 200): lymphocytic cell infiltration and lamellar epithelial hypertrophy, hyperplasia with degenerative changes in the gill epithelium; H (x 200): Mc: myocardium; Ec: endocardium, degeneration and lymphocyte cell infiltration in subendocardial cells.

In the infection experiment, the mortality of the groups after 36 hours of injection was determined and found to be 80 % and 90 % in the 0.85 x 10^8 CFU ml^{-1} and 1.28 x 10^8 CFU ml^{-1}groups respectively. The symptoms of the disease were observed to be ascites, and hemorrhages on the body surface and internal organs,and the causative pathogen was reisolated from the kidneys, spleens and livers of the IP-injected fish. Death stopped in the sixth day of the experiment, and no clinical or pathological signs of the disease were detected in the control groups.

DISCUSSION

This study investigated the disease caused by *A.veronii* in farmed sea bass in the Aegean Region of Turkey and examined it in detail through biochemical and molecular identification, histopathological study, and the determination of pathogenicity. European sea bass production reached 116,915 tonnes in 2018 in Turkey (TUIK, 2019), and it thus became the primary species in the marine aquaculture industry. This paper highlights the significance of the presence of a new bacterial disease in this area. *A.veronii* was isolated from the commercial-sized diseased fish showing clinical signs and mortality. Determining the impacts of *A.veronii* infection on seabass is important for the future of aquaculture production in the Mediterranean region in order to prevent further outbreaks that could cause several pathological disorders and mass mortality.

Uzun and Ogut (2015) reported *A.veronii* biovar-*sobria* from European sea bass in their study of the occurrenceand frequency of bacterial pathogens in sea bass in the Black Sea Region of Turkey, which sampled two different fish farms monthly between 2009 and 2011. Overall, *A. veronii* bv *sobria* was identified as the most common pathogen (65.2%), although it was isolated from only 13 fish with signs of disease. The study was the only statement of the presence *A.veronii* in Turkey and there was no significant evidence that *A.veronii* was a pathogenic disease agent in that area that could cause an epidemic causing mass mortalities and pathologic disorders in cages. Previous reports have shown the existence of disease caused by *A.veronii* on the Greek coast of the Aegean Sea (Smyrli et al., 2017). The present study verifies the presence and the effects of the pathogen on the Turkish side of the Aegean Sea as a primary causative disease agent.

Abdominal distention, hemorrhages on different parts of the head, and ulcerative lesions were all observed in the diseased fish, while multiple granulomas in the major organs were noted as the main pathologic

findings of the disease. Smyrli et al. (2017) observed epidermal lesions that became ulcerated and reddened fins in European sea bass as the clinical signs of the disease. Furthermore, hemorrhages, lesions, necrotic foci, and granulomas were detected on spleen, kidney, and liver tissue in infected fish. Hassan et al. (2017) reported hemorrhages on the surface of the body, detached scales, ulcerations, and abdominal dropsy in Nile tilapia (*O.niloticus*). Moreover, hemorrhagic spots in the liver, bloody exudates in the abdominal cavity, hepatosplenomegaly, and the gall bladder filled with bile were documented as the post-mortem examination results of *A.veronii* infection. The most obvious clinical and pathological symptoms were stated to be hemorrhages in the mouth and fins, enlarged spleen, hemorrhages, and necrosis in the spleen, kidney, and liver in cultured channel catfish (*I. punctatus*) (Liu et al., 2016). In addition, Yu et al. (2010) found ulcerative lesions on the skin, abdominal dropsy, and enlarged spleen and kidney from *A.veronii* infection in farmed carp (*C. carpio*). These research findings are indicated that the pathogen causes similar clinical and pathological symptoms in several fish species.

Isolated *A.veronii* strains were observed with brown pigment-producing colonies on TSA. Smyrli et al.(2017) isolated two different *A.veronii* strains; pigment-producing isolates and non-pigment producing isolates. However, Uzun and Ogut (2015), Yu et al. (2010) and Cai et al. (2012) did not provide any information about the pigment-production of strains. The biochemical test results of the isolated *A.veronii* strains are compared with related research in Table 2. Some indicators, such as arginine dihydrolase (ADH), ornithine decarboxylase (ODC) and tryptophan deaminase (TDA) vary among different isolates, but the parameters are not essential criteria for biochemical identification of *A.veronii*(Abbott et al., 2003).

Table 2. Morphologic and API 20E biochemical test results

	Present isolate	Yu et al. 2010[a]	Zheng et al. 2012[b]	Liu et al. 2016[c]	Smyrli et al. 2017[d]
Gram stain	-	-	.	-	-
Motility	+	+	.	+	+
Oxidase	+	+	.	+	+
Catalase	+	+	.	.	+
O/129	-	-	.	.	.
OF	+	+	.	+	.
ONPG	+	+	.	+	+
ADH	-	+	+	-	-
LDC	+	+	+	+	+
ODC	+	-	.	+	+
Citrate utilization	+	+	.	.	+
H_2S	-	-	.	-	-
Urease	-	-	-	-	-
TDA	+	-	-	.	+
Indole	+	+	.	+	+
VP	+	+	.	+	+
Gelatin	+	+	.	+	+
Glucose	+	+	.	.	+
Mannitol	+	+	+	+	+
Inositol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Rhamnose	-	-	.	-	-
Sucrose	+	+	+	+	+
Melibiose	-	-	.	-	-
Amygdalin	-	-	.	.	-
Arabinose	-	-	-	-	-

a: Isolated from Israeli carp (*Cyprinus carpio*); b: Isolated from snakehead fish (*Ophiocephalus argus*); c: Isolated from channel catfish (*Ictalurus punctatus)*; d: Isolated from European sea bass (*Dicentrarchus labrax*)

Smyrli et al. (2017) reported a systemic infection from European sea bass characterized by chronic granulomatous inflammation; necrotic lesions with granulomas contained the foci of rod-shaped bacteria. Yu et al. (2010) observed hepatocellular vacuolar degeneration and congestion in sinusoids, and pulps in the spleen, which had numerous bacterial invasions, in *A. veronii*-infected *Cyprinus carpio*. In naturally-infected Nile tilapia (*O.niloticus*) vacuolar degeneration in the liver, depletion of hemopoietic tissue in the spleen, and degenerative changes in the parenchymatous organs including the liver and spleen were noted by Hassan et al. (2017). Similarly, in this study,degenerations in the spleen tissue, vacuolar degeneration, hemorrhages between cells, and bacterial colonies in the liver tissue were found among the histopathological results for the European sea bass.

Previous studies on the mortality rates of *A.veronii* infection experiments have demonstrated acute mortalities at different concentrations. Yu et al, (2010) tested the effects of *A.veronii* on carp (*Cyprinus carpio*) and reported that a 10^5 CFU fish^{-1} dose caused 16.7% mortality within 10 day, while a 10^6 CFU fish^{-1} dose raised this percentage to 65% within nine days, and 10^7 CFU fish^{-1} injected fish all died within seven days. All the dead fish indicated typical external and internal signs of the disease. Smyrli et al. (2017) tested *A.veronii* infection in sea bass with two different challenge tests: 10^4 CFU fish^{-1} by injection intraperitoneally and 10^5 CFU fish^{-1} bacterial suspension by immersion. All the fish in the bath-challenge group were reported dead within 10 days, and displayed lethargic swimming, reddening on the skin and fins, diffused hemorrhages in the peritoneum cavity and on the surfaces of internal organs, as well as lesions on the spleen and liver. The IP-injected fish group died within four days with diffused hemorrhages in the peritoneum cavity and internal organs. Sreedharan et al. (2011) tested the pathogenicity of the *A.veronii* strain on goldfish (*Carassius carassius*). They calculated the LD_{50} values to be $10^{5.071}$ CFU/ml and observed loss of scales with hemorrhagic scale pockets in the fish tested. Hassan et al. (2017) established the LD_{50} dose of *A.veronii* strain for the Nile tilapia (*O. niloticus*) 5.2 x 10^6 CFU/ml and determined 78% mortality in the challeng egroups,observing hemorrhage and redness at scale pockets, enteritis and the filling of the intestine with transparent content. Many recent studies have shown that *A.veronii* causes similar clinical and pathological symptoms in different fish species and the present study provides similar results in sea bass.

Photobacterium damselae subsp. *piscicida* infection was described as "pseudotuberculosis" by Egusa (1993), due to its having similar symptoms and histopathological characteristics as tuberculosis, including numerous tubercles in the internal organs, especially in the kidney and spleen. The clinical and pathological symptoms of the disease caused by *A.veronii* are very similar to those of pasteurellosis, including the appearance of severe granulomas in the internal organs, and the disease in this current case could be considered as "pseudopasteurellosis".The pathological symptoms were generally confused with pasteurellosis infection in cage farms, especially in Turkey but this study verifies the presence and pathological effects of *A.veronii* in sea bass.

In conclusion, this is the first study to our knowledge to report *A.veronii* infection in European sea bass from the Turkish coast of the Aegean Sea. The study has determined the presence of the pathogen as a disease agent in seabass in this area through clinical, pathological, biochemical, molecular, and histopathological findings, and has also confirmed the results with an infection experiment. Overall, these findings highlight the potential for anout break in a region that is one of the main seabass producers in the world, which could lead to massive economic loss. More research is required to fully understand the prevalence of *A.veronii* in the Aegean Sea and to explore its effects on other fish species and the longer-term economic implications.

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CONFLICT OF INTEREST STATEMENT

None declared by the authors.

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Research article
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Seroprevalence and risk factors associated with *Salmonella* Dublin presence in Algerian dairy farms

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ABSTRACT: *Salmonella* Dublin is a causative agent of a gastrointestinal bacterial infection prevalent in many cattle herds worldwide. Hence, the goal of this research was to evaluate the prevalence of *Salmonella* Dublin carriage in fecal and milk samples from dairy cattle from Algeria, and to investigate potential risk factors associated with the presence of *S*. Dublin antibodies. A total of 307 cows from 39 farms were analyzed in this study. Bacteriological and immunological methods were used to isolate and detect *S.* Dublin antibodies in feces and cow's milk. Antimicrobial susceptibility testing was performed using the disc diffusion method. Logistic regression was used to study risk factors associated with *S*. Dublin antibodies. The bacteriological results showed the absence of*S*. Dublin and a prevalence of 0.97 %(3/307) (IC 95% 0 - 2.08)for *S*. Mbandaka. The immunological analysis of milk by the ELISA technique showed a prevalence of 36.33% (95% CI 30.44 - 42.22) for *S*. Dublin. Final multivariate regression models showed that the breed, the region and introduction of purchased cattle were associated with the presence of *S.* Dublin antibodies. This study is the first that reports the seroprevalence and risk factors associated with *S*. Dublin infection in Algeria and could be considered as a comparison point for further studies in Algeria.

***Keywords*:** Cattle, Milk, Risk factors, *Salmonella* Dublin, Seroprevalence.

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INTRODUCTION

Salmonella infections are a major concern for the various animal productions and for public health(Agren et al., 2016). Salmonellosis is one of the most common diseases in cattle but also poses a significant zoonotic risk (Camart-Périéet al., 2007). Cattle is the main reservoir of *Salmonella enterica* subsp. *Enterica* serovar Dublin (*Salmonella* Dublin) which is considered to be the most frequent cause of *Salmonella* infection in cattle (Henderson and Mason, 2017).

These carrier animals are responsible for propagating infection in dairy herds via *S.* Dublin shedding in feces and milk (Holschbach and Peek, 2018),and are transmitted to humans, usually through the consumption of beef meat and cow's milk (Molla et al., 2003; Rodrigez-Rivera et al., 2014).Veterinarians have also been infected from skin contact with the bacteria, especially following obstetric maneuvers and inseminators (Visser, 1998). In addition, *S.* Dublin is the serovar of most economic concern, because of its particularly invasive nature, causing acute diarrhea and mortality, mainly observed in calves between 2 weeks to 3 months of age, septicemia and reproductive disorders, including abortions. Moreover, with this serovar, some animals remain infected for life without manifesting clinical signs (asymptomatic carriers) (Radostits et al., 2007).Therefore, the presence of these asymptomatic carriers of *S.* Dublin in cattle herds is a major concern because they shed the bacteria continuously or intermittently for years in milk and/or faeces, resulting in environmental contamination and infections in other animals (Holschbach and Peek, 2018). However, the use of bacteriological examination for the detection of the Dublin serovar has lower sensitivity rate compared with serological methods (Nielsen, 2013; Nyman et al., 2013). Therefore, the most used tests for *S.* Dublin detection include enzyme-linked immunosorbent assays (ELISAs) used for the detection of immunoglobulins against *S.* Dublin in serum and in milk samples, and bacteriological culture of fecal samples (Veling et al., 2002; Nielsen and Ersbøll, 2004). In Algeria, the prevalence of *S.* Dublin has not yet been studied.To date,only two studies were published about *S.* Dublin in Algeria (Ayachi et al., 2012; Derdour et al., 2017),but no study was done on risk factors associated with the presence of *S.* Dublin antibodies. Therefore, the aims of our work were (i) to investigate the prevalence of *S.* Dublin carriage in dairy cattle, (ii) to identify potential risk factors that could be associated with the presence of *Salmonella* Dublin antibodies, and (iii) to compare the ELISA test with bacteriological methods in detection of serovar Dublin from the dairy herd.

MATERIALS AND METHODS

Study area

This study was carried out in Khenchela region. This region is located in the east of Algeria, and it is characterized by a large number of cattle (4478 cows in 2018), and a promising milk sector (27 million liters of milk per year). The altitude range is from 1050 to 1710 meters and the daily average temperature ranges from -2°C to 42°C.

Sampling

We calculated the sample size using the formula for simple random samples recommended by Thrusfield (2007):

$$n = (1.96)^2 \frac{Pexp(1-Pexp)}{d^2}$$

where n = required sample size; Pexp = expected prevalence; d = desired absolute precision; 1.96 was the Z value for the selected confidence level (95%). According to this formula, the minimum sample size for an infinite population was 139 cows using an expected individual prevalence of 10% (according previous studies in this region), a desired absolute precision of 5% and a confidence level of 95%. The sample size was increased to 307 in order to increase the absolute precision and compensate for 5% attrition. A total of 39 farms were randomly selected, from which, 307 fecal samples were taken and analyzed. About 25g of individual fecal samples of cows were collected directly from the rectum using disposable gloves, and then stored in sterile pots. Samples were then sent for analysis on the same day. On the other hand, milk from 256 cows (10 mL) among the 307 cows selected for bacteriological analysis, was collected in vacutainer tubes and stored at -80°C until serological analysis (Fifty-one cows were in the dry period, and they were not included in milk sampling).

The minimum number of cattle to be tested on each farm was established as 10 (Cannon and Roe, 1982), the number of cattle to be sampled on each farm was defined on the basis of the total number of cattle in the farm: the farm consisted of less than 10 cattle, in which case all cattle were harvested or the farm contained more than 10 cattle and, in this case, at least 10 individuals were taken.

Questionnaire survey

During this study, a questionnaire was established-to determine potential risk factors. The variables included as potential risk factors at the farm level were as follows: Farm location (El Hamma, Baghai, El Mahmal, Kais, Remila), age (between 2 to 10 year), breed (Montbéliarde, Holstein, crossed breed, Brown Swiss, Fleckvieh, Normande, Limousin), general hygiene (good, average, bad), introduction of new purchased animals (yes/no), water supply (networks,-drilling), water quality (bad/clean), gestation (yes/no), gestation stage (between 1 to 9 month), parity(uniparous, multiparous), clinical signs at the time of collection(diarrhea, mastitis, respiratory problem, arthritis, eye infection, no sign, abortion (yes/no),stage of abortion (between1-9 month)).

BACTERIOLOGICAL CULTURE

Isolation of *Salmonella* spp.

The isolation was performed according to the AFNOR standard (NF U: 47-100) (2007). 25g of individual fecal samples were mixed with 225 mLof buffered peptone water (Condalab, Spain)and incubated for 24h at 37°C. Then, 1 mL of the pre-enriched culture was transferred to Müller Kauffmann Tetrathionate-novobiocin broth (Bio-Rad, France) and 0.1mLof the same pre-enriched culture was transferred to Modified Semisolid Rappaport Vassiliadis Medium (MSRV;Condalab, Madrid, Spain)and incubated at 37°C and 42°C for 24h respectively. A loopful from each culture was streaked into selective xylose-lysine-deoxycholate agar (Condalab,Spain) and Hektoenagar plates (HK; InstitutPasteur Algeria (IPA)),and incubated at 37°C for 24h. The initial biochemical tests were performed on a 24h pure culture using Triple Sugar Iron (TSI; IPA) agar slant, indole urea reagent (IPA), Lysine Decarboxylase (LDC; IPA) reagent and ortho-NitroPhenyl-β-galactoside (ONPG; IPA).Then, the API 20E system (BioMérieux, France)**.**

Serotyping of *Salmonella*

Salmonella serovars were identified serologically by slide agglutination test using diagnostic polyvalent and monovalent O and H *Salmonella* antisera (Bio-Rad, France),according to Kauffman-White scheme (Grimont and weill, 2007).

Antimicrobial susceptibility testing

The agar disk diffusion method was used to determine the antimicrobial susceptibility patterns of *Salmonella* isolates according to the Clinical and Laboratory Standards Institute guidelines, (CLSI)(2018) Using Mueller- Hinton agar (IPA, Algiers, Algeria). The isolates were tested for the following antibiotics (disk content): ampicillin (10 µg), piperacillin (100 µg), ticarcillin (75 µg), amoxicillin/clavulanate (20 µg/10 µg), ceftazidime (30 µg), cefotaxime (30 µg), aztreonam (30 µg), imipenem (10 µg), sulfonamides(300 µg), trimethoprim (5 µg), cortrimoxazol (25µg), nalidixic acid (30 µg), norfloxacin(10 µg), ciprofloxacin (5 µg),colistin (10 µg), furans (300 µg), chloramphenicol (30 µg) and tetracycline (30 µg), the results were evaluated after 24h of incubation at 35°C.

ELISA TEST

The ELISA test is based on the detection of antibodies against *Salmonella* lipopolysaccharide (LPS) antigens, and it was performed according to the manufacturer's instructions (PrioCHECK *Salmonella* Antibody ELISA Dublin; Thermo Fisher Scientific, Waltham, MA). Milk samples were heated for one hour at 37°C. Briefly, the upper layer of fat was pulled out, and the undiluted skim milk samples were inoculated in 96 microtiter plate and the optical density (OD) was measured at 450nm using ELISA reader (Bio-Rad, USA).

STATISTICAL ANALYSIS

Statistical differences in proportions were compared using the Chi-square test. The association between the presence of serovar Dublin in milk and possible risk factors was tested using logistic regression (SPSS software version 20). The farm was included as random effect due to repeated measurements, P value equal to or less than 0.25 during simple regression were forwarded to multiple regression analysis, and only variables with P value ≤ 0.05 were included in the final model of risk factors. Specificity, sensitivity, Kappa, McNemar test and confidence intervals were calculated with the use of Winepiscope 2.0. Values of$P< 0.001$ and $P<0.05$ were considered as statistically significant.

RESULTS

Bacterial isolation and serotyping of *Salmonella* isolates

Three out of 307 (0.97%) collected fecal samples were positive for *Salmonella*, all the three serotyped *Salmonella* were *Salmonella* Mbandaka. However, serovar Dublin was not found in any fecal cultures.

Antibiotic susceptibility testing

The antimicrobial susceptibility pattern of the three isolates indicated that all isolates were susceptible to the all antibiotics used.

Serology of milk samples

The ELISA results showed, that out of the 256 milk sample examined, 93 (36.33%) were positive at 95% with a confidence interval between30.44 to 42.22for *S.* Dublin antibodies in milk samples, while 163 (63.67%) were found negative. The difference of *S.* Dublin individual seroprevalence between regions (municipalities) was statistically significant (P<0.05) (Table1).The comparison of the prevalence of *S.* Dublin using bacteriological methods (0%) and ELISA (36.33%) indicated clearly that those methods were significantly different (P<0.01). The capability to detect a positive animal is significantly higher for ELISA.

Risk-factors analysis

Risk factors (Table 2) with P≤ 0.25 in the univariable analysis (Univariable regression results table is included as supplementary material S1) were included in the final model of regression: Age, hygiene, gestation, stage of gestation, parity, and clinical signs at the time of collection. Cows from the Remila region were less susceptible of having *Salmonella* antibodies in milk, than cows in El Hamma region (OR=0.027, IC: 0.003-0.256), and the introduction of new purchased animals reduced the risk of having *Salmonella* antibodies in milk (OR=0.06, IC: 0.008-0.510). However, Brown Swiss cows were 15 times more susceptible of having *Salmonella* Dublin antibodies in milk than the Montbeliarde (OR= 15.66, IC: 1.679-146.15).

DISCUSSION

Diseases caused by *Salmonella* spp. constitute a real problem of public health and animal production in the world (Smith et al., 2004). *S.* Dublin is a serotype adapted and concern to cattle in several countries due to its ability to induce abortions, reduced milk production and its significant economic losses (Visser et al., 1997).

Table 1. Individual serological prevalence of *Salmonella* Dublin in milk by region

Region	Farm	Samples (%)	Seropositive	Prevalence % 95% CI[a]	P value
El hamma	14	105 (41.01)	42	40 (30.63- 49.37)	
Baghai	2	12 (4.68)	7	58.33 (30.44-86.23)	
El mahmal	6	24 (9.37)	19	79.77 (62.92-95.41)	
Kais	11	69 (26.95)	17	24.64 (14.47-34.81)	< 0.0001[b]
Remila	6	46 (17.96)	8	17.39 (6.44-28.34)	
Total	39	256	93	36.33 (30.44-42.22)	

[a]Confidence interval (95%CI),[b]< 0.0001: The results are very significant in every single region.

Table 2. Final multivariable logistic regression model; for identifying the association between risk factors and the presence of *Salmonella* Dublin in milk

Risk factors	Level	OR[a]	95% CI[b]	Pvalue
Cow breed	Montbéliarde	-[c]	-	
	Brown Swiss	15.66	1.679-146.15	0.016
Region	El Hamma	-	-	-
	Remila	0.027	0.003-0.256	0.002
the introduction of purchased cattle into a farm	Yes	0.06	0.008-0.510	0.010
	No	-	-	-

[a]Odds ratio at cow level (OR), [b]Confidence interval (95%CI), [c]Reference Category

In this study, based on the Bacterial isolation, three *Salmonella* spp. were isolated from 307 fecal samples (0.97%), similar results were previously reported in Spain (0.9%) (Adesiyun et al., 1996), Egypt (0.97%) (Mohamed et al*.,* 2011), Iran (1.25%) (Halimi et al.,2014), and in Turkey (1.74%) (Hadimli et al., 2017). However, the prevalence was much higher in other countries such as the USA (10.1%)(Cummings et al., 2010), Ethiopia (7.6%) (Eguale et al., 2016), and in Ivory Coast (20%) (Yao et al., 2017).These differences could be explained by seasonal variation in *Salmonella* shedding of animals, other factors such as herd size and age could be responsible for these differences (Fossler et al., 2005). Moreover, most of the farms visited in the current study had small herd size, and *Salmonella* fecal shedding by cattle is commonly intermittent (Warnick et al., 2003;Cummings et al., 2010). Moreover, the region can also influence the frequency of isolation from one study to another (Callaway et al., 2005).

In our study, *S*. Dublin was not detected. However, the isolates detected in fecal samples belonged to Mbandaka serovar, this serovar is not frequently reported from cattle. Nevertheless, in one study conducted in the USA, it was found to be one of the most prevalent serovars at slaughter houses (Wells et al., 2001), which can indicate that *S.*Mbandaka can colonize cattle and could be transmitted to the slaughterhouse environment.

Milk collected from 256 cows was analyzed by ELISA serology to evaluate the presence of serovar *S.* Dublin. A positivity rate of 36.32%(93/256) was recorded; this prevalence was similar to that found in Ireland (49%) (Doherty et al., 2013). However, our results were higher than those found in the USA (14.1%) (Smith et al., 1989), Denmark (11%) (Nielsen, 2009) and in Sweden(3%) (Agren et al., 2015). The differences in the seroprevalence rates of *S.* Dublin in milk from dairy cows may also be attributed to the geographical location and herd size that can influence significantly the seroprevalence of salmonellosis in the dairy cattle (Kabagambe et al., 2000).

The comparison between the direct detection technique of *S.* Dublin (Fecal culture), and the indirect detection technique (ELISA test), shows different results,by the absence of this bacteria in fecal culture, and the presence of its antibodies in milk, which can indicate that the bacteriological method is less sensitive than the immunological method.Nevertheless, the two methods indicated two different results. The bacteriological method showed the presence of alive *Salmonella* in feces (at least one bacteria per 25 g). On the other hand, ELISA detected the presence of anti-*Salmonella* antibodies in milk. This can indicate that *Salmonella* antibodies will persist even in absence of alive *Salmonella* in cows. These results were similar to those reported by Nielsen (2013) who found a low number of *S.* Dublin, and they were isolated from 0.7% (46/6614) of dairy cattle. The immunological method is based on the presence of specific antibodies in milk, the persistence and the level of detectable antibodies seems to be higher than the presence and amount of *S*. Dublin in feces.*S*. Dublin in feces also might be caused by the existence of latent carries with persistent antibodies and intermittent shedding of *S*. Dublin in feces (Smith et al., 1989; House et al., 1993).Therefore, bacteriological culture tests are not ideal, because of their lack of sensitivity (Nielsen and Dohoo, 2012). However, some differences in detection limits may be found between types of *Salmonella* and between feces types, *S*. Dublin may have a poor analytical sensitivity than other types of *Salmonella*, and its detection limits in cow feces may be higher, because of some factors such as structure of the fecal matter and competing ruminal microflora(Nielsen and Dohoo, 2012). Moreover, the sensitivity of the bacteriological culture tests are known to be best for recently infected animals (1-15 days post-infection), untreated, diseased animals and carrier cows during the peripartum period where shedding is most likely to occur due to stress following for instance hormonal changes (Nielsen et al., 2004).

The study of risk factors allowed the identification of some factors that can be associated with the presence of *S.* Dublin antibodies in milk. A strong association was found between the Brown Swiss cows and the presence of *S*. Dublin antibodies in milk,these cows are characterized by a low milk production level. However, they are rich in protein content which create a good environment for bacteria proliferation (De Marchi et al.,2007).

Moreover, the Remila region was more likely to be infected with *S.* Dublin than El Hamma region, this finding is in agreement with a study conducted in Wales and North-west England (Davison et al., 2006), and another study in USA (Ruzante et al., 2010) that showed that differences between regions can be found. In addition, there was a significantly negative association between the introduction of new purchased ani-

mals and the presence of *S.* Dublin antibodies in milk. Where the purchase of animals reduced the amount of antibodies in milk, which is not in accordance with other studies who found that the purchase of animals is a significant risk factor for the development of *Salmonella* infections in herds (Van Schaik et al., 2002; Nielsen and Dohoo, 2012).

CONCLUSIONS

We have detected *S.* Dublin antibodies from 36.33% of milk samples, indicating that it is widely distributed in the region of Khenchela. Moreover, we have found thatthe indirect method (ELISA test) is more sensitive that the direct method (bacteriological culture) for the detection of *S.* Dublin. Moreover, the region, the breed and the purchase of new animalsare important risk factors associated with the presence of *S.* Dublin antibodies in milk.This work could be considered as pioneer and a comparison point for further studies in Algeria. However, additional epidemiological data using more cattle herds are needed to determine the distribution of these serovars in Algeria.

CONFLICT OF INTEREST

We have no conflict of interests to declare

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Research article
Ερευνητικό άρθρο

Investigation of Stem Cell Applications on *In Vitro* Fertilization in Rats

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ABSTRACT: We aimed to search the effects of bone marrow-derived mesenchymal stem cell-conditioned media on in vitro fertilization by investigation of lifetime of germ cells cleavage, degeneration rates and embryo quality. For this purpose, firstly MSCs were isolated from femurs and tibias of the rat, and cells were cultured until the fourth passage. Sperm and oocytes were collected from male and female rats. Oocytes were added in Human Tubal Fluid Media (HTFM), Single Step Media (SSM), Alpha-MEM Media (AMM) and Bone Marrow-Derived Mesenchymal Stem Cell-Conditioned Media (CM). Thousand sperm were added into the media which including oocytes. Embryos were allowed to produce by IVF. The development of the embryos was followed until the 11th day, and the arrest, degeneration rates and alive embryos were established. The embryos reached 2, 4, 8, 16 cells stages and morula stage in the CM. While AMM had a negative effect on fertilization and embryo development, the most favourable effect was shown to be caused by CM in comparison with the other medias. These results have shown that the beneficial effects of CM in IVF would be a significant increase in the rate of fertility and development of embryos.

Keywords: Bone Marrow Mesenchymal Stem Cell, In Vitro Fertilization, Oocyte, Sperm, Superovulation.

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INTRODUCTION

Infertility has become an important problem for both men and women in recent years, and approximately 8-12% of married couples of reproductive stage have infertility problems. In the literature, the definition of infertility is as follows: It is the absence of pregnancy for 12 months or more during an unprotected sexual intercourse (Gunes et al., 2020). Considering the infertility rates of couples who apply to IVF centers, the rate of infertility caused by women is 33-41%, while the rate caused by men is approximately 25-39%. The infertility problem caused by both women and men is 9-39%. These rates of infertility may differ according to the geography and socio-economic status of the countries (Agarwal et al., 2020; Wasilewski et al. 2020).

When the reasons of infertility are investigated, it has been determined that the diseases that cause the dysfunction in the reproductive organs, such as hormone dysregulation, uterine or cervical diseases or cancers, endometriosis, polycystic ovary syndrome, premature ovarian insufficiency, may result in infertility. In men, hormone and sperm motility disorders, hypogonadism, infection or varicocele are the causes of infertility. The rate of unexplained cases in infertility is 30% (Agarwal et al., 2020; Del Giudice et al., 2020; Wasilewski et al. 2020).

Fertilization is a complex event in which many molecular signaling pathways come from oocyte and spermatozoa are involved (Yao et al., 2019). On the other hand, it is important to provide a suitable media or microenvironment in the in vitro fertilization (IVF) laboratory together with appropriate physical conditions. Since the conventional IVF procedures are insufficient in some cases of infertility, there is tendency to alternative methods (Osman et al., 2018). One of these is mesenchymal stem cell (MSC) applications (Zhu et al., 2020; Karimaghai et al., 2018; Virant-Klun et al. 2019; Manuel, 2020). Stem cells hold promise in many areas due to its plasticity and ability to differentiate into other cells, and also they secrete many growth factors (Yang et al., 2019). Despite this potential, clinical studies on reproductive organs in humans are limited because of ethical reasons. In a clinical research of stem cells, adipose tissue-derived mesenchymal stem cells (ADSCs) were used to trigger oogenesis in the ovary. While only 2 oocytes were harvested as a result of ovarian stimulation treatment with homones from a woman diagnosed with low idiopathic ovarian response, it has been reported that 14 oocytes were obtained after the application of ADSCs with hormone therapy (Estuardo et al. 2020). Mozafar et al., stated that bone marrow derived mesenchymal stem cell (BMSC) application showed an effective result by stimulating spermatogenesis in mice created azoospermia model (Mozafar et al., 2018). A similar study was carried out with hamsters and in the azoospermia model created with busulfan, it was found that ADSCs repaired the tissue damage in the testicle and induced the spermatogenesis (Karimaghai et al., 2018).

As can be seen, the stem cell itself was used in these studies. Therewithal, it has been reported that the conditioned medium (CM) of mesenchymal stem cells could support the maturation of oocytes and sperm motility. Because, the MSCs secreted the cytokines and growth factors into the CM, and it is thought taht the CM will have an improvement effect on the reproductive cells and organs (Ullah et a., 2015; de Olivera Bezerra et al., 2019; Yang et al., 2019). Bader et al., reported that the CM of ADSCs rised the sperm motility in vitro condition, whereas Akbari et al., appointed that CM of human umblical cord mesenchymal stem cells prometed the oocyte maturation and the formation of blastocyst (Akbari et al., 2017; Bader et al., 2018).

In our study, we intended to reveal whether some growth factors secreted by stem cell into the medium have an effect on IVF and embryo developmet. And, we aimed to search the effects of BMSC-conditioned media on IVF and compare with other conventional mediums by investigation of lifetime, germ cells cleavage, embryo quality, and degeneration rates.

MATERIALS AND METHODS

Informed consent was obtained from all individual participants included in the study. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (Manisa Celal Bayar University (date: 27.11.2013, ethic approval number: 77.637.435-71).

Experimental Animals

In this study, we realized our animal experiment by receiving animal ethics certification approval from Manisa Celal Bayar University (date: 27.11.2013, ethic number: 77.637.435-71). Rats were provided by Experimental Animal Centre from Manisa Celal Bayar University. We used 20 female Wistar albino rats and 10 male Wistar albino rats which has 250 ±

50 gr weight and without inbreed animals. Rats have waited in 25°C room temperature, 12 hours dark and 12 hour light condition. Rats were fed by *ad-libitum*. The subjects were kept in a separate room in the other isolated laboratory to avoid stress due to factors such as noise and heat.

BMSC Isolation and Characterization

BMSCs which are collected from rat tibias and femurs, and cells were cultured in alpha-MEM medium (F0915, Biochrom, Berlin, Germany) containing 15% fetal calf serum (S0113, Biochrom, Berlin, Germany), 50 µg/ml gentamycin (A2712, Biochrom, Berlin, Germany), 100 UI/ml. penicillin and 100 UI/ml. streptomycin (A2213, Biochrom, Berlin, Germany), amphotericin (A2612, Biochrom, Berlin, Germany) and 200 mM L-glutamine (K0282, Biochrom, Berlin, Germany) at 37°C and 5% CO2. Then, non-adherent cells were removed from the flask, and adherent cells were maintained until passage 4 (P4) by changing the media every two days (Aydemir et al., 2018).

BMSCs were dyed immunocytochemically for characterization using Stro-1, CD45, CD90 and CD105 markers at P4 (Demirayak et al., 2016). Cells were passaged into the 35mm2 petri dishes, and were seeded to be 70-80% confluent. After fixation with 4% paraformaldehyde (1.04004, Merck, Darmstadt, Germany), cell were permeabilized with 0.1% Triton-x100 (T8787, Sigma, St. Louis, USA), they were washed in phosphate buffer saline (PBS). The blocking serum was applied to the cells for 1 h, and cells were incubated in primary antibodies, Stro-1 (MAB4315, Millipore), CD45 (ab10558, Abcam), CD90 (ab92574, Abcam) and CD105 (ab11414, Abcam) for 18 h. For the negative control, some of cells were treated with PBS instead of primary antibodies. Then cells were treated with biotin-streptavidin hydrogen peroxidase secondary antibody (85-9043, Invitrogen®-Histostain Plus Bulk Kit, CA, USA) for 30 min. Diaminobenzidine (DAB, 00-2020, Zymed, CA, USA) was used to make the immunoreactivity visible, and the counterstaining was performed with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany). After washing in distilled water, cells were mounted using aqueous media AML060, Scytek, Logan, Utah, USA) (Özdal-Kurt et al., 2016). The immunoreactivities were evaluated under a light microscope (BX43, Olympus, Japan) according to H-score method. The intensity of immunoreactivity was determined as; weak (+), moderate (++) and strong (+++) respectively, and the stained cells were counted in five fields for each intensity. The H-score values were obtained using the formula: Pi (intensity of staining +1). The percentage of stained cells was indicated as Pi (varying from 0% to 100%) (Aydemir et al., 2018).

Following the characterization, BMSCs at P4 were cultered and after two days, the media taken from the flask and it was used to experiments. This media called as Conditioned Media (CM).

Ovary and Oocyte Collection

Superovulation

For the superovulation (SO) method, the female rats at estrous cycle were identified by analyzing vaginal smear. The vaginal smear was performed between 15.00-16.00 p.m. the smear samples were dyed with Giemsa, and the samples were examined under a light microscope (Aydemir et al., 2018). 10 Female rats at estrous cycle were applied to the superovulation process, Follicular Stimulating Hormone (FSH, Pregon, Organon) ve Human Chorionic Gonadotropin (HCG, Pregnyl, Organon). Firstly, the 25 IU FSH was injected to the them, after 48 hours, superovulation was completed by doing 20 IU HCG injection. After 48 hours from HCG injection (intraperitoneally (ip)), the ovaries were dissected from female rats under anesthesia (75 mg/kg ketamine and 10 mg/kg xylazine by ip). One of two ovaries was used for oocyte collection, other ovary was used for the histological examination (Honda et al., 2019).

Histochemistry

After superovulation, one of two ovaries was evaluated for the histological structure and formation of follicles. The ovaries were fixed in the 10% formalin for 48 h and the routin paraffin embedding protocol was applied. 5 µm thick sections were cut paraffin blocks. The samples of ovary were dyed with routin heamatoxylin and eosin (H&E), and they were examined in terms of the presence of oocyte cumulus complex (OCC) and number of follicles under a light microscope.

Oocyte collection

One of two ovaries was used to obtain oocytes. The ovaries with tuba uterina were removed by using sterile forceps and scissors and then were put in RPMI (Biochrom 0844B) medium. We put tuba uterine and ovary in Petri dishes (No. 1016 falcon Petri dishes). To collect oocytes, the ovaries and tuba uterina from the RPMI medium were shredded by ppd injectors

in laminar airflow. Shredded ovary and tuba uterine samples were taken outside and then oocytes were collected with Pasteur pipette (Isolab 225 mm, cat. no: 084.01.002) under a stereomicroscope (Olympus SZX12, Shanghai, China). Oocyte cumulus complex (OCC) came out of the tuba uterina. Oocytes were collected with cumulus complexes (aid with Pasteur pipette) (Hino et al., 2020). These groups were divided into four groups which are Alpha-MEM Media (AMM), Human Tubal Fluid Media (HTFM), Single Step Media (SSM) and Conditioned Media (CM) obtained from BMSCs.

Sperm Collection and IVF Method

Male rats were anesthetized (ketamine and xylazine) and sacrificed with the method of cervical dislocation in laboratory conditions. After sacrification, we removed male rat testes and put in RPMI media which included 10% fetal bovine serum (FBS). The testis samples were put in Petri dishes in laminar airflow. Excess fat around of testes was removed. After we removed excess fat tissue, testes were put in fresh media. The end of epididymis from testis was cut with a sterile surgical knife and then testis was milked (Kumar et al., 2016). Later, sperm in the medium was collected and centrifuged at 1000 rpm for 3 minutes. After centrifugation, sperm from supernatant portion was spread on a slide (we only take 5 µl to count) and sperm count was made. After we saw sperm on the slide, sperm which is in 15 ml tube was collected with 1000 µl pipette and then 1000 sperm (almost 30 µl) were put in drops which include oocytes. Then we investigated embryo development at 0, 3, 6 and 9. day.

Statistical analysis

The findings were analyzed statistically with one-way analysis of variance (ANOVA) by Tukey-Kramer multiple comparisons test on GraphPad (GraphPad Software, San Diego, CA, USA). using one-way analysis of variance (ANOVA). The results were given as mean ± standard deviation. The statistical significance was considered as $P \leq 0.05$ (Aydemir et al., 2018).

RESULTS

BMSCs characterization

Under cell culture laboratory conditions, BMSCs which were collected from rat tibia and femur were cultured until its confluent in AMM containing other supplements. The **characterization** of BMSCs were made immunocytochemically at the P4. After immunocytochemistry assay, we stated that the positivities of Stro-1 and CD105 (***p<0.001), and also negativities of CD45 and CD90 (***p<0.001) (Figure 1).

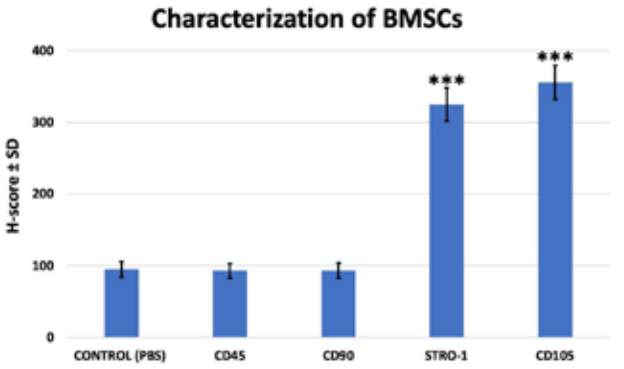

Figure 1. The H-score results of BMSCs characterization using CD45, CD90, Stro-1 and CD105

Histological evaluation

The ovary samples from 10 female rats treated with SO, were examined under a light microscope, and the follicles were counted from each rat ovary samples (five area) (Figure 2). It was seen that SO significantly increaesed the number of follicles (16 ± 3,1) compared to non-treated with SO (5 ± 1,4) method (Figure 3). However, the OCCs were found in all follicles.

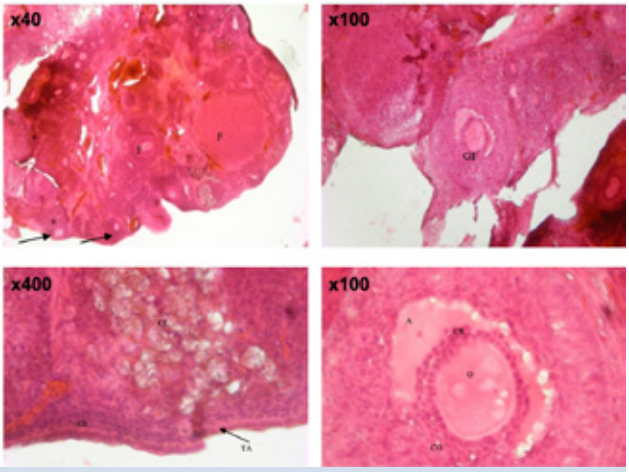

Figure 2. The images of ovaries of female rats that have been given FSH and HCG. We have shown that ovaries which have been induced with FSH and HCG application was increased the follicles and bloody supply amount. H&E staining, F: Follicle, primordial follicles are indicated by the arrow, GF: Graff Follicle, CL: Corpus Luteum, O: Oocyte, TA: Tunika Albuginea, GE: Germinal Epithelium, CR: Corona Radiata, CO: Cumulus Oophorus, A: Antrum

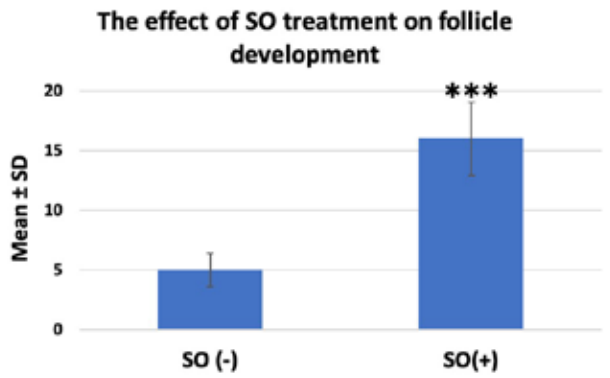

Figure 3. The evaluation of development of ovarian follicles after the treatment of SO.

Evaluation of IVF

After SO procedure, we collected oocyte and put in HTFM, SSM, AMM ve CM media which have been incubated one night before. Firstly, we determined the cell division stages in the oocytes as prophase I (PI), metaphase I (MI), and metaphase II (MII) (Figure 4).

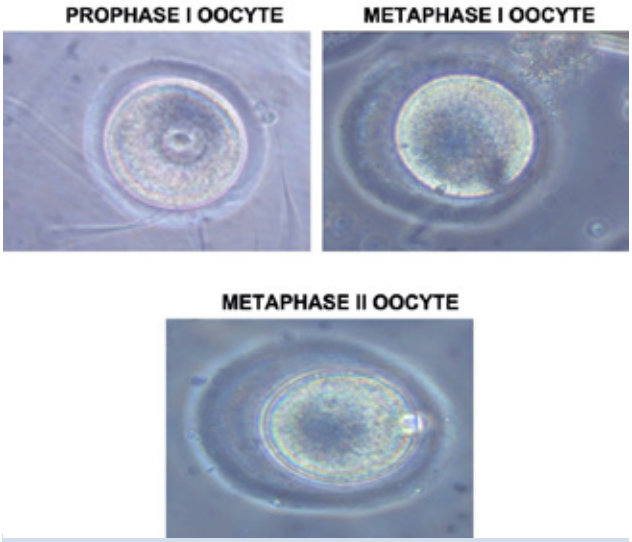

Figure 4. Rats Prophase I (PI), Metaphase I (MI) and Metaphase II (MII) oocytes examples. Magnification: x200.

After we added sperm, we took sperm and oocytes photos (Figure 5). We have indicated that PI oocytes in CM were matured into MII oocytes (Figure 6). Whereas oocytes in AMM weren't seen fertilization, oocytes in CM, SSM ve HTFM were seen fertilization. (Figure 6 and 7).

We observed that oocytes and embryos in HTFM were been healthy at the end of the 6th day. Degeneration was observed in 20% of oocytes in HTFM. On the contrary HTFM and SSM, oocytes and embryos in CM were seen healthy (Figure 8). We have shown that all oocytes in AMM degenerated on the 9th day. Moreover, oocytes and embryos in SSM and HTFM degenerated. Only one oocyte in SSM was seen as healthy. We have indicated that almost half of embryos and oocytes in CM were degenerated (Figure 9). Oocyte maturation and healthy process were indicated at (Table 1).

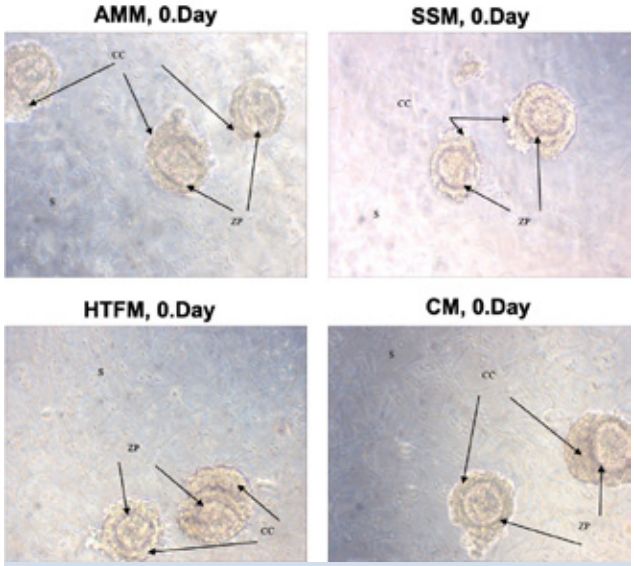

Figure 5. Images of oocytes which were collected from SO+ ovary in a different medium. Morphological differences between sperm and oocyte were not observed. CC: Cumulus Cells, ZP: Zona Pellusida, S: Sperm, Magnification: x100.

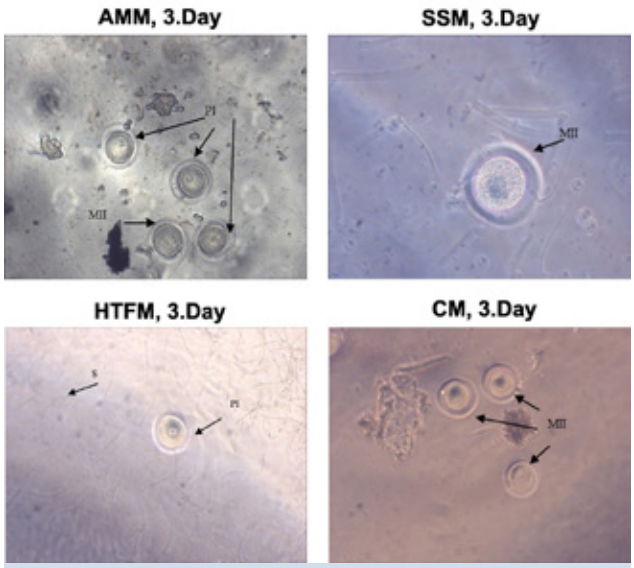

Figure 6. Images of non-fertilized oocytes on culture condition. PI: Prophase I, MII: Metaphase II, S: Sperm. Magnification: x100.

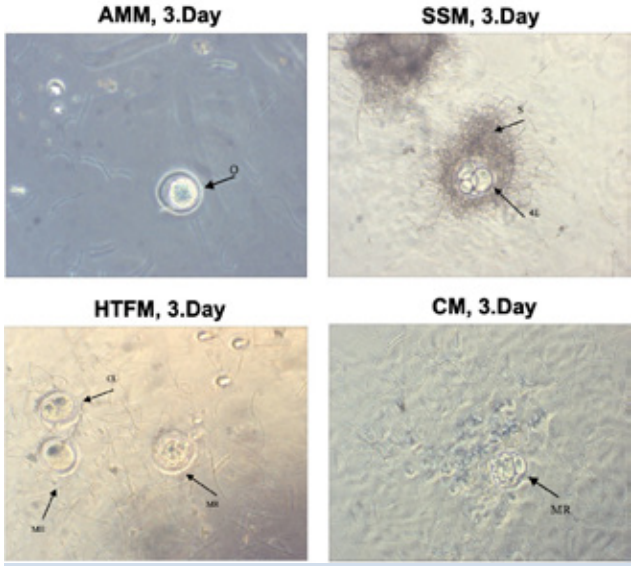

Figure 7. Images of oocyte and embryo at the end of the 3th day. Oocytes in AMM haven not been fertilized. One oocyte in CM has been fertilized. We showed that it has been reached the morula stage. We also showed that one oocyte in HTFM was fertilized and reached the compaction stage. 4E: 4 Cell Embryo, S: Sperm, MII: Metaphase II Oocyte, O: Oocyte, MR: Morula, CL: Embryo from cleavage. Magnification: x100.

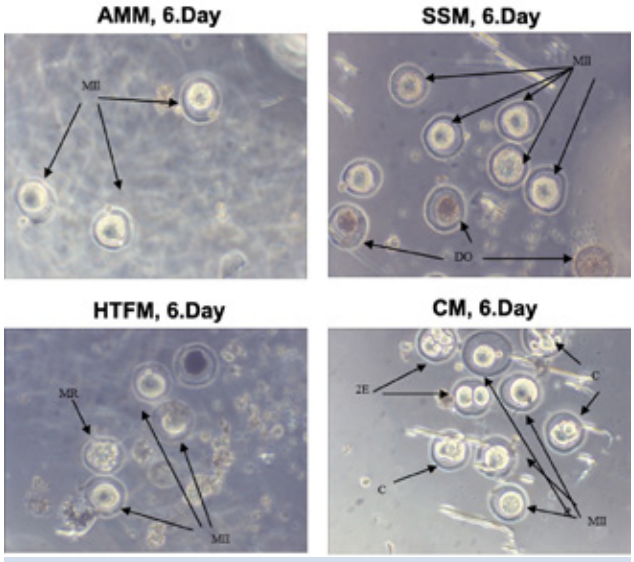

Figure 8. Images of oocytes and embryos on the 6th day. Oocytes in AMM were seen healthy. Whereas two oocytes in CM were degenerated, other oocytes in CM were healthy. We saw that four oocytes in HTFM and three oocytes in SSM were degenerated. 2E: 2 cell embryo, MR: Morula, MII: Metaphase II, DO: Degenerated Oocyte, C: Compaction. Magnification: x100.

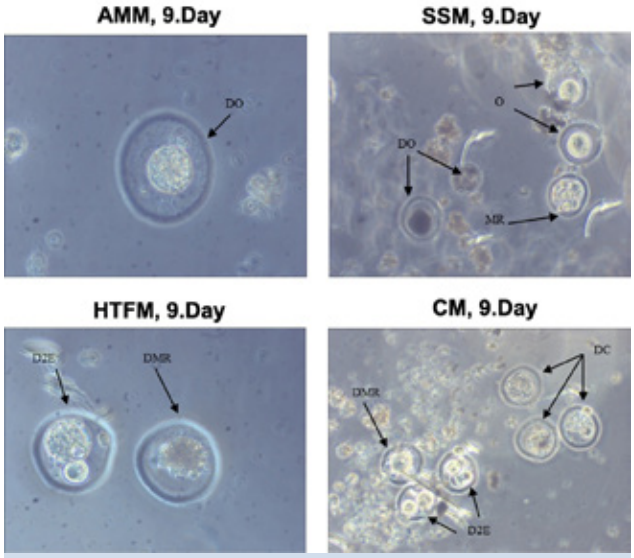

Figure 9. Images of oocytes and embryos on the 9th day. We showed that all embryos in HTFM and SSM degenerated on the 9th day. Only four oocytes in SSM were seen healthy. All oocytes in AMM were degenerated. Nine oocytes in BMSCM were seen healthy whereas three oocytes in HTFM were seen healthy. In addition to, three embryos in BMSCM were arrested at compaction stage. MR: Embryo from morula, D2E: Degenerated 2 cell embryo, DC: Degenerated embryo from compaction, MII: Metaphase II oocyte, DO: Degenerated Oocyte, O: Oocyte, DMR: Degenerated embryo from morula stage. Magnification: x100.

Table 1. The lifetime of oocytes in AMM, CM, SSM, HTFM in 0th, 3rd, 6th, 9th and 11th days.

The Number of Oocyte	AMM	SSM	HTFM	CM
0th DAY	14	15	16	17
3th DAY	11	13	15	16
6th DAY	9	10	11	14
9th DAY	1	4	3	9
11th DAY	0	1	0	3

DISCUSSION

In the current study, we investigated the effects of CM of BMSCs on the oocyte lifetime, germ cells cleavage, embryo quality, and degeneration rates, comparing the other convesional media, Alpha-MEM Media (AMM), Human Tubal Fluid Media (HTFM), and Single Step Media (SSM). We performed the evaluation of parameters morphologically in the culture conditions. We ascertained that the CM showed a positive effect on the development of reproductive cells and embryos.

With the discovery of stem cells, experimental or clinical researches on their use in the treatment of many diseases have increased. The stem cell itself has been used in many previous studies, and then many studies have been carried out with the conditioned medium from stem cells due to its ability to secrete many

factors. It has been established that the CM contained many growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), macrophage stimulating protein (MSP), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), transforming growth factors (TGFs) and interleukins (ILs), also microvesicles (Ma et al., 2014; Ullah et al., 2015; Bhardwaj et al., 2016; Noverina et al., 2019; de Oliver Bezerra et al., 2019; Yang et al., 2019). Nowadays, infertility is a major subject that causes people not to have children and gives important problems about having a good quality of life and peace. One of the most important problems with infertility is that oocytes and sperm which not enough maturation can create a fertilization problem (Mirnamniha et al., 2019). Despite some successful IVF trials, the success rate is still around 30% (Wasilewski et al. 2020). For this reason; some researcher has been trying to create new medium and they have been providing critical achievement.

In the previous studies, the BMSC-conditioned media was used the evaluate the development of follicles and oocyte maturation (Ling et al., 2008; Feng et al., 2009; de Olivera Bezerra et al., 2019). According to an experiment, 48 h of CM of BMSCs was compared with HTFM, AMM, Dulbecco's modified Eagle's medium (DMEM) using 120 oocytes of mice for each medium. CM was exhibited two times efficiency than other media which was included 4 times tubal fluid. More importantly, embryos development until blastocyst were increased between 2 and 8 times by CM (Ling et al., 2008). Similar to previous study, Feng et al., (2009) stated the effects of CM of BMSCs on development of oocytes and meiosis in mice. The CM has been found to support parthenogenetic development of oocytes. It is thought that not only CM can play an important role in oocyte activation, but also it can affect pronuclear formation by using Ca+2 (Feng et al., 2009). Likewise, CM of Wharton's jelly-derived mesenchymal stem cells exhibited the similar effect on the growth of ovine oocytes morphologically. Though, mitochondrial activity of oocytes was enhanced in the AMM and AMM+CM conditions, whereas the level of reactive oxygen species (ROS) was decreaesed with the AMM+CM and CM treatments (de Olivera Bezerra et al., 2019). Microvesicles, one of the agents secreted by mesenchymal stem cells, have been observed to trigger follicle development and oocyte maturation in mice with premature ovarian insufficiency (Yang et al., 2019). The findings we obtained in our study were in parallel with these studies.

In a clinical study, the CM of human umblical cord mesenchymal stem cells (hUCMSc) was indicated that it triggered the maturation of oocytes which harvested from infertile women, and it was compared with AMM. Together, the maturation effect of these two media on vitrified and non-vitrified oocytes was evaluated. It has been demonsrated that the 48 h of CM has a greater maturation effect (85.18%) on non-vitrified oocytes than the AMM (79.24%). The parthenogenesis induced with ionomycin was enhanced in the presence of CM in comparison with AMM. In the AMM, there was no seen the develeopment of blastocyst, whereas 5% blastocyst development was reported in the CM in the non-vitrified oocytes (Akbari et al., 2017). In another clinical study, the effects of CM of ADSCs on sperm motility and viability were searched. Oxidative stress-treated and non-treated sperm were incubated with 24, 48 and 72 h of CM for 24 h, and the parameters of motlility, vacuolization and DNA fragmentation were ameliorated in the presence of 24 h of CM. Especially, vacuolization and DNA fragmentation of sperm were diminished significantly. This clinical study contains promising results in the case of male infertility (Bader et al., 2018). We only analyzed the effect of CM on oocyte and fertilization, and we found that CM supported the oocyte maturation and quality. At the 9th day, we detected the morula stage in the CM. However, it was seen that CM delayed the oocyte degradation.

CONCLUSIONS

In the other studies and our experiment, the CM was exposed to the oocytes or sperm without isolation of any growth factors. The findings in our study were obtained only as a result of morphological evaluations under the inverted microscope, and they have been supported that the CM from BMSCs may have a beneficial effect in the treatment of infertility. Our study is stated that mesenchymal stem cells and their niche are very important in this process. Because CM has been much more effective than other media in embryonic development which happens until two cells embryo stage to the compaction stage. Although these findings are promising in the future clinical use, the molecular analyzes are needed to fully understand the effects of the CM of mesenchymal stem cells.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

The anxiolytic and antidepressant effect of *Buxus hyrcana* in the pentylenetetrazole kindled rat

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ABSTRACT: Pentylenetetrazole (PTZ) is a chemical substance which largely used for induction of seizure and epilepsy in the animal model, and it can also, disrupts free radicals balance and causes oxidative stress in the body with a negative impact on behavioral statuses like anxiety and depression. In this study, the medicinal plant *Buxus hyrcana*, was used to evaluate its effect on oxidative stress, anxiety and depression caused by PTZ in the rat. Twenty-four male rats were randomly allocated to 4 groups: control negative under treatment with PTZ (sub-threshold dose 35 mg/kg for one month), control positive under treatment with phenobarbital (PB-30 mg/kg), and two PTZ groups under treatment with *B. hyrcana* extract (BHE-300, and -600 mg/kg). For anxiety parameters, the elevated plus maze (EPM) was used. The forced swim test (FST) and rotarod test were employed to assess the antidepressant and balance potential, respectively. After behavioral evaluation, rats were anesthetized, brains were removed, and following preparation of brain homogenates, oxidative stress was evaluated using specified methods. BHE administered at the doses of 300, and 600 mg/kg, reduced immobility time in the FST exerting antidepressant-like activity. In the EPM test, BHE at the same doses, produced the anxiolytic-like effect. Also, the rats which received BHE had a significant improvement in rotarod test in contrast to control groups. In addition, brain catalase activity and superoxide dismutase level were significantly greater versus PTZ group BHE-300 treated PTZ group was significantly lower and. BHE could prevent anxiety and depression and ameliorate oxidative stress in PTZ-kindled rats.

***Keywords*:** Elevated Plus Maze; Epilepsy; Forced Swim Tests; Kindling; *Buxus hyrcana;* Pentylenetetrazole

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INTRODUCTION

One of the most prevalent chronic neurological diseases is epilepsy which is associated with reversible seizures and can affect the patients both psychologically and behaviorally (Kowski et al. 2016; Saha and Chakrabarti 2014). Oxidative stress and free radicals are among the most important mechanisms that can contribute to disorders such as epileptic seizures (Waldbaum and Patel 2010). Epileptic seizures have also been shown to increase the amount of reactive oxygen species (ROS) and the production of superoxide anion (O_2-) in the brain (Costello and Delanty 2004). Oxidative stress is the result of an imbalance between the antioxidant defense system and the formation of ROS (Stadtman 2001). Among the antioxidant defense system there are two key enzymes which is very important in the first line of defense against production of ROS. These enzymes are catalase and superoxide dismutase (SOD) which they are very fast in neutralizing any molecule with the potential of developing into a free radical or any free radical with the ability to induce the production of other radicals (Ighodaro and Akinloye 2018).

Nowadays, antiepileptic drugs (AEDs) such as barbiturates and a sundry of sedatives are adopted to ward off or treat epilepsy. Although in recent decades, myriad drugs have been familiarized for the dealing of epilepsy, they continue to show adverse and even deleterious effects that in most cases, even with a sufficient dosage, fail to properly stave off the seizures. Therefore, they would pose a great negative impact on one's quality of life both psychologically and behaviorally (Kowski et al. 2016; Saha and Chakrabarti 2014). In effect, to discover and develop sufficient AEDs have been a desideratum. To this end, kindling has been posited as an efficacious model for the clinical facets of epilepsy at biochemical, electrophysiological and behavioral levels (Dhir 2012). Also, one of the common approaches in the discovery and development of AEDs - which is the backbone for the cure of patients with seizures and epilepsy - is the utilization of natural substances with medicinal properties (Pahuja et al. 2012). One of the herbs that has evinced beneficial medicinal properties and has been studied in traditional medicine is *Buxus hyrcana* (Buxaceae family), which grows in various part of the world (Babar et al. 2006; Choudhary et al. 2006). Previous studies have demonstrated many medicinal properties for *B. hyrcana* such as being antimalaria, anticancer, immunosuppressive due to its active ingredients, antifungal and antileishmania; it, further, has anti-inflammatory and antioxidant characteristic because of its triterpenoids and alkaloids (Ata et al. 2010; Babar et al. 2006; Choudhary et al. 2006; Ebrahimzadeh et al. 2010; Esmaeili et al. 2009; Mesaik et al. 2010). Moreover, it has recently been documented that it has anticonvulsive and neuroprotective properties in experimental animal models (Azizi et al. 2018).

Given the behavioral manifestations associated with epilepsy and the beneficial effects of *B. hyrcana* on above mentioned disorders, it was hypothesized that the *B. hyrcana* extract (BHE) would have positive effect on behavioral parameters, related to stress, depression, and balance in pentylenetetrazole (PTZ)-kindled rats. The aim of this study, therefore, was to evaluate the effects of BHE on behavioral test (related to stress, depression and balance), in order to assess behavioral disruptions resulted from epilepsy and seizures in adult male rats.

MATERIALS AND METHODS

Animals and ethics

This experiment was conducted at Shahid Beheshti University (Tehran, Iran) on twenty-four male adult Wistar rats (200 ± 20 g, 8 weeks old) obtained from Shahid Beheshti University of Medical Sciences (Tehran, Iran). Animals were kept for 1 week in a room with constant conditions (12 h light/dark period with lighting starting at 7 a.m., 22 ± 2°C, 55 ± 5% relative humidity) in standard cages (42×27×15 cm; Tajhiz Gostar-e Omid Iranian Co.) made from polycarbonate in order for them to adapt to a new environment. The animals had free access to water and feed for rats (Pars Animal Feed, Iran) throughout the experiment. Animals were randomly (simple randomization) given to experimental groups (each group containing 6 rats) after one week of adaptation to the laboratory environments. Each animal was used only once through the trial, and efforts were made to reduce the animal suffering and at the same time to obtain reliable scientific data. All experiments were carried out between 09:00 and 15:00. All conducted experiments pertaining to animal rights and conservation in this study were in accordance with the standard ethical guidelines (European Communities Directive 2010/63/EU) and were approved by Local Ethics Committee at the Shahid Beheshti University (ethical code: IR.REC.SBU.1397.156).

Obtaining the plant and preparation of the extract

The *B. hyrcana* plant was obtained from the Insti-

tute of Medicinal Plants of Shahid Beheshti University. The extract was prepared according to the references and previous work (Azizi et al. 2018). Thus, about 100 g of the aerial part of the plant was prepared, cleaned, dried in the shade, and powdered by mechanical shredder. To prepare the hydroalcoholic extract, the plant powder was drenched in 1L of ethanol 80% for 72 h, then the resulting mixture was filtered and concentrated in vacuum at 45°C using a rotary apparatus (EYEL A, Japan). The resultant extract was 37.8% dry and stored in the refrigerator at 4°C until the experiment. The dose required for intraperitoneal (i.p.) injection was based on animal weight.

Medications and treatments

Pentylenetetrazole from Sigma Company (USA), phenobarbital sodium (PB) from Chemidarou Pharmaceutical Company (Iran), ketamine and xylazine from Alfasan Company (the Netherlands) were obtained. PTZ was prepared as a 1% v/w solution in saline. Phenobarbital sodium was used in current experiment as a conventional anticonvulsant drug. Phenobarbital sodium was also dissolved in the physiological saline solution and administrated intraperitoneally in a dose of 30 mg kg^{-1} of rat weight to the animals. BHE was continued in normal saline solution via ip in doses of 150 and 300 mg kg^{-1} with PTZ during the experiment until complete animal kindling. Animals were randomly alienated into four groups of six: (1) negative control group receiving normal saline (0.5 mL/rat), (2) positive control group receiving PB (30 mg kg^{-1}), (3 and 4) groups receiving BHE (300 and 600 mg kg^{-1}, respectively). Thirty min after vehicle injection, PB and BHE (300 and 600 mg kg^{-1}) animals were challenged with the sub-threshold dose of PTZ (35 mg kg^{-1} of body weight). All injections were administered in the form of fresh solutions in a constant volume of 0.5 mL/rat throughout the study (Azizi et al. 2018).

PTZ-induced kindling test

The study was conducted in a double-blind manner (the experimenter was unaware of which animal belonged to which group). Pentylenetetrazole was injected in a sub-threshold dose of 35 mg kg^{-1} every 48 h interval for a period of one month. In order to record and measure seizure behavior, the animals were transferred individually to transparent plastic boxes and were immediately observed for one hour after PTZ administration and recorded by a computer-connected camera. Seizure threshold was measured on a 6-step scale (Table 1). Animals were considered kindled if they showed stages 4 or 5 in two consecutive trials. Animals were given the PTZ challenge (70 mg kg^{-1}) 7 days after the kindling development. Meanwhile, the animals underwent the anxiety, depression and rotarod test. Anxiety, depression and rotarod test were carried out after the PTZ challenge (Pahuja et al. 2013).The experimental schedule and intervals for the estimation of various parameters is shown in Figure 1.

Table 1. Adapted Racine's scale for pentylenetetrazole (PTZ)-induced seizure in rats.

Stage	Seizure intensity
0	No behavioral fluctuations
1	Ear and facial twitching
2	Nodding of the head,head clonus and myoclonic jerks
3	Unilateral forelimbclonuswith lorditic posture
4	Bilateral forelimb clonus with rearing and falling
5	Generalized tonic-clonic seizure (GTCS) with loss of postural tone

Elevated plus maze (EPM) test

EPM test was used to determine the anxiety-like behavior in rats. The EPM consists of two open arms without walls (50×10 cm) and two enclosed arms with high walls (50×10×40 cm), extending from a common central platform (10×10 cm). Each rat was individually placed in the center of the maze, its head facing an open arm and was allowed for five min of free exploration. All sessions were videotaped and behavior was scored using "EthoVision XT" software. After each test, the floor was cleaned with ethanol (10%) and dried. Measurements were made from the frequencies of total open and closed arm entries (arm entry = all four paws into an arm) and the time spent in open, closed and central parts of the maze. The latency to open arm entries as the standard index of anxiety-like behaviors were calculated (Iwamoto et al. 2007).

Forced swim test (FST)

The modified forced-swim test was performed according to the modified method described earlier (Cryan et al. 2005). All testing was carried out in a 20-min test with no preswim session in order to negate any confounding aspect of an induction procedure.The rats were placed in a glass cylinder (45 cm diameter and 50 cm high; Borj Sanat Azma Co.) filled to a depth of 30 cm with water (23°C). The immobility time during the last 5 min of a 20-min swim test was defined as the absence of active/escape directed movements. After the test, animals were removed from the water, dried with a towel then carried back to their home cages.

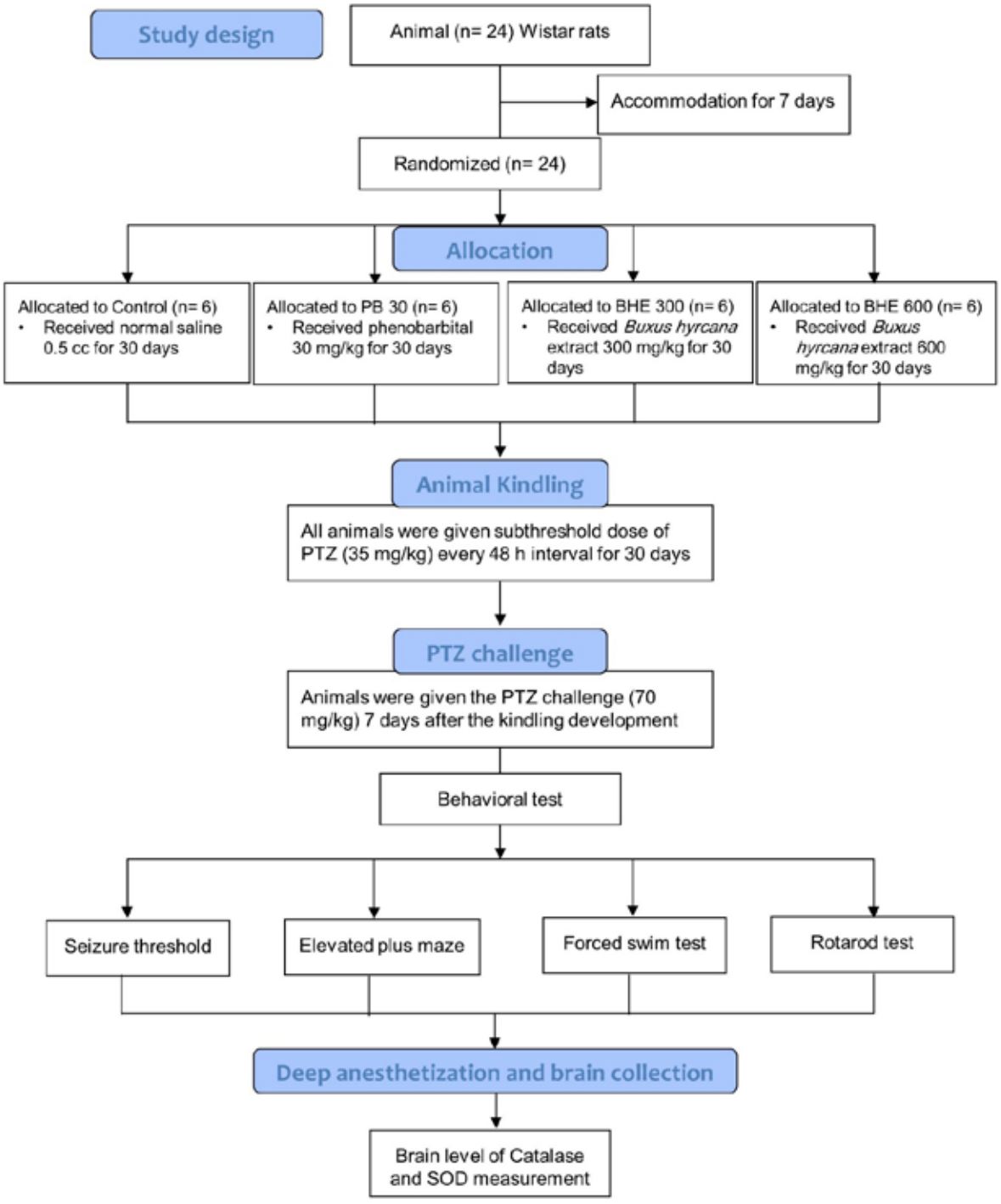

Figure 1. Flow chart of the experimental design. Schematic timeline representation for PTZ kindling, administration of drugs and behavioral test in experimental groups.

Rotarod test

The rotarod test is a widely used test to measure coordinated motor skills. It requires animals to balance and walk on a rotating cylinder. The rotarod (49 cm diameter and 45 cm width; Borj Sanat Azma Co.) unit consisted of a rotating rod, which divided into four parts by compartmentalization, which allows examining four rats at a time. When the rats fell down from rotating rod, the time automatically stopped. In this study, the rotating speed of rotarod was constant (15 rpm). After training, the time for each rat to remain on the rotating rod (rotarod latency) was recorded for three trials at 30 min intervals. The maximum time for each trial was 90 s. The rotarod latency is directly dependent on the movement and balance skill of the animal. Twice daily training for two consecutive days was done before the test day (Khan et al. 2013).

Biochemical measurement

After the behavioral test, the rats were quickly beheaded under deep anesthesia by a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg), and their brains were removed, washed with ice saline, and kept at minus 80°C for subsequent analyses.

Measurement of brain catalase (CAT) activity

Catalase activity of brain tissue was performed according to the previous work (Milanizadeh et al. 2018). In short, 50 mL of phosphate buffer is removed and 0.05 mL is H_2O_2 is added. To study the changes in the optical density of the CAT enzyme in the samples of different groups, after combining them, the optical density of the CAT enzyme activity was measured at 240 nm wavelength for 2 min by the spectrophotometric device.

2.8.2. Measurement of brain superoxide dismutase (SOD) activity

In this section, the previous reference was also used to measure SOD activity of brain tissue (Milanizadeh et al. 2018). Briefly, 0.43 g of Na_2HPO_4 is dissolved in 61 mL of distilled water and 0.3 g of $NaHPO_4$ is dissolved in 39 mL of distilled water. The two solutions were poured together in a beaker and the pH of the solution is brought to 7. Fifty mL from previous solution was mixed with ethylenediaminetetraacetic acid (EDTA) (0.0018 g), and pyrogallol (0.003 g). For comparison purposes, the optical density changes of the SOD enzyme in the sample were calculated at 420 nm wavelength for 4 min by the spectrophotometric device.

Statistical Analysis

All statistical evaluations and graphs were run using the GraphPad Prism software (version 8). All behavioral and biochemical tests were articulated as mean ± standard error from the mean (SEM) or median (min, max). The normality test was carried out to show the data distribution is normal. All data met ANOVA assumptions for normality and homogeneity of variance. One-or two-way ANOVA was done to compare the means of the statistics. The Tukey *post-hoc* test was used where data were significant to compare the groups by pairs. The significance level was considered $P < 0.05$ for all the study groups.

RESULTS

The effect of BHE on seizures in PTZ-induced kindled rats

Analysis of variance showed a significant increase in the mean of seizure threshold [$F(3,20) = 15.65$, $P<0.001$] between the experimental groups. Further analysis with Post *hoc* test showed a significant increase ($P = 0.001$) in the seizure threshold mean in the BHE treatment group in a dose of 600 mg kg^{-1} compared to control group. However, BHE-treated groups in dose of 300 failed to show significant changes when compared to the control group. Also, a significant increase in the mean of the seizure threshold was observed in the PB 30-treated group in comparison with control group ($P < 0.001$) (Fig.2).

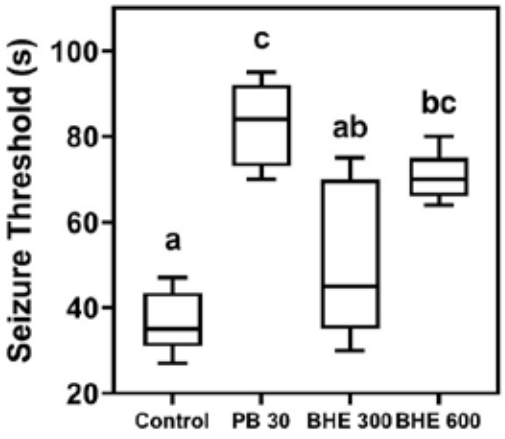

Figure 2. Effect of BHE on seizure threshold in PTZ-induced kindled rats. The box plots showing the median (min, max) is related to six male Wistar rats. Different small letters in the box indicate the significance of differences at $P<0.05$.

Effect of BHE on EPM in PTZ-induced kindled rats

As shown in Figure 2. a-e, and confirmed by analysis of variance, there were a significant differenc-

es between the experimental groups with respect to the time spent in the open arms [F (3,20) = 13.90, P <0.001], the time spent in the closed arms [F (3, 20) = 26.86, P <0.001], the number of open arm entries [F (3,20) = 10.64, P <0.001], number of closed arm entries [F (3,20) = 26.54, P <0.001] and latency to enter open arms [F (3,20) = 4.84, P =0.013] in the EPM test.

