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ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ

EUROPEAN COLLEGES OF VETERINARY SPECIALISTS
ΕΥΡΩΠΑΪΚΑ ΚΟΛΕΓΙΑ ΕΙΔΙΚΕΥΜΕΝΩΝ ΚΤΗΝΙΑΤΡΩΝ

				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†
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General Secretary:	Athanassios E. Tyrpenou
Spec. Secretary:	Konstantinos Chandras
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The Board of Directors and the Editorial Board of the Journal of the Hellenic Veterinary Medical Society, warmly thank the reviewers that substantially contributed in the successful publication of the **70th volume 2019** of the J Hellenic Vet Med Soc, the names of which are sited below in alphabetical order:

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Prevalence and new histopathological aspects of *Haemoproteus spp.* in pigeons from Iran

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ABSTRACT. *Haemoproteus spp.* is pathogenic protozoan that effecting blood circulatory system of birds.
The present study was undertaken to evaluate the presence of *Haemoproteus spp.* in pigeons from Iran and associated histopathological changes. A total of 108 blood samples were taken from pigeons to investigate *Haemoproteus spp.* presence by blood smear and semi-nested PCR targeting the cytochrome b gene methods. Also, to evaluate histopathological changes 12 infected pigeons to *Haemoproteus* were sacrificed and studied. 34.2% of pigeons infected with *Haemoproteus* showed macro and microgametocytes in their erythrocytes while based on the molecular method 63.8% were infected. Focal lymphocytic aggregates, pigmentation and cell swelling were the main histopathological lesions in infected livers. Multifocal non- suppurative interstitial nephritis, pigmentation and splenic lymphoid hyperplasia were also seen in the infected pigeons. Mild lymphocytic myocarditis in the heart of one pigeon was the other finding. No histopathological changes were seen in brain, intestine, and pancreas. Schizonts with variable shapes and sizes were detected in infected livers, lungs, kidneys, and spleens but megaloschizonts were not found. This study also reports the molecular prevalence of *Haemoproteus spp.* in Iran

Keywords: Haemoproteus spp., Pigeon, Semi- nested PCR, Histopathology, Iran

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INTRODUCTION

Species of the apicomplexans *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* comprise a diverse group of vector transmitted parasites that infect red blood cell (in the case of *Leucocytozoon spp.*, also white blood cells) and other organs within their vertebrate hosts (Atkinson & Van Riper, 1991; Valkiūnas 1993). These parasites have served as model organisms for studies on many aspects of parasite-host interactions, including parasite-host evolution (Perkins & Schall, 2002; Ricklefs & Fallon, 2002), host life-history trade-offs (Richner et al., 1995), and sexual selection (Hamilton & Zuk, 1982).

Haemosporidian protozoa of the genus *Plasmodium* (Marchiafava et celli, 1885) and *Haemoproteus* (Kruse, 1890) have a broad geographic distribution and a diversity of vectors and have been described throughout the world as parasitizing several hosts, including birds, mammals, and reptiles (Garnham 1966; Valkiūnas 2005). In birds, these parasites, has been described as a potential cause of extinction and population decline (Van Riper III et al. 1986, Atkinson et al. 1995, 2000, Massey et al. 1996), reducing the fitness of their hosts (Lefèvre et al., 2008) and may sometimes lead to death (Donovan et al. 2008, Cannell et al. 2013). Severe infections by haemosporidian can lead to death and involve different physiopathological phenomena such as anemia, thrombocytopenia, and inflammation (Macchi et al., 2013; Cannell et al., 2013).

Haemoproteus spp. is characterized by schizogony (i.e., merogony) within visceral endothelial cells, typically of the lung, liver, or spleen, and gametocyte development in circulating erythrocytes (Bermudez 2003; Campbell 1995). The organisms are transmitted by biting flies, characteristically louse flies (Hippoboscidae) and biting midges (Ceratopogonidae). Sexual stages of *Haemoproteus spp.* occur in the intermediate host (i.e., insects) with asexual stages in the bird. *Haemoproteus spp.* infection is usually subclinical but can cause mild clinical signs (Earle et al., 1993; Macwhirter 1994; Merino et al., 2000). When clinical disease occurs, it is typically associated with anemia because of erythrocytic parasitism, (Cardona et al., 2002) frequently in immunocompromised hosts. The true extent of pathology and mortality caused by *Haemoproteus spp.* parasites remain unclear because the severe hemoproteosis and death of infected birds occur mainly during the tissue stage of parasite development, before the appearance of parasitemia. Recent PCR- based findings indicate that species of

Haemoproteus are responsible for some instances of mortality in birds. Due to the application of molecular diagnostic methods, the traditional opinion about the harmlessness and insignificant veterinary importance of avian hemoproteids is an ongoing partial reconsideration. *Haemoproteus spp.* parasites are worth more attention in veterinary medicine and in bird conservation projects (Valkiūnas 2015). Natural infection with *Haemoproteus spp.* has been reported in pigeons from Iran. For example, Tavassoli et al., (2017) examined 93 blood samples of pigeons for *Haemoproteus spp.* infection. In their study, 13 (13.97%) samples were positive in stained blood smears while 27 (24.73%) were positive by PCR. In Nourani et al., investigation (2018), 37.5% of passerine birds from the East of Iran have been detected as harboring Haemoproteus spp.

The distribution and nature of histopathological lesions and the types of schizonts present in the tissues in different species of *Haemoproteus* vary in reports published to date.

In this study, in addition to the evaluation of *Haemoproteus spp.* prevalence in Iranian pigeons, the aim was to investigate histopathological changes of different tissues. Possible existence of megaloschizonts in different tissues infected with *Haemoproteus spp.* was investigated. Some new findings in histopathological changes in pigeons infected with *Haemoproteus spp.* are discussed.

MATERIALS AND METHODS

The study was conducted in Khuzestan, a south-western province of Iran from November 2015 and November 2016. Khuzestan province has an area of about 64 236 km² (Statistical book of Khuzestan province, 2006). The province has hot and wet summers, a mild spring and cold winters.

Preparation of blood smears and morphological analysis

For the observation of blood hemoparasites, samples were collected from 108 pigeons between November 2015 and November 2016. The volume of blood collected was almost 50 µL, not exceeding 1% of the live weight of the animal, as recommended by SISBIO, Campbell (1995) and Clark et al., (2009). The blood was collected by puncture of the brachial vein and was used for the preparation of blood smears and/or for PCR testing. Thin blood smears were prepared, fixed with absolute methanol (1 min), stained with 10% Giemsa solution (30 min) and examined under oil immersion lens. More than 60 microscopic

fields of blood films at a magnification of ×1000 were examined. When no parasite was observed, another smear was examined to confirm the result. Length and width of infected and non- infected RBCs were recorded. Also, length, width, and number of granules in macro- and microgametocytes were evaluated. The infection intensity was estimated by scoring the infections as weak (<10 parasites/1000 RBCs), moderate (10-100 parasites/1000 RBCs), and severe (>100 parasites/1000RBCs) infections.

DNA extraction and molecular analysis

DNA was extracted using a genomic DNA purification kit (Cinna Gen, Iran). Partial amplification of a 390-base-pair (bp) fragment of the cytochrome b (cyt b) gene of the parasites was accomplished by PCR using the nonspecific primers PALU-F (59-GGGT-CAAATGAGTTTCTGG-39) and PALU-R (59-DG-GAACAATATGTARAGGAGT-39) as described in Martınez et al., (2009). This set of primers is unable to distinguish between *Plasmodium* and *Haemoproteus* genera, so to confirm mixed infections formed by both *Haemoproteus* subgenera, the following forward primer PALU-F1 (59-TAGTTAGCGACCCAACAC-39) was designed and used with the reverse primer PALU-R, to amplify specifically a DNA fragment of 301 bp from the *Haemoproteus* subgenera. PCR reactions included a negative control, consisting of the reaction mix and 2 μl of DNase/RNase-free water and a positive control that consisted of a DNA sample from the blood of a pigeon with positive blood smear. All PCR reactions were performed in a 20 μl mixture consisting of 10 μl Taq master mix, 1 μM primers and 5 μl DNA template. PCR cycling included an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s. This was followed by a final extension step at 72 °C for 10 min. At least 20% of the samples were randomly selected, and the amplification reaction was repeated to ensure the reproducibility of the technique. PCR products were electrophoresed in 1.5% agarose in Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet light. The samples were assigned to: (1) Parahaemoproteus subgenus when the results were positive for the first set of primers and negative for the second set, (2) *Haemoproteus* subgenus when the results were the opposite (negative and positive, respectively), and (3) unknown when both sets of primers yielded a positive result. Amplified fragments corresponding to the expected size taken from five pigeons were purified using a PCR purification kit (Vivantis, Revongen Corporation Center, 47600 Subang Jaya, Selangor Darul Ehsan, Malaysia). The PCR fragments were sequenced using specific primers and the Big Dye Terminator V.3.1 Cycle Sequencing kit in an ABI 3130 Genetic Analyzer (Applied Biosystems, 850 Lincoln Centre Drive Foster City, CA 94404, USA). Multiple sequence alignment analysis between sequences taken from samples and those from submitted to the GenBank (http://ncbi.nlm.nih.gov) was performed using nBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Histopathological analysis

In this study, we tried to investigate histopathological changes in pigeons that were apparently healthy, and they did not show any clinical symptoms of bacterial or viral infections. Also, any findings that were suspicious of other infections were excluded from the study. Finally, twelve pigeons with positive blood smears the infections of which confirmed with the molecular method were selected for histopathological studies. Pigeons were euthanized in a glass desiccator jar for open-drop anesthesia with chloroform following standard animal ethics guidelines of Iran. All experiments were performed according to the requirements of the animal welfare committee of Shahid Chamran University of Ahvaz following the Iranian Veterinary Medical Association guidelines. For histopathological analysis, tissue samples were taken from liver, heart, lung, kidney, spleen, brain, intestine and pancreas. The samples were fixed in 10% neutral buffered formalin, dehydrated in graded ethanol, embedded in paraffin wax, sectioned at 5 μm, stained with hematoxylin and eosin and Congo red, and studied with a light microscope.

Statistical analysis

Student T. test was used to compare changes of length and width of infected and non- infected erythrocytes and their nucleus. *P*-values of < 0.05 were considered statistically significant.

RESULTS

Blood smear examination

A total of 108 thin blood smears were examined and 37 (34.2%) were infected with *Haemoproteus spp.* macro and microgametocytes. The intensity of the parasitemia was 1-254 infected cells in 60 microscopic fields. Among the infected pigeons 14, 19 and four showed weak, moderate and severe infections, respectively (Fig 1).
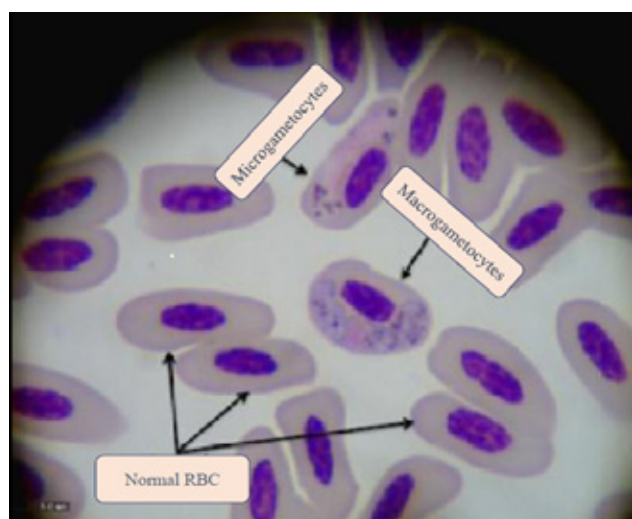
Fig 1. Normal RBCs, macrogametocytes and microgametocytes of *Haemoproteus spp.* in the blood smears of infected pigeons.