The post-test indicated that the PB 30 receiving group caused a significant increase in the mean of the time spent in the open arms (P <0.001; Fig.3.a) and the number of open arm entries (P <0.001; Fig.3.c) and a significant decrease in the time spent in the closed arms (P <0.001; Fig.3.b), number of closed arm entries (P <0.001; Fig.3.d), and latency to enter open arms (P =0.011; Fig.3.e) in comparison to the PTZ group. There was also a significant increase in the group receiving BHE in the doses of 300 and 600 compared to the control group in the time spent in the open arms (P =0.006 and P <0.001, respectively; Fig.3.a) and the number of open arms entries (P =0.063 and P =0.006, respectively; Fig.3.c) and a significant decrease in the time spent in the closed arms (P <0.001; Fig.3.b), number of closed arm entries (P <0.001; Fig.3.d). However, in concern to latency to enter open arms parameter there is no significant changes in the group receiving BHE in a dose of 300 compared to the control group (Fig.3.e)

Effect of BHE on FST in PTZ-induced kindled rats

Significant differences were observed in the mean of immobility [F (3,20) = 54.92, P<0.001] and swimming [F (3,20) = 24.36, P<0.001] factors between the different groups. The post *hoc* analysis suggested that the immobility time mean was significantly increased in the BHE-treated groups in doses of 300 and 600 compared to the control group (P<0.001) (Fig.4.a). Also, there was a significant increase in the mean of swimming time in the BHE group in doses of 300 and 600 (P =0.029 and P =0.002, respectively; Fig.4.b) compared to the control group.

The Effect of BHE on Rotarod test in PTZ-induced kindled rats

Analysis of variance showed a significant difference in the mean of latency to fall in rotarod test between the different groups in 30 min [F (3,20) = 6.37, P=0.004], 60 min [F (3,20) = 17.07, P<0.001] and 90 min [F (3,20) = 33.38, P<0.001] after treatments. The mean latency to fall in the BHE group in 300 and 600 doses at 30 min (P=0.175 and P=0.020, respectively), 60 min (P=0.021 and P<0.001, respectively) and 90 min (P=0.001 and P<0.001, respectively) after treatments was significantly higher than that of the control group (Fig.5).

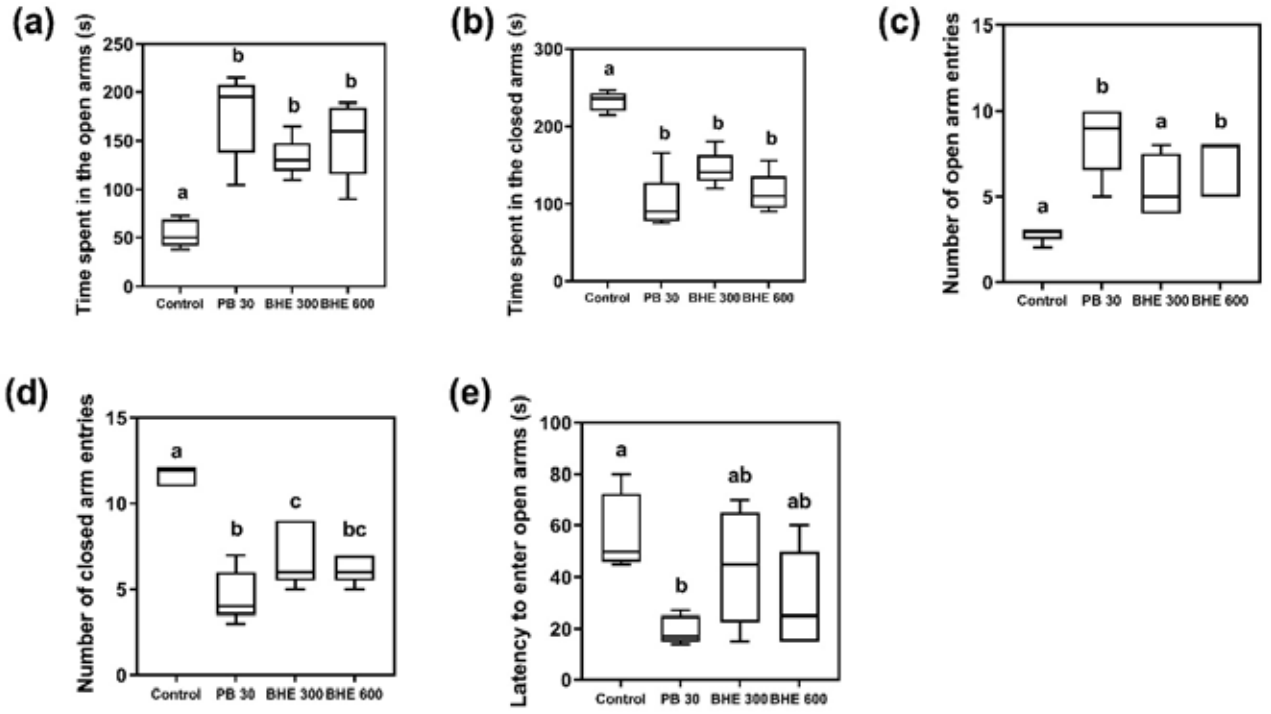

Figure 3. Effect of BHE on time spent in the open arms (a), time spent in closed arms (b), number of open arm entries (c), number of closed arm entries (d), and latency to enter open arms (e) in PTZ-induced kindled rats. The box plots showing the median (min, max) is related to six male Wistar rats. Different small letters in the box indicate the significance of differences at P<0.05.

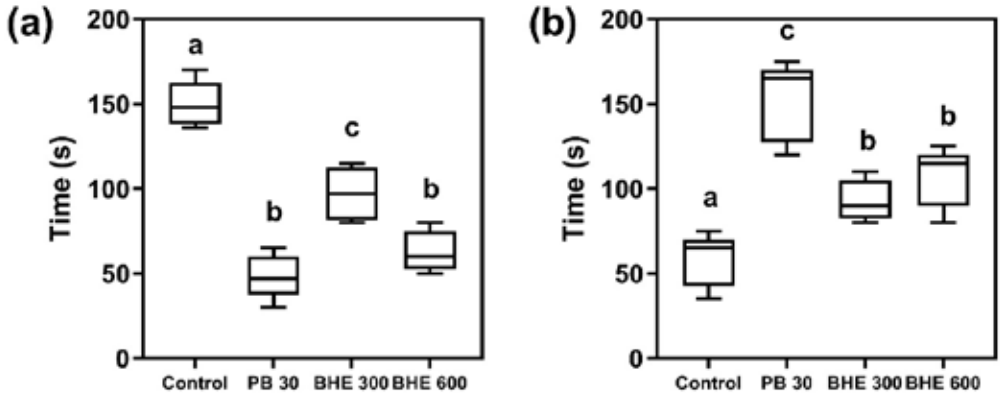

Figure 4. Effect of BHE on immobility (a), and swimming (b) in PTZ-induced kindled rats. The box plots showing the median (min, max) is related to six male Wistar rats. Different small letters in the box indicate the significance of differences at P<0.05.

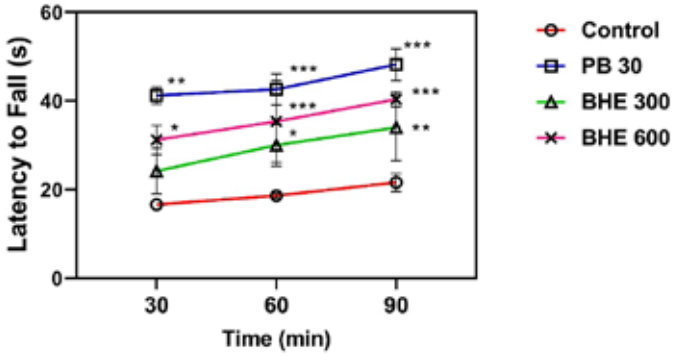

Fig.5. Effect of BHE on latency to fall in rotarod test in PTZ-induced kindled rats. Data represents mean ± SEM, * *P* <0.05, ** *P* <0.01, *** *P* <0.001, as compared to control group.

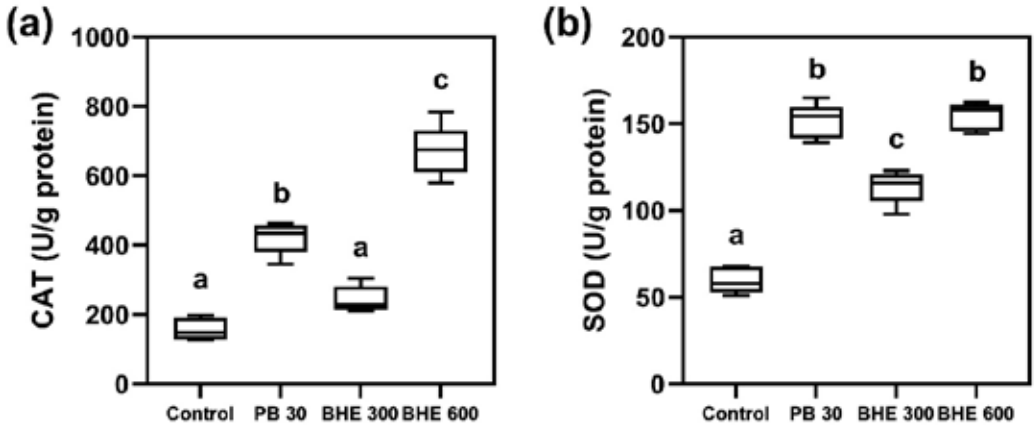

Figure 6. Effect of BHE on CAT (a), and SOD (b) levels in PTZ induced kindling in rats. The box plots showing the median (min, max) is related to six male Wistar rats. Different small letters in the box indicate the significance of differences at P<0.05.

Effect of BHE on oxidative stress parameters

Effect of BHE on brain CAT levels

Significant differences were observed in the mean of CAT levels in the brains of the rats between the different groups [F (3,20) = 101.6, P<0.001]. The *post-hoc* analysis suggested that the CAT level mean was significantly increased in the BHE-treated groups in dose of 600 compared to the control group(P<0.001) (Fig.6.a). However, there is no significant change in the group receiving BHE in a dose of 300 compared to the control group.

The effect of BHE on brain SOD levels

Analysis of variance showed a significant difference in the mean of SOD total content of the brain between the different groups [F (3,20) = 123.3,

P<0.001]. The SOD level mean in the BHE group in 300 and 600 doses was significantly (P<0.001) higher than that of the control group (Fig.6.b).

DISCUSSION

This study identified the positive effect of BHE on stress and depression in the experimental model of kindling induced by PTZ. The data showed that pretreatment with BHE raises seizure threshold in PTZ-induced kindling. In addition, our findings revealed a significant decrease in epileptic stress and depression in pre-treated BHE rats. In the present study, as the results revealed, BHE at doses of 300 and 600 mg kg^{-1} significantly elevated seizure threshold in kindled rats *vis-à-vis* the control group. In line with this study, Azizi et al. (2018) observed that *B. hyrcana* exhibits neuroprotective and anticonvulsant characteristicin Wistar rats in the PTZ-induced seizure model. They have also documented that the dose of 600 mg kg^{-1} of the extract has the greatest effect, which is in harmony with the present study (Azizi et al. 2018).

In the current work, the EPM and FST tests were adopted to evaluate the anti-anxiety and antidepressant properties. The outcomeof the study confirmedthat doses of 300 and 600 mg kg^{-1}BHE increased the frequency and duration of entry into open arms and reduced it in the closed arms compared to the control group. It also significantly reduced the delay in open arm entry in the EPM test, indicating a decrease in anxiety in BHE-receiving rats. Furthermore, the BHE treatment significantly reduced the period of non-movement and significantly increased the time of swimming compared to the control group in the FST-treated rats. In line with our results, in the recent studies on *B. hyrcana*, it has been observed that this planthaving steroidal alkaloids, inhibits acetylcholinesterase activity (AChE) -an enzyme which degrade acetylcholine by hydrolytic cleavage- and by this means can be used to cure Alzheimer's disease, which is a neurological progressive deficit illness(Babar et al. 2006; Choudhary et al. 2003; Choudhary et al. 2006). In the most recent experiment in mouse model, it has been shown that nicotinic acetylcholine receptor can excite the release of the gamma-aminobutyric acid (GABA) (Aracri et al. 2017). Although the most conventional models of seizure studies are induction by PTZ, the mechanism underling of this procedure was not completely acknowledged. Nevertheless, there is a public agreement that one of the mechanisms of the PTZ can cause the seizure is inhibition of the ion channel GABA type A complex and disruption in the neural inhibitory pathway (Mandhane et al. 2007). Consequently, according to the above-mentioned reason, at least in part the anti-seizure properties and consequently anxiety and depression amelioration observed in this study are due to the presence of the steroidal alkaloids in the BHE. These are due to the anti-AChE activity as well as the rise in the levels of ACh and subsequently rise in the release of the GABA, the competition with the PTZ on the active site of the GABA receptors and increase in the inhibitory activity in the neural cells.

In this study, to measure the motor activity, rotarod performance test was used. As the results show, BHE at the doses of 300 and 600 mg kg^{-1} had the highest anticonvulsant effect, as this effect rate was also evident in the locomotor activity of animals treated with BHE and was significantly increased compared to the control group. Since this study examined the positive effects of BHE in the rotarod motor testing for the first time, therefore, it is not possible to compare it with previous works. That said, to confirm this hypothesis cogently, further research is warranted.

In this study, we examine the antioxidant activity of BHE, therefor the total brain level of the CAT and SOD were evaluated. As the results show, BHE at the doses of 300 and 600 mg kg^{-1} significantly increase the CAT and SOD level, as compared to the control group. Pentylenetetrazole also disrupts the blood-brain barrier (BBB) and disrupts brain function by creating free radicals (Choudhary et al. 2013). The brain is very sensitive to the damage of free radicals, because it contains a lot of fatty acids and it has a high rate of oxidative metabolism (Mariani et al. 2005). Reactive oxygen species are involved in the pathogenesis of various types of neurodegenerative diseases (Perry et al. 2002). Oxidative stress may play an important role in causing neurological damage due to seizures (Sudha et al. 2001). Prolonged administration of PTZ induces free radicals, leading to seizure activity in animals. The oxidative stress induced by PTZ leads to tonic-clonic seizures and subsequent neurological death (Zhao et al. 2014). Inhibition of brain neurons and production of free radicals by PTZ is one of the major causes of epilepsy in animal models of epilepsy. In epilepsy, seizure activity is always associated with increased levels of reactive oxygen species (Rauca et al. 1999). Studies have focused on elucidating whether prolonged seizure activity in animals leads to increased ROS production and whether

oxidative damage leads to seizure-induced brain injury. Therefore, this study is consistent with the theory that in animals induced by PTZ, oxidative stress is probably one of the parameters involved in the pathophysiology of epilepsy (Nassiri-Asl et al. 2013).

Chemically, flavonoids and iso-flavonoids destroy the free radicals and reduce oxidative stress by single electron transfer. The accumulation of free radicals by flavonoids can be one of the reasons for the protective effect of this substance on nerve cells (Bors et al. 1990). There is also ample evidence that flavonoids are involved in preventing the destruction of nerve cells caused by oxidative stress (Ishige et al. 2001). It has been shown that in the epileptic condition the neural cells the level of oxidative stress markers rise up and the levels of antioxidants will decrease (Cárdenas-Rodríguez et al. 2014). Based on studies on the active ingredients of the *B. hyrcana* have confirmed that it has characteristic to block the oxidative stress. As noted above, BHE has numerous flavonoids, which exert anti-inflammatory and antioxidant properties (Wollenweber and Rustaiyan 1991); therefore, at least partially, the antiepileptic and ultimately the improvement in anxiety and depression observed in this study may be due to the presence of flavonoids present in BHE, as well as the control of CAT and SOD status in the brain.

Our study has some limitations that should be considered. As limitations, our study did not evaluate the other biochemical and molecular parameters which very important such as MDA, nitric oxide, inflammatory cytokines, gene expression of enzymes or receptors responsible for epilepsy or stress pathway due to limited funding, and sample size. Therefore, supplementary studies need to justify the result and beneficial effect of BHE on the epilepsy or stress conditions. It is also suggested that in order to determine the role of the inflammatory cytokines and the effects of BHE in the pathogenesis of epilepsy and stress, other pathways such as the inflammation pathway and the expression of genes in the relevant pathways should be examined.

Overall, the results of this study indicated that the BHE has anticonvulsant properties in PTZ-kindled rats in that it increases seizure threshold in the groups receiving the extract. Moreover, given the improvement of seizure symptoms, it was observed that the behavioral markers of the rats (such as anxiety, depression and movement) receiving the extract was improved compared to the PTZ group. These effects can be attributed to the improvement of oxidative stress status, including increased in CAT and SOD activity.

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CONFLICT OF INTEREST

The Authors declare that they have no conflicts of interest to disclose.

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Research article
Ερευνητικό άρθρο

Anticoccidial potential of *Ageratum conyzoides* and its effect on Blood parameters of experimentally infected Broiler Chickens

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ABSTRACT: Coccidiosis is an infectious parasitic disease of poultry which causes heavy economic losses to commercial poultry industry. Coccidiosis has been generally controlled by using different synthetic anticoccidial drugs but, due to development of resistance to these drugs this method is losing its effectiveness. Exploration of novel and alternative compounds against avian coccidiosis is need of time now a days. In this regard, medicinal plants can serve as substitute to these synthetic anticoccidials. Thus, to find out alternative novel agents, current research was designed to evaluate the anticoccidial potential of *Ageratum conyzoides* extract (ACE). For *in vivo* experiment, 105 broiler chicks were purchased and further divided into 7 sub-groups (15 birds in each group). At 7th day of experiment, Groups A, B and C were fed with plant extract at 100, 200 and 300 mg/kg respectively. Group D was supplemented with Vitamin-E while, Group E and F remained as infected medicated and infected un-medicated control groups and served with standard medicine (Baycox®) and Phosphate Buffer Saline (PBS) respectively. Moreover, Group G served as normal control group. At day 14th of experiment, all groups except rom Group G were orally infected with 60,000 sporulated oocysts of mixed *Eimeria* species. Anticoccidial potential of ACE was evaluated on the basis of lesion scores, fecal scores, oocyst scores and feed conversion ratio. Furthermore, the effect of ACE on the serum chemistry was also evaluated to check toxicity of plant extract if any. The results were compared with standard medicine (Baycox®). On the basis of results, ACE showed anticoccidial activity by reducing fecal, lesion and oocyst scores in infected chicks ($P<0.05$). ACE also improved FCR of infected chicks. Moreover, ACE exhibited positive effects on serum chemistry of broiler chickens ($P<0.05$).

***Keywords*:** Resistance, Sporulated oocyst, *Eimeria*, Medicinal plant

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INTRODUCTION

Avian coccidiosis is caused by single cell parasitic protozoa of genus *Eimeria*, which belongs to Phylum Apicomplexa having seven types with multiple life cycle stages (Abbas *et al.*, 2015, 2019). Among all species pathogenic ones are *E. tenella* and *E. necatrix* that cause cecal coccidiosis in chickens. *Eimeria* species affect intestine of the bird and cause bloody diarrhea, poor Feed Conversion Ratio (FCR) and mortality which lead to heavy economic losses to poultry industry (Chapman, 2014; Shahid *et al.,* 2020; Khater *et al*., 2020). The coccidiosis infection starts with the ingestion of sporulated oocysts by the chicken from contaminated feed and water (Qamar *et al.,* 2020; Zhang *et al.,* 2020). Coccidian oocysts produce millions of oocysts by asexual and sexual multiplication within the host. So, it is not easy to protect the birds from infection (Fall *et al.*, 2016). Synthetic anticoccidial drugs are generally used to control coccidiosis. Sulfanilamide was the first anticoccidial drug that was used against coccidiosis in poultry birds. Later on, a variety of anticoccidial drugs has been developed to control and treat coccidiosis. However, due to the irrational use of anticoccidial drugs, resistance has been developed in *Eimeria* species (Abbas *et al.*, 2019). There is need of time to find out and explore some other novel agents and compounds for the sustainable effective control of coccidiosis. In the last decade, many plants and their compounds have been reported to have excellent potential as anticoccidials and immunomodulators (Khan *et al.,* 2017; Yi *et al.,* 2020). Use of antioxidants rich plants have gained special importance (Ijaz *et al.,* 2020; Masood *et al.,* 2020; Alshamiri *et al.,* 2021), therefore, plants rich in antioxidants (phenols, flavonoids, tannins and saponins) are being used as an alternative strategy to treat coccidiosis (Abbas *et al.* 2019). *Ageratum conyzoides* also called as goat weed bears various pharmacological properties against different parasitic and bacterial diseases of humans and livestock due to its various antioxidants and other useful compounds (Nweze and Obiwulu, 2009). On the basis of presented literature including antioxidant properties of various botanicals, present study was planned to assess the anticoccidial potential of *Ageratum conyzoides* against experimental *Eimeria* infection in chickens.

MATERIALS AND METHODS

Plant material

Leaves of *Ageratum conyzoides* were procured from local market. The plants material was dried out and then with the help of electric mill converted into powder form. Aqueous methanolic extract of *Ageratum conyzoides* (ACE) was prepared using Soxhlet's apparatus (Velp Italy) following method described by Abbas *et al.* (2017). The suspension was evaporated in rotary evaporator (Heidolph Germany) at 50°C. The prepared ACE was stored at 4°C in refrigerator after freeze drying until further use.

Parasite

Parasitic material (*Eimeria* oocysts) was collected from guts of naturally infected chicken and outbreak cases in Faisalabad. Sporulation of *Eimeria* oocysts was done in Potassium dichromate solution (2.5%) at 25-29°C under 60-80% humidity and examination was done following Ryley *et al.* (1976). The sporulated oocysts were counted by Modified McMaster Technique. The chambers of McMaster slide were filled with the sample and allowed to stand undisturbed for 2-3 minutes so that the sporulated oocysts float and come to same focus level. The slides were examined under microscope at low (10x) and high magnification (40x).

Experimental design

A total of 105 day old chicks broiler chicks were purchased and reared by adopting proper management practices. At seven days of age chicks were divided in 7 equal groups (A, B, C, D, E, F and G) having 15 birds in each group. Groups A, B and C were given (orally) different doses of ACE @ 100, 200 and 300 mg/kg of body weight. At 14th day of experiment, all groups were given (oral route) infection with 50,000 oocysts of mixed *Eimeria* species except group G which remained as un-infected, un-medicated control group. Group D was fed with Vitamin E (Aquasol E®) which was administered in water @ 87 mg/kg of body weight. Group E served as Baycox® treated group and Group F served as infected un-treated group. Baycox® (A&K Pharmaceuticals, Faisalabad) was administered in group E @ 1ml/l of water.

Evaluation of Anticoccidial Activity

Anticoccidial potential of ACE was evaluated on the basis of parameters such as FCR, lesion score, (Johnson and Reid 1970), oocyst score (Hilbrich 1978) and fecal score (Youn *et al.*, 1993).

Analysis of Serum Chemistry

To access toxicity of plant extract in infected chicks serum enzymes levels including Alanine trans-

ferase (ALT), Lactate dehydrogenase (LDH) and creatinine levels were carried out using the commercially available diagnostic kits (Merck, Germany) according to manufacturer instructions. (Abbas *et al.*, 2017, 2019).

Statistical Analysis

Statistical analysis was done by ANOVA and DMR tests using SAS (statistical analysis software) (SAS, 2004). The data were considered statistically significant with *P* value <0.05.

RESULTS

All the groups administered with ACE showed the improvement in FCR in dose dependent manner. There was no statistical analysis for FCR due to group feeding of birds. The group which was administered with higher dose of ACE exhibited better FCR as shown in Table 1.

Lesion scores of different groups have been shown in Table 2. The groups treated with ACE showed the reduced lesion score in infected groups in dose dependent manner. Lesion score of group treated with higher dose was non significantly different to Baycox® and Vitamin E (P>0.05) and were significantly different to infected non medicated group (P<0.05).

Table 1: Feed Conversion Ratio (FCR) of different treated groups

Groups	Feed consumed	Weight gain	FCR
A. conyzoides @ 100 mg/kg	1632.11	620.11	2.63
A. conyzoides @ 200 mg/kg	1660.20	660.30	2.51
A. conyzoides @ 300 mg/kg	1644.18	706.10	2.32
Vitamin E	1602.19	761.13	2.10
Baycox®	1594.15	780.12	2.04
Infected, non-medicated	1690.14	581.70	2.90
Non-infected, non-medicated	1801.12	900.42	2.00

*Because of group feeding statistical analysis was not achievable

Table 2: Lesion score of different treated groups (n=6)

Groups	0	+1	+2	+3	+4	Mean±SD
A. conyzoides@ 100 mg/kg	-	1	2	2	1	2.50±0.5^{b}
A. conyzoides@ 200 mg/kg	-	1	2	3	-	2.33±0.5^{b}
A. conyzoides@ 300 mg/kg	-	2	2	2	-	2.00±0.5^{c}
Vitamin E	-	3	1	2	-	1.83±0.5^{c}
Baycox®	-	3	2	1	-	1.66±0.5^{c}
Infected, non-medicated	-	-	1	2	3	3.33 ±0.5^{a}
Non- infected, non-medicated	-	-	-	-	-	-

Means with the different letters (a, b, c) are significantly different (*P*<0.05)

All ACE administered groups showed minimal oocyst score, however, the better results were observed in group treated with higher dose of ACE which were non significantly different to Baycox® and Vitamin E (P>0.05) as shown in Table 3.

Fecal scores of different groups are shown in Table 4. Minimal fecal score was observed in ACE treated groups. The fecal score of groups treated with higher dose of ACE was non significantly different to Baycox® and Vitamin E (P>0.05) and were significantly different to infected non medicated group (P<0.05).

Table 3: Oocyst score of different treated groups (n=6)

Groups	0	+1	+2	+3	+4	+5	Mean±SD
A. conyzoides@ 100 mg/kg			1	2	2	1	3.5±0.5^{ab}
A. conyzoides@ 200 mg/kg		1	1	2	2		2.83 ±0.75^{b}
A. conyzoides@ 300 mg/kg	1	1	3	1			1.6±0.5^{c}
Vitamin E	1	3	1	1			1.33±0.5^{c}
Baycox®	2	2	2				1.00±0.5c^{c}
Infected, non-medicated				2	2	2	4.00±0.50^{a}
Non- infected, non-medicated	-	-	-	-	-	-	-

Means with the different letters (a, b, c) are significantly different (P<0.05)

Table 4: Fecal score of different treated groups (n=6)

Groups	4th day	5th day	6th day
A. conyzoides@ 100 mg/kg	3.21±0.8^{b}	3.30±0.7^{a}	2.89±0.6^{b}
A. conyzoides@ 200 mg/kg	3.15±0.5^{b}	3.22±0.7^{a}	1.90±0.5^{bc}
A. conyzoides @ 300 mg/kg	3.00±0.5^{bc}	3.08±0.75^{bc}	1.50±0.6^{c}
Vitamin E	2.01±0.8^{bc}	2.00±0.5^{bc}	1.52±0.5^{c}
Baycox®	1.71±0.8^{c}	1.66±0.5^{c}	1.37±0.5^{c}
Infected, non-medicated	3.80±0.44a	3.89±0.5^{a}	3.00±0.88^{a}
Non- infected, non-medicated	0.0±0.1^{d}	0.0±0.0^{d}	0.0±0.0^{d}

Means with the different letters (a, b, c) are significantly different (P<0.05)

ACE treated groups showed the improvement in serum chemistry of infected groups. Mean serum enzyme values i.e. ALT, LDH and Creatinine of ACE treated groups at higher dose were non significantly different to Baycox® and Vitamin E (P>0.05) and were significantly different to infected non medicated group (P<0.05) as shown in Table 5. In current study, lower serum enzyme values ALT, LDH and creatinine were observed in ACE treated groups as compared to control groups suggesting that the plant extract was nontoxic. Stabilization of ALT and LDH levels may indicate that the extracts are not hepatotoxic. The significant decrease in serum creatinine levels across different doses may suggest that extract are not nephrotoxic.

Table 5: Serum enzymes values of different treated groups (n=4)

Groups	ALT	LDH	Creatinine
A. conyzoides@ 100 mg/kg	14.90 ± 1.23^{b}	575.76 ±16.74^{b}	0.36 ±0.03^{a}
A. conyzoides@ 200 mg/kg	12.90 ± 1.71^{b}	556.91±18.30^{b}	0.28 ±0.04^{b}
A. conyzoides@ 300 mg/kg	12.80 ±1.14^{c}	533.03 ±24.35^{c}	0.26 ±0.03^{c}
Vitamin E	12.01 ±1.04^{c}	524.51 ±21.13^{c}	0.18 ±0.03^{c}
Baycox®	11.50 ±1.11^{c}	518.50 ±20.13^{d}	0.17 ±0.01^{c}
Infected, non-medicated	25.61 ± 2.32^{a}	889.94 ±22.16^{a}	0.54 ±0.03^{a}
Non- infected, non-medicated	8.63.08±1.8^{d}	494.43±12.66^{d}	0.11±0.07^{d}

Means with the different letters (a, b, c) are significant different (P>0.05) ALT (Alanine transaminase), LDH (Lactate dehydrogenase)

DISCUSSION

Several scientific reports have shown that plants and their antioxidant compounds (phenols, flavonoids, tannins and saponins) are being used as an alternative strategy for improving the health status of the animals (Jang *et al.*, 2007; Molan *et al.,* 2009; Abbas *et al.,* 2019; Raheel *et al*., 2019; Elghobashy *et al*., 2020; Hassan *et al*., 2020; Juman *et al*., 2020; Khaskheli *et al*., 2020; Ragab *et al*., 2020; Zhang *et al.,* 2020). On the basis of results of current research it can be concluded that *Ageratum conyzoides* has anticoccidial activity as positive effects were seen on different parameters including lesion, oocysts, fecal score and. Results were non-significantly different to standard medicine (Baycox®) and Vitamin E (P>0.05). ACE also improved FCR of infected chicks. Results of this study revealed that ACE @ 100, 200, 300 mg/kg showed anticoccidial activity in dose dependent

manner against mixed *Eimeria* infection. Similar type of anticoccidial effects have also been reported in previous studies on the evaluation of anticoccidial potential of different botanical extracts (Dkhil *et al.,* 2011; Yang *et al.,* 2015; Wang *et al.*, 2016). In one study, ethanolic extract from *Carica papaya* (leaves) showed remarkable positive effect on the weight gain in broiler chickens infected with *Eimeria* (Nghonjuyi *et al*., 2015).

In another study, ACE extract was administered orally to chicks and administration of ACE showed positive effect on red blood cells count, white blood cells and packed cell volume in challenged treated birds. Thus ultimately oocyst excretion and level of infection was also reduced (Nweze and Obiwulu, 2009). Likewise, the *in vivo* anticoccidial potential of *Trachyspermum ammi* (ajwain) and its effect on serum chemistry has also been reported in broiler chickens. Supplementation of *Trachyspermum ammi* into the feed of broiler reduced lesion, oocyst and fecal scores, and improved serum chemistry of birds (Abbas *et al.,* 2019). Abbas *et al.* (2017) has also reported similar type of anticoccidial effects of *Beta vulgaris* (sugar beet) extract in broiler chicks. *Beta vulgaris* extract reduced the lesion score, oocysts count and also improved hematological parameters of birds.

In another recent study, oral treatment with methanolic leaf extract of *Lannea schimperi* reduced the oocysts shedding and growth of *Eimeria tenella* in experimenatlly infected chickens and concluded that *Lannea schimperi* possess anticoccidial potential against cecal coccidiosis (Mikail *et al.,* 2019).

Ageratum conyzoides is enriched with various antioxidant compounds which include phenols, flavenoids, conyzorium, methexnebilitin and quercetin. Antioxidants compounds of *A. conyzoides* may decrease the oxidative stress level which is produced due to *Eimeria* parasite (Nweze and Obiwulu 2009). The anticoccidial potential of *A. conyzoides* on different paramters can be due to the action of different active and antioxidant compounds contained *A. conyzoides* which can reduce *Eimeria* infection level by interfering with lipid peroxidation process.

In current study, stability in different tested serum enzyme values indicates that the extract has non toxic effects on liver and kidney of birds. ALT, LDH and creatinine refer to hepatotoxicity while serum creatinine and urea refer the nephrotoxicity. So, on the basis of above mentioned studies it may be concluded that plants derived extracts like *Ageratum conyzoides* may be helpful in controlling coccidiosis and toxicity in chicken.

CONCLUSION

Present study reports the anticoccidial activities of *A. conyzoides* extract in broiler chickens. *A. conyzoides* showed anticoccidial activity against coccidiosis in dose dependent manner. It suggests that *A. conyzoides* derived compounds may be helpful in treatment and controlling coccidiosis in chicken. Further studies, are needed to characterize the active compounds of *A. conyzoides* which are involved in enhancing the anticoccidial potential against avian coccidiosis.

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Research article
Ερευνητικό άρθρο

Differences in antimicrobial resistance of *Salmonella* spp. isolated from humans, animals and food products in Kazakhstan

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ABSTRACT: The aims of this study were twofold: 1. to isolate *Salmonella* spp. from different sources in Kazakhstan, to determine their serovars and resistance profiles and 2. to evaluate similarities in antimicrobial susceptibility of *Salmonella* spp. isolated from humans, animals and food products. From the10 212 samples tested *Salmonella* spp. were isolated in 47 cases. The predominant serovar isolated from humans and food products was *S.* Enteritidis. Although different animal species were tested the *Salmonella* spp. were isolated mainly from chickens and ducks. *S.* Enteritidis and *S.* Typhimurium were the most prevalent serovars in raw poultry meat. The most frequent resistances were those to nalidixic acid, ampicillin and tetracycline. Human isolates demonstrated lower resistance compared to animal and food isolates. The genes encoding antimicrobial resistance in human isolates in most cases were absent except for some isolates which harboured *tet*, *sul*, *stre* and *bla* TEM genes encoding resistance to the oldest antimicrobial classes. The situation in animals and food products was less beneficial as besides different genes, integrons associated with the horizontal gene transfer were detected. The findings suggest that antimicrobials in animal sector should be used more strictly, paying attention to critically important antimicrobials for humans and possible horizontal transfer of pathogens and their genetic determinants.

Keywords: *Salmonella* Paratyphi, One Health, salmonellosis, zoonoses

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INTRODUCTION

Salmonella spp. are one of the most frequently isolated foodborne pathogens. It is a major worldwide public health concern, accounting for 93.8 million foodborne illnesses and 155,000 deaths per year (Eng et al., 2015). Gastroenteritis is the most common manifestation of *Salmonella* infection, followed by bacteraemia and enteric fever (Majowicz et al. 2010). Non-typhoidal *Salmonella enterica* infection,-caused by different serovarsfrom contaminated food, is an important cause of both sporadic gastroenteritis and outbreaks internationally. In the European Union, over 91,000 human cases are reported each year (EFSA, 2018). In Australia, the incidence of infection due to *Salmonella* spp. in the community is estimated to be 185 infections per 100,000 population per year (Kirk et al., 2014). Enteric fever that is caused by *Salmonella enterica* Typhi causes illness in south-central Asia, Southeast Asia, and southern Africa by more than 100 cases per 100,000 person-years) (Marks et al., 2017). It is also prevalent in many other parts of the world. For instance, approximately 200 to 300 cases of *S.* Typhi were reported in the United States each year (Lynch et al, 2009).

Epidemiological data and incidence cases of salmonellosis are registered in many countries including the USA, EU, China, Australia, India and others while some countries still are white spots in the map according to the prevalence of salmonellosis (Eng et al., 2015). In Kazakhstan the incidence of diagnosed salmonellosis in 1999 and 2000 was about 3500-4000 cases per year, while typhoid fever incidence was about 30-110 per year (WHO 2001).

Main factors associated with *Salmonella* outbreaks include incomplete cooking of food products, improper storage and direct contact with raw ingredients (Lynch et al. 2006). The food products that are predominantly associated with the outbreaks include animal products such as milk, poultry and eggs, as well as food products such as chocolate and peanut butter (Eng et al., 2015). Animals are the main carriers and reservoir of non-typhoid *Salmonella*. The transmission of *Salmonella* usually occurs through gastrointestinal tract by ingestion of food or water contaminated with infected animals. Direct contact with infected animals also constitutes a risk of infection (Swanson et al., 2007). The worldwide incidence rate of *Salmonella* infection is high as the strains can be found naturally in the environment and in both domestic and wild animals including cats, dogs, amphibians, reptiles and rodents (Eng et al., 2015). The diversity of possible reservoirs of infection results in significant challenges for public health authorities to control the infections (Swanson et al. 2007; Dione et al. 2011).

Salmonella resistance to at least 3 antibiotics started to be reported in 1960s (Dyson et al. 2019). Since then, the frequency of resistant isolates has increased in many countries; however, the spectrum of resistance or the rate of multidrug resistant isolates depends on country, source of isolation and *Salmonella* serovar. For instance, in the USA *Salmonella* spp. recovered from poultry were resistant to streptomycin (30.9%), gentamicin (12.6%), sulfadimethoxine (20.9%), tetracycline (13.9%), and trimethoprim-sulfamethoxazole combination (8.6%). Among these isolates, 20% were multi-drug resistant, showing resistance to three or more antibiotics; noteworthy is that 67% of *S.* Heidelberg and 54% of *S.* Kentucky isolates have shown resistance to five or more antibiotics (Liljebjelke et al., 2017). In China high rates of resistant *salmonellae* from chicken were found to sulfisoxazole (76.1%), tetracycline (75.3%), ampicillin (48.0%), and ofloxacin (44.7%). Notably, antimicrobial susceptibility tests have identified resistance to polymyxin B (2.0%) and imipenem (0.5%) (Zhang et al., 2018). In Poland the resistance of salmonella isolated from non-meat food products was low with the highest prevalence to nalidixic acid (35.2%), sulphonamides (6.6%), ampicillin (4.9%), amoxicillin/clavulanic acid (2.5%), streptomycin, cefoxitin, gentamicin and tetracycline (1.6%) (Mąka et al., 2015). Although an official data about salmonellosis prevalence in Kazakhstan is collected,there is no current systematic data about the situation of antimicrobial resistance of the strains that are prevalent in humans and in other sources including animals, food products or feedstuffs. Thus, the aims of this study were twofold: 1. to isolate *Salmonella* spp. strains from different sources in Kazakhstan, to determine their serovars and resistance profiles and 2. to evaluate similarities among antimicrobial susceptibility of *Salmonella* spp. isolated from humans, animals and food products.

MATERIALS AND METHODS

Place and sampling

Bacteriological investigations were performed at the Institute of Applied Biotechnology, Kostanay University during 2018-2019. The aim was to obtain wide spectrum of *Salmonella* isolates prevalent in Ka-

zakhstan. Animal carcasses and raw meat including poultry, lamb, beefwere collected in supermarkets and markets at a retail level. Clinical and pathological material of animals was collected at slaughterhouses and animal farms. *Salmonella* spp. strains already isolated from humans (in hospitals and other health-care facilities) were delivered to the department of Microbiology of Kostanay University as isolates without the data about their concrete source (name of the patient) of isolation. Ready to use, raw and frozen food products were obtained at retail markets and supermarkets. In total 10212 samples were tested from which 1006 samples were from live animals, animal carcasses and milk, 383 food samples and 8823 human samples.

Isolation and identification of *Salmonella*

Isolation of *Salmonella* was performed according to EN ISO 6579-1 (ISO, 2017) procedure for *Salmonella* detection. Xylose Lysine Deoxycholate (XLD) agarand Salmonella Shigella (SS) Agar (Oxoid, UK) were used as plating media after the enrichment procedure. Isolates obtained from health-care facilities were re-cultivated on XLD agar.

Identification of the isolates was performed using classical biochemical testing including fermentation of carbohydrates, production of hydrogen sulfide, indole, production of lysine decarboxylase, urease, oxidase, catalase, MR-VP as well as other conventional tests.

Serotyping was performed using slide agglutination test with sera to O and H antigens (Petsal, Russia) as well as Wellcolex Colour salmonella serogroup identification latex test (Remel, UK) according to the manufacturer's instructions.

Susceptibility testing

Antimicrobial susceptibility testing was performed using the disc diffusion method according to Kirby-Bauer (Hudzicki, 2009). The following discs with antimicrobials (μg) (Thermo Fisher, UK) were used: ampicillin (10), cefoperazone (75), cefoxitin (30), streptomycin (10), kanamycin (30), gentamicin (10), tetracycline (30), doxycycline (30), chloramphenicol (30), ciprofloxacin (5), nalidixic acid (30), gemifloxacin (5), enrofloxacin (5), ofloxacin (5) and sulfamethoxazole-trimethoprim (25). Interpretation of the results was carried out using CLSI clinical breakpoints (CLSI, 2019) set for *Salmonella* except for ofloxacin, gemifloxacin and nalidixic acid for which breakpoints set for Enterobacteriaceae were used.

Detection of the genes encoding antimicrobial resistance

Molecular testing of the isolates was performed at the Microbiology and Virology Institute, Lithuanian University of Health Sciences. DNA material for molecular testing was obtained after bacterial lysis according to the extraction protocol prepared by the EU Community Reference Laboratory for Antimicrobial Resistance with slight modifications (Ruzauskas et al., 2014). Detection of the genes encoding antimicrobial resistance was performed by PCR. Annealing temperatures and oligonucleotides used are presented in Table 1.

Table 1. Antimicrobial resistance genes tested and oligonucleotide primers used in the study

Primer name	Sequencece(5'-3')	Size, bp and t (°C)	Target gene	Source
*bla*TEM-F	GAGTATTCAACATTTTCGT	857 (50)	*tem*	Maynard et al., 2003
*bla*TEM-R	ACCAATGCTTAATCAGTGA			
*bla*SHV-F	TCGCCTGTGTATTATCTCCC	768 (60)	*shv*	Ojdana et al., 2014
*bla*SHV-R	CGCAGATAAATCACCACAATG			
*oxa*1-F	TCAACAAATCGCCAGAGAAG	276 (55)	*oxa* group I	Bert et al., 2002
*oxa*1-R	TCCCACACCAGAAAAACCAG			
*oxa*group 5-F	AGCCGCATATTTAGTTCTAG	644(56)	*oxa* group V	Bert et al., 2002
*oxa*group 5-R	ACCTCAGTTCCTTTCTCTAC			
*ctx*M-F	ATGTGCAGYACCAGTAARGT	593 (50)	*ctxM*	Pagani et al., 2003
*ctx*M-R	TGGGTRAARTARGTSACCAGA			
ctxM group 2-F	ATGATGACTCAGAGCATTCGCCGC	876 (56)	*ctxM2*	Celenza et al., 2006
ctcM group 2-R	TCAGAAACCGTGGGTTACGATTTT			
*cmy*2-F	GCACTTAGCCACCTATACGGCAG	758 (58)	*cmy*	Hasman et al., 2005
*cmy*2-R	GCTTTTCAAGAATGCGCCAGG			

PER-1-F	ATGAATGTCATTATAAAAGCT	927 (48)	*per*	Celenza et al., 2006
PER-1-R	TTAATTTGGGCTTAGGG			
PER-2-F	ATGAATGTCATCACAAAATG	927 (49)		Celenza et al., 2006
PER-2-R	TCAATCCGGACTCACT			
*tet*A-F	GTGAAACCCAACATACCCC	888 (55)	*tet*A	Maynard et al., 2003
*tet*A-R	GAAGGCAAGCAGGATGTAG			
*tet*B-F	CCTTATCATGCCAGTCTTGC	774 (55)	*tet*B	Maynard et al., 2003
*tet*B-R	ACTGCCGTTTTTTCGCC			
aadB-F	ATGGACACAACGCAGGTCGC	534 (55)	*aad*B	Asadollahi et al., 2012
aadB-R	TTAGGCCGCATATCGCGACC			
*aad*A-F	GTGGATGGCGGCCTGAAGCC	528 (68)	*aad*A	Asadollahi et al., 2012
*aad*A-R	AATGCCCAGTCGGCAGCG			
*rmt*B-F	ATGAACATCAACGATGCCCT	769 (55)	*rmt*B	Yan et al., 2004
*rmt*B-R	CCTTCTGATTGGCTTATCCA			
*arm*A-F	CAAATGGATAAGAATGATGTT	774 (55)	*arm*A	Galimand et al., 2003
*arm*A-R	TTATTTCTGAAATCCACT			
*aph*A1-F	AAACGTCTTGCTCGAGGC	500 (55)	*aph*A1	Frana et al., 2001
*aphA*1-R	CAAACCGTTATTCATTCGTGA			
*aacA*4-F	ATGACTGAGCATGACCTTGCG	487 (55)	*aacA4*	Odumosu et al., 2015
*aacA*4-R	TTAGGCATCACTGCGTGTTCG			
aac(3)II-F	TGAAACGCTGACGGAGCCTC	369 (65)	*aac(3)II*	Sandvang et al., 2009
aac(3)II-R	GTCGAACAG GTAGCACTGAG			
*str*A-F	CCTGGTGATAACGGCAATTC	546 (55)	*str*A	Lanz et al., 2003
*str*A-R	CCAATCGCAGATAGAAGGC			
*str*B-F	ATCGTCAAGGGATTGAAACC	509 (55)	*str*B	Lanz et al., 2003
*str*B-R	GGATCGTAGAACATATTGGC			
*cat*II-F	ACACTTTGCCCTTTATCGTC	495 (55)	*cat*II	Vassort-Bruneau et al., 1996
*cat*II-R	TGAAAGCCATCACATACTGC			
*cml*A-F	TTGCAACAGTACGTGACAT	293 (55)	*cml*A	Keyes et al., 2000
*cml*A-R	ACACAACGTGTACAACCAG			
*sul*1-F	TTCGGCATTCTGAATCTCAC	822 (55)	*sul*1-F	Christabel et al., 2012
*sul*1-R	ATGATCTAACCCTCGGTCTC			
*sul*2-F	CGGCATCGTCAACATAACC	722 (50)	*sul*2-F	Pereten et al., 2003
*sul*2-R	GTGTGCGGATGAAGTCAG			
*sul*3-F	GAGCAAGATTTTTGGAATCG	792 (51)	*sul*3-F	Pereten et al., 2003
*sul*3-R	CATCTGCAGCTAACCTAGGGCTTTGA			
dfr1-F	ACGGATCCTGGCTGTTGGTTGGACGC	254 (55)	*dfr*1	Gibreel et al., 1998
dfr1-R	CGGAATTCACCTTCCGGCTCGATGTC			
dfr5-F	GCBAAAGGDGARCAGCT	394 (44)	*dfr*5	Seputiene et al., 2010
dfr5-R	TTTMCCAYATTTGATAGC			
dfrA7-F	AAAATTTCATTGATTTCTGCA	471 (44)	*dfr*7	Navia et al., 2003
dfrA7-R	TTAGCCTTTTTTCCAAATCT			
mcr1-F	CGGTCAGTCCGTTTGTTC	309 (58)	*mcr1*	Liu et al, 2015
mcr1-R	CTTGGTCGGTCTGTAGGG			
mcr2-F	TGTTGCTTGTGCCGATTGGA	567 (58)	mcr2	Xavier et al., 2016
mcr2-R	AGATGGTATTGTTGGTTGCTG			
qnrA-F	ATTTCTCACGCCAGGATTTG	516 (53)	qnrA	Robicsek et al., 2006
qnrA-R	GATCGGCAAAGGTTAGGTCA			
qnrB-F	GATCGTGAAAGCCAGAAAGG	469 (53)	qnrB	Robicsek et al., 2006
qnrB-R	ACGATGCCTGGTAGTTGTCC			
qnrS-F	ACGACATTCGTCAACTGCAA	417 (53)	qnrS	Robicsek et al., 2006
qnrS-R	TAAATTGGCACCCTGTAGGC			
qepA-F	CAGTGGACATAAGCCTGTTC	218 (60)	qepA	Liu et al., 2008
qepA-R	CCCGAGGCATAGACTGTA			
teg1-F	TTATTGCTGGGATTAGGC	164 (55)	integrase 1 class	Chen et al., 2013
teg1-R	ACGGCTACCCTCTGTTATC			
teg2-F	ACGACATTCGTCAACTGCAA	233 (50)	integrase II class	Goldstein, 2001
teg2-R	TAAATTGGCACCCTGTAGGC			

Table 2. Characteristics of *Salmonella* isolated from humans in Kazakhstan

Isolate number	Serovar	Phenotypic Resistance	Genotypic Resistance	Integrons
733	*S.* Tshiongwe	CIP		-
735	*S.* Enteritidis	NAL, CIP		-
737	*S.* Blegdam			-
739	*S.* Blegdam			-
990	*S.* Enteritidis	NAL, ENR		-
991	*S.* Enteritidis	CIP		-
993	*S.* Enteritidis	TET		-
994	*S.* Enteritidis	STR, DOX	*strB*	-
998	*S.* Enteritidis	ENR, NAL, CIP		-
999	*S.* Enteritidis	NAL, ENR, CIP, GEMI,		-
1106	*S.* Enteritidis	NAL, CIP		-
1107	*S.* Enteritidis			-
1108	*S.* Enteritidis			-
1109	*S.* Enteritidis			-
1110	*S.* Enteritidis			-
1111	*S.* Enteritidis	NAL, CIP		-
1112	*S.* Enteritidis			-
1113	*S.* Enteritidis	TET	*tet B*	-
1205	*S.* Enteritidis	NAL, ENR, CIP, GEMI		-
1206	*S.* Enteritidis	AMP, TET, DOX, NAL, ENR, CIP, OFX, SXT	*blaTEM, tet A, tet B, sul3*	+ (class I)
1280	IIIc 4p	NAL, ENR, CIP		-

NAL - nalidixic acid; CIP - ciprofloxacin; ENR - enrofloxacin; TET - tetracycline, DOX - doxycycline; GEMI - gemifloxacin; AMP - ampicillin; OFX - ofloxacin; STRE - streptomycin; SXT - sulfamethoxazole/trimethoprim

Table 3. Characteristics of *Salmonella* isolated from animals in Kazakhstan

Serovar	Source of Isolation	Phenotypic Resistance	Genotypic resistance	Integrons
*S.*Typhimurium	duck	STR, KAN, ENR		-
*S.*Typhimurium	duck	STR, CHL, TET, ENR,		-
S. Typhimurium	chicken			-
S. Typhimurium	chicken			-
S. Paratyphi C	chicken	CHL, TET, DOX, NAL, CIP, SXT	*tetA, sul3, cmlA, catII*	+ (class I)
*S.*Enteritidis	chicken	NAL, CIP		-
*S.*Enteritidis	chicken	NAL, CIP		-
*S.*Enteritidis	chicken	AMP, STR, SXT	*Bla TEM, aadA, dfr1*	-
*S.*Enteritidis	chicken	STR, TET, DOX, ENR, CIP, GEMI, NAL, SXT	*tet A, sul 2, dfr1, strA, str B*	-
*S.*Enteritidis	chicken	ENR, NAL, CIP		-
*S.*Enteritidis	chicken	AMP, STR, ENR	*bla TEM, aacA4,*	-
*S.*Enteritidis	chicken	NAL, ENR, CIP		-
*S.*Moscow	chicken	NAL, ENR, CIP		-
S. Paratyphi C	chicken (imported)	FOX, STR, TET, DOX, NAL, ENR, CIP, OFX, GEMI	*aadA, tet A, qnr B*	+ (class I)

NAL - nalidixic acid; CIP - ciprofloxacin; ENR - enrofloxacin; TET - tetracycline, DOX - doxycycline; GEMI - gemifloxacin; AMP - ampicillin; OFX - ofloxacin; SXT - sulfamethoxazole/trimethoprim; CHL - chloramphenicol; STR - streptomycin; KAN - kanamycin

Table 4. Characteristics of *Salmonella* isolated from food products in Kazakhstan

Serovar	Source of isolation	Phenotypic Resistance	Genotypic resistance	Integrons
S. Enteritidis	Roasted chicken drumstics			-
S. Enteritidis	Roasted chicken drumstics	NAL, ENR, CIP		-
S. Enteritidis	Sausage	NAL, ENR, CIP		-
S. Enteritidis	Cake			-
S. Enteritidis	Ice-cream	NAL, ENR, CIP		-
S. Enteritidis	Cake	NAL, ENR, CIP		-
S. Enteritidis	cake		tet B	-
S. Enteritidis	Refrigerated chicken drumstics	AMP, STR, KAN, TET, DOX, ENR		-
S. Enteritidis	Sausage	AMP, STR, KAN, CHL, DOX, ENR		-
S. Enteritidis	Refrigerated chicken drumstics	AMP,STR, KAN, CN, TET, DOX		-
Salmonella group C	Force meat	AMP, STR, CHL, TET, DOX, NAL, ENR, CIP, OFX, GEMI, SXT	aadB, tet A, tet B, sul3, catII, qnrA	+ (Class I)
S. Tennessee	Meat in paste	AMP, CFP, KAN, CHL, TET, DOX, NAL, ENR, CIP, SXT	ctxM, aphA1, aadA, tet A, sul3, cmlA, catII	+ (ClassI, Class II)

NAL - nalidixic acid; CIP - ciprofloxacin; ENR - enrofloxacin; TET - tetracycline, DOX - doxycycline; GEMI - gemifloxacin; AMP - ampicillin; CN - gentamicin; OFX - ofloxacin; SXT - sulfamethoxazole/trimethoprim; CHL - chloramphenicol; STR - streptomycin; KAN - kanamycin; CFP - cefpodoxime

Data analysis

Occurrence of multi-resistant isolates was calculated by dividing the number of isolates resistant to at least 3 antimicrobial classes by the total number of obtained isolates. Statistical analysis was performed using IBM SPSS Statistics package, version 20.

Comparison between categorical variables was calculated using the chi-square test. Results were considered statistically significant if $P \leq 0.05$.

RESULTS

From total 10 212 samples tested (1006 samples from live animals, animal carcasses and milk; 383 food and 8823 human samples)*Salmonella* were isolated in 47 cases (0.46%). Twenty one (21) isolates (0.24%) were obtained from humans, fourteen (14) (1.39%) - from live or slaughtered animals and twelve (12) isolates (3.13 %) - from ready to use, raw or refrigerated food products (Tables 2-4).

As it could be seen from Table 2, the predominant *Salmonella* serovar isolated from humans was *S.* Enteritidis (81% from all isolates). Susceptibility varied among the strains: seven isolates were susceptible to all tested antimicrobials while a single isolate was resistant to eight (8) of the tested antimicrobials. The most frequent resistance was towards nalidixic acid, fluoroquinolones and tetracyclines. The prevalence of the genes encoding resistance was low. Integrons were detected in a single multi-resistant isolate of *S.* Enteritidis.

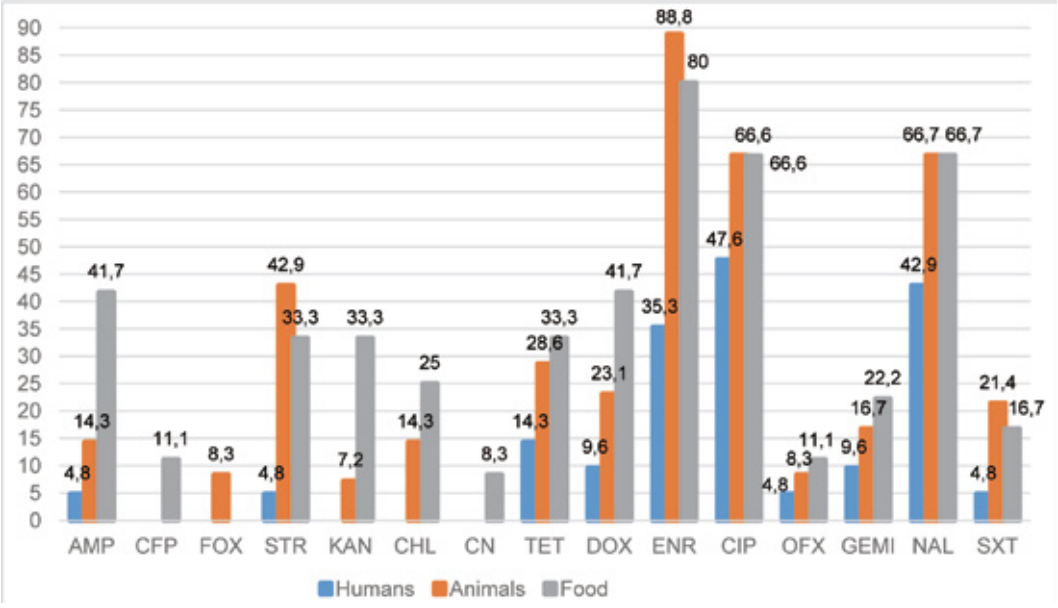
Although different animal species were tested the *Salmonella* were isolated only from chickens and ducks (Table 3). The serovar variety in poultry was higher to those of humans. The predominant serovar were *S.* Typhimurium and *S.* Enteritidis. Two isolates were identified as *S.* Paratyphi C. One of those isolates was resistant to tetracyclines, nalidixic acid, ciprofloxacin, chloramphenicol, sulfonamides and trimethoprim and harboured class I integrons while the other - to 2d generation cephalosporins, streptomycin, tetracyclines and all fluoroquinolones tested. Two (2) strains out of fourteen (14) isolated from animals were susceptible to all antimicrobials tested while six (6) isolates were multi-resistant.

S. Enteritidis was the predominant serovarin food products (81% from all isolates) however, two other serovars- (*Salmonella*group C and *S.* Tennessee) had much wider spectrum of resistance. Five (5) isolates out of twelve (12)of food origin were multi-resistant, two (2) strains were susceptible to all antimicrobials and four (4) had similar resistance patterns being resistant only to (fluoro)quinolones. The isolate of *S.* Tennessee was a single strain harboured integrons of both I and II class.

The frequency of antimicrobial resistance in *Sal-*

monella isolates obtained from different sources is presented in Fig 1.

The most frequent resistance among *Salmonella* isolates was demonstrated to fluoro(quinolones), ampicillin, streptomycin and tetracycline. Resistance to critically important antimicrobials for humans was also detected and varied depending on the source of isolation. Human isolates demonstrated lower resistance compared to animal and food isolates. Food isolates were more frequently resistant to antimicrobials compared to animal isolates except for streptomycin and enrofloxacin which were more frequently non effective against animal isolates and for nalidixic acid for which the number of resistant isolates from animals and foods had the same range. The correlation between the strains isolated from different sources regarding their resistances to separate antimicrobials is presented in Table 5.



NAL - nalidixic acid; CIP - ciprofloxacin; ENR - enrofloxacin; TET - tetracycline, DOX - doxycycline; GEMI - gemifloxacin; AMP - ampicillin; CN - gentamicin; OFX - ofloxacin; SXT - sulfamethoxazole/trimethoprim; CHL - chloramphenicol; STR - streptomycin; KAN - kanamycin; FOX - cefoxitin; CFP - cefpodoxime

Fig 1. Antimicrobial resistance (% of resistant isolates) of *Salmonella* isolated from humans, animals and food products

Table 5. Statistical differences among resistances in *Salmonella* isolates from different sources

Antibiotics	Human vs Animals			Human vs Food			Animals vs Food		
	Value	df	P - value	Value	df	P - value	Value	df	P - value
Ampicillin	0.972	1	0.324	6.991	1	**0.008**	2.462	1	0.117
Cefoperazone	constant			2.414	1	0.120	1.400	1	0.237
Cefoxitin	1.805	1	0.179	constant			0.788	1	0.375
Streptomycin	10.862	1	**0.001**	7.966	1	**0.005**	0.248	1	0.619
Kanamycin	1.544	1	0.214	7.966	1	**0.005**	2.854	1	0.091
Gentamicin	constant			1.805	1	0.179	1.213	1	0.271
Levomycetin	3.182	1	0.074	5.775	1	**0.016**	0.478	1	0.490
Tetracycline	2.146	1	0.143	2.910	1	0.088	0.069	1	0.793
Doxycycline	1.176	1	0.278	4.721	1	**0.030**	0.991	1	0.319
Enrofloxacin	6.801	1	**0.009**	5.040	1	**0.025**	0.281	1	0.596
Ciprofloxacin	0.638	1	0.424	0.524	1	0.469	0.000	1	1.000
Ofloxacin	0.171	1	0.679	0.408	1	0.523	0.046	1	0.830
Gemifloxacin	0.366	1	0.545	0.879	1	0.348	0.103	1	0.748
Nalidixic acid	1.733	1	0.188	1.429	1	0.232	0.000	1	1.000
Sulfamethoxazole/ trimethoprim	2.305	1	0.129	1.310	1	0.252	0.094	1	0.759

DISCUSSION

This study describes the prevalence of *Salmonella* spp. in different sources in Kazakhstan. Up to date only the data from humans were collected by national and international responsible institutions within the country. Salmonellosis is one of the leading causes of foodborne infections in humans with animals being reservoirs of *Salmonella*. *Salmonella* colonizes the gastrointestinal tract of food animals (Andino et al., 2015) and is shed via feces (Narváez-Bravo et al., 2013). Although the clinical manifestation of salmonellosis in different food-producing animal species can be unequally expressed, all animal species can be a source of foodborne infection (Chaney et al., 2017; Evangelopoulou et al., 2015; Pande et al., 2016).

Knowledge ofthe prevalence and diversity of *Salmonella* serovars in animals and food can provide important information necessary to develop preventive measures and strategies at different stages of the food chain such as application hazard analysis and critical control programs in meat production industries to ensure food safety (Tietjen and Fung, 1995; Gutema et al., 2019). According to this study the most prevalent serovar of *Salmonella* in Kazakhstan was *S.* Enteritidis which was the most frequently detected both in humans (81%) and food products (83%). According to the World Health Organization, *S.* Enteritidis is one of the most frequently isolated *Salmonella* serovars (along with *S.* Typhimurium) from countries involved in the Global Foodborne Infections Network (Hendriksen et al., 2011). The other serovars isolated from humans included *S.* Blegdam and *S.* Tshiongwe. *S.* Blegdam which is genetically similar to *S.* Enteritidis was isolated for the first time in the USA in the middle of last century from a patient suffering with the enteric type of fever and extensive erythema during the fifth week after onset (Holt and Newton, 1948). There is a lack of data about its prevalence during past decades, although *S.* Blegdam was recently isolated from broilers in Egypt (Ammar et al., 2019). *S.* Tshiongwe was described as a pathogenic serovar for both humans and animals and was also isolated from foods (Thong et al., 2004; Mshelbwala et al., 2017). This study demonstrated that the variety of serovars prevalent in animals in Kazakhstan was not wide; besides *S.* Enteritidis, which prevalence was 50% of all serovars, *S.* Typhimurium (29%) was also common. This serovar was recovered from poultry. The disease caused by *S.* Typhimurium is of public health significance, as it is associated with food poisoning in humans (Dar et al., 2017). The other highly pathogenic to man serovar-*S.* Paratyphi C was isolated from chicken carcasses in two cases. This serovaris associated with human disease although the carriers can be different animal species (ECDC, 2019). Although paratyphoid fever is a rare disease in Europe and the USA it is still prevalent in Asia (Ekdahl et al., 2005).

Interesting data was also obtained according to the antimicrobial susceptibility testing: the isolates from humans were less frequently resistant to different antimicrobials comparing to animal and particularly food isolates, although it is known that salmonellosis is a foodborne human infection. All of the isolates obtained from humans, *in vitro* were susceptible to chloramphenicol, cephalosporins and aminoglycosides (except one isolate which was resistant to streptomycin), while the isolates from animals and foods had different susceptibility patterns. Although aminoglycosides are poorly effective against salmonellosis *in vivo* (Kihlstrom and Andaker, 1985), these antimicrobials are good indicators testing *in vitro* when comparing differences in antimicrobial susceptibility of different isolates, particularly isolated from different sectors. As it is important to understand the chain of salmonellosis “from farm to table” the data obtained in this study is interestingly enough demonstrating quite big differences among susceptibility in salmonella isolates from animals, foods and humans. The most frequent resistance in the isolates irrespective from the source of isolation was towards (fluoro) quinolones, aminopenicillins, streptomycin, tetracyclines and sulphamethoxazole/trimethoprim. These antimicrobials are frequently used both in humans and animals. Comparing the resistance between animal and food isolates the differences were less obvious except for beta-lactams and kanamycin with the highest resistance frequency being in food isolates. Isolates from animals had the highest resistance towards enrofloxacin compared to isolates from other sources. This is probably associated with the frequent use of enrofloxacin for animal treatment in Kazakhstan as it is known that broad use of fluoroquinolones has been followed by emergence of resistance to this class of antimicrobials (Hooper, 2001). Although all food products from which *Salmonella* were isolated contained ingredients of animal origin (meat, milk products or eggs) only a part of the products were originated from Kazakhstan while some of them were imported (data are not presented). Resistance to 3d generation cephalosporins was detected only in the isolates from foods but not from humans and animals, meaning that *salmonellae* circulating in Kazakh-

stan are quite specific and isolated. This fact is also proved by the low overall prevalence of *Salmonella* and by the poor variety of serovars circulating within the country. Imported food however, may change the situation in case of foodborne infections appear. The current situation regarding antimicrobial resistance in humans is beneficial with possibility to treat salmonellosis with antimicrobials of the 1st choice. The best known genes encoding antimicrobial resistance in human isolates mostly were absent except for some isolates which harboured *tet*, *sul*, *stre* and *bla*TEM genes encoding resistance to the oldest antibiotic classes. The situation in animals and food products was less beneficial as besides different genes, integrons associated with the horizontal gene transfer were detected more frequently. These findings suggest that antimicrobials in animal sector should be used more strictly particularly paying attention to critically important antimicrobials for humans and possible horizontal transfer of pathogens and their genetic determinants between environment (animals, food ingredients) and humans. Since multi-resistant highly pathogenic to humans serovars,as *S*. Paratyphi C, were detected in animal carcasses, monitoring of *Salmonella* prevalence in animals should be implemented on a regular basis and measures for *Salmonella* eradication should be foreseen.

In conclusion, food import is one of the key factors changing the *Salmonella* variety and its antimicrobial resistance patterns in Kazakhstan. Usage of enrofloxacin in veterinary medicine however, is the factor of increasing salmonella resistance to fluoroquinolones that may have negative impact on treatment of salmonellosis infections.

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CONFLICT OF INTERST

The authors declare no conflict of interest.

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Research article
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Effects of carprofen and/or CIDR administration on pregnancies per artificial insemination around pregnancy recognition in lactating dairy cows

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ABSTRACT: Effect of carprofen and/or CIDR on pregnancies per AI (P/AI) 14 days after artificial insemination (AI) in lactating cows were investigated. Following detection of corpus luteum (CL) by ultrasonography (USG) 14 days after AI, cows (*n*=853) were randomly allocated to CARP (carprofen, 1.4 mg/kg; Rimadyl®XL), CIDR (progesterone, 1.38g, for seven days), CARP+CIDR and CONT (control) groups. CL was re-determined with USG 21 days after AI to monitored the maintenance of CL. Rates of maintenance of CL did not differed among CARP (79.6% [168/211]), CIDR (86.0% [196/228]), CARP+CIDR (80.0% [172/215]) and CONT (74.9% [149/199]) groups; however, the interaction effect of treatment by body condition score (BCS) at AI ($P<0.05$) were existed. In this matter, among cows with BCS≤2.5, chances of maintenance of CL was lower in CONT (70.7%, [111/157]) group compared to those in CARP (81.7%, [125/153]), CIDR (83.6%, [153/183]) and CARP+CIDR (80.1%, [129/161]) groups. Whereas, among cows with BCS>2.5, chances of maintenance of CL were lower in CARP (74.1%, [43/58]) and CARP+CIDR (79.6%, [43/54]) groups compared to those in CONT (90.5%, [38/42]) and CIDR (95.6%, [43/45]) groups. For the P/AI at 28-32 days after AI, there was no difference among CARP (48.8% [103/211]), CIDR (50.9 [116/228]), CARP+CIDR (47.4% [102/215]) and CONT (44.7% [89/199]) groups. Pregnancy losses between 28-32 and 55-60 days did not differ among CARP (3.9%, [4/103]), CIDR (4.3%, [5/116]), CARP+CIDR (5.9%, [6/102]) and CONT (6.7%, [6/89]) groups. However, there was a significant ($P<0.05$) interaction effect of treatment by the number of services on pregnancy losses. In this regard, pregnancy losses were higher in cows inseminated thrice and four or more times in CONT (11.8% and 16.7%) group compared to those in CARP (0% and 4.8%), CIDR (0% and 6.9%), CARP+CIDR (0% and 11.1%); respectively. Consequently, no effects of carprofen or CIDR around pregnancy recognition on P/AI were observed despite a higher maintenance rate of CL in lactating cattle. Furthermore, fewer pregnancy losses in cows following three or more services could indicate the beneficial carry-over effects of carprofen and/or CIDR administration around maternal recognition of pregnancy.

Key words: Carprofen; cow; pregnancy; progesterone.

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INTRODUCTION

Over decades, lower conception rates have been reported in lactating dairy cows(Lucy, 2001; Santos et al., 2004; Demetrio et al. 2007).In this matter, almost 40% embryonic losses have been reported between days 8 and 17 of pregnancy (Thatcher et al., 1994). Similarly, approximately 30% of pregnancy losses have been reported in lactating dairy cowsfrom days 8 to 27 of pregnancy during which embryo elongation and maternal recognition of pregnancy occur (Wiltbank et al., 2016).

Sufficient progesterone (P4) secretion via maintenance of CLis crucial to advance early pregnancy in cows (Mann and Lamming, 1995; Okuda et al., 2002). Embryos produce a trophoblastic protein, IFN-Ƭ, to prevent luteolysis caused by a pulsatile release of PG-$F_{2\alpha}$ from the endometrium (Bazer, 1992;Demmers et al., 2001). In this matter, IFN-Ƭdiminishes the oxytocin-dependent pulsatile release of $PGF_{2\alpha}$ from the endometrium by suppressing oxytocin receptor expression; thereby, leading to maternal recognition of pregnancy between days 15 and 16 of pregnancy in cattle (Bazer et al., 1997; Farin et al., 1990; Thatcher et al., 1995).

Nonsteroidal anti-inflammatory drugs (NSAID), organic acids in the constitution, diminish the release of prostaglandins by blocking cyclooxygenase (COX) enzyme in the arachidonic acid cascade (Vane, 1971; Espinasse et al., 1994). As an NSAID, carprofen non-selectively inhibits COX-1 and COX-2 in cattle (Brentnall et al., 2012). In this regard, the effective suppression ofPGF2α release following a single dose of carprofen (0.7 mg/kg) administration has been reported in the postpartum cow (Thun et al., 1989).With regard to carprofen administration 15 days after artificial insemination (AI), no beneficial effect on conception rates to the first service was reported in lactating dairy cows (von Krueger and Heuwieser, 2010). Based on current literature, there were controversial results with regard to use of NSAID to increase pregnancies perartificial insemination (P/AI) in cattle. In this matter, Guzeloglu et al. (2007) reported higher P/AI following flunixin meglumine administration 15 and 16 days after AI in Holstein heifers. Similarly, flunixin meglumine administration approximately 14 days after AI just prior to transportation stress increased P/AIin beef cows regardless of transportation (Merrill et al., 2007). In contrast, no beneficial effects of two injections of flunixin meglumine 14/15 or 15/16 days (Von Krueger and Heuwieser, 2010), and 15.5 /16 days (Rabaglino et al., 2010) after inseminations on P/AIwere reported in Holstein heifers. Likewise, a single administration of flunixin meglumine approximately 13 days (10 to 15 days in range) after AI did not increase P/AI in beef cows and heifers (Geary et al., 2010). Moreover,Erdem and Guzeloglu (2010) reported lower P/AI upon meloxicam administration 15 days after AI in Holstein heifers.

Another strategy for the maintenance of early pregnancy is P4 supplementation during maternal recognition of pregnancy. Garcia-Ispierto et al. (2016) reported improvement in conception rates following P4 supplementation around the time of pregnancy recognition after AI in high-producing dairy cows. Nevertheless, Alnimer and Lubbadeh (2008) reported that pregnancy rates (PR) on day 28 did not differ; however, pregnancy losses between days 28 and 45 tended to be lower in lactating dairy cows following administration of CIDR between 14 and 21 days after AI. Likewise, El-Zarkouny and Stevenson (2004) reported that PR on day 29 did not differ; however, pregnancy losses between days 29 and 57 were lower in lactating Holstein cows following insertion of used CIDR between 13 and 20 days after AI. Similarly, increased PR due to reduced pregnancy losses were reported following CIDR insertion between 14 and 21 days after AI in lactating dairy cows (Chebel et al. 2006). No effects of CIDR insertion fromday 14 to 23 (Bartolome et. al., 2009) or from day 14 to 21 (Galvao et al., 2007) after AI were reported on PR or pregnancy losses in lactating dairy cows.

In this study, it was aimed to use a single administration of carprofen due to its longer lasting effect and poorly excretion in milk in cows (Lohuis et al., 1991; Ludwig et al., 1989). Moreover, there is no study testing the potential additive effect of P4 supplementation and NSAID administration around the time of pregnancy recognition on P/AI in lactating dairy cows. Therefore, objective of this study was to investigate effect of carprofen and/or CIDR administration 14 days after AI onP/AI in lactating dairy cattle.

MATERIAL AND METHODS

Animals and Treatments

This study was approved by Local Ethical Committee for Animal Experiments of Erciyes University, Kayseri, TURKEY. In four commercial dairy farms, lactating Holstein dairy cows were housed in free stall barns, milked two or three times a day. Cows were fed a TMR prepared according to the National Research

Council (NRC) recommendations (NRC, 2001), and the cows were fed twice or thrice per day. In one of the dairy farm, AI was done following the detection of natural estrus; whereas, AI was performed following synchronization of estrus by $PGF_{2\alpha}$ (Enzaprost-T®, Dinoprost tromethamine, 5 mL, Ceva Animal Health, Turkey) injection following detection of corpus luteum (CL) or Ovsynch protocol with the use of GnRH (Receptal®, Buserelin acetate, 2 mL, MSD Animal Health, Turkey) and $PGF_{2\alpha}$ (Enzaprost®) in two farms (selective protocol), and AI was performed at detection of synchronized estrus following intravaginal P4 (CIDR®;1.38 g P4, Zoetis Animal Health, Turkey) insertion for seven days and $PGF_{2\alpha}$(Dynolitic®, Dinoprost tromethamine, 5 mL, Zoetis Animal Health, Turkey) injection one day prior to CIDR removal or detection of natural estrus at the last farm. It means that this experiment was performed following either the first service or multiple services. After the last AI, no heat detection was performed during the study.

Following the detection of mature visible CL by ultrasonography (USG) 14 days after AI, lactating Holstein dairy cows (*n*=867)with CL and with/without postpartum health disorder prior to initiation of the study were randomly allocated to four groups in each farm. Due to missing data or health disorders including clinical mastitis, metritis, laminitis etc. during the study, 853 cows completed the experiment. In CARP group I (*n*=211), carprofen (1.4 mg/kg; Rimadyl®XL [5% carprofen], Zoetis Animal Health, TURKEY) was administered subcutaneously14 days after AI. In CIDR group (*n*=228), CIDR® was inserted intravaginally 14 days after AI and removed 21 days after AI for seven days. In the CARP+CIDR group (*n*=215), carprofen and CIDR were administered as in CARP and CIDR groups. No administration was done in CONT (*n*=199) as a control group. Cows were examined for the presence of visible CL by USG 21 days after AI to monitored the maintenance of CL. Pregnancies were diagnosed with USG and palpation per rectum 28-32, and 55-60 days after AI, respectively. Pregnancy losses between two pregnancy diagnosis by USG and rectal palpation were also determined. Body condition scores (BCS; 1-5 scale) were recorded at the last AI, 28-32 and 55-60 days after AI. BCS were accepted as poor if BCS <=2.5 or optimal if BCS is >2.5. Moreover, changes in BCS between AI and the first pregnancy diagnosis, and between two pregnancy diagnosis were calculated. Cows were classified as low (<=31.5 L) or high (>31.5 L) milk producer based on median value (31.5 L).