Length and width of infected and non- infected erythrocytes and their nucleus are presented in Table 1. There were not significant differences in the length of infected erythrocytes and their nucleus but there were significant differences in the width of erythrocytes infected with macro (P=0) and microgametocytes (P=0.03) in comparison with non- infected RBCs. Furthermore, nucleus width of erythrocytes infected with macro (P=0.001) and microgametocytes (P=0.004) were significantly decreased. Length, width and number of granules were 14.26 μm, 3.18 μm, 23.86 μm and 11.91 μm, 3.13 μm and 8.43 μm in macro and microgametocytes, respectively. Table 2 showed the mean of length, width and number of granules found in *Haemoproteus spp.* macro and microgametocytes.

Table 1. Length and width of infected and non- infected RBCs by *Haemoproteus spp.* in pigeons. Results are expressed as mean ± SD.

	Non infected RBC		Infected RBC with macrogametocytes		Infected RBC with microgametocytes	
	Cell	nucleous	Cell	nucleous	Cell	nucleous
Length (μm)	13.4 ± 0.7	6.7±0.5	14.2±1.3	6.6±0.6	13.7±0.9	6.7±0.4
Width (μm)	6.7±0.6	3.06±0.3	7.6±0.5	2.6±0.2	7.33±0.5	2.6±0.2

Table 2. Length, width and number of granules of *Haemoproteus spp.* in pigeons. Results are expressed as mean ± SD.

	Length	width	No. granules
Macrogametocytes (μm)	14.2±1.7	3.1±0.4	23.8±9
Microgametocytes (μm)	11.9±1.4	3.1±0.3	8.4±2.6
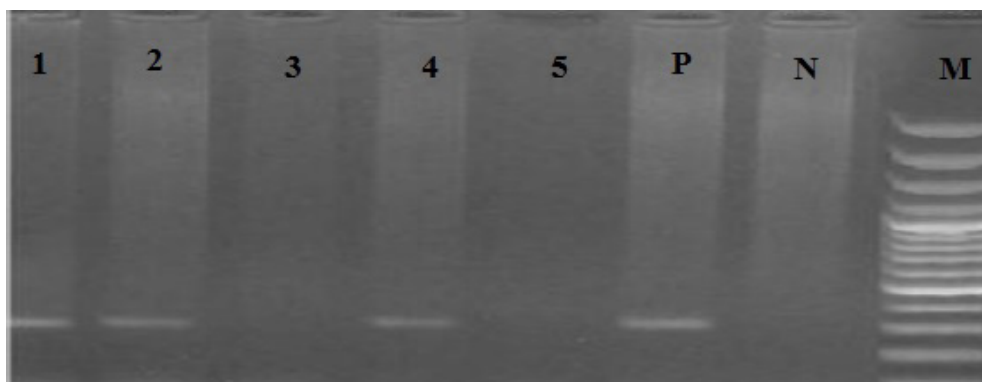
Fig 2. Amplification of *Haemoproteus spp.* DNA. Lane M is a 100-bp ladder. Lane P is a positive control DNA lane. Lane N is a negative control DNA lane. Lanes 1, 2, and 4 represent positive field samples. Lanes 3 and 5 represent negative field samples.

Molecular analysis

In 69 samples of these 108 examined samples, the PCR was positive and a band of approximately 300bp was seen on the agarose gel which considered as infection with *Haemoproteus spp.* (63.8%) (Fig. 2). All sequenced samples were found by BLAST analysis to be closest to the *Haemoproteus spp.* gene in GenBank with a similarity of ≥ 98%.

Pathological findings

The gross pathological changes of the liver were characterized by darkening and enlargement of the organ. Histopathologically, focal hepatic lymphocytic aggregations with variable sizes were seen in infected birds (Fig 3). Also, mild infiltration of heterophils was seen in the aggregates in three samples. Based on the number of aggregations, liver involvement was

categorized in weak (1-3), moderate (4-7) and severe (>7). One, five and six livers had weak, moderate and severe lymphocytic aggregations. Furthermore, in the most severe cases, accumulation of lymphocytes was also seen in many sinusoids. Hepatic pigmentation was detected in all infected pigeons. Yellow to dark brown refractile intracytoplasmic particles were detected in RBCs, endothelial cells, Kupffer cells, leukocytes and hepatocytes (Fig 4). Cell swelling, mac-

rovesicular lipidosis, multifocal heterophilic hepatitis (Fig 5) and telangiectasis were other findings. Hepatc cirrhosis was diagnosed in one of the infected birds. Fibrosis, biliary ducts hyperplasia, nodular regeneration of hepatocytes, pigmentation, lipidosis, diffuse cell swelling and infiltration of heterophils, macrophages and lymphocytes were seen in this specimen. Different microscopic lesions of livers and their frequencies are presented in Table 3.

Table 3. Frequency of histopathological lesions found in liver of pigeons infected by *Haemoproteus spp.*

Histopathological lesions	Frequency (%)
Focal lymphocytic aggregates	12 (33.3)
Pigmentation	12 (33.3)
Cell swelling	8 (22.2)
Lipidosis	1 (2.8)
Multifocal heterophilic hepatitis	1 (2.8)
Telangiectasis	1 (2.8)
Cirrhosis	1 (2.8)
Total	36 (100)
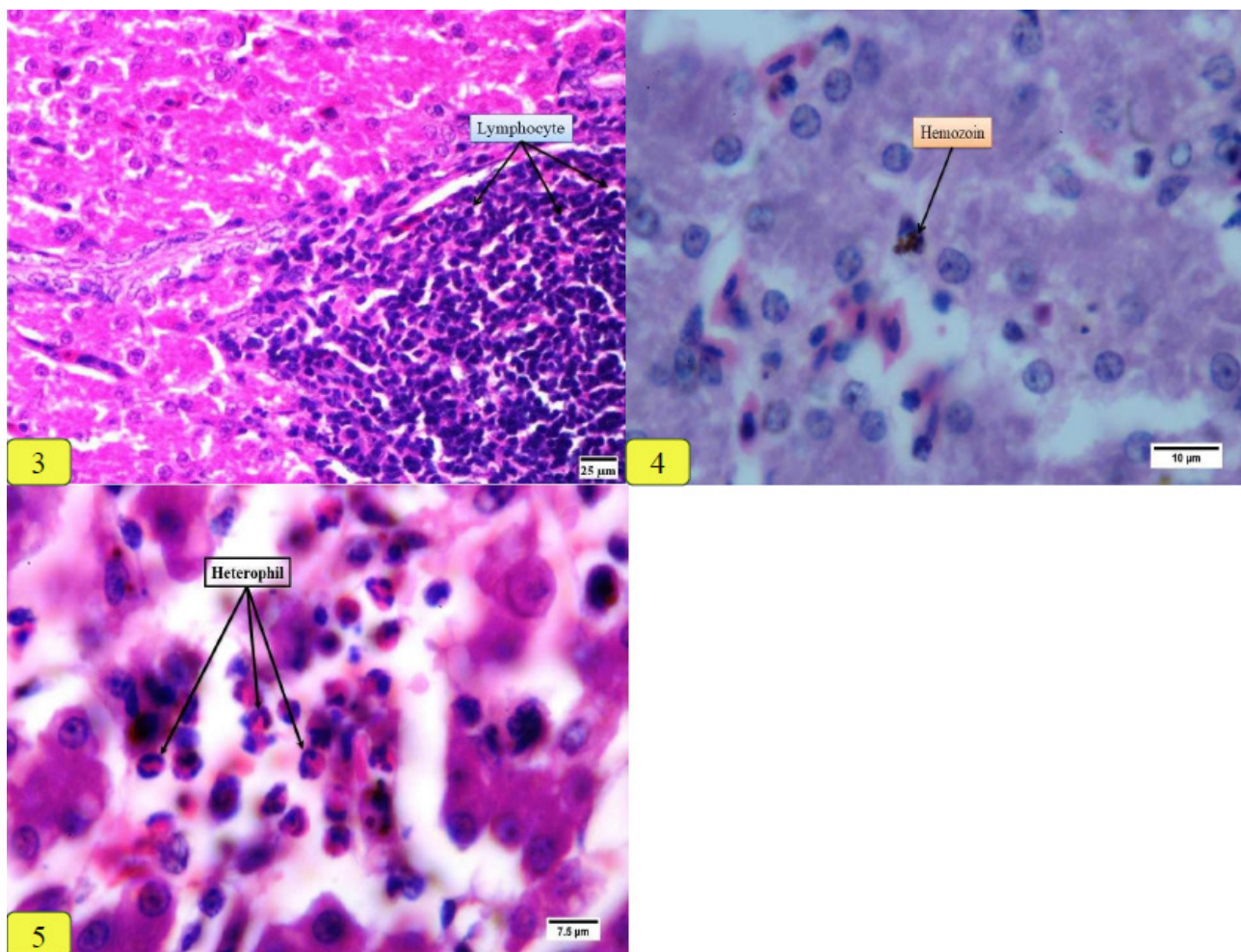
Fig 3-5. Cross sections of pigeon's liver infected by *Haemoproteus spp.* (H&E). (3) Tissue section indicating focal lymphocytic aggregation. (4) Tissue section indicating hemozoin pigment in a Kupffer cell within a sinusoid. (5) Tissue section showing focal heterophilic hepatitis.

There were no obvious gross changes in kidneys, but histopathological study showed multifocal non-suppurative interstitial nephritis (Fig 6).
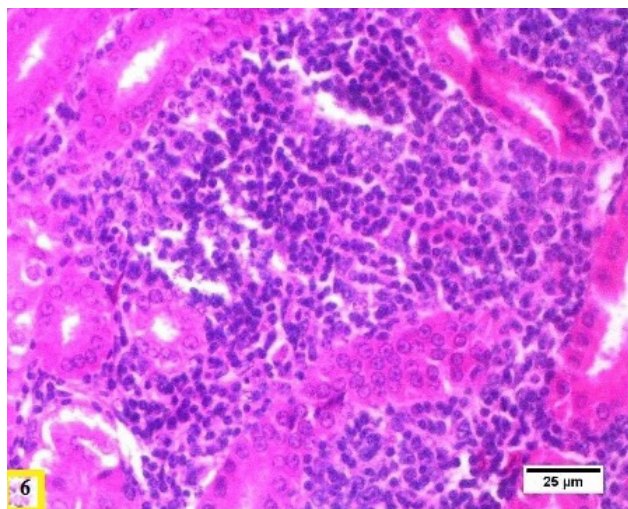
Fig 6. Tissue sections of pigeon's kidney infected by *Haemoproteus spp.*. (6) Tissue section showing non- suppurative interstitial nephritis with infiltration of mononuclear leukocytes (mainly lymphocytes) between tubules (H&E).