Statistical analyses

The median value for milk production was determined by the univariate procedure of SAS (SAS, Software Version 9.3; 2002-2010 by SAS Institute Inc., Cary, NC, USA).Data were analyzed withthe logistic regression-stepwise selection procedure of SAS to obtain an estimated odds ratio with 95% confidence interval (CI).

The statistical model for risk of the presence of CL 21 days after AI included milk yield at AI,parity, BCS at AI, postpartum disorder, the season of enrollment, type of reproductive management at the last AI (heat detection versus synchronizations), the total previous number of AI services, treatment and two-way interactions. The statistical model for risk of pregnancy at 28-32 days after AI included milk yield at AI,parity, BCS at the first pregnancy diagnosis, changes in BCS between AI and the first pregnancy diagnosis, postpartum disorder,season of enrollment,type of reproductive management at the last AI, the total previous number of AI services, treatment and two-way interactions. The statistical model for risk of pregnancy losses between days 28-32 and 55-60 after AI included milk yield at AI, parity, BCS at the second pregnancy diagnosis,changes in BCS between two pregnancy diagnosis, postpartum disorder,the season of enrollment, type of reproductive management at the last AI, the total previous number of AI services, treatment and two-way interactions.

RESULTS

Maintenance of of CL at 21 days after AI did not differ among CARP (79.6%, [168/211]), CIDR (86.0%, [196/228]), CARP+CIDR (80.0%, [172/215]) and CONT (74.9%, [149/199]) groups. For the maintenance of CL 21 days after AI, there are significant effects of type of reproductive management at the last AI (P<0.01), number of services (*P*<0.01), and interaction of treatment by BCS at AI (*P*<0.05). In this matter, rates of maintenance of CL 21 days after AI did not differ (odds ratio:1.96 [1.17-3.31]) between cows inseminated following CIDR and $PGF_{2\alpha}$ based synchronization program (83.3% [170/204]) and those inseminated following the detection of estrus (83.3% [434/521]). Whereas, the rate of maintenance of CL 21 days after AI in cows inseminated following $PGF_{2\alpha}$ or Ovsynch protocol (63.3% [81/128]) was 0.36 (0.23-0.57) times lower compared to those inseminated following the detection of estrus. Cows with only one service (74.7%, [280/375]) had 0.45 (0.26-0.78) times less chance for the maintenance of CL 21 days

after AI than those with four or more services (84.0%, [152/181]). There were no differences for the maintenance of CL 21 days after AI in cows with two (85.7%, [156/182]) versus (odds ratio: 1.32 [0.72-2.42]) four or more services, or three (84.4%, [97/115]) versus (odds ratio: 1.14 [0.59-2.23]) four or more services. With regard to interaction effect of treatment by BCS at AI, among cows with BCS≤2.5, chances of maintenance of CL was lower (odds ratio:1.62 [1.10-2.40]) in CONT (70.7%, [111/157]) group compared to those in CARP (81.7%, [125/153]), CIDR (83.6%, [153/183]) and CARP+CIDR (80.1%, [129/161]) groups; whereas, among cows with BCS>2.5, chances of maintenance of CL were lower in CARP (74.1%, [43/58]) and CARP+CIDR (79.6%, [43/54]) groups compared to those in CONT (90.5%, [38/42]) and CIDR (95.6%, [43/45]) groups (Figure 1).

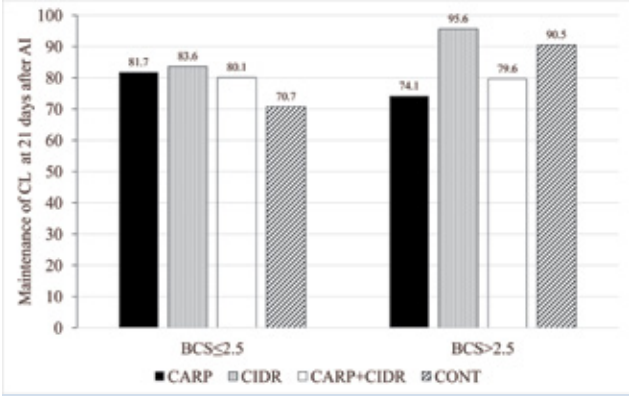

Figure 1. Interaction effect of treatment by BCS on maintenance of CL at 21 days following AI.

For the P/AI at 28-32 days after AI, there was no difference among CARP (48.8% [103/211]), CIDR (50.9 [116/228]), CARP+CIDR (47.4% [102/215]) and CONT (44.7% [89/199]) groups. For the P/AI at 28-32 days after AI, there were significant effects of milk production at AI (P<0.01) and the number of services (P<0.05). Low milk-producing cows (57.0%, [245/430]) had3.31 (1.73-6.36) times the higher chance for pregnancy at 28-32 days after AI compared to high milk producing cows (39.0%, [165/423]). Cows with two services (55.5%, [101/182]) had a 1.75 (1.17-2.80) times the higher chance for pregnancy than those with four or more services (47.5%, [86/181]). Based on odds ratios, there were no differences for the risk of pregnancy at 28-32 days after AI in cows with one service (42.9%, [161/375]) versus four or more services(odds ratio:0.81 [0.51-1.28]), or three services (53.9%, [62/115]) versus four or more services (odds ratio:1.51 [0.93-2.44]).

Pregnancy losses between 28-32 and 55-60 days did not differ among CARP (3.9%, [4/103]), CIDR (4.3%, [5/116]), CARP+CIDR (5.9%, [6/102]) and CONT (6.7%, [6/89]) groups. For the pregnancy losses between 28-32 and 55-60 days, there are significant effects of type of reproductive management at the last AI (P<0.01) and interaction of treatment by the number of services (P<0.05). Pregnancy losses in cows inseminated following $PGF_{2\alpha}$ or Ovsynch protocol (16.7% [10/60]) were 11.78 (3.03-45.82) times higher than those inseminated following the detection of estrus (3.2% [8/248]). Pregnancy losses between cows inseminated following CIDR and $PGF_{2\alpha}$ based synchronization program (2.9% [3/102]) and those inseminated following the detection of estrus did not differ (odds ratio: 1.39 [0.30-6.40]). With regard to the interaction effect of treatment by the number of services, pregnancy losses were higher in cows inseminated thrice and four or more times in CONT (11.8% and 16.7%) group compared to those in CARP (0% and 4.8%), CIDR (0% and 6.9%), CARP+CIDR (0% and 11.1%), respectively (Figure 2).

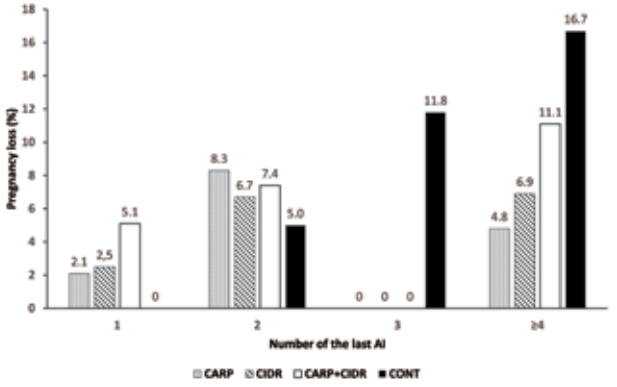

Figure 2. Interaction effect of treatment by number of the last AI on pregnancy loss between days 28-32- and 55-60-days following AI.

DISCUSSION

Because cows had visible CL at 14 days after AI were included in the study, all cows were assumed to be ovulated after AI. Detection of visible and mature CL 21 days after AI could indicate maintenance of CL; however, some of the cows could have longer estrous cycles which may lead to overestimation of the maintenance of CL. In this matter, Remnant et al. (2015) reported longer range of interservice intervals in modern dairy cows and higher unexplained variation for cycle length within individual cows rather than between cows or herds. Therefore, some ofthe assumptions for the maintenance of CL based on the presence of CL could be overestimated due to lon-

ger estrous cycles at 21 days after AI in current study. However, differences in rates of CL maintenance based on the presence ofCL among treatment groups on day 21 after AI could not be solely attributed to longer estrous cycles because cows were randomly assigned for each treatment group in this study. Lower rates of maintenance of CL 21 days after AI in cows inseminated following $PGF_{2\alpha}$ or Ovsynch protocol compared to those inseminated following the detection of estrus or CIDR and $PGF_{2\alpha}$ based synchronization program could indicate lower ovarian stimulation following $PGF_{2\alpha}$ or Ovsynch protocol. Moreover, cows following the first service had lower rates of maintenance of CL 21 days after AI compared to those following subsequent inseminations. Probably, at the first service cows could not maintain the CL due to impotent embryonic development and its signal and/or impotent luteal development for the sake of lactation. The interaction effect of treatment by BCS at AI could indicate the beneficial effects of carprofen and/or progesterone treatments on maintenance of CL among cows with BCS≤2.5 could be due to ovarian stimulation among cows with poor BCS. In contrast, there were detrimental effects of carpofen treatment with or without progesterone supplementation among cows with BCS>2.5. Cows with optimal BCS could have better quality and larger embryos than those with poor BCS. In this matter, the effect of BCS on the quality of preimplantation embryos recovered from dairy cows was reported (Makarevich et al., 2016). Similarly, it has been reported that improvement of BCS at AI is required to increase embryonic survival in lactating dairy cows (Santos et al., 2009). In this case, carprofen administration with or without P4 supplementation could compromise PGE_2 secretion in cows with optimal BCS, and faster growing embryos could not be developed during attachment process.

With regard to P4 supplementation 14-21 days after AI in the current study, no beneficial effect was found on P/AI similar to no effects of CIDR insertion from day 14 to 23 (Bartolome et. al., 2009) or from day 14 to 21 (Galvao et al., 2007; Alnimer and Lubbadeh, 2008) and from day 13-20 days after AI (El-Zarkouny and Stevenson, 2004) after AI on PR in lactating dairy cows.In contrast, Garcia-Ispierto et al. (2016) reported an increase in PR following P4 supplementation from days 15 to 17 after AI in cows without retained placenta. In addition, a 1.4 times higher chance for pregnancy following P4 supplementation from days 15 to 17 following AI was reported by Garcia-Ispierto and Lopez-Gatius (2017). This discrepancy between current and previous studies could be due to the longer length of P4 supplementation following AI, and probably P4 supplementation could have been implemented just during maternal recognition of pregnancy but not further in current study. Perhaps, longer P4 supplementation could accelerate embryonic growth and cause asynchrony between embryo and uterus leading to no benefit for fertility. In this regard, slight reduction in PR was reported following CIDR insertion between 14 and 21 days after AI in lactating dairy cows (Chenault et al., 2003).

Current results could indicate that carprofen administration 14 days after AI had no effect on P/AI in lactating dairy cows similar to carprofen administration 15 days after AI in a study conducted by von Krueger and Heuwieser (2010). Therefore, the administration of carprofen as an NSAID one day prior to previous report could reveal neither beneficial nor detrimental effect on embryo survival in lactating dairy cows. However, in the current study and a previous study conducted by von Krueger and Heuwieser (2010) all experimental cows were healthy. If the same studies would perform on cows with subclinical health problems, these results would have been different, and this phenomenon warrants further research. Perhaps, lower pregnancy losses in repeat breeder cows in the CARP group could be attributable to the beneficial effect of carprofen in repeat breeder cows with subclinical or chronic disorders.

Unexpectedly, a combination of CIDR and carprofen did not have an additive effect on embryo survival. In this regard, CIDR supplementation could fasten embryonic growth and larger embryos could be dependent on PGE_2 secretion for embryonic development during carprofen administration in this study. Because carprofen administration could non-selectively suppress PGE_2 secretion, an additive effect of the combination of CIDR and carprofen applications could not be existed in current study. In this regard, it has been known that PGE_2 promotes luteal function (Kennedy, 1983; McCracken et al., 1999) and involves the establishment and maintenance of gestation in mammals (Bazer, 1992; Bazer et al., 1997). Moreover, it has been postulated that IFN-t could stimulate PG-endoperoxide synthase-2 expression leading to alteration of the prostaglandin production cascade from $PGF_{2\alpha}$ to PGE_2 in ruminants (Bazer et al, 1997; Spencer et al., 1996).Since carprofen non-selectively suppress both PGE_2 and $PGF_{2\alpha}$, embryos dependent on PGE_2 could not be developed; thereby, no additive

effect of CIDR and carprofen on P/AI was obtained in the current study.

Significantly higher pregnancy losses between 28-32 and 55-60 days in cows inseminated following $PGF_{2\alpha}$ or Ovsynch protocol compared to those inseminated following the detection of estrus or following CIDR and $PGF_{2\alpha}$ based synchronization program could reveal that higher fertility following natural or induced estrus compared to timed artificial insemination or selective estrus induction with $PGF_{2\alpha}$. With regard to the interaction effect of treatment by AI number on P/AI, higher pregnancy losses in cows inseminated thrice and four or more times in the CONT group could indicate the beneficial effects of progesterone and/or NSAID treatment (Figure 2). However, among repeat breeder cows, pregnancy losses were lower in carprofen and CIDR treatment but not its combinations compared to in the CONT group. This detrimental effect of combined treatment of NSAID and P4 on pregnancy maintenance was not expected in repeat breeder cows. Unlike the study reported by Amiridis et al. (2009), a combination of NSAID and P4 supplementation did not increase P/AI in repeat breeder cows; however, pregnancy loss was significantly lower in carprofen and progesterone treatment. This disagreement between the current study and the results of Amiridis et al. (2009) could be the experimental design. In the present study, all repeat breeder cows were supposed to be cycling since all of them had CL 14 days after the last AI, and induction of cyclicity would not be the problem. Moreover, a possible reason for this discrepancy could be differences for NSAID administration between present study and Amiridis et al. (2009). In this regard, Amiridis et al. (2009) administered meloxicam 16, 17 and 18 days after AI; whereas, carprofen was administered only once at 14 days following the last AI. Perhaps, higher pregnancy losses in the CARP+CIDR group could be due to the carryover effect of the disruption of embryonic growth in repeat breeder cows.In this matter, carprofen administration could suppressed PGE_2 which is needed for faster-growing embryo following P4 supplementation. Unlike current results, Amiridis et al. (2009) reported higher P/AI following a combined protocol including GnRH, P4 and meloxicam; however, they suggested that determination of the relative contribution of each of the treatment was very difficult.Moreover, Khoramian et al. (2011) reported higher P/AI inthe following used CIDR administration 5-6 days after AI for 10 days in repeat breeder cows. Similarly, repeat breeder cows were benefited from CIDR administration between days 14 and 21 after AI with lower pregnancy loss in current study.

CONCLUSIONS

In conclusion, neither beneficial nor detrimental effects of carprofen or CIDR administration around the time of maternal recognition of pregnancy were observed based on P/AIin lactating dairy cattle. However, pregnancy losses were significantly lower following carprofen or CIDR administrations, but not its combinations, in repeat breeder cows. No increase in P/AIfollowing carprofen administration during the critical period of pregnancy recognition in this study warrants further research focusing on specifically suppression of $PGF_{2\alpha}$ and stimulate embryonic growth in lactating dairy cows.Furthermore, use of NSAID during maternal recognition of pregnancy in cows with subclinical and clinical disorders warrants further research to increase fertility. Moreover, higher rate of presence of CL following P4 administration; whereas, a lower rate of maintenance of CLfollowing NSAID with or without P4 administration in cows with optimal BCS could indicate that BCS should be considered for the cause of asynchrony of embryonic development among cows for the different therapeutic approaches in dairy herd fertility programs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Table 1. Treatment and selected variables by logistic regression stepwise elimination analyses for risk of presence of CL 21 days after AI.

	% and proportion (*n*/*n*)		Odds ratio (95% CI)	*P*-value
Treatment				
CARP vs CON	79.6 (168/211) vs 74.9 (149/199)		2.06 (1.18-3.61)	<0.09
CIDR vs CON	86.0 (196/228) vs 74.9 (149/199)		2.67 (1.57-4.55)	
CARP+CIDR vs CON	80.0 (172/215) vs 74.9 (149/199)		1.48 (0.91-2.42)	
Type of reproductive management				
CIDR-$PGF_{2\alpha}$ vs Estrous detection	83.3 (170/204) vs 83.3 (434/521)		1.96 (1.17-3.31)	<0.01
Ovsynch or $PGF_{2\alpha}$ induced estrus vs Estrous detection	63.3 (81/128) vs 83.3 (434/521)		0.36 (0.23-0.57)	
Number of services				
1 vs 4 or more	74.7 (280/375) vs 84.0 (152/181)		0.45 (0.26-0.78)	<0.01
2 vs 4 or more	85.7 (156/182) vs 84.0 (152/181)		1.32 (0.72-2.42)	
3 vs 4 or more	84.4 (97/115) vs 84.0 (152/181)		1.14 (0.59-2.23)	
Treatment by BCS at AI	Poor BCS	Optimal BCS		
CARP	81.7 (125/153)	74.1 (43/58)		
CIDR	83.6 (153/183)	95.6 (43/45)	1.62 (1.10-2.40)	<0.05
CARP+CIDR	80.1 (129/161)	79.6 (43/54)		
CON	70.7 (111/157)	90.5 (38/42)		

Table 2. Treatment and selected variables by logistic regression stepwise elimination analyses for risk of pregnancy at 28-32 days after AI.

	% and proportion (*n*/*n*)	Odds ratio (95% CI)	*P*-value
Treatment			
CARP vs CON	48.8 (103/211) vs 44.7 (89/199)		NS
CIDR vs CON	50.9 (116/228) vs 44.7 (89/199)		
CARP+CIDR vs CON	47.4 (102/215) vs 44.7 (89/199)		
Milk yield at AI (Low vs High)	57.0 (245/430) vs 39.0 (165/423)	3.31 (1.73-6.36)	<0.01
Number of services			
1 vs 4 or more	42.9 (161/375) vs 47.5 (86/181)	0.81 (0.51-1.28)	<0.05
2 vs 4 or more	55.5 (101/182) vs 47.5 (86/181)	1.81 (1.17-2.80)	
3 vs 4 or more	53.9 (62/115) vs 47.5 (86/181)	1.51 (0.93-2.44)	

Table 3. Treatment and selected variables by logistic regression stepwise elimination analyses for risk of pregnancy losses between 28-32 days and 55-60 days after AI.

	% and proportion (*n*/*n*)				Odds ratio (95% CI)	*P*-value
Treatment						
CARP vs CON	3.9 (4/103) vs 6.7 (6/89)					NS
CIDR vs CON	4.3 (5/116) vs 6.7 (6/89)					
CARP+CIDR vs CON	5.9 (6/102) vs 6.7 (6/89)					
Type of reproductive management						
CIDR-$PGF_{2\alpha}$ vs Estrous detection	2.9 (3/102) vs 3.2 (8/248)				1.39 (0.30-6.40)	<0.01
Ovsynch or $PGF_{2\alpha}$ induced estrus vs Estrous detection	16.7 (10/60) vs 3.2 (8/248)				11.78 (3.03-45.82)	
Treatment by number of services	Number of Services					
	1	2	3	>3		
CARP	2.1 (1/48)	8.3 (2/24)	0 (0/10)	4.8 (1/21)		
CIDR	2.5 (1/40)	6.7 (2/30)	0 (0/17)	6.9 (2/29)	1.12 (1.00-1.25)	<0.05
CARP+CIDR	5.1 (2/39)	7.4 (2/27)	0 (0/18)	11.1 (2/18)		
CON	0 (0/34)	5 (1/20)	11.8 (2/17)	16.7 (3/18)		

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Research article
Ερευνητικό άρθρο

Neosporosis: a neglected abortifacient disease in Egypt, seroprevalence and farmers' knowledge, attitudes and practices

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ABSTRACT: Neosporosis has emerged as a serious disease of cattle and dogs worldwide, causing abortions, neonatal mortalities and massive economic losses in dairy herds. The current study aimed to investigate the burden of *Neospora* (*N.*) *caninum* infection among cows that aborted and to assess knowledge, attitudes, and practices (KAPs) of farmers toward neosporosis in Kafrelsheikh governorate, Egypt. A total of 92 cows that aborted and 25 heifers born from cows that aborted and seropositive for *N. caninum* infection from 15 dairy herds in different districts of Kafrelsheikh governorate were examined serologically against *N. caninum* infection using ELISA. A structured questionnaire was built and distributed to 41 farmers in the study area. The overall seroprevalence of *N. caninum* infection among the examined cows that aborted was 38.04% (35/92). On the other hand, the prevalence of *N. caninum* infection among the 25 examined heifers born from seropositive cows that aborted was 28% (7/25). The KAPs analysis showed that farmers lack the required information on *N. caninum* infection and its consequences on dairy farms. The farmers performed risky practices which are responsible for disease entrance and spread on the farm; buy and keeping animals after abortion as well as their heifers. In addition, risk management practices were widespread in dairy farms such as: free movement of dogs, especially stray ones, in 100% of the farms, dogs were able to access and defecate in cattle feed and drinking water sources and had the chance to eat placentas and abortion materials. The current study points out neosporosis as a neglected cause of abortion among cattle in Kafrelsheikh governorate, as well as there is a lack of knowledge and risky practices by the local farmers. These findings may be extrapolated to nearby areas and countries of the same cattle husbandry practices, and veterinary services in such countries should consider neosporosis in their surveillance and control programs.

Keywords: *Neospora caninum*; cattle; Egypt; knowledge, attitudes and practices; abortion

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INTRODUCTION

Neospora caninum is an intracellular apicomplexan protozoan parasite recently discovered in Norway in 1984 (Bjerkas et al., 1984). It infects carnivores as definitive host and cattle, buffalo, sheep, chickens, and rabbits as intermediate hosts (Dubey and Schares, 2011). Neosporosis in cattle is associated with massive economic losses because of reproductive problems such as abortions, stillbirths, infertility, loss of milk production and premature culling (Bartels et al., 2006, Reichel et al., 2013, Wilson et al., 2016).

Cattle may acquire the infection in two ways, first: horizontally through the ingestion of oocysts that are shed in the feces of infected dogs and this is considered the principal route to the entrance of *N. caninum* infection in dairy herds (Dijkstra et al., 2001a, Gondim et al., 2002). The Second way of infection is the transplacental transmission of *N. caninum* where the parasite passes from mother to fetus via the placenta, and this route is considered the most important for sustaining the infection through consecutive pregnancies by congenital infection (Williams et al., 2009, De Aquino Diniz et al., 2019, Japa et al., 2019).

Several risk factors have been proven to be associated with the seroprevalence of neosporosis in dairy herds. The most prominent risk factor is the presence of dogs and their number (Collantes-Fernández et al., 2008, Ribeiro et al., 2019). The presence of rabbits, poultry, foxes, rodents and cats, as well as old age animals was found to be a risk factor for neosporosis in cattle and buffalo (Haddad et al., 2005, Barburas et al., 2019, Olmo, 2019). Moreover, feeding of decayed food has an immunosuppressive effect and was associated as well with a seroprevalence of neosporosis (Bartels et al., 1999). Furthermore, other risk factors for *N. caninum* infection such as handling of cows that aborted and the introduction of new cattle to the herd were also reported (Darío et al., 2013, Llano et al., 2018).

Despite its important role in abortion among cattle herds and the associated economic losses, neosporosis in Egypt has drawn little attention from both farmers and researchers because brucellosis is well known to be the main cause of abortion in dairy farms and this fact obscures the importance of other causes of abortion (El-Diasty et al., 2016, Shalaby et al., 2019). Furthermore, the diagnosis of *N.caninum* infection is hard and expensive (Ahmed et al., 2017). On the other hand, animal husbandry systems in Egypt, where most farms located near to rural areas of villages sustain the favorable environment for *N. caninum*, due to the presence of dogs with high numbers roaming freely around and enter the cattle farms and the lack of veterinary attention. The scarce researches on *N. caninum* in Egypt showed that it is endemic at moderate levels; 68%, in buffaloes (Dubey et al., 1998), 3.6% in camels (Hilali et al., 1998), 20.43% in cattle, 1.85% in rabbits and 7.92% in human in Northern Egypt (Ibrahim et al., 2009), 14.75% in chickens in Kafrelsheikh (Ibrahim et al., 2013), and 18.9% in cattle in southern Egypt (Fereig et al., 2016).

Antibodies in the serum of infected animal are very important for diagnosis of *N. caninum* infection, which could be detected by different serological tests, including: immunoblotting (IB), direct agglutination test (DAT), indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISAs) (Söndgen et al., 2001, Moraveji et al., 2012, Moore et al., 2014, Hamidinejat et al., 2015). Serological surveys for capturing sero-prevalence data are much required for control neosporosis (Fereig et al., 2016). Therefore, a sero-survey for *N. caninum* infection among cows that aborted in dairy herds may highlight the importance of the neglected role of such protozoan for abortion in Egyptian farms and withdraw the attention towards different causes of abortion other than brucellosis.

The objectives of the current study were to determine the seroprevalence of *N. caninum* among cows that aborted in Kafrelsheikh governorate. Furthermore, for the first time up to our knowledge, to determine, the knowledge, attitude, and practices of the farmers towards *N. caninum* infection in dairy herds which may help in the spread of the disease.

MATERIAL AND METHODS

Study Area

Kafrelsheikh is an agricultural governorate which lies in the Northern part of Egypt. It is bordered in the North by the Mediterranean Sea, in the West by Rosetta branch of the River Nile, that stretch by 85 km till its mouth in the Mediterranean Sea, in the East,by Dakahlia governorate, and in the South by Gharbia governorate. Total area estimated as (3466.69 Km2) while, the total population estimated as (3,386,270). The governorate has 10 districts: Kafrelsheikh, El Hamool, Baltim, Biyala, Desouk, Fuwwah, Metoubes, Qallin, El Reyad, and Sidi Salem. The map of the study area is illustrated in Figure 1 and was created using Quantum GIS (Quantum GIS Development Team 2017).

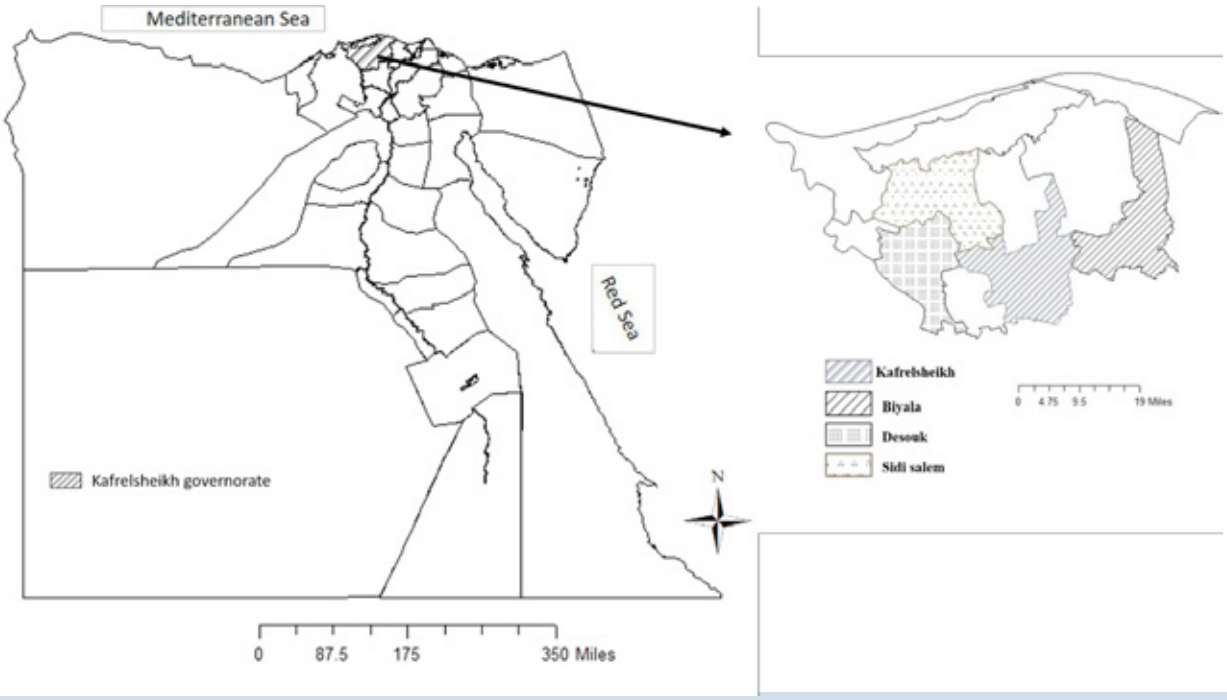

Figure 1: A choropleth map of Egypt showing the administrative boundaries of Kafrelsheikh governorate on the left side and the study area districts at this governorate on the right side

Animals and sampling

Fifteen cattle herds in four districts of the Kafrelsheikh governorate (Figure 1) were included in this study with a total number of 935 cows. These herds were suffering from the repeated occurrence of endemic and sporadic abortion in the late gestation period, of unidentified etiology. A total of 92 cows that aborted in these herds, and 25 heifers born from cows that aborted and seropositive for *N. caninum* infection were blood-sampled and examined in this study. All Kafrelsheikh University and international guidelines for the care and use of animals in scientific research according to Vasbinder and Locke (2016) were followed-up. The collected plain blood samples (n= 117) were immediately transported to the diagnostic laboratory. Serum was separated after centrifugation at 3000 rpm for 10 minutes and was kept at -20 °C till be used in ELISA test.

Data collection

A structured questionnaire was used for collecting data on the knowledge, attitudes, and practices of cattle farmers in the study area regarding neosporosis. Knowledge of the farmers regarding neosporosis and source of infection was assessed through both direct and indirect closed questions on the owner's awareness of the diseases. Other questions to identify farmers' attitudes and practices related to *N. caninum* infection and transmission were included in the questionnaire. The questions focused on what is the role of dogs in the transmission of the disease, are the farmers protecting water sources and feedstuff from contamination with dog feces, how could they handle cows that aborted and the abortion materials, what is the behavior of cows toward the abortion materials, and finally how do the dogs, particularly stray dogs, behave upon entrance to the farms. The questionnaire was developed in English and the main author of the work was responsible to deliver it to farmers, explain the questionnaire aims and contents in Arabic to the farmers, fill in the questionnaire, stored collected data on Microsoft excel 2007 and carried out the statistical calculation on excel using its built-in functions.

Serological examination

All sera were tested for antibodies against *N. caninum* by using a commercially available ELISA kit (IDEXX Laboratories, United States) coated with *N. caninum* antigen. 10 µl of each serum sample as well as the provided positive and negative controls was diluted in 90 µl of sample diluent. The microtiter plate was incubated at 37 °C for 60 minutes. After three washing steps, 100 µl of the conjugate was added to each well and incubated under the same conditions. The washing steps were repeated and 100 µl of TMB substrate was added into each well and incubated at

20°C for 15 minutes. The reaction was stopped by adding 100 µl stopping solution. The microtiter plate was read at a wavelength of 450nm. The results were interpreted according to the producer equation:

$$\text{S/P \%} = 100 \times \frac{\text{Sample OD-Negative control OD}}{\text{Positive control OD-Negative control OD}}$$

Where OD is the optical density of samples

Sample of S/P % ≥40% is considered a positive sample to *N. caninum* infection.

RESULTS

The overall seroprevalence of *N. caninum* in the examined cows that aborted was 38.04% (35/92), the distribution of seropositive cows that aborted by the district is shown in (Table 1). On the other hand, the prevalence of *N. caninum* among the 25 examined heifers born from seropositive cows that aborted, was 28% (7/25) (Table 2).

Results of KAPs assessment

The results of the questionnaire survey to assess the farmers' KAPs in Egypt towards neosporosis are shown in (table 3). All of the farmers who participated in the study (N= 41) do not know about *N. caninum* infection and its role in the abortion of their cows. Furthermore, they confirmed that they do not know the role of dogs in disease transmission to their animals.

Table 1: Seroprevalence of *N. caninum* infection among cows that aborted at Kafrelsheikh governorate, Egypt.

District	Number of examined farms	Number of cows that aborted	Number of seropositive animals/%
Kafrelsheikh	5	37	14 (37.8%)
Biyala	4	23	11 (47.8%)
Desouk	3	15	7 (46.7%)
Sidi salem	3	17	3 (17.6%)
Total	15	92	35 (38.04) %

Table 2: *N. caninum* infection seroprevalence among heifers born from seropositive dams at Kafrelsheikh governorate districts, Egypt.

District	Number of heifers born from seropositive aborted dams	Number of seropositive heifers %
Kafrelsheikh	17	5 (29.4%)
Biyala	7	2 (28.6%)
Total	25	7 (28.0%)

Table 3: Knowledge, attitude and practices of cattle farmers' towards *N. caninum* at Kafrelsheikh governorate, Egypt.

Topics	Yes
Quarantine for newly purchased animals on the farm	2.4 %
Asking of the history of abortion of new abortion cases	0.0%
Buy cows previously known to have abortion, for breeding	7.3%
Source of drinking water in your farm	
Ponds and lakes	24.4 %
Tap water supply	75.6 %
Type of reproductive service	
AI	9.7 %
Natural mating	48.8 %
Both of them	41.5 %
History of abortion last year	100%
Prevalence of abortion on the farm	
< 10%	80.5%
> 10%	19.5%
Identification of the causative agents of abortion	7.3%
Vaccination against infectious abortifacient agents like RVF, Brucellosis….etc	0.0%
Percentage of cows with repeated abortion on the farm	58.5%
Knowledge of *Neospora caninum*	0.0%
Reduction in milk of animals suffered from abortion	9.8%
Decreasing in the fertility in animals had abortion	58.5%
Keep heifers born from previously had abortion and *N. caninum* seropositive cows for breeding	100.0%
Cows eating placentas in farms	19.5%
Cows licking in abortion uterine discharge on the farm	92.7%

Abortion occurs mainly in	
Heifers	2.4%
Cows	85.4%
Both	12.2%
Dealing with cows that aborted	
Keep on the farm	87.8%
Sell or slaughter	12.2%
Handling of aborted feti, materials and placentas on the farm	
Throw them outside the farm	73.2%
Present/leave them to dogs on the farm	22.0%
Burial	2.4%
Burning	2.4%
Noticing disease complication in calves, like nervous manifestation and paralysis	26.8 %
Handling of milk of cows that suffered from abortion	
Normally use and sell it	92.7%
Get rid of it	7.3%
Provide suckling calves with milk from dams	100%
Entrance of dogs to the farm	100%
Fences around farms to prevent stray dogs' entrance	75.6%
Dogs behavior on the farm	
Freely rooming on the farm	80.5%
Short visits	19.5%
Type of dogs entered the farm	
Owned dogs	68.3%
Stray dogs	31.7%
Age of the dogs entered the farm	
Adult dogs only	90.3%
Both of Bubbies and adult dogs	9.7%
Observing of bitches giving birth on the farm	19.5%
Observing of dogs eating abortion materials and placenta on the farm	100%
Dogs defecate on silage and green fodders	92.7%
Dogs defecate on drinking water supply	29.3%
Putting the remnant fodder to heifers on the farm	48.8%
Knowledge of the role of dogs for transmission of infectious diseases to his cows	0.0%
Noticing disease complication in these dogs, like nervous manifestation and paralysis	0.0%

All farmers declared that their cows suffered from abortion in the last year before the current study. The prevalence of abortion in 80.5% of the herds was < 10% and 58.5% of farmers experienced repeated abortions among their animals. All farmers kept heifer born from animals with a previous abortion for breeding and they do not ask for the history of abortion for newly purchased animals, but 7.3% only of the farmers will still buy cows that aborted if they knew their abortion history. A total of 92.6% of the farmers noticed that cows that abort lick their abortion discharges and 19.5% of the farmers confirmed that the cows eat their placentas after abortion. The decrease in the fertility and milk production after abortion was recorded by 58.5% and 9.8% of the farmers, respectively. Abortion was recorded mainly among adult cows in 58.4% of the farmers' answers. None of the farmers vaccinated against the causative agents of abortion and only 7.4% of them identified the microbial causative agent of abortion in their herds. The cattle that abort are kept on the farm by 87.8% of the farmers and 95% and 100% of them use and/or sell the milk of these animals and offered this milk to suckling calves, respectively. On the other hand, 22.0% of the farmers presented or leave the abortion materials to dogs.

Dogs are owned by 68.3% of the farmers and kept on the farm/herd. On the other hand, 100% of farmers see the dogs in their herds - mainly adult dogs- either owned or stray despite the presence of fences in 75% of these farms to prevent external dog entrance. Around 92.6%, 92.2% and 19.2% of the farmers noticed that dogs defecate on feedstuff and in water supply for cattle, and giving birth inside the farm, re-

spectively. The decayed feedstuff is being provided to heifers as reported by 48.8% of the farmers.

DISCUSSION

N caninum infection is a major cause of abortion in dairy herds and so it hinders the attempts to progress livestock productivity in the main source of income to low-middle income countries such as Egypt (Perry and Grace, 2009, Reichel et al., 2013, Semango et al., 2019). There is a little attention given to *N. caninum* in the problem of abortion in cattle in Egypt. This is up to our knowledge the first sero-epidemiological report of *N. caninum* infection among cows that aborted in Egypt's dairy cattle herds and the first KAPs analysis of the farmers towards such infection.

The results of the current study declared that *N caninum* infection is a neglected widely spread problem in dairy herds of Kafrelsheikh governorate, Egypt. The existence of all suitable environmental and management aspects of disease spread as obtained from the answers of the farmers to the questionnaire on the farmers' KAPs towards *Neospora* infection is the reason for this high prevalence obtained.

Environmental aspects of disease spread in Kafrelsheikh governorate represented in its topography as an agricultural governorate with a lot of rural areas in and around the farms and herds which encourage the presence of dogs with high numbers. In the current study, farmers confirmed the usual presence of dogs, including stray dogs rooming-in their farms, and declared that dogs can defecate in water and feedstuff which represents a major risk for infection because *N.* caninum oocysts release with feces of dogs (Lindsay et al., 1999, Dijkstra et al., 2001b, De Souza et al., 2002).The oocyst-contaminated ration and water are the principal routes of horizontal transmission of *N.* caninum and they are responsible for the entrance of infection to neosporosis-free dairy herds (Dubey et al., 2007).

The lack in knowledge of the farmers about the disease, its sources of infection and routes of transmission are responsible for the risk practices which they perform and increase the chance of neosporosis spread. Keeping cows that abort, buying animals that had aborted, breeding of heifers born from cows that aborted and offering the abortion materials to dogs which completes the cycle of *N. caninum* infection are management practices increase the risk of horizontal and vertical transmissions of *N. caninum* in dairy herds. Such findings are compatible with Dijkstra et al. (2001a), Gondim et al. (2002), Williams et al. (2009), Trees and Williams. (2005), who confirmed that farms with bad hygienic management with cows that aborted and their related materials as (abortion placenta, and aborted fetuses and uterine discharges) increase probability of *Neospora* infection because these materials constitute the sources of infection especially for dogs which eat these materials. Also, keep the cows that aborted in the farms increase the probability of calves being seropositive due to transplacental infection with *N caninum*. Another risky management factor for neosporosis spread in dairy herds is the ignorance of the veterinarians to identify the causative agents responsible for abortion in the farms either by bacteriological or serological examinations. Farms without policies of regular serological testing and culling of cows that aborted and vaccination toward diseases causing an abortion, increase the probability of *N caninum* infection by increasing the number of seropositive cows in these farms (Hall et al., 2005, Weston et al., 2012, Aguado-Martínez et al., 2019).

Semango et al. (2019) found a positive association between within-herd *N. caninum* seroprevalence and abortion rates among cattle in Tanzania. The resulted high seroprevalence of *N. caninum* infection among cows that aborted indicates the neglected role of *N. caninum* infection as the causative agent of abortion in dairy herds in Egypt. Previous studies showed lower seroprevalence values in cattle such as Ibrahim et al. (2009) and Fereig et al. (2016) who reported seroprevalences of 20.43% and 18.9% in northern and southern Egypt, respectively. Similarly, lower prevalences were reported in other countries such as in Tanzania by Semango et al. (2019), who reported a prevalence of 21.5% and such as in other African countries such as Ghalmi et al. (2012), who reported prevalence of 10.7% and 19.6% in cows. The higher seroprevalence found in the current study than that reported by others is because the sampling in the current study was only from cows suffered from abortion. Other factors which influence the prevalence of neosporosis in cattle include the temporal and geographical distribution of different studies, the type of the used serological test, cattle husbandry practices and immune response of animals upon exposure to infection (Innes et al., 2002, Santolaria et al., 2011, Bartley et al., 2013).

The vertical mode of the disease transmission contributed to the maintenance of *N. caninum* infection

in the herd over several following bovine generations (Ortega-Mora et al., 2007). The present study showed that the high prevalence of *N. caninum* among heifers born from cows that aborted and *N. caninum* seropositive was (28%).

Significantly, some previous surveys also recorded a very high rate of congenital transmission in seropositive cows as 94% (French et al., 1999), 36.8% (Vianna et al., 2008), 67.53% (Andreotti et al., 2010), and 55.5% as reported by Lagomarsino et al. (2019).

The obtained results in the current study could be widely upscaled to other neighboring countries with similar animal production systems and lack of information on *N. caninum* infection in dairy herds.

CONCLUSIONS

This study results indicated a high prevalence of *N. caninum* infection among cows that aborted in dairy herds in Egypt. Despite this high prevalence, neosporosis is still a neglected problem among farmers and incorrect farm management practices widely exists which contributed to the spread of *N. caninum* infection entire dairy herd. Therefore, there is a must supply for the data about *N. caninum* infection to increase knowledge for the managers of farms and herd in Egypt about this parasite. Finally, obtained results emphasize the need to implement prevention programs of *N. caninum* infection and strategies for the culling of seropositive cows in dairy herds in Egypt.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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Research article
Ερευνητικό άρθρο

Epidemiosurveillance of *Brucella* infection in humans, non-ruminants and wildlife from Pakistan perspective (2000-2020)

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ABSTRACT: This review aimed at providing an overview of the prevalence and epidemiosurveillance of brucellosis in non-ruminants and humans in Pakistan during 2000-2020. Sero-prevalence of brucellosis has been reported in non-ruminants such as camels, equines, dogs and humans with the range of 0.5-21%, 16.23-62.6%, 9.2-63.8% and 2.0-70% respectively. Non-target species like Avian, reptiles and amphibians were also reported with the prevalence of 2.5%, 24.9% and 25% respectively. Ignorance and indifference make it endemic in ruminants and much-neglected disease in non-ruminants with less or no studies reported in canines. Vaccines are available and being used for ruminants while none is available for non-ruminants, which may serve as an important source of spreading disease in animals and humans. In Pakistan, it is considered as ignored disease in non-ruminants lacking effective policies for control and eradication. This review guides policymakers to draw guidelines regarding brucellosis control and eradication using one health approach.

Keywords: Brucellosis; non-ruminants; endemic; zoonosis; epidemiology; Pakistan

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INTRODUCTION

Brucellosis is a zoonotic disease affecting both animals and humans caused by bacteria of the genus *Brucella* (Karthik et al., 2016). This contagious disease poses a heavy economic impact on the livestock industry and also has serious health hazards. *Brucella* is a facultative intracellular, non-motile, non-spore-forming coccobacillus (Shahzad et al., 2017). *B. melitensis*, *B. abortus*, and *B. suis* cause abortion and infertility in domestic animals, while *B. canis* causes infection in canines (Lopes et al., 2010; Godfroid and KaÈsbohrer, 2002; Karthik et al., 2016). Camel is considered susceptible to *B. abortus* and *B. melitensis* (Shahzad et al., 2017). Humans can be infected by *B. melitensis, B. abortus, B. suisbiovars 1-4* and *B. canis*making it a public health concern. *B. melitensis*is considered the most pathogenic and invasive species for humans followed by *B. abortus, B. suis*and *B. canis* in descending order (Lopes et al., 2010). *Brucella* species and their potential to infect humans are presented in Table 1.

Infected domestic animals are the main source of infection as well as the natural reservoir of these bacteria. They are excreted in milk, urine, semen and fetal fluids of the infected animals and transmitted through the conjunctiva, oral, nasal and sexual routes (Alfattli, 2016). The practice of rearing mixed livestock species can facilitate the spread of the disease (Radostitis et al., 2007). Humans are most frequently infected via direct contact with infected reproductive material, through inspecting and whipping slaughtered animals and consuming rawmilk (Earhart et al., 2009; Liu et al., 2014). Person-to-person transmission is very rare. Breastfeeding, blood transfusion, organ transplantation, and accidental self-inoculation of *Brucella* vaccine strains can result to disease in humans.

Brucella is a facultative intracellular microorganism, which multiplies and escapes the host immune mechanism simultaneously by developing inside phagocytic cells (Gorvel and Moreno, 2002). Maintaining the chronic infection by this pathogen lies in its ability to survive and replicate within the macrophages (Neta et al., 2010; Roop et al., 2004).

The disease is of great economic importance having the potential effects on the production and reproductive status of animals including infertility and cessation of milk production after abortion (Wadood et al., 2009). In animals, the main clinical signs are abortion, low milk production, infertility, weak offsprings and death due to acute metritis and retained fetal membranes.The clinical signs of brucellosis in camels can vary from asymptomatic to abortion. Retention of fetal membranes, infertility, and delayed sexual maturity have been documented. Males may suffer from orchitis and arthritis accompanied by acute lameness (Sprague et al., 2012). In equines, the clinical manifestations of brucellosis are poll-evil and fistulous withers due to the inflammation of supraspinous bursa and connective tissue, leading to abscess formation and fistulation in the affected regions.

Table 1: Currently described Brucella species and their zoonotic potential (Sprague et al., 2012)

Species	Biovars	Animal host	Human disease
Brucella (B.) abortus	1-9	Cattle, bison, buffalo, elk, yak, camel	Yes
B. melitensis	1-3	Sheep, goat, cow, camel	Yes
	3	Nile catfish; dog	
B. suis	1	Horse	Yes (biovars 1-4)
	1,2,3	Pig, wild boar	
	2	European hare	
	4 (B. rangiferi)	Caribou, reindeer	
	5	Rodents	Yes
B. ovis		Ram	Not reported
B. neotomae		Rodent	Not reported
B. canis		Canines	Yes (rarely)
B. ceti		Whale, dolphin, porpoise	Yes
B. pinnipedialis		Seal	Not reported
B. microti		Common vole, red fox; (soil)	Not reported
B. inopinata		Human	Yes
Baboon isolate		Baboon	Not reported
BO2		Unknown	Yes
Australian rodent strains		Rodents	Yes

Occasionally abortions and other reproductive problems are also reported (Megid et al., 2010).The typical sign in female dogs is late abortion followed by a mucoid, serosanguineous, brownish, or gray vaginal discharge that persists for up to six weeks (Hollett, 2006; Shin and Carmichael, 1999). The clinical signs in males are severe epididymitis, orchitis, and prostatitis (Hollett, 2006; Wanke, 2004). Natural infections of birds with *Brucella* and transmissions of disease from aborting cows to birds were discussed many times in the literature (Shahzad et al., 2018; Wareth et al., 2020). However, birds often show no clinical signs but they do occur, symptoms frequently include enteritis and diarrhea (Wareth et al., 2020). In the case of amphibians, pathologic changes ranging from individual, localized disease manifestations (e.g. subcutaneous abscess, skin lesions, swollen paravertebral ganglia, panopthalmitis) to systemic bacterial infections with high mortality were observed (Mühldorfer et al., 2017). The clinical manifestation of brucellosis in humans is an undulant fever in which the temperature can vary from 37ºC in the morning to 40ºC in the afternoon. Other symptoms like night sweats, chills and weakness are also reported. Malaise, insomnia, anorexia, headache, arthralgia, constipation, sexual impotence, nervousness, and depression are also common in patients (Megid et al., 2010).

The gold standard test for diagnosis of brucellosis is isolation and identification of the organism. It takes a longer time, which makes this method laborious and time-consuming. Infection risk is a major hurdle in culturing *Brucella* due to its zoonotic potential (Karthik et al., 2014). A presumptive diagnosis can be made by a different serological test like Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT) and Enzyme-Linked immune sorbent assay (ELISA) (Nicoletti, 2007). Other screening tests include Buffered Plate Agglutination Test (BPAT), Milk Ring Test (MRT), Complement Fixation Test (CFT), and Fluorescence Polarization Test (FPT) (Acha and Szyfres, 2003; Godfroid *et al.,* 2010). The use of PCR to identify the *Brucella* species up to biovars level has increased the efficiency of the test. It is an easy, cost-effective and less time-consuming method to identify the organism (Fernando et al., 2010).

Expansion of the animal industry, lack of hygienic conditions on the farm and improper food processing makes brucellosis a public health risk. It spreads from one region to others due to international travel, importation of animals and their derived products. Being a zoonotic disease, it is considered an occupational hazard for those persons having direct contact with infected animals like farmers, veterinarians, and butchers (Dil et al., 2017). Brucellosis has been eradicated from many developed countries but still endemic in Africa, the Middle East, the Mediterranean, Asia and Latin America (Geering et al., 1995; Refai, 2002). In Pakistan, there is little information on animal and human brucellosis. The epidemiosurveillance and bacteriological isolations of *Brucella* are very scarce. In the last few decades, brucellosis in ruminants has become a focused point for researchers resulting in a huge research gap for other vulnerable species. It is one of the most ignored diseases with respect to non-ruminants in Pakistan. Despite the detection of brucellosis in all domestic and wild animals, Pakistani people lack awareness regarding the zoonotic potential of this disease with their existing habit of raw milk consumption and close contact with infected animals. This review aims to describe the prevalence and epidemiology of brucellosis and encourage interested researchers to understand the brucellosis situation in Pakistan in a better way. For this purpose, available epidemiological data from 2000 to 2020 on non-ruminants and human brucellosis in Pakistan were analyzed using various search engines such as google scholar, Pubmed, Scopus and Web of Science. The geographical distribution of Brucellosis in non-ruminants and humans in Pakistan is illustrated in figure 1.

CAMEL BRUCELLOSIS

Investigations confirmed the presence of brucellosis in camels. Studies reported that seroprevalence of brucellosis may range from 0.51 % (Ullah 2015) to 21.0% (Baloch et al., 2016) in Pakistan. Fatima et al (2016) examined 200 camel sera using random and multi-cluster sampling from the lower Punjab of Pakistan. They found 5%, 2% and 1.5% sera positive using RBPT, cELISA and real-time PCR respectively. Shehzad et al., (2017) investigated 761 camel serum samples for brucellosis using RBPT and found 3.1% positives. The prevalence of brucellosis in camels belonging to various regions of Pakistan is summarized in table 2. A higher prevalence of brucellosis was recorded in the nomadic production system than in the organized production system. The seroprevalence of brucellosis was higher among adult camels than young ones and also higher in females compared to males (Fatima et al., 2016; Shahzad et al., 2017). With the increase in age, an increase in the level of hormones and erythritol may enhance the growth of

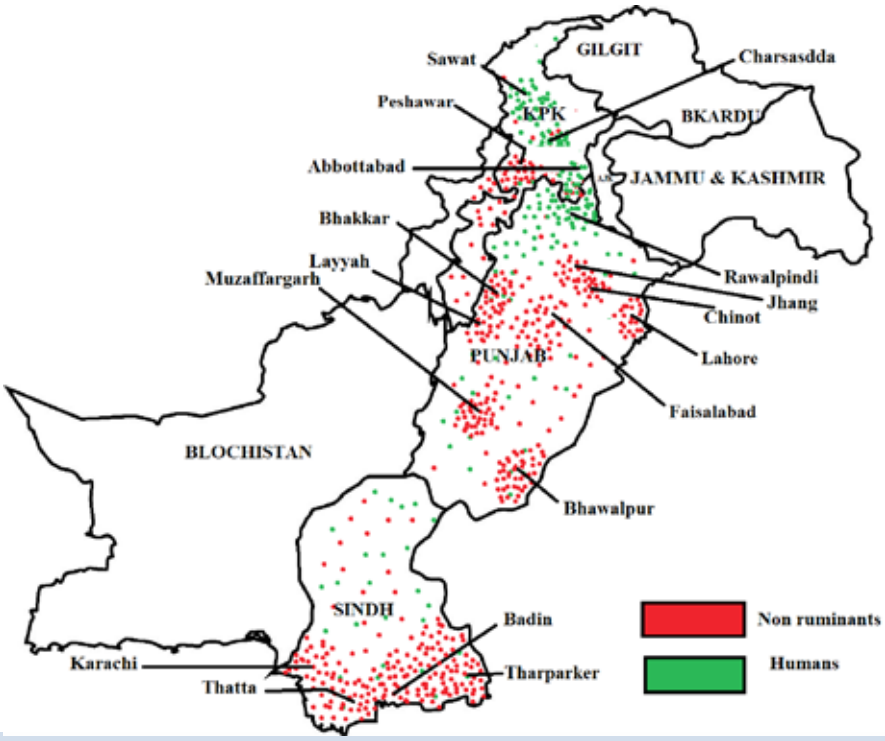

Fig 1: Geographical distribution of non-ruminant and human brucellosis in Pakistan

this pathogen (Poester et al., 2013). The high seropositivity of brucellosis was noticed in the animals with poor health status followed by moderate and good health status (Shahzad et al., 2017). The animals having more parity numbers were found more infected compared to the animals with fewer parity numbers (Shahzad et al., 2017). Fatima et al. (Fatima et al., 2016) suggested that brucellosis cases decrease in summer and spring and increase in winter because the pathogen does not survive in hot weather and cannot withstand direct exposure to sunlight. The study also described that sharing of common pastures and water points with infected animals had enhanced the transmission of brucellosis to camels.

EQUINES BRUCELLOSIS

Very few seroprevalence-based studies of equine brucellosis conducted in Pakistan. The prevalence range of equine brucellosis in Pakistan was reported as 16.23% (Gul et al., 2013) to 62.6% (Safirullah et al., 2014). Wadood et al., (2009) investigated 300 serum samples from equines and used RBPT and SAT. The overall prevalence was 20.7% by RBPT and 17.7% by SAT. All studies about the prevalence of equine brucellosis are presented in table 2. Females were more prone to this disease than males (Gul et al., 2013; Safirullah et al., 2014; Wadood et al., 2009). Wadood et al., (2009) observed that 9.6% of stallions and 17.7% mares were found infected with brucellosis. The higher prevalence in mares might be due to their close association with reproductive discharges passed after abortion or parturition by infected mares which can infect the healthy ones. Sexually matured animals are more susceptible to *Brucella*infection than sexually immature animals of either sex (Radostitis et al., 2007). Higher seroprevalence was found in those groups with the age of greater than 5 years than those groups with the age of less than or equal to 4 years (Gul et al., 2013; Safirullah et al., 2014; Wadood et al., 2009). Brucellosis prevalence was highest in the 6-10 years age group (20.26%) followed by 13.75% and 10.66% in 11-15 and 1-5 years age groups (Gul et al., 2013). The low prevalence can be explained on the basis that young animals may harbor the organism without expressing any detectable antibodies until their first parturition or abortion. Generally, the disease was more prevalent in animals of poor health condition while less in healthy animals (Safirullah et al., 2014; Wadood et al., 2009). Wadood et al., (2009) observed that 9.7%, 13.0% and 20.0% of seropositivity rate was found in good, fair and poor conditioned animals respectively.Wadood et al., (2009) also documented that desi breed was highly infected with brucellosis followed by Thoroughbred, Crossbred and Arabian horses in descending order. This study also revealed that chances of brucellosis increase with the increase of parity number in mares.

Table 2: Prevalence of brucellosis based on various diagnostic tests in non-ruminants and humans of Pakistan (2000-2020)

Year of studying	Diagnostic method	Camel	Equine	Dogs	Human	Avian	Reptiles	Amphibian	References
2001-2002	SAT	775 (1.8)							(Siddiqui, 2016)
2008	RBPT ELISA				300 (14) 300 (11)				(Ali *et al.,* 2013)
2008	ELISA				360 (21.7)				(Mukhtar and Kokab, 2008)
2009	RBPT SAT		300 (20.7) 300 (17.7)						(Wadood *et al.,* 2009)
2012	SPAT, PCR		500 (62.6)						(Safirullah *et al.,* 2014)
2012-2013	SPAT STAT PCR				300 (3.66) 300 (2) 300 (2.66)				(Ahmad *et al.,* 2017)
2013	RBPT SAT cELISA	100 (21) 100 (21) 100 (13)							(Baloch *et al.,* 2016)
2013	RBPT, SAT				262 (6.9)				(Hussain *et al.,* 2018)
2013	RBPT				429 (5.8)				(Ali *et al.,* 2016)
2013	RBPT SAT		308 (20.13) 308 (16.23)						(Gul *et al.,* 2013)
2014	SPAT PCR				200 (10) 200 (7.5)				(Perveen and Raqeebullah, 2015)
2014	SAT PCR				95 (38.94) 95 (14.7)				(Asif *et al.,* 2014)
2014-2015	RBPT qPCR				446 (10.1) 446 (5.8)				Saddique et al., 2019
2015	RBPT PCR	387 (0.51) 387 (0)							(Ullah, 2015)
2015-2016	SAT ELISA qPCR			87 (9.2) 87 (10.3) 87(1.15)					Jamil et al., 2019
2015-2016	SAT			94 (63.8)					
2016	RBPT cELISA qPCR	200 (5) 200 (2) 200 (1.5)							(Fatima *et al.,* 2016)
2016	RBPT, ELISA				250 (16)				(Sultan Ali *et al.,* 2018)
2016	RBPT					79 (2.5)	34 (24.9)	4 (25)	(Shahzad Ali *et al.,* 2018)
2016	SPAT RBPT PCR				73 (24.6) 73 (6.84) 73 (12.3)				Khan et al., 2018
2016	SPAT RBPT PCR				50 (30) 50 (4) 50 (18)				Khan et al., 2018
2017	SPAT PCR				200 (6) 200 (2)				(Khan *et al.,* 2017)
2017	RBPT	761 (3.41)							(Shahzad et al., 2017)
2017	SAT				70 (70)				(Malik *et al.,* 2018)
2018	SPAT				100 (23)				Maria Saif et al., 2018
2018	RBPT cELISA PCR				183 (8) 183 (13) 183 (33)				(Waheed *et al.,* 2018)

Figures in parenthesis indicate percentage and outside show total no. of samples examined

CANINE BRUCELLOSIS

The first-ever report on *B. canis* and *B abortus* in dogs of Pakistan reported by Jamil et al., (2019) showed the 9.2% and 10.3% serological prevalence of brucellosis in dogs of Faislabad district of Pakistan by SAT and ELISA respectively. Only one Elisa positive sample being founded positive for *B. abortus* through real-time PCR. They also reported 63.8% seroprevalence in dogs of Bahawalpur district of Pakistan with none of the sample was positive by Elisa and real-time PCR. One-year-old stray dogs were found positive against *B. canis* with poor body conditions. Moreover, *B. abortus* was detected from wounds present on the animal body. These findings highlight a risk of disease transmission from stray, wild and domestic dogs to livestock and humans and vice versa.

AMPHIBIANS, REPTILES AND BIRDS BRUCELLOSIS

To date, the epidemiology of *Brucella* infections in cold-blooded hosts is largely unknown. Shahzad et al., (2018) examined 117 blood samples from birds, amphibians and reptiles collected from the Sindh (Karachi) and Punjab (Pattoki) provinces of Pakistan. They found 11.11% samples seropositive for *Brucella* antibodies. More specifically, 25% avian, 29.4% reptiles and 25% amphibian samples were found seropositive using RBPT. In avian species, 6.25% peafowl and 9.1% Indian blue rock pigeons were diagnosed positive against brucellosis. In the case of amphibians and reptiles, 25% of Indian bullfrog and 32.3% yellow-spotted mud turtles were seropositive for *Brucella* antibodies respectively. These animals had no clinical signs of disease but could be a non-target species of brucellosis and can serve as a potential source of disease spread in marine, ground and flying birds. They can also pose a great risk to zoo laborers, veterinarians and shopkeepers.

HUMAN BRUCELLOSIS

The prevalence of brucellosis in humans may range from 2.0% (Ahmad et al., 2017) to 70% (Malik et al., 2018) in Pakistan. Waheed et al., (2018) collected 183 blood samples of occupationally exposed humans and tested using RBPT, cELISA and PCR and results revealed 8%, 13% and 33% samples positive respectively. Asif et al., (2014) examined 95 blood samples collected from veterinary professionals, livestock farmers and butchers. They found 38.94% and 14.7% of sample positives using SAT and PCR respectively. All prevalence-based studies are illustrated in table 2. Generally, it is found to be more common in males as compared to females. Sultan Ali et al., (2018) observed that the prevalence of brucellosis was higher in males (24%) than females (8%). In contrast, few studies reported that the disease is more common in females as compared to males (Ahmad et al., 2017; Khan et al., 2017; Malik et al., 2018). It was suggested that age constituted an important epidemiological risk factor for human brucellosis.Mukhtar and Kokab, (2008)reported that the age group of 51-60 years had the maximum seropositivity. While, Malik et al., (2018) documented that the majority of *Brucella*-positive individuals belonged to the age group 21-40 years. In another study, the highest prevalence of brucellosis was found in the age group ranging from 40-60 (Perveen and Raqeebullah, 2015). Young people showed higher prevalence than older because they were more engaged in working with livestock and also exposed to other occupational risks. In a study, 5.8% of pregnant Pakistani women were found to be seropositive and indeed women from rural areas were more often seropositive than those from urban areas (Ali et al., 2016). This study further revealed that pregnant women consuming raw milk were more often seropositive (76.5%) compared to those never consuming raw milk (2.9%). Sultan Ali et al., (2018) reported that individuals living in rural areas were 2.3 times more likely to be *Brucella* seropositive as compared to urban areas.Malik et al., (2018) found brucellosis among patients presenting with nonspecific symptoms. Symptoms were malaise, headache, insomnia, and fever. Lack of education and proper awareness about health and diet is the major reason for infections in the area. When the presence of infection was measured with education level, 83% of total patients were found to be illiterate (Ahmad et al., 2017). A high prevalence of *Brucella* infection was reported in those people having direct contact with animals and/or their products. The slaughterhouse workers are generally more susceptible to contract brucellosis by virtue of their direct exposure to viscera, gravid uterus and fetal membranes of infected animals (Mukhtar and Kokab, 2008). This study further revealed that brucellosis was found to be more common among individuals who had been involved in calf deliveries and had handled placenta. It is a significant finding that raw milk is a constant source of disease spread to farmers, milking men and general users. Disease prevalence was more in people associated with milking activities possibly due to the use of raw milk (Waheed et al., 2018).

Policy, response and control strategies

Treatment of brucellosis is not effective in animals. Career animals should be quarantined to limit the further spread and infected ones need culling based on screening and confirmatory tests (Falagas and Bliziotis, 2006). Brucellosis can be treated using a multi-drugs approach but treatment failure and relapse rates are very high (Pal et al., 2017). Antibiotic treatment can be used for genetically superior animals but due to uncertain outcomes, it is not recommended (Radostitis et al., 2007). Human treatment is possible and effective if this disease is diagnosed at an early stage and the patient gets effective drugs for an adequate length of time. A combination of doxycycline, rifampin, sulphamethoxazole, and trimethoprim are being used for treatment in humans (Alp et al., 2006; Khuri-Bulos et al., 1993; Yilmaz et al., 2004).

Most of the countries, particularly developed states, follow test and cull policy for infected animals to limit the spread of this disease among animal and human populations. This policy is not practicable in Pakistan due to limited resources to compensate the farmers for the slaughtering of infected animals(Mukhtar, 2010). Measures need to be followed for regular screening of the herd using RBPT, MRT, and PAT. Farmers should use screening tests while purchasing new animals from the market, if any animal is found positive, then avoid buying such an animal. A national eradication program has yet to propose for brucellosis in the country. The main hurdles limiting the control of brucellosis are the security of the country, shortage of funds, laboratory facilities and trained manpower. A human vaccine against brucellosis is not developed yet, therefore, the control of human brucellosis can only be possible by keeping animalsbrucellosis-free(Ning et al., 2013). In order to reduce the brucellosis burden in humans,mass vaccination not only in ruminants but also in non-ruminants can increase the resistance to infection in animals. To date, the brucella vaccine for dogs is not commercialized yet and trials are being conducted at the research level. Preventive measures for Brucella infection in dogs include spaying or neutering, giving antibiotics for several months, and frequent blood tests to monitor treatment progress.Pasteurization of milk and dairy products is another preventive measurement for humans. The development of powerful tactics is necessary to raise awareness in people about brucellosis, its zoonotic potential, the economic impact on the livestock industry and preventive measurements using extension services, leaflets, posters and other mass media. Mass vaccination not only in ruminants but also in non-ruminants can increase the resistance to infection in animals. Seroprevalence and epidemiology-based studies at the country level can help policymakers to propose a brucellosis eradication program in the country. Brucellosis control and eradication program should be initiated as in Egypt and Palestine (Eltholth et al., 2017; Awwad et al., 2018). In Egypt, two strategies are used. One is to test the animals and cull the infected ones based on positive serological tests. Another strategy is vaccination of the animal population. In Palestine, the brucellosis control program includes mass vaccination of animals and strengthening of their institutions for controlling and monitoring the disease.

CONCLUSION

Brucellosis poses a significant impact on human and animal health as well as socio-economic impact, particularly in those countries where rural income relies largely on livestock production and dairy products.Sharing of pastures, water and feeding points with infected animals should be avoided. High stocking density, the introduction of untested livestock animals from the market, lack of quarantine policy, and mixing of different species at the same farm are associated with a high prevalence of brucellosis in non-ruminants of Pakistan. In humans, males are found more susceptible as compared to females due to their occupational risks. The rural population of Pakistan harbors more infection than urban due to consumption of raw milk. Screening animals for brucellosis in villages and slaughterhouses is necessary and further attempts should be made to control this disease. Non-ruminants spread disease to humans as people are engaged at animal farms, treating animals and providing assistance in births. People keep dogs as a pet in their homes and they can be a source of infection, highlighting the need to screen pets on a routine basis. Detection of brucellosis in non-target species (reptiles, amphibians and birds) indicates the diverse host range of *Brucella* in Pakistan and increases the risk of infection not only for livestock animals but also for workers engaged with farm and wild animals. Vaccination in dairy animals is successfully being used but there is a dire need of time to vaccinate non-ruminants which may serve as an infection source for humans and animals. Programs to educate the agricultural people about brucellosis and food hygiene are needed to reduce the disease incidence. Due to the high prices of animals, the test and slaughter

policy is not an effective approach for the eradication of brucellosis in Pakistan. Testing, isolation, and management of infected animals in a quarantine system is the only viable approach to limit the spread of brucellosis. However, the impact of such policy in Pakistan has yet to be demonstrated.

CONFLICT OF INTEREST STATEMENT

None declared.

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Research article
Ερευνητικό άρθρο

Assessment for the passage of tylosin into the milk of Anatolian buffaloes

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ABSTRACT: Tylosin is a broad-spectrum macrolide antibiotic commonly employed in veterinary medicine to treat bacterial infections. The present study assessed the milk-passage patterns of tylosin up to the 16th milking after a single intramuscular injection at the dose of 10 mg/kg/b.w. to Anatolian buffaloes. The residue levels of tylosin in milk samples of each animal were analysed by LC-MS/MS. The detection and determination limits of the employed method were 0.19 μg/kg and 0.64 μg/kg, respectively. The highest level of tylosin was found to be at the second milking. At the ninth milking, tylosin residue level decreased under the maximum residue limit of 50 μg/kg. Additionally, the employed LC-MS/MS method is used to assess tylosin residue in 40 milk samples and all of the samples were found to be tylosin free. In conclusion, this study determined the milk passaged levels of tylosin into milk of Anatolian buffaloes using an LC-MS/MS method.

***Keywords*:** Anatolian buffaloes, tylosin, milk, LC-MS/MS.

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INTRODUCTION

Macrolide antibiotics are commonly employed in veterinary medicine to treat several diseases such as enteric infections and respiratory diseases in sheep, cattle, poultry and swine due to their effects on both gram-positive and some gram-negative bacteria (Draisci et al., 2001).

Milk is an indispensable part of human nutrition due to its valuable ingredients. It provides a high diversified composition with essential roles in metabolism such as minerals, fat and lactose, proteins and vitamins. In addition, milk is a source of significant trace elements i.e. zinc, copper, iron and manganese, which are vital in various physiological functions of human body (Acaroz et al., 2019). On the other hand, Buffalo milk as the second most consumed milk after the cow milk is an inevitable source of nourishment in many regions of the world and this milk type is widely used in the production of different milk products. Also, Turkey is one of the major buffalo milk producers with Italy and Bulgaria in Europe (Acaroz et al., 2020; Pasquini et al., 2018).

Milk provides many beneficial health effects; however, it can contain some hazardous substances such as antibiotic residues due to improper usage or misuse of antibiotics to livestock animals (Cháfer-Pericás et al., 2010; Vishnuraj et al., 2016). The presence of antibiotic residues in milk should be taken into consideration due to its negative impacts on human health including transiently disturbing intestinal flora or inducing allergic reactions which may be ended up even with anaphylaxis. In addition, the development of antibacterial resistance may be caused by antibiotic residues. Lastly, the production of fermented milk products such as yoghurt and cheeses could be inhibited by means of these residues (Graham et al., 2014; Quintanilla et al., 2018). In case of macrolide antibiotics, although negative effects on human health were rarely reported for these antibiotics, they have been also associated with serious adverse effects for pregnancy by increasing risk of cardiovascular malformation, miscarriage, major malformations in observational studies (Fan et al., 2019).

To minimize possible food-associated health risks of a macrolide antibiotic tylosin, a maximum residue limit for milk at the level of 50 µg/kg was established by European Union (EU No37/2010). To control the residues of tylosin in many foodstuffs, different methods such as immunoassay (Burkin and Galvidis, 2012), quantum dot-based immunoassay (Le et al., 2015), HPLC (Dudriková et al., 1999), LC-MS/MS (Wang et al., 2006) were established. LC-MS/MS is considered as one of the most promising methods because of its ability to quantify and confirm macrolides at trace levels (Wang and Leung, 2007).

Some studies were carried out to assess the milk passage of macrolide antibiotics of different animal species including cows (Avci and Elmas, 2014), ewes (Al-Wabel, 2008) and goats (Quintanilla et al., 2018). However, there is no available information on residual patterns of tylosin in Anatolian buffalo milk. Therefore, this study aimed to evaluate the milk passage of tylosin into the milk of Anatolian buffaloes using a LC-MS/MS method.

MATERIAL AND METHODS

Tylosin was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents of analytical grade were obtained from commercial sources.

Healthy female Anatolian buffaloes (n=5) that were weighing 400-500 kg were employed for the study. The Anatolian buffaloes were provided by Afyon Kocatepe University, Veterinary Faculty Research and Application Farm. Additionally, the ethical approval was given by Afyon Kocatepe University Animal Experiments Local Ethics Board with an approval number of 49533702/95. Experimental animals were given standard ration and water under similar conditions. Each buffalo was intramuscularly injected with tylosin at the dose of 10 mg/kg (Tylan 200, Elanco). Then, milk samples were taken for 8 consecutive days at the 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192 hours and stored stored at -20°C for further residue analysis Also, a blank sample was collected before applying the drug to each experimental animal.

Forty buffalo milk samples were collected from Afyonkarahisar province of Turkey from September to December in 2017. The samples were purchased from local markets and producers and then directly transported to the laboratory under cold chain conditions. The samples were kept at -20°C for further residue analysis.

The stock solution of tylosin was prepared in methanol at the concentration of 1 mg/mL. The respective stock solution was diluted in milk to produce calibration curve (0.5, 1, 2, 5, 10, 20, 50 ng/ml) and calibration standard samples of tylosin.

Methods

The extraction of tylosin from milk was carried out in line with Dudriková et al. (1999). Briefly, 30 ml of homogenized buffalo milk sample was spiked with the related concentration of tylosin and then fat layer was separated by a centrifugation step at 3000 rpm, 10 min and 4°C. 10 ml of the skim layer was mixed with 20 ml of acetone in another centrifuge tupe. After this step, a centrifugation at 3400 rpm, for 10 min at 4°C was performed. The obtained extract was diluted with water to 100 ml and a solid-phase extraction step was done with a Sep-Pak C18 cartridge. Firstly, the activation of the cartridge was carried out with methanol (2 ml) and water (5ml). Then, the extract applied to SPE cartridge and filtered under vacuum. The elution was done with 0.1M ammonium acetate solution in methanol (2x2 ml).The obtained eluate was given to 6 ml phosphate buffer (of 0.01M, pH 6.0) in a separatory funnel. This mixture was shaken for 5 min after the addition of dichloromethane (20 ml). The organic layer was evaporated using a vacuum evaporator. The remaining residue was dissolved in mobile phase (2 ml) and transferred to HPLC vials. Then, the analysis of milk samples was performed with Agilent Technologies 1200 series (Waldbronn, Germany), attached with a binary high-pressure gradient pump. LC separation was carried out by Zorbax Eclipse XDB-C8 (150 mm × 4.6 mm, 1.8 µm Agilent Technologies) at 45°C. The mobile phases consisted of solvent A (aqueous solution of 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). The gradient profile was performed as follows: 0.0 min, A/B (100/0); 1.0 min, A/B (100/0); 3.0 min, A/B (20/80); 7.50 min, A/B (5/95); 8.10 min, A/B (100/0), 12.00 min, A/B (100/0). The flow rate of mobile phase was 0.6 ml/min and the injection volume was 10 µl. Mass spectrometry analysis was conducted on Agilent 6460 LC/MS Triple Quadrupole instrument equipped with an ESI (Waldbronn, Germany). The employed nitrogen generator (Balston, Haverhill, MA, USA) produced nebulizer and drying gas (350°C). MS parameters including nebulizer gas, capillary voltage, sheath gas temperature, and flow were as 40 p.s.i., 4000 V, 400°C, and 10 l/min, respectively. MS analysis was performed on positive ion mode. The retention time of tylosin was detected as 5.608. Molecular weight, precursor ions (m/z), and product ions (m/z) of tylosin were 916.5, 174.1, 101.2 respectively. The method validation was done by spiking milk samples. The quality parameters were as follows; intra- and inter-day precisions recovery, limit of detection (LOD), limit of quantification (LOQ) and linearity range. The residual levels of tylosin in milk samples were determined by means of the calibration curve for which a series of standard solutions (0.5, 1, 2, 5, 10, 20, 50 ng/ml) were prepared and calculated. To establish the equation of calibration, data fitted on a line and the obtained equation was emplyed to determine the level antibiotic residue in unknown samples. Additionally, the strength of linear regression was expressed by the coefficient of determination (r^2). The lowest concentration that the analytical process can confidingly differentiate from background levels (signal-to-noise ratio≥3) was defined as LOD whereas lowest concentration that can be quantified (signal-to-noise ratio ≥10) was decribed as LOQ.

RESULTS

The chromatogram of tylosin was presented in Figure 1. In addition, as the validation parameters of the used LC-MS/MS method, accuracy, recovery, linearity, precision, LOQ and LOD were employed. The calibration curve for tylosin was given in Figure 2. The linearity of related curve was in the range from 0.5 to 50 µg/kg and showed good the coefficient of determination (r^2=0.997). Additionally, the sensitivity of the method was quite high and LOD and LOQ parameters were presented in Table 1. Relative standard deviation (RSD%) was employed for the overall precision of the method. These values were found to be lower than 5.89 %. The accuracy was expressed by intra-day and inter-day recoveries at the concentrations of 45, 90, 135 µg/kg. Recovery and RSD values were presented in Table 1.

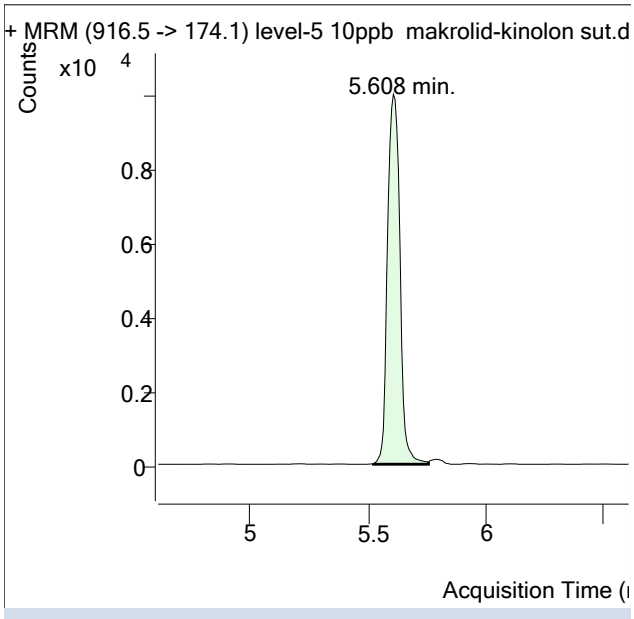

Figure 1. Chromatogram for tylosin standard.