Table 4 represents kidneys histopathological changes with their frequencies. Pneumoconiosis was diagnosed in the lungs. Table 4 represents pulmonary histopathological changes and their frequencies. Various degrees of splenomegaly and darkening were noted in spleens in gross study. Severe (6 samples) and moderate (6 samples) hyperplasia of splenic white pulp accompanied with pigmentation were detected in 12 infected animals (Fig 7,8). Also, white pulps contained lymphoid follicles that were dilated or transformed into hyaline blocks. In heart of one infected pigeon mild lymphocytic myocarditis was detected. Brain, intestine and pancreas had no gross or histopathological changes.
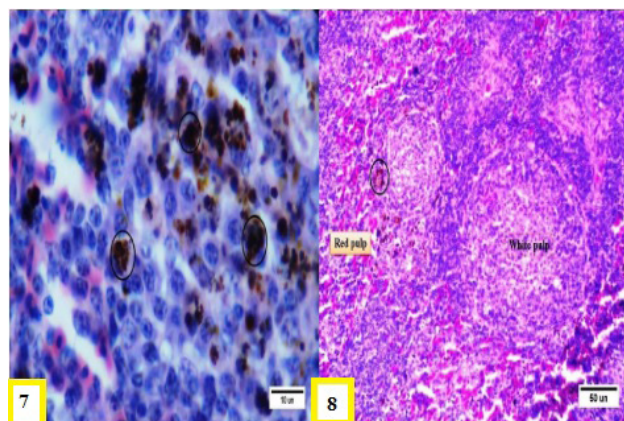
Fig 7 and 8. Cross sections of pigeon's spleen infected by *Haemoproteus spp.* (H&E). (7) Tissue section with hemozoin pigments (encircled) and few erythrocytes within the white pulp. (8) Tissue section showing white pulp hyperplasia and hemozoin pigments among red pulp.

In histopathological study of different tissues, schizonts with variable shapes and sizes were detected in endothelium of microcirculation of livers, lungs, kidneys and spleens in infected pigeons (Fig 9).
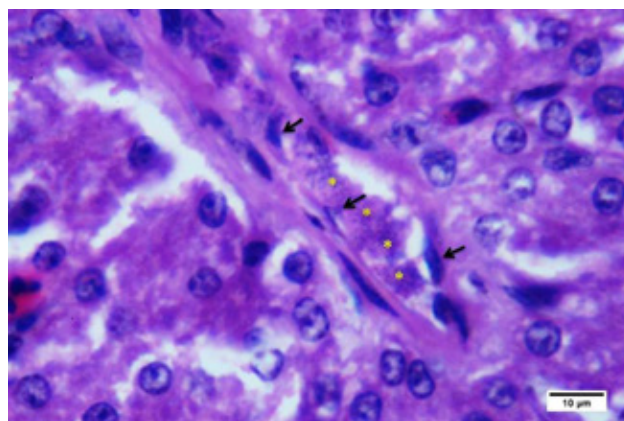
Fig 9. Schizonts of *Haemoproteus spp.* (stars) in the hepatic microcirculation of infected pigeon (short arrows show endothelial cells) (H&E).

Table 4. Frequency of histopathological lesions found in different tissues (kidney, lung and spleen) of pigeons infected by *Haemoproteus spp.*

Tissue	Histopathological lesions	Prevalence (%)
Kidney	Multifocal non suppurative interstitial nephritis	11 (84.6)
	Suppurative interstitial nephritis	1 (7.7)
	Amyloidosis	1 (7.7)
	Total	13 (100)
Lung	Pneumonoconiosis	8 (100)
	Total	8 (100)
Spleen	Pigmentation	12 (50)
	White pulp hyperplasia	12 (50)
	Total	100

DISCUSSION

Throughout the world, the prevalence of *H. columbae* in pigeons in different geographical locations varies from 14 to 100 %. It has been reported to be 65.8 % infection in San Juan, Puerto Rico (Mclaughlin 1968), 70.4 % in Izmir, Turkey (Tolgay & Cesitli, 1972), 76.5 % in Kampala, Uganda (Dranzoa et al., 1999), 80 % from Sebele (Mushi et al., 2000), 57 % in wild pigeons of Ankara (Gicik & Arslan 2001), 37 % in domestic pigeons of Morogoro municipality of Tanzania (Msoffe et al., 2010) and 17.47 % from Ganbad, Golestan Province of North Iran (Youssefi et al., 2010). Most of the current data on haemosporidians, their distribution, vectors, parasite- host interactions and seasonality of infections have been collected primarily by microscopy (Atkinson & van Ripper 1991; Valkiūnas 2005). New molecular techniques have been considered to improve the detection of vector-borne blood parasites (Bensch et al., 2000; Ricklefs & Fallon 2002; Waldenstorm et al., 2004). Based on the results of the present study 34.2% of pigeons were infected with *Haemoproteus spp.* macro and microgametocytes by microscopic examination while, semi-nested PCR method detected 63.8%. Tavassoli et al., (2018) examined 93 blood samples from Iranian pigeons for *Haemoproteus spp.* In their study, 13 (13.97%) samples were positive in stained blood smears for *Haemoproteus spp.* and 27 (24.73%) were positive by PCR. Their results also revealed that PCR had higher sensitivity in detecting *Haemoproteus spp.* in pigeons. According to some studies, and based on the results of the present study, PCR tests were many-fold better than microscopy for detecting chronic blood parasite infections (Jarvi et al., 2002; Durrant et al., 2006). It has recently been shown that relative to microscopy methods, the PCR- based molecular methods generally provide higher estimates for *Haemoproteus spp.* (Garamszegi 2010).