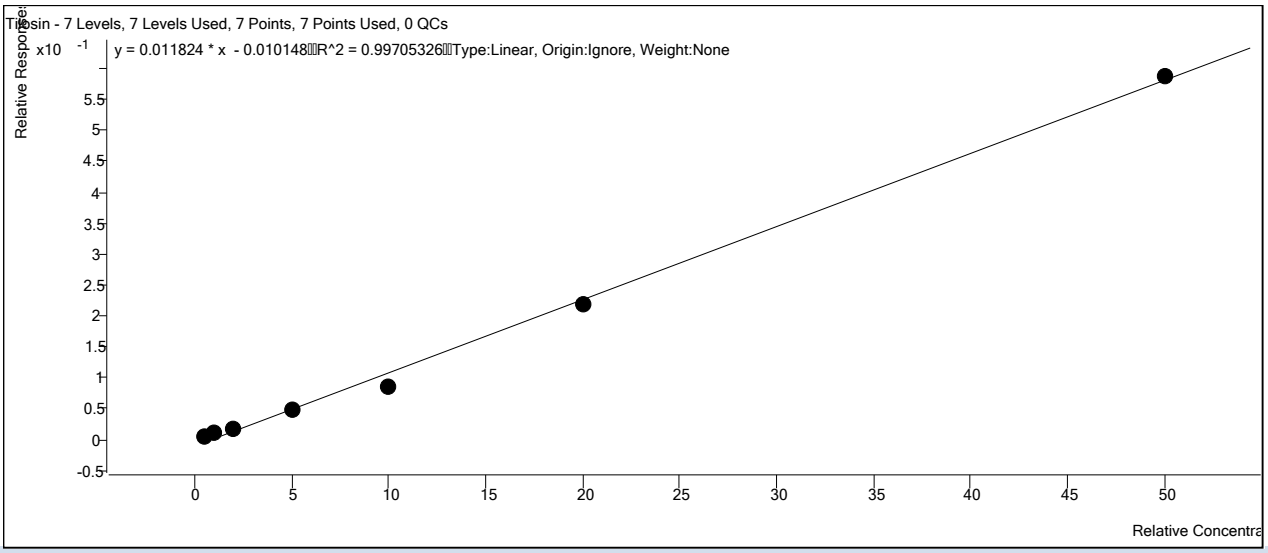

Figure2. Calibration curve for tylosin

Table 1. Intra- and inter-day precisions for tylosin in buffalo milk samples.

Spiked (ppb)	Intra-day Assays (n=6)		Inter-day Assays (n=6)	
	Percentage Recovery±CV	RSD (%)	Percentage Recovery±CV	RSD (%)
45	91.70±4.55	4.95	91.49±3.88	4.24
90	94.36±4.52	4.80	90.66±3.89	4.29
135	95.59±3.03	3.17	89.22±5.25	5.89

The results of the study exhibited that the highest level of tylosin was detected at the second milking with a mean concentration of 1625.1±358.97 µg/kg (Fig. 3). Also, the level of tylosin in milk was decreased after the second milking continuously and its level was lower than the maximum residue limit (50 µg/kg) at ninth milking with a mean concentration of 38.26±5.29 µg/kg (Fig. 3).

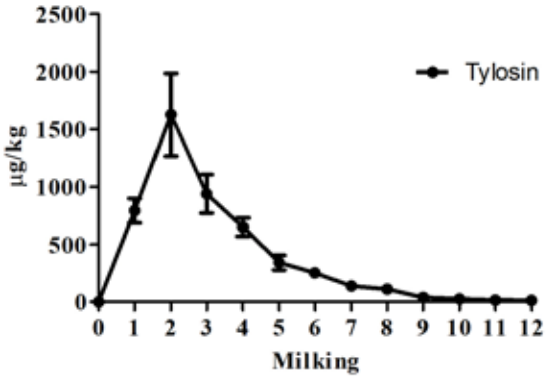

Figure 3. Passage of tylosin into the milk of buffaloes.

Also, 40 buffalo milk samples offered for consumption in Afyonkarahisar were analysed for the presence of tylosin. According to results of the analysis, all of the milk samples were found to be tylosin-free.

DISCUSSION

Macrolides are widely used in veterinary medicine based on their broad-spectrum. They show activity activity against Gram-positive and some of Gram-negative bacteria (Rigos et al., 2003; Sugawara et al., 2017).

Some quality parameters including the limit of detection and determination values and also recovery rates of tylosin in similar studies for milk samples were presented in Table 2. The LOD values of the selected methods were ranged from 0.024 µg/kg (Şanli et al., 2011) to 1 µg/kg (Aguilera-Luiz et al., 2008; Bogialli et al., 2007) whereas LOQ values were reported between 0.007 µg/kg (Şanli et al., 2011) and 3 µg/kg (Aguilera-Luiz et al., 2008). In addition, recovery rates of tylosin in milk samples were found to be between 88% and 109 % (Dubois et al., 2001; Wang et al., 2006; Wang and Leung, 2007). The results of the previous reports are in line with the result of the employed study for which LOD and LOQ values were determined as 0.19 µg/kg and 0.64 µg/kg, respectively while recovery rates ranged from 89 % to 95 %.

Table 2. Selected chromatographic methods for the residue analysis of tylosin in milk samples.

Method Type	Matrix	LOD µg/kg	LOQ µg/kg	Recovery (%)	Reference
LC-MS/MS	Buffalo Milk	0.19	0.64	89-95	Current Study
HPLC-DAD	Sheep Milk	0.024	0.007	90	Şanli et al. (2011)
LC-MS/MS	Bovine Milk	NA	NA	97-100	Dubois et al. (2001)
LC-MS/MS	Bovine Milk	0.02	NA	96-109	Wang and Leung (2007)
LC-MS/MS	Bovine Milk	0.06	NA	99-105	Wang et al. (2006)
LC-MS/MS	Bovine Milk	1	2	88-97	Bogialli et al. (2007)
UPLC-MS/MS	Bovine Milk	1	3	90-95	Aguilera-Luiz et al. (2008)

NA: Not available.

Several studies investigated the passage of tylosin in the milk of livestock animals. Al-Wabel (2008) evaluated the milk passage of tylosin in lactating Najdi ewes after a single intramuscular injection of this antibiotic (10 mg/kg) by microbiological agar plate assay and measurable residual levels of tylosin were reported in all animals up to 72 h following the treatment. In another study conducted by Avci and Elmas (2014), pharmacokinetic and amount of residue in milk of tylosin for healthy Holstein breed cows were determined after a single intramuscular injection at the dose of 17.5 mg/kg. The mean tylosin concentration was reported as 0.20±0.09 µg/ml (96 h after administration) which was higher than the established MRL of 50 µg/kg and they concluded the withdrawal period was inadequate to ensure the elimination of this drug based on the determined half-life of 26.36 ± 5.55 h in milk for the related study. Quintanilla et al. (2018) administered macrolide antibiotics including tylosin (0.5 ml/10 kg bw.), spiramycin (0.5 ml/10 kg bw.) and erythromycin (1 ml/10 kg bw.) three consecutive days to dairy goats for an *in vivo* experiment. Then, they produced ripened cheeses from contaminated milk samples. After 24 hours of injection, milk residues of the related antibiotics were found to be relative higher than the respective MRL for erythromycin 234.9 ± 52.7 µg/kg; tylosin 198.7 ± 57.8 µg/kg and spiramycin 1539.8 ± 469.4 µg/kg and making the cheese from these milk were not possible. However, the seven-day period was enough to clear tylosin and erythromycin from goat milk, only spiramycin was found to be at the concentration of 79.6 ± 19.2 µg/kg. In addition, no antibiotic residues were determined in the cheeses after these time period. They recommended seven days days of withdrawal time to ensure milk safety regarding these antibiotics. Our results are compatible with the above mentioned studies, and the results of the present study showed that highest levels of tylosin were detected at the second milking (1625.1±358.97 µg/kg) and residue level decreased under the maximum residue limit of 50 µg/kg at ninth milking (38.26±5.29).

CONCLUSIONS

This study determined the milk passage of tylosin for the Anatolian buffaloes by employing a precise, reliable, and accurate LC-MS/MS method. The method was able to determine tylosin within the related MRL for milk due to its low LOD and LOQ values. The present study gives information on the withdrawal period of tylosin in the milk of Anatolian buffaloes. Also, real milk samples were evaluated for the presence of tylosin and determined as safe regarding residue risk of this antibiotic in Afyonkarahisar Province.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Management of congenital flexural tendon contractures with stretching in calves

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ABSTRACT: It has been reported that mild to moderate congenital flexural limb contractures of the calves can be treated with stretching. This study was carried out on eleven calves with mild to moderate flexural foot - tendon contractures (three mild and eight moderate cases). The mild cases were treated successfully with only splints or wooden hoof block/PVC application adhered to the ground under the sole of the hoof with acrylic adhesive. The moderate cases, however,were treated successfully with the combined application in which a splint was placed on an entire limb to correct it and then a wooden hoof block/PVC was adhered to the ground under the sole of the hoof with an acrylic adhesive.

***Keywords*:** Calf, tendon, flexural contracture, management

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INTRODUCTION

The reports suggest that congenital flexor tendon contractures are a common disorder in cattle that can occur in many breeds (Leipold et al., 1993; Leipold et al., 1987) and cause significant economic losses (Steiner et al., 2014; Weaver et al., 2014*)*. Previous research showed that environmental and genetic factors or both may be the cause of some of these disorders, however, the causes of many of them have not been fully clarified (Leipold et al., 1993; Akın et al., 1976). In addition to the inherited factors, it has been suggested that malnutrition during fetal development in the uterus, misposition of the fetus or its large size compared to the uterus play a role in the etiology of this disease (Adams and Santschi, 2000). It is also suggested that this disease is associated with species, race, geographic region, season and other environmental factors.Congenital flexural tendon deformities usually occur around the carpal or heel joint and can range from mild flexion of one joint to severe flexion of several joints. Flexural deformities are primarily caused by the musculotendon units that are shorter than the skeletal structure (Steiner et al., 2014).

Flexural limb deformities of calves are classified as mild, moderate and severe. In bilaterally affected cases,if it is a mild one, the calf can walk by standing the tips of the hoof, but the heel is off the ground.In the moderate cases, the calf can stand upright on the dorsal aspect of the hooves and it can stand without assistance. In severe cases, the animal lies on its side andit usually has difficulty in standing on the dorsal side of the carpus and walking (Adams and Santschi, 2000).

This study was carried out to demonstrate the effectiveness of the physical stretching technique performed by applying pressure bandage and / or wooden hoof block / PVC adhered to the hooves of the calves with first and second degree congenital flexural tendon contractures.

MATERIALS AND METHODS

The study material consisted of eleven calves with flexural limb contractures. Of these cases,three of them were mild and eight were moderate. The calves from various breeds and genders, between 1-day-old to 5-day-old, were brought to Ondokuz Mayıs University Veterinary Faculty Animal Hospital between 2017 and 2019.

After obtaining an anamnesis and performing physical examination, the calves were evaluated to see whether there was a problem with the rotation of the feet and ability to get up and stand. The calves were examined further to rule out any other problems, such as joint disorder, cleft palate and nervous disorder and then, radiography was carried out in suspected cases.

The calves diagnosed with congenital flexural tendon contracture were administered with 0.2 mg / kg xylazine IM and 0.3 mg / kg meloxicam SC before applying each bandage.The calf was placed on its side for bandage and after wrapping the leg with cotton, PVC was placed on the palmar / plantar side of the extremity and a splint was applied on the entire leg. The owners were told that the calf needed help lying down and getting up and instructed accordingly.The bandage application was sustained3 to 6 times with an interval of 4-7 days until the foot joint returned to its proper position.

When the metacarpophalangeal or metatarsophalangeal joints became flat with the bandage and the calves were able to stand on the tip of the toes,the wooden hoof block / PVC were glued to the sole of the hooves with acrylic glue to prevent the hooves from bending back and tohelp the calf gain balance on its feet and also to extend the hooves by 3-4 cm (Figure 1).Support bandages were placed to prevent back bending in those who bent back despite the wooden hoof block/ PVC. (Figure 2).

Figure1. PVC application on the sole of the hooves with acrylic

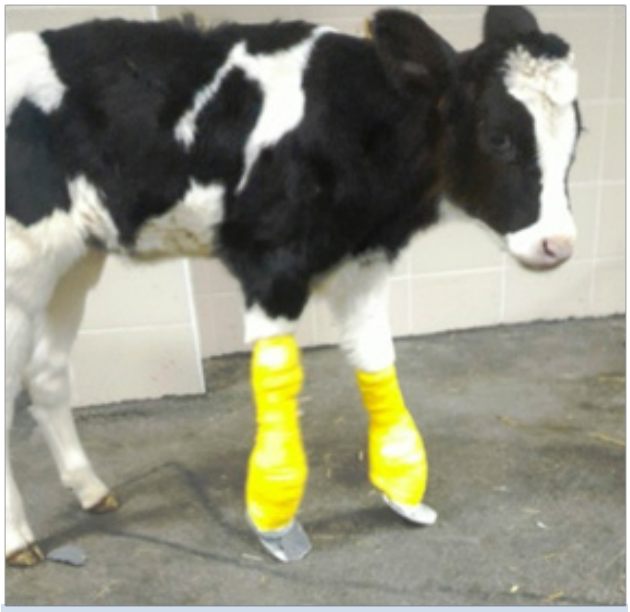

Figure2. Support bandage application/PVC to the foot joint of the calf glued to the sole of the hooves

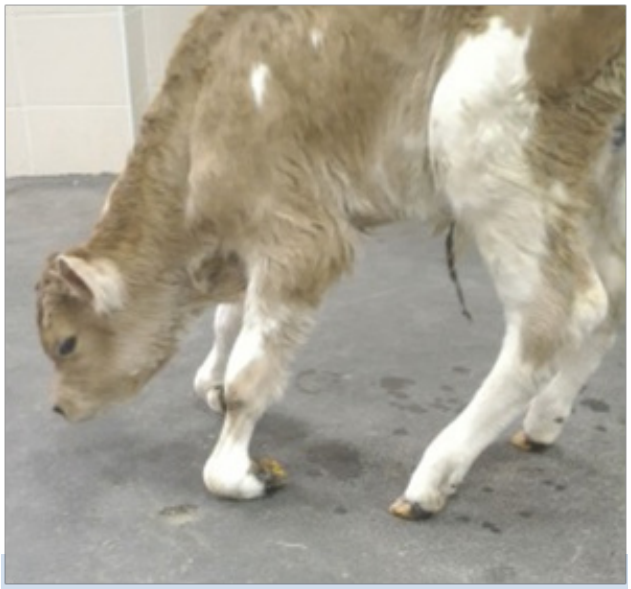

Figure 3. Moderate contracture on the forefoot

The heat generated by the acrylic use was relieved by applying cold water and wet cotton.

A 2-cm-thick wooden block was used in three cases and a 0.5-mm thick PVC pipe was used in eight cases. These procedures were sustained until the hoof-ground contact was observed. When the hoof-ground contact was observed, the procedure was terminated by breaking the acrylic mold.

RESULTS

The application of pressure bandage prevented back bending of the foot in three calves with light - grade flexural tendon contractures. The heel touched the ground with weight gain after two or three bandages. Afterwards, these calves did not show any problems.

When the contracted tendon in the mild - grade (Figures 3 and 4) case extended and the foot became flat (Figure 5) after the application of splint, the calf(n=8) had difficulty standing on the tips of toes and in this case the feet were bent back again. The calf was unable to walk properly.

As a second application, a wooden wedge / PVC were adhered to the sole of hooves to prevent the foot from bending back (Figure 6). In this case, the heels were lifted and were off the ground. The calf was able to walk with some difficulty in a hesitant manner (Figure 7). Afterwards, it was able to lie down and suck its mother. The calf got used to this new state after 3 to 4 days.

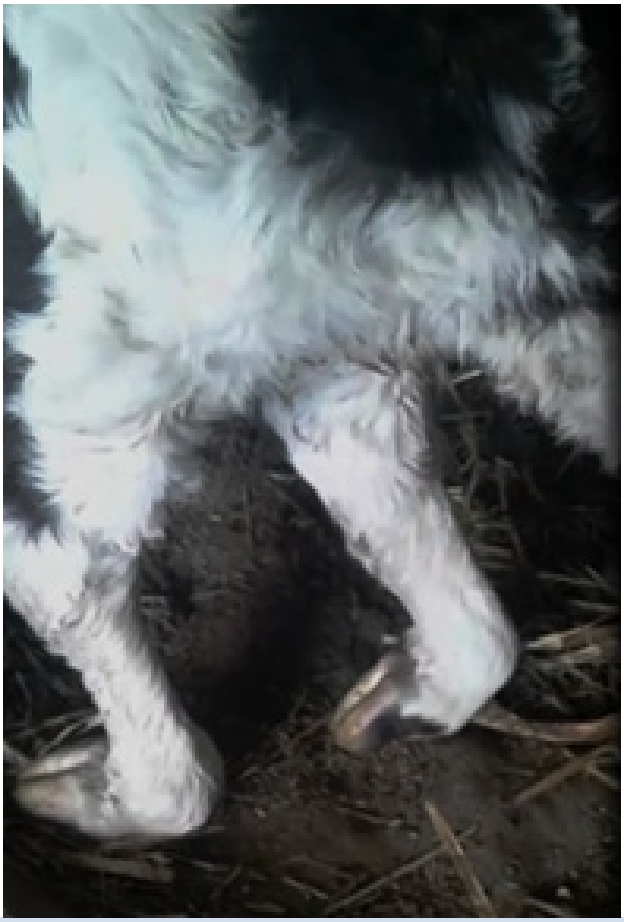

Figure 4. Moderate contracture on the hind legs

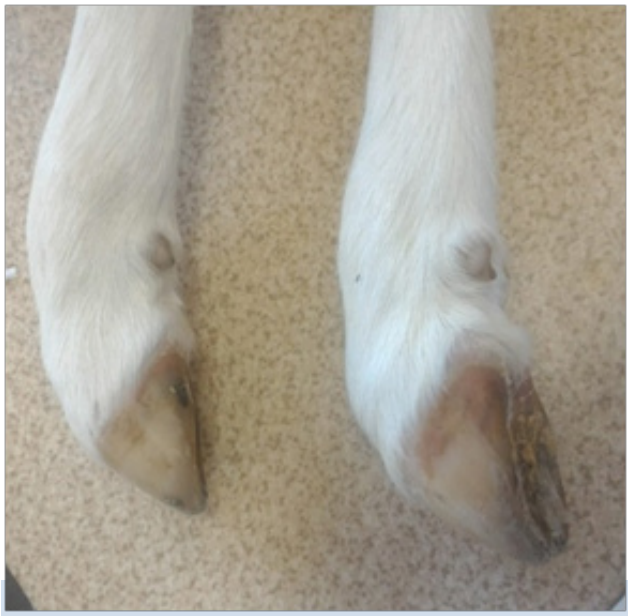

Figure 5. Foot and hooves' joint after the splint

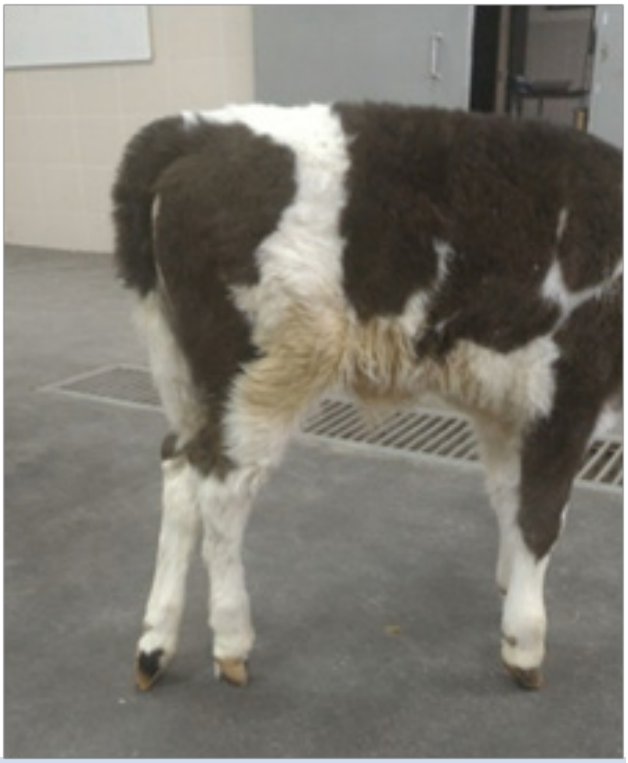

Figure 6. Gait after bandage application

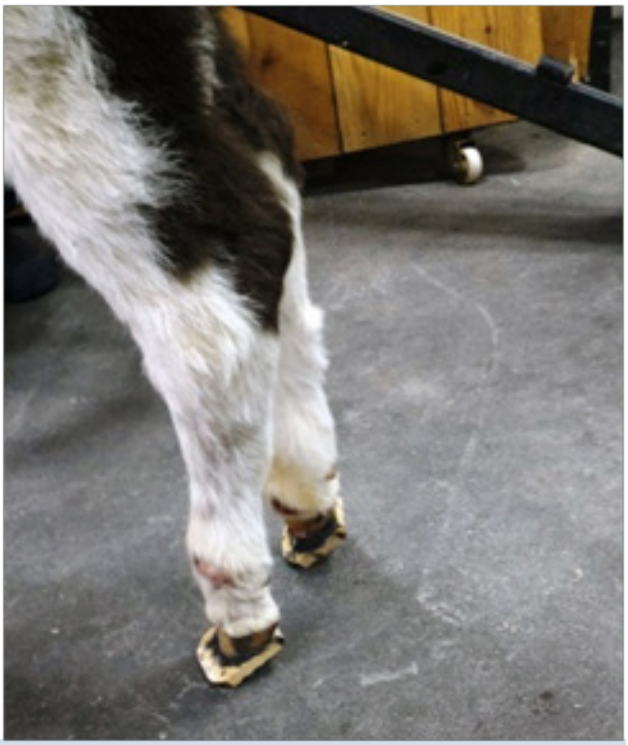

Figure 7. Wooden block application after split bandage use

The calves preferred to lie down more than 3 to 4 days after the wooden block / PVC were glued. During the feeding period, they stood up and lay down after a while.

In two cases, the foot was bent back despite the wooden block/ PVC. Support bandages with the wooden block / PVC were placed on the feet of these calves to make sure they wouldn't bend back (Figure 2). However, the wooden blocks used in both hooves in one case and PVCs in three cases were broken within 5 to 10 days. A new one was reapplied in the place of the broken wood block and PVC.

After the hoof-ground contact within 3-4 weeks, acrylic and wooden block / PVC were broken and therefore, removed (Figure 8). Although there were mild contractures in a few cases after the application, the calves were able to walk without bending their feet (Figure 9).

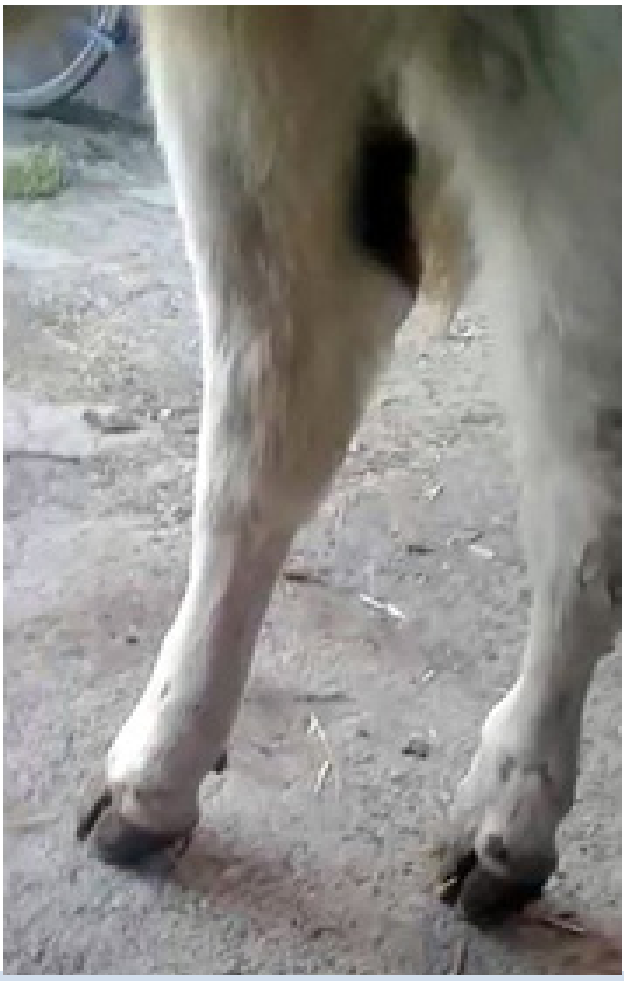

Figure 8. Hind leg after the recovery

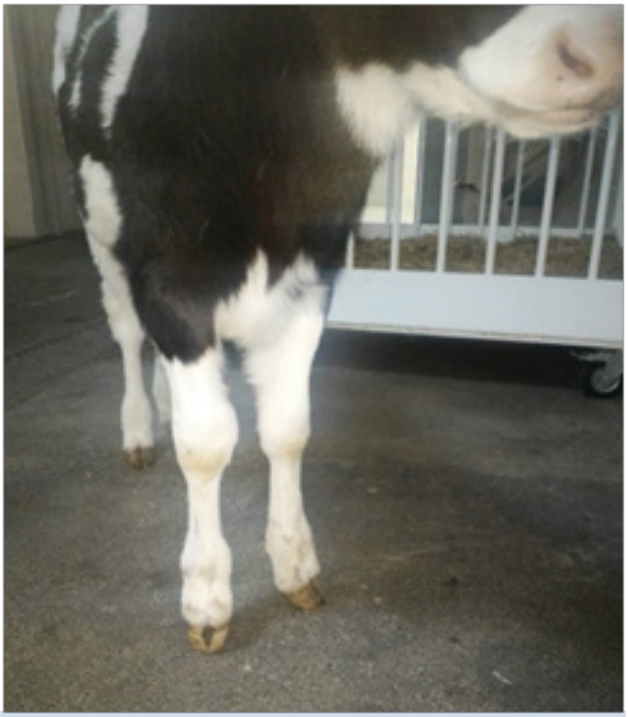

Figure 9. Forefront after the recovery

DISCUSSION

It is reported that mild and moderate congenital flexural contractures occurring in the limbs of calves can be treated with aluminum, PVC or plaster casted splints and it would be useful for extending the joints (Rashmi et al., 2018; Steiner et al., 2014).Additionally, starting the treatment soon after the diagnosis is advised, otherwise the contracted tissues may become less responsive to the treatment (Gençcelep et al., 2017; Fazili et al., 2014; Steiner et al., 2014).

In the present study, it was observed that the three mild cases of flexural contractures improved with the help of splints or wooden hoof block / PVC application.

Some authors recommend leaving the hooves out of the bandage to apply weight pressure and increase tendon tension (Steiner et al., 2014). However, the results in the current study revealed that leaving the hooves out of the bandage did not contribute to walking and standing, on the contrary, the hooves were bent back again in moderate deformities.As the hooves were bent back, it was concluded that the splint application did not create enough tension in the feet and distal phalangeal joint tendons and ligaments.

As a result of the application of the splints to the entire leg to deliver enough tension, the feet and distal phalangeal joint tendons were elongated and straightened (Figure 4).At the end of this stretching process, the calf could only press on the tips of the toes, and this was not enough to walk, the tendons were re-contracted over time and the joint angle was also narrowed.

After the wooden base wedge / PVC were glued to the sole of the feet (Figures 1, 2 and 7) to prevent tendons and ligaments from re-contracting and to help the extension of tendons until the distal phalanx joint gained its normal angle, the calves were able to walk and their heelswere off the ground. But they were not able to stand for a long time and preferred to lie down after a while.

Even though wooden block/ PVC were adhered following splints, the occurrence of re-contracture in two of the calves was thought to be associated with the long periods of lying down and lack of exercise. However, they recovered in two weeks after the bandage application was repeated, in which the foot was prevented from bending.

It is thought that the reason for the wooden blocks

/ PVCs adhered to the sole of the hooves to break within 5-10 days may be due to the softness of the hoof tissue or its softening after the hooves got wet.

CONCLUSIONS

As a result, this study reveals that applying splints or wooden hoof blocks/ PVC to the sole of the hooves of the calves in mild cases of congenital flexural limb contracture could lead to successful results. Also, again successful results can be achieved by applying a splint on the entire leg of a calf and gluing a wooden hoof block/ PVC to the sole of the calf's hoof immediately after the moderate cases of congenital flexural limb contracture.

Overall, the current study provides a compelling evidence that application of wooden hoof block / PVC to the sole of the hooves of the calves may be routinely used in the clinical settings for the treatment of congenital flexural limb contracture.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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Research article
Ερευνητικό άρθρο

Age related changes in testicular histomorphometry and spermatogenic activity of bulls

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ABSTRACT: The aim of the present study was to evaluate age related changes in testicular histomorphometry and spermatogenic activity of bulls during their sexual development. A total of 36 bulls were selected and divided into four groups (n=9 in each) according to their age. Bulls included in Groups I, II, III and IV were 10, 12, 14 and 16 months old respectively. Left testes of bulls were subjected to histomorphometry after slaughter. Statistical analysis revealed that the secondary spermatocytes, round and elongated spermatids increased significantly ($P<0.05$) with the age of bulls. Likewise, both sertoli and leydig cell numbers increased significantly ($P<0.05$) with the age of bulls. However, the number of spermatogonia and primary spermatocytes did not change ($P>0.05$) due to age. The mean tubular diameter increased from 200.70±5.45 µm (10 months of age) to 227.30±9.16 µm (16 months of age) and the total volume of seminiferous tubule per testis from 69.63±1.50 % (10 months of age) to 84.64±2.53 % (16 months of age). A positive linear relationship ($P<0.05$) was found between meiotic index (Y) and the age (X, in month), which was characterized by the equation 0.048X+3.135 and a coefficient of correlation (R) of 0.396. The correlation between age and sertoli cell efficiency was 0.482 with a regression equation Y= 0.141X+7.696. It is concluded that histomorphometric parameters of the bulls' testes and spermatogenic activity are correlated with the age, so these parameters provide a reliable tool for the assessment of the reproductive state and sperm production capacity of a bull in a breeding program.

***Keywords*:** histomorphology; spermatogenic efficiency, testis; age; bulls.

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INTRODUCTION

Fertility in bulls is a complex trait that is made up of several physiological processes such as the development of the reproductive system from birth to puberty, spermatogenesis, ejaculation and mating behaviour (which involves libido and copulation). For optimal semen quality, all these physiological processes should be coordinated. Since the bull has more genetic influence (80- 90%) on calves it sires, fertile bull selection can be the most powerful method for improvement of the herd (Rezende et al., 2018)

Anatomical studies on the male genital system at various ages, particularly the testis and its tubular system, are important to understand the anatomical growth, development and physiology of the bulls. To select a breeding bull the anatomy of the testes of young animal and their gradual development is very important. If any anatomical defect is observed at young age, the animal shouldn't be reared as a breeding bull in spite of good genetic characters. Selection for precocious sexual maturation in bulls can decrease production costs, reduce generation interval, and increase genetic gains and overall productivity (Britao et al., 2004)

The relationship between age and reproductive efficiency in bulls has already been evaluated based on testicular biometry parameters, sexual behavior and sperm production and quality (Pant et al., 2003; Luz et al., 2013; Ahmad et al., 2010; Genedy et al., 2019). It is known that the quantification of spermatogenesis, which consists of the numerical observation of different types of cells and constituents of the seminiferous epithelium and ratios of different cell populations, is an important method of sperm production analysis (Berndtson and Picket, 1987). However, quantitative data on spermatogenesis efficiency, measurements of the tubular diameter, seminiferous epithelium thickness, during sexual development are still inadequately documented. The quantitative histology of the testicles, efficiency of sertoli cells, and general morphometry of the seminiferous tubule may be related to the quantity of spermatozoa in the ejaculate, as well as reproductive efficiency (Kalwar et al., 2020). Information on the process of spermatogenesis should provide a basis for experimental work to promote increased spermatogenic activity in the testes and to achieve puberty at an earlier age. The aim of the present study was to quantify the changes in the seminiferous tubules during sexual development and relate them with age of bulls. The data of testicular activity can be used for the definite selection of bulls in future breeding programs.

MATERIALS AND METHODS

Location

The experiment was carried out in Batna province in Algeria, which is located between 35°33′21″ North latitude and 6°10′26″ East longitude and at an altitude range from 968 m mean sea level. Climate in Batna is semi-arid, with four distinct seasons, mean temperature varies between 4C° (January) to 35°C (July) and mean annual rainfall is 329 mm.

General procedures

A total of 36 local breed bulls (non-descript) were selected and divided into four groups according to their age and each group consisted of 9 bulls i.e., Group I: 10 months, Group II: 12 months, Group III: 14 months, and Group IV: 16 months.

Testicular histology

Within 20 minutes after slaughter, the scrotum was incised, the paired testicular weight was estimated by digital scale. Small pieces were taken from three regions (proximal, middle and distal) of the left testes. Immediately fixed in fixed in formaldehyde 10% for 24 hour, and dehydrated subsequently by submerging in series of concentrated ethanol (70%, 95% and 100%). Afterward dehydrated tissues were cleared and infiltrated by placing in liquid paraffin for embedding procedure. Five alternate sections of 5µm thickness from each slide were cut and stained with hematoxylin and eosin. From each section, at least ten essentially circular tubular cross sections for each bull were assessed to determine tubular diameter and epithelial height using AxioVision Rel 4.6 (Carl Zeiss, Thornwood, NY).

The testicular weight (g) for both testes was directly converted into volume (VT), since the volume density of the testes in mammals is very close to one as reported previously by Lunstra et al. (2003). Relative volume (Vr) of seminiferous tubules (surface occupied by the tubules divided by total surface of the field), was determined using the AxioVision software (Carl Zeiss, Thornwood, NY). Total seminiferous tubules volume per testis (VTS) (%) was measured by multiplying Vr by VT. The volume of the intertubular tissue (VIT): was estimated by subtracting the volume of seminiferous tubules from the total testicular volume.

Seminiferous tubule diameter (STD) was measured at X 200 magnification, using a digital camera (MICROCAM MA88-500) attached to the ocular of a light microscope (ZEISS, Germany, Axioplan) and connected to a computer. The epithelium height (GEH) was obtained in the same tubules used for tubular diameter measurements.

Concerning the assessment of total length of seminiferous tubules (LST); the seminiferous tubules were assumed to form a single cylinder with a radius r and a volume VTS; using the equation $VTS=\pi r^2$ LST (Sarma and Devi, 2017).

The different types of germ cells nuclei and sertoli cell nucleoli per tubular cross-section were evaluated at 600 X magnification using light microscopy on10 roundish randomly selected tubular cross-sections per testis. These counts were corrected for section thickness and nucleus or nucleolus diameter according to a previous report (França and Godinho, 2003). For this purpose, 10 nuclei or nucleoli diameters were measured for each cell type analysed per animal. Numerical correction (Nc) of spermatogonia, primary and secondary spermatocytes, round and elongated spermatids, and leydig cells, was performed by using the equation: Nc=COxE/(E+D) where CO is the number of cell nuclei per unit area, E is the average thickness of the section (5 μm), and D is the average nuclear diameter (measured via AxioVision software). Total number of spermatogonia, primary and secondary spermatocytes, round and elongated spermatids, sertoli cells, and leydig cells per testis were determined by multiplying Nc (for each cell type) by VT. This procedure is more precise for the different types of germ cells and round spermatids because they have nuclei with spherical shape. On the other hand, it is less precise for elongated spermatids and sertoli cells of bulls because these cell nuclei have shapes that are not quite spherical (Hien et al., 2011).

The efficiency of the spermatogenesis process was evaluated through several quantitative parameters based on the counting of various cells of the seminiferous tubules, as reported by Segatelli et al. (2004) and Pintus et al. (2015):

Ratio	Functional aspects
i) Primary spermatocytes / spermatogonia	To estimate the coefficient of efficiency of spermatogonial mitosis
ii) Round spermatid/spermatogonia	To obtain an overall spermatogenesis yield
iii) Round spermatids /Primary spermatocytes	To obtain rate of germ cell apoptosis (loss) during meiosis (Meiotic Index)
iv) Elongated spermatids/ Round spermatids	To estimate the post-meiotic germ loss
v) Round spermatids/ sertoli cells	To estimate sertoli cell efficiency

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (SPSS Inc, Chicago, IL, USA). Observations were grouped according to the age of animals, and means and standard error (SE) were determined. The Kolmogorov-Smirnov and Levene's tests were used to check data normality and homogeneity of variance, respectively. Means were analysed by one way analysis of variance between the different age groups of bulls, followed by Tukey's post hoc test to determine significant differences between the groups. One-tailed Pearson correlation test and regression analysis were used to assess the relationship between various observed parameters and age. A value of $P\leq0.05$ was considered statistically significant. Furthermore, according to the level of statistical significance, results of the present study are presented as follows: $P<0.05$: (*), $P<0.01$: (**) and $P<0.001$: (***), respectively.

RESULTS

Age related changes in histomorphometric testicular parameters

The results of different germ cells number at different age of bulls are presented in Table 1. These results revealed that spermatogonia, primary and secondary spermatocytes, round and elongated spermatid, sertoli cells and leydig cells were present in bulls of all ages (10, 12, 14 and 16 months).

According to age, the sertoli cell number, second-

ary spermatocytes and round and elongated spermatids increased significantly (P<0.05): the maximum numbers of these cells per testis were found in the 16 years age group, followed by the 14, 12, and 10 month age groups. Similarly, mean values for leydig cells number was lower (P<0.01) in the groups I (10 months) compared to group IV (16 months) bulls. However, there was no difference in the mean values of the spermatogonia and primary spermatocytes number between the experimental groups (P>0.05).

The relationship between age (X) and the different germ cells number (Y) is shown in Table 2. The sertoli cell number showed a linear relationship with the age of bulls and was characterized by the equation Y=0.164X+1.848, R=0.543 (P<0.01).

A positive linear relationship (P<0.01) was found between leydig cells number and the age of bulls (Table 2).

Table 1. Number (Mean ± SE) of different cells per testis and per gram of testes of male bulls at different ages.

Age	Paramaters						
	Sertoli cells	Spermatogonia	Primary spermatocytes	Secondary spermatocytes	Round spermatids	Elongated spermatids	Leydig cells
Total number per testis ($x10^9$)							
GI	2.80±0.39*	2.72±0.39 ^{NS}	7.73±0.65 ^{NS}	6.39±0.52*	20.57±1.42*	10.90±4.5*	3.72±0.11**
GII	3.23±0.23*	3.11±0.23^{NS}	8.28±1.10 ^{NS}	7.54±0.88*	21.76±2.4*	17.93±2.1*	3.94±0.19**
GIII	3.92±0.24*	3.81±0.24 ^{NS}	9.55±0.69 ^{NS}	9.07±0.55*	23.09±1.5*	20.17±3.4*	4.93±0.12**
GIV	4.14±0.39*	3.98±0.39 ^{NS}	8.24±1.10 ^{NS}	9.54±0.84*	28.46±2.6*	26.96±4.5*	5.29±0.20**
Total	3.60±0.14	3.47±0.14	8.32±0.39	7.97±0.35	21.62±8.43	23.04±2.11	4.38±0.12
Total number per gram of testis ($x10^6$)							
GI	4.93±0.68 *	4.61±0.42 ^{NS}	10.36±1.41^{NS}	10.48±0.80*	13.98±7.94**	27.72±2.51*	6.126±0.42*
GII	4.77±0.43 *	4.69±0.67 ^{NS}	11.41±0.88^{NS}	12.76±1.34*	28.88±4.72**	27.09±4.04*	6.212±0.42*
GIII	5.42±0.41*	5.22±0.40^{NS}	12.42±0.84^{NS}	11.01±0.84*	32.49±4.95**	33.38±2.40*	6.748±0.26*
GIV	5.56±0.68*	5.37±0.67 ^{NS}	14.16±1.41^{NS}	12.57±1.34*	62.84±7.94**	41.49±4.04*	7.037±0.25*
Total	5.26±0.23	5.06±0.23	11.97±0.49	11.58±0.48	31.71±1.49	34.11±3.40	6.43±0.23

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01; ***: Significant at P <0.001.

Table 2. Relationship between the different cells in testis and age of bulls.

Dependent variable (Y)	Regression equation (X=Age)	R
Sertoli cells	Y=0.164X+1.848	0.543**
Spermatogonia	Y= 0.159X+1.777	0.225^{NS}
Primary spermatocytes	Y=0.295X+5.226	0.213^{NS}
Secondary spermatocytes	Y=0.552X+2.247	0.539**
Round spermatids	Y=2.775X+15.447	0.621***
Elongated spermatids	Y=0.291X+19.971	0.465**
Leydig cells	Y=0.163X+2.698	0.438**

^{NS}: Not significant; **: Significant at P <0.01. ***; Significant at P <0.001.

Age related changes in morphometric testicular parameters

Data corresponding to values obtained for total seminiferous tubule volume, total length and diameter of the seminiferous tubules, interstitium volume and seminiferous epithelial height during testicular development from 10 to 16 months of age are presented in Table 3.

The volume percent occupied by the seminiferous tubule differed significantly between the four groups (P<0.05). The seminiferous tubules volume ranged from 69.63±1.50 to 84.64±2.53 %.

The diameters of seminiferous tubule were significantly larger (p < 0.05) in the testes of bulls at 16 months compared to those at 10 and 14 months. The seminiferous tubule diameters at age of 10, 12, 14, and 16 months were 200.7±5.45, 202.0±9.16, 213.2±5.71, and 227.3±9.16μm, respectively. Although the length of seminiferous tubules increased with age, no significant differences was observed between the age groups (P>0.05).

The interstitium volume decreased significantly (P<0.01) with increase in the age of bull whereas the height of germinal epithelium in testes increased with the increase in the age of bulls (P<0.05; Table 3).

Table 3. Morphometrical values (Mean ± SE) of seminiferous tubules of testes in male bulls at different ages.

Age	Parameters				
	Total seminiferous tubule volume per testis (%)	Seminiferous tubules diameter (μm)	Length of seminiferous tubules (m)	Interstitium volume (%)	Germinal epithelium height of testes (μm)
GI	69.63±1.50*	200.70±5.45*	3043.76±201.05^{NS}	20.57±0.74**	79.43±0.75*
GII	70.46±2.53*	201.96±9.16*	3436.45±337.84NS	15.88±1.27**	83.40±1.27*
GIII	84.64±1.57*	213.23±5.71*	3234.25±210.68NS	13.55±0.79**	84.11±1.27*
GIV	84.64±2.53*	227.30±9.16*	3526.43±337.84NS	16.59±1.27**	86.44±0.79*
Total	83.01±0.65	209.01±3.45	3251.71±115.59	16.98±0.65	76.88±1.59

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01.

Table 4. Relationship between the morphometric data of testes and age of bulls.

Dependent variable (Y)	Regression equation (X= Age)	R
Total seminiferous tubule volume per testis (%)	Y= 1.158X+71.046	0.626***
Seminiferous tubuli diameter (μm)	Y= 1.741X+191.010	0.520**
Length of seminiferous tubules (m)	Y= 65.013X+2579.391	0.196^{NS}
Interstitium volume (%)	Y= -1.158X+28.954	-0.616***
Germinal epithelium height of testes (μm)	Y= 1.984X+56.342	0.499**

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01; ***: Significant at P <0.001.

Relationship of age with morphometric testicular parameters of bulls is presented in Table 4. A positive relationship (P<0.001) was found between seminiferous tubule volume and the age of bulls. This relationship was linear and was characterized by the equation Y= 1.158X+71.046, where Y equals total seminiferous tubule volume in percent, and X denotes the age in month. The coefficient of correlation (R) between these parameters was 0.626 (P<0.001).

Age related changes in the efficiency of the spermatogenesis

Data corresponding to values obtained for the efficiency of the spermatogenesis are presented in Table 5. The efficiency coefficient showed uniformity in the average values found, ranging from 2.34 in GI to 2.55 in G IV (P>0.05). The maintenance of the efficiency of spermatogonial mitosis among the different experimental groups may be confirmed further through the results of the corrected number of cells in a testis on per gram basis as listed in Table 1.

The overall spermatogenic yields was significantly higher (p < 0.01) in the testes of 16 months old bull compared to those of 10 and 14 months old bulls. Again, the Meiotic Index (ratio between round spermatids and pachytene primary spermatocytes) recorded in the present study in bulls ranged from 2.3 ± 0.20 at 10 months to 3.0 ± 0.20 in 16 month-old bulls. The overall Meiotic Index in bulls was recorded as 2.6 ± 0.07. The ratio of elongated spermatids to round spermatids differed significantly (P<0.01) between groups and the measurements increased significantly by the advancement of bull's age. The efficiency of sertoli cells (ratio of round spermatid and sertoli cell nuclei) showed a significant (P < 0.01) increase between 10 and 16 months of age (Groups-I & IV). Relationship of age to efficiency of the spermatogenesis in bulls is presented in Table 6.

Table 5. Efficiency of the spermatogenesis (Mean ± SE) in bulls of different ages.

Age	Parameters				
	Efficiency Coefficient	Meiotic index	Overall Spermatogenesis Yield	Elongated spermatids /Round spermatids	Sertoli cell efficiency
GI	2.34±0.30^{NS}	2.29±0.20*	5.40±0.80**	0.52±0.25**	5.21±0.75**
GII	2.44±0.18^{NS}	2.66±0.11*	6.45±0.43**	0.82±0.25**	5.89±0.45**
GIII	2.43±0.16^{NS}	2.97±0.12*	6.10±0.47**	0.87±0.15**	6.17±0.40**
GIV	2.45±0.30^{NS}	3.04±0.20*	8.88±0.80**	0.94±0.14**	8.46±0.75**
Total	2.48±0.10	2.63±0.07	6.50±0.30	0.78±0.10	6.23±0.28

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01; ***: Significant at P <0.001.

Table 6. Relationship between the efficiency of spermatogenesis and age of bulls.

Dependent variable (Y)	Regression equation (X= Age)	R
Efficiency Coefficient	Y= 0.012X+2.611	0.039^{NS}
Meiotic index	Y= 0.048X+3.135	0.396*
Overall Spermatogenesis Yield	Y= 0.157X+8.125	0.378*
Elongated spermatids /Round spermatids	Y= 0.109 X+2.261	0.473**
Sertoli cell efficiency	Y= 0.141X+7.696	0.482**

^{NS}: Not significant; *: Significant at P <0.05; **: Significant at P <0.01.

DISCUSSION

The present study estimates the spermatogenic efficiency and histomorphometry of bull's testis structure during the sexual development in a local non-descript cattle breed. These standards can be used for selection of bulls maintained for breeding programs to eliminate the problem of infertility and could be help in minimizing the age at puberty.

In the present study, the total number of secondary spermatocytes and round and elongated spermatids increased with age of the male (Table 1). Our findings were consistent with those of Karmore et al. (2001), who reported that the number of these cells was lower in prepubertal than pubertal and post-pubertal animals. Sun et al. (2017) reported that cattle yak had similar histomorphological structures at 10, 12, and 14 months of age. In pigs, the number of germ cell per seminiferous cord/tubular cross section was very low from birth to 4 months of age. A very dramatic increase in various populations of germ cells per cross section of seminiferous tubule occurred from 4 to 5 months of age, but the number of various germ cells showed a tendency to stabilize after 7 months of age (França et al., 2000).

In this study, the relationship between bull's age and Sertoli cell number was highly significant. In horses, it has been reported that the Sertoli cells have a stable population size in adults because evidence of Sertoli cell division and Sertoli cell death is not obvious in adults. However, the age-related loss of Sertoli cells in humans was not accompanied by obvious degeneration of Sertoli cells, and mitotic activity at the base of seminiferous epithelium is generally considered spermatogonial in nature (Johnson et al., 1994). Based on the fact that each Sertoli cell supports a limited number of germ cells in a species-specific manner (Almeida et al., 2006), and the number of Sertoli cell is established before puberty; hence, it determines the rate of sperm production in sexually mature animals (Johnson et al., 2008).

Most authors report a decrease in the number of Leydig cells with age (Gofur et al., 2008; Petersen et al., 2015). This stands in contrast with our finding for total number of Leydig cells, which did not decline with age. Karmore et al. (2001) reported that the number of Leydig cells was less in prepubertal than in pubertal and post-pubertal goats. In most mammals, other than seasonal breeders, the Leydig cells undergo a phase of proliferation from resident stem cells during pre-puberty, followed by differentiation, which results in a fixed number of Leydig cells persisting with only modest attrition throughout life (Teerds and Huhtaniemi, 2015). Because the body and testicular weights increased substantially during development (Lee et al., 2004), the demands for steroids and other substances secreted by Leydig cells were probably higher, requiring more Leydig cells per testis.

In the current study, the total seminiferous tubule volume showed changes during testicular development. This might be attributed to cellular proliferation, secretion of tubular fluid, and appearance of the tubular lumen. A similar growth pattern has been reported by Genedy et al. (2019). In general, there is 70 to 90% of the seminiferous tubule volume in mammals (França and Russel, 1998) and the observed seminiferous tubules volume (84.64%) is situated around the mean range observed for mammals.

Our findings revealed that the seminiferous tubule diameter increased with advancing age. The significant increase in diameter between 10 and 16 months indicated fast development of the tubules before sexual maturity. Similar findings were documented in Assam goats (Sarma and Devi, 2017) and cattle (Wrobel et al., 1988). Ibrahim et al. (2013) reported that an increase in the process of spermatogenesis led to an increase in the thickness and diameter of seminiferous. Based on these observations, it is implied that the reproductive capacity of bulls in group IV might be

greater than group I, II and III due to larger seminiferous tubules (Gofuret al., 2008).

Ratio estimation between primary spermatocytes and spermatogonia in the present work revealed that the efficiency of spermatogonial mitosis did not vary significantly between the age groups. Sarma and Devi (2017) revealed that the efficiency of spermatogonial mitosis increased noticeably from 4 months of age onward, with the maximum being in 10-month-old goats. In mammals, only 2 or 3 of 10 expected spermatozoa are produced from differentiated type A spermatogonia, and the highest level of germ cell apoptosis occurs during the spermatogonial phase through a density-dependent regulation and during meiosis due to chromosomal damage (Almeida et al., 2006).

Again, the Meiotic Index recorded in the present study in bulls, denoting a decrease in germ cell loss during meiosis with increasing age of the male. However, further works on this aspect is required as no available literature could be traced to compare with the present findings. The meiotic index total was 2.63±0.07. Similar data have been reported for the goat (2.8±0.3; Leal et al., 2004), gerbil (2.8±0.1; Segatelli et al., 2004), cat (2.6±0.2; Neubauer et al., 2004), jaguar (2.66±1.11; Pintus et al., 2015), despite the fact that in these studies only spermatocytes in prophase were considered.

The expected ratio of elongated spermatids to round spermatids is theoretically 1.0 because spermiogenesis does not involve further cellular divisions (Pintus et al., 2015). It was observed that this ratio was only 0.52±0.25 in the experimental Groups I and was significantly lower compared to the Group IV. The lowest ratio of elongated spermatids to round spermatids suggests that a greater selective spermiogenesis during the breeding season may be useful to minimise low quality sperm cells in the ejaculate. However, no such reports were found available in the literature to compare the present findings.

The efficiency of Sertoli cells increased from 10 to 16 months of age in bulls. However, the present mean value for efficiency of Sertoli cell was relatively low when compared with other species such as Assam goats and gerbil (Segatelli et al., 2004; Sarma and Devi, 2017). The noticeable flexibility among species in the number of spermatids supported by a single Sertoli cell shows that, in general, species in which the ratio of spermatids to Sertoli cells is higher may also have a higher spermatogenic efficiency (Sharpe, 1984; França and Russell, 1998).

CONCLUSION

In the present investigation, we obtained several fundamental data regarding the testis structure and several indices that quantify testicular activity in the bull. In conclusion, histomorphometric parameters of the bulls' testes and spermatogenic activity can vary depending on age, but the spermatogonia, primary spermatocytes number and the length of seminiferous tubules were stable during sexual development. It can be recommended that the relationship between the stereological testicular parameters and age may be a reliable tool for the assessment of the reproductive state and sperm production capacity of bull. Thus testicular activity can be used for the definite selection of bulls in future breeding programs.

CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research article
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Screening of AmpC-/ESBL-producing *Escherichia coli* isolates from livestock for STEC/EHEC virulence genes

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ABSTRACT: Livestock is an important reservoir of Shiga toxin-producing *Escherichia coli* and enterohemorrhagic *E. coli* (STEC/EHEC) strains and acts as a significant source of transmission to humans. In addition to the virulence of STEC/EHEC isolates, antibiotic resistance is also an escalating problem in these bacteria and increases the risk to public health. Therefore, the present study aimed to explore *E. coli* O_{157}:H_7 serotype and STEC/EHEC virulence genes in AmpC- and extended-spectrum beta-lactamase (ESBL)-producing *E. coli* isolates from cattle, chicken and sheep. A total of 61 confirmed AmpC- or ESBL-producing *E. coli* isolates were screened for the virulence genes (*stx1, stx2, eae*, *ehxA, espP*, *katP* and *saa*) and *E. coli* O_{157} (*rfbO*$_{157}$) and H_7 (*fliCH*$_7$) genes by polymerase chain reaction (PCR). None of the ESBL-producing *E. coli* was positive for these genes, but six multidrug-resistant AmpC-producing *E. coli* were positive for the *fliCH*$_7$ gene only. When considering the function of the H_7 flagellar antigen of *E. coli*, it may be concluded that the development of ESBL/AmpC beta-lactamase production in the *E. coli* isolates with H_7 flagella, which reside in the chicken intestine, may be potentially important for public health regarding both virulence and antimicrobial resistance.

Keywords: AmpC, ESBL, *Escherichia coli*, *fliCH*$_7$, multidrug resistant

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INTRODUCTION

Escherichia coli is an important bacterial species containing both commensal strains of intestinal microflora and pathogenic strains causing infections in various parts of the body of both humans and animals. Therefore, *E. coli* strains are divided into two main pathogenic groups: intestinal and extraintestinal pathogenic strains. Intestinal pathogenic strains in humans have been classified into several pathotypes based on their virulence characteristics and infection mechanisms. Six main intestinal pathogenic *E. coli* strains have been described; namely,enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli*(EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). Intestinal pathogenic *E. coli* strains originate from domestic animals and are transmitted to humans by contaminated food, water, or by direct contact (Haiko and Westerlund-Wikström, 2013). EHEC strains are responsible for bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in humans (Bugarel et al., 2011). All of them cause life-threatening infections in humans. Livestock is known as a reservoir of STEC/EHEC strains, and cattle are notably recognized as carriers of O_{157}:H_7 and STEC/EHEC strains as revealed by several studies (Venegas-Vargas et al., 2018).

The World Health Organization (WHO) reported that extended-spectrum beta-lactamase- (ESBL) producing *E. coli* are resistant bacteria that have been classified as presenting a high risk to public health (WHO, 2017). Reports indicate an increasing prevalence of ESBL-/AmpC-producing *E. coli* as both commensal and pathogenic strains in livestock. On the other hand, the zoonotic potential of ESBL-/AmpC-producing *E. coli* (from farm animals to humans) has been proved (Huijbers et al., 2014). Therefore, it is worth investigating the virulence potentials of ESBL- and AmpC-producing *E. coli* strains isolated from healthy animals. In this study, the presence of serotype O_{157}:H_7 and STEC/EHEC virulence genes were investigated in ESBL-/AmpC-producing fecal *E. coli* isolates from cattle, chicken and sheep.

MATERIALS AND METHODS

In the present investigation, a total of 61 AmpC- or ESBL-producing *E. coli* stock isolates from previous studies were used. The above isolates originated from fecal samples of cattle, chicken and sheep in Burdur, Turkey were subjected to ESBL confirmatory test (phenotypically) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines while the presence of genes (TEM, SHV and CTX-M), plasmidic AmpC genes (ACC, CIT, DHA. EBC. FOX and MOX families) and the phylogenic group(A, B1, B2, and D)were detected by PCR.The AmpC-producing *E. coli* isolates had been isolated from 2 chicken farms and ESBL-producing *E. coli* isolates had been isolated from 8 cattle, 3 sheep and 2 chicken farms (Pehlivanoglu et al., 2016; Pehlivanoglu et al., 2017; Pehlivanoglu 2017). Information about the source and phylogenetic characteristics of the isolates are presented in Table 1.

The presence of *E. coli* serotype O_{157}:H_7 and STEC/EHEC virulence genes were determined by PCR. PCR was performed for *rfbO*$_{157}$, *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2), *eae* (intimin),and *ehxA* (enterohemolysin) according to Bai et al. (2012), for *espP* (extracellular serine protease) and *katP* (catalase peroxidase) according to Posse et al. (2007),for *saa* (autoagglutinating adhesin) genes according to Paton and Paton (2002), and for *fliCH*$_7$gene according to Osek (2003). Primer sequences used in the determination of virulence genes are presented in Table 2. PCR products were run on a 1.0 % agarose gel, visualized, and photographed under UV light.

RESULTS

In the present study, 46 ESBL-producing *E. coli* were not carriers of the STEC/EHEC virulence genes investigated. Amongst the AmpC beta-lactamase-producing *E. coli* isolates (n = 15), six isolates that had been isolated from chickens were positive for the *fliCH*$_7$ gene only. The other genes investigated were also absent in the AmpC-producing *E. coli* isolates. All the *fliCH*$_7$ gene-positive *E. coli* isolates (n = 6) were from B1 phylogenetic group and one chicken flock. The antibiotic susceptibility pattern was the same for six of them, and they were multidrug-resistant (MDR) isolates (resistant to streptomycin, sulfamethoxazole-trimethoprim, nalidixic acid, enrofloxacine, and tetracycline). All the *fliCH*$_7$-positive *E. coli* isolates were CIT type pAmpC beta-lactamase producers (Table 3).

Table 1. Origins of *E. coli* isolates (Pehlivanoglu et al. (2016); Pehlivanoglu et al. (2017); Pehlivanoglu (2017).

Animal species	Beta- lactamase type	Herd / flock (n)	*E. coli* isolates (n)	Phylogenetic group						
				A_0	A_1	B1	$B2_2$	$B2_3$	D_1	D_2
Cattle	ESBL	8	31	2	15	8	-	-	3	3
Chicken	ESBL	2	12	-	-	5	-	-	1	6
Chicken	AmpC	2	15	-	8	6	-	-	1	-
Sheep	ESBL	3	3	-	2	1	-	-	-	-
Total		15	61	2	25	20	-	-	5	9

n: number of isolates

Table 2. The primer sequences for STEC/EHEC virulence genes and O_{157}:H_7 type of *E. coli*.

Target gene	Primer sequence (5'----------3')	Amplicon (bp)	Reference
$rfbO_{157}$	F-CAGGTGAAGGTGGAATGGTTGTC R-TTAGAATTGAGACCATCCAATAAG	296	Bai et al. (2012)
fliCH_7	F-GCTGCAACGGTAAGTGAT R-GGCAGCAAGCGGGTTGGT	948	Osek (2003)
stx1	F-TGTCGCATAGTGGAACCTCA R-TGCGCACTGAGAAGAAGAGA	655	Bai et al. (2012)
stx2	F-CCATGACAACGGACAGCAGTT R-TGTCGCCAGTTATCTGACATTC	477	Bai et al. (2012)
eae	F-CATTATGGAACGGCAGAGGT R-ACGGATATCGAAGCCATTTG	375	Bai et al. (2012)
ehxA	F-GCGAGCTAAGCAGCTTGAAT R-CTGGAGGCTGCACTAACTCC	199	Bai et al. (2012)
espP	F-GATTACAGCACGCATTCATGGTAT R-TCCAGGCATCCTCAGTGACA	73	Posse et al. (2007)
katP	F-GAAGTCATATATCGCCGGTTGAA R-GTCATTTCAGGAACGGTGAGATC	73	Posse et al. (2007)
saa	F-CGTGATGAACAGGCTATTGC R-ATGGACATGCCTGTGGCAAC	119	Paton and Paton, (2002)

Table 3. PCR results for STEC/EHEC virulence genes and O_{157}:H_7 type specific genes

Animal	*E. coli*isolates	n	STEC/EHEC virulence genes and O_{157}:H_7 serotype spesific genes (n)								
			rfbO$_{157}$	*fliCH*$_7$	*stx1*	*stx2*	*eae*	*ehxA*	*espP*	*katP*	*saa*
Cattle	ESBL	31	-	-	-	-	-	-	-	-	-
Chicken	AmpC	15	-	6	-	-	-	-	-	-	-
	ESBL	12	-	-	-	-	-	-	-	-	-
Sheep	ESBL	3	-	-	-	-	-	-	-	-	-

n: number of isolates

DISCUSSION

E. coli contains peritrichous flagella. The flagellum of *E. coli* has a heterogeneous character, and *E. coli* strains are classified into H-serotypes according to the seroreactivity of the variable antigenic domain of FliC (Haiko and Westerlund-Wikström, 2013). In this investigation, we detected only one gene, the-*fliCH*$_7$ gene, coding for the H_7 type flagella. Therefore, our discussion focused on the H_7 type flagella.

So far, 53 H flagellar antigens (numbered from 1 to 56, excluding 13, 22, and 50) were characterized serologically from *E. coli* species (Wang et al., 2003). On the other hand, molecular identification of the flagellum type of *E. coli* is based on the sequence of the *fliC* gene, which encodes the flagellar filament protein. Differences in the amino acid sequence in the central part of the FliC protein determine the different H types because the N and C terminal parts of the FliC protein are highly conserved, and the central part is exposed to the surface and is highly variable (Reid et al., 1999). In this study, primers specific to the H_7

type FliC protein were used. Therefore, we could determine the *E. coli* strain(s) carrying the H_7 type flagella.

The flagella of *E. coli* have been shown to play essential roles in motility and adhesion. Especially in intestinal pathogenic *E. coli* strains, H_7 flagella act as adhesins at the initiating step of EHEC infections but did not have any functions during later phases. In O_{18}:K_1:H_7*E. coli* (extraintestinal), the serotype responsible for newborn meningitis, the H_7 flagellum is involved in infection pathogenesis and the invasion of brain microvascular endothelial cells. Reports indicate that the expression of H_7 flagella by both EHEC and newborn meningitis causing *E. coli* is upregulated after contact with host cells (Haiko and Westerlund-Wikström, 2013).

CONCLUSION

In conclusion, to the best of our knowledge based on our PubMed search, this report is the first publication of an AmpC-producing *E. coli* with a *fliCH*$_7$gene present in healthy chicken. In the current study, the pAmpC-producing *E. coli* isolates that were positive for the *fliCH*$_7$ gene did not belong to the O_{157} serotype and were not STEC strains. However, there are many prevalent *E. coli* strains from several O serotypes with H_7 flagellum and cause extraintestinal infections in both humans and animals. For example, O_1:K_1:H_7 and O_2:K_1:H_7 cause urinary tract infections, septicemia, and neonatal meningitis; and O_{18}:K_1:H_7 serotype causes neonatal meningitis (Delannoy et al., 2017). Therefore, more virulence factors should be investigated in ESBL-/AmpC-producing *E. coli* isolates in the present study to be able to evaluate their pathogenic potential better (for example, for O_{55}:H_7 as an EPEC strain, ETEC, and others).

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CONFLICT OF INTEREST

The author declares no conflict of interest

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Research article
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Effect of supplementing flaxseed oil on growth and carcass traits of Friesian bulls

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ABSTRACT: This study aimed to evaluate effects of supplementing flaxseed oil (FSO) on growth and carcass traits, as well as meat chemical composition, quality, and fatty acids (FA) profile in Friesian bulls. The bulls (n = 30) were randomly divided into 3 groups (G1-G3, n = 10/group). In G1 (control), animals fed basal diet, while in G2 and G3, they were supplemented with 2% and 4% FSO, respectively, for ~ 7 months. The obtained results revealed that bulls fed diet supplemented with 2% (G2) and 4% (G3) FSO had significantly higher TDN intake ($P < 0.01$) and average daily gain ($P < 0.05$) than G1. Additionally, G3 showed significantly higher hot carcass weight ($P<0.001$), dressing % ($P<0.05$), fat weight ($P<0.05$), boneless meat weight ($P<0.001$), 9-11th ribs cut weights ($P<0.05$), DM ($P<0.01$), CP ($P<0.05$), and CF ($P<0.05$) in eye muscle, and general cooked meat quality ($P<0.05$) than G1. However, meat of G3 had significantly ($P<0.05$) lower water-holding capacity than G1. Meat contents of C20:0 and C22:0 SFAs were significantly higher in G3 ($P<0.05$) than G1, while C14:0, C15:0, and C17:0 were significantly ($P<0.05$) lower in G3 and G2 than G1. C16:1 trans-9 MUFA was significantly higher in G3 ($P<0.01$) and G2 ($P<0.05$) than G1, while C18:1 cis-9 +trans-13-14 and C20:1 cis-11 were significantly lower in G3 ($P<0.001$) and G2 ($P<0.05$) than G1. Among the 2 treated groups, only G3 had significantly higher C17:1 cis-9 ($P<0.05$), C18:1 cis-11+trans15 ($P<0.01$), and C18:1 cis-15+trans-16 ($P<0.01$) and significantly lower C16:1 cis-7 ($P<0.05$) and C18:1 trans-12 ($P<0.01$) than G1. Meat contents of C18:3 n-3 (ALA), C22:5 n-3 (EPA) and C22:6 n-3 (DHA) was significantly higher in G3 ($P<0.0001$) and G2 ($P<0.05$) than G1. The total n-3 FAs content in meat was significantly ($P<0.0001$) higher in G3 and G2 than G1, while only G3 showed significantly higher total PUFA ($P<0.05$) than G1. The n-6:n-3 ratio was significantly ($P<0.0001$) lower in G3 and G2 than G1. With these results, we could conclude that flaxseed oil supplementation in bull diets could improve growth performance, and carcass quality and increase omega-3 FA in animal meat.

Keywords: Friesian bulls; carcass traits; meat quality; fatty acid profile.

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INTRODUCTION

It is beneficial for our health to consume meat with low content of saturated fatty acids (SFA) and high content of polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs). Among the most important PUFAs, omega-3 FAs come at the top due to their role in decreasing bad cholesterol (LDH) and increasing good cholesterol (HDL), thereby reducing cardiovascular diseases. Omega-3 FAs are essential fats because human and animal body can not build them, and the only source is food (Palmquist, 2009). The most common types of omega-3 FAs are alpha-linolenic acid (ALA, C18:3 n-3), docosahexaenoic acid (DHA, C22:6 n-3), and eicosapentaenoic acid (EPA, C20:5 n-3). Additionally, conjugated linoleic acid (CLA, C18:2 cis-9, trans11+trans-7, cis-9+trans-8, cis-10) and linoleic acid (LA, C18:2 n-6) are two important members of omega-6, which are also essential FAs and are very important for our health (Baba et al., 2016). The recommended ratio of omega-6 (n-6) to omega-3 (n-3) in human food is 4: 1. If this ratio increased above 5:1, human body would be more susceptible to hypercholesteremia and cardiovascular diseases. Hence, it is crucial to elevate PUFA:SFA and to decrease n-6:n-3 ratio to get healthy meat (Scollan et al., 2006).

Ruminant meat is rich in high quality proteins, fats (especially omega-3 FAs), vitamins and minerals (Ebrahimi et al., 2014; Oliveira et al., 2012; Scollan et al., 2014). The main source for meat omega-3 FAs is the consumption of diet supplemented with omega-3 FAs-enriched vegetable oils (Deng et al., 2017). Additionally, Palmquist, (2009) and Scollan et al., (2006) found that cattle fed diet supplemented with n-3 PUFA or LA had significantly higher n-3 PUFA and CLA contents in their meat fat. Similarly, animals fed diets supplemented with linseed oil (LSO), which contains a high content of ALA, had significantly higher ALA content in their meat fat (Mach *et al*., 2006; Herdmann *et al*. 2010). Moreover, dietary supplementation of vegetable oils also enhances rumen metabolism, nutrients digestibility, and growth performance (Kowalsk *et al*., 1997). Indeed, dietary supplementation of sunflower oil increased the final body weight and average daily gain in goats (Saqhiret al. 2012).

Flaxseed oil (FSO), which is rich in PUFAs particularly ALA, is considered as the main constituent of some enzymes involved in the metabolism of protein and growth of organs, and thus it could be beneficial for growth performance and immune response (Abu El-Hamd et al., 2019). FSO can also effectively enhance the nutritional value of PUFAs (Scollan et al. 2014) and increase carcasses grading choice and marbling scores in beef (LaBrune, 2000). Moreover, the meat of bulls fed diet supplemented with FSO had significantly higher EPA and DHA content than bulls fed control diet (Renna et al., 2018). Herdmann et al. (2010) and Simopoulos (2011) also found that cattle fed FSO had a lower n-6:n-3 PUFA ratio than control cattle.

Apart from these previous studies which mainly performed on cattle breeds other than Friesian, little is known regarding the effect of FSO supplementation on growth performance, carcass characteristics, meat quality, and fatty acids profile of Friesian bulls. Therefore, this study was conducted to evaluate this effect.

MATERIALS AND METHODS

The present study was carried out at Sakha Animal Production Research Station, belonging to the Animal Production Research Institute, Agricultural Research center, Ministry of Agriculture, and Animal Production Department, Faculty of Agriculture, Kafrelsheikh University during the period from August 2016 to March 2017. This study was conducted after an agreement from the Animal Care and Ethics Committee of Kafrelsheikh University, Egypt (license number, KFS1345/10).

Experimental design

A total of 30 Friesian bulls, with an average live body weight of (LBW) 265.35±12.36 kg and age of 8 months. All bulls were free of physical defects and diseases and had normal external genitalia. The animals were kept freely under semi-open sheds and were managed according to the recommendations of Animal Production Research Institute (APRI, 1997).

Animals were randomly divided into three groups (G1-G3, n = 10 per group). Each 10 animals per group were subdivided into 3 replicates, each replicate had 3 animals except the last one contained 4 animals. Each animal replicate (3-4 animals) was individually housed in a free-stall unit. Bulls in G1 (control) were fed a basal diet, while those in G2 and G3 were fed basal diet supplemented with 2% and 4% DMI flaxseed oil (FSO), respectively, for 208 days (~ 7 months). The basal diet contained concentrate feed mixture (CFM), corn silage (CS), berseem hay (BH),

and rice straw (RS) and was formulated based on the recommendation of the NRC (1980). CFM included 37.5% yellow corn, 20% soybean meal, 15 % corn gluten, 22.5% wheat bran, 3% molasses. 0.5% premix and 1.5% common salt. The chemical composition of feedstuffs and experimental rations (DM, CP, CF, EE, NFE, and ash) was analyzed according to A.O.A.C. official method (2012) and the results were shown in Tables 1 and 2. The NDF, ADF, and ADL were analysed according to Mertens (2002), AOAC (2004) and Van Soest et al. (1991), respectively. Dry matter (DM) intake was determined by weighing feed refusals from each animal replicate on 2 consecutive days weekly.

At the end of the experimental period (at the age of 14 months), 6 bulls (2 from each replicate) in each group with an average of 475±23.50 Kg LBW were slaughtered after fasting for 16 h (Sharawy, 2005).

Growth traits

The initial and final body weight of bulls was recorded at the beginning (at the age of 8 months) and end (at the age of 14 months) of the experiment, respectively. Bulls were weighed before morning feeding and after holding off feed and water for 16 h. The total body weight gain was then calculated by subtracting initial body weight from final body weight. The average daily gain (ADG) of bulls was calculated by dividing the total body weight gain by experiment duration (208 days)

Carcass traits

Fasting body weight was recorded before slaughter. Subsequently, weights of hot carcass and edible organs (liver, kidney, heart and, spleen) were recorded. Each carcass was divided into 4 quarters (2 fore and 2 hind quarters) between the 11th and 12th ribs. Dressing % and boneless meat for each carcass were estimated according to the following formulas: dressing % without edible offal = carcass weight / fasting weight x 100. Dressing % with edible offal = carcass weight + edible offal (liver + heart + kidneys) / fasting weight X100.

Table 1. Chemical composition of feedstuffs.

Feedstuffs	Chemical composition on dry matter basis					
	Ash	NFE	EE	CF	CP	OM
CFM	8.50	64.11	2.48	8.41	16.50	91.50
CS	9.09	61.62	2.56	17.21	9.52	90.91
BH	11.88	43.57	2.38	29.01	13.16	88.12
RS	15.49	49.06	1.10	31.82	2.53	84.51

Data was presented as averages.
NFE: nitrogen free extract; EE: ether extract; CF: crude fiber; CP: crude protein; OM: organic matter; CFM: concentrate feed mixture; CS: corn silage; BH: berseem hay; RS: rice straw

Table 2. Chemical composition of experimental rations on dry matter basis.

Chemical composition	Experimental groups		
	G1	G2	G3
OM	90.28	90.49	90.22
CP	13.38	13.39	13.33
CF	14.63	14.58	14.62
EE	2.31	4.01	6.07
NFE	59.69	58.51	56.20
Ash	9.92	9.51	9.56
NDF	39.19	39.54	39.44
ADF	20.30	20.46	20.41
ADL	4.07	4.12	4.38

Data was presented as averages.
OM: organic matter; CP: crude protein; CF: crude fiber; EE: ether extract; NFE: nitrogen free extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent liginin.

The eye muscle area was determined by a planimeter from tracing taken on the cut surface over the 9th rib. The 9 -11th rib samples were weighed cold and used for chemical analysis and estimation of meat quality. Tenderness and water-holding capacity were measured as previously described (Owen et al. 1982). The color intensity of the meat samples was calculated at 542 nm by a spectrophotometer. The pH of *Longissimus dorsi* muscle was determined in 2.5 cm thick muscular slices by a digital pH-meter (Jenway, 3010) at room temperature (Suksombat et al., 2016). The 9, 10, and 11th rib cuts were dissected into lean, fat, and bone and their weights were recorded. Chemical composition (DM, CP, CF, ash, and moisture) of the *Longissimus dorsi* muscle minced samples was determined based on A.O.A.C. official method (2012).

Meat quality

Meat quality parameters, including flavor, juiciness, color, taste, and tenderness of cooked meat were evaluated by 10 members of our lab using a five-point scale (Chambaz, et al. 2003). General meat quality was calculated according to the following formula: general meat quality = (taste grade + flavor grade + juiciness grade + tenderness grade + color grade) /5.

Fatty acids composition

Total lipids in *Longissimus dorsi* samples were extracted using chloroform-methanol as previously described (Suksombat et al., 2016). Preparation of fatty acids methyl esters (FAME) was performed as previously described (Ostrowska et al., 2000). Based on this method, hexane was used to extract FAME. The fatty acids profile of meat samples was measured by gas chromatography (GC, Perkin-Elmer Auto syst-X.L) equipped with silica capillary column. Injector and detector temperatures were 240°C. The column temperature was kept at 70°C for 4 min, then increased at 13°C/min to 175°C and held at 175°C for 27 min, then increased at 4°C/min to 215°C and held at 215°C for 17 min, then increased at 4°C/min to 240°C and held at 240°C for 10 min. Results were expressed as g/100 g total fatty acids.

Statistical analysis

Statistical analysis of the obtained data was analyzed by one-way analysis of variance (ANOVA) using the Statistical Analysis System (SAS). Duncan multiple range test had used to determine the significant differences among groups. A significant level of $p<0.05$ was used.

RESULTS AND DISCUSSION

Effect of flaxseed oil dietary supplementation on feed intake

Bulls fed diet supplemented with 2% and 4% flaxseed oil (FSO) in G2 (6.02±0.12 kg/h/d, $P < 0.01$) and G3 (6.34±0.11 kg/h/d, $P < 0.0001$) had significantly higher TDN intake than G1 (5.46±0.13 kg/h/d) (Table 3). However, no significant ($P > 0.05$) difference was noticed in DM and DCP intake or feed conversion among the three groups. These results agreed with Huerta-Leidenz et al. (1991) and Suksombat et al., (2016) who found no significant difference in DM and DCP intake and feed conversion among animals fed diet supplemented with linseed oil (LSO) and whole cotton seed, respectively, with fat contents below or equal to 5.0%. On the other hand, other previous studies showed that fat contents above 5% of the diet significantly reduced DM intake and digestion (Rule et al. 1989).