It is known that after an initial acute phase of infection by blood parasites, the host develops chronic, low intensity parasitemia that is regulated by host cellular and humoral immunity. Whereas high-intensity acute parasitemias are typically easy to monitor and quantify by inspecting blood smears (Valkiūnas et al., 2008), the use of microscopy for diagnosing chronic, low-intensity infections may considerably underestimate parasite prevalence (Jarvi et al., 2002). Our results support conclusions of previous studies about insufficient sensitivity of microscopy when parasitemia is low. The low numbers of *Haemoproteus spp.* from microscopy samples may be explained mainly by difficulties to detect the patent infections of this haemosporidian genus during examining of blood smears of exceptionally light infections when just a few parasites are present in samples.

It is important to emphasize that, in most cases, the identification of haemosporidian species occurs through features observed by microscopy, such as the erythrocytic stages, including the length, width, area, size and number of hemozoin granules (Garnham 1966; Valkiūnas 2005; Martinsen et al., 2006). Therefore, this technique is still of great importance for the diagnosis of haemosporidian and should continue to be used together with molecular analyses.

In the present study histopathological changes of different tissues of naturally infected pigeons were investigated. Because schizogony does not occur in erythrocytes, infections cannot be experimentally transmitted by blood transfer and tissue transmission attempts rarely have been successful (Bierer et al., 1959). Host specificity, difficulties associated with experimentally infecting birds, limiting the number of feasible experimental models and few identified vectors also have restricted laboratory studies on the adverse effects of these parasites. Therefore, similar to our study most of the research about *Haemoproteus spp.* are based on natural infections. Histopathological lesions associated with *Haemoproteus* species occur in the pre-erythrocytic stages, which result from the formation of schizonts that can occur in a variety of cell types, and their presence causes tissue damage that may lead to the death of birds (Atkinson et al., 1986, 1988; Cardona et al., 2002; Donovan et al., 2008; Cannell et al., 2013). The following changes have been reported as related to *Haemoproteus spp.* infection: splenomegaly, hepatomegaly, multifocal hemorrhages in liver and spleen, necrosis (hepatocellular, liver, splenic, and cardiac), deposition of pigment (in Kupffer cells, hepatocytes, and macrophages of the lung and spleen), tissue displacement and inflammation (in the spleen, liver, lungs, and heart), and the presence of megaloschizonts in the liver and spleen, which were surrounded by a hemorrhagic inflammatory infiltrate composed of macrophages, heterophils, giant cells, and red blood cells (Atkinson et al., 1988; Peirce et al., 2004; Donovan et al., 2008; Cannell et al., 2013). Therefore, these results indicate that the histopathological changes found in this study may be related to *Haemoproteus spp.* But there were some differences in our study. Our main difference is the absence of megaloschizonts. Numerous uninucle-

ar merozoites, which are asexual stages of spreading with the vertebrate host, develop in exoerythrocytic meronts. The latter develop mainly in the endothelial cells and probably in fixed macrophages, while in some species the meronts mature in myofibroblasts. There are several generations of the exoerythrocytic development, during which the parasite gradually adjusts to the host. In the present study the meronts most frequently were found in livers of infected pigeons and less often in spleens, lungs, kidneys and hearts and they were variable in shape and size. Although most of the investigations have shown that the lungs have the high level of schizonts. Also, in most of the studies megaloschizonts of *Hamoproteus* have been detected while none of the infected pigeons in our study showed megaloschizonts. Albeit it should be mentioned that Valkiūnas (2015) believed that some species like *H. handai* and *H. mansoni* (syn. *H. meleagridis*) are able to produce huge meronts in the endothelial cells of capillaries, in myofibroblasts of the skeletal musculature, in the heart muscle, and sometimes in other muscular organs. Earle et al., (1993) suggested that all species of *Haemoproteus* are probably capable of forming schizonts in a variety of tissue and that the number of different tissues containing schizonts depends on the density of infection. In Dey et al., (2010) study comma shaped schizonts of *Haemoproteus spp.* were found in liver parenchyma accompanied with reactive cells. In their study the schizont like structures was seen in liver parenchyma which was surrounded by neutrophils. Hepatic cords were found to disappear in the affected area. The morphology of the schizonts identified here as those of *H. columbae* resemble those described in the literature by several authors including Baker (1966). However, Earle et al., (1993) described a range of morphological forms including megaloschizonts, from what was claimed to be *H. columbae* from a Bleeding-heart Dove in South Africa. As discussed by Lederer et al., (2002), the host from which Earle et al., (1993) described the schizonts is not endemic to Africa, and no such forms have been described from indigenous columbiform hosts. Thus, the conclusions were drawn by Earle et al., (1993) should be interpreted with caution as other parasites may have been involved. Although large schizonts have been recorded, the only true megaloschizonts confirmed for a species of *Haemoproteus* based on controlled experimental studies are for *H. meleagridis* in turkeys (Atkinson et al., 1986). The status of schizonts in psittacid tissues is unclear. Large megaloschizonts described

from tissues of imported psittacines in Europe were initially thought to be an aberrant form of *Leucocytozoon*, even though no blood forms were ever observed (Walker & Garnham 1972; Peirce and Bevan 1977). However, subsequent studies suggested that these large multilocular schizonts were in fact *Besnoitia spp.* (Bennett et al., 1993; Peirce 1993). Schizonts with similar morphology have been observed in Pied Currawongs (Lederer et al., 2002). Overall, based on the results of the present study it seems that we should not expect megaloschizonts in pigeons infected with *Haemoproteus*.

In the present study focal lymphocytic aggregates, pigmentation, cell swelling and lipidosis were the histopathological findings in the liver. Nermean et al., (2016) claimed that liver histopathological changes of pigeons infected with *H. columbae* revealed a granuloma-like round cell infiltration formed mainly of small lymphocytes, many plasma cells, and schizonts of the parasite in and around the granuloma. As a rule, granuloma refers to a mass consisting mainly macrophages and its related cells (epitheloid and multinucleated giant cells), therefore it seems that in fact, the intended granuloma in their study was lymphocytic aggregates. Histopathological findings of the present study revealed pigmentation of liver, spleen, kidney, and lungs. The hem groups released from the digestion of the hemoglobin of infected red blood cells are aggregated into an insoluble material called hemozoin (Pagola et al. 2000). The α-hematin (ferriprotoporphyrin IX), which is toxic to the parasite, is released during hemoglobin digestion. However, and most possibly as a protection strategy, the parasite transforms α-hematin into hemozoin (chemically identical to β-hematin), a molecule with paramagnetic properties (Orjih 2001). In the present investigation, histopathological analysis of kidneys showed multifocal non-suppurative interstitial nephritis. Blockage of vessels, destruction of renal tubules and nephritis (consisting of granulocytes, macrophages and lymphocytes) were reported by Peirce et al., (2004) as histopathologic findings of *Haemoproteus spp.* infection. White pulp hyperplasia and pigmentation were the main histopathological changes in spleens of the infected pigeons of the present study. It should be mentioned that Tostes et al., (2015) reported alterations such as disorganization, intense congestion of the red pulp, hyperplasia, and hypertrophy of macrophages, brownish pigmentation, and splenic cords transformation into fibrous bands in caracaras infected with *Haemoproteus spp.* In the present study heart

of one infected pigeon showed mild lymphocytic myocarditis. In the study of Tostes et al., (2015) there were signs of myocardial and pericardial congestion in tissue sections of the hearts of infected caracaras. Overall, based on the molecular method 63.8% of pigeons were infected with *Haemoproteus spp.*. By comparing the pathological lesions of the present study with the results of other investigations, some similarities and differences were found. The differences may be related to different species of parasite, different hosts or even misdiagnosis of lesions. Focal lymphocytic aggregates, pigmentation and cell swelling were the main histopathological lesions in infected livers. Multifocal non- suppurative interstitial nephritis, pigmentation and splenic white pulp hyperplasia were also seen in the infected pigeons. Mild

lymphocytic myocarditis in the heart of one pigeon was the other finding. No histopathological changes were seen in brain, intestine, and pancreas. Schizonts with variable shapes and sizes were detected in infected livers, lungs, kidneys, and spleens but megaloschizonts were not found. The main finding of this study was the absence of megaloschizonts of parasite in different infected tissues.

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CONFLICT OF INTEREST
None declared.

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Development of Enzyme Linked Immunosorbent Assay for humoral immune response and infection monitoring of anthrax

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ABSTRACT. Immune assays were taken into consideration to diagnose and quantify metabolites such as antigen and antibody. Enzyme-Linked Immunosorbent Assays (ELISAs), which are used to detect antigens and antibodies, generated several periods of infectious and vaccination conditions. There is an extensive range of commercial infectious disease ELISA kits useful for the detection of human and animal IgG, IgA, IgM antibodies and microorganism antigens. Anthrax is one of the serious infectious diseases caused by rod-shaped, gram-positive bacteria known as *Bacillus anthracis*. Subunit or attenuated vaccines applied against anthrax disease increase the antibody against the Protective Antigen (PA) which has a critical role as a toxin of *B. anthracis*. Herein, the ELISA was developed using PA domain 4 and anthrax Lethal Factor to detect IgG antibody in serum. Besides, the level of anti-LF antibodies were determined as a complementary test to measure variance in antibody titers associated with vaccination or infection that leads to detection of anthrax in livestock. The results show that we developed high-quality ELISA kit that can be used to test immunogenicity of vaccines and infections in mice. We tried to develop the Anti- PA4 ELISA kit and conduct the validation studies to evaluate the fluctuation level of the antibody in the anthrax vaccine and distinction between disease and vaccination in mice.

Keywords: PA4; Anthrax; Humoral immunity; validation; ELISA.

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INTRODUCTION

Enzyme-linked Immunosorbent Assays (ELISAs) as an immune assays approach are applied to detect antigens and antibodies throughout the infectious and vaccination situation. Nowadays, a broad range of commercial ELISA kits is being utilized to detect human and animal IgG, IgA, IgM antibodies and microorganism antigens. Since its discovery 80 years ago, the live attenuated Sterne strain (34F2) of *B. anthracis* has been successfully used as the predominant method to immunize livestock against anthrax, leading to the control of several positive cases of anthrax in different species (Tadayon et al., 2016). The mechanism employed by such vaccines is to trigger the immune system against the protective antigen (PA) of the *B. anthracis* (Dumas et al., 2017). Anthrax toxin is composed of protective antigen (PA), lethal factor (LF) and edema factor (EF). All of which play a significant role in boosting the immune system's response to such vaccines; however, the PA (among the three component PA, LF and EF) is the most effective factor in increasing the immunogenicity in *B. anthracis* toxin (Zai et al., 2016). Although the existing vaccines such as AVA are safe and effective, there has been a surge in the need for anthrax vaccines due to the increased terroristic attacks in the recent decades. Furthermore, the prevalence of such bacteria among humans has highlighted the necessity of research and developing methods of producing subunit vaccines (Malik et al., 2018). PA is one of the most important antigens in the anthrax which is used in the design of vaccines and several commercial diagnostic kits. It plays a key role in the pathogenesis of *B. anthracis*, and if the antibody against PA is produced, it can prevent the binding of PA to the cell surface, and eventually the function of *B. anthracis* toxin will be impaired{Male, 2017 #109} (Goldsteinet al., 2017). The anthrax PA is a four-domain protein that each part plays a specific role (Male et al., 2017). The fourth domain maintains the structure and binding to the host receptor Capillary morphogenesis protein 2 (CMG2) which leads PA to bind to the cell. So the PA domain 4 (PA4) has a critical role in the function of PA and the toxin of *B. anthracis* (Mamillapalli et al., 2017). Subunit vaccines or attenuated vaccines applied against anthrax disease increase the immune systems' antibody against the PA. Therefore, the cornerstone of a vaccine showing its protection and efficacy in curbing the disease is the power of antibody applied against the PA (Sim et al., 2017). {Sim, 2017 #113}