Effect of flaxseed oil dietary supplementation on growth performance

Bulls fed diet supplemented with a higher dose of FSO (G3) had significantly ($P<0.05$) higher final live body weight (LBW) than other groups (Table 4). G2 (1.002±0.02 kg/h/d, $P<0.05$) and G3 (1.124±0.03 kg/h/d, $P<0.0001$) had also significantly higher average daily gain (ADG) than G1 (0.900±0.03 kg/h/d) (Table 4). These results are consistent with those reported by Abu El-Hamd et al. (2015), who also found significantly higher LBW and ADG in Friesian calves fed on ration supplemented with FSO than those fed on the control diet. Similarly, Khattab et al. (2011) found that buffalo calves fed on a black seed oil diet grew faster than those fed on a basal diet. Moreover, Maddock et al. (2004) reported that feeding beef cattle on the whole (rolled or ground) flaxseed significantly increased total body weight gain. Additionally, dietary supplementation of 10% and 20% LSO significantly improved growth traits as compared to the control group (Abuelfatah et al. 2013). In contrast, He and Armentano (2011), Noci et al. (2007) and Suksombat et al., (2016) did not find any significant difference in final LBW or ADG in animals fed diet supplemented with 5%LSO or sunflower oil (SFO), or a mixture of FSO and SFO at 5% of diet. This could be attributed to balance of the total net energy consumption by treatments.

Table 3. Effect of flaxseed oil dietary supplementation on bull feed intake

	G1	G2	G3
Total intake (kg/h/d)			
DM	9.37±0.45	8.97±0.49	9.06±0.39
TDN	5.46±0.13	6.02±0.12[a]	6.34±0.11[b]
DCP	0.877±0.04	0.867±0.03	0.892±0.04
Feed conversion			
Kg DM /kg gain	9.83±0.67	9.72±0.70	8.53±0.82
Kg TDN / kg gain	6.89±0.45	6.52±0.48	5.97±0.51
Kg DCP / kg gain	0.986±0.05	0.939±0.04	0.840±0.06

Data are presented as means ± SEM (n = 10/group). [a] P < 0.01, [b] P < 0.0001 (vs G1).
DM: dry matter; TDN: total digestible nutrients; DCP: digestible crude protein.

Table 4. Effect of flaxseed oil dietary supplementation on bull growth performance

	G1	G2	G3
Duration (day)	208	208	208
Initial body weight (kg)	265.5±10.2	264.8±11.4	264.5±10.8
Final body weight (kg)	452.7±9.6	473.3±8.5	498.3±12.6[a]
Total gain (kg)	187.2±8.4	205.5±7.8	233.8±9.5[b]
Average daily gain (kg/h/d)	0.900±0.03	1.002±0.02[a]	1.124±0.03[c]

Data are presented as means ± SEM (n = 10/group). [a] P < 0.05, [b] P < 0.01, [c] P < 0.0001 (vs G1). ADG: average daily gain

Table 5. Effect of flaxseed oil dietary supplementation on bull carcass traits

	G1	G2	G3
Fasting body weight (kg)	446.67±6.5	463.3±5.6	493.3±5.1[cB]
Hot carcass weight (kg)	242±5.7	261±6.7	290±8.1[bA]
Dressing % without edible offal	54.18±1.2	56.33±1.1	58.79±1.1[a]
Dressing % with edible offal	59.61±1.4	62.25±1.7	65.69±1.8[a]
Boneless meat weight (kg)	197.7±6.8	212±7.4	244±7.8[bA]
Fat weight (kg)	22.4±1.4	25.6±2.4	29.5±1.9[a]
Bone weight (kg)	44.3±2.7	49±3.1	46±3.4
Boneless meat (%)	81.69±1.4	81.23±1.3	84.13±1.4
Bone (%)	18.31±1.3	18.77±1.4	15.86±2.1
Boneless meat: bone ratio	4.46	4.33	5.31

Data are presented as means ± SEM (n = 6/group). [a] P < 0.05, [b] P < 0.001, [c] P < 0.0001 (vs G1); [A] P < 0.05, [B] P < 0.01 (vs G2).

Effect of flaxseed oil dietary supplementation on carcass traits

Effect of FSO supplementation on carcass traits (fasting body weight, hot carcass weight, dressing % with or without edible offal, boneless meat weight and %, bone weight and %, fat weight and %, and boneless meat: bone ration) were presented in Table 5. The obtained results showed that G3 had significantly higher fasting body weight (493.3±5.1kg; P<0.0001 vs G1 and P<0.01 vs G2) than G1 (446.67±6.5kg) and G2 (463.3±5.6kg). Additionally, G3 showed a significantly higher hot carcass weight (290±8.1kg; P<0.001 vs G1 and P<0.05 vs G2) than G1 (242±5.7kg) and G2 (261±6.7). G3 had a significantly higher dressing % without (58.79±1.1%, P<0.05) or with edible offal (65.69±1.8%, P<0.05) and fat weight (29.5±1.9kg; P<0.05) than G1 (54.18±1.2% and 59.61±1.4%, respectively). Besides, G3 had significantly higher boneless meat weight (244±7.8kg; P<0.001 vs G1 and P<0.05 vs G2) than G1 (197.7±6.8kg) and G2 (212±7.4kg). However, FSO treatment did not significantly affect other carcass characteristics (boneless meat %, bone weight and %, and boneless meat: bone ratio). Consistent with these findings, LaBrune, (2000) found that FSO supplementation to basal diet of finishing cattle increased the percentage of carcasses grading choice and improved marbling scores due to its high content of α-linolenic acid. In contrast, Noci et al. (2007) and Suksombat et al., (2016) reported that cattle fed a mixture of 150 g/d SFO and 150

g/d LSO or 5% LSO, respectively, showed no significant differences in carcass weight and dressing%.

Effect of flaxseed oil dietary supplementation on characteristics and chemical composition of eye muscle

The region of the eye (*Longissimus dorsi*) muscle is often utilized as a meaningful indicator of the size, quality and muscle mass distribution. Longissimus dorsi is the most relevant muscle for chemical analysis because it is a late mature muscle and easy to measure. Effect of FSO supplementation on carcass characteristics of the eye muscle (9-11th ribs cut weights and their contents of meat, bone, and fat, water-holding capacity, and pH) and chemical composition were presented in Table 6. G3 had significantly higher 9-11th ribs cut weights (13.9±0.3kg, P<0.05) than G1 (12.0±0.5kg). In contrast to our findings, Zinn et al. (2000) and Suksombat et al., (2016) did not find significant effects on eye muscle area in steers fed diets supplemented with LSO or animal fat.

Water-holding capacity have significantly decreased in G3 (6.74±0.08 cm^2, P<0.05) as compared to G1 (7.12±0.09 cm^2). In contrast, no significant change in pH was observed among the three groups. Consistent with these findings, Suksombat et al., (2016) also did not observe a significant change in the pH in the eye muscle of steer following LSO dietary supplementation.

The chemical composition of *Longissimus dorsi* of FSO-supplemented bulls was better than the control animals. G3 had significantly higher DM in eye muscle (27.95± 0.82%, P<0.01 vs G1 and P<0.05 vs G2) than G1 (25.34±0.78%) and G2 (27.56±0.62%). Moreover, G3 had significantly higher CP (82.65±1.7%, P<0.05), and CF (13.68±0.9%, P<0.05) in eye muscle than G1 (76.48±1.6% and 10.52±0.7%, respectively). However, no significant differences were noticed in moisture and ash percentages among the three groups. Consistent with our findings, previous studies also showed significantly higher CF and CP in the meat of animals fed diet supplemented with FSO as compared to those fed the control diet (Corazzin et al. 2012; Barahona et al. 2016, Abdel-Gawad and El-Emam 2018). However, other researchers did not find any significant changes in chemical composition of eye muscle between LSO-fed steers and non-supplemented steers (Suksombat et al., 2016).

Table 6: Effect of flaxseed oil dietary supplementation on characteristics and chemical composition of *Longissimus dorsi* muscle of bulls

	G1	G2	G3
Weight of the 9th -11th ribs cut (Kg)	12.0±0.5	13.4±0.6	13.9±0.3^a
Meat (kg)	8.45±0.7	9.47 ±0.8	9.96±0.5
Bone (kg)	2.15±0.21	2.40±0.23	2.32±0.24
Fat (kg)	1.40±0.12	1.53±0.14	1.62±0.11
Water-holding capacity (cm^2)	7.12±0.09	6.92±0.08	6.74±0.08^a
pH	5.54±0.24	5.65±0.32	5.47±0.26
Chemical composition of eye muscle			
Dry matter (%)	25.34±0.78	27.56±0.62^a	27.95± 0.82^b
Crude protein (%)	76.48±1.6	77.14±1.5	82.65±1.7^a
Crude fat (%)	10.52±0.7	11.73±0.8	13.68±0.9^a
Ash (%)	3.33±0.24	3.59±0.21	3.76±0.15
Moisture (%)	74.66±1.6	72.44±1.5	72.05±1.7

Data are presented as means ± SEM (n = 6/group). ^{a}P < 0.05, ^{b}P < 0.01 (vs G1).

Table 7. Effect of flaxseed oil dietary supplementation on bull meat quality

	G1	G2	G3
Taste	7.7±0.5	7.6±0.5	8.12±0.4
Color	6.12±0.4	8.7±0.6	8.56±0.5
Flavor	7.46±0.4	7.78±0.4	8.42±0.4
Drumming	7.6±0.5	7.62±0.3	8.24±0.6
Exterior	7.62±0.4	7.84±0.4	8.3±0.5
General meat quality	7.30±0.2	7.91±0.2	8.33±0.3^a

Data are presented as means of grades ± SEM (n = 6/group). ^{a}P < 0.05 (vs G1).

Effect of flaxseed oil dietary supplementation on meat quality

Effect of FSO dietary supplementation on bull meat quality (taste, flavor, juiciness, tenderness, and color) was presented in Table 7. G3 showed significantly higher general meat quality (8.33±0.3 grade, P<0.05) than G1 (7.30±0.2 grade). This infers that meat consumers prefer meat produced from G3 than G1. Among the different meat quality parameters, the color of meat gains a particular interest. Redness of meat is one of most important factors taken in consideration during buying meat, as consumers correlate it with freshness status of meat (Kerry et al., 2000). In consistence with our results, a previous study by Scheeder et al. (2001) also founded that the meat of animals fed various sources of fat are juicier and have more attractive smell than animals fed control diet. This favorable meat quality could be due to higher contents of n-3 PUFA which can induce odor precursors release during heating (Scheeder et al. 2001). In contrast, Suksombat et al., (2016) reported insignificant changes in meat quality after feeding steers diets supplemented with LSO. Similarly, Nuernberg et al., (2005) found no significant changes in meat quality of bulls feeding diet supplemented with cracked linseed. Meat tenderness is accepted if the value of shear strength less than 8 N (Swan et al., 1998) regardless of lipid supplementation adopted (Santana et al. 2014). Dietary fat can positively affect fat content, growth, and carcass quality in ruminants (De Brito et al., 2017).

Effect of flaxseed oil dietary supplementation on fatty acid profile in Longissimus dorsi muscle

Table 8 shows effect of FSO dietary supplementation on SFA and MUFA. C20:0 SFA was significantly higher in G3 (0.12±0.006%, P<0.01 vs G1 and P<0.05 vs G2) than in G1 (0.09±0.004%) and G2 (0.10±0.005%). C22:0 was also significantly higher in G3 (0.15±0.006%, P<0.05) than G1 (0.12±0.005%), but no significant difference with G2 (0.14±0.006%). However, C14:0; C15:0; and C17:0 were significantly lower in G3 (1.38±0.03%, P<0.001; 2.28±0.02%, P<0.05; and 1.05±0.03%, P<0.0001, respectively) and G2 (1.43±0.02%, P<0.01; 2.29±0.03%, P<0.05; and 1.32±0.03%, P<0.05, respectively) than G1 (1.63±0.04%; 2.41±0.03%; and 1.49±0.05%, respectively). In contrast, the contents of *de novo* synthesized SFA (C10:0, C12:0, and C16:0) showed insignificant differences between the three groups. Previous studies reported a decrease in individual SFA (Mach et al. 2006; Corazzin et al. 2012), however, Rennaet al., (2018) denoted an increase in C18:0 and C20:0 following FSO supplementation.

On the other hand, meat content of C16:1 trans-9 MUFA was significantly higher in G3 (1.12±0.07%, P<0.01) and G2 (1.04±0.07%, P<0.05) than in G1 (0.73±0.06%), while C18:1 cis-9 +trans-13-14 and C20:1 cis-11 were significantly lower in G3 (17.21±0.24%, P<0.0001 and 0.10±0.004%, P<0.001, respectively) and G2 (19.05±0.25%, P<0.05 and 0.11±0.004%, P<0.05, respectively) than in G1 (20.05±0.27% and 0.13±005%, respectively). In agreement with these results, Choi et al. (2015) reported that C18:1 cis-9+trans-13-14 downregulated the expression of adipogenic gene in intramuscular preadipocytes. Among the two treated groups, only G3 had significantly higher C17:1 cis-9 (19.05±0.25%, P<0.05), C18:1 cis-11+trans15 (1.76±0.13%, P<0.01), and C18:1 cis-15+trans-16 (19.05±0.25%, P<0.01) than G1 (1.32±0.11%, 1.13±0.11 and 0.79±0.03%, respectively) and significantly lower C16:1 cis-7 (1.33±0.11%, P<0.05) and C18:1 trans-12 (1.44±0.11%, P<0.01) than G1 (1.85±0.13% and 1.13±0.11, respectively).

Table 9 shows effect of FSO dietary supplementation on PUFA. The meat content of C18:3 n-3 PUFA (ALA) was significantly higher in G3 (1.64±0.02%, P<0.0001) and G2 (1.32±0.02%, P<0.0001) than in G1 (0.76±0.01). These results agreed with Albert et al. (2014), Conteet al. (2019) and Renna et al., (2018), who found that LSO and FSO dietary supplementation resulted in a significant increase in ALA content of beef fat as compared to bulls fed control diet. It is well known that FSO is rich in ALA, and it therefore has been used effectively to enhance the nutritional impact of PUFAs in bull meat (Scollan et al. 2014). Piedmonti beef had a larger n-3 PUFA, including ALA, amount than the overall FAs, making their meat leaner but healthier than other breeds (Aldai et al. 2006; Sevane et al. 2014). In contrast, Choi et al. (2015) found that ALA downregulated the expression of adipogenic gene in intramuscular preadipocytes and Bessa et al., (2007) and Suksombat et al., (2016) found a significant decrease in ALA level in beef following LSO supplementation.

Table (8): Effect of flaxseed oil dietary supplementation on SFA and MUF in *Longissimus dorsi* muscle of bulls

Name	G1	G2	G3
Individual and total SFA (g/100 g TFA)			
C10:0	0.13±0.02	0.12±0.02	0.13±0.02
C12:0	0.09±0.004	0.08±0.003	0.09±0.004
C14:0	1.63±0.04	1.43±0.02^{b}	1.38±0.03^{c}
C15:0	2.41±0.03	2.29±0.03^{a}	2.28±0.02^{a}
C15 *anteiso*	0.18±0.01	0.17±0.01	0.16±0.01
C16:0	18.02±0.31	18.05±0.33	18.20±0.32
C17:0	1.49±0.05	1.32±0.03^{a}	1.05±0.03^{dC}
C17 iso	0.38±0.02	0.36±0.02	0.35±0.02
C18:0	13.94±0.03	14.21±0.04	14.05±0.04
C18 iso	0.39±0.04	0.37±0.03	0.36±0.02
C20:0	0.09±0.004	0.10±0.005	0.12±0.006 ^{bA}
C22:0	0.12±0.005	0.14±0.006	0.15±0.006^{a}
Total SFA	38.87±2.7	38.64±2.4	38.32±2.5
Individual and total MUFA (g/100 g TFA)			
C14:1 cis-9	0.61±0.02	0.63±0.02	0.65±0.02
C16:1 trans-9	0.73±0.06	1.04±0.07^{a}	1.12±0.07^{b}
C16:1 cis-7	1.85±0.13	1.50±0.12	1.33±0.1^{1a}
C16:1 cis-9	0.42±0.08	0.38±0.07	0.36±0.07
C17:1 cis-9	1.32±0.11	1.49±0.12	1.94±0.13^{bA}
C18:1 trans-9	0.75±0.11	0.69±0.09	0.67±0.09
C18:1 trans-11	0.34±0.05	0.37±0.06	0.41±0.06
C18:1 trans-12	2.02±0.13	1.92±0.11	1.44±0.11^{bA}
C18:1 cis-9 +trans-13-14	20.05±0.27	19.05±0.25^{a}	17.21±0.24^{dC}
C18:1 cis-11+trans15	1.13±0.11	1.51±0.12	1.76±0.13^{b}
C18:1 cis-12	0.43±0.09	0.51±0.10	0.58±0.11
C18:1 cis-15+trans-16	0.79±0.03	0.85±0.04	1.05±0.04^{cB}
C20:1 cis-11	0.13±005	0.11±0.004^{a}	0.10±0.004^{c}
Total MUFA	30.56±2.12	30.05±2.00	28.62±2.15

Data are presented as means ± SEM (n = 6/group). ^{a}P < 0.05, ^{b}P < 0.01, ^{c}P < 0.001, ^{d}P < 0.0001 (vs G1); ^{A}P < 0.05, ^{B}P < 0.01, ^{C}P < 0.001 (vs G2).
SFA: saturated fatty acids; TFA: total fatty acids; MUFA: monounsaturated fatty acids

We did not find a significant difference in C18:2n-6 (LA) levels in meat fat following dietary supplementation with FSO, however Suksombat et al., (2016) found that LA levels were significantly decreased with increasing LSO and attributed this inverse effect to partial conversion to C18:0 in the rumen. Choi et al. (2015) found that LA inhibited the expression of adipogenic gene in intramuscular preadipocytes. On the other hand, the meat content of C20:5 n-3 (EPA), C22:6 n-3 (DHA), and C22:5 n-3 (DPA) was significantly higher in G3 (0.46±0.02%, 0.06±0.006%, P<0.0001, 0.93±0.09%, P<0.05, respectively) and G2 (0.42±0.02%, P<0.0001, 0.05±0.005% and 0.88±0.08%, P<0.05 respectively) than G1 (0.23±0.01%, 0.03±0.003%, and 0.56±0.07% respectively). Consistent with these findings, Renna et al., (2018) and Suksombatet al. (2016) reported that FSO- or LSO-fed bulls had significantly higher EPA and DHA contents in their meat than non-supplemented bulls.

In the present study, no significant change was noticed in CLA meat content following FSO supplementation. In contrast, Suksombat et al. (2016) found a significant decrease in the CLA content of *Longissimus dorsi* and *Semimembranosus* muscles after feeding animals on a ration containing LSO. However, Noci et al. (2007) reported higher CLA level in meat fat in heifers supplemented with SFO and LSO. The total SFA, MUFA, and n-6 FAs were also not affected by FSO treatment. Similarly, Raes et al., (2004), Albert et al., (2014), and Rennaet al., (2018) also found no statistical differences in total SFA and MUFA between FSO-fed bulls and the control bulls. Although PUFA/SFA ratio was not significantly changed among the three groups, its value matches the suggested

Table 9: Effect of flaxseed oil dietary supplementation on PUFA and PUFA/SFA ratio in *Longissimus dorsi* muscle of bulls.

Individual and total PUFA (g/100 g TFA)			
C18:2 cis-9, trans-13+cis-9, trans-14	0.20±0.01	0.32±0.0^{1d}	0.52±0.01^{dD}
C18:2 cis-9, trans-12+trans-8, cis-13	0.45±0.05	0.63±0.06	0.88±0.08^{cA}
C18:2 trans-9, cis-12	0.21±0.03	0.25±0.03	0.33±0.04
C18:2 trans-10, cis-15+trans-11, cis-15	0.36±0.08	0.66±0.10	0.80±0.1^{1a}
C18:2 n-6 (LA)	21.4±0.33	21.0±0.34	22.1±0.35
C18:2 cis-9, cis-15	0.35±0.05	0.29±0.04	0.23±0.03
C18:2 cis-9, trans11+trans-7, cis-9+trans-8, cis-10 (CLA)	0.17±0.04	0.16±0.03	0.13±0.03
C18:3 n-6 (GLA)	0.13±0.04	0.14±0.05	0.14±0.05
C18:3 n-3 (ALA)	0.76±0.01	1.32±0.02^{d}	1.64±0.02^{dD}
C20:2 n-6	0.46±0.02	0.39±0.03	0.32±0.03
C20:3 n-9	0.09±0.01	0.12±0.01	0.14±0.01^{b}
C20:3 n-6	0.71±0.04	0.62±0.04	0.42±0.03^{cB}
C20:3 n-3	0.04±0.006	0.05±0.007	0.05±0.007
C20:4 n-6	4.02±0.36	3.69±0.32	3.68±0.30
C20:5 n-3 (EPA)	0.23±0.01	0.42±0.0^{2d}	0.46±0.0^{2d}
C22:4 n-6	0.48±0.06	0.32±0.05	0.23±0.03
C22:5 n-3 (DPA)	0.56±0.07	0.88±0.08^{a}	0.93±0.09^{a}
C22:6 n-3 (DHA)	0.03±0.003	0.05±0.005^{a}	0.06±0.006^{dD}
Total n-6	27.13±1.8	26.16±1.9	26.89±1.5
Total n-3	1.62±0.06	2.72±0.08^{d}	3.14±0.10^{dB}
n-6:n-3 ratio	16.75±0.57	9.62±0.43^{d}	8.56±0.40^{d}
Total PUFA	30.58±0.63	31.31±0.55	33.06±0.6^{1a}
PUFA/ SFA ratio	0.787±0.05	0.810±0.04	0.863±0.03

Data are presented as means ± SEM (n = 6/group). $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, $^{d}P < 0.0001$ (vs G1); $^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$ (vs G2).

PUFA: polyunsaturated fatty acids; TFA: total fatty acids; LA: linoleic acid; CLA: conjugated linoleic acid; GLA: γ-linolenic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid; SFA: saturated fatty acids.

value (0.4-1) for humans (Jimenez-Colmenero et al. 2012). While, the total PUFA was significantly higher in G3 (33.06±0.61%, P<0.05) than G1 (30.58±0.63%). The positive dietary effects on total PUFA in meat fat indicates that LSO supplementation can affect rates of ruminal lipolysis. On the other hand, the total n-3 FAs content in meat was significantly higher in G3 (3.14±0.10%, P<0.0001) and G2 (2.72±0.08%, P<0.0001) than G1 (1.62±0.06%). The higher levels of n-3 PUFA in beef fat coincided with the higher contents of C18:3n-3 in the LSO diets (Suksombat et al. 2016).

The n-6:n-3 ratio was significantly lower in G3 (8.56±0.40%, P<0.0001) and G2 (9.62±0.43%, P<0.0001) than G1 (16.75±0.57%). In support, Herdmann et al. (2010), Simopoulos (2011), and Suksombatet al. (2016) also found a similar reduction in this ratio in animals fed ration supplemented with FSO or LSO. Our data and those obtained by Herdmann et al. (2010) and Simopoulos (2011) revealed a n-6:n-3 PUFA ratio of 4:1, which is close to that recommended for human consumption. On the other hand, FSO-fed Belgian Blue cattle showed a higher ratio above 5:1 (Raes et al. 2004). Moreover, Quinn et al. (2008) found that feeding animals on diet supplemented with 4% LSO resulted in significant increase in levels of C18:2n-6 (LA) and C18:3n-3 (ALA) in meat fat of steers, but C20:4n-6 was reduced. However, Suksombatet al. (2016) reported an increased C18:3n-3 and decreased C18:2n-6 and C20:4n-6 levels in meat fat of steers following LSO supplementation. In the present study, we only found a significant increase in C18:3n-3 (ALA) following FSO supplementation.

These findings suggest that feeding animals with FSO could be essential to get healthier meat with higher PUFA content and lower n-6:n-3 PUFA ratio. FSO could facilitate the deposition of n-3 PUFA in beef muscle tissues to produce healthy meat (Baba et al. 2016). Nevertheless, PUFA enhancement may provoke more susceptibility to lipid oxidation, which can decrease organoleptic value, especially color and taste, of meat (Guyon et al. 2016).

CONCLUSION

Flaxseed oil supplementation in bulls finishing diets could improve growth performance, and carcass quality, and increase omega-3 fatty acids.

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CONFLICT OF INTEREST STATEMENT

No potential conflict of interest was reported by the authors.

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Research article
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The antioxidant effect of *Michauxia campanuloides* on rat ovaries

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ABSTRACT: This study investigated how Michauxia campanuloides affects the Proliferating Cell Nuclear Antigen (PCNA) expression in granulosa cells, the ovarian histomorphology and serum total antioxidant capacity (TAC) in rats. Rats were divided into control (C), treatment 1 (T 1) and treatment 2 (T 2) groups. The rats in the T 1 and T 2 groups received aqueous extract of M. campanuloides at doses of 20 mg/kg/day and 40 mg/kg/day orally for 21 days, respectively. Serum TAC levels, follicles counts including primordial, primary, preantral, antral and atretic follicles, and PCNA expression in granulosa cells were evaluated. Numbers of preantral follicles increased in T 1 and T 2 groups compared to C group ($P < 0.05$). TAC and numbers of preantral and antral follicles increased in T 2 group compared to T 1 and C groups ($P < 0.05$). PCNA expression in granulosa cells was increased in T 2 group compared to T 1 and C groups ($P < 0.01$). In conclusion, treatment with M. campanuloides had positive effects on antioxidant activity, follicular dynamics, and PCNA expression of granulosa cell in rats.

***Keywords*:** Antioxidant, *Michauxia campanuloides,* ovarian, PCNA, rat.

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INTRODUCTION

Antioxidant substances are molecules that can reduce or prevent cellular damage caused by oxidant substances (Abuelo et al., 2015). Oxidant substances are classified as reactive nitrogen species and reactive oxygen species (ROS) (Zhong and Zhou, 2013) and are generated as a natural by-product during cell metabolism in the biological system (Puppel et al., 2015). Antioxidants are divided into two groups, endogenous and exogenous. Exogenous antioxidants are very important because of their roles on the production and structure of endogenous antioxidants (Sen and Chakraborty, 2011). If the production of oxidant substances exceeds the defense capacity of antioxidant substances, oxidative stress occurs (Puppel et al., 2015). Oxidant substances have detrimental effects on cellular structures such as lipid, DNA and protein. Therefore, they can lead to low fertility and infertility due to cell apoptosis, disruption of ovarian steroidogenesis mechanism and other pathological effects on reproductive tracts (Agarwal et al., 2005). Oxidant production increases due to the metabolic activity of granulosa cells during follicular development. Increased ROS production can cause granulosa cell degeneration, which leads to a decrease in ovulation rate and oocyte quality (Wang et al., 2017). Activations of cells are associated with DNA replications and the level of DNA replications can be observed by Proliferating Cell Nuclear Antigen (PCNA), a protein of DNA polymerase-δ enzymes required for DNA synthesis during replication (Strzalka and Ziemienowicz, 2011). PCNA is an important regulator of the cell cycle and has a role in DNA repair (Oktay et al., 1995; Tománek and Chronowska, 2006). PCNA expression increases in granulosa cells during follicular development (Oktay et al., 1995). Exogenous antioxidant supplements have positive effects on ovarian follicular dynamics and cell activities (Zhong and Zhou, 2013). Plants are one of the most important natural sources of exogenous antioxidants (Luximon-Ramma et al., 2002). In addition, antioxidant substances can be synthetically produced (Sen and Chakraborty, 2011). However, it was reported that many synthetic antioxidant substances can have toxic or mutagenic effects (Fejes et al., 1998; Sen et al., 2010; Al-Amiery et al., 2012; Ćilerdžić et al., 2013). Therefore, natural plant origin antioxidants are better than synthetic antioxidants (Sen et al., 2010; Zhong and Zhou, 2013). There are many plants containing antioxidants (Krishnaiah et al., 2007; Ćilerdžić et al., 2013; Zhong and Zhou, 2013; Hamidpour et al., 2017). *Michauxia campanuloides* L'Hér. (Campanulaceae) is one of the antioxidants containing plants (Hürkul and Köroğlu, 2019; Koca et al., 2020). *M. campanuloides* grows naturally in Mersin (Turkey) province. Plants are biennial, 25-200 cm long, bodied, strong herbaceous. Also, its root or stem can be eaten after cooking or peeling and the leaves of *M. campanuloides* are used externally in traditional medicine, as wound healer by applying directly on the wound in Kahramanmaras shire (Hürkul and Köroğlu, 2019; Damboldt, 1978; Güvenç et al., 2012). In previous study, the antioxidant activity and total phenolic contents of *M. campanuloides* was determined by various methods. The strongest antioxidant activity in 1,1-diphenyl-2-pikrylhydrazyl (DPPH) of *M. campanuloides* was found in aqueous extract of its aboveground part (Güvenç et al., 2012).

The aim of this study was to investigate the effect of aqueous extract of aerial parts of *M. campanuloides* on PCNA expression in granulosa cells, ovarian histomorphology and total antioxidant capacity (TAC) in Wistar Albino female rats.

MATERIALS AND METHODS

Plant materials and extraction procedure

The plant, *M. Campanuloides,* material used in the study was collected from Mersin province (Turkey, 2010). A voucher specimen was deposited in the Ankara University Faculty of Pharmacy Herbarium (Herbarium number AEF 25892). 100 g of the powder of *M. Campanuloides* was boiled with 500 ml of distilled water for 30 minutes, filtered and lyophilized, and finally 12 g lyophilized extract was obtained. In the previous study, total contents of the phenolic compounds in the extract were determined by the Folin-Ciocalteu method as gallic acid equivalent. The total phenol content of the aqueous extract was found 107.3 mg equivalent to gallic acid per gram extract (Güvenç et al., 2012). Some results in the previous study on current extract are presented in the discussion section (Güvenç et al., 2012).

Animal diets and management

18 female Wistar Albino rats (8 weeks old) weighing 170-220 g were used in this study. This study was approved by the Experimental Research Ethics Committee of Bingöl University (Bingol University Ethical Council Number: 2019/02-01/04). In this protocol, there are no *in vitro* or other alternative methods that could prevent the use of animals for the ovarian histomorphology study. The use, numbers and man-

agement of animals in this study are in accordance with the European Community Guidelines of ethical use and care of lab animals (Directive 2010/63). The rats were obtained from the Experimental Research Center of Bingol University and were housed at 12 hours dark, 12 hours light and temperature of 23 ºC with constant humidity. The animals were fed with a standard pellet diet and water ad libitum during the experimental period.

Experimental design

The rats were randomly divided into three groups as control (C; n = 6), treatment 1 (T 1; n = 6) and treatment 2 (T 2; n = 6) groups. Then the rats were weighed and the adaptation process was performed for 7 days. No treatment was made, only daily checks were done during this period. The rats in the T 1 and T 2 groups were given aqueous extract of *M. campanuloides* at doses of 20 mg/kg/day and 40 mg/kg/day orally using a gavage for 21 days, respectively. The volume of aqueous extract administered to both groups was 1 ml per day. Similarly, rats in group C received the same volume of distilled water. The dose of extract of *M. campanuloides* was determined considering its proliferative effect on sperm concentration (Koca et al., 2020) and phenolic antioxidant level in the previous studies (Güvenç et al., 2012). In this protocol, the total phenol concentrations of the extract administered in T 1 and T 2 were 2.14 mg/kg and 4.29 mg/kg/day per rat, respectively.

Sample collection and tissue preparation

All rats were anaesthetized by intramuscular administration of xylazine (Rompun®, Bioveta, Czech Republic) and ketamine (Ketasol®; Richterpharma ag, Austria) at the same timepoint of the study. Blood samples (2 ml) were collected from the tail vein into vacutainer tubes (Hema & Tube®, Italy) with clot activator. Immediately after this process and while being in general anesthesia, all rats were euthanized by decapitation (Van Rijn et al., 2011). The blood samples were centrifuged at 1500 g for 10 minutes and the serum samples were immediately stored at -80 °C until TAC analysis. Ovarian tissues were completely removed by making a ventral midline incision in all rats and one of ovaries of each rat was weighed. The other ones were rapidly frozen at -80 °C for further examinations. Immunohistochemistry studies were performed on frozen tissues according to the instructions of Shi et al. (2008). Before starting the study, these ovaries were thawed, fixed in 10% neutal buffered formalin solution, dehydrated with ethanol series and clarified by using xylene. The fixed ovaries were embedded in paraffin blocks. Then serial sections of 5 µm thickness were obtained with a rotary microtome. A total of 7 sections were taken, leaving a distance of 45 µm between the sections. The first 6 slides were stained with hematoxylin and eosin (H & E), and used for follicle counting (Tománek and Chronowska, 2006). The 7th slides were immunohistochemically stained with PCNA antibody, and used to determine both PCNA index in granulosa cells (Strzalka and Ziemienowicz, 2011) and the number of follicles (Tománek and Chronowska, 2006).

Measurement of total antioxidant capacity (TAC)

The serum TAC levels of each rat were measured by autoanalyser using commercial kits (LOT: OK18095A, Rel Assay Diagnostics, Gaziantep, Turkey).

Follicle counting

The counting of ovarian follicles was manually performed under a light microscope at 200 magnification (Nikon H550L, Japan) by a blind observer. To avoid counting more than once the same follicles, the follicle was counted only when the nucleus was clearly identified (Rajaei et al., 2019). Primordial (single layer of flattened granulosa cells), primary (single layer of cuboidal granulosa cells), preantral (at least two layers of cuboidal granulosa cells, no antral cavity) and antral follicles (numerous layers of cuboidal granulose cells with an antral cavity) were characterized according to granulosa cells and layers around the oocyte. If there are vacuolization in the follicles, pycnotic nuclei in granulosa cells and shrinkage in the oocyte, they were characterized as atretic follicles (Rajaei et al., 2019).

PCNA immunohistochemistry

In this study, we investigated the PCNA index to determine the effect of treatment on granulosa cell viability between different experimental groups. For this purpose, sections of 5 µm taken from the ovarian tissues were passed through xylol and decreasing alcohol series and washed in phosphate buffer saline (PBS) for 3 X 5 minutes. The sections were heated in Citrate buffer (pH: 6.0) to remove antigen blockage and washed again 3 X 5 minutes in PBS. The sections were kept for 30 minutes in 3% H_2O_2 prepared in methanol for endogenous peroxidase blockade and were washed again in PBS. After applying the

serum to the samples, PCNA antibody (Santa Cruz, sc-25280) prepared with 1/300 dilution was instilled on sections and left overnight at + 4 °C. After these procedures, secondary antibody and streptavidin peroxidase administrations were performed. These methods were conducted with UltraVision Large Volume Detection System according to the manufacturer's instructions (Thermo, Cat no: TP-125-HL). Then the sections were washed again in PBS and DAB chromogen (Thermo, TL-125-HD) was applied. The sections were examined under the microscope until it reacted with the DAB chromogen. When the reaction was observed, the sections were taken to PBS and counterstained with hematoxylin. Then they were kept for 5 minutes in increasing alcohol series and were passed from two xylol series for 15 minutes. Finally, the sections were covered with entellan and examined under a microscope.

PCNA positive granulose cells were counted under the light microscope at 400 magnification (Nikon H550L, Japan) and microphotographs were captured with NIS elements software version 3.22 (Nikon, Japan) depending on whether the granulosa cells were DAB positive or negative. The expression of PCNA positive granulosa cells was evaluated for each follicle individually. The PCNA index was determined for all follicles (excluding atretic follicles) with or without nuclei.

Statistical analysis

In the present study, the group sizes were determined as 6, according to the results of the power analysis using 80% power and 5% margin of error. All evaluated datasets were analysed statistically with SPSS version 24.0 (IBM, USA). Before the statistical analyses, the normality test was performed with Kolmogorov-Smirnov test. Primordial and preantral follicle counts, serum TAC levels and PCNA expression positive cell index data which were found to be normally distributed were evaluated by parametric One-Way Anova test. On primary, antral and atretic follicle counts were not found to be normally distributed, the Non-parametric Kruskal Wallis test was performed. In both statistical analyses, multiple comparisons were performed with the Post-hoc Tukey and the Tamhane's T 2 tests, respectively. P-value less than 0.05 was considered statistically significant. The results were shown as mean ± standard error of the mean (SEM).

RESULTS

Serum TAC

Serum TAC level was higher significantly in T 2 group (0.87 ± 0.14 mmol/L) compared to T 1 (0.58 ± 0.04 mmol/L) and C (0.28 ± 0.08 mmol/L) groups ($P < 0.05$).

Ovary weight

Treatment with *M. campanuloides* did not significantly affect ovary weights in the T 1 (0.06 ± 0.01 g), T 2 (0.06 ± 0.01 g) and C (0.06 ± 0.01 g) groups ($P > 0.05$) (Figure 2).

Ovarian histomorphology

In the ovarian tissue sections of the C, T 1 and T 2 groups, primordial, primary, preantral, antral and atretic follicles and corpus luteum presence were observed. No pathological finding was detected in the morphological examination (Figure 1). The numbers of preantral follicles increased significantly in T 1 and T 2 groups compared to C group ($P < 0.05$). Antral follicles increased significantly in T 2 group compared to the other groups ($P < 0.05$). However, there were no significant differences in the number of primordial, primary and atretic follicles in T 2 group compared to T 1 and C groups ($P > 0.05$). Besides, no statistical difference was observed in any follicle types when T 1 group was compared to C group ($P > 0.05$). The follicle count results are presented in Table 1.

Immunohistochemistry

Distribution of nuclear positive and negative signals in granulosa cells was observed in ovarian sections of all groups. Weak positive signals were observed in interstitial cells and ovarian capillaries endothelium. When the positive signals were compared with negative control signals of interstitial cells and ovarian capillaries endothelium, it was found to have a similar distribution. However, DAB positivity was not seen in negative control samples of granulosa cells (Figure 2). Immunohistochemically, PCNA expression positive granulosa cells index increased significantly in T 2 group (64.98 ± 1.67 %) compared to T 1 (60.45 ± 1.47 %) and C (55.87 ± 1.87 %) groups ($P < 0.01$). At the same time, although there was a numerical increase in PCNA expression of T 1 group compared to C group, no statistical difference was found between these groups ($P > 0.05$) (Figure 6).

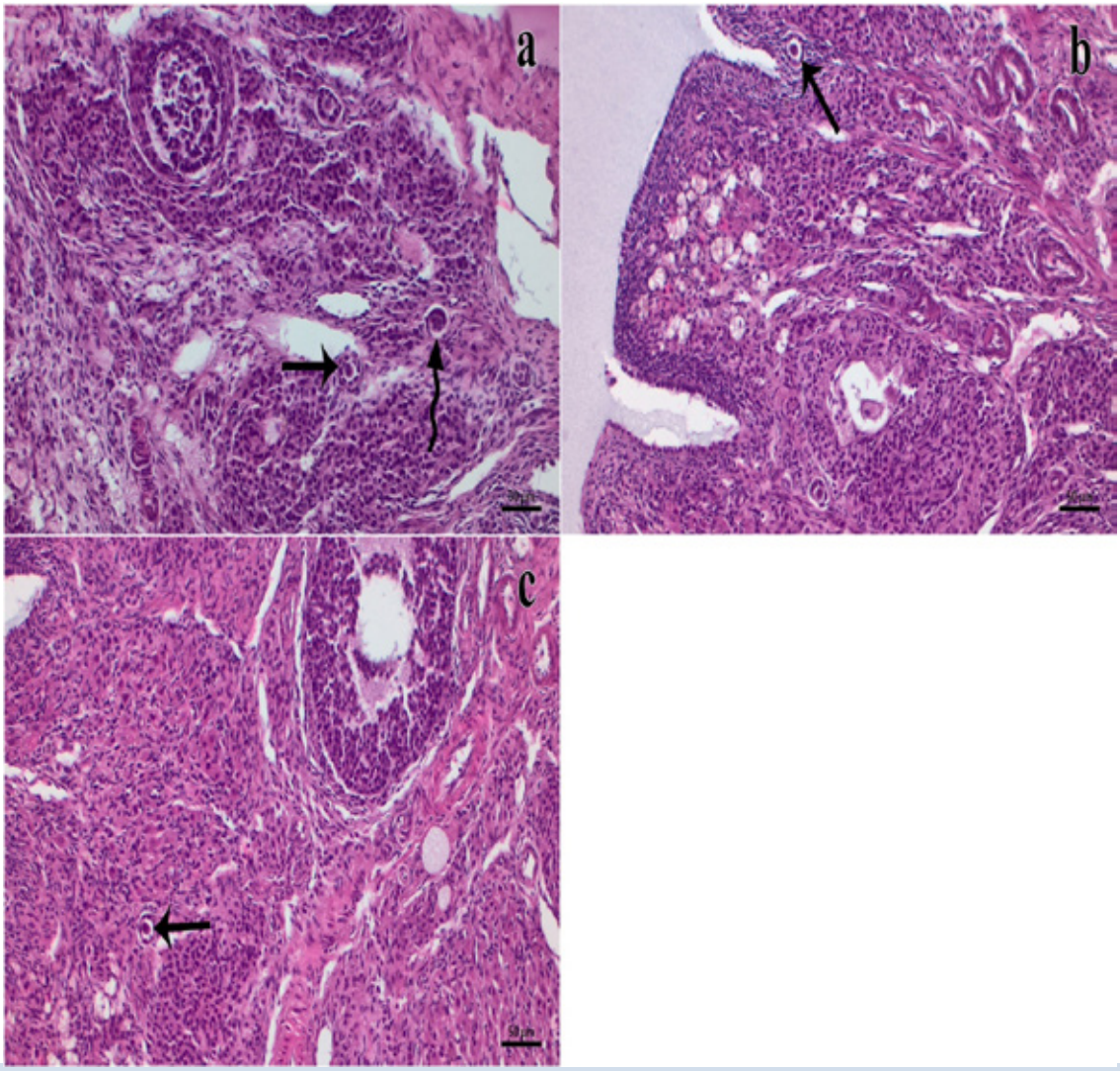

Figure 1: H & E microphotographs of the groups. a: Control, b: Treatment 1, c: Treatment 2. The microphotographs show developing (arrow) and atretic (curved arrow) follicles. Staining: H & E. Bar: 100 μm.

Table 1. Comparison of the number (Mean ± SEM) of primordial, primary, preantral, antral and atretic follicles in Control, Treatment 1 and Treatment 2 rats. Different superscript at the same line indicates the statistical difference.

Follicles	Control	Treatment 1	Treatment 2	P
Primordial	13.17 ± 1.42^{a}	14.00 ± 0.58^{a}	14.00 ± 0.73^{a}	>0.05
Primary	5.17 ± 0.54^{b}	6.17 ± 0.17^{b}	6.50 ± 0.43^{b}	>0.05
Preantral	4.17 ± 0.40^{c}	6.67 ± 0.92^{d}	7.67 ± 0.56^{d}	<0.05
Antral	3.67 ± 0.33^{e}	3.50 ± 0.22^{e}	5.50 ± 0.34^{f}	<0.05
Atretic	3.50 ± 0.43^{g}	3.00 ± 0.45^{g}	2.17 ± 0.31^{g}	>0.05

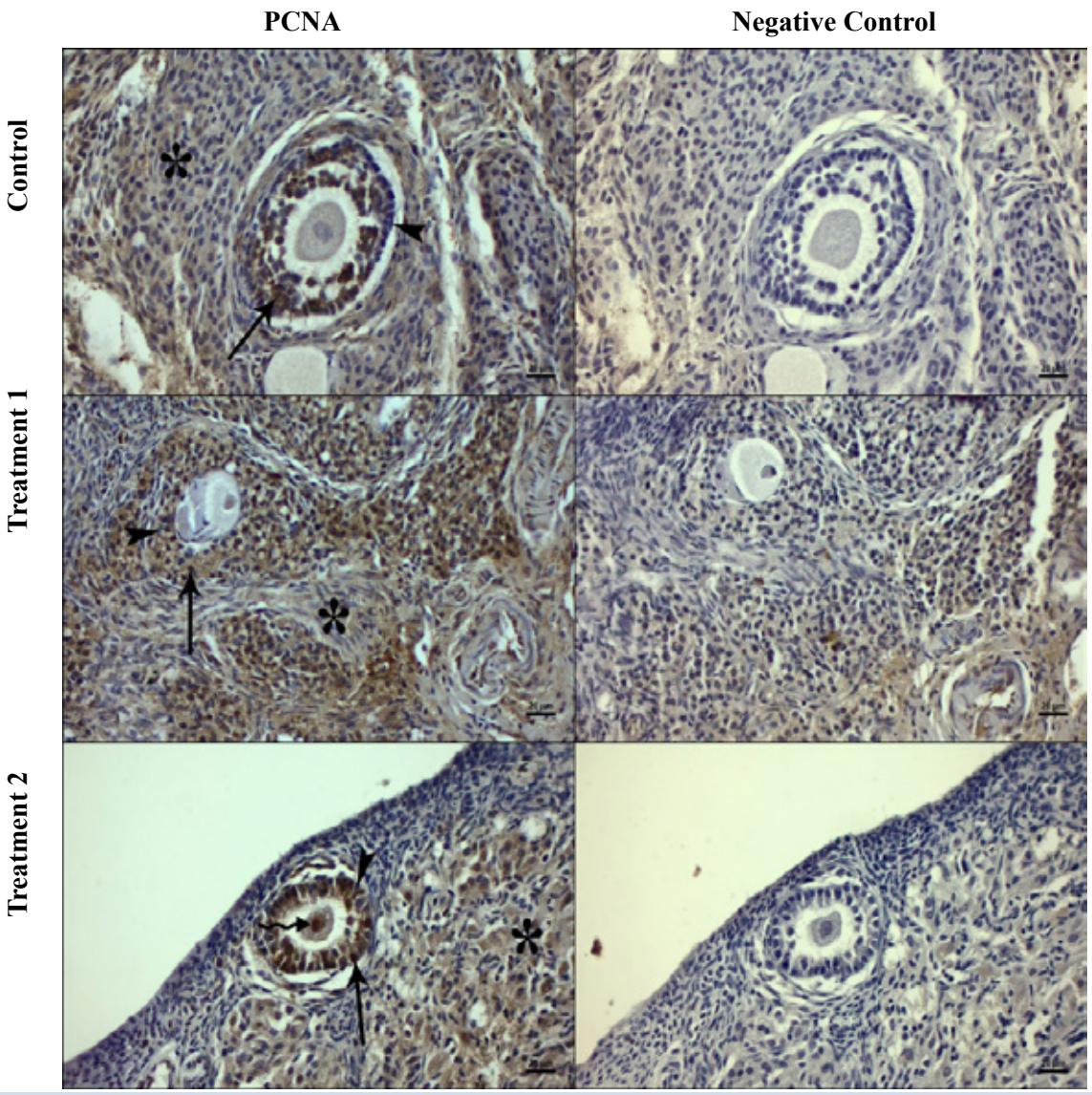

Figure 2: PCNA microphotographs of the groups. The microphotographs show positive (arrow) and negative (arrowhead) signals in granulosa cells of preantral follicles, weak nuclear positivity in interstitial cells (*) and positive signal in the oocyte of treatment 2 group (curved arrow). Staining: PCNA immunohistochemistry, Bar: 20 µm.

DISCUSSION

It was reported that plants containing phenolic compound have strong antioxidant properties and exhibit high antioxidant activity (Asif, 2015). *M. campanuloides* extract has an antioxidant property with phenolic content (Hürkul and Köroğlu, 2019). In the previous study conducted on the current extract, the highest antioxidant content of *M. campanuloides* was observed in the ethanolic extract, 439.0.5 ± 18.70 mg/g as gallic acid equivalents. The half-maximal inhibitory concentrations (IC_{50}) value of the aqueous extract in the thiobarbituric acid test for antioxidant activity was found 464.98 ± 2.21 mg/ml. On the other hand, its the strongest antioxidant activity in DPPH was found in aqueous extract (Güvenç et al., 2012). It has been observed that its antioxidant activity changes according to the extract type. In the present study, we used aqueous extract because of its powerful antioxidant activity. Besides, a recent study demonstrated that aqueous extract of *M. campanuloides* improved some semen parameters in rats (Koca et al., 2020).

Khaki et al. (2009) have reported that antioxidant containing plant significantly increased the level of TAC in rats. In our study, TAC levels were similar in C and T 1 groups. However, a significant increase in TAC levels was observed in T 2 group compared to the other groups. It was determined that the dose of administration had an effect on TAC levels. However, the antioxidant concentrations may have been

partially metabolized by combating ROS in treatment groups. Therefore, it is thought that measuring serum oxidant level is important in antioxidant studies. However, oxidant levels were not measured in this study because our primary aim was to investigate its antioxidant effect in rats. Antioxidant substances have a beneficial effect on energy metabolism (Maritim et al., 2003) and can have an effect on ovarian tissue mass (Chuffa et al., 2011). However, several researchers reported that treatment with antioxidant had no effect on ovary weight (Rajaei et al., 2019; Soleymanı et al., 2010). Similarly, in our study, no significant difference was observed between the groups in terms of ovarian weights ($P > 0.05$).

It was reported that administrations of plant extract containing antioxidant increased the numbers of follicles and follicular activity in animals (Zhong and Zhou, 2013). Besides, it was declared that animals with low fertility have insufficient antioxidant capacity (Stier et al., 2012). Some researchers showed that antioxidant agents improved folliculogenesis (Soleymanı et al., 2010). Abdollahifar et al. (2019) reported that antioxidant has a positive effect on the total number of primordial, primary and antral follicles in mice. In the presented study, a significant increase in the number of antral (T 2) and preantral (T 1 and T 2) follicles was observed. So, it is understood that *M. campanuloides* has a beneficial effect on developed follicles. However, it did not affect the numbers of primordial and primary follicles. When these results are considered, it is concluded that it did not affect the emergence of new follicles from the follicular pool of the ovary.

During the development process of follicles, approximately 99% of them can be atresia (Sugino, 2005). It was noted that antioxidants have a protective effect against apoptosis in rat antral follicles cultured (Ciani et al., 2009). Yener et al. (2013) reported antioxidant administrations reduced atresia of antral follicles. Similarly, Özcan et al. (2015) reported that antioxidants have the effect of reducing follicular atresia. In the present study, we found that numbers of atretic follicles were similar between the groups. It was reported that PCNA expression increases during granulosa cell proliferation (Can et al., 2018). However, follicular atresia is associated with apoptosis of granulosa cells (Sugino, 2005), and PCNA expression gradually decreases with increased atresia (Can et al., 2018). In a previous study, it was indicated that antioxidants have positive effects on the functions of granulosa cells during folliculogenesis (Tománek and Chronowska, 2006). In our study, *M. campanuloides,* as an antioxidant agent, provided an effective improvement in PCNA expression in granulosa cells. However, it was observed that its positive effect on granulosa cells was associated with serum TAC levels because no significant differences were observed in PCNA expression between the T 1 and C groups. However, there was a significant increase in PCNA expression in the T 2 group compared to the other groups.

CONCLUSION

Treatment with aqueous extract of *M. campanuloides* had a positive effect on antioxidant activity, follicular dynamics and PCNA expression of granulosa cell in female Wistar Albino rats. Despite the fact that the treatment dose caused differences on these results, it still evidenced its positive potential in the rat model. It is believed that multifaceted research will provide further information on the toxicological and pharmacological potential of *M. campanuloides*.

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Research article
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Dim blue light colour reduces the activities and improves the performance of Indian River broilers under Egyptian conditions

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ABSTRACT: Poultry producers accustomed to use light colours in broiler production as atrial to increase their productivity, especially in the developing countries to cover the animal protein gap. This experiment was planned to study the effect of different blue light colour intensities (high, medium and dim) on a recently imported Indian River (IR) broilers to Egypt. In this study, 270 one-day old Indian River broiler chicks were used. The birds were exposed to high blue light intensity (HBLI), medium blue light intensity (MBLI) and dim blue light intensity (DBLI), through a monochromatic lighting system that was installed in different rooms for 24 hours daily. The birds were randomly divided and housed into three well controlled pens of 5.46 m^2 with three replicates of 30 each using a density of 17 birds/m^2 in the room. The results showed that the broilers reared under DBLI had a significantly ($p< 0.05$) higher body weight, body weight gain, Newcastle disease virus antibody titer and foot pad dermatitis with obviously, economic Feed conversion ratio (FCR) and low activities and heterophyil/lymphocyte ratio in comparing with (MBLI) and (HBLI). In conclusion, poultry producer can use dim blue light in their farms to reduce the activities and increase the productivity of the birds.

Keywords: behaviour; blue light; IR broilers; light intensity.

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INTRODUCTION

Nowadays, the search for good welfare conditions is a global tendency in animal production (Moura et al., 2006). The transition from backyard to intensive poultry production was done to overcome the animal protein gap in most of the developing countries.This contributes can be done by manipulating and modulating the critical environmental items such as, light intensity and light colour as it controls many physiological and behavioural processes (Olanrewaju et al., 2006). As, the light helps in the establishment of circadian rhythms and synchronization of various essential physiological functions that influence the growth (Manser, 1996) and improve the poultry welfare (Mousa-Balabel et al., 2017).

The Indian River (IR) broiler has recently been introduced to Egypt, and debate persists about the perfect regime of light intensities and colour is unknown. The preferences of broilers to light intensity differed according to their age (Davis et al., 1999).The light intensity and wavelength affected the broilers behaviour due to the different preferences of birds to the type of light spectra and illuminant used (Barber et al., 2006: Kristensen et al., 2007). Generally, using of brighter lighting results in increased bird activity. But, the use of lower light intensities can help in the controlling of aggressive actions (Olanrewaju et al., 2006). Regarding the broiler performance under different light wavelength, it has been reported that the green and blue lights stimulate the growth, while red light boosts the pecking (Rozenboim et al., 2004). Moreover, the use of monochromatic green light accelerates the muscle growth (Halevy et al., 1998). Several studies show that broilers kept under blue or green light colours were significantly heavier in the body weight than those reared under red or white light (Rozenboim et al., 2004; Mousa-Balabel et al., 2017) without any significant impacts on total feed intake, food conversion ratio and mortality percent (Cao et al., 2008).

The behavioural expression was found to be reduced when exposed to dim light intensity as found by Alvino et al. (2009) for 5 lux and Newberry et al. (1988) for 6 lux *vs.* 180 lux. The authors concluded that the failure of high intensity blue light to increase standing and walking acts indicates that the pineal gland's sensitivity to long wave length light is essential to affect the bird activity.

In addition, broilers were found to be more active at high light intensity (30 lux) (more ground pecking, wing stretching and walking) than at low light intensity (10 lux). Further, rearing broilers in bright light early in life increased activity and reduced leg disorders (Prayitno et al., 1997). In addition, the implications of blue light intensity on IR broiler behaviour and bird welfare remain relatively unknown. Thus, this study was carried out to shed light on the beneficial effect of different blue light colour intensities (high, medium, dim) on the performance, behaviour and welfare status of IR broilers under Egyptian conditions to achieve the optimum one.

MATERIALS AND METHODS

Birds, experimental design and diet

The present study was conducted under the temperate climatic conditions of Kafrelsheik Governorate, Egypt, during the months of February and March, 2019. In this study, two hundred and seventy unsexed day old commercial Indian River (IR) broiler chicks were well selected and obtained from a reputable local commercial hatchery in El-Gharbyia Governorate, Egypt. Their average body weight was 44 ±2.3 gand brooded under standard brooding conditions (All birds were kept under an intensity of 40 lux and 24 h light length from 1 to 7 days of age) according to Mousa-Balabel et al. (2017). After 7 days of age, the light-dark cycle was 23 hours:1 hour. From d 8 to d 35 of age, the chicks were randomly distributed into3 equal separate environmental light proof rooms (2.6X2.1m each) with three replicates of 30 chicks each.

The birds were exposed to 3 different blue light intensity (BLI) treatments of incandescent bulb following their identification with wing rings according to Senaratna et al. (2016). The chicks in the first pen (90 chicks) were reared under high intensity (320 lux) of blue light colour (HBLI). While, the chicks in the second pen (90 chicks) were kept under medium intensity (20 lux) of blue light colour (MBLI) and the chicks in the third pen (90 chicks) were kept under dim (low) intensity (5 lux) of blue light colour (DBLI). These treatments were used to assess the effect of various intensities of blue light colours (high, medium and dim) on IR broiler performance and behaviours under the Egyptian conditions with a trial end stocking density of 34 kg/m^2 (equivalent to 17 chicks /m^2) based on chick placement numbers (Rozenboim et al., 2004). Throughout the duration of the study, all birds in the different treatments were given identical care and management (Xie et al.,

2008). The chicks were grown on a deep litter system with *adlibitum* water and fed on a commercial ration (El-Wadi Company, Egypt); broiler starter (metabolizable energy [ME] = 3,000 kcal/kg, crude protein [CP] = 23%); broiler finisher (ME = 3,100 kcal/kg, CP = 20%). The starter ration was used for feeding all broiler chicks from day 1 to day 21 of age and the finisher ration was used for feeding all broiler chicks from day 22 to day 35 of age. The light intensity was recorded three times weekly at the bird's eye height from different three positions in each experimental room using lux meter (Conrad, Hirschau, Germany) to guarantee approximate light intensity which altered due to the dust accumulation on the light bulbs.The dimming bulbs were done by dimmer switches.

Newcastle disease virus (NDV) vaccine (Pfizer Company, Egypt) was administered twice in the drinking water at 7 and 17 days old using live vaccine strains. All procedures performed in the study involving birds were in accordance with the ethical standards of the Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, Kafrelsheikh University, Egypt, at which this study was conducted.

Data collection

At the day of birds arrival, the body weight (BW) of the birds was recorded individually and subsequently at the end of each week. Body weight gain (BWG), total feed intake (TFI) and water intake (WI) were calculated every week. Also, feed conversion ratio (FCR) was determined for relevant time periods. Pens were tested twice daily (8 a.m. and 8 p.m.) for dead birds and the total mortalities were calculated as a percentage of live birds at the start of each treatment according to El-Husseiny et al. (2000). Blood samples (5 ml) were aspirated weekly from wing vein by disposable needle and transferred to heparinized vacuum tubes without delay to assess some blood parameters as Heterophyil/Lymphocyte (H/L) ratiousing May-Grunwald, Giemsa stains (Evrim et al., 2017) and Newcastle Disease Virus (NDV) antibody responses by the Haemagglutination Inhibition (HI) test At 28 days old only as, the peak of antibodies was achieved after 10-11 days from the second time of vaccination (Xie et al., 2008).

Behaviour observation and welfare indicator

The bird behaviour was recorded using a fixed digital camera over the experimental pens. The scan sampling technique from an electronic compact discwas used to monitor the bird behaviour (Bowden et al., 2008). For documenting the different behavior trends, each group was monitored three days a week (twice a day; each of 30 minutes) for for the duration of the entire experimental period at 7 am and 4 pm for reporting the different behavioural patterns. Individual birds (small pen) involved in feeding (at feeder), drinking (under the drinker), resting, pecking of feather, preening, stretching of wing and legs and wing flapping were counted at every 10 minutes of the observation period. Resting and sleeping, was listed as a rest behaviour (Rierson,2011). The welfare indicator was assessed using an internationally accepted score system used by Kestin et al. (1992) for the presence of foot pad dermatitis (FPD), hock burning damage (HBD), and bumble foot (BF). Both HBD and BF were graded on a three point scale (1, signs of deterioration without redness; 2, signs of deterioration with the presence of redness; and 3, an evident lesion or score). In addition, FPD was scored on a two point scale where 1 describing normal footpads without lesions, whereas a score of 2 was given for obvious sores on the footpads (Ekstrand and Carpenter, 1998).

Statistical Analysis

Data were reported as means ± SEM and analyzed by one-way ANOVA using Graph Pad prism 5. The significance of difference among the different groups was evaluated by Tukey's post hoc multiple comparison test. The significance level was set at $P < 0.05$.

RESULTS

Data in table (1) reflects the mean output values of broiler performance that were held under various intensities of blue light colour. Results showed that, the BWG at 35 days of age was higher in the birds kept under DBLI group (2052.15±2.028 g) compared to those kept under MBLI group (1890.22±3.786 g) and under HBLI (1897.83±1.764 g).

Regarding the feed intake and mortality percent, table (1) reveals that the overall consumption of feed intake did not vary in different treatments for blue light intensities (5 lux: 3662.1±0.624 g, 20 lux: 3657.1±0.328 g and 320 lux: 3658.8±0.290 g). The most economic FCR was recorded in birds kept under DBLI group (1.785±0.057) compared to those kept under MBLI group (1.934±0.088) and underHBLI (1.927±0.115). However, the lowest percentage of mortality was reported in broilers held under DBLI group then MBLI group (4.5 and 8.1%, respectively) compared to 10.8% for HBLI

Light intensity had a significant effect (P<0.05) on most broiler behaviours. Birds reared at low light intensity (5lux) displayed the lowest frequencies of feeding, drinking, wing flapping, and feather picking behaviours (Table 2).

Concerning leg problems, data in table (3) shows that the prevalence of FPD and HBD was significantly(P<0.05) differed by different light intensities. Whereas, FPD and HBD were more common in broilers kept at 5 lux compared to broilers kept at 20 and 320 lux.

Moreover, birds reared at low light intensity environment (5 lux) had the lowest H/L ratio (0.43±0.006) compared to the other light intensity groups which had 20 lux; 0.58±0.006 and 320 lux; 0.63±0.004 as shown in table (4). Also, table 4 reveals that the highest concentration of total NDV antibody titer was recorded in high stocked broilers kept under dim blue light in this experimentin comparison with other treatments.

Table 1: Means ± SE of IR broiler performance under different blue light color intensities.

	HBLI	MBLI	DBLI	P-Value
IBW (g)	44.00±2.309	44.00±1.155	44.00±1.165	0.4219
7 day (g)	145.01±1.480	146.00±2.140	144.91±1.680	0.6217
35 day (g)	2042.84±1.501	2036.22±1.057	2197.06±9.970	0.0158
BWG (g)	1897.83±1.764	1890.22±3.786	2052.15±2.028	0.0442
TFI (g)	3658.8±0.290	3657.1±0.328	3662.1±0.624	0.5312
FCR	1.927±0.115	1.934±0.088	1.785±0.057	0.0390
WI (ml)	871.63±1.379	841.29±2.901	953.81±5.604	0.0114
M %	10.8	8.1	4.5	0.0421

HBLI: high blue light intensity; MBLI: moderate blue light intensity; DBLI: dim blue light intensity; IBW: Initial body weight; BWG: Body weight gain; TFI: Total feed intake; FCR: Feed conversion rate; M%: Mortality percent; SE: Standard error.

Table 2: Means ± SE of some broilers behaviors (Frequencies) kept under different blue light color intensities.

Behavior	HBLI	MBLI	DBLI	P-Value
Resting	7.33±1.202	10.33±1.453	19.00±0.577	0.0009
Feeding	3.00±1.155	10.00±1.155	1.66±0.881	0.0032
Drinking	5.00±0.547	10.00±0.574	1.00±0.577	0.0001
Pecking	5.33±0.819	2.66±0.881	2.00±1.155	0.0316
Preening	3.00±0.574	2.00±1.155	8.00±1.155	0.0114
Wing and leg stretching	2.00±1.155	7.00±1.165	8.00±1.158	0.0217
Wing flapping	3.00±0.819	2.33±0.574	1.00±0.544	0.0219

HBLI: high blue light intensity; MBLI: moderate blue light intensity; DBLI: dim blue light intensity; SE: Standard error.

Table 3: Means ± SE of some broilers behaviors (Frequencies) kept under different blue light color intensities.

Leg problems	HBLI	MBLI	DBLI	P-Value
FPD (%)	1.8	6.3	9	0.0121
HB (%)	2.7	13.5	18	0.0354
BF (%)	0	0	0	0.6032

HBLI: high blue light intensity; MBLI: moderate blue light intensity; DBLI: dim blue light intensity; FPD: foot pad dermatitis; HB: hock burns; BF: bumble foot; SE: Standard error.

Table 4: Means ± SE of some blood parameters of broilers kept under different blue light color intensities

	HBLI	MBLI	DBLI	P-Value
NVD Titer	2.09±0.076	2.82±0.094	3.08±0.056	0.0308
H/L	0.63±0.004	0.58±0.006	0.43±0.006	0.0011

HBLI: high blue light intensity; MBLI: moderate blue light intensity; DBLI: dim blue light intensity; NVD: Newcastle Viral Disease antibody; H/L: Heterophyil/ Lymphocyte; SE: Standard error.

DISCUSSION

Poultry is more affected by light intensity, and by using the retina of the eye, they can distinguish-between light colours with varying degrees of sensitivity (Lewis and Morris, 2000). The improvement in the performance of high stocked broilers reared under DBLI group may be attributed to a calming effect of blue light and the positive effect of this light color on feed intake and feed utilization. These results are in line with the findings of Downs et al. (2006); Velo and Ceular (2017) who reported that bird's performance was enhanced by low light intensity. Davis et al. (1999) reported similar findings, that low light intensity increased the bird body weight, feed intake, feed conversion and weight gain.

Concerning the broilers feed intake,there was no significant difference in the overall consumption of feed intake under different treatment for blue light intensities, these findings are supported by the findings of Rault et al. (2017) who noted that there was no significant difference in feed intake between broilers kept at 20 lux and 5 lux. But the obtained data are in disagreement with the results of Davis et al. (1999) who mentioned that the birds that were held under high light intensity ate and drank more than that those kept at the low light intensity. Also, Mosa et al. (2015) explained that blue light has a calming effect on birds making them less active than in white light and the bird preferes to spend more time in resting under blue light with a filled crop and gizzard content.I n addition, the best FCR was recorded inbirds reared under dim blue light. A possible explanation for why broilers prefer to consume more feed under white light due to it helps them to identify texture differences which they cannot see under other colors.

Adopting a strategy allowing broiler chicks to feed under white light and rest under blue light (Abu-Tabeekh et al., 2015).

These results are consistent with the findings of Downs et al. (2006) who stated that the lower light intensities may improve FCR due to reduced activity and stimulated muscle growth.

Furthermore, these results are partly supported by the findings of Buyse et al. (1996) who tested for FCR under 5 *vs*. 51 lux and Lien et al. (2008) who tested for FCR under 1.75 *vs*. 162 lux and they summarized that the decrease in LI had a significant improvement effect on FCR.

The highest mortality percent was reported in birds group held under HBLI group. Such findings are recorded by Newberry et al. (1988) and Buyse et al. (1996) who foundt hat the mortality rate increased with the increasing in the light intensity. But, Evrim et al.(2017) found that light intensity did not have any major impact on the mortality percent.

Behavioural studies are of great significance for enhancing animal cognition and understanding.

Light may be the most critical for chickens as it controls many behavioral patterns (Olanrewaju et al., 2006). Poultry producers are concerned with raising poultry in improved and comfortable conditions (Harper and Henson, 2001). The mean values of broiler behaviors were affected by different light intensities. These findings are in agreement with the results of Khalil et al. (2016) who observed thatdim light reduced eating and drinking behaviour. But, it is contrary to the findings of Newberry et al. (1988) who claimed that the light intensity had no effect on the behaviour of eating and drinking. In addition, in the current study the frequency of preening, rest, as well as leg and wing stretching was higher in birds kept under low light intensity than those kept under high light intensity. The rest behaviour is essential for poultry as it allows for energy preservation, tissue restoration and growth (Blokhuis,1984). These results are compatible with the findings of O'connor et al., (2011) who observed that the highest frequency of preening, dust bathing, leg and wing stretching, and body shaking was recorded in birds held under low light intensity (5 lux) when compared with the other groups (50 and 250 lux). This can be attributed to the birds kept under low light intensity in this investigation having been less involved with other aggressive activities and appearing to be less scared at low intensity (Davis et al., 1999).

If the bird held under high light intensity, it showed higher activity which needed high energy and therefore triggered the aggressive behavior (Newberry et al., 1988). So, low light intensity embraces to relegate cannibalism, aggression and pecking behaviour (Blatchford et al., 2009).

Sejian et al. (2011) described the animal welfare by the ability of an animal to adapt with its environmental stimuli either physiologically or behaviorally. The highest leg problems (FPD and HBD) were reported in broilers kept at 5 lux. This may be attributed to the lower activity of broilers kept under low intensity (5

lux), because the foot and hock burns are generally correlated with a reluctance to walk (McKeegan, 2010). Similar results were recorded byDeep et al. (2010) who reported that the prevalence of FPD was decreased by the increasing of light intensity from 1 to 40 lux. On the other hand, BF wasn't observed in all groups.

The H/L ratio is a sensitive stress indicator, because the H/L ratios in birds subjected to environmental stressors have increased (Evrim et al., 2017). The lowest H/L ratio and highest concentration of total NDV antibody titer were recorded in broilers kept underlow light intensity environment (5 lux). These findings were contrary to Evrim et al. (2017) who reported that light intensity had no impact on H/L ratios. These results proved that broiler chickens were more sensitive to light intensity. Whereas, when boilers reared at low light intensity environment, they had a low level of stress and strong light is considered a stress factor in poultry production (Guo et al., 2018). Increased percentage of NDV antibody titer under dim blue light in this experiment suggested that low light intensity improved the cellular and humoral immune responses of broilers. This could be explained by increasing melatonin secretion (Abbas et al., 2007), or by activating the peripheral T and B lymphocyte proliferation to produce antibodies (Abbas et al., 2008). The opposite results were obtained by Olanrewaju et al. (2016) who found no difference in immune parameters in broilers reared under different light intensities. Light intensity is routinely kept lowin the broiler production sector (usually 5 lux) to minimize bird activity to save energy (Prescott et al. 2003). But, commercially, there is an insight that very low light intensities improved feed efficiency and decreased carcass damage by the reduction of activity (Downs et al., 2006).

CONCLUSION

Dim blue color was a good atmosphere for keeping IR broilers less active, safe and increased their performance. In addition, dim blue light not only improved the performance, but also improved the ability to anti-stress as well as immune function. On the other hand, high intensity of blue light deemed to be detrimental to the birds welfare.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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Research article
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First study on oxyuriosis in horses from Algeria: Prevalence and clinical aspects

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ABSTRACT: The present study was carried out the Tiaret region, on horses belonging to the Chaouchaoua National Haras, ONDEEC and on two private farms during the period from February 05th to April 17th, 2019. It aims to assess the overall prevalence of equine oxyuriosis in the area and to describe the most dominant clinical signs of this parasite infection.

Scotch tape test was applied on 176 horses randomly selected and the microscopic observation of slides was carried out at the parasitology laboratory of the Veterinary institute of Tiaret.

The overall prevalence of *Oxyuris equi* was 38.64% with females being more infected (37.04%) than males (20.45%). The prevalence of oxyuriosis was higher in fillies (70%) than in foals (48.39%).The high prevalence of *Oxyuris equi* was recorded in the private farms with a percentage of 85% and 44% in farm 1 and 2, respectively. The more commonly clinical signs were tail rubbing in 42% and scratching in 32% of positive horses.Equine oxyuriosis is a common infection in the study area and requires the application of hygienic measures with more therapeutic and preventive care.

***Keywords*:** Oxyuriosis, horses, Algeria, prevalence

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INTRODUCTION

Horses are animals of great global economic importance and can be used for a wide variety of activities, such as sport, leisure and work (Rosa et al., 2018).The most common internal parasites of the horse are nematodes, among which strongyles (*Strongylus spp*.), ascarids (*Parascarisequorum*), pinworms (*Oxyuris equi*) and bots (*Gasterophilus spp*.) have the highest prevalence (Bulgaru and Tudor, 2016). These animals are considered very susceptible to helminths, which affect their health and decrease their performance (Rosa et al., 2018). Pinworms are the most frequent nematodes that affect horses of all ages and the infection is more common in stabled horses than in those at pasture because the eggs are poorly resistant in external conditions (Bussiéras and Chermette, 1995).

Oxyuris equi is a fairly large nematode (~1-6 cm in length) that resides as an adult in the small colon and dorsal colon of equids (Uquhart et al., 1996).*Oxyuris* is known as a pinworm because the tail end of the female is sharply pointed (Nielson et a., 2014).The predominant clinical sign of pin worm infection is the intense perianal pruritus caused by the sticky eggs deposited on the skin of perianal region (Beugnet et al., 2005).

Although oxyuriosis is of great importance in equine production, no study has been conducted in Algeria. For this reason, the present study was performed to assess the prevalence of this parasite infection in Tiaret province.

MATERIALS AND METHODS

The present study was conducted in Tiaret region (Algeria) on horses belonging to the Chaouchaoua National stud farm, to ONDEEC and two private stables from February to April2019.

Study area description

Tiaret province is located in the west of Algeria (35 ° 15 ‘N of latitude and 1 ° 26’ E of longitude), 300 km to the southwest of Algiers. Its relief varies, with altitudes between 800 and 1200 m. It is an agropastoral zone with a Mediterranean continental type of climate with harsh winter, and hot and dry summer, and the rainfall is 300-400 mm per year on average. The maximum thermal average (26°C) is recorded in August and the minimum average (6 °C) in January. The hotdry season can extend over six months (from May to October) (Boulkaboul, 2003; Houssou et al., 2018).

Animals

A total of 174 Barb and Arabian purebred horses of different sex and age were included in this study (Table1). Most of horses (127) belong to the Chaouchaoua National Haras of Tiaret, which is of great importance in equine breeding.This stud farm was created in 1877on a surface of 922 ha and it is the first source of horses used in racing. Other horses belonging to the national Office of Development of Equine and Camels Farms (O NDEEC) and two private stables were used in this study. All horses used in this study have not been dewormed two months at least prior to sampling.

Table 1. Number of horses examined

	Mares	Stallions	Foals	Fillies
Chaouchaoua National Haras	44	34	20	31
ONDEEC	01	10	0	0
Private stud farm 1	09	0	0	0
Private stud farm 2	27	0	0	0
Total	**81**	**44**	**20**	**31**

Clinical exam

All horses included in this study were subjected to clinical examination of the tail and the perineal region for any sign of pinworm infection.

Scotch tape test

This method is simple, fast and inexpensive (Gevrey, 1971). A transparent adhesive tape was applied to the skin of the perianal region, then removed and examined microscopically to identify the characteristic oxyurid eggs (Thienpont et al., 1979) (Fig. 1 and 2).

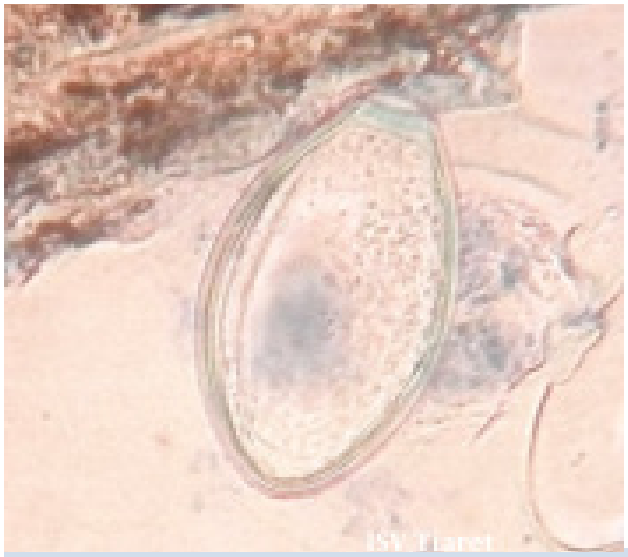

Figure 1: *Oxyuris equi eggs* at morula stage (without coloration) (G.X40).

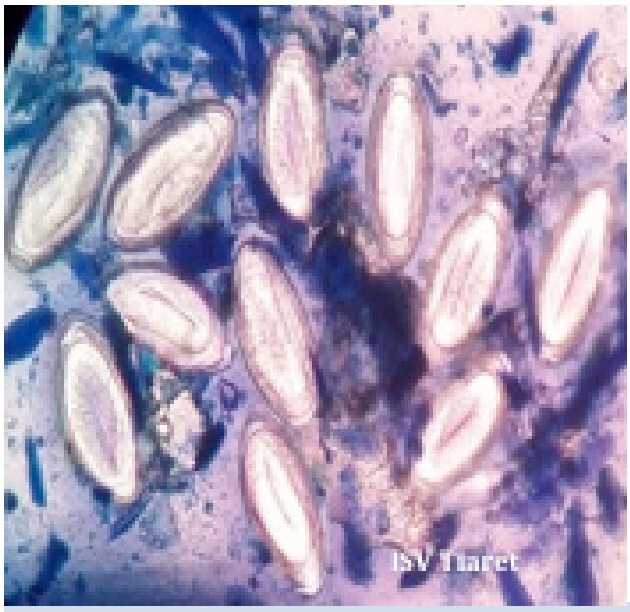

Figure 2: *Oxyuris equi eggs* at larval stage (G.X10).

RESULTS AND DISCUSSION

The present study conducted on the horses belonging to the national stud farm of Chaouchaoua, to the ONDEEC and two private stud farms has allowed the following results:

Overall Prevalence of pinworms infections among horses in Tiaret

The prevalence of horse pinworms in Tiaret is presented in Table 2.

Table 2. Overall prevalence of oxyuriosis in horses from Tiaret

Total number of samples	176
Positive samples	68 (38,64%)
Negative samples	108 (61,36%)

According to the results showed in table 2, the overall prevalence of pinworm infection in horses was 38.64%. A percentage of infection close to 36% was registered byGawor (1995) in Poland. In contrast, high prevalence of 56.8% was reported by Torbert et al. (1986). Moreover, a low prevalence of oxyuriosis varying from 0.7 to 2% was recorded in Turkeyand was mainly attributed to the sampling technique. In fact the eggs of *Oxyuris equi*are attached to the perianal skin and their research by coprological flotation technique in fecal samples taken from the rectum might not give the exact prevalence of the infection (Tolossa and Ashenafi, 2013; Sheferaw and Alemu, 2015).

Prevalence of horsepin worms based on sex and age group

The prevalence of *Oxyuris equi* based on sex and age group of animals is presented in table 3.

Table 3. Prevalence of *Oxyuris equi* in horses based on sex of animals and age groups

Horses	No. of animal examined	Positive	Prevalence
Stallions	44	9	20.45%
Mares	81	30	37.04%
Foals	31	15	48.39%
Fillies	20	14	70%
Total	176	68	38.64%

The results of table 3 indicated that the highest prevalence of *Oxyuris equi* was recorded in fillies with 70% followed by 48.39% in foals, 37.04% in mares and 20.45% in stallions. Our results are consistent with those of Collobert et al. (1996) who found that foals of less than 2 years of age are more susceptible to oxyurid infections than adults with a prevalence of 80% and 56.6%, respectively. In contrast, Belay and al. (2016) reported a prevalence of 2.1% in horses less than 2 years old and 2.3% prevalence in adult horses between 2 and 10 years old. Our findings disagree also with the work of Alanazi et al.(2011) who reported a prevalence of 2.22% and 6.66% in foals and adults,respectively in a study carried out in Saudi Arabia.

Regarding the sex of animals examined, the current study indicated that sex had influence on the prevalence of *Oxyuris equi* being females more infected than males and this agrees with the finding of Hassan et al. (2013) who have reported a prevalence of 54% in females and 46% in males. In contrast, the prevalence of *Oxyuris equi*was higher (65.90%) in males than females (35.55%) as reported by Alanazi et al. (2011).

Prevalence of *Oxyuris equi* in the different stables included in the study

Horses included in the current study belonged to four different stables and the rate of infection in each stable is presented in table 4.

Table 4. Prevalence of *Oxyuris equi* in horses based on their origin

Stable	No. of examined animals	No.of positive	Prevalence
Chaouchaoua National Haras	129	40	31.01%
ONDEEC	11	1	9.09%
Private stable 1	9	4	44.44%
Private stable 2	27	23	85.19%
Total	176	68	38.64%

Data represented in table 4 showed that the greatest percentage of horses with oxyurids belonged to both private stables with 85.19% for stable 2 and 44.44% for stable1 and the lowest prevalence (9.09%) was obtained in horses from ONDEEC. However, the Chaouchaoua National Haras has registered a prevalence of 31.01%.The observed difference could be attributed to the conditions of housing and some management practices applied in each stable.

In fact, the highest prevalence recorded in the two private stables could be explained by the poor hygienic conditions and the neglected veterinary care given to theses horses. It is well known that *Oxyuris equi* has direct life cycle where adult female worms living within the cæcum and colon shed eggs which are excreted in faeces, then the larvae develop, hatch and moult to the infective third stage (L3) which serve as a source of contamination of housing facilities, pasture and feedstuff resulting thereby in infection or re-infection of susceptible horses (Wosu and Odubi, 2014).

Therefore, the main way to inhibit parasite from completing its life cycle and to prevent horses from infection is to apply some simple management practices such as regular removal of faeces from stables (several times daily or at least once a day), cleaning thoroughly water and feed buckets (Proudman and Matthews, 2000). Unfortunately, these conditions were not respected in the private stables. Other management problems could play an important role in the prevalence of oxyurid infection in horses belonging to these stables such as use of a single rectal sleeve for rectal examination of many mares during pregnancy diagnosis, use of the same stall by multiple horses and irregular change of bedding.

Clinical signs of horse's pinworms

Table 5. Principal signs observed in infected horses

Clinical signs	Number of animals	Prevalence (%)
Scratching	50	31,65
Tail rubbing	67	42,41
Yellow-grey egg masses on the perianal skin	33	20,89
Injuries on the perineal region	08	5,06
Total	**158**	**100**

Results shown in table 5 revealed that the more observed sign was tail rubbing (Fig. 3) with a prevalence of 42.41%followed by scratching (31.65%) and the presence of grey-yellowish egg masses on the perianal skin (20.89%) while the presence of injuries on the perineal region (Fig. 4) was only recorded in 5.06% of infected animals. Our results revealed that 86.76% of infected horses presented more than one sign (47.06% presented two signs, 33.82% three signs and 5.88% four signs). Beugnet et al. (2005) reported that severe itching of the perineal skin was the more common clinical sign. The infected animal rubs very frequently against any object in its environment causing consequently the break off hairs and giving the tail a rat tail appearance (Fig. 5).

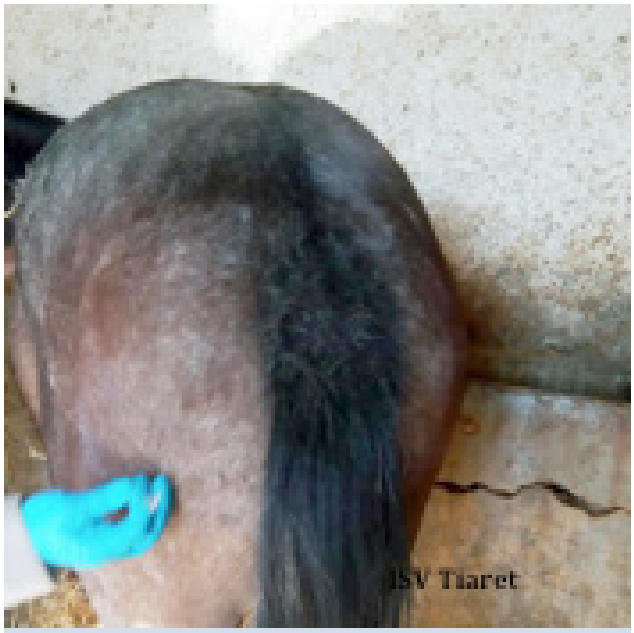

Figure 3: Tail rubbing.

Figure 4: Injuries and lesions on the perianal skin

Figure 5: Rat tail.

CONCLUSION

The present study conducted on equine oxyuriosis in Tiaret showed the high occurrence of *Oxyuris equi* pinworms in this region. However, the attention given to this disease often confused with scabies and pthiriosis and affecting the well-being and productivity of stabled horses remains insufficient and needs more importance.

This is the first study on equine oxyuriosis in Algeria, so further investigations are needed in order to know the different risk factors affecting the prevalence of *Oxyuris equi* infection in horses and other equids.

In the light of our findings, regular screening of *Oxyuris equi* infection in stabled horses is highly recommended. In addition, strategic deworming programmes should be applied regularly. Also, it is well recommended to improve housing and to apply some management practices based primarily on appropriate hygienic conditions. Further, each newly purchased animal must be quarantined, and properly screened to prevent the spread of parasite infection.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this study.

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Research article
Ερευνητικό άρθρο

Influence of dietary protein levels and some cold pressed oil supplementations on productive and reproductive performance and egg quality of laying Japanese quail

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ABSTRACT: The present study was carried out to examine the effect of dietary protein levels (18, 20, and 22%) and addition of cold-pressed oils (no addition, 1 g thyme, 1 g oregano, and 0.5 g thyme + 0.5 g oregano /kg diet) on the productive and reproductive performance and egg quality of Japanese quail at the laying period. 324 mature ten-week-old Japanese quails (216 females and 108 males) were used. Quails were randomly distributed to 12 treatment groups, 27 in each group, with 3 replicates of 9 quails (6 females and 3 males) per group. Feed conversion ratio (FCR) significantly improved in the layers fed with the diets containing 20 or 22% CP in all experimental periods, except in 3- to 4-month-old birds. The diets supplemented with thyme only or interaction with oregano improved FCR significantly compared to the control or oregano -administered group in all experimental periods, except in 2- to 3-month-old birds. Dietary supplementation with thyme oil caused the egg number and egg mass to increase significantly in all experimental periods compared to the other dietary groups. Fertility percentages were significantly higher in the groups which had oregano than in the control group. Dietary supplementation with thyme oil significantly increased the egg number ($P<0.05$) and egg mass ($P<0.01$) in all experimental periods compared to other dietary groups. Fertility and hatchability percentages were not significantly affected due to varying dietary protein levels throughout the experiment. In conclusion, the quails fed with a diet containing 20 to 22% CP or supplemented with oregano or thyme oil exhibited an improved productive and reproductive performance without any detrimental impacts on the other parameters studied.

Keywords: Cold pressed oil, Protein, Performance, Production, Reproduction, Quails

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INTRODUCTION

Nutritional costs account for about 65-70% of the total costs of poultry production (FAO, 2006; Alagawany et al., 2016). Protein is one of the most important and expensive nutrients in the rations of poultry - so much so that its quality and quantity are considered as the main limiting factors to the productivity and efficiency of poultry. Shrivastav et al. (1994) concluded that laying quail performed well when given 19% dietary protein and 2750 kcal ME/kg and when early diets contained 24 and 20% CP in the starter and finisher periods, respectively. The protein and energy requirements of grower and layer Japanese quail have been reported under different conditions (Alagawany et al., 2014a,b; Reda et al., 2015; Reda et al., 2020).

The effects of some aromatic medicinal plants and their extracts and cold pressed oils on broiler activity have been studied, and the addition of feeds or water has been found to improve the feed intake (FI), and feed conversion ratio (FCR) (Alagawany and Abd El-Hack, 2015; Alagawany et al., 2017). Thyme (*Thymus vulgaris* L.) and oregano (*Origanum vulgare)* and their effective components improve the activity of digestive enzymes such as protease, amylase and lipase, which results in a reduced nutrient digestibility (Abd El-Hack and Alagawany, 2015; Badiri and Saber, 2016). Additionally, these grasses of the Labiatae family are used to improve digestion and absorption. These effects are able to increase the length of intestine and the depth and width of villi, which creates better good conditions for dietary absorption. Nonetheless, while there are abundant studies on thyme essential oils, there is limited knowledge about the effects of this plant alone.

Phytogenic feed additives such as thyme played an active role in forming chelates with metal ions and preventing or decreasing the oxygen formation (Dhama et al., 2015; Mohamed et al., 2017). Regarding the combination of protein and herbal oils, the quails fed with a diet containing 24% or 26% CP or the diets enriched with oregano or thyme oils or both exhibited an improvement in performance, antioxidant capacity, and immunity (Mohamed et al., 2019). But, in the literature, there is a limited number of studies in this regard. So, this study aimed to examine the effects of supplementation with dietary protein levels and cold-pressed oils (thyme and oregano) on the productive and reproductive performance and egg quality of Japanese quail in the production stage.

MATERIALS AND METHODS

Experimental Design and Animal Husbandry

completely random design with factorial arrangement (3×4) was performed to reveal the effect of three levels of CP (18, 20, and 22%, Table 1) with the addition of cold-pressed oils (no addition, 1 g/kg thyme, 1 g/kg oregano and 0.5 g/kg thyme + 0.5 g/kg oregano) on the productive and reproductive indices and egg quality criteria of laying Japanese quail in the production stage. The oil types were purchased from the company named "El Hawag for Natural Oils", Cairo, Egypt. A total of 324 mature ten-week-old Japanese quails (216 females and 108 males) were used. Quails were randomly distributed into 12 treatment groups, 27 in each group, with 3 replicates of 9 quails (6 females and 3 males) per group. Birds were housed in a naturally ventilated house measuring 5×3m^2 equipped with laying cages measuring 144×70×30cm^3. All birds were reared under similar managerial and hygienic conditions at a temperature of 35-38°C and a humidity of about 60-80%. Feed and water were provided *ad libitum* throughout the experiment.

The diets were formulated according to NRC (1994) and the required amount of cold pressed oil supplement for each diet was initially blended with vegetable oil of the diet, then mixed with small amount of the diet, and finally mixed with the remaining of the diet. The cumulative and per-period average daily feed intake (FI), body weight gain (BWG), and FCR were calculated using these data. Feed lost was recorded every day, and the information was used to estimate the feed utilization. Animal care and maintenance were performed in accordance with the guidelines of the Egyptian Research Ethics Committee and the Guide for the Care and Use of Laboratory Animals (2011).

Data Collection

FI was recorded weekly, calculated as grams of diet consumed per 7 days, and divided by the number of birds in each replicate group. On the other hand, the feed conversion of feed (g feed/ g egg) was calculated as the rate of the feed intake to the egg mass. The eggs were weighed and the egg number was counted every day to measure the egg mass (egg number ×egg weight) (Alagawany et al., 2014). The egg characteristics parameters were recorded monthly using three eggs from each replicate. The external and internal egg quality characteristics (yolk percentages, albumen, and shell; shape index of egg (ESI); thickness of shell; unit surface shell weight (USSW), and Haugh unit) were indicated according to Romanoff and Romanoff (1949).

Table 1. Ingredient and nutrient contents of experimental diets of laying Japanese quail.

	Crude protein levels (%)		
Items	18	20	22
Ingredient (%)			
Yellow corn	65.20	58.70	53.00
Soybean meal 44%	22.80	27.50	31.00
Corn gluten meal 62 %	3.50	4.60	6.22
Vegetable oil	0.8	1.8	2.46
Limestone	5.5	5.54	5.52
Di-calcium phosphate	1.27	1.20	1.20
Salt	0.30	0.30	0.30
Premix*	0.30	0.30	0.30
L-Lysine%	0.18	0.06	0.00
Dl-Methionine %	0.07	0.00	0.00
Total	100	100	100
Calculated composition**			
ME, Kcal /Kg	2902	2901	2900
Crude protein %	18.00	20.00	22.00
Calcium %	2.50	2.50	2.50
Nonphytate P %	0.35	0.35	0.35
Lysine %	1.00	1.00	1.03
TSAA %***	0.70	0.70	0.77

*Layer Vitamin-mineral Premix, Each 1Kg consists of vit. A, 8000 IU ;vit. D3, 1300 ICU, vit. E 5 mg ; vit. K, 2 mg ; vit B1 ,0.7 mg; vit. B2, 3 mg; vit. B6, 1.5 mg; vit. B12, 7 mg; Biotin 0.1 mg; Pantothenic acid , 6 g; Niacine, 20 g ; Folic acid, 1 mg , manganese , 60 mg ; Zinc, 50 mg, Copper , 6 mg ; Iodine, 1 mg , Selenium, 0.5 mg ; Cobalt, 1mg

**Calculated according to NRC {30}.

***TSAA: Total sulfur amino acids

Fertility and hatchability

At the end of first, second, and third month of each experimental period, 20 eggs were collected from each replicate. The eggs were then incubated at 37.6°C and 65% RH in an automatic incubator and turned 45° every 1 h. Beginning on the 14th day of incubation, the eggs were maintained at 37.5°C and 70% RH without turning until hatching. After hatching, chicks were counted and unhatched eggs were broken to determine the percentages of fertility and hatchability. The hatchability was expressed as hatched chicks from the total egg set. Fertility and hatchability percentages were calculated as follows: fertility percentage = (number of fertile eggs / total eggs set) × 100; hatchability percentage = (number of hatched chicks / total number of fertile eggs) × 100; hatchability percentage = (number of hatched chicks / total eggs set) × 100 (Alagawany and Attia, 2015).

Statistical analysis

The data of productive and reproductive performance as well as egg quality criteria were analyzed with a generalized linear model using the normal distribution and the identity link function (SAS Institute Inc., 2001). The model used included the protein levels and cold pressed oils, as well as the interaction effects:

$$Y_{ijk} = \mu + A_i + S_j + AS_{ij} + e_{ijk},$$

Where Y_{ijk} = an observation, μ = the overall mean, A_i = effect of protein level (j = 18, 20 and 22%), S_j = effect of cold pressed oils (0, 1 g/kg thyme, 1 g/kg oregano and 0.5 g/kg thyme + 0.5 g/kg oregano), AS_{ij} = interaction effect between CP levels and cold pressed oils supplementation (j = 1, 2 … and 12) and $e_{(ijk)}$ = random error. The differences between means were calculated using the *post hoc* Tukey's test. The statistical significance was set at $P<0.05$ unless otherwise stated.

RESULTS AND DISCUSSION

Feed intake and Feed Conversion Ratio

The average FI and FCR of Japanese quails were affected by dietary protein values, cold pressed oils and their mixture in the laying period, which is presented in Table 2. The different protein levels did not have a significant effect on FI in all experimental periods, except in 3- to 4-month-old birds; while feeding the laying quails with a diet containing 22% CP

increased FI significantly compared to the diets with lower CP content. The FCR significantly improved in the layers fed with the diets containing 20 or 22% CP in all experimental periods, except in 3- to 4-month-old birds in which the dietary protein levels did not significantly affect the FCR. These results were in line with the results of Abd-Elsamee et al. (2001) who showed that the average FI values did not differ significantly depending on the increasing dietary CP. On the other hand, the lowest feed intake values were observed in the groups that took low levels of CP. The protein consumption and protein efficiency ratio differ significantly depending on strain, protein level, and age (Hammouda et al., 2001). Alagawany et al. (2011) asserted that the FI in 20- to 30-week-old laying hens improved and the feed conversion ratio in 22- to 34-week-old hens at the laying period also improved when the birds were fed with a diet containing 20% CP compared to the hens fed with the diets containing 16% and 18% CP. Protein efficiency rates were significantly higher for the 16% and 18% CP diets than that for the 20% CP diet in almost all the experimental periods. These results were in line with the results of Dean et al. (2006) who reported a reduced gain/feed ratio when CP levels were below 22%.

The results presented in Table 2 show that FI showed no significant response to cold pressed oils throughout the entire experimental period. The diets supplemented with thyme only or interaction with oregano improved the FCR significantly compared to the control or oregano-administered group in all experimental periods, except in 2- to 3-month-old birds in which the herbal oil supplementation had no significant effect on FCR. Sengul et al. (2008) found that supplementation with thyme oil significantly lowered the feed intake compared to the antibiotic-supplemented and control diets. These results may have stemmed from the good taste of the phenolic components in thyme oil. Parlat et al. (2005) reported that the thyme oil supplementation improved FCR. Oils of plant origin can stimulate the activity of enzymes and improve the absorption of feed, and thyme oil has a toxin binding effect and effectively diminishes the detrimental effects of aflatoxin on performance (Parlat et al., 2005). Also, Osman et al. (2010) reported that chicks fed with the basal diets containing 0.5 g/kg rosemary, oregano or sweet basil supplementation consumed less food and had an improved FCR ($P<0.05$) compared to the control. This may be due to the ability of oregano to increase the efficiency of digestion by increasing saliva, the amount of digestive enzymes; quieting the stomach and digestive system; improving craving; curing or preventing basic intestinal infections; and relieving diarrhea and constipation**,** thereby maximizing the benefit of feed without increasing FI (Badiri and Saber, 2016). The interaction between dietary protein levels and herbal oil supplementation had no significant effects on FI and FCR in any experimental period, except for the effects on FCR during the whole laying period (2- to 5-month-old birds), when the layers fed with a diet containing 22% CP supplemented with thyme oil. In this combination, FCR improved significantly compared to the other combinations.

Table 2. Feed intake and average feed conversion ratio (n=3) of laying Japanese quails as affected by dietary protein levels, cold pressed oils and their interaction during the laying periods (2-5 months of age).

	Feed intake (g)				Feed conversion ratio (g feed/ g egg)			
Items	2-3 months	3-4 months	4-5 months	2-5 months	2-3 months	3-4 months	4-5 months	2-5 months
Dietary protein level								
18	30.09±0.28	32.16±0.35^{b}	30.83±0.34	31.03±0.25	3.22±0.03^{a}	3.12±0.06	3.19±0.05^{a}	3.17±0.03^{a}
20	30.11±0.25	32.02±0.27^{b}	30.97±0.33	31.03±0.21	3.02±0.05^{b}	2.97±0.06	2.90±0.06^{b}	2.96±0.02^{b}
22	30.27±0.31	33.36±0.38^{a}	31.56±0.22	31.37±0.21	3.05±0.05^{b}	2.96±0.10	2.89±0.08^{b}	2.96±0.07^{b}
Cold pressed oils (g/kg diet)[1]								
0.0	30.01±0.26	32.65±0.33	31.41±0.31	31.36±0.26	3.14±0.043	3.27±0.06^{a}	3.16±0.08^{a}	3.19±0.05^{a}
1 Org	30.33±0.32	32.73±0.48	30.88±0.35	31.32±0.30	3.09±0.07	3.07±0.08^{b}	3.04±0.07^{ab}	3.06±0.05^{b}
1 Thy	30.01±0.30	32.73±0.43	31.14±0.37	31.29±023	3.03± 0.06	2.88±0.06^{c}	2.83±0.08^{c}	2.91±0.^{06c}
0.5 Org + 0.5 Thy	30.28±0.41	31.95±0.47	31.05±0.42	31.09±0.35	3.14±0.06	2.85±0.08^{c}	2.95±0.09^{bc}	2.97±0.05^{bc}
Probabilities								
Dietary protein	N.S	*	N.S	N.S	**	N.S	**	**
Cold pressed oils	N.S	N.S	N.S	N.S	N.S	**	**	**
Interaction	N.S	N.S	N.S	N.S	N.S	N.S	N.S	*

Means in the same column within each classification bearing different letters are significantly different. **($P \leq 0.01$), *($P \leq 0.05$) and NS = not significant.

[1]Org = oregano; Thy = Thyme

Table 3. Average egg number and egg weight (n=3) of laying Japanese quails as affected by dietary protein levels, cold pressed oils and their interaction during the laying periods (2-5 months of age).

	Average egg number (h/month)				Average egg weight (g)			
Items	2-3 months	3-4 months	4-5 months	2-5 months	2-3 months	3-4 months	4-5 months	2-5 months
Dietary protein level								
18	21.04±0.14^{b}	22.83±0.27^{b}	21.90±0.13^{b}	65.78±0.30^{c}	12.42±0.06	12.68±0.09^{b}	12.37±0.13^{b}	12.49±0.04^{b}
20	22.19±0.27^{a}	23.15±0.24^{b}	23.00±0.27^{a}	68.35±0.46^{b}	12.66±0.05	13.07±0.12^{a}	13.04±0.18^{a}	12.90±0.07^{a}
22	22.36±0.28^{a}	24.14±0.62^{a}	23.07±0.54^{a}	69.57±1.37^{a}	12.46±0.07	13.16±0.11^{a}	13.34±0.12^{a}	12.99±0.06^{a}
Cold pressed oils (g/kg diet)[1]								
0.0	21.52±0.20^{b}	21.78±0.23^{c}	21.70±0.38^{c}	64.10±0.63^{d}	12.45±0.03	12.87±0.16	12.86±0.22	12.73±0.12
1 Org	21.44±0.35^{ab}	23.15±0.37^{b}	22.22±0.18^{c}	67.31±0.63^{c}	12.57±0.79	12.92±0.13	12.85±0.22	12.80±0.10
1 Thy	22.39±0.39^{a}	24.48±0.50^{a}	23.67±0.46^{a}	70.54±1.28^{a}	12.42±0.78	13.04±0.14	13.05±0.21	12.84±0.90
0.5 Org + 0.5 Thy	21.61±0.32^{b}	24.09±0.33^{a}	23.04±0.42^{b}	68.74±0.86^{b}	12.53±0.09	13.06±0.12	12.88±0.22	12.82±0.10
Probabilities								
Dietary protein	**	**	**	**	NS	**	**	**
Cold pressed oils	**	**	**	**	NS	NS	NS	NS
Interaction	**	**	**	**	NS	NS	NS	NS

Means in the same column within each classification bearing different letters are significantly different. **($P \leq 0.01$), *($P \leq 0.05$) and NS = not significant.

[1]Org = oregano; Thy = Thyme

Egg Production

The results in the Tables 3 and 4 show an increase in egg number and egg mass for the 2- to 3-month-old and 4- to 5-month old birds fed with the diets containing 20 and 22% CP compared to the birds fed with 18% CP. Moreover, the egg number and egg mass significantly and gradually increased as the dietary CP levels increased, up to 22%. The egg weight significantly improved in the birds fed with the diets containing 20 and 22% CP in all experimental periods ($P<0.01$), except in the 2- to 3-month-old birds, compared to those fed with the diets supplemented with 18% CP (Tables 3 and 4). Alagawany et al. (2011) found that the egg number in 26- to 30-week-old birds and the egg mass in 22- to 34-week-old laying hens increased significantly ($P<0.01$) in response to the diets containing 20 and 18% CP compared to the birds fed with diets containing 16% CP. These results were in line with Novak et al. (2006) who found that the egg production decreased by 2% in response to feeding with a low-protein diet, whereas the same values were recorded for the same characteristics in the hens fed with high and medium levels of protein diets. According to Bunchasak et al. (2005), the dietary protein levels did not affect egg production, but the diets with higher protein levels (16 and 18% CP) tended to yield a good egg production percentage than the diets with 14% CP. In the study in question, the hens fed with 16 and 18% CP diets yielded a significantly higher egg mass than those fed with a 14% CP diet due to heavier egg weight.

The results in the Tables 3 and 4 reveal that the dietary supplementation with thyme oil significantly increased the egg number and egg mass in all experimental periods compared to the other dietary groups. On the other hand, the egg weight did not differ significantly in response to the cold pressed oils supplementation in all experimental periods**.** According to Botsoglou et al. (2005), the volatile components in the extracts may cause the hens fed a diet with thyme or its extracts to give heavier eggs. It was reported that carvacrol and thymol in thyme oil increased the nutrition metabolism in hepatocytes and could also improve the activity of antioxidants (Liu, 2011). Radwan et al. (2008) reported that adding 0.5% oregano to the feed of laying hens increased both egg weight and egg mass. Furthermore, the power of this effect increased significantly as the level of oregano was increased to 1.0%. The pure components of essential oils were found to reduce the activity of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CO A) reductase, which is a regulatory enzyme in the synthesis of cholesterol (Crowell, 1999). Ali et al. (2007) reported that adding thyme to hens' feed increased their egg number. However, Cetingul et al. (2009) indicated that egg weight was not significantly affected when oregano was added to the diets of quails. Regarding the combination between dietary protein values and herbal oil, it could be concluded that a diet with 22% CP and supplemented with thyme would produce the highest egg number and egg mass. In contrast, egg weight showed no significant response to the interaction between dietary protein levels and herbal oil supplementation.

Table 4. Average egg mass and percentages of fertility (n=60) of laying Japanese quails as affected by dietary protein levels, cold pressed oils and their interaction during the laying periods (2-5 months of age).

	Average egg mass				Percentage of fertility			
Items	2-3 months	3-4 months	4-5 months	2-5 months	2-3 months	3-4 months	4-5 months	2-5 months
Dietary protein level								
18	261.30±1.47^{b}	289.57±4.10^{c}	271.00±3.53^{b}	821.63±5.24^{c}	81.94±1.73^{b}	84.03±1.61	81.25±1.49	82.40±1.29
20	279.67±3.67^{a}	302.73±4.64^{b}	299.86±5.92^{a}	881.69±6.19^{b}	88.19±1.91^{a}	84.72±2.01	78.47±2.04	83.79±1.77
22	278.68±3.50^{a}	318.10±9.74^{a}	307.93±8.21^{a}	904.13±19.63^{a}	87.50±1.92^{a}	85.41±1.81	79.86±2.16	84.26±1.50
Cold pressed oils (g/kg diet)[1]								
0.0	268.06±3.03^{b}	280.13±3.80^{c}	279.23±6.92^{c}	827.46±11.40^{d}	81.48±2.31^{b}	80.55±1.96^{b}	75.00±1.96^{b}	79.01±1.47^{c}
1 Org	275.10±5.40^{a}	299.30±6.65^{b}	285.70±6.05^{bc}	860.86±13.67^{c}	83.33±1.96^{b}	82.40±1.67^{b}	80.55±2.78^{ab}	82.01±1.80^{bc}
1 Thy	278.16±5.30^{a}	319.30±9.40^{a}	309.46±9.77^{a}	906.35±21.53^{a}	91.67±1.96^{a}	89.81±1.85^{a}	85.18±1.85^{a}	88.89± 0.92^{a}
0.5 Org + 0.5 Thy	270.67±3.92^{ab}	314.85±6.17^{a}	297.30±9.47^{ab}	881.92±15.30^{b}	87.04±1.46^{ab}	86.11±1.39^{a}	78.70±1.46^{ab}	83.95±0.77^{b}
Probabilities								
Dietary protein	**	**	**	**	*	N.S	N.S	N.S
Cold pressed oils	*	**	**	**	**	*	*	**
Interaction	**	*	NS	**	N.S	N.S	N.S	N.S

Means in the same column within each classification bearing different letters are significantly different. **($P \leq 0.01$), *($P \leq 0.05$) and NS = not significant.
[1]Org = oregano; Thy = Thyme

Fertility and Hatchability Percentage

The results shown in the Tables 4 and 5 reveal that the fertility percentage, hatchability percentage from the total egg set and the hatchability percentage from fertile eggs did not vary significantly in response to varying dietary protein levels in all periods. However, increasing the dietary protein level to 20 or 22% CP significantly increased the fertility percentage and hatchability percentage from the total egg set in 2- to 3-month-old birds. Alagawany et al. (2014a) found that the high fertility percentages were observed in the quails fed with a diet having a reduced level of CP, followed by those fed with the same diet supplemented with valine. In contrast, a diet containing 16% CP supplemented with an amino acid mixture resulted in the lowest fertility value in the first trial period, but no differences were observed in fertility percentage in 4- to 5-month-old birds or during the overall feeding period. The same results were also found by Zeweil et al. (2011). In contrast, no differences ($P>0.05$) were detected in the hatchability percentage of total eggs or that of fertile eggs in the other periods.

The results presented in the Tables 4 and 5 show that the dietary supplementation of thyme alone or in combination with oregano improved the fertility percentage significantly in all experimental periods, except in the whole period (2- to 5-month-old birds), when the thyme supplementation produced the highest fertility percentage, followed by the supplementation with oregano alone or in combination with thyme, compared to the control group. The hatchability percentage from the total eggs set significantly improved in all experimental periods, except in 4- to 5-month-old birds, when dietary thyme oil produced the highest hatchability percentage from total eggs set, compared to the other groups. In contrast, hatchability percentage from fertile eggs did not vary significantly in response to dietary cold pressed oils supplementation (Tables 5 and 6).

Fertility percentages were significantly higher in the groups which had oregano than in the control group. The increase in fertile eggs in the oregano-treated groups could be due to a significant increase in sperm motility, live sperm percentage, ejaculate volume, sperm concentration, total sperm/ejaculate, and total live sperm/ejaculate compared to those in the control group (Daghigh et al., 2016; Alagawany et al., 2018, 2020a,b; Mbaye et al., 2019). Additionally, hatchability percentages (hatchability of total and fertile eggs) were significantly higher in oregano groups than in the control group. This increase may be due to the increased egg shell thickness in most treated groups compared to the control group (Soliman et al. 2016).

Table 5. Average percentages of hatchability from total eggs set and hatchability from fertile eggs (n=60) of laying Japanese quails as affected by dietary protein levels, cold pressed oils and their interaction during the laying periods (2-5 months of age).

	Percentage of hatchability from total eggs set				Percentage of hatchability from fertile eggs			
Items	2-3 months	3-4 months	4-5 months	2-5 months	2-3 months	3-4 months	4-5 months	2-5 months
Dietary protein level								
18	68.75±1.49^{b}	71.53±2.16	68.75±2.32	69.68±1.47	84.08±1.74	85.19±2.31	84.43±1.69	84.57±1.33
20	76.39±2.01^{a}	72.92±2.08	67.36±1.91	72.22±1.64	86.71±1.68	86.41±2.63	86.41±2.81	86.51±1.99
22	75.00±2.29^{a}	71.53±2.40	66.67±2.05	71.06±1.98	85.90±2.41	83.90±2.72	83.99±3.00	84.60±2.07
Cold pressed oils (g/kg diet)[1]								
0.0	68.52±1.85^{b}	66.67±1.96^{c}	62.04±2.02^{b}	65.74±1.22^{c}	84.46±2.74	83.14±3.18	83.46±4.19	83.68±2.61
1 Org	69.45±1.39^{b}	68.52±1.85^{bc}	67.59±2.17^{b}	68.52±1.30^{bc}	83.69±2.74	83.64±3.60	84.33±2.83	83.89±2.64
1 Thy	79.63±2.82^{a}	77.78± 1.96^{a}	74.07±2.17^{a}	77.16±1.29^{a}	86.84±2.20	86.82±2.55	87.17±2.71	86.94±1.34
0.5 Org + 0.5 Thy	75.92±1.67^{a}	75.00±2.40^{ab}	66.67±1.39^{b}	72.53±1.57^{a}	87.27±1.53	87.07±2.33	84.81±1.72	86.39±1.44
Probabilities								
Dietary protein	**	N.S	N.S	N.S	N.S	N.S	N.S	N.S
Cold pressed oils	*	**	**	**	N.S	N.S	N.S	N.S
Interaction	N.S	N.S	N.S	N.S	N.S	N.S	N.S	N.S

Means in the same column within each classification bearing different letters are significantly different. **(P ≤ 0.01), *(P ≤ 0.05) and NS = not significant.
[1]Org = oregano; Thy = Thyme

Table 6. External and internal egg quality characteristics (n=9) of laying Japanese quails as affected by dietary protein levels, cold pressed oils and their interaction during the whole laying period (2-5 months of age).

	External egg quality					Internal egg quality			
Items	Shell percent	Shell thickness (mm)	ESI[1]	USSW (mg/cm^2)[2]	Yolk percent	Albumen percent	Albumen height (mm)	Yolk index	Haugh Unit
Dietary protein level:									
18	11.46 ± 0.09	0.237 ± 0.002	78.08 ± 0.51	47.59 ± 0.05	30.88 ± 0.39	57.70 ± 0.40	4.43 ± 0.10^{c}	46.64 ± 0.41	88.16 ± 0.56^{b}
20	11.45 ± 0.10	0.24 0± 0.003	77.11 ± 0.79	47.90 ± 0.08	31.79 ± 0.67	56.80 ± 0.68	4.67 ± 0.06^{b}	46.65 ± 0.36	89.27 ± 0.36^{b}
22	11.78 ± 0.21	0.242 ± 0.001	77.19 ± 0.35	48.09 ± 0.08	31.70 ± 0.47	56.56 ± 0.56	4.94 ± 0.09^{a}	47.45 ± 0.57	90.60 ± 0.48^{a}
Cold pressed oils (g/kg diet)[3]									
0.0	11.96 ± 0.23	0.239 ± 0.003	78.23 ± 0.35	47.65 ± 0.09^{b}	31.17 ± 0.49	56.91 ± 0.55	4.66 ± 0.11	46.98 ± 0.52	89.43 ± 0.57
1 Org	11.56 ± 0.10	0.242 ± 0.002	76.64 ± 0.48	48.02 ± 0.11^{a}	31.20 ± 0.70	57.27 ± 0.76	4.84 ± 0.10	46.88 ± 0.64	90.14 ± 0.51
1 Thy	11.35 ± 0.12	0.236 ± 0.003	77.46 ± 0.92	47.86 ± 0.07^{a}	31.49 ± 0.77	57.19 ± 0.82	4.45 ± 0.12	46.31 ± 0.49	88.04 ± 0.66
0.5 Org + 0.5 Thy	11.39 ± 0.13	0.239 ± 0.003	77.51 ± 0.77	47.91 ± 0.12^{a}	31.95 ± 0.45	56.71 ± 0.52	4.76 ± 0.12	47.49 ± 0.45	89.77 ± 0.63
Probabilities									
Dietary protein	NS	NS	NS	NS	NS	NS	**	NS	**
Cold pressed oils	NS	NS	NS	*	NS	NS	NS	NS	NS
Interaction	NS	NS	NS	NS	NS	NS	NS	NS	NS

Means in the same column within each classification bearing different letters are significantly different. **(P ≤ 0.01), *(P ≤ 0.05) and NS = not significant.
[1]ESI = egg shape index; [2]USSW= unit surface shell weight
[3]Org = oregano; Thy = Thyme

Radwan et al. (2008) who showed that the use of 1% oregano to hens' feed significantly increased (by 8.23%) the percentages of fertility in comparison to those in the hens fed with control diets. This improvement can be explained by the fact that oregano**,** which has antioxidant activities, decreased malondialdehyde (MDA) formation in egg yolk and, consequently, improved the semen characteristics. The addition of 1% thyme to hens' diets significantly increased the percentages of hatchability in comparison to those of the hens fed with control diets for fertile fresh eggs. The addition of thyme to hens' diets significantly increased the percentages of fertility and hatchability of eggs compared to those of the hens fed with control diets. This improvement in hatchability in response to thyme supplementation can be explained by the effect of thyme on decreasing plasma total lipid (TL), and consequently, the lipid and oxidized compounds pass to egg (Ali et al., 2007). The results presented in the Tables 4 and 5 reveal that the dietary herbal

oil supplementation had no significant effects on the fertility percentage or hatchability percentage from total egg set or the hatchability percentage from fertile eggs due to the interaction between protein levels and herbal oils.

Egg Quality

The results presented in the Table 6 revealed that no egg quality parameters were significantly affected by dietary protein levels, except Haugh unit and albumen height. Where, increasing the dietary protein level to 22% increased the Haugh unit and albumen height in comparison to the low CP level. In this respect, the increases in CP did not improve the Haugh unit score, egg shell percentage or shell thickness (Junqueira et al., 2006; Alagawany, 2012). No significant effect was observed on all egg quality characteristics due to herbal oil supplementation, except USSW. Ali et al. (2007) found that the addition of thyme insignificantly increased the percentage of ESI, shell thickness, and shell weight compared to those in the birds fed with a free diet. The addition of 0.5% oregano in the diets of laying hen insignificantly decreased the albumen weight percentage and increased the yolk weight percentage. The addition of 1.0% thyme had a significant effect additionally; the yolk index percentage was significantly higher in the birds fed with the diet having 0.5-1.0% oregano (Radwan et al., 2008).

Additionally, Ali et al. (2007) found that the use of thyme increased the percentage of shell weight and the thickness of shell compared to those of the hens fed with a control diet. Because thyme is known as an antioxidant, it may improve the environment of the uterus (site of calcium deposition) and also increase the shell weight and shell thickness. On the other hand, Arpasova et al. (2013) suggested that no egg yolk quality parameters (egg yolk weight (g) and egg yolk index) were significantly influenced by oregano oil. In the present study, none of the studied external egg quality characteristics were significantly influenced by the combination between dietary protein traits and herbal oil supplementation in any experimental laying period.

CONCLUSION

In the present study, it was found that the quails fed with a diet containing 20 to 22% CP or supplemented with oregano or thyme oil had improved productive and reproductive performances without any detrimental impacts on the other parameters studied. It could be concluded that a diet with 22% CP and supplemented with thyme would produce the highest egg number and egg mass. Dietary supplementation of thyme alone or in combination with oregano improved the fertility percentage significantly in all experimental periods, except in the whole period (2- to 5-month-old birds).

CONFLICT OF INTEREST STATEMENT

The author has declared that no competing interest exists.

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Research article
Ερευνητικό άρθρο

The supplementation of ovine oocyte maturation medium with triiodothyronine affects the embryo development and apoptotic gene expression

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ABSTRACT: Triiodothyronine (T3) plays an essential role in different animal species' embryonic development. The present research was designed to identify the effect of triiodothyronine on the in vitro ovine embryonic development and the expression of apoptotic genes.A total of 436 immature cumulus-oocyte complexes (COCs) were cultured for 24 *h* in the oocyte maturation medium supplemented with two concentrations of T3 (T-10 and T-100 *ng/mL*) or without T3(T-0: control group). Oocyte maturation, cleavage, and blastocyst rates were assessed under an inverted microscope as crucial indicators of embryo development.The relative mRNA abundance of BCL-2-associated X protein (*BAX*) and anti-apoptotic B-cell lymphoma-2 (*BCL2*) were determined at blastocysts (day 8 after IVF on day 0)by quantitative reverse transcription PCR.The data were analyzed by logistic regression using the GLIMMIX procedure followed by Chi-Square, and one-way ANOVA tests. The higher concentration of T3(100 *ng/mL*) significantly decreased cumulus expansion and blastocyst rate compared to controls ($P<0.001$). Additionally, a significantly higher expression level of *BAX*($P<0.001$) and a dramatically lower expression level of *BCL2* ($P<0.01$) were detected in the T-100*ng/mL* group compared to the controls.However, the relative mRNA level of *BCL-2* was significantly higher in the T-10 *ng/mL* group compared to the control group ($P<0.01$).It appears that the supplementation of ovine oocyte maturation medium with T3 at high concentration (100 *ng/mL*) suppresses the ratio of blastocyst formation.

Keywords:Embryo, Gene expression,In vitro oocyte maturation,Triiodothyronine

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INTRODUCTION

One of the goals of improving in vitro maturation conditions is to achieve optimum production rate, in order to produce embryos that can be frozen or transferred (Hashimoto, 2009). Improvement of culture medium composition increases the number of blastocysts during oocyte maturation (Alsalim et al., 2018;Laskowski et al., 2017). The oocytes are entirely dependent on the biophysical conditions of the culture system(Gardner and Michelle,2003;Flood et al., 1993). In general, the in vitro culture of livestock oocyte is associated with reduced embryogenesis, potential and altered gene expression (Lane and Gardner, 2007). Several studies have documented the beneficial effects of enriching culture medium with hormones, such as growth hormones, melatonin, and leptin, which improve the efficiency of oocyte development(Arias-Alvarez et al.,2011;El-Raey et al.,2011;Pers-Kamczyc et al.,2010).In addition, it has been reported that the thyroid hormone (TH), triiodothyronine (T3), plays a key role in the embryogenesis of fish (Detlaf and Davydova, 1974) and birds (Sechman et al.,2009).In vitro studies in mammals have shown contradictory outcomes about the effects of T3 on the ovary, perhaps due to genetic variations or differences in THs doses (Komninou et al., 2016; Ashkar et al.,2010a). According to previous findings, triiodothyronine (T3) may reducethe FSH-driven aromatase activity in cumulus oophorus cells collected from infantile and adult rodents'follicles leading to a reduction in estradiol production (Cecconi et al.,1999). In another report, rodent follicles lost their ability to form an antrum when co-cultured with high doses of T3 and consequently, oocytes represented a poorer tendency to pass via meiosis beyond germinal vesicle breakdown (GVBD) (Vissenberg et al., 2015). The previous study showed that when bovine granulosa and theca cells were cultured in vitro (IVC) in media supplemented with T3 and T4, the production of steroids was increased. For instance, in the presence of luteinizing hormone and insulin, both THs could increase and rostenedione production by theca cells (Spicer et al.,2001). Several *in vivo* and *in vitro* studies have demonstrated that THs are involved in the induction and inhibition of programmed cell death through mitochondrial pathways (Shi et al., 2001; Asahara et al., 2003; Laoag-Fernandez et al., 2004). However, the effect of THs on oocyte maturation and blastocyst formation rates has not yet been established in sheep. It appears that THs are involved in the regulation of early embryogenesis and expression of various apoptotic genes;thus, it is assumed that THs may play a role in sheep embryo development. Therefore, this research aimed to evaluate the possible beneficial or detrimental effects of adding triiodothyronine to oocyte maturation medium on the ovine blastocyst formation as well as the expression of pro-apoptotic (*BAX*) and anti-apoptotic (*BCL-2*) genes.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis. MO, USA) or Gibco (Grand Island, NY, USA) unless otherwise stated. All protocols in this study were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, the University of Tabriz, with ethical code **(**IR.FVM.REC.1396.139).

Experimental design

To assess the effect of T3 on the meiosis process during IVM, the maturation medium was supplemented with exogenous triiodothyronine (Sigma cat No.709719) in concentrations of 10 or 100 *ng/mL.* COCs were randomly divided into three groups (T-0, T-10, T-100*ng/mL*).Each group was assessed for the stage of nuclear maturation after IVM. The number of blastocysts was recorded after IVF and IVC (day 8 - day 0: IVF).In addition, the expression of apoptosis-related genes was examined in the blastocysts obtained at the end of IVC period, to assess the effect of different concentrations of T3 on apoptosis.

Collection of ovine oocytes and in vitro maturation (IVM)

Ovine oocytes(*n*=450) were collected as described earlier(El-Raey et al.,2011). Initially, ovine ovaries were gathered from an abattoir and delivered to the research lab in normal saline supplemented with streptomycin (100*μg/mL*) and penicillin (100*IU/mL*) at 35-37°C within 2*h*. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles with a diameter of 2-6 mm using an18-gauge needle connected to 5-10 mL disposable syringe. HEPES-buffered tissue culture medium 199 (H-TCM199), complemented with 10%FBS, 0.2 mM sodium pyruvate, 100 µl/mL heparin and 5µg/mL gentamicin, was used for manipulation of the COCs before culture (Davachi et al., 2018). Only the oocytes with at least three layers of cumulus granulosa cells and homogeneous cytoplasm were used in three independent repeats, allocating about 50COCsper treatment (T-0, T-10 and T-100 ng/mL).After washing, the COCs were transferred to50

μLdrops of in vitro maturation medium(IVM)consisting of modified tissue culture medium (mTCM) supplemented with 10 or 100 ng/mL of T3 and 10 % FBS, 1mL glutamine, 2.5mM Na pyruvate, follicle stimulating hormone(FSH;*10μg/mL*),human menopausal gonadotropin (HMG;*0.075IU/mL*), estradiol17-β(*1μg/mL*) and,gentamycin sulfate (5 *μg/mL),*under mineral *oil* and cultured for 24 *h* under a maximum humidified atmosphere of5 % CO_2in the air, at 38.5°C.

Evaluation of oocyte maturation

The scale of cumulus cell expansion was subjectively measured by the stereomicroscope after 24*h* of IVM; the COCs were categorized as fully expanded(G1), partially expanded(G2) or not expanded(G3),as previously described by Marei et al.(2009). The nuclear maturation of oocytes was identified after aceto-orce in staining. Briefly,a total of 45oocytes were used in three independent replicates, allocating 5 oocytes per treatment(T-0, T-10 and T-100 ng/ml), and the cumulus cells were removed by gentle pipetting; denuded oocytes were fixed in fixation solution(acetic acid: ethanol; 1:3; v/v) at room temperature for 24 *h*. Then, the samples were put on a slide and covered with a square veneer of four drops of a paraffin-vaseline mixture (1:40).Next, the oocytes were stained with 1% aceto-orcein for 2 minutes, followed by rinsing with acetic acid,glycerol and water (1:1:3).The morphology of nucleus was observed under a stereomicroscope (LABOMED, USA).The extrusion of polar body 1(PB1) was examined under a stereomicroscope and considered as an indication of oocyte nuclear maturation for data analysis) Ni et al., 2015). Three replications were performed.

Sperm processing and *in vitro* fertilization (IVF)

Fresh semen from a ram of proven fertility was used for IVF. The motility was assessed using an inverted microscope and the motile spermatozoa were selected using the swim-up method as described by Di Francesco et al. (2011). At the end of IVM, the COCs (*n*=250)were completely denuded rom granulosa cells by gentle pipetting in H-TCM 199 consisting of 1mg/mL of hyaluronidase.Then,approximately15oocyteswereculturedin50 μL fertilization medium (Fert-TALP), containing 90mMNaCl, 25mM $NAHCO_3$, 12mM KCL,10mM sodium lactate,05mM $MgSO_4$, 0.5mM NaH_2PO_4, sodium pyruvate (0.018g/100mL), $CaCL_2$(0.147g/100mL), 3 mg/mL BSA (fatty acid free) and 50μg/mL gentamicin; 1×10^6 spermatozoa/mLwas added to the fertilization droplet. The gametes were co-incubated for 18 *h*at 38.5°C under 5% CO_2 in humidified air (Davachi et al., 2018).

In vitro culture

Eighteen hours after IVF, approximately 20presumptive zygotes per treatments were rinsed three times in culture medium and transferred to 50μL of synthetic oviductal fluid (SOF) droplets complemented with 2% basal medium Eagle's essential amino acids, 1% minimum essential medium nonessential amino acids, 1mmol/L glutamine, and 6 mg/mL bovine serum albumin (BSA) in a dish, coated with paraffin oil and incubated till day 8(day 0: IVF) at 38.5°C in a humidified atmosphere of 5% CO_2.The culture medium was renewed every 48 *h*. Cleavage was determined after 48*h*of cultivation, and the percentages of morula and blastocyst were estimated on days 4 and 8 (day 0 = IVF), respectively.Five replications were performed.

Quantitative reverse transcription PCR

The relative expression levels of *BAX a*nd *BCL-2* in blastocysts were assessed using quantitative reverse transcription PCR. Total RNA was extracted from pools of blastocysts(a total of 45 blastocysts were used in three independent replicates, allocating 5blastocysts per treatment)in each group utilizing Trizol reagent (Invitrogen, USA) and dissolved in 20 μl of RNase free water. The concentration and quality of the RNA were assessed by spectrophotometer (NanoDrop, USA). Briefly, DNase treated RNA was converted to cDNA using solvents and Takara guidance. The RT-qPCR solution was achieved in 20μL reaction with SYBR Green Mix (Takara, Japan) by Rotor-Gene 6000 RT-PCR (Corbett Research, Sydney, Australia). Primer sequences and average sizes of the amplified fragments are available in Table 1. The melt curve test was conducted during each run to test the existence of non-specific PCR products and primer dimers. Standard efficiency curves for each primer pair were calculated using a five 10-fold dilution sequence of positive control cDNA as a reference. The efficacy of the assays (E) was ≥95% and the standard curve R2 was ≥0.999. The relative expression levels of *BAX* and *BCL-2*were normalized to the endogenous normalizer (GAPDH), and $2^{-\Delta\Delta Ct}$ formula was applied for relative quantification (Livak andSchmittgen, 2001)

Table 1. Details of the primary sequences, the gene bank accession numbers, and the expected product size of the genes used for quantitative RT-PCR.

Gene	Sequence 5′→3′	Annealing Temperature (°C)	Amplicon Length (bp)	Accession No.
BAX	F:5-TGCAGAGGATGATCGCAGCTGTG-3′ R:5′-CCAATGTCCAGCCCATGATGGTC-3′	60	198	NM_173894
BCL-2	F:5′- ATGTGTTGGAGAGCGTCA-3′ R:5′-AGAGACAGCCAGGAGAAATC-3′	60	182	NM_001166486.1
GAPDH	F: 5′-CCTGAGACAAGATGGTGAAGGT-3′ R: 5′-ATGGGTGGAATCATACTGGAAC-3′	60	164	NM_001190390

F: forward; R: reverse; apoptotic *BAX*, anti-apoptotic *BCL-2,* and *GAPDH:* glyceraldehyde-3-phosphate dehydrogenase

Table 2. Effect of different concentration of Triiodothyronine on cumulus expansion in ovine COCs (three replicates)

Groups	Total COCs	%(N) of oocytes with cumulus cells		
		G1 %(n)	G2 %(n)	G3 %(n)
T-0 ng/mL	150	72%(108)[a]	21.3%(32)[a]	6.67%(10)[a]
T-10ng/mL	150	65.33% (98)[a]	16.67%(25)[a]	18%(27)[b]
T-100ng/mL	150	36%(54)[b]	34%(51)[b]	30%(45)[c]
df		2	2	2
Chi-Square		45.38	12.99	29.12
P-value		<.0001	0.0015	<.0001

[a, b, c] Different superscripts within the same column indicate a significant difference (P<0.001). T: Triiodothyronine, n; number; G1: Full expansion, G2: Partial expansion and G3: Without expansion (logistic regression; The GENMOD Procedure followed by chi-square test).

Table 3. Effect of Triiodothyronine during IVM on ovine oocyte maturation, cleavage and blastocyst rate.

Treatment	Total Oocytes	Cleavage, (n)		Morula, (n)		Blastocysts, (n)	
T-0	149	88.64±6.02[a]	(132)	60.41±5.35[a]	(90)	40.92±8.50[a]	(61)
T-10	145	87.59±3.93[a]	(127)	68.28±5.66[b]	(99)	42.07±7.48[a]	(61)
T-100	142	48.69±7.54[b]	(69)	31.67±5.41[c]	(45)	22.51±3.81[b]	(32)
P-value		*P*<0.001		*P*<0.001		*P*<0.01	

Data are presented as absolute values (mean of percentage±standard deviation) (five replicates).[a,b,c]Different superscripts letters indicate significant differences among treated groups. One-way ANOVA and Duncan's multiple range tests for post hoc were used to test the differences between the treatments.

Statistical analysis

Normally distributed variables were tested using student´s t-test. If data could not be normalized on log transformation, the Mann-Whitney test was used. Logistic regression modelsusing the GLIMMIXprocedure followed by Chi-Square and one-way ANOVA tests were used for categorical variables (SAS version 9.2, Inc., Cary, NC, USA).Significance was set at 0.05 in all cases (P<0.05).

RESULTS

Effects of exogenous T3 on IVM

The highest full cumulus expansion in COCs at the end of IVM was observed in the control group (T-0) (Table2). No significant differences were noticed between the 10*ng/mL*T3 and the control group (Table2). However, the highest concentration (100*ng/mL*) of T3 significantly reduced the proportion of COCs with the fully expanded cumulus cells compared to T-0 and T-10 groups (P<0.001).At the end of IVM, the percentage of oocyte in the metaphase II (MII,based on extrusion of polar body 1)was higher inthe control (62.41 %) compared to 100*ng/mL* of T3 group(41.54%, P<0.0004). The addition of 100*ng/mL* T3 to the maturation medium substantially decreased (P<0.0004) the rate of nuclear maturation to MII compared to the addition of 10*ng/mL* of T3(41.54% vs 67.58 %, respectively, P<0.0004). Nevertheless, the rate of nuclear maturation of oocytes under the IVM medium with 10*ng/mL*T3 was not significantly different from the control group (67.58%vs.62.41, respectively).

Effects of exogenous T3 on blastocyst development and quality

The results of cleavage and blastocyst formation are summarized inTable 3. Our findings showed that the addition of the high concentration (100*ng/mL*) of T3 to IVM medium dramatically decreased the cleavage rate compared with that of theT-10and control groups. The proportion of cleaved embryos developed to the morula stage at day 4 (day 0 - IVF) in the T-100*ng/*mL group was lower ($P< 0.001$) than the control and T-10*ng/mL* groups. The highest rate of blastocyst formation at day 8was recorded in the T-10*ng/mL*group. However, no statistically significant differences in the mean cleavage and blastocyst formation rate were observed between the T-10*ng/mL*and control embryos (Table3).

Effect of exogenous T3 on apoptotic genes expression in blastocysts

A significantly higher expression level of *BAX* ($P<0.001$) and a dramatically lower expression level of *BCL2* ($P< 0.01$) were recorded in the T-100*ng/mL* group compared to the control group (Figure1). Furthermore, the relative mRNA levels of *BCL2* and *BAX* were significantly higher in the T-10*ng/mL* group compared to the control ($P<0.01$, Figure1).In addition, the ratio of the *BAX: BCL2*expression in blastocysts was significantly decreased in the T-10 compared with the T-100ng/mL group (Figure 1).

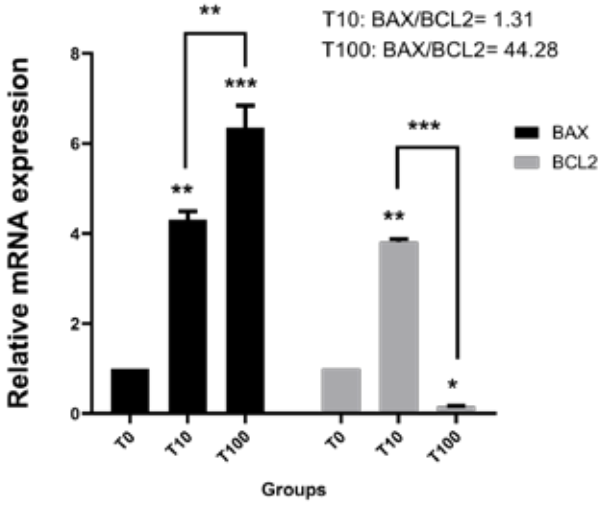

Figure 1. Effect of different concentration of Triiodothyronine (T3) on the relative mRNA expression of *BCL2* and *BAX* in ovine blastocysts. T: Triiodothyronine. Bars with star mark (*) represent groups that were different from control and between treatments group (*$P<0.05$, ** $P<0.001$;***$P<0.0001$).

DISCUSSION

Several studies reported that THs might influence ovarian metabolism and embryogenesis on mouse (Cecconi et al., 1999) and bovine (Costa et al., 2013). In this research, the effects of T3 supplementation to the IVM media on the meiosis process, expansion rate of cumulus cells, and blastocyst formation rate of ovine oocytes were investigated. Our results indicate that the treatment of ovine oocytes with 100 *ng/mL* of T3 during IVM decreases the nuclear maturation rate. The results were in line with the study of Ashkar et al. (2010b), which have noted that high concentrations of THs during bovine oocyte maturation reduced progression from MI to MII. However, when IVM media were supplemented with low concentrations of THs the percentage of MII stage oocytes was not different from that of controls.It seems that there is a cut-off point above which the effects of T3 are detrimental for embryo development.

The current research indicates that the addition of 10 *ng/mL*T3 to the IVM medium results in higher oocyte maturation, cleavage, and blastocyst rate when compared with 100*ng/mL* T3. Consistent with our results, Costa et al. (2013) revealed that the addition of 50ng/mL of T3tothe maturation medium could promote the nuclear maturation and fertilization rate of bovine oocytes.It has been indicated that T3 might foster oocyte maturation by transcribing and accumulating certain mRNA molecules (Regassa et al.,2011). Therefore, TH scan promote transcription after the maturation phase and may enhance the efficacy and quality of the rainbow trout embryonic development(Raine et al.,2004).

In contrast, Cecconi et al.(1999)stated that supplementation with THs had no beneficial effects on murine embryonic development. THs treatment could probably impose an adverse effect on follicle cells or oocyte metabolism by a decline in aromatase activity, inability of follicles to progress through the antral stage, or delayed meiotic process following GVBD(Cecconi et al.,1999; Cecconi et al., 2004).Although these results may be species-specific, dose-dependent effects of THs supplementation on blastocyst formation and hatching rate of bovine oocytes have been reported (Ashkar et al., 2010b)as well as in our study.

Ashkar et al. (2010b) reported that the supplementation of IVM medium with different concentrations of T3 or a combination of T3 and T4 in bovine did not influence cleavage and blastocyst rates.In the present study, there was no significant difference between the

supplementation of ovine oocytes maturation medium with low-dose (10 ng/ml) of T3 and the control group. Although the low-dose (10 *ng/mL*) supplementation improved blastocyst formation rate, this improvement was not significant. In line with our results,Ashkar et al.(2010a) indicated that T3 supplementation at the dose of 50 *ng/mL*enhanced the blastocyst formation rate in dairy cattle.It has been demonstrated that the apparent improvement in embryonic development might be in part related to up-regulatory effects of THs on certain responsive genes involved in cellular proliferation, improvement in mitochondrial function and adequate production of energy (Ashkar et al.,2010b;Cecconi et al.,2004). However, we observed that the addition of 100*ng/mL* of T3 during IVM was harmful to oocyte development and exerted an inhibitory effect on maturation, cleavage and blastocyst rate *in vitro*, indicating that the effects of maturation medium supplementation with T3 could be species-specific and dose-dependent.

In contrast with our result, Ashkaret al.(2010b) demonstrated that the addition of 100 *nM*of T3 to the maturation medium increased the rate of bovine blastocysts on the eighth day of culture. It has been shown that higher THs concentrations in IVM medium were more effective at inducing progesterone and and rostenedione production in cultured bovine follicle cells(Spicer et al.,2001).We agree with Ashkar et al., (2010b) who reported that it is difficult to make direct comparisons between studies, because these findings might be species-specific and the effects of THs might be dose dependent.

Apoptosis is a basic physiological mechanism and plays an important role in renewing the normal cells and eliminating the abnormal cells for the multicellular organism. *BCL2*family proteins are involved in the modulation of apoptosis and expected to combine signals from survival-inducing and death-promoting mechanisms(Burlacu,2003).It has been reported that, THs inhibit apoptosis and granulosa cells' death through mitochondrial pathways (Asahara et al.,2003). To elucidate the molecular mechanisms of THs mediated improvement in blastocyst quality, we analyzed the mRNA expression of *BAX* and *BCL2* genes in blastocyst stage of embryos.The results of the present study clearly showed that the addition of 10 ng/mL of T3 to maturation medium increased the expression of anti-apoptosis related genes (*BCL-2)* in the blastocysts.Feugang et al. (2011)indicated that anti-apoptotic genes (*BCL2-like1*) are important for the survival of embryos and *BCL2-like1*/*BAX* ratios were always in the direction of *BCL-2 like1* transcripts, which is favorable to embryonic survival. Our results revealed that the relative expression level of *BAX* was significantly higher in the T-100 *ng/mL*group compared to the T-10*ng/mL*.Moreover, a decrease in *BAX*/*BCL2*ratio was observed in the T-10 treatment compared to T-100*ng/mL*.It was also noticed that the expression of *BAX*was lower in the T-10 group than in the T-100*ng/mL* group, and the ratio of*BAX to BCL-2*transcripts was up-regulated in T-100*ng/mL* group. Rao et al., (2012) demonstrated that the ratio of *BAX* to *BCL2*might be used to measure the tendency of oocytes and embryos towards either survival or apoptosis,and it might also be associated with the ability of oocytes to complete nuclear maturation as reported by Filali et al.,(2009).In other words, the addition of high doses ofT3tothe IVM medium led to a significantly lower rate of blastocyst formation and promoted ovine blastocyst cell apoptosis.

CONCLUSION

In general, the addition of thyroid hormone to the IVM medium had controversial effects on the development of ovine oocytes. The lower dose of T3 (10 *ng/mL*) could reduce the rate of apoptosis in blastocysts; whereas the higher dose (100 *ng/mL*) imposed detrimental effects.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Serological and immuno-histopathological detection of *Paramphistomum epiclitum* infection in large ruminant population in Punjab, Pakistan

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ABSTRACT: Recent molecular identification of *Paramphistomum epiclitum* in Pakistan raises concerns about its epidemiology and pathologies in infected tissues of ruminants. The present study aimed to find the seroepidemiological and histopathological record of *Paramphistomum epiclitum* from cattle and buffaloes. Indirect ELISA on animal sera and histology of infected rumen with hematological and biochemical analyses were performed. The overall prevalence of *P. epiclitum* was noted as 15.3% in the abattoir survey and 37.6% in the serological examination. The sensitivity and specificity of the diagnostic test were 100% and 83.3% respectively. The paramphistomosis was significantly (p= 0.001) higher during August (6.4%) followed by September (5.4%), whereas the lowest prevalence was recorded during April (0.4%). The hematological and biochemical variations showed significant increase in total leukocyte count (p= 0.002), alanine aminotransferase (p= 0.05), glucose (p= 0.01) and cholesterol (p = 0.024) levels. However, significant decrease in the level of erythrocyte (p= 0.019), hemoglobin (p= 0.001), mean corpuscular hemoglobin concentration (p= 0.05), mean corpuscular volume (p= 0.038) and platelets count (p= 0.048) was observed. The histopathology of rumen tissue showed haemorrhages, atrophy of ruminal papillae, sloughed mucosa, cellular vacuolation, and infiltration of lymphocytes inflammatory cells. The present study provides the prevalence and histopathological record of *P. epiclitum* in Pakistan for the first time in order to take control measures in the country.

Keywords: Indirect ELISA; histopathology; prevalence; *P. epiclitum*; Pakistan

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INTRODUCTION

Paramphistomum digenean trematodes belonged to Paramphistomatidae family are the most prominent parasites, infecting rumen, and reticulum of livestock (Mogdy *et al.,* 2009). *Paramphistomum epiclitum* is one of the most important gastrointestinal trematodes that cause infection in ruminants with a diverse range of geographical distribution (Singh *et al.,* 2012). In the life cycle of paramphistomes, mammals and snails are the definitive and intermediate hosts respectively. Passive ingestion of metacercariae by mammals causes infection. Immature development of the fluke occurs in the small intestine while later development up to the adult stage occurs in the rumen. Paramphistomosis caused by these flatworms is responsible for heavy economic losses to several thousand crore rupees annually to the livestock industry (Ozdal *et al.,* 2010). The highest numbers accounted for in tropical and sub-tropical regions of the world (Ozdal *et al.,* 2010).

Paramphistomosis causes fatal diarrhea, weakness, dehydration, enteritis, low milk yield, sub-maxillary edema of the clinically ill hosts, and deaths (Bianchin *et al.,* 2007). The immature flukes penetrate the duodenal mucosa causes severe damage and increased mortality rate (Millar *et al.,* 2012). In the small intestine, the juvenile *Paramphistomum* causes erosions, petechiae, necrotic areas, mucus, and eosinophilic infiltrate. Due to intestinal discomfort loss of appetites occurs and some animals showed anorexia. Other symptoms are the loss of albumen, in severe cases, hypoalbuminemia occurs with oedema in several body parts. The clinical signs are mostly related to juvenile helminths; however, after two weeks of post-infection, the adults also cause problems. Mature paramphistomes are associated with ruminal papillae atrophy and ulceration at the point of attachment (Fuertes *et al.,* 2015). Heavy adult infestation rate is characterized by anemia, leucopenia, diarrhea, loss of appetite, loss of body weight, and malabsorption (Rolfe and Boray, 1993).

Immature stages of flukes are incapable to lay eggs and cause severe pathogenicity (Magdy *et al.,* 2009), while adult release eggs with faeces and does not harm the animals severely (Kelly and Henderson, 1973). Early detection methods are required for its treatment and control before irreversible damage occurs (Wang *et al.,* 2006). Immunodetection with the standard parasitological technique could play a dependable means for monitoring the infection (Anuracpreeda *et al.,* 2008).

In Pakistan prevalence of paramphistomes were 75.07% and 50.7% in buffaloes and cattle, respectively (Cheema *et al.,* 1997). These highest incidences may occur due to the canal system extensions in the country, where buffaloes and cattle were exposed to infective larvae and metacercariae of helminths (Misra *et al.,* 1997). Recently, we reported the genome and morphology of this parasite for the first time in Pakistan (Khan *et al.,* 2020). To the best of our knowledge, no study has previously been conducted on the seroepidemiology and histopathology of *Paramphistomum epiclitum* in the country. Therefore, the present study was aimed to find the epidemiological and histopathological records of *P. epiclitum* in Pakistan to reduce the impact of disease in livestock.

MATERIALS AND METHODS

Study area

This study was carried out from September 2017 to February 2019, which comprised of local abattoir of Pothwar region in north-eastern Pakistan, forming the northern part of Punjab Province (Figure. 1). This area has a semi-arid and sub-humid climate with four different seasons namely hot (April-Jun), cold (December-March), monsoon (July-September), and post-monsoon (October-November).

Collection of adult worms

Adult worms were collected from the rumen of slaughtered cattle and buffaloes. A total of 215 slaughtered animals were thoroughly examined for the collection of adult *P. epiclitum*. The worms were identified with morphological and molecular analysis (part of other reported study by Khan *et al.* (2020). The detail of animals including host type, sex, age, breed, body condition and month of infection were recorded. Worms were collected from rumen with the help of forceps to avoid any type of damage to the parasite. Collected parasites were washed several times with 0.01M phosphate buffer saline (PBS- pH-7.2) and immediately transferred to the laboratory.

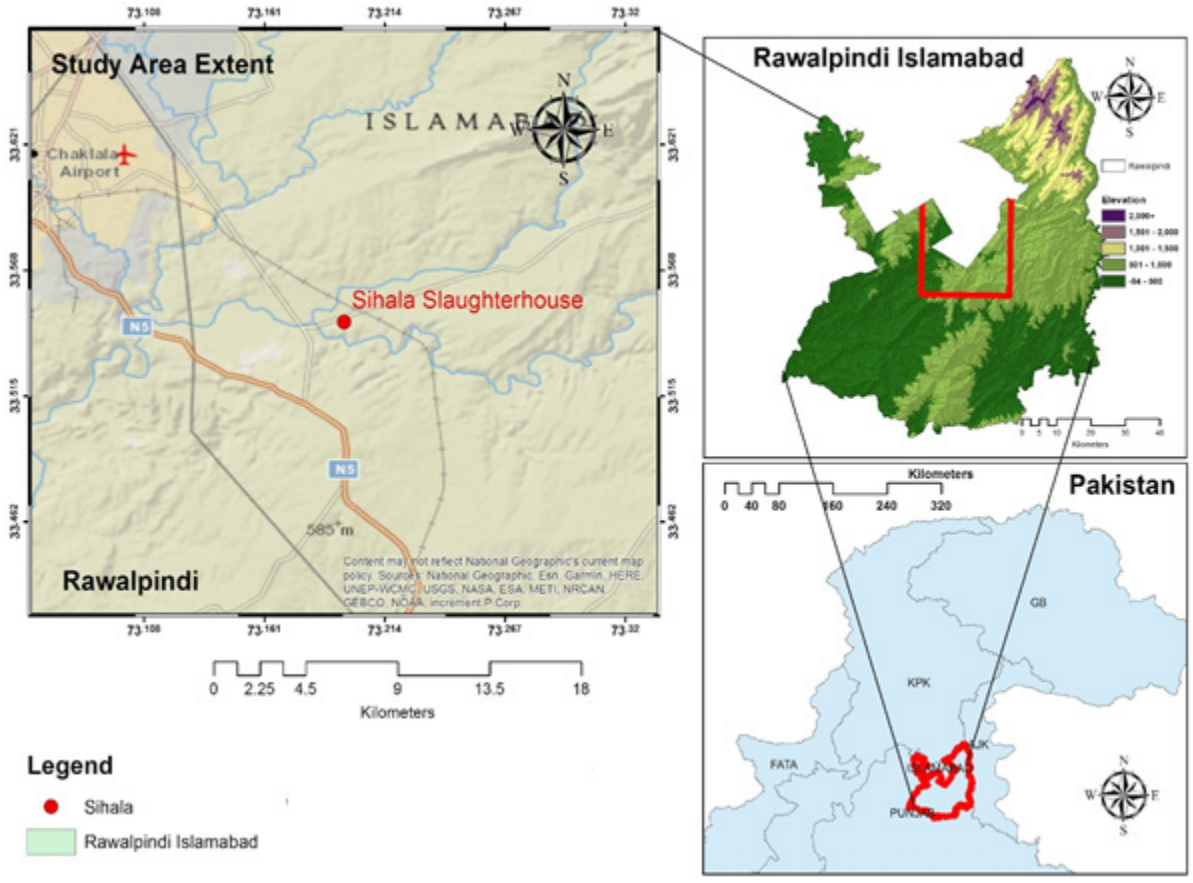

Figure 1: Study area indicating location of slaughterhouse in Rawalpindi district of Punjab Province, Pakistan.

Preparation of somatic antigen

For the extraction of somatic antigen, the parasite (whole worm) was homogenized via tissue grinder in tissue lysis buffer (50 mM HEPES- Free Acid (Roth), 150 mM Sodium Chloride (Sigma), 0.02% Sodium Azide (Sigma, 0.1% SDS (Reidel de hein), 1% Triton X-100, 0.1 mg/ml PMSF. According to the weight of the parasite, approximately 10 µL/mg of lysis buffer was added. Then the homogenate was centrifuged in an Eppendorf tube at 10000 rpm for 10 min at 4 ºC. The supernatant was taken, and the pellet was discarded. Bradford's method (Bradford, 1979) was applied to determine protein concentration and was stored at - 20 ºC until used.

Blood sample collection

A total of 500 blood samples were randomly collected from farm animals to find out the seroepidemiology of paramphistomosis in cattle and buffaloes. The sera were separated and stored at - 20 ºC until used. For establishment of in-house ELISA, control sera were collected from confirmed positive animal with *P. epiclitum* (n= 27), *Fasciola hepatica* (n= 10), *Gigantocotyle explanatum* (n= 10) and from negative control (n= 30) obtained from 2-week-old kids born to herd having history of stall feeding.

Indirect ELISA

ELISA was performed on a polystyrene microtiter plate having 96 wells with previously determined all incubation time by checkerboard titration and followed the method described by Faghemi *et al.* (1997) and Ferre *et al.(*1997). Briefly, the antigen was diluted in coating buffer in (1:1), coated (200 µl) in each well of a microtiter plate, and kept at 4 ºC overnight. The next day, the plate was washed 3 times with 0.01 M PBS Tween-20. The plate was filled with (200 µL) blocking solution (10 ml PBS+ T-20 (0.02% + 0.2g BSA (2%) and incubated at room temperature for 2 hours. Then the plate was washed 3 times with 0.01M PBS, Tween-20. Each well of microtiter plate was treated with 100 µL of diluted primary antibodies (infected bovine serum) and incubated for 2 hours at room temperature. Again, the washing was done 3

times followed by the addition of 100 µL secondary antibodies (Goat Anti-Bovine IgG (H+L) Antibodies) diluted 1:5000 to each well, incubated for 120 minutes at room temperature. Then the plate was washed 3 times with 0.01M PBS Tween-20 and to each well, 100 µL of PNP (para-Nitrophenyle Phosphate) substrate was added and incubated at room temperature for 15-30 minutes in dark. The reaction was stopped by adding 50 µL stopping solution (2M H_2SO_4) to each well. Results were analyzed by ELISA reader at OD value 405 nm.

Hematological and Biochemical Analysis

The blood (2 ml) was directly taken from the external jugular vein of cattle and buffaloes through a syringe in EDTA coated vacutainers and stored at 4 ºC till analyzed through an auto hematology analyzer (URIT - 2900Vet Plus, China). The complete blood profile i.e. red blood cells count (RBCs), total leucocytes count (TLCs), platelets count (PLT), hemoglobin concentration (HGB), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and hematocrit (HCT) level was measured. For biochemical analysis, sera were analyzed for liver function tests, total cholesterol, and triglycerides concentrations through a semi-auto-chemistry analyzer. The activities of alanine aminotransferase (ALT) and glucose were measured at 37 ºC and the results were presented in respective units.

Histological Studies

A portion of rumen tissues from infected animals was separated and preserved in 10% (w/v) formaldehyde at room temperature for histological study. For histopathology, preserved rumen tissues were fixed overnight in 10% buffered formalin, dehydrated in different alcoholic grades and then placed in xylene with two changes and transferred to molten paraffin wax preheated to 59 ºC and kept overnight. Second wax infiltration was done, and tissues were finally embedded in paraffin wax. After removing bubbles from wax, solidify, trimmed and mounted on wooden blocks for section cutting. Albumin coated slides were prepared and stained with haematoxylin and eosin (H&E) following standard histopathological procedures (Stevens, 1996). Sections were photographed on a compound photomicroscope (Leica, Germany).