Testing the potency of anthrax vaccine entails us-

ing a virulent strain of *B. anthracis* so that the real immunization of the vaccine would be determined (Moazeni et al., 2007). Conducting bioassay and lethal challenge on laboratory animals with the virulent strain entails the use of specialized containment equipment including class-3 facilities, proper environment and special laboratory tools. Such facilities are not available everywhere (Milleret al., 2012). On the other hand, testing the immunization of a vaccine in field and clinical studies entails the adoption of a safe and simple method (Ionin et al., 2013). The existing ELISA kits measure the practicality of antibodies against PA. The amount of antibody produced against PA shows the stimulated cases, (human or animal) possibility of exposure to the microorganism or the vaccination (Laws et al., 2016). However, determination of the subjects' exposure to the microbe or vaccine is not possible with the available ELISA kits.

Studies show that the most specific antibody against the *B. anthracis* is the antibody produced against the PA4. That is because the generated anti-PA4 prevents PA's binding to the receptors in the cell surface and consequently prohibits the LF and EF from entering the cell and causing the disease. Therefore, measuring the titer of anti-PA4 following the injection of PA4 to mice can provide a safe and simple method to show the potency of such vaccines (Williamson et al, 2015). On the other hand, the use of the PA4 in the ELISA kit design can be indicative of an effective immune response against the disease as compared to other commercial types.

The present study tries to use the new recombinant antigen PA4 to survey the pattern of humoral immunity responses kinetically. It is done after the injection of AVA, Razi anthrax vaccine and PA4 antigen with aluminum hydroxide adjuvant. In order to differentiate the infection caused by the *B. anthracis* and the immunity obtained from injecting several anthrax vaccine, virulent strain *B. anthracis* (17JB) with a sub lethal dose was used to survey the rise of produced antibody against PA4 and LF. The Anti-PA4 ELISA was developed and the validation studies were conducted to evaluate the antibody titer in the anthrax vaccine in mice. The validation purpose of this approach is to show that such method suits our study. All of the needed instructions have been followed to validate the study based on the international Conference of harmonization (ICH). (Pombo et al., 2004).

MATERIALS AND METHODS

Antigens and Vaccines

The new recombinant PA4 and the LF antigens were supplied from Razi Vaccine and Serum Research Institute's Immunology Department (Karaj, Iran) and kept at the temperature of -70 °c. The AVA vaccine was supplied from Biothrax Company. The Razi anthrax vaccine, which is a *B. anthracis* live spore vaccine, and the *B. anthracis* strain 17JB were supplied from Razi Vaccine and Serum Research Institute.

Reference Serum

The domestic reference serum dubbed 'Pool' used in the research as the PA4 antigen (25 µg) was mixed with the aluminum hydroxide adjuvant 2.5% V/V, which was diluted 1/10 with PBS, and 0.25mL of the mixture was injected subcutaneously to ten mice Balb/c in the timespan of thirty days. The blood sampling was conducted in the weeks 1, 2, 4, 6, 8, and 10 and the serum was collected afterwards. The serum attained from 3 mice with the most OD (>2.5) during those dates was selected and mixed together. The serum pool of its antibody was conventionally set as 800 EU/mL. It was divided into 0.2 mL micro tubes and kept at the temperature of -70 °C (Pombo et al., 2004).

Samples

Experimental Balb/C mice were allocated into five groups, each containing ten animals. Mice of the first three groups were treated in the days 1 and 15 with 0.25mL AVA vaccine, 0.25mL of The Razi Anthrax vaccine and 25µg PA4 antigen with aluminum hydroxide adjuvant, respectively. The fourth group contains the virulent strain of *B. anthracis* (17JB) in which 150 *B. anthracis* live spore was injected subcutaneously; the sampling was carried out on third and fifth weeks. For the last group, Normal Mouse Serum (NMS) was applied to be used as a negative control (Xiang et al., 1994). All serums kept at the temperature of -70 °C. All experimental protocols were conducted according to the principles described in guidelines for care and use of laboratory animals and approved by the Research and Ethics Committee of Babol University of Medical Sciences (MUBABOL.REC.1395.175).

ELISA for anti-PA4 and anti-LF antibody measurement

A checkerboard titration was carried out to settle the optimum antigen concentration and conjugation was diluted by blocking buffer just before being used in the ELISA. Antigen concentrations considered in the study included 200 ng/well of PA4 and 100 ng/well LF in 0.05 M Carbonate-Bicarbonate, and pH 9.6 that were coated on 96 well ELISA plates (Jet bio, Canada) and incubated overnight for 24 h at 4°C (amount of proteins were determined by Bradford assay). Plates were washed once with phosphate buffered saline (PBS) and then they were blocked with 200 µL