Statistical Analysis

The data on the prevalence of *P. epiclitum* infected cattle and buffaloes with respect to age, sex, breed, and months were calculated by using the chi-square test. The hematological and biochemical data were analysed by t-test and presented with mean ± S.E values. The level of significance was set at $p < 0.05$. The O.D value of the serum sample was observed at 405 nm and compared with the cut-off value. The cut-off was calculated by the mean optical density (OD) of the negative reference serum, plus three times standard deviations (0.11+3*0.01=0.14). The cut-off value was set at 0.14, and sera with OD value higher or equal to 0.14 consider as positive. The sensitivity (a / a + b) and specificity (d / c + d) were calculated, where (a) is the number of cases in the diseased group that test positive and negative (*b*); and the number of cases in the non-diseased group that test positive (*c*) and negative (*d*).

RESULTS

Prevalence of *P. epiclitum*

The sensitivity and specificity of the diagnostic test for *P. epiclitum* were 100% and 83.3%, respectively. The kappa value calculated for the test was 0.81 and revealed that the strength of agreement was 'perfect' (Table 1). The value of R^2 calculated from the linear regression equation was 0.96 and the protein concentration of somatic antigens was determined (Figure. 2). The OD value of control sera used in the validation of ELISA is given in Figure 3.

The overall prevalence of *P. epiclitum* was noted as 15.3% in the abattoir survey and 37.6% in serological examination (Table 2). In the abattoir survey, the highest rate of infection was found 12.6% in females, while in the seroprevalence method male had a higher infection (24.6%), while statistically non-significant ($p > 0.05$). Higher prevalence of infection was noted 6% in 5-7 year of the old age group in the abattoir survey, and the serologically highest rate of infection was 13.6% in 8-10 years age group, while statistically not significant ($p > 0.05$). The animals with semi-extensive grazing habit showed a 7% infection rate in the abattoir survey, while ELISA was 15.6% in animals with intensive grazing habit. However, the difference was not significantly ($p > 0.05$) associated with the infection. The abattoir survey revealed a significantly ($p = 0.001$) higher infection rate of 11.2% in animals with poor body condition as compared to healthy animals. Similarly, a higher seroprevalence rate was noted 22.2% in poor body condition animals, while not significantly ($p > 0.05$) different. Among

breeds paramphistomosis based on abattoir survey was 5.6% in Kundhi fallowed by Nili Ravi 4.7%, Azi Kheli 2.8% breed of buffaloes, while 1.4% in Sahiwal breed of cattle. The seroprevalence of *P. epiclitum* was 11.2% in Kundhi and 7.2% in Sahiwal breed of buffalo and cattle respectively. However, the breed did not show significant ($p > 0.05$) association with disease. The infection was found higher in buffaloes (11.6%, 25.8%) as compared to cattle (3.7%, 11.8%) with abattoir and serological survey, respectively; however, the difference was not significant ($p > 0.05$). The animals having contact with pond water showed a high prevalence (6%, 12.6%), but the association was not significant ($p > 0.05$). The paramphistomosis was significantly ($p = 0.001$) higher during August (6.4%) followed by September (5.4%), whereas the lowest prevalence was recorded during April at 0.4%.

Table 1: Validation parameters of indirect ELISA developed for identification of specific antibodies against *Paramphistomum epiclitum.* Isolation of adult flukes from rumen of slaughtered animals was considered as gold standard.

Parameters	Results	Kappa correlation index
Number of tested sera	500	
Number of truly positive sera	27	Kappa = 0.811 almost perfect agreement
Number of truly negative sera	30	SE of kappa = 0.072
Number of false-positive	6	95% confidence interval:
Number of false negatives	0	From 0.669 to 0.952
Cut-off	0.14	
Sensitivity (%)	100	
Specificity (%)	83.3	
*Accuracy (%)	89.6	
*Positive Predictive Value (%)	52.01	
*Negative Predictive Value (%)	100	
*Disease prevalence (%)	15.3	

*These values are dependent on disease prevalence.
Positive predictive value = Sensitivity x Prevalence / Sensitivity x Prevalence + (1- Specificity) x (1- Prevalence)
Negative predictive value = Specificity x (1 − Prevalence)/ (1-Sensitivity) x Prevalence + Specificity x (1- Prevalence)
Accuracy = Sensitivity × Prevalence + Specificity × (1 − Prevalence)+

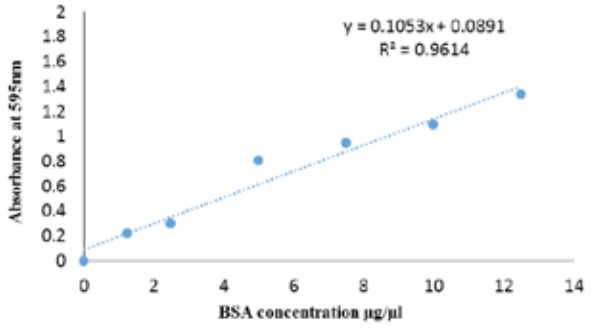

Figure 2: Standard protein curve to determine the protein concentration of antigen used in establishment of indirect ELISA.

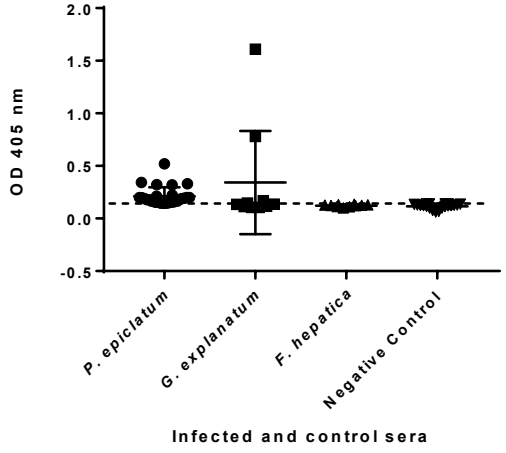

Figure 3: The optical density values (OD) of positive and control sera for identification of specific antibodies against *Paramphistomum epiclitum.* The cut-off value was set at 0.14.

Table 2: Prevalence of *Paramphistomum epiclitum* based on postmortem examination and indirect ELISA in Pakistan.

	Abattoir Survey				Seroprevalence				
Variable	**No. examined**	**Positive n (%)**	**χ^2**	**p-value**	**No. examined**	**Positive n (%)**	**OD Mean ± SD (Min-Max)**	**χ^2**	**p-value**
Gender									
Male	60	6(2.8)	1.83	0.176	315	123(24.6)	0.24±0.14 (0.14-0.72)	0.76	0.383
Female	155	27(12.6)			185	65(13.0)	0.23±0.13 (0.14-0.80)		
Age									
2-4 year	33	3(1.4)	2.93	0.403	84	36(7.2)	0.23±0.13 (0.14-0.58)	1.44	0.696
5-7 year	67	13(6.0)			115	44(8.8)	0.23±0.14 (0.14-0.80)		
8-10 year	80	10(4.7)			187	68(13.6)	0.22±0.13(0.14-0.72)		
11-12 year	35	7(3.3)			114	40(8.0)	0.24±0.15 (0.14-0.60)		
Grazing Habitat									
Intensive	70	11(5.1)	5.54	0.063	202	78(15.6)	0.22±0.12 (0.14-0.58)	0.34	0.846
Extensive	22	7(3.3)			162	58(11.6)	0.26±0.17 (0.14-0.80)		
Semi-Extensive	123	15(7.0)			136	52(10.4)	0.21±0.11 (0.14-0.58)		
Body Condition									
Poor	65	24(11.2)	33.38	0.001**	291	111(22.2)	0.21±0.11 (0.14-0.59)	0.09	0.767
Good	150	9(4.2)			209	77(15.4)	0.25±0.16 (0.14-0.80)		
Breed									
Kundhi	70	12(5.6)	5.37	0.251	153	56(11.2)	0.23±0.12 (0.14-0.58)	3.66	0.453
Azi Kheli	66	6(2.8)			112	47(9.4)			
Nili Ravi	40	10(4.7)			69	30(6.0)			
Sahiwal	20	3(1.4)			104	36(7.2)			
Dhani	19	2(0.9)			62	19(3.8)			
Host Type									
Buffalo	148	25(11.6)	0.78	0.351	331	129(25.8)			
Cattle	67	8(3.7)			169	59(11.8)			
Water Bodies									
Canal	40	6(2.8)	3.92	0.270	132				
Reservoir	55	5(2.3)							
Pond	58	13(6.0)							
All Type	62	9(4.2)							
Month	na	na	na		na				
April						2(0.4)	0.17±0.16 (0.16-0.18)	74.47	0.001**
May						16(3.2)	0.18±0.06 (0.14-0.31)		
Jun						12(2.4)	0.21±0.13 (0.14-0.59)		
July						26(5.2)	0.24±0.16 (0.14-0.72)		
August						32(6.4)	0.30±0.18 (0.14-0.80)		
September						27(5.4)	0.22±11 (0.14-0.52)		
October						5(1.0)	0.20±0.06 (0.14-0.20)		
November						25(5.0)	0.19±0.08 (0.14-0.47)		
December						22(4.4)	0.27±0.15 (0.14-0.65)		
January						21(4.2)	0.19±0.10 (0.14-0.51)		

**Significant (p <0.05); na data not calculated

Table 3: Hematological and biochemical analysis of *Paramphistomum epiclitum* infected cattle and buffaloes along with control groups.

Parameters	Infected (n=28) Mean ± SEM	Control (n= 10) Mean ± SEM	p-values
Hematological Analysis			
TLC/mm3	9.35 ± 0.67	7.20 ± 1.96	0.002**
TEC × 106µL	5.41 ± 0.26	6.62 ± 0.34	0.019**
HCT%	30.10 ± 1.62	33.45± 1.32	0.087^{NS}
HGB (g/ dl)	9.42 ± 0.60	11.48 ± 0.46	0.001**
MCH (pg)	15.65 ± 0.93	17.82 ± 0.55	0.05*
MCHC (g / dl)	31.00 ± 0.95	33.60 ± 0.43	0.21^{NS}
MCV (FL)	49.56 ± 1.91	53.33 ± 1.50	0.038*
PLT ×10^9/L	129.00 ± 11.98	149 ± 9.70	0.048*
Biochemical analysis			
ALT (U/I)	50.50 ± 3.06	41.12 ± 3.05	0.005**
Sugar (mg/dl)	66.75 ± 2.73	62.37 ± 2.41	0.01*
Cholesterol (mg/dl)	118.50 ± 7.6	103.87 ± 5.01	0.024*
Triglycerides (mg/dl)	126.12 ± 3.09	126.12 ± 3.09	0.42 ^{NS}

^{NS} non-significant (p >0.05), *,** Significant (p <0.05; p <0.01)

Hematological and Biochemical Analysis

Hematological parameters showed significant increase in number of TLC (p = 0.002) and decrease in the level of TEC (p = 0.019), HGB (p = 0.001), MCH (p = 0.05), MCV (p = 0.038) and PLT (p = 0.048) in infected animals than controls. Significantly increased level of ALT (p = 0.05), glucose (p = 0.01) and cholesterol (p = 0.024) was observed in infected as compared to non-infected animals (Table 3).

Histopathology of Rumen

The infected rumen tissue with *P. epiclitum* fluke microscopically showed haemorrhages and atrophy at attachment site to the ruminal papillae (Figure. 4), necrosis of epithelial papilla, and thickening of the wall and ulceration. The most prominent histopathological changes were sloughed mucosa, cellular vacuolation, submucosal edema, and disruption of the stratified epithelium with dilated lymphatics in the submucosa. Focal hyperplasia of the ruminal epithelium in different regions was also prominent and infiltration of lymphocytes inflammatory cells were observed around them. The pathogenicity was associated with the number of parasites sucking the ruminal mucosa.

DISCUSSION

Paramphistomosis is usually diagnosed by examination of parasite eggs in host feaces, but immature paramphistomes that cause pathogenicity are incapable of laying eggs. ELISA is an immunodiagnostic tool used for the detection of early infection in the host (Ahmad *et al.,* 2014). To develop a reliable and specific diagnostic tool many investigations have been performed to detect a related trematode infection in the host body (Raina *et al.,* 2006). In the present study antigen prepared from somatic extract were subjected to ELISA test for early diagnosis of paramphistomiasis. Similarly, reported that the antigen of *P. cervi* has been used to detect antibodies of paramphistomosis (Alabay, 1981; Keller, 1983). Diaz *et al.* (2006) conducted a study utilizing ELISA to analyze IgG antibody response in cattle against *Calicophoron daubneyi.*

The present study recorded the seroprevalence and abattoir-based prevalence of *P. epiclitum* in cattle and buffalo to find out the accurate status of the disease in the country. So far the reported prevalence of other species of *Paramphistomum* in Punjab was 22.29% (Javed *et al.,* 2006), and 11% in buffalo, and 7% in cattle (Raza *et al.,* 2007). In the current study, the overall prevalence of *P. epiclitum* was observed 15.3% on slaughterhouse-based investigation and 37.6% with serological technique. The present result showed a higher prevalence than other reported studies of which 11.25% in Sudan (Alkareem *et al.,* 2012), 7% in Spain (Sanchís *et al.,* 2013) and 1.99% to 3.4% in India (Shabih and Juyal, 2006). However, a higher prevalence as compared to present work was reported 53.1% in Bangladesh (Paul *et al.,* 2011) and 45.8% in Ethiopia (Yeneneh *et al.,* 2012). These differences in results could be explained with sample size, geographical region, and practicing of communal traditional grazing and examination techniques.

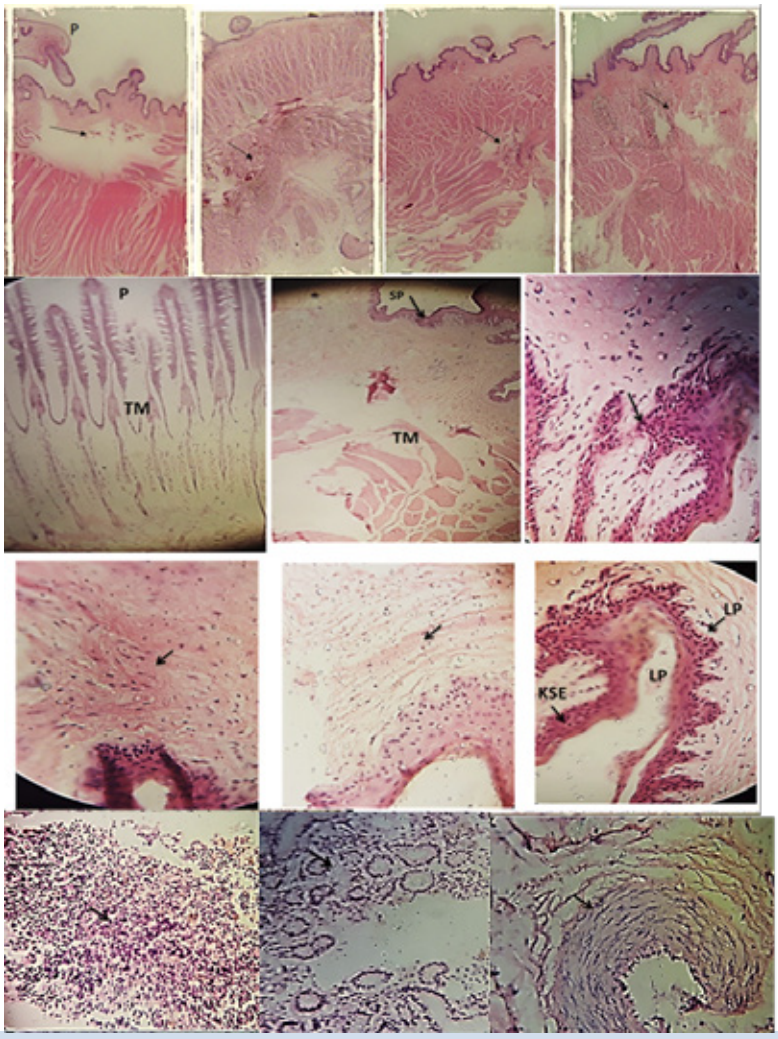

Figure 4: Histopathology of rumen tissues of buffaloes (H&E; Scale Bar = 100 μm), showing fluke(P) attached to rumen papilla, keratinized stratifies squamous epithelium (KSE) divisible into basal dark and light superficial layer, Lamina propria (LP), Long papilla (LP), short papilla (SP) and tunica muscularis (TM). Necrosis of squamous epithelial cells at the tip of the papilla and submucosal neutrophil and edema fluid accumulations and an infiltrate of a few lymphoid inflammatory cells into the epithelial layer are present. Rumen papilla of buffaloes showing infiltrates of lymphoid inflammatory cells form a dense aggregate, cellular vacuolation.

In the present abattoir survey, the prevalence of *P. epiclitum* was higher in females, in agreement with Alim *et al*. (2012). This may be due to changes in the physiological condition of female animals at the time of pregnancy, lactation, low resistance, or temporarily near parturition the loss of acquired immunity. Our findings with seroprevalence of *P. epiclitum* showed higher antibody titer in males, in agreement with Khedri *et al.* (2015). The reason is due to the social practice of females, which keep under better feeding and good management condition as compared to the male which are let loose in posture for freely grazing (Iqbal *et al.,* 2013).

The occurrence of *P. epiclitum* was found higher in old aged animals, which corroborates the observation of Iqbal *et al.* (2013). An increase in worm burden is the reason due to, exposure to grassland, high grazing, and absence of helminthic drugs (Iqbal *et al.,* 2013). This may also be explained by environmental factors, faulty management, and increased disease incidence (Javed *et al.,* 2006). The present result showed a higher infection rate in animals with semi-extensive (7%) and intensive (15.6%) grazing management, which is not in agreement with the study by Bilal *et al.* (2009). However, studies reported the chances to ingest the eggs of parasites by free-grazing animals are more due to their increased exposure to contaminated pastures (Bilal *et al.,* 2009).

The body condition of animals was recorded poor with high *P. epiclitum* infection in the current investi-

gation, consistent with Bashier (2014). This could be explained by the fact that ruminants have high protein loss and lower resistance to fluke (Bashier, 2014).). The present result of breed and host type did not significantly associate with *P. epiclitum* infection. Tehmina *et al.* (2016) recorded different results and found a significant association of breed with *Paramphistomum cervi* infection. The present result showed the highest prevalence of paramphistomosis in buffaloes as compared to cattle. Similarly, Raza *et al.* (2009) observed high incidences of infection in buffalo as compared to cattle. The reason may be due to moistening areas and water-loving habit of buffaloes, whereas intermediate host (snail) is more than grassland for transmission of the disease (Raza *et al.*, 2009). In the current investigation, no significant interaction was found between the rate of infection and water bodies. Although, it is suggested that among water bodies risk of infection is variable, generally shallow-water provides a favorable habitat.

In the current study, the month of August (6.4%) and September (5.4%) showed a higher prevalence of *P. epiclitum* agrees with other researchers. Ozdal et al. (4) recorded *Paramphistomum* spp. infection in cattle and sheep during the September (14.1%) to November (8.33%). Hassan *et al.* (2005) in domesticated ruminant documented the highest infection 8.06% in July to October and least in November to December (0.49%). Tariq *et al.* (2008) recorded the maximum intensity of infection in sheep during the late summer and drier autumn season. A study in Mexico recorded infection in cattle in early winter as animals mainly pick infection in rainy and windy summer and appeared in early autumn (Rangel-Ruiz *et al.,* 2003). Similar findings were recorded by Rolfe *et al.* (1991) in eastern Australia. Soulsby (1968) described the paramphistomosis outbreak usually occur in early winter or drier months. The results highlighted that moisture and temperature are the two main factors, affecting the hatching of parasites ova, snail's population, and accessibility of encysted cercaria which ultimately increase the incidence of paramphistomosis in animals (Soulsby, 1968).

The cattle and buffaloes infected with *P. epiclitum* had low TEC, HGB, MCH, MCV, and PLT, suggesting anemia. The increased TLC showed an inflammatory response caused by the fluke, results showed agreement with other researchers. Chauhan *et al.* (1972) recorded a significant reduction in the mean hemoglobin and total erythrocyte count in infected animals. Singh *et al.* (1984) reported decrease in TEC ($P < 0.05$), Hb ($P < 0.01$), and PCV ($P < 0.01$), while an increase in eosinophilic count ($P > 0.01$) as compared to the healthy animals was observed. Chhabra *et al*. (1972) conducted a study on amphistomes infected cross-bred calf and reported a reduction in hemoglobin. The possible cause of anemia may be due to worms, which feed on the host resulting in the depletion of nutrients and improper digestion.

The current pathology of rumen tissues showed an increase in the level of liver enzymes (ALT), glucose, and cholesterol. The possible cause of higher ALT results attributed to liver damage; could be necrosis of the liver due to toxemia from the damaged rumen mucosa (Garry, 2002). The increase in glucose level may be due to stress leading to adrenocorticosteroid release, which has the glycogenolytic effect, causing hyperglycemia. The Lipoproteins are synthesized in the liver and hepatic dysfunction may results in a disturbance in serum cholesterol levels.

The current histopathology results showed necrosis of epithelial papilla and increased infiltration of lymphocytes inflammatory cells, consistent with other researches. Rolfe *et al.* (1991) reported rumen with increased infiltration of eosinophils in the mucosa. Love & Hutchinson (2003) found adult flukes do not cause pathogenicity, although heavy fluke infestation is associated with chronic ulcerative rumenitis with atrophy of ruminal papillae. Singh *et al.* (1984) reported severe damage in the duodenal tissue caused by immature flukes, whereas the adult form inflicted mild tissue damage in the rumen.

CONCLUSION

In conclusion, this study provides the prevalence rate of *P. epiclitum* in Pakistan for the first time. The infected rumens were microscopically examined and showed damage of epithelial papilla and infiltration of lymphocytes inflammatory cells, resulting in hematological and biochemical disturbance associated with liver dysfunctions. In order to take control measures and determine precisely the prevalence of *P. epiclitum* in Pakistan, further seroepidemiological studies are required to determine the infection caused by immature flukes.

CONFLICT OF INTERESTS

The author(s) declare no potential conflicts of interest.

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Research article
Ερευνητικό άρθρο

Effects of pennyroyal (*Mentha pulegium* L.) dietary supplementation on performance, carcass quality, biochemical parameters and duodenal histomorphology of broilers

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ABSTRACT: In this study, the effects of (*Mentha pulegium* L.) dietary supplementation on performance, carcass characteristics, some biochemical parameters, and intestinal histology were investigated in broiler chickens. Four groups were formed as control and groups treated with pennyroyal at different levels (0.25%, 0.50%, and 1.00%). In the experiment. Each group had eight replicates. A total of 192 broilers were used in the study with six broilers in each replicate. Water and feed were *ad libitum* provided. Adding different levels of pennyroyals to broiler rations significantly affected performance parameters; an increase in final body weight and carcass yield and a decrease in total feed intake and the conversion rate was observed ($P<0.05$). Serum cholesterol, malondialdehyde, and glutathione values were also affected by the addition of pennyroyal ($P<0.05$). Pennyroyal was effective in vitro against S. enteritidis, E. coli, S. aureus, S. abortus ovis, B.anthracis sterne strains. Besides, it increased the duodenum villus' length compared to the control group ($P<0.05$). On the other hand, pennyroyal did not affect carcass and visceral organ weights, several serum biochemical values ($P>0.05$). As a result, it was concluded that pennyroyal at the level of 0.50% in ration was effective on health and growth performance of broilers.

***Keywords*:** Broiler; pennyroyal; performance; serum parameters; villus.

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INTRODUCTION

The essential amino acids that are necessary for the human nutrition can be found in foods of animal products (meat, milk, eggs). Most of the essential amino acids needed in the people's healthy nutrition can be met with foods of animal origin (Tuncer, 2007). Healthy and balanced nutrition are possible by covering the animal protein deficit. To bridge the gap in animal production, several studies were implemented in the late 1940s (Emborg et al., 2001; Tuncer, 2007). The effects of antibiotics that were first used in animal health for treatment and protection have been discovered concerning supporting growth and decreasing death rates in farm animals (Emborg et al., 2001; Barreto et al., 2008; Abedini et al., 2017) and have been used in mixed feed as feed additives since then (Hashemi and Davoodi, 2011; Abedini et al., 2017; Kostadinović and Lević, 2018). Research on broiler chickens also coincides with these periods (Moore et al., 1946; Dibner and Richards, 2005; Popović et al., 2019).

However, the use of antibiotics as feed additives has led to the development of antibiotic-resistant bacteria in animals. Many reports state that pathogenic bacteria have gained antibiotic resistance worldwide (Ferket, 2004). Taking antibiotics together with feed, even at low doses, causes drug residues to be left in animal products (meat, milk, eggs) and bacteria to cross immune against the antibiotics used, as well as transferring the resistance between bacteria (Özkan and Açıkgöz, 2007; Adiyaman and Ayhan, 2010; Erhan, 2015). Thus, the use of all antibiotics as feed additives is prohibited with the decision made by the European Committee in 2002 (directive 70/524 / EEC, directive 82/471 / EEC and regulation 1831/2003 / EC) (Cheli et al., 2013). Following the banning of antibiotics, research on new and alternative growth factors which will increase growth performance, reduce costs and prevent the colonization of pathogenic bacteria in animals' digestive system, protect against diseases and improve digestion have accelerated (Karasu and Öztürk, 2014; Erhan, 2015; Abedini et al., 2017). Alternatives to antibiotics in the livestock sector, especially natural yield enhancers have recently found many areas of use (Goodarzi and Nanekarani, 2014; Karasu and Öztürk, 2014). These substances mainly are probiotics, prebiotics, enzymes, and medical aromatic plants (Yörük and Bolat, 2003; Yörük et al., 2008; Christaki et al., 2012; Vuong et al., 2016; Şahin et al., 2020). These substances have been used as drugs, food, and incense since ancient times and according to WHO, there are around 70.000 species in the world, and Turkey's vegetation is composed of 12.000 species and subspecies of the genus in 1251 from 174 families. The use of these substances as feed additives have brought the Medicinal and Aromatic Plants (MAP) forward (Kirici, 2015). These plants have gained importance as alternative feed additives in food animal nutrition, particularly poultry, due to their natural characteristics that do not harm the animal and human health (Çetin, 2016; Ghaly et al., 2017). Studies have shown that MAP increases feed conversion, body weight gain and protect intestinal health (Duru and Şahin, 2015; Erhan, 2015). One of the most widely used plants is Mentha from the Labiatae (*Lamiaceae*) family with 20 different subspecies. One of the most well-known types is Pennyroyal (*Mentha pulegium* L.) (Erhan et al., 2012; Goodarzi and Nanekarani, 2014; Abedini et al., 2017). It is also known by different names such as squaw mint, Mosquito plant, and Pudding grass. It has been determined that the plant spreads from the Mediterranean region to Iran, and its appearance, leaf shape, and hairiness vary as it has many varieties (Davis, 1982). In Turkey, it has been recognized as one of the most easily distinguished species of mint with its morphological structure and its peculiar smell. The chemical structure of pennyroyals includes terpenes and phenolic compounds. Due to these compounds, the plant has antioxidant and antimicrobial effects (Erhan and Ürüşan, 2015). Also, when compared with antibiotics and probiotics, (*Mentha pulegium* L.) was determined to have a positive effect on growth performance and feed conversion values and that it could support growth as an alternative to antibiotics (Abedini et al., 2017).

The purpose of this study was to examine the effects of the aromatic herb pennyroyal that can be obtained naturally and in an inexpensive way on body weight, body weight increase, feed intake, feed conversion rate, carcass traits, some internal organ weight, serum biochemical and oxidative stress parameters and intestinal histology as a growth factor added to the broiler rations at different levels.

MATERIAL AND METHODS

Ethical approval

The experimental protocol and animal care in this study were approved by animal experiments from the local ethics committee (KAÜ-HADYEK/2016-136) of Kafkas University.

Pennyroyal

Pennyroyal (*Mentha pulegium* L.), used as a feed additive in the study was collected in July 2016, from the Kars province (40 ◦ 48'21.2 "N 42 ◦ 53'37.8" E / Google Earth) in Turkey. Botanists performed the species determination of the pennyroyal. The essential oil was obtained by water vapor distillation from the material obtained after drying and grinding the pennyroyal. The essential oil composition of the pennyroyal was determined by the gas chromatography method. Each compound's structure was defined using mass spectra with the Xcalibur program (Wiley 9) (Table 1).

Table 1. Bioactive compounds of Pennyroyal essential oil.

Compounds	Retention time (RT)	International code (Cas)	%
Linalool	21.10	78-70-6	13.61
p-Menthone	24.01	89-80-5	10.56
Terpinen-4-ol	24.71	562-74-3	0.28
p-Menthan	25.36	89-80-5	6.19
Levomenthol	25.53	89-78-1	0.20
α-Terpineol	27.53	470-08-6	0.13
Isopulegone	28.82	29606-79-9	0.11
Pulegone	29.39	89-82-7	4.45
Piperitenone oxide	33.38	35178-55-3	3.07
Thymol	39.36	499-75-2	0.49

Table 2. Nutrient composition and chemical analysis of rations

Ingredients, %	Starter	Grower	Finisher
Corn	55.45	62.60	69.95
Soybean meal (44%)	22.50	10.65	5.00
Corn gluten (60%)	16.25	20.55	20.60
Marble dust	2.38	2.40	1.10
DCP (dicalcium phosphate)	1.93	2.13	1.78
Salt	0.25	0.25	0.23
Vit (K_3A)	0.15	0.15	0.15
Soda	0.10	0.10	0.08
Vit E	0.63	0.65	0.63
Lysine	0.33	0.48	0.50
Methionine	0.05	0.05	-
Chemical Analysis			
ME, Kcal/kg	3025	3150	3210
Dry matter %	89.20	87.90	87.7
Crude protein %	23.80	21.20	20.4
Lysine %	1.20	1.20	1.10
Methionine %	0.66	0.50	0.49

Animals and experimental design

One hundred and ninety-two Ross-308 broiler chicks were used in the study. Control and experimental groups were created by allocating 48 chicks to each group homogeneously. Each group consisted of eight replicates with six chicks. While the broilers in the control group were fed only with mixed feed, pennyroyal was added at the rate of 0.25%, 0.50%, 1.00% in the other experimental groups. Feed and water were *ad libitum* offered. In the experiment, after all the broilers were allotted into groups during the acclimatization period (1 week), they were fed with a starter diet (I) for a week, then fed with a grower diet (II) between the 15th and 28th days, and finally with a finisher diet (III) between the 29th and 42nd day. The nutrient content and chemical analysis results of the feeds are shown in Table 2. All the chicks in the experiment groups excluding those in the control group were provided feed mixed with pennyroyal from the 7th day. The pennyroyal, dried, and powdered was added to the diets by mixing through manual methods. The coop heating was provided with radiators, and electric radiants were used when needed.

The ambient temperature was kept at 32-33˚C for

the first two days, while it was decreased by 1-2°C every week starting on the third day and set to 22-23°C which was maintained until the end of the study. Lighting was provided in the coop 23 hours a day. Wood shavings were used as the bedding. The broilers were observed daily during the study period, mortality was recorded, and their survival rate was determined at the end of the study.

Performance

All broilers were weighed weekly on days 7, 14, 28, 35 and 42 and body weights (BW) were recorded. The daily body weight gain (DBWG) of the broilers were calculated by calculating the difference of total weights between two weighings and dividing by the number of days and broilers. Feed intake (FI) was measured together with weekly weighings, and the daily average feed intake was determined by dividing the weekly average feed intake (AFI) by the number of broilers and days. Using the daily feed intake and daily body weight gain values of the broilers in the subgroups, the feed conversion rate (FCR) was calculated.

Sampling and analysis

The broilers were fasted the day before the end of the study and ten broilers from each group were selected (near to mean weight) for slaughtering. Serums extracted from blood drawn during slaughter were kept at +4°C until the day of analysis. The broilers were sacrificed by cervical dislocation method and their blood was shed, and their internal organs were removed and weighed on a sensitive scale. After removing the internal organs, hot and cold carcass (after kept at -20°C for 24 hours) weighings were made.

After the serums were thawed, some biochemical (cholesterol, HDL, LDL, VLDL, triglyceride, glucose, total protein, albumin, globulin, uric acid) parameters were determined by a spectrophotometric device using commercial ELISA kits (Erba Mannheim-Germany). Malondialdehyde (MDA) from antioxidant parameters was analyzed according to Placer et al. (1966) and glutathione (GSH) was analyzed according to Sedlak and Lindsay (1968).

The antimicrobial effect of the essential oil obtained from the pennyroyal was determined in vitro, and minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were calculated again by spectrophotometric reading.

The measurements of the small intestine duodenum taken during the slaughtering process were examined and photographed under the imaging microscope after detection, blocking, cross-sectioning, and staining (Hematoxylin-Eosin), and villus measurements were performed using the ImageJ (1.46r-Wayne Rasband National Institutes of Health, USA) program.

Statistical analysis

SPSS 18 (IBM SPSS, Chicago, IL) program was used for statistical analyses. Results are presented as means and standard errors (±). A one-way variance (ANOVA) analysis was used to determine the differences between the groups in performance, slaughter and carcass characteristics, serum biochemical measurements, oxidative stress parameters, and histological villus lengths. A Duncan multiple comparison test was used to check the significance of the differences. The significance of the factors in the statistical results was tested at the level of $P<0.05$.

RESULTS

The survival rate of the control and pennyroyal supplemented (0.25%, 0.50%, 1.00%) groups were 91.67%, 89.52%, 89.58% and 95.83%, respectively.

The effects of pennyroyal on performance parameters of broilers are presented in Table 3. It has been shown that the addition of pennyroyal at all levels increases the BWG, lowers the daily AFI, and lowers the FCR. Moreover, pennyroyal dietary supplementation increased total body weight gain (TBWG) and decreased total feed intake (TFI) compared to the control group. At the end of the study, it was determined that the highest BW was in the group with the addition of pennyroyal at the level of 0.50%.

Table 3. Effect of Pennyroyal dietary supplementation on growth performance parameters (g)

Items	Control	0.25% Pennyroyal	0.50% Pennyroyal	1.00% Pennyroyal	P
IBW[1]	178.83± 1.15	176.88± 2.66	180.84± 0.85	180.75± 0.88	0.258
FBW[2]	2165.53± 11.80[b]	2230.78± 19.65[a]	2264.48± 19.81[a]	2259.42± 27.38[a]	0.007
BWG[3]	56.76± 0.32[b]	58.68± 0.53[a]	59.53± 0.57[a]	59.39± 0.77[a]	0.007
AFI[4]	110.25± 1.28[a]	106.71± 0.48[b]	105.70± 0.72[b]	106.63± 1.20[b]	0.002
FCR[5]	1.94± 0.02[b]	1.82± 0.01[a]	1.79± 0.02[a]	1.81± 0.02[a]	0.000
TBWG[6]	1986.71± 11.33[b]	2053.90± 18.59[a]	2083.63± 19.94[a]	2078.66± 27.12[a]	0.014
TFC[7]	3858.93± 44.76[a]	3734.83± 14.74[b]	3699.57± 25.16[b]	3732.22± 42.14[b]	0.014

[1]IBW: Initial body weight
[2]FBW: Final body weight
[3]BWG: Body weight gain
[4]AFI: Average feed intake
[5]FCR: Feed conversion ratio
[6]TBWG: Total body weight gain
[7]TFI: Total feed intake
[a-b:] The difference between means is significant at the level of P <0.05.

The effect of pennyroyal on hot and cold carcass yields was significant (P<0.05), and the highest yield rates were observed in groups with added pennyroyal at the levels of 0.25% and 0.50%. No significant differences were found among groups on thigh, breast, wing, and back-neck relative weights (P>0.05). In addition, pennyroyal did not affect the weights of heart, gizzard, liver, and spleen (Table 4).

Table 4. Effect of Pennyroyal dietary supplementation on carcass traits

Items	Control	0.25% Pennyroyal	0.50% Pennyroyal	1.00% Pennyroyal	P
Hot Carcass, (%)	71.01±0.14[c]	73.95±0.56[a]	72.82±0.25[b]	71.97±0.30[bc]	0.000
Warm Carcass, (%)	70.28±0.14[c]	72.76±0.65[a]	71.95±0.23[ab]	71.19±0.32[bc]	0.001
Thigh, (g)	453.47±6.05	458.17±14.70	455.14±10.24	453.96±8.57	0.989
Breast, (g)	483.83±21.39	502.52±15.12	502.11±13.11	492.97±22.46	0.705
Wings, (g)	170.62±2.87	172.50±4.01	177.25±1.98	169.85±2.47	0.299
Back-Neck, (g)	426.13±6.47	429.99±7.86	449.40±7.07	436.86±6.36	0.116
Heart, (g)	16.56±0.64	16.41±0.64	17.15±0.91	17.15±0.65	0.835
Gizzard, (g)	40.68±1.88	47.64±3.41	44.23±1.95	42.60±2.13	0.237
Liver, (g)	44.68±1.17	41.35±1.71	46.63±2.23	44.17±1.22	0.171
Spleen, (g)	2.99 ± 0.26	3.43 ± 0.30	3.57 ± 0.26	2.95 ± 0.24	0.267

[a-c:] The difference between means is significant at the level of P<0.05.

The effects of pennyroyal on biochemical parameters are presented in Table 5. As indicated, pennyroyal dietary supplementation at the level of 0.50 and 1.00% increased the high-density lipoprotein (HDL) values, while it decreased the low-density lipoprotein (LDL) and the very low-density lipoprotein (VLDL) at the level of 0.25 and 1.00% (P<0.05).

Table 5. Effect of Pennyroyal on blood serum parameters

Parameters	Control	0.25% Pennyroyal	0.50% Pennyroyal	1.00% Pennyroyal	P
Cholesterol mg/dl	123.00 ± 1.22[a]	115.70 ± 2.27[b]	123.00 ± 1.25[a]	122.20 ± 1.57[a]	0.070
Triglycerides mg/dl	56.20 ± 3.53	57.10 ± 4.40	53.50 ± 1.83	58.20 ± 3.92	0.812
HDL[1] mg/dl	55.90 ± 1.36[b]	56.60 ± 1.20[b]	58.60 ± 1.23[a]	61.80 ± 1.72[a]	0.023
LDL[2] mg/dl	41.55 ± 1.65[a]	33.15 ± 1.73[b]	40.08 ± 1.45[a]	33.94 ± 1.97[b]	0.002
VLDL[3] mmol	55.86 ± 1.56[a]	47.68 ± 1.98[b]	53.70 ± 1.26[a]	48.76 ± 1.50[b]	0.002
Total Protein g/dl	3.02 ± 0.05	2.97 ± 0.11	3.22 ± 0.05	2.96 ± 0.16	0.292
Albumin mg/dl	0.93 ± 0.03	0.96 ± 0.05	1.07 ± 0.03	1.03 ± 0.06	0.137
Globulin mg/dl	2.09 ± 0.04	2.01 ± 0.08	2.15 ± 0.04	1.94 ± 0.11	0.217
Glucose mg/dl	235.10 ± 4.91[a]	223.20 ± 2.03[b]	220.70 ± 2.79[b]	227.00 ± 4.23[a]	0.046
Uric acid mg/dl	6.48 ± 0.65	6.19 ± 0.56	7.15 ± 0.56	9.82 ± 1.79	0.070

[1]HDL: High-density lipoprotein
[2]LDL: Low-density lipoprotein
[3]VLDL: Very low-density lipoprotein.
[a-b:] The difference between means is significant at the level of P<0.05.

The results showing the effect of pennyroyal on MDA and GSH, one of the antioxidant parameters, are presented in Table 6. According to the results obtained, it has been determined that pennyroyal improved MDA and GSH levels (P<0.05).

Table 6. Effect of Pennyroyal on MDA and GSH (nmol/ml)

Parameters	Control	0.25% Pennyroyal	0.50% Pennyroyal	1.00% Pennyroyal	P
MDA[1]	2.40±0.07[b]	2.03±0.08[a]	1.97±0.08[a]	2.32± 0.09[b]	0.010
GSH[2]	0.17±0.01[b]	0.18±0.02[a]	0.18±0.03[a]	0.18± 0.02[a]	0.010

[1]MDA: Malondialdehyde, [2]GSH: Glutathione.
[a-c:] The difference between means is significant at the level of P<0.05.

It was determined that pennyroyal inhibited the reproduction of *Salmonella enteritidis*, *Escherichia coli* (0157h7), *Escherichia coli* (laboratory strain), *Staphylococcus aureus, Salmonella abortus ovis*, *Bacillus anthracis Sterne* bacteria. In terms of inhibitory effects, the bacteria that pennyroyal affects at the lowest concentration (2µl / ml) was *B. anthracis Sterne* and the bacteria that it inhibits at the highest concentration (128 µl / ml) was *S. enteritidis*. Pennyroyal did not have any inhibitory or bactericidal effect on *P. aeruginosa* (Table 7).

Table 7. The MIC ve MBC effects of Pennyroyal (µl/dl)

Bacteria	MIC[1]	MBC[2]
S. enteritidis ATCC 13076	128	128
E. coli O157H7	16	32
E. coli (laboratory strain)	16	32
S. aureus ATCC 6538	8	64
S. abortus ovis	32	32
B.anthracis sterne	2	32
P. aeriginosa	-	-

[1]MIC: Minimum inhibitory concentration.
[2]MBC: Minimum bactericidal concentration.

As shown in Figure 1, the addition of pennyroyal in different doses to the diet increased the duodenum villus lengths (Table 8) (P<0.05). Duodenum villus height of broilers fed diets supplemented with pennyroyal at the rate of 0.50% (1013.26 µm) and 1.00% (984.05 µm) were higher than that of the control group (830.78 µm) and the group with 0.25% pennyroyal additive (942.01 µm).

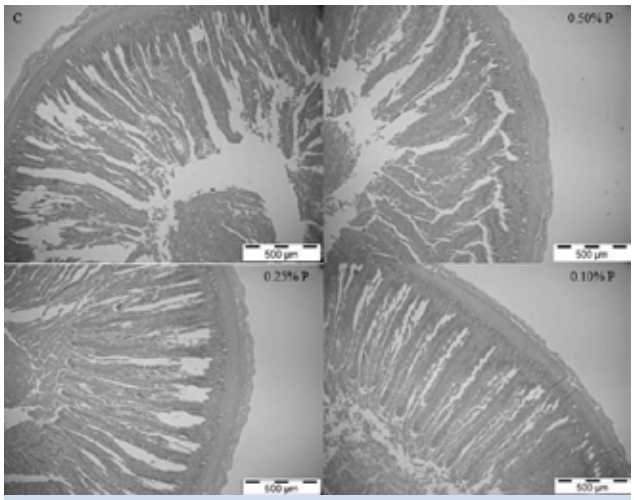

Figure 1. Duodenal villi of Broilers

C: Control group, 0.25% P: 0.25% Pennyroyal, 0.50% P: 0.50% Pennyroyal, 1.00% P: 1.00% Pennyroyal.

Table 8. Effect of Pennyroyal on duodenal villus lengths (µm)

Groups	Villus Lengths
Control	830.78± 26.04[b]
%0.25 Pennyroyal	942.01 ± 41.56[ab]
%0.50 Pennyroyal	1013.26 ± 60.03[a]
%1.00 Pennyroyal	984.05 ± 27.05[a]
P	0.021

[a-b:] The difference between means is significant at the level of P <0.05.

DISCUSSION

The similarity of body weights on the seventh day (start of the experiment) was expected due to the random distribution of chicks in groups. At the end of the experiment, the mean body weights in the control and pennyroyal supplemented groups were 2165.53, 2230.78, 2264.48 and 2259.42 g, respectively, and the body weight values of the groups with additives at different levels were determined to increase by 3.02%,

4.56% and 4.33%, respectively, in comparison to the control group (P<0.05). Average daily body weight gain values compared to the control group were found to increase by 3.38%, 4.88%, and 4.63%, respectively, and this increase was also significant (P<0.05). Feed intake was higher in the control group compared to pennyroyal supplemented groups(P<0.05). The feed conversion rates in the pennyroyal groups decreased significantly compared to the control group (P<0.05). The feed conversion rates were not affected by the addition of the pennyroyal according to the weekly values, and the differences between the groups remained at the numerical level (P>0.05). However, when the overall study was taken into consideration, pennyroyal dietary supplementation has significantly improved the feed conversion rate (P<0.05).

It is suggested that the positive effects of pennyroyal addition to broiler rations on body weight, body weight increase and feed conversion rate are due to improvement of nutrient digestibility, increase of lactic acid bacteria in the environment and inhibition of coliform bacteria, increase of the absorption of feed substances and stimulation of bile retention capacity (Erhan et al., 2012; Abedini et al., 2017). In the present study, feed conversion rate was improved as an expected result of the significant decrease in the rate of feed intake and the to be increased in body weight.

The pennyroyal additive significantly improved both hot and cold carcass yields (P<0.05). The results obtained from the present study are in agreement with previous researchers (Durrani et al., 2008; Erhan et al., 2012; Mondal et al., 2015). It has been reported that the pennyroyal has stimulating effects on pancreas by increasing the secretions of digestive enzymes, and it causes nutrients and especially amino acids to be digested and absorbed more efficiently, thereby increasing feed efficiency (Aminzade et al., 2012). On the other hand, there are also studies that provide contradictory results (Nobakht et al., 2011; Abedini et al., 2017). It was found that thigh, breast, wing, and back-neck ratios did not change with the addition of pennyroyal (P> 0.05). While the results obtained from this study are in agreement with that of a previous one (Shafiei et al., 2014), the majority of the existing literature provides different results (Goodarzi and Nanekarani, 2014; Shamlo et al., 2014; Abedini et al., 2017). At the end of the study, no statistically significant difference was found between the groups in the weights of the heart, gizzard, liver, and spleen in the slaughtered broilers (P>0.05). The numerical increases observed in the pennyroyal-added groups resulted from the fact that the slaughter weights were also high as a result of the high increase in body weight in these groups. The current results are similar to the results of studies showing that the effects of pennyroyal and similar aromatic plants on heart, liver, gizzard, and spleen weights are not significant (Erhan et al., 2012; Ghalamkari et al., 2012; Shokraneh et al., 2016). However, in contrast to the current study, there are studies reporting that different types of mint have changed internal organ weights (Al-Kassie, 2010; Aminzade et al., 2012).

As indicated, pennyroyal dietary supplementation decreases LDL and increases HDL levels. It is thought that the inhibition of 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme that controls cholesterol synthesis, by the plant extracts is effective in pennyroyal lowering the cholesterol levels (Elson and Qureshi, 1995). These results are similar to those obtained by Shamlo et al. (2014) and Abdulkarimi et al. (2012) in terms of cholesterol, LDL, and HDL levels. It is reported that pennyroyal decreases the serum glucose levels in comparison to the control group due to the stimulation of pancreatic activity by medicinal aromatic herbs including pennyroyal and thus, reducing the glucose levels (Tekce and Gül, 2015; Ulbricht, 2016). Nobakht et al. (2011), in their study conducted with pennyroyal, had similar results to the current study in terms of serum triglyceride, total protein, albumin, and uric acid values, but had different results in terms of glucose.

While the organism keeps the antioxidant functions in a balanced manner with the agents that create oxidative stress in physiological conditions, it causes lipid peroxidation by affecting the unsaturated fatty acids in the structure of membranes due to the increase of free radicals as a result of stress. The resulting lipid peroxides break down to form reactive carbon compounds. The increase in MDA, which is the most important of these, is directly proportional to the increase in oxidative damage. In previous studies (Çöteli et al., 2013; Meral et al., 2017), it has been determined that the effect of pennyroyal on MDA values is more intense than that of many medicinal plants. Due to this fact, pennyroyal is thought to decrease the level of MDA in the current research with its sweeping effect that inhibits lipid peroxidation. GSH is the reduced form of glutathione which is the most important antioxidant substance in the cell. GSH has important duties such as transport of amino acids,

reshaping some antioxidants, regulation of vitamins C and E. Glutathione defends the organism against oxidative damage by reacting with free radicals and peroxides (Çöteli et al., 2013; Karabulut and Gülay, 2016). In the current study, while the serum GSH level increased with the addition of pennyroyal, serum MDA level decreased. Therefore, pennyroyal is considered to be a good source of antioxidants. The current study results show similarities with the results of the studies conducted by Gumus et al. (2017) and Ri et al. (2017) reporting the positive effects on serum antioxidant parameters. There are also studies that show results that are not similar to that of the current in terms of antioxidant parameters (Alagawany and Abd El-Hack, 2015; Imaseun and Ijeh, 2017).

It was determined that the bacteria species in which the essential oil of pennyroyal is effective at the lowest concentration of inhibition activity is *B. anthracis Sterne* while *S. enteritidis* (ATCC13076) is the species that pennyroyal is effective at the highest concentration. It was also found that pennyroyal had no effects on P. aeruginosa. It is suggested that the resistance of this bacteria is caused by the lipopolysaccharide barrier structure on the outer membrane (Sbayou et al., 2014). The results related to the bacteria species affected by the antibacterial effect of the pennyroyal are compatible with the results of several studies using the pennyroyal (Abd El Azim et al., 2014; Abedini et al., 2017). When the results of the study were examined, it was found that the essential oil of the pennyroyal showed more efficacy on Gram (+) bacteria. It has been stated that the antimicrobial activity of the pennyroyal is due to the phenolic compounds it contains such as pulegone, piperitone, and piperitenone and that Mentha species can be a good alternative to antibiotics (Sbayou et al., 2014; Amalich et al., 2016).

When the villus lengths of the groups with pennyroyal additive was measured, an increase by 13.39%, 21.96%, and 19.45%, respectively, were found compared to the control group ($P < 0.05$). Erhan et al. (2012) reported that the pennyroyal increased the number of intestinal lactic acid bacteria linearly ($P<0.001$) in comparison to the control group, attributing the effect of the addition of pennyroyal to the increase of duodenal villus lengths in the current study. The results of the research were in accordance with the reports of Hamedi et al. (2017) and Rajput et al. (2013). There are also data that are not in accordance with the findings of the current study (Viveros et al., 2011; Yakhkeshi et al., 2011).

CONCLUSION

It was found that the pennyroyal improves performance, optimizes serum cholesterol values, has an antioxidant effect, increases the surface area by extending the intestinal villus, and can be safely used as an alternative feed additive to antibiotics due to its antimicrobial activity. However, it is suggested that there is a need to increase the studies that can serve as national and international resources in the future as there is limited literature information.

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Research article
Ερευνητικό άρθρο

Molds and Aflatoxins in Traditional Moldy Civil Cheese: Presence and Public Health Concerns

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ABSTRACT: The purpose of this study was to detect the mold biota, the contamination levels of total aflatoxin (AFB1, AFB2, AFG1, AFG2) and aflatoxin M1 (AFM1) in moldy civil cheese. A total of 100 moldy civil cheese were collected from randomly selected retailers. Mold biota was determined with conventional and ITS sequence analysis, and Aflatoxin (AF) analysis was performed usingEnzyme-Linled Immunosorbent Assay (ELISA). In the analyzed samples, *Penicillium roqueforti* (100%) was isolated as the dominant species followed by *P. verrucosum* (83%), *Aspergillus flavus* (17%). Fifteen (15 %) of moldy civil cheese samples contained AF with levels ranging from 12 to 378 ng/kg.Likewise, AFM1 was found in 25 (25 %) of samples (ranging from 5.46 to 141.56 ng/kg), among which 5 (5 %) were above the legal limits. Considering the presence of *A. flavus,* total AF and AFM1 contamination in the analyzed cheese samples it could be emphasized that moldy civil cheese might pose a hazard for public health.

Keywords: AFM_1, ELISA, Moldy civil cheese, Total Aflatoxin

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INTRODUCTION

Moldy civil cheese is a mold-ripened variety of cheese manufactured mainly in eastern Turkey. This cheese is generally made from non-pasteurized and skimmed cow and/or sheep milk using home-made calf rennet at 30-32 ºC without adding starter culture. For moldy civil cheese production, 70-75% civil cheese and 25-30% curd cheese, obtained from whey, are mixed, salted,and pressed into plastic drums or goat skins and ripened for three months or longer in the refrigerator or at chilly conditions(Cambaztepe et al., 2009;Cakmakci et al., 2012, 2015; Celikel et al., 2019). Molds which are naturally growing on the surface of and within the cheese during the ripening are considered important to lipid and protein breakdown that play active roles in the sensorial properties, texture, and flavor of the cheese (Benkerroum, 2016; Fox et al., 2017; Anelli et al. 2019; Ritota and Manzi 2020). However, molds might also be lead to undesirable changes, such as toxic secondary metabolite production, during cheese ripening and the presence of these metabolites in dairy products might result in a considerable hazard on public health (Panelli et al., 2012; Garnier et al., 2017; Anelli et al. 2019).

Mycotoxins are secondary metabolites naturally produced by certain types of molds including *Aspergillus* spp., *Penicillium* spp. etc. These metabolites especially aflatoxins are known to be potent cause of acute or chronic toxications (teratogenic, carcinogenic, and mutagenic) in humans and animals (Becker-Algeri et al., 2016; Garnier et al., 2017; Kowalska et al., 2017;El-Tawab et al., 2020). Aflatoxins, especially Aflatoxin B1 (AFB1), are immensely toxic secondary metabolites produced by some fungi, mostly by *Aspergillus flavus*, *A. parasiticus* and less often by *A. nomius* (Elkak et al., 2012; Sottili et al., 20117; Peles et al., 2019; Reinholds et al. 2020). In ruminants, ingested AFB1 with contaminated feed is biotransformed in the liver to AFM1, which then passes into the milk of lactating mammals (Bakirci, 2001;Bilandzic et al., 2014; Fontaine et al., 2015; Garnier et al., 2017; Peles et al., 2019). European Commission (EC, 2006) has defined the maximum residue level (MRL)of AFM1 as 50 ng/L in milk. On the other hand, according to the Turkish Food Codex, the MRL of AFM1 is defined as 0.050 µg / kg in raw milk, heat-treated milk, and milk used for milk products (TFC, 2011). Moldy cheese is a commonly produced traditional food in Turkey. However, no regulated standard is available for the production of civil cheese yet (Cambaztepe et al., 2009). In this regard, the aim of this study was to identify mold biota and the total aflatoxins (B1, B2, G1, and G2) and AFM1 in moldy civil cheese obtained from local bazaars in Erzurum/Turkey.

MATERIALS AND METHODS

Samples

A total of 100 moldy civil cheese samples were collected from randomly selected retailers from local bazaars in Erzurum between September and November 2015 with two-week of intervals. The samples were takenaseptically, brought to the laboratory under the cold chain and examined within 1-2 h.

Isolation and characterization of molds from moldy civil cheese

The mold isolation in cheese samples was performed according to the standard ISO 21527-2:2008. Briefly, ten grams of sample was suspended in 90 mL of 0.1% peptone water and mixed in a stomacher for 2 min (BagMixer 400 P, Interscience, France). Serial dilutions were prepared from 10^{-2} to 10^{-5} from each sample and spread onto Dichloran rose Bengal chloramphenicol agar (DRBC, Oxoid, UK). After incubation at 25 °C for 7 days, single colonies were selected based on their morphological properties (shape, size, and color) and examined according to the previous studies of Pitt and Cruickshank (1990) and Samson and Frisvad (2004).

DNA isolation and sequencing of the ITS1-5.8S-ITS2 rDNA region

Genomic DNA was extracted from the isolates using UltraCleanTM microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, California, USA) with a procedure from that of the manufacturer's. The amplification of the ITS1-5.8S-ITS2 region was carried out using ITS1 (5'-TCCGTAGGTGAACCTGCGG- 3') and ITS4 (5'-TCCTCCGCTTATTGATATGC- 3') primer pairs conducted by previous studies (White et al.,1990; Cardoso et al., 2007;Cakmakci et al., 2012). Ampliconswere purified with a GeneJet PCR purification kit (Thermo Scientific, Waltham, MA) andsubjected to sequence analysis by a commercial company (Atlas Biotechnologies, Co., Ltd., Turkey). The BLAST program was used to align the resulting sequences (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Detection of Aflatoxin levels ofcheese samples with ELISA

Chemicals and equipment

To determine total aflatoxin (AF) and AFM1 levels in moldy cheese samples, RIDASCREEN® AF Total kit (R-Biopharm, R4701, Germany) and RIDASCREEN® AFM1 test kit (R-Biopharm, R 1101 Germany) were used. Each kit contained standards, 1 x Microtiter plate with 96 wells, wash buffer salt Tween20, conjugate, antibody, substrate/chromogen, and stop solution. Other chemicals (dichloromethane, methanol, and n-heptane) were purchased from Merck (Darmstadt, Germany). Also, the immunoaffinity column (Rida Aflatoxin Column Art. No: R5001/5002) was used for the clean-up of the sample prior to the analysis of total aflatoxin and AFM1 in cheese.

Extraction of Aflatoxin from cheese samples

The extraction of aflatoxin from cheese samples was conducted using dichloromethane according to the procedure applied by Ertas et al (2011).

ELISA Test Procedures

The existence and concentrations of total AF and AFM1 in moldy civil cheese samples were determined using ELISA in compliance with the manufacturer guidelines of the commercial kits (R-Biopharm GmbH, Germany). For the AFM1 measurements,100 µL of each the standards and extraction solutions were added per separate wells (in duplicate) of the microtiter plate and incubated at 24 °C in the dark for 60 min. The liquid discarded from wells and washed two times with 250 µL of washing buffer. Then, 100 µL of diluted enzyme conjugate was added to each well and incubated for a further 60 min at 24 °C in dark. The washing procedure was repeated three times and then 100 µL substrate/chromogen was put into each well and incubated for 30 min at room temperature in dark. Finally, the reaction was stopped by adding 100 µL stop solution into the wells. The optical absorbance of each well was read at 450 nm absorbance within 15 minutes in an ELISA plate reader (ELX800, Bio-Tek Instruments, USA).

In the total aflatoxin test protocol, 50 µL of each standard and extraction solutions were separately added to each well. The plates were gently mixed and incubated for 30 min at 24 °C. Following the incubation period, wells were washed with 250 µL of washing solution two times. Then, 100 µL of substrate/chromogen were added to the wells and incubated for 30 min at 24 °C again. Finally, 100 µL stop solution was added and measured absorbance of each well at 450 nm as soon as possible after adding a stop buffer.

Evaluation of the results

RIDA®SOFT Win/RIDA®SOFT Win.net (Art. No. Z9996) software was used in the evaluation of the results obtained from ELISA analysis to determine the total aflatoxin and AFM_1 content of moldy cheese samples. According to the manufacturer guidelines of the RIDASCREEN kit for cheese; the detection limit, recovery rates and the coefficient variation (CV) were 5 ng/kg, 102% and 11%, respectively.

RESULTS AND DISCUSSION

In this study, the mold and yeast count of moldy cheese samples were ranging from 8×10^3 to 1×10^6cfu/g in cheese samples.A total of 175 mold isolates which mainly belonged to two different genera (Penicillium and Aspergillus) were obtained from 100 moldy civil cheese samples. 155 of 175 isolates were determined as *Penicillium* spp., and the rest of the isolates were Aspergillus spp. based on their morphology. ITS sequence analysis revealed that the isolates identified morphologically as Penicillium included *P. roqueforti* (62%) and *P. verrucosum*(27%), while the remaining isolates detected as Aspergillus included only *A. flavus* (11%) (Table 1). Consequently, *P. roqueforti* was isolated from all (100%) samples analyzed. In 83(83%) samples were isolated *P. roqueforti* plus *P. verrucosum*, while *P. roqueforti* plus *A. flavus* isolated from the remaining 17 (17%) samples (Table 2).

In the study, total aflatoxins (B1, B2, G1, G2) were found in 15 (15%) of 100 moldy civil cheese samples, ranging from 687.10 ng/kg to 8273.5 ng /kg (Table 2). AFM1 was found in 25 (25 %) of samples (ranging from 5.46 to 141.56 ng/kg), from which 5 (5 %) were above the limit set by TFC (50 ng/kg) and European Community (50 ng/kg). The contamination level was determined as 54.95 ng/kg, 57.7 ng/kg,74.38 ng/kg, 80.23 ng/kg, and 141.56 ng/kg in these five samples (Table 2).

Table 1. Distrubition Mold isolates obtain from moldy civil cheese samples

Mold species			
Morphological	**Number of isolates (%)**	**ITS sequencing**	**Number of isolates (%)**
Penicillium sp.	155 (89%)	*P. roqueforti*	108 (62%)
		P. verrucosum	47 (27%)
Aspergillus sp.	20 (11%)	*A. flavus*	20 (11%)
Total	175	**Total**	175

Table 2. Mold species and total aflatoxin and AFM1 concentrations in moldy cheese samples

Mold species	Positive number of samples (n=100)	Number of total AF positive samples	Number of AFM1 positive samples	Total Aflatoxin level (ng/kg)			AFM1	Limit according to TFC (> 50 ng/kg)	Range of AFM1 of positive Samples (ng/kg)		
				X±SE	Min	Max			X±SE	Min	Max
P. roqueforti	100 (100%)		-								
P. roqueforti + *P. verrucosum*	83 (83%)	-	16 (16%)	4120.6±699.4	687.1	8273.5	28 (%28)	5(5%)	36.58±1.6	5.46	141.56
P. roqueforti + *A. flavus*	17 (17%)	15 (%15)	12 (12%)								

X±SE: Mean±Standart Error

In this study,*Penicillium* spp., especially*P. roqueforti,* was the dominant moldstrainin the cheese samples similar to previously other studies (Kivanc, 1992;Gobbetti et al., 1997; Erdogan et al., 2003; Montagna et al., 2004;Hayaloglu andKirbag, 2007;Fernández-Bodega et al.,2009; Cakmakci et al., 2012). Although several species of the genus Penicillium play the main role in the ripening stage of cheese production as a starter culture, these organisms can also cause cheese spoilage resulting in significant economic losses (Kivanc, 1992; Lund et al., 1995; Erdogan et al., 2003; Hayaloglu and Kirbag, 2007).*P. roqueforti* is the major ripening culture in some cheeses such as Roquefort, Stilton, and Gorgonzola (Gripon, 1987; Fox et al., 2017)ensuring the formation of crumbly texture and small air holes in moldy cheese. However, it might also be a spoilage and mycotoxin producer agent, especially for PR toxins and patulin, in some other cheeses (Panelli et al., 2012). Nevertheless, some other earlier studies (Aran and Eke, 1987; Kivanc, 1992; Larsen et al., 2001) have reported *P. verrucosum* as the dominant flora which causes mold growth on the cheese surface and has the ability to produce mycotoxins such as ochratoxin-A and citrine.

Aspergillus flavus contamination, the producer of highly toxic and carcinogenic aflatoxins, has also been reported in several previous studies in cheese (Barrios et al., 1997; De Santi et al., 2010; Baranyi et al., 2015).On the contrary, Aran (1993) reported *A. flavus* or *A. parasiticus*contamination was not found in tulum or civil cheeses.Mycotoxin producing fungi are regarded to be among the most important contaminants in foods from the point of public health, food safety, and the economy, as mycotoxins are resistant to industrial processes (Pitt, 2000; Abdel-All et al., 2008; Zhang et al., 2013).

Compared to our results, Ozgoren (2012) reported higher values with the mean contamination level of total AFas 6896.73ng / kg, ranging from 3148.11 to 13603 ng /kg in moldy cheese samples. Moreover, in a study presented by Abdel-All et al. (2008) the content of AF B1-B2, G1 and G2 of 126 cheese samples were 3100-13000 ng/kg, 2000-12000 ng/kg and 2300-12000 ng/kg, respectively. Abd Alla et al. (2000) found AFB_1 and G_1 in only one cheese sample at levels of 10000 ng/kg and 4000 ng/kg, respectively. On the contrary, Guley et al. (2013) reported no total AF in the tested moldy cheese samples. In this study, it is remarkable that *A. flavus* was isolated from total AF-contaminated cheeses.The contamination of total AF might be associated with the growth of *A. flavus* on the surface and inside the cheese during the ripening period.No limit values have been determined for total AF in cheeses in TFC. However, the results of this study and other previous studies (Abd Alla et al., 2000; Abdel-All et al., 2008; Ozgoren, 2012) show that consuming these cheeses, contaminated with AF, may pose a potential public health concern. Therefore, it is important to standardize the moldy cheese production process using non-toxic strains as starter

cultures to prevent contamination of unwanted mold species and the formation of mycotoxins in moldy cheese.

Very limited data are available on the AFM1 contamination of moldy cheese. In this study, the analyzed AFM1 incidence of moldy cheese samples were lower than that reported by Ozgoren and Seckin (2016) who detected AFM1 in 52 % of moldy cheese samples with concentrations ranging from 10.61 to701.54 ng/kg. In contrast, Guley et al. (2013), Kokkonen et al. (2005) and Fontaine et al. (2015) found no AFM1 in the analyzed moldy cheese samples. The difference in AFM1 concentrations in cheese in other previous studies may be related to various reasons including contamination levels in milk, cheese manufacturing procedures, type of cheese, condition of cheese ripening, different geographical regions, sampling methods and the analytical methods employed (Galvano et al., 1996; Bakirci,2001; Nakajima et al., 2004).

CONCLUSION

In this study,the dominant mold species in moldy civil cheese samples analyzed was *P.roqueforti* followed by *P. verrucosum* and *A. flavus*.Considering the mycotoxin producing capability of these species and the presence of *A. flavus*in aflatoxin determined cheeses, it could be concluded that moldy cheese might pose a public health concern as these cheeses are produced and ripened under uncontrolled conditions without using any pure culture in Turkey. Thus, to protect consumer health, further studies areneeded to determine whether these mold isolates are toxic or nontoxic. Moreover, considering the preventive measures be taken for AFM1 in cheeses, the best way to protect the consumer against AFM1 induced health hazards is the monitoring andavoiding AFB1 contaminationin feed due totheclose relationship between the AFM1 concentration in milk products and AFB1 concentration in the feed.

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CONFLICT OF INTEREST STATEMENT

The authors declared that have no conflicts of interest with anyone

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Research article
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The importance of professional based questionnaire in the dog's acute and chronic pain evaluation

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ABSTRACT: The measurement of pain levels is made differently depending if acute or chronic pain is diagnosed, objective (e.g. cortisol, prolactin, serotonin, catecholamines, or the cardiac frequency and arterial pressure evaluation) or subjective methods being imagined. All subjective methods are including questionnaires and specific additional methods. The aim was the verifying the effectiveness of drug and physiotherapy combinations by using an owner based questionnaire for the chronic patients and a veterinary professional based questionnaire for the acute pain patient group. In this study a total of 20 dogs with observable pain were selected and two groups (n = 10 / group), constituted: G1 - chronic pain, and G2 - acute pain. The treatment of dogs with acute signs of pain was made oral or injectable with NSAIDs administration and for dogs with signs of chronic pain, physiotherapy treatment and drug therapy was administered. The owners of the dogs with *chronic pain* received the *HCPI questionnaire* in order to evaluate their dog's pain level subjectively. For the dogs with *acute pain* the veterinarian filled out the short form of *Glasgow Composite Measure Pain Scale* (CMPS-SF). After initiating a Paired *t*-test in Excel 2010 with the scores obtained with HCPI and CMPS-SF, there was observed a significant reduction of pain after associated drug administration and physiotherapy and no significant evidence of acute pain after drug therapy. The used physiotherapy and drug combinations delivered a significant reduction of chronic pain, both clinically and visually mirrored in score reduction after treatments. The HCPI questionnaire could be considered a valuable tool for evaluating chronic pain in patients in the clinic environment. The CMPS-SF has also proven to be a very useful questionnaire in diagnose of acute pain and evaluation of the effectiveness of drug therapy used.

Keywords: dog, pain level, pain scales, HCPI, CMPS-SF, significance.

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INTRODUCTION

Pain can be defined as the sensation felt by a patient and it represents a function of the nervous system that takes place in three different areas: perception, emotion and psychological area (Goldberg, 2019; Epstein et al., 2015; Hellyer et al., 2007; Kopf and Patel, 2010; Reddi et al., 2013).

Animals have neurologic paths and neurotransmitters for building, transmitting and modulating pain similar to humans (Sabiza et al., 2019; David and Millis, 2014). Most often the physiotherapy method used for lameness treatment is the underwater treadmill. This allows the establishment of a precise therapeutic protocol regarding distance, speed and inclination. The intensity of the exercises should increase gradually in order not to intensify oedema and acute pain (Schmidt and Müller, 2014).

After surgical intervention on the limbs, pain can lead to immobility or even osteoarthritis (Bockstahler et al., 2004). A study made by Monk et al. (2006), pointed out the beneficial combination between passive and active physiotherapy measures, underwater treadmill included, after TPLO *(Tibial Plateau Levelling Osteotomy),* surgical procedure in patients diagnosed with cranial cruciate ligament rupture (Monk et al., 2006).

Massage and passive range of motion exercise (pROM) are used as supplementary physiotherapeutic measures. Increased blood flow, activation of the lymphatic system and adhesion treatment are the most important results obtained by these methods (Castelli et al., 2019; Callaghan, 1993; Zink and Van Dyke, 2013).

Electrotherapy is also used in veterinary medicine in order to treat acute and chronic pain in patients with orthopaedic and neurologic signs. Alternating currents of low frequency are usually used in veterinary medicine, like NMES (*Neuromuscular Electrical Stimulation*) and TENS *(Transcutaneous Electrical Nerve Stimulation)* (Hussain et al., 2019; Dumitrescu and Cristina, 2015; Samoy et al., 2016).

The response to pain will be diminished or inexistent but also the sensation of pain is lacking according to gate control theory on which TENS is based (Kopf and Patel, 2010; Reddi et al., 2013). Opioid like substances also play a role (Canapp et al. 2009).

TENS is recommended in osteoarthritis. NMES can be an alternative for TENS after surgical treatment of cranial cruciate ligament rupture (Hyytiäinen, 2015).

Laser therapy is used in veterinary medicine for the treatment of pain and inflammation. Therapeutic lasers have wavelengths of over 500 mW, or between 600-1070 nm. They interfere with *c*-cytochrome and this leads to ATP production, which modifies pain transmission mechanisms. The dosage used is measured between 2-20 $J \times cm^{2-1}$ (Pryor and Millis, 2015; Redondo, 2015; vetmanual.com)

The measurement of pain levels is made differently depending if acute or chronic pain is diagnosed, objective or subjective methods being imagined. Acute or chronic pain can be analysed through the ascertained serologic parameters like: cortisol, prolactin, serotonin, catecholamines, or the cardiac frequency and arterial pressure evaluation, all subjective methods including questionnaires and specific additional methods (Lafuente et al., 2019; Mathews et al. 2014).

For instance, the *Helsinki Chronic Pain Index* (HCPI) evaluates dogs radiologically and clinically diagnosed with osteoarthritis throughout the owner, who will answer to the questions about the mobility of their dog (there exists also a psychometric HCPI, but only validated in Finnish language) (Hielm-Björkman et al., 2003; Martello et al., 2019).

For the evaluation of acute pain after surgical interventions, a dependable tool is the *Glasgow Composite Measures Pain Scale* (CMPS-SF), a short form of it, the *Glasgow Short Form*, being useful to be easier used in the clinical environment (Murrell et al., 2009).

Another objective pain measurement method would be *gait analysis*, which can be made with the force plates, special roads sensitive to: pressure, treadmills and opto-electronic digital systems. Kinetic gait analysis can be less useful in arthrosis, which coexists in multiple locations (Rialland et al. 2012; Sharkey, 2013)

In the last decade, treatment of chronic and acute pain in dogs has become a more important aspect for veterinarians to be considered. Physiotherapy alone or in combination with allopathic drug treatment has proven to be a good alternative to drug treatment by itself. Especially the long-term effects of physiotherapy are to be taken more into consideration.

The present study has the aim of verifying the ef-

fectiveness of drug and physiotherapy combinations by using an owner based questionnaire for the chronic patient group and the effectiveness of drug therapy with a veterinary professional based questionnaire for the acute pain patient group.

MATERIAL AND METHODS

Animals

In this study a total of 20 dogs were included, whose owners have consented in written to participate in this one year clinical trial. The dogs were regular patients of two veterinary practices: Gesundheitszentrum für Kleintiere, Passau Germany and Praxis Dr. Pauli, Waldkirchen, Germany.

Dogs from the study have been previously dewormed and the vaccines status of each pacient verified. All owners have been previously detailed informed and all consented of the therapeutic and recovery protocol to be used, which all fitted to the veterinary methods used in practice.

In Table 1 the breed, age, weight and sex / cathegory of the dogs inluded in the study is presented.

Table 1. Dogs included in the study.

No.	Breed	Age (years)	Weight (kg)	Sex / cathegory
1.	Entlebucher Sennenhund	8	25.0	m
2.	Golden Retriever	8	36.0	nm
3.	Labrador Retriever	1	29.8	nm
4.	Labrador Retriever	11	48.0	nm
5.	Malinois	7	12.0	f
6.	Mixed breed	0,7	6.2	nf
7.	Mixed breed	2	18.9	nf
8.	Mixed breed	12	20.0	nm
9.	Mixed breed German Shepherd	10	35.0	nf
10.	Mixed breed Husky	4	20.0	f
11.	Mixed breed Labrador Saint Bernard	2	19.0	nm
12.	Mixed breed Malteser	8	13.0	nm
13.	Mixed breed Pitbull	5	30.0	nm
14.	Mixed breed Pitbull	1	13.0	m
15.	Mixed breed Romanian mioritic Shepherd	7	32.7	nf
16.	Mops	8	11.6	nm
17.	Pudel	9	8.1	m
18.	Pudel	10	5.0	nm
19.	Saint Bernard	2	50.0	f
20.	Terrier Mix	11	10.0	nm

f-female; m-male; nf-neutered female; nm-neutered male.

Dogs identified with pain belonged to following breeds: Mops (n = 1), Pudel (n = 2), Golden Retriever (n = 1), Labrador Retriever (n = 1), Pudel (n = 1), Saint Bernard (n = 1), Malinois (n = 1), Mixed breed (n = 11), Entlebucher Sennenhund (n = 1), of which 4 neutered males, 9 males and 7 females. Depending on diagnostic, the dogs were separated into two groups: with chronic pain (G1) and with acute pain (G2). In the Table 2 the findings of the clinical examination are described for the chronic and acute pain groups.

Table 2. Dogs diagnosed with chronic and acute pain.

No.	Breed/Sex	Clinical examination/Diagnose
Chronic pain		
1.	Labrador Retriever (m)	Femoral head luxation right leg and subluxation left leg, hip dysplasia (femoral head resection on the right side). Moderate to severe pain. Physiotherapeutic evaluation: at 10 days after surgical treatment. Stands on all 4 legs at walk and trot, stabilises at the sacrum his position.
2.	Pitbull (m)	Moderate pain of the lumbar spine, careful gait, slower gait, Kibbler impossible, shakes incompletely.
3.	Mixed breed Husky (f)	Moderate pain at the cervical spine. Neck rotation to the right is not completely possible because of the pain.
4.	Mixed breed Labrador-Sain-Bernard (m)	Hip dysplasia grade 2-3 on the right, grade 1 on the left, moderate pain of the spine. Physiotherapeutic evaluation: moderate chronic pain with accutisation at T13-L1.
5.	Mixed breed Pitbull (nm)	Moderate pain at the lumbar spine. Physiotherapeutic diagnose: Kibbler negative, partial shake.
6.	Mixed breed (f)	Intermittent lameness lower members, extension and rotation of both posterior members reduced. Physiotherapeutic evaluation: Kibbler positive, shakes rarely but completely, back in persistent contraction.
7.	Mixed breed (nm)	Moderate pain of the lumbar vertebral column, Kibbler impossible.
8.	Mops (m)	Intermittent ataxia, intermittent lameness, intermittent paraplegia. Spontaneous recovery after a few seconds. Moderate to severe pain at thoracolumbar transition site. Delayed proprioception al the posterior right leg. Radiological diagnose: narrow intervertebral spaces T6-7, T10-11. Diagnose: discopathy.
9.	Pudel (nm)	Chronic pain at the lumbar vertebral column, reluctance to move, shakes incompletely
10.	Saint Bernard (f)	Moderate pain at the right hip, intermittent lameness during increased effort, extension of the right posterior leg not completely possible in comparison with the left leg. Presumptive diagnosis: Hip dysplasia. Recommendation: Radiographic consult under anaesthesia.
Acute pain		
1.	Entlebucher Sennenhund (m)	Lameness of the right hind limb, drawer sign positive, cranial cruciate ligament rupture (surgical treatment).
2.	Golden Retriever (m)	Drawer sign positive, moderate pain, cranial cruciate ligament rupture of the left leg, (surgical intervention).
3.	Labrador (m)	Moderate pain at the lumbar vertebral column.
4.	Malinois (f)	Lameness at the right posterior leg, acute pain, ligament injury
5.	Mixed breed German shepherd (f)	Drawer sign positive, lameness at the posterior right leg, moderate pain, cranial cruciate ligament rupture.
6.	Mixed breed Malteser (nm)	Lameness, acute pain at both posterior limbs, drawer sign positive, cranial cruciate ligament rupture (surgical treatment).
7.	Mixed breed Mioritic shepherd (f)	Lameness, moderate acute pain at the knee joint of the left leg, articular effusion, reduced patellar crepitation with slight possibility to luxate towards lateral.
8.	Mixed breed (f)	Lameness, severe acute pain at the knee joint right leg, ligament injury
9.	Pudel (m)	Lameness of the posterior right legt, reduced to moderate pain, drawer sign positive, cranial cruciate ligament rupture, patellar luxation to medial, (surgical treatment).
10.	Terrier Mix, male	Lameness, moderate pain, cranial cruciate ligament rupture right leg, (surgical treatment).

Legend: f-female; m-male; nf-neutered female; nm-neutered male.

Administered treatments

The treatment of dogs with acute signs of pain was made oral or injectable administration with the following NSAIDs:

- Robenacoxib *(Onsior, Elanco Tiergesundheit AG, Germany)* 1-2 mg×kg.bw.$^{-1}$
- Firocoxib *(Previcox, Merial, France)* 5.0 mg×kg.bw.$^{-1}$

- Meloxicam *(Metacam, CP-Pharma mbH, Germany)* 0.2 mg×kg.bw.$^{-1}$
- Carprofen *(Carprotab, CP-Pharma mbH, Germany)* 4.0 mg×kg.bw.$^{-1}$

Physiotherapy methods used

Dogs with signs of chronic pain, whose owners agreed with physiotherapy treatment were treated with the physiotherapy measures considered appropriate by the clinics physiotherapist. The diagnosis was made and complementary drug therapy was prescribed by the veterinarian, depending on the chosen physiotherapy protocol.

Physiotherapy equipment

For the application of physiotherapy the following equipments were used:

- Underwater treadmill (*Physio Tech*, Germany).
- Laser Laserdusche Power Twin 21 (*MKV Lasersystem* GmbH, Germany), Po 21x50 mW, λ 785 nm.
- MultiProg 2510Laser (*Bio Medical Systems,* Germany).
- Electrotherapy Vetri-combi (*Physiomed Elektromedizin*, Germany).

In Table 3 the NSAID administration and particularized physiotherapy protocol for chronic pain dogs is presented.

Table 3. NSAID administration and physiotherapy protocol for the dogs categories included in the study.

No.	Breed/Sex	Administered drug / individual therapy / doses
Chronic pain		
1.	Labrador Retriever (m)	Electrotherapy (ET) -amplitude modulated current 4000Hz, massage, hydrotherapy ET 100-250Hz, 30 mA. ET 1-250Hz, 22mA. pROM exercises. Hydrotherapy with hip high water level: Session S1:11 m walk, 3× break with water reaching knees. S2:0.6 km×h^{-1}, slow walk, 61m, 10min, 3× break. S3:identical. S4:0.6 km×h^{-1}, 137 m, 20min. 3× break. S5:identical, S6:0.6 km×h^{-1}, 140m, 20min. S7: = 188 m without break. S8:0.8 km×h^{-1}, 403m, 32min. S9:0.8 km×h^{-1}, 500m, 40min. S10: 550, 40min.
2.	Pitbull (m)	pROM exercises, massage, laser. Laser Nog E, Frequency 2.400Hz, amplitude 80%, 240 secconds. Drug treatment 10 days Previcox (Firocoxib) 227 mg 0,5x1/day
3.	Mixed breed Husky (f)	Massage and drug treatment for 10 days with Metacam (Meloxicam) 5mg×mL^{-1}, 0.8 mL injectable way 1× then Previcox 0.5×1/day for 9 days.
4.	Mixed breed Labrador - Sain-Bernard (m)	ET - amplitude modulated current 4000Hz, hydrotherapy, laser. Laser F-L: NogC, 2J 10 sessions. ET 100-250Hz, 16mA at the spine T13-L1, ET 1-250, 4.4KHz, 14.5mA the whole spine. Hydrotherapy with hip high water level: S1: 100m walk, 2× break with knee high water level. S2: 0.6 km×h^{-1} slow walk, 170m. S3: identical S4: 0.8 km×h^{-1}, 425m. S5: identical. S6: 0.7 km×h^{-1}, 47 m. S7: identical. S8:0.8 km×h^{-1}, 54 m. S9: 0.8 km×h^{-1}, 700m. S10: identical.
5.	Mixed breed Pitbull (nm)	Laser Nog E, Frequency 2400Hz, amplitude 80%, 240 sec. Drug treatment 10 days with Metacam 5mg×mL^{-1}, 1.2 mL injectable way 1× then Previcox 1×1/day 9 days
6.	Mixed breed (f)	ET-amplitude modulated current 4000Hz, hydrotherapy. ET 100-250Hz, 20mA at the hips. Hydrotherapy with water reaching the hips: S1: 105m walk, 1× pause with water reaching knees. S2: 0.6 km×h^{-1} slow walk, 163m. S3: identical S4: 0.8 km×h^{-1}, 345m. S5: identical. S6: 0.7 km×h^{-1}, 467m. S7: identical. S8: 0.8 km×h^{-1}, 530m. S9: 0.8 km×h^{-1}, 660m. S10: identical but after 8 minutes with a cuff.
7.	Mixed breed (nm)	Laser and massage Laser Nog E, Frequency 2400Hz, amplitude 80%, 240 sec. Drug treatment for 10 days Onsior 40mg, Tabl 1×1/day

8.	Mops (m)	ET - amplitude modulated current 4000Hz, massage, hydrotherapy, laser. ET 100-250Hz, 16 mA, 10 sessions. Laser F-L: NogE, 3J. 1 session. Hydrotherapy with hip high water level: S1: habituation to walking (reticent patient), S2: 0.6 km×h^{-1} slow walk, 94m, S3: 100m, hypermetria posterior members, S4: 170m S5: identical. S6: 0.6 km×h^{-1}, 225m, S7: 188m walk, S8: 0.6 km×h^{-1}, 200m, S9: 0.6 km×h^{-1}, 200m, S10: 220m for 26min.
9.	Pudel, neutered (m)	Laser F-L: NogE, 3J. 10 sessions Onsior (Robenacoxib) 20 mg×mL^{-1} inj. 0.50mL 1 day, Onsior 10mg Tabl 1×1/day
10.	Saint Bernard (f)	pROM exercises, drug treatment Previcox 227mg 1½ ×1/day, 10 days
Acute pain		
1.	Entlebucher Sennenhund (m)	Drug treatment:7 days with Onsior 20 mg×mL^{-1} injectable 2.5mL 1 day, afterwards Previcox (Firocoxib), Tabl 227 mg 1×1/day.
2.	Golden Retriever (m)	Onsior (Robenacoxib) 20 mg×mL^{-1} inj. 3.80 mL, for 2 days, afterwards Previcox. Tabl 227 mg 1×1/day.
3.	Labrador (m)	Metacam (Meloxicam) 5mg×mL^{-1}, 1.9mL injectable 1×, afterwards Previcox, Tabl 227 mg 1½ ×1/day.
4.	Malinois (f)	Metacam 5 mg/mL^{-1}, 0.5mL injectable 1×, afterwards, Previcox, Tabl 227mg 0.5×1/day.
5.	Mixed breed German shepherd (f)	Metacam 5 mg×mL^{-1}, 1.4 mL injectable way 3×, afterwards Previcox, Tabl 227mg 1×1/day
6.	Mixed breed Malteser (nm)	Onsior 20 mg×mL^{-1} inj. 1.3 mL for 2 days, Onsior 20 mg Tabl 1×1/day
7.	Mixed breed Mioritic shepherd (f)	Onsior 20 mg×mL^{-1} inj. 3.30mL, afterwards Previcox (Firocoxib). Tabl 227mg 1×1/day.
8.	Mixed breed (f)	Onsior 20 mg×mL^{-1} injectable 0.65mL, afterwards Onsior Tabl 10 mg. 1×1/day.
9.	Pudel (m)	Onsior 20 mg×mL^{-1} inj. 0,90mL 2 days, Onsior 20 mg. Tabl 1×1/day
10.	Terrier Mix (m)	Metacam 5mg×ml^{-1} 0.4mL injectable way, for 2 days, afterwards, Carprotab (Carprofen) 50 mg 1×1/day

Legend: f-female; m-male; nf-neutered female; nm-neutered male.

Subjective pain assessment methods

Classification of the patients in the acute or chronic pain group respectively classification of the intensity of pain in reduced, moderate or severe was made by veterinarian in charge after clinical examination without taking in consideration the level of pain suggested by the owner.

The owners of the dogs with *chronic pain* received the *HCPI questionnaire* in order to evaluate their dog's pain level subjectively. These patients were part of the group that received physiotherapy.

For the dogs with *acute pain* the veterinarian in charge used and filled out the short form of *Glasgow Composite Measure Pain Scale* (CMPS-SF)© (University of Glasgow 2008. Licensed to New Metrica Ltd). All the pacients had obtained scores ≥6, with a maximum of 14. According to test interpretation analgesic treatment is initiated starting with scores of 6.

Statistical analysis

Since observations are taken from the same or matched subjects before-after situation, the paired *t*-test two tailed analyzed in Excel 2010, to compare two paired values it was used. The results plotted as a matrix are indicating the result for each pair as a confidence interval *p* value where p varied from: $^{ns}p>0.05$, non-significant to $^{***}p$ and $^{****}p<0.001$, highly-significant (99.9% confidence).

RESULTS AND DISCUSSION

Pain of the locomotor system exclusively at the legs was present at (n = 11) patients, pain localised at the vertebral column combined with pain of the locomotor system was present at (n = 9) dogs. Clinical examination revealed posture and gait modification of the dogs belonging to chronic or acute pain groups. Scores of 18, 20, 34, 24, 25, 20, 19, 30, and 25, can be classified into the group of chronic pain (G1), which needed drug therapy.

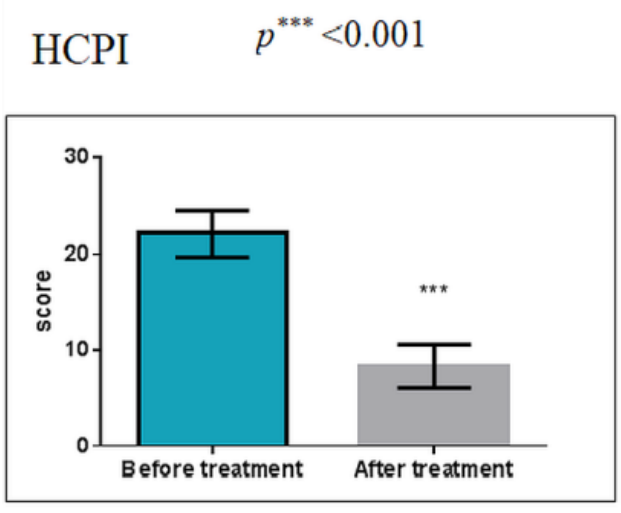
At the third patient, the initial HCPI score of 6 was considered normal in test interpretation. After treatment, all of the patients showed an improvement mirrored in the HCPI score, seven patients had scores within normal values at the second evaluation, considering the superior limit = 11. The third patient with normal HCPI scores before physiotherapy treatment is, according to the owner, a dog that arrived from a dog shelter and had an unknown prior history. In this case, even if the HCPI score was normal, the registered clinical signs of lameness were evident, this leading to the start of physiotherapy.

The clinical signs were present for more than three weeks before consult and didn't improve sole with drug treatment.

The fourth, sixth and ninth patients showed considerable improvement on HCPI questionnaire but the examination 10 days after treatment showed that the treatment failed to reach normal interpretation levels for these patients.

After initiating a Paired *t*-test in Excel 2010 with the scores obtained with HCPI, there was observed a significant reduction of pain after associated drug administration and physiotherapy, as Figure 1 shows.

Figure 1. The *t*-Test for HCPI questionnaire, for chronic pain group (G1)

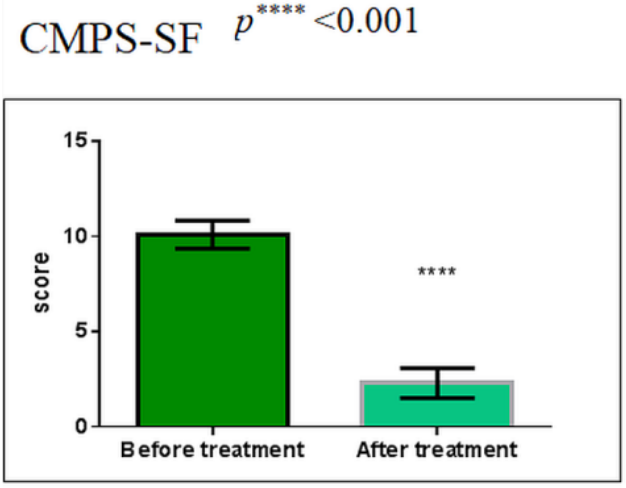


the *t*-Critical two-tail value, high-significantly reduced compared ($^{***}p$ <0.001) to *t* Stat and value p <α. Subsequently H_0 hypothesis can be rejected.

For the acute pain group (G2) the CMPS-SF was made at the study initiation time during consultation and diagnoses settlement. During the clinical examination, after 10 days of treatment, there was made a new CMPS-SF test.

Eight dogs had scores within normal values and two dogs had a score = 6, with a reduction of the initial value with at last one half. After initiating a Paired *t*-test in Excel 2010 with the scores obtained with CMPS-SF, there was no significant evidence of acute pain after drug therapy, as Figure 2 shows.

Figure 2. *t*-test CMPS-SF results, for acute pain group (G2)



the *t*-Critical two-tail value is high-significantly reduced ($^{****}p$ <0.001) compared to *t*-Stat and value p <α. Subsequently H_0 hypothesis can be rejected.

The two patients that presented scores = 6, received a prolonged treatment and the symptoms disappeared completely after prolongation.

Rogatko et al. (2017) studied the effects of low level laser therapy applied preoperatively before tibia plateau levelling osteotomy in dogs by using force plate analysis. The collective was found an improved peak vertical force that could correlate with low level laser therapy, therefore this type of treatment was recommended (Rogatko et al., 2017).

Draper et al. (2012), in a study used laser therapy with 25 $W \times cm^{2\,-1}$ delivered to the skin and obtained a decreased time to ambulation in dogs undergoing hemilaminectomy (Draper et al., 2012).

In our study laser therapy was added to the physiotherapy treatment protocol in 6 of the 10 cases from the G1, in order to obtain fast reduction of clinical symptoms such as lameness and pain, subjectively assessed by HCPI questionnaire and clinical exam-

ination.

Krstic et al. (2010) obtained a significant pain reduction after using TENS in dogs with ankylosing spondylitis. Pain reduction was observed through VAS *(Visual Analogue Scale)* (Krstic et al., 2010).

Our *electrotherapy* protocol was variable depending on the diagnosed cause of lameness and pain. The physiotherapist in charge managed the electrotherapy treatment depending on the clinical findings of the veterinarian in order to obtain best possible clinical improvement of the symptoms. We found that for the chronic pain group the treatment acceptance was higher when combined physiotherapy with drug therapy. Two pet owners refused although drug therapy and the dogs received just physiotherapy.

Hydrotherapy with the underwater treadmill was also part of the physiotherapy methods used. We found a high acceptance of this therapy method in both patients and owners, with exceptional clinical improvement of the symptoms and quality of dog's welfare. In this aim, physical therapy alone or combined with surgical methods can lead to full recovery based on examinations by gait analysis and owners questionnaires (Wucherer et al., 2013).

HCPI questionnaire can indicate the presence of chronic pain in dogs with craniate crucial ligament repair (Mölsä et al., 2013).

Acupuncture and *carprofen* used in the treatment of dogs with hip dysplasia lead to a reduction of scores in case of The Canine Brief Inventory and VAS scale, but failed to differ significantly when compared to placebo group (Teixeira et al., 2016).

We selected for our study the HCPI questionnaire. The HCPI questionnaire was rapidly accepted and the scores correlated with clinical findings in 10 dogs from G1.

CMPS-SF is a sensitive test used regularly for acute pain in dogs and can indicate accurately the severity of pain (Murrell et al., 2008). Della Rocca et al. (2018), also proved ICMPS-SF to be a reliable tool in assessment of acute pain in dogs. (Della Rocca et al., 2018).

In this study we used the CMPS-SF. We had a good workflow by using the questionnaire and it rapidly helped us in choosing the most appropriate treatment modality. Also we are confident, that the English version of the CMPS-SF can be used also by non-native English speakers in order to sensitively categorise acute pain in dogs. To this we agree that synoviocentesis could have been an additional tool to assess the signs of inflammation in the joint in case of cranial cruciate ligament rupture (Kennedy et al., 2018).

Treatment protocol of acute pain included usage of robenacoxib, firocoxib, meloxicam and carprofen. The drug treatment was adapted according to administration preferences of the pet owner (oral vs. injectable) and known hypersensitivity or adverse effects seen in the dog to one of the drugs listed. When comparing the use of robenacoxib and meloxicam, Gruet et al. (2011), reached the conclusion, that both controlled adequately acute pain in dogs undergoing orthopaedic surgery. Evaluation was made with modified Glasgow index (Gruet et al., 2011).

Also robenacoxib compared to carprofen had similarly good effects on acute pain in dogs with osteoarthritis (Reymond et al., 2012), and firocoxib may be a better choice in pain induced by arthritis in dogs (Hazewinkel et al., 2008).

Even tough, the evaluation of the patients before and after treatments showed a significant reduction of pain scores for acute and chronic pain groups, there should nevertheless follow more extensive studies, in order to generalise the results. As an objective observation, it has to be stated that the patients had different types of pain and the study would have been more relevant if they all have had the same disease type. But in clinic's conditions, this is the real "environment" in which the pain is evaluated in dogs and, in this reality the vet is interested, that was why the questionnaires used couldn't be equalised.

Also the questionnaires have been filled out by the veterinarian in charge in the clinic. Maybe it would have been a lower influence if the same doctor would have evaluated the patient regarding pain and if he wouldn't have known the history of his clinic's patients before filling out the forms. In this aim a more restrictive study in terms of diagnosis and therapy combination should follow, to be capable to confirm with greatest accuracy what are the most effective physiotherapy and drug treatment combinations for success.

CONCLUSIONS

The used physiotherapy and drug combinations delivered a significant reduction of chronic pain, both

clinically and visually mirrored in score reduction after treatments.

The HCPI questionnaire could be considered a valuable tool for evaluating chronic pain in patients in the clinic environment. We recommend the use of the HCPI questionnaire to evaluate the evolution of the chronic pain status in dogs undergoing physiotherapy associated with drug therapy in the veterinary practices.

The CMPS-SF has also proven to be a very useful questionnaire in diagnose of acute pain and evaluation of the effectiveness of drug therapy used in this study. We recommend the use in a clinical setting of this questionnaire for the acute pain in dogs.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Influence of dietary olive paste flour on the performance and oxidative stress in chickens raised in field conditions

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ABSTRACT: Olive oil contains a variety of antioxidants, including vitamin E. Its consumption protects against oxidative stress, which is involved in many pathological conditions, affecting animals' development and their general welfare. The present study aimed to investigate the effect of olive paste flour (OPF) on the antioxidant status and performance of broiler chickens raised in field conditions. Total of 18.000 broilers was randomly allocated in equal numbers in two poultry houses. The chickens were grouped according to their diet as follows: **Control group**: chickens fed commercial poultry feed, and **OPF group**: chickens fed control dietsin the starter period, but they got a supplement of OPF in grower, and finisher 1 and 2periods, respectively. The birds were raised under identical field conditions (ventilation, vaccination, lighting, etc.). Antioxidant status was assessed by measuring the concentration of vitamin E in plasma, as well as the total antioxidant capacity (TAC) in plasma and muscle tissue. According to the statistical analysis of the results, the addition of OPF to chickens' diet significantly enhanced plasma α-tocopherol concentration ($p \leq 0.05$). TAC did not show any significant differences in chicken plasma nor muscle tissue($p > 0.05$).Feed intake (FI) was higher in OPF group, while Body weight (BW) was lower. Liveability was similar for the two groups. The feed conversion ratio (FCR) was higher, and the European production efficiency factor (EPEF) was lower in broilers of the OPF group compared to those of the control group.Samples of roasted breast from chickens of both groups were used for organoleptic characteristics evaluation. Results showed that samples of the OPF group smelled more intensely and were more tasteful than those of the control group.However, other organoleptic characteristics did not differ. In conclusion, the results demonstrated that although the addition of OPF to chickens' diet can cause growth retardation, it can significantly increase the plasma α-tocopherol concentration. Further studies are needed to optimize the concentration of OPF in poultry feed in order to avoid growth retardation or even to promote growth in broiler chicks.

***Keywords*:** Olive paste flour, α-tocopherol, total antioxidant capacity, performance, field conditions

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INTRODUCTION

Olive oil is obtained from olive tree fruit (*Olea europaea* L.) and is an essential constituent of Mediterranean diet, which is widely accepted as a healthy diet. Many of the beneficial effects of olive oil on human and animal health are attributed to the presence of several bioactive compounds such as mono/polyunsaturated fatty acids, phenols, phytosterols, carotenoids and tocopherols (Cárdeno et al., 2013; Scoditti et al., 2014). Olive oil production generates a significant amount of wastes, whose disposal causes severe environmental problems (Rinaldi et al., 2003; Venieri et al., 2010). The exploitation of such wastes could be an ideal solution in avoiding environmental pollution and toxicity and meets the targets of a circular economy to eliminate waste and the continual use of resources.

Toward this direction, some wastes of olive oil production (olive paste and olive oil mill wastewater, OMWW) could be incorporated into animal feed due to their significant antioxidant content (Anniva and Tsimidou, 2009; Cardinali et al., 2010; Frankel et al., 2013; Gerasopoulos et al., 2015; Padalino et al., 2018; De Bruno et al., 2021).To be more specific, olive paste contains vitamin E (Anniva and Tsimidou, 2009; Padalino et al., 2018; De Bruno et al., 2021), among other essential antioxidants such as polyphenols.

Vitamin Eis the most active antioxidant ingredient of the olive oil, in terms of antioxidant and anti-stroke activity and can protect against lipid peroxidative damages (De Luca, 1978; Gimeno et al., 2002). It is known that the term vitamin E denotes two groups of related compounds, having similar biological activity: α, β, γ and δ-tocopherols and α, β, γ, and δ-tocotrienols. The greatest physiological interest is represented by tocopherols (Hansen and Warwick, 1966), especially α-tocopherol, one of the most often encountered in nature. It is the only one present in animal bodies.

The incorporation of olive-based byproducts into animals' diets has shown contradictory results concerning the animals'performance. For instance, the supplementation of broiler diet with5% of olive cake (OC) showed the best performance compared to the broilers fed with 10% OC, and the control. Particularly, the body weight gain (BWG), FI,and FCR were higher for the broilers fed with 5% OC, except for EPEF which was higher in broilers fed with 10% OC (Al-Harthi, 2016). Pappas et al. (2019) reported that broilers fed with olive pulp up to 5% performed well and maintained good health, final body weight, and carcass yield without impacting to feed:gain ratio. Remarkably, broilers fed 8% of olive pulp showed meaningfully poorer FCR and numerically higher feed intake (FI). Also, the addition of 2.5% or 5% of olive pulp in the starter period and5% in the finisher periodpresented the best performance results. Furthermore, Shafeyet al. (2013) showed that the replacement of15g or 30 g of wheat bran with olive leavesdid not significantly influence the growth performance and carcass characteristics of the chickens fed the olive leaves. However,the replacement of 50 g/kg of wheat bran with olive leaves reduced theB-WG but increased FI and significantly increased FCR.

The effect of olive byproducts on animals' redox status is also debatable.Gerasopoulos et al. (2015) proposed that incorporation of OMWW retentate into broiler diet may improve the redox status of chickens and reduce the oxidative stress by scavenging free radicals, as well as improve meat nutritional value. This study assessed the antioxidant effects of supplementation with OMWW by measuring oxidative stress biomarkers (protein carbonyl levels, TBARS; TAC; GSH;catalase, CAT) in broiler blood and different tissues (i.e. muscle, heart and liver). This research concluded that OMWW is a supplement that can enhance the polyphenolic content of animal feed and thus protect animals against various pathologies.

By contrast, Pappas et al. (2019) reported that the addition of 5% olive pulp into chicken diet did not significantly affect the total antioxidant activity measured in blood by several biochemical markers (GPx, GST, SOD, GR, CAT, ABTS, FRAP).Furthermore, it was reported that differences in antioxidant activity between studies could be attributed to the experimental conditions, but also to the different content of polyphenols between the examined olive-based products. The latter is further justified by the fact that indeed there are differences in phenolic content during olive processing, oil extraction and storage (Frankel et al., 2013). Finally, Pappas et al. (2019) stated that their study was applied in small-scale production and suggested that commercial conditions in large-scale production may affect the analyzed biochemical markers.

In this work, the diet of commercial broiler chickens was supplemented on top with an olive paste-based product, rich in antioxidant substances, coming from wastes of olive oil production. Particularly, we aimed to estimate the influence of olive paste flour on the redox status and performance of broiler chick-

ens raised in field conditions in large-scale production systems. The chosen dosage of 2.5% and 5% OPF in grower, and finisher 1 and 2 periods was based on previous studies (Shafeyet al., 2013; Al-Harthi 2016; Pappas et al., 2019),which showed that olive byproducts up to 5% affected positively the performance in broilers raised in small-scale production.The total antioxidant capacity was studied in chicken plasma and muscle extract, while α-tocopherol levels were determined in broiler plasma.Organoleptic attributes were evaluated by tasters. BW and liveability were recorded daily, FI was calculated at the end of each period, whereas FCR and EPEF were calculated at the end of the experiment for estimating growth performance.

MATERIALS AND METHODS

All the procedures were performed in accordance with PINDOS welfare guidelines.

Materials

Olive paste flour (Sparta INNOLIVE) used on top of the commercial chicken diet was purchased from Sparta Life S.A (Greece). Sodium Phosphate used in muscle tissue homogenisation was purchased from Fluka (Switzerland). Hexane, Ethanol and Methanol used in evaluation of α-tocopherol and TAC respectively, were obtained from Fisher Scientific (USA). α-tocopherol, used to prepare the Standard solution of vitamin E, as well as DPPH used in TAC evaluation, were purchased from Sigma Aldrich (Germany).

Animals and their diet

Eighteen thousand broiler chicks were randomly allocated equally in two poultry houses, as described below: house 1that hosted the control group, which served as the negative control, and house 2that hosted the OPF group, whose diet was supplemented with 5% olive paste flour. The birds were as hatched and originated from the same parent stock and hatchery. They were raised under the same field conditions (equipment, ventilation, vaccination program, lighting program, etc.). Two production cycles were performed for each treatment. Chickens were offered from Agricultural Poultry Cooperative of Ioannina "PINDOS" (Greece).

To meet the nutrient requirements of the broiler chicks during the experimental period, four complete basal diets (Table 1) were formulated each one for the starting (0-10d), growing (11-24d), finishing 1 (25-39d) and finishing 2 (40-slaughter) periods, respectively. Feed formulation and chemical analysis of rations are presented in Table 2. The addition of 2.5 and 5% OPF was done on top in grower and finisher rations. Chemical analysis of OPF is illustrated in Table 3. No antibiotic growth promoters, organic acids and phytobiotics were used. Feed and drinking water were offered to all birds ad libitum throughout the experiment.

Table 1. Diet formulation of the tested groups

Ingredient (kg/ton)	Starter 0-10 days		Grower 11-24 days		Finisher 1 25-39 days		Finisher 2 40 day-slaughter	
Corn	200 [a]	200 [b]	200 [a]	200 [b]	0 [a]	0 [b]	0 [a]	0 [b]
Wheat	385 [a]	385 [b]	391 [a]	401 [b]	660 [a]	700 [b]	689 [a]	719 [b]
Soya-meal	340 [a]	340 [b]	312 [a]	327 [b]	220 [a]	230 [b]	195 [a]	215 [b]
Sparta INNOLIVE	0 [a]	- [b]	25 [a]	- [b]	50 [a]	- [b]	50 [a]	- [b]
Palm oil	5 [a]	5 [b]	12 [a]	12 [b]	22	22 [b]	25 [a]	25 [b]
Soya oil	30 [a]	30 [b]	27 [a]	27 [b]	17 [a]	17 [b]	13 [a]	13 [b]
Limestone	14 [a]	14 [b]	13 [a]	13 [b]	12 [a]	12 [b]	12 [a]	12 [b]
Premix	26 [a]	26 [b]	20 [a]	20 [b]	19 [a]	19 [b]	16 [a]	16 [b]

[a] OPF group; [b] conventional group.

Table 2. Dietary ingredients and chemical composition[1] of broiler feed

		Starter		Grower		Finisher 1		Finisher 2	
Age	days	0-10		11-24		25-39		40-slaugther	
Metabolizable Energy	kcal/kg	3000		3100		3200		3200	
AMINO ACIDS		**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**	**Total**	**Digest**
Lysine	%	1.44	1.28	1.29	1.15	1.15	1.02	1.08	0.96
Methionine+Cystine	%	1.08	0.95	0.99	0.87	0.90	0.80	0.85	0.75
Methionine	%	0.56	0.51	0.51	0.47	0.47	0.43	0.44	0.40
Threonine	%	0.97	0.86	0.88	0.77	0.78	0.68	0.73	0.64
Valine	%	1.10	0.96	1.00	0.87	0.89	0.78	0.84	0.73
Isoleucine	%	0.97	0.86	0.89	0.78	0.80	0.70	0.75	0.66
Arginine	%	1.52	1.37	1.37	1.37	1.21	1.09	1.14	1.03
Tryptophan	%	0.23	0.20	0.21	0.18	0.18	0.16	0.17	0.15
Leucine	%	1.58	1.41	1.42	1.27	1.26	1.12	1.19	1.06
Crude Protein	%	23.0 [a] 23.0 [b]		21.5 [a] 21.5 [b]		19.5 [a] 19.1 [b]		18.3 18.2	
Crude Fat	%	5.1 [a] 5.1 [b]		6.5 [a] 5.8 [b]		6.4 [a] 5.6 [b]		6.4 [a] 5.2 [b]	
Crude Fiber	%	2.4 [a] 2.4 [b]		3.2 [a] 2.4 [b]		3.5 [a] 2.3 [b]		3.4 [a] 2.2 [b]	
Moisture	%	11.5 [a] 11.5 [b]		11.5 [a] 11.5 [b]		11.3 [a] 11.3 [b]		12.0 [a] 11.5 [b]	
Ash	%	5.5 [a] 5.5 [b]		5.1 [a] 5.0 [b]		4.9 [a] 4.6 [b]		4.8 [a] 4.4 [b]	
MINIMUM PECIFICATION									
Choline per kg	mg	1700		1600		1500		1450	
Linoleic Acid	%	1.25		1.20		1.00		1.00	

[1]Ingredients and chemical composition was noted according to feed formulation and NIR tests for verification; a: OPF group; b: conventional group; MINERALS % (Minerals are those used on diet): Calcium: starter: 0.96; grower: 0.87; finisher 1: 0.78; finisher 2: 0.75; Available Phosphorus: starter: 0.480; grower: 0.435; finisher 1: 0.390; finisher 2: 0.375; Magnesium: starter; grower; finisher 1; finisher 2: 0.05-0.50; Sodium: starter; grower: 0.16-0.23; finisher 1; finisher 2: 0.16-0.20; Chloride: starter; grower; finisher 1; finisher 2: 0.16-0.23; Potassium: starter: 0.40-1.00; ADDED TRACE MINERALS PER KG (mg): Copper: starter; grower; finisher 1; finisher 2: 16; Iodine: starter; grower; finisher 1; finisher 2: 1.25; Iron: starter; grower; finisher 1; finisher 2: 20; Manganese: starter; grower; finisher 1; finisher 2: 120; Selenium: starter; grower; finisher 1; finisher 2: 0.30; Zinc: starter; grower; finisher 1; finisher 2: 110;ADDED VITAMINS PER KG (IU): Vitamin A: starter: Wheat based feed: 13,000; Maize based feed: 12,000; grower: Wheat based feed: 11,000; Maize based feed: 10,000; finisher 1: Wheat based feed: 10,000; Maize based feed: 9000; finisher 2: Maize based feed: 10,000; Maize based feed: 9000; Vitamin D3: starter: Wheat based feed; Maize based feed: 5000; grower: Wheat based feed; Maize based feed: 4500; finisher 1: Wheat based feed; Maize based feed: 4000; finisher 2: Maize based feed; Maize based feed: 4000; Vitamin E: starter: Wheat based feed; Maize based feed: 80; grower: Wheat based feed; Maize based feed: 65; finisher 1: Wheat based feed; Maize based feed: 55; finisher 2: Maize based feed; Maize based feed: 55; Vitamin K (Menadione) (mg): starter: Wheat based feed; Maize based feed: 3.2; grower: Wheat based feed; Maize based feed: 3.0; finisher 1: Wheat based feed; Maize based feed: 2.2; finisher 2: Maize based feed; Maize based feed: 2.2; Thiamin (B1) (mg): starter: Wheat based feed; Maize based feed: 3.2; grower: Wheat based feed; Maize based feed: 2.5; finisher 1: Wheat based feed; Maize based feed: 2.2; finisher 2: Maize based feed; Maize based feed: 2.2; Riboflavin (B2) (mg): starter: Wheat based feed; Maize based feed: 8.6; grower: Wheat based feed; Maize based feed: 6.5; finisher 1: Wheat based feed; Maize based feed: 5.4; finisher 2: Maize based feed; Maize based feed: 5.4; Niacin (mg): starter: Wheat based feed: 60; Maize based feed: 65; grower: Wheat based feed: 55; Maize based feed: 60; finisher 1: Wheat based feed: 40; Maize based feed: 45; finisher 2: Maize based feed: 40; Maize based feed: 45; Pantotheninic Acid (mg): starter: Wheat based feed: 17; Maize based feed: 20; grower: Wheat based feed: 15; Maize based feed: 18; finisher 1: Wheat based feed: 13; Maize based feed: 15; finisher 2: Maize based feed: 13; Maize based feed: 15; Pyridoxine (B6) (mg): starter: Wheat based feed: 5.4; Maize based feed: 4.3; grower: Wheat based feed: 4.3; Maize based feed: 3.2; finisher 1: Wheat based feed: 3.2; Maize based feed: 2.2; finisher 2: Maize based feed: 3.2; Maize based feed: 2.2; Biotin (mg): starter: Wheat based feed: 0.30; Maize based feed: 0.22; grower: Wheat based feed: 0.25; Maize based feed: 0.18; finisher 1: Wheat based feed: 0.20; Maize based feed: 0.15; finisher 2: Maize based feed: 0.20; Maize based feed: 0.15; Folic Acid (mg): starter: Wheat based feed; Maize based feed: 2.20; grower: Wheat based feed; Maize based feed: 1.90; finisher 1: Wheat based feed; Maize based feed: 1.60; finisher 2: Maize based feed; Maize based feed: 1.60; Vitamin B12 (mg): starter: Wheat based feed; Maize based feed: 0.017; grower: Wheat based feed; Maize based feed: 0.017; finisher 1: Wheat based feed; Maize based feed: 0.011; finisher 2: Maize based feed; Maize based feed: 0.011.

Table 3. Chemical analysis of the complementary OPF

Chemical Analysis	%
Proteins	9.5
Total Fat	16.0
Total Carbohydrates	62.9
Sugars	0.7
Total Ash	6.4
Total Fibers	22.5
Salt	0.46
Moisture	5.23
Lysine	0.105
Threonine	0.468

Performance

The body weight (BW) was measured at the end of the experiment on 45th day of age, while the feed conversion ratio (FCR) was calculated cumulatively for the total production period as feed intake divided by body weight gain. Mortality was recorded daily, while European Production Efficiency Factor (EPEF) was calculated at the end of the experiment, according to the type: (BW (kg) x Liveability (%) x 100)/ (FCR x slaughter age (d)). Feed intake was calculated per growing phase.

Antioxidant status

Blood and muscle tissue samples were collected in the slaughterhouse at the 45th day and stored at -80 °C, until needed for processing. In particular, blood samples from 20 birds per group were collected by jugular vein-puncture in ethylene diamine tetra-acetic acid (EDTA) tubes, centrifuged in 1.500 rpm for 10 minutes in 4 °C, while plasma was collected in eppendorf tubes. Homogenized muscle tissue samples were received after treating them with buffer, containing 1 M NaCl, 2 mM EDTA, 50 mM Tris-HCl (pH 7.3), 0,5 v/v Triton X-100, sonicated in ice and centrifuged in 20.000 x g for 30 min at 4 °C. The supernatant was collected for further processing, while the pellet was discarded.

Determination of Total Antioxidant Capacity

The estimation of total antioxidant capacity was based on the measurement of DPPH in 520 nm. The DPPH reduction assay was performed by adding 20 μl of blood plasma or 40 μl homogenate muscle extract to 480 or 460 μl of 10 mM sodium phosphate buffer (pH 7.4) respectively. Then the total volume of the samples (500 μl) was added to 500 μl of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanol solution. Then the samples were incubated for 30 minutes at 21 °C. Absorbance was measured at 520 nm according to standard protocol (Janaszewska and Bartosz, 2002). Total Antioxidant Capacity (TAC) represents mmol of DPPH reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH:H) by the existing antioxidants in plasma or muscle tissue. Evaluation of antioxidant capacity of the samples, either plasma or muscle extract, was achieved with calculation of % Radical Scavenging Activity which is derived from the type: $[(Absorbance_{Control}-Absorbance_{Sample})/Absorbance_{Control})] *100$.

Determination of α-tocopherol

The evaluation of α-tocopherol levels was based on the measurement of the fluorescence of the extracted α-tocopherol, into hexane, at specific excitation and emission wavelengths. Initially, a Standard and a Working solution of vitamin E were made: Standard solution of vitamin E, 2000 μg/ml: 500 mg of α-tocopherol were dissolved in 100 ml of filtered ethanol and then to 250 ml. Working solution of vitamin E, 20 μg/ml: 1 ml of vitamin E standard solution was dissolved in 100 ml of filtered ethanol.

Then a variety of solutions were prepared in 15 ml centrifuge tubes (with stopper): 1. Blank: in 1.2 ml water, 2 ml of filtered ethanol was added under stirring. 2. Pattern: in 0.2 ml Working solution, 1.2 ml of water was added and mixed for 30 sec. Then 1.8ml of filtered ethanol was added under stirring. 3. Unknown: in 0.2ml plasma of each sample, 1ml of water was added and mixed for 30 sec. Then 2 ml of filtered ethanol were added under stirring.

Then all tubes were mixed for 30 sec, 5 ml hexane was added, and they were shaken well for 5 min by hand and Vortex. Next the tubes were centrifuged for 5min at 2000 rpm. The hexane layer of each tube was transferred to fluorescence cuvettes. The absorbance was measured at 330 nm. The results were processed by the equation: μg of free vitamin E/ml = $[(F_x-F_b)/(Fs-Fb)] *20$. Where F_x = absorption of unknown, F_s = standard absorbance, F_b = blank absorption (Hansen and Warwick, 1966).

Evaluation of organoleptic attributes

At the end of the experiment (45th day), 30 birds per group were slaughtered at PINDOS slaughterhouse for evaluating the organoleptic characteristics. Chicken breasts of each group were roasted at 200 °C for 30 min and served to different people to investigate the possible organoleptic differences. The sam-

ples were presented one pair at a time and in random order to each taster. Tasters were asked to judge about "more", "less", or "no difference" for taste, odour, colour, consistency, and texture of the meat. None of the animals was destined to be marketed/consumed.

Statistical Analysis

Assays were performed in a series of samples equally for both groups in duplicate. Results are shown as mean, while standard deviation (SD), standard error of the mean (SEM) and statistical significance (*p* value) were determined according to normality and calculated with independent t test, using the software IBM® SPSS® Statistics 26.

RESULTS

Performance

The BWat slaughter age was significantly lower (p≤0.05) in the OPF groupcompared to the control group (Table 4). FI, liveability,FCR, and EPEFwere calculated on a farm level and statistical analysis and evaluation was performed. However, the high number of birds per farm as well as the application of identical farm management practices and micro-environmental conditions could allow to draw out some conclusions. The FI and FCRwere higher and the EPEF was lower in the OPF group compared to the control group, while liveability was similar between experimental groups (Tables 2,4).

Antioxidant status

As it is shown in Table 5 (Figure 1), α-tocopherol could pass from the diet in chicken plasma. The amount of α-tocopherolin plasma of broilers fed with OPF was significantly higher (p≤0.05) than α-tocopherol content of plasma of broilers fed with the control diet (control group).TAC of chicken plasma and muscle tissue was not significantly different as it is conducted from % RSA (p>0.05) (Table5, Figs. 2, 3).

Table 4. Performance of broilers during the experimental period

	Control group	**OPF group**
Final BW (kg)	2.73	2.50
SD	0.29	0.13
SEM	0.09	0.04
P value	≤0.05	
Liveability	94.88	95.70
SD	2.12	1.77
SEM	0.67	0.56
FCR	1.73	1.83
SD	0.06	0.03
SEM	0.02	0.01
EPEF	332.90	285.50
SD	22.59	20.18
SEM	7.14	7.38
Feed Intake kg/broiler		
Starter	0.40 [b]	0.41 [a]
Grower	1.14 [b]	1.21 [a]
Finisher 1	1.56 [b]	1.73 [a]
Finisher 2	0.88 [b]	1.02 [a]

BW: Body Weight; FCR: Feed Conversion Ratio; EPEF: European Production Efficiency Factor; SD: Standard Deviation; SEM: Standard Error of Means.

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Table 5. Effect of OPF on a-tocopherol in broiler plasma and ontotal antioxidant capacity in broiler plasma and muscle tissue

	α-tocopherol				total antioxidant capacity							
	plasma				plasma				muscle tissue			
	Vitamin E (μg/ml)	SD	SEM	P value	% RSA	SD	SEM	P value	% RSA	SD	SEM	P value
Control	9.0	4	0.8	≤0.05	70.6	4.8	2	>0.05	81.6	1.67	0.53	>0.05
OPF	13.2	6.7	1.5		64	11.9	3		81.8	2.05	0.65	

RSA: Radical Scavenging Activity; SD: Standard Deviation; SEM: Standard Error of Means.

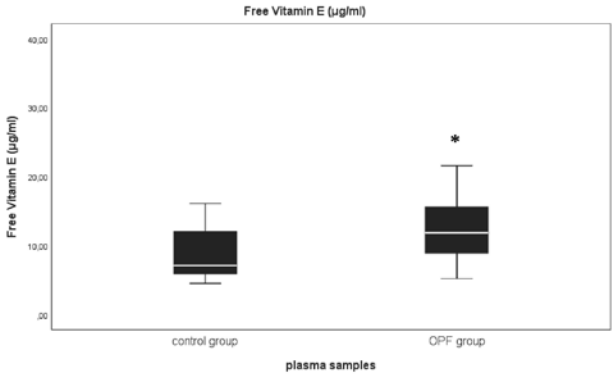

Figure 1. Free Vitamin E (µg/ml) in chicken plasma fed with OPF compared to control. *Significantly different from the values of the control group at the same sampling time (p≤0.05).

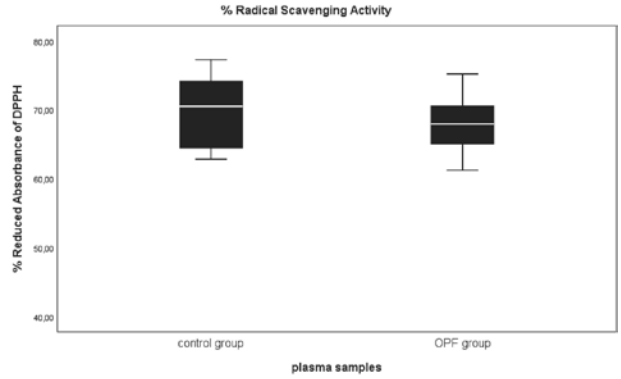

Figure 2. Total antioxidant capacity of broiler plasma against DPPH. (p>0.05).

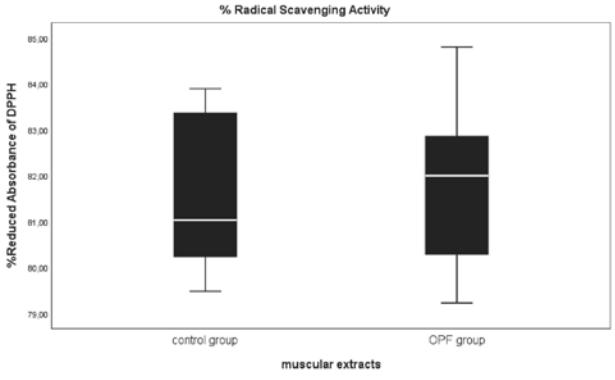

Figure 3. Total antioxidant capacity of broiler muscle tissue against DPPH. (p>0.05).

Evaluation of organoleptic characteristics

In the context of organoleptic control, chicken meat from both groups was roasted and tested by 10 people to determine organoleptic differences. Concerning the taste sensory, the two different groups of chickens presented differences. Broilers fed with OPF were tastier and had increased smell attributes than the conventional ones. However, no other organoleptic characteristic like meat appearance, namely colour, consistency and texture were differentiated in the two different groups.

DISCUSSION

Vitamin E is the most important lipophilic antioxidant in mammals. It can provide protection against lipid peroxidation (Pompeu et al., 2018) and thus improve meat quality (Nam and Ahn, 2003; Arshad et al., 2013). Also, vitamin E can increase the antioxidant status thus results in protection from pathological problems or mitigation of their symptoms (Deaton et al., 2004; Lykkesfeldt and Svendsen, 2007; Abuelo et al., 2015).Natural antioxidants are safer than the synthetic ones, and they have been used widely in broiler feed for reducing lipid oxidation (Arshad et al., 2013; Starčević et al., 2015).Olive paste and OMWW are sources of natural antioxidants as it has been shown previously (Anniva and Tsimidou, 2009; Cardinali et al., 2010; Frankel et al., 2013; Gerasopoulos et al., 2015; Padalino et al., 2018; De Bruno et al., 2021). Furthermore, olive paste contains vitamin E (Anniva and Tsimidou, 2009; Padalino et al., 2018; De Bruno et al., 2021), which is one of the dominant antioxidants presented in olive oil (Gimeno et al., 2002). So, it is conducted that products stemming from olive oil production like olive paste could be an excellent supplement for animal feed.

The findings of the current study propose that the incorporation of olive paste into the feed increases α-tocopherol levels in poultry plasma.The results suggest that α-tocopherol may be transferred from olive to the processed feed (olive paste flour) and finally to broiler plasma.Results of the present work are in accordance with a study conducted by Paiva-Martins et al., (2009) who found that incorporation of 5% or 10% of olive leaves into pig diet increases α-tocopherol content of the meat. Furthermore, similarly to our results, various studies (Guo et al., 2001; Surai and Kochish, 2019) reported that supplement of vitamin E to broiler diets increases α-tocopherol concentration either in chicken plasma or in tissues.

Total antioxidant capacity did not exhibit significant differences between the two groups in broiler plasma (p>0.05). These findings agree with a study (Pappas et al., 2019)conducted under conditions of small-scale production which showed that total antioxidant activity did not alter in animal blood after the addition of olive pulp in broiler diet in various proportions. Vossen et al. (2011) also reported that the plas-

ma redox status and lipid oxidation in broilers have not changed by incorporating natural antioxidants into broiler diet. Contrary to our results, it has been suggested that olive oil mill wastewater (OMWW) retentate with 1% olive oil might improve the redox status of chickens (Gerasopoulos et al., 2015). This could indicate that the supplemented diet used in the above study maybe contain more antioxidant compounds and vitamin E than the OPF used in this work.

Moreover, our data did not show significant differences in TAC of muscle tissue extracts. An explanation could be that the role of vitamin E on the tissue depends on the age of the birds (Jankowski et al., 2016). To investigate the specific issue, studies at earlier ages of birds would have to be conducted.

The effect of the supplemental vitamin E in TAC values varies among studiesmaybe due to the variance of amounts and duration of the supplemental olive-based productor vitamin E, as well as other poultry management factors such as food composition, scale production, living conditions, polyphenolic content, etc.(Pappas et al., 2019; Surai and Kochish, 2019). The most important issue which affects TAC, may bethe differences in phenolic content between the examined olive-based products due to olive processing, oil extraction, and storage (Frankel et al., 2013). The addition of vitamin E (>100 mg/kg) from the beginning growing period until slaughter, or just for a greater period than this study, might improve poultry total antioxidant defense (Surai and Kochish, 2019). In the present work, there was a gradual decrease in the standard commercial supplementation of vitamin E from the starter to the finisher. This might indicate that even though plasma α-tocopherol concentration was elevated by the addition of OPF, still it was not such efficient in increasing TAC.

In addition, the quality evaluation of chicken meat of both groups showed that OPF enhanced ones had higher taste and smell attributes. Maybe this is due to increase in fatty acids, which is known to affect nutritional value of poultry meat (Wood et al., 2004). Furthermore, both groups presented the same meat colour as well as consistency and texture. Maybe the similarity of appearance characteristics is attributed to the production system used in rearing of the chicks (Arreza, 2019) at the poultry farm, but also could be attributed to theirgenotype, processing procedure of the meats, slaughter conditions, and the overall health and nutrition of the poultry animals (Mir et al., 2017).

The results of the study illustrate that introduction of olivepaste flour, into broiler diet, increases the FCR, which could be attributed to the higher FI as well as to the relative final body weight of the OPF group (Masouri et al., 2015; Al-Harthi, 2016; Yi et al., 2018; Pappas et al., 2019). Moreover, EPEF value was decreased in OPF group compared to Control group. This difference could be due to the higher FCR or the significantly lower BW of the OPF group. Decreased EPEF, however, could not be ascribed to the liveability as this parameter was not significantly different in two groups. Higher FI, FCR and lower EPEF values in this studyare in agreement with other reports (Masouri et al., 2015; Pappas et al., 2019). The inclusion of 5% of olive byproducts into broilers' diet has been tested several times and it was reported as one of the best portions to be added into broilers' feed regarding the performance (Shafey et al., 2013; Al-Harthi, 2016; Pappas et al., 2019). Similarly to our results, Pappas et al. (2019) and Shafey et al., (2013) reported higher FI, FCR, and lower BW in broilers fed with 5% of the corresponding byproduct. Also, Al-Harthi (2016) stated that broilers fed with 5% of OC as olive byproduct presented increased FI and FCR, however,in contrast to our results, the EPEF was lower and BW was higher. The differences in FCR and EPEF,that olive byproducts have displayed, among studies, may be due to the variations on α-tocopherol concentration in broiler feed, but also due to the different chicken raising conditions (Rama Rao et al., 2011; Masouri et al., 2015).Also, the differences in performance could be ascribed to the different content of the crude fiber among different olive byproducts, given that certain fiber content may be related to better digestibility and other performance parameters (Al-Harthi, 2016; Pappas et al., 2019).Hence, the high content of the crude fiberin the OPF supplement that was used in the present study, could justify theincreased FI and consequently the higherFCR in the OPF group.

CONCLUSION

In conclusion, the findings of the present study show a significant increase in α-tocopherol in broiler plasma fed with OPF compared to the control group. This indicates that α-tocopherol could pass from olive and olive paste to broiler plasma through diet. TAC did not exhibit significant differences between the two groupsneither inplasma norin muscle tissue. Furthermore, the organoleptic characteristics presented a difference in taste and smell attributes, but color, consistency and texture remained the same for both

groups. In addition, the OPF group showed higher FI and FCR,but decreased BW and EPEF compared to the control group. Finally, given that this study took place in a poultry farm unit under field conditions, these findings are considerable because of their application in large-scale poultry production. Further studies are needed to optimize the concentration of olive paste flour in poultry feed in order to avoid growth retardation or even to promote growth in broiler chicks.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Research article
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Oxidative Stress and Acute-Phase Response Status During Treatment in Premature Calves with Respiratory Distress Syndrome

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ABSTRACT: This study aimed to determine lipid peroxide (LPO), antioxidant capacity (AOC), and acute-phase protein changes before and after different nebulization treatments in premature calves with respiratory distress syndrome (RDS).

Thirty-six premature calves were divided into equal number of 6 groups. Group 1 was labelled as negative control and received standard treatment. Group 2 was labelled as positive control; Groups 3, 4, 5, and 6 were labelled as trial groups. These groups received nebulizer treatment. Nebulizer drug combinations were as follows: Group 2 (fluticasone), Group 3 (formoterol + fluticasone), Group 4 (ipratropium bromide + fluticasone), Group 5 (fluticasone + furosemide) and Group 6 (formoterol + ipratropium bromide + furosemide + fluticasone). Venous blood was taken from all calves before (0 hour) and after treatment (24th, 48th, 72nd hour). It was concluded that total LPO levels gradually decreased while AOC levels increased during treatment but there was no difference in the serum amyloid A (SAA) and fibrinogen levels within groups.

In conclusion, supportive and nebulizer treatments to improve function of lungs were demonstrated to alleviate oxidative stress. However, in order to reveal the effects of local nebulizer applications on oxidative stress, further studies are required to investigate oxidation parameters in the bronchoalveolar fluid.

***Keywords*:** respiratory distress syndrome, premature calf, oxidative stress markers, acute-phase response

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INTRODUCTION

As a widespread complication in the world and Turkey, premature calf birth is among the leading causes of mortality (Guzelbektes et al., 2012; Aydogdu et al., 2016; Yildiz and Ok, 2017; Yildiz et al., 2019; Ok et al., 2020). The majority of perinatal calf deaths are related to premature birth which causes failing to complete organ development (Meyer et al., 2001; Johanson and Berger, 2003). Premature calves are born without completing the ordinary developmental process, thus they may have substantial problems in circulation, nervous system, metabolism, and especially respiration (Yildiz and Ok, 2017; Yildiz et al., 2019; Ok et al., 2020). The most critical problem in these cases is respiratory distress syndrome, which is caused by the insufficient development of the lungs in the absence of surfactant. In premature calves with RDS, the lungs collapse with the development of hypoxia, interstitial inflammation, pulmonary hypertension due to excessive strain, and interstitial edema as a result of impaired air exchange due to decreased pulmonary compliance and surfactant deficiency. Death occurs in a short period if the cases are left untreated (Avery and Merrit, 1991). In most premature calves with RDS (Yildiz and Ok, 2017), respiratory acidosis (Ok et al., 2020), hypercapnia (Sustronck et al., 1996; Konduri and Kim, 2009), and hypoxemia (Yildiz and Ok, 2017, Yildiz et al., 2019) develop. Respiratory difficulties also lead to significant hypoxia in tissues. This increases the production of oxidative stress factors and causes a decrease in antioxidant capacity (Mutinati et al., 2014).

Hypoxia associated with lung diseases increases oxidative stress products in tissues and massive amounts of free radicals are also formed in the organism. Lipids are the most sensitive biomolecules to the free radical effects. When the radicals develop at rates exceeding antioxidant capacity, the unsaturated bonds of cholesterol and fatty acids in the cellular membranes react easily with free radicals to produce peroxidation products (Gutterridge, 1995; Mutinati et al., 2014). Therefore, lipid peroxidation is the basis of cellular damage and can be used as an indicator of oxidative stress in tissues and cells (Lichtenstern et al., 2011, Maden et al., 2012). Total LPO measurement determines all peroxide products in the organism. Antioxidant capacity (AOC) or total antioxidant protection AOP measurement carries out a crucial role in biological events. The presence of antioxidants is effective in preventing the harmful effects of free radicals. Antioxidants include superoxide dismutase, catalase, glutathione peroxidase enzymes and albumin, ferritin, ceruloplasmin, ascorbic acid, α-tocopherol, ß-carotene, and uric acid (Gutteridge, 1995, Halliwell, 1997; Carole et al., 2007; Ighodaro and Akinloye, 2018; Gulcin et al., 2018; Gulcin, 2020). Oxidative stress is the cause of impaired balance between oxidants and antioxidants in cells in favor of oxidants. In case of oxidative damage in the cell, similar to the tissues, damage or death occurs (Grobben et al., 1997; Lykkesfeldt ve Svendsen, 2007). In hypoxia-causing diseases (lung diseases), a significant amount of oxidative stress occurs and antioxidant levels decrease (Deaton, 2006). Respiratory distress syndrome is the most important cause of mortality in preterm children. As a result of surfactant insufficiency, vascular permeability due to pulmonary hypertension increases causing pulmonary edema and ischemic damage (Avery and Merrit, 1991). While the antioxidant levels decrease in hypoxia-related ischemic damage, an increase in free radical LPO levels occurs (Auten and Davis, 2009; Kim et al., 2012). In newborns and premature, free radicals are reported to bear a very high risk of creating oxidative damage and oxidative stress (Bounocore et al., 2002; Mutinati et al., 2014).

Inflammation and edema develop in the interstitial tissue due to forced functioning of the lungs and hypertension in the pulmonary vessels as a result of the surfactant insufficiency. Moreover, due to the difficulties in air exchange, breathing difficulties and hypoxia occur in tissues. Acute-phase protein profile can be changed as a consequence of inflammation, edema, and hypoxia in the lung tissue. The most important positive acute-phase proteins in ruminants are fibrinogen, haptoglobin (Hp), serum amyloid A (SAA), alpha-1 acid glycoprotein, and ceruloplasmin (Murata et al., 2004; Eckersall and Bell, 2010, Yun et al., 2014). The number of acute-phase proteins increases in inflammation, edema, hypoxia, bacterial infection, or trauma and may also increase in transplantation and other stress situations (Murata et al., 2004; Lomborg et al., 2008). Serum fibrinogen, serum amyloid A and haptoglobin levels increases significantly during the inflammation process (Martinez-Subiela et al., 2004; McGrotty et al., 2005). Acute-phase proteins are a group of glycoproteins produced primarily in the liver by leukocytes and macrophages in response to inflammatory mediators during (AOP) tissue damage. Acute-phase proteins carry out many roles in free oxygen radicals, immunoglobulin production, and tissue repairment (Martinez-Subiela et al., 2004).

This study aimed to determine changes in LPO, AOC, and acute-phase proteins before and after different nebulizer treatments in premature calves with RDS.

MATERIALS AND METHODS

Materials

In this study, only premature calves of spontaneous birth that fulfilled the premature criteria and at least three criteria of respiratory distress syndrome were included. The criteria for premature and respiratory distress syndrome were determined according to the studies of Yildiz and Ok (2017) and Ok et al. (2020). All the calves were admitted from those referred to the clinic of the Department of Internal Medicine at Selcuk University, Faculty of Veterinary Medicine. Study was approved (2014/05) by the Ethics Board (SUVFEK) of Selcuk University Faculty of Veterinary Medicine.

Animals

The material of the study included 36 spontaneously born premature calves (27 Holsteins, 5 Simmentals, and 4 Montafon breeds) with respiratory distress syndromes. The calves were evenly randomly divided into 6 groups: Group 1 as a negative control, Group 2 as a positive control, Groups 3, 4, 5, and 6 as experimental groups.

Clinical Examinations and Diagnosis

The first clinical evaluation was carried out to diagnose the premature status of the calves referred to the clinic. History of the calves and premature criteria determined in the previous related studies were taken into consideration in the evaluation of premature status. Calves that met premature criteria were evaluated whether they fulfilled the criteria of respiratory distress syndrome. As a result of these evaluations, 36 calves, which fulfilled the criteria of respiratory distress syndrome, from 43 premature calves were included in the study.

Collection and Storage of Blood Samples

Venous blood samples with and without anticoagulants were collected for biochemical measurement from calves in all groups before (0 hour) and after treatment (24th, 48th, 72nd hour) from Vena Jugularis according to the study of Ok et al. (2020). Blood samples were centrifuged at 5000 rpm, plasma and serum samples were collected. Plasma and sera were kept at -80 °C until further analysis. Venous blood $SatO_2$ were analysed within 15 min at 0, 24, 48 and 72 h using a blood gas analyser (GEM Premier 3000, Instrumentation Laboratory, Lexington).

Methods

The Treatment Protocols

Premature calves with respiratory distress syndrome were evenly and randomly divided into 6 groups and the treatment protocols below were applied.

1. Group negative control (G1): Standard treatment protocol
2. Group positive control (G2): Standard treatment protocol + fluticasone
3. Group 3 (G3): Standard treatment protocol + fluticasone + formoterol
4. Group 4 (G4): Standard treatment protocol + fluticasone + ipratropium bromide
5. Group 5 (G5): Standard treatment protocol + fluticasone + furosemide
6. Group 6 (G6): Standard treatment protocol + fluticasone + formoterol + ipratropium bromide + furosemid

Standard Treatment Protocol for Premature Calves

Standard treatment including oxygen supplementation and supportive treatment were provided to each calf in all the groups.

Oxygen Therapy

All the premature calves received oxygen therapy via a mask (Figure 2) at the rate of 5 - 6 L / min per calf. The flow rate of oxygen was decreased to 3 to 4 L / min after 3h. Oxygen supplementation was discontinued when $SatO_2$ was > 80% after 24 hours of intranasal oxygen administration and if $SatO_2$ was < 80%, oxygen supplementation was reinstituted.

Supportive Treatment and Clinical Care

Vitamin A - D - E combination (1 ml / day / IM - Ademin, Ceva Animal Health Limited), calcium (0.2ml / kg / day / SC - Kalsimin, Vilsan Veterinary Pharmaceuticals), phosphorus (3 ml / day / SC, Catosal, Bayer Animal Health), erythromycin (10 mg / kg / day / IM - Erivet, Mira Ilac ve Yem Katki Mad. Gida San. Tic. Ltd. Sti.), vitamin C (3 ml / day / SC

- Vitce, Sanovel Pharmaceuticals) were administered every day for 3 days, along with a single dose of selenium - vitamin E combination (1 ml / IM, Yeldif, Ceva Animal Health Limited) was administrated to the each calf. Further, a single dose of hyperimmune serum (15 ml / SC, Septicol, Vetal Animal Health, containing Escherichia Coli serotypes K99, F41 and F (Y) piluses.) was administered to each premature calf. Intravenous fluids (0.9% NaCl and 5% dextrose) were administered slowly as required.

Nebulizer Treatment Protocol for Premature Calves

While calves in the experimental group received nebulizer drugs for 5 min (Figure 3), calves in the control group received only 2.5 mL saline solution (12h, during 72h) for 5 min via a nebulizer machine. The nebulizer drug combinations were administered as follows: fluticasone for Group 2 (G2), fluticasone + formoterol for Group 3 (G3), fluticasone + ipratropium bromide for Group 4 (G4), fluticasone + furosemide for Group 5 (G5) and fluticasone + formoterol + ipratropium bromide + furosemide for Group 6 (G6). Formoterol (15 μg totally / 12h, during 72h, Ventofor, Bilim Pharmaceuticals), ipratropium bromide (2 μg / kg / 12hr, during 72h, Atrovent, Boehringer Ingelheim International), furosemide (1 mg / kg / 12h, during 72h, Lasix, Sanofi Pharmaceuticals) and fluticasone (15 μg / kg / 12h, during 72h, Flixotide, GlaxoSmith-Kline Pharmaceuticals) each diluted with 2.5 mL saline solution, was administered for 5 min using a nebulizer machine sequentially at regular intervals during the study period (72h). The 0h represents the time just before the initiation of the treatment and immediately after the collection of blood sample the nebulized drugs were administered over a course of 5-20 min depending on the drug combination used.

Measurement of Total Lipid Peroxide

Lipid peroxidation products were determined from plasma by a spectrophotometric method with commercial LPO-586™ (Oxis Research, TM, Bioxytech, CA, 92202, USA).

Principle: The analysis is based on the reaction of chromogen substance N-methyl-2-phenylindole at 45 °C with MDA and 4-hydroxialkenal (LPO). One mole MDA or 4-hydroxialkenal reacts with 2 molecules of N-methyl-2-fenilindol in acetonitrile, resulting in a durable chromophore at 586 nm. The results were defined as μMol.

Measurement of Total Antioxidant Capacity

Total AOC was determined from plasma by spectrophotometric method with antioxidant capacity kit (Antioxidant Assay Kit, Sigma Aldrich CS O790, Germany).

Principle: The reaction is based on the formation of ferrilmyoglobin, which is formed by hydrogen peroxide and myoglobin and constitutes radical $ABTS^+$ cation from ABTS (2.2'-azinobis 3 ethylbenzthiazoline-6-sulfonic acid). Radical $ABTS^+$ cation is a soluble green chromogen that can be determined at 405 nm. In the presence of antioxidants, radical $ABTS^+$ cation formation is inhibited and color density decreases. As standard, a vitamin E analog, trolox, was used. The results were defined as mMol.

Acute-Phase Protein Measurement

Bovine serum Amyloid A (Cat. No: 201-04-0126) and Bovine fibrinogen (Cat. No: 201-04-0086) levels were measured on Synergy HT multi-mode microplate reader (BioTek Inc. USA) with commercial ELISA kit (SunRed®, China) method. The measurable sensitivity and test range of the SAA test was 0.15 μg/ml to 40 μg/ml and the measurable sensitivity and test range of the fibrinogen test was 0.2 mg/ml to 60 mg/ml.

Statistical Analysis

All data were presented as mean and standard errors (SEs) values (mean ± SEM). The distribution of the data was determined using the Kolmogorov-Smirnov test. One way Anova (Posthoc Duncan) was used for statistical analyses of the parameters of within groups by using the SPSS 21.0 software (USA). Values that differed significantly from the control were indicated as $P < 0.05$.

RESULTS

Lipid Peroxide

Averages and within group statistical significance values of the LPO levels of calves in all groups are presented in Table 1.

Statistically significant decreases were detected in all groups at 24th, 48th, and 72nd hours compared to 0-hour sampling time in all groups (Table 1).

Antioxidant Capacity

Averages and within group statistical significance values of the AOC levels of calves in all groups are

presented in Table 2.

Statistically significant increases were detected in all groups at 24th, 48th, and 72nd hours compared to 0-hour sampling time in all groups (Table 2).

Acute-Phase Proteins

Serum amyloid A and fibrinogen levels measured in calves of all groups at all time points and their within group statistical significance values are presented in Table 3. There was no statistical difference in the SAA and fibrinogen parameters of the calves in all groups after the treatments compared to 0-hour sampling time (Table 3).

$SatO_2$ Percentages of Venous Blood Samples

Averages and within group statistical significance values of the $SatO_2$ percentages of venous blood samples of calves in all groups are presented in Table 4. There was no statistical difference within G1 and G6. However, statistically significant increases were detected within G2 at 24. and 48th. hour, G3, G4 and G5 at 72nd. hour after the treatments compared to 0-hour sampling time within the groups (Table 4).

Table 1. Lipid peroxide product levels, standard errors and statistical significance between the groups of the premature calves (Mean ± SE).

LPO (µMol / L)	0h	24h	48h	72h
G1	0.89 ± 0.15^{a}	0.51 ± 0.12^{ab}	0.42 ± 0.10^{b}	0.30 ± 0.06^{b}
G2	0.53 ± 0.06^{a}	0.37 ± 0.08 ^{ab}	0.43 ± 0.09^{ab}	0.21 ± 0.01^{b}
G3	1.04 ± 0.14^{a}	0.57 ± 0.09^{b}	0.50 ± 0.07^{b}	0.46 ± 0.06^{b}
G4	0.87 ± 0.12^{a}	0.78 ± 0.17^{ab}	0.47 ± 0.05^{ab}	0.43 ± 0.04^{b}
G5	1.27 ± 0.21^{a}	0.73 ± 0.10^{b}	0.69 ± 0.17^{b}	0.57 ± 0.06^{b}
G6	1.02 ± 0.20^{a}	0.61 ± 0.13^{ab}	0.47 ± 0.03^{b}	0.41 ± 0.02^{b}

$^{a. b. c}$: Means in the same row with different letters differed significantly ($P \leq 0.05$). Group negative control (G1). Group positive control (G2). Group 3 (G3). Group 4 (G4). Group 5 (G5). Group 6 (G6).

Table 2. Averages of total antioxidant capacity levels, standard errors and statistical significance between the groups of the premature calves (Mean ± SE).

AOC (mMol / L)	0h	24h	48h	72h
G1	0.54 ± 0.02^{b}	0.59 ± 0.03^{ab}	0.65 ± 0.01^{a}	0.64 ± 0.02^{a}
G2	0.51 ± 0.04^{b}	0.57 ± 0.01^{ab}	0.62 ± 0.02^{a}	0.61 ± 0.02^{a}
G3	0.48 ± 0.02^{b}	0.55 ± 0.03 ^{ab}	0.55 ± 0.03 ^{ab}	0.58 ± 0.02^{a}
G4	0.58 ± 0.02^{b}	0.66 ± 0.01^{a}	0.73 ± 0.04^{a}	0.71 ± 0.03^{a}
G5	0.64 ± 0.03^{b}	0.71 ± 0.03^{ab}	0.74 ± 0.03^{a}	0.71 ± 0.02^{ab}
G6	0.54 ± 0.05^{b}	0.64 ± 0.02^{a}	0.71 ± 0.01^{a}	0.67 ± 0.02^{a}

$^{a. b. c}$: Means in the same row with different letters differed significantly ($P \leq 0.05$).

Table 3. Serum amyloid A and fibrinogen levels, standard errors and statistical significance between the groups of the premature calves (Mean ± Statistics kısmında SEs yazmışız).

Parametreler		0h	24h	48h	72h
SAA (µg / ml)	**G1**	0.40 ± 0.03	0.50 ± 0.06	0.32 ± 0.07	0.50 ± 0.04
	G2	0.43 ± 0.03	0.49 ± 0.02	0.46 ± 0.02	0.47 ± 0.02
	G3	0.50 ± 0.14	0.47 ± 0.06	0.45 ± 0.06	0.50 ± 0.14
	G4	0.55 ± 0.03	0.60 ± 0.08	0.54 ± 0.06	0.55 ± 0.07
	G5	0.50 ± 0.10	0.62 ± 0.04	0.64 ± 0.06	0.58 ± 0.04
	G6	0.42 ± 0.11	0.48 ± 0.13	0.50 ± 0.12	0.51 ± 0.12
Fibrinojen (mg / ml)	**G1**	0.35 ± 0.01	0.39 ± 0.05	0.42 ± 0.04	0.42 ± 0.03
	G2	0.39 ± 0.02	0.41 ± 0.01	0.40 ± 0.01	0.39 ± 0.01
	G3	0.36 ± 0.20	0.37 ± 0.16	0.41 ± 0.03	0.44 ± 0.13
	G4	0.33 ± 0.11	0.41 ± 0.14	0.43 ± 0.05	0.35 ± 0.16
	G5	0.43 ± 0.03	0.53 ± 0.05	0.51 ± 0.05	0.48 ± 0.03
	G6	0.42 ± 0.02	0.42 ± 0.07	0.47 ± 0.02	0.43 ± 0.01

* No statistical difference within the groups.

Table 4. $SatO_2$ percentages of venous blood samples, standard errors and statistical significance between the groups of the premature calves (Mean ± SE).

$SatO_2$ %	0h	24h	48h	72h
G1	15,67 ± 3,48	28,00 ± 6,86	21,00 ± 4,49	29,17 ± 5,33
G2	22,00 ± 3,08[b]	41,50 ± 4,98[a]	40,00 ± 6,07[a]	35,00 ± 4,29[ab]
G3	25,67 ± 2,95[b]	37,83 ± 4,28[ab]	36,67 ± 5,68[ab]	41,17 ± 5,19[a]
G4	26,00 ± 4,96[b]	37,83 ± 3,69[ab]	38,60 ± 4,4[ab]	44,20 ± 6,95[a]
G5	15,33 ± 6,26[b]	19,50 ± 3,15[ab]	26,33 ± 2,22[ab]	32,83 ± 6,61[a]
G6	20,50 ± 4,46	28,67 ± 6,15	27,20 ± 4,29	31,00 ± 7,82

[a. b. c]: Means in the same row with different letters differed significantly ($P \leq 0.05$).

DISCUSSION

In this study, changes in LPO, AOC, and acute-phase protein levels were evaluated before and after treatment in premature calves with respiratory distress syndrome (Table 1, 2, 3). Following the treatment of premature calves with RDS, the total LPO levels decreased and antioxidant levels increased gradually at 24th, 48th, and 72nd hours. A decrease in LPO levels and increase in AOC levels following the treatments (Tables 1 and 2) were probably due to improved lung function, normal gas exchange, and elimination of hypoxia in the organism. These results were in accordance with the results of Ballard et al. (2008). Premature infants are extremely susceptible to oxidative stress since they have both increased reactive oxygen species (ROS) formation and underdeveloped antioxidant defenses (Ahola et al., 2004). In this study, it was realized that premature calves were as highly susceptible to oxidative stress as premature infants (Table 1, 2). Increased need for oxygen due to immature lungs and infections is thought to contribute to increased oxygen radical formation (Moison et al., 1996; Laborie et al., 2000; Hirano et al., 2001; Dennery, 2004; Kapoor et al., 2006; von Dessauer et al., 2010). Oxidative stress levels increase in premature infants in the first day and following weeks after birth (Buonocore et al. 2002; Perone et al., 2007).

While ROS formation increases, the antioxidant defense is stated to be not fully mature or present at birth in premature infants (Frank, 1992; Buonocore et al. 2002; Georgeson et al., 2002; Ahola et al., 2004; Davis and Auten, 2010). It has been reported that antioxidant consumption increases after birth and therefore AOC decreases in premature infants (Buhimschi, 2003). It has also been shown that the concentration of glutathione (GSH) in premature infants declines rapidly in the early days of life (Jain et al., 1995; Jean-Baptiste and Rudolph, 2003; Ahola et al., 2004]. This is considered to be most likely due to increased GSH consumption associated with oxidative stress. Glutathione is one of the most important non-enzymatic intra-cell antioxidants. Glutathione in the epithelial layer fluid (ELF) of the lower respiratory tract is thought to be the first line of defense against oxidative stress (Lomaestro and Malone, 1995). The ELF concentration of GSH is 140 times higher than the serum concentration with a redox ratio of > 9:1 (Cantin et al., 1987). In this study, the decrease in total LPO levels and the increase in AOC levels during the treatment of calves in both standard treatment and nebulizer treatment group (Table 1, 2) and no differences in serum total LPO levels and AOC levels in the different treatment groups may be due to the nebulizer applications locally in the lung (Yildiz and Ok, 2017; Ok et al., 2020). The most satisfactory explanation of this condition can be the presence of glutathione (Cantin et al., 1987), which is found to be 140 times more in the lungs than in the serum. In the present study, bronchoalveolar fluid sampling was not possible because premature calves had severe RDS symptoms which could have risked different complications. Therefore, local effects of different nebulizer treatment applications on oxidation parameters could not be evaluated, which was considered to be the limitation of this research.

Hypertension in the pulmonary vessels and similar complications caused by surfactant insufficiency lead to the development of inflammation and edema in the interstitial tissues. As a result of inflammation, edema, and hypoxia in the lung tissue, changes in acute-phase proteins, cytokines, and coagulation profiles can occur (Murata et al., 2004; Eckersall and Bell, 2010). Acute-phase proteins may increase tissue damages caused by inflammation, edema, hypoxia, bacterial infection or trauma, and following transplantations and other stress situations (Murata et al., 2004; Lomborg et al., 2008). Significant changes in serum fibrinogen, serum amyloid A and haptoglobin levels have been reported in inflammations (Martinez-Subiela et al., 2003; McGrotty et al., 2005). In this study, SAA and

fibrinogen parameters were not statistically different at 24th, 48th, and 72nd hours compared to 0 hours in all groups. No changes in the acute-phase protein levels in the present study could be due to the fact that the treatments did not cause a significant change in serum acute-phase protein levels.

In conclusion, supportive and nebulizer treatment protocols were demonstrated to decrease the total lipid peroxide levels and improve the antioxidant capacity in premature calves with RDS. However, further researches are needed to evaluate the local effects of these treatments on lung tissue and also to compare these effects in calves born at term without any complications.

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CONFLICT OF INTEREST STATEMENT

None declared.

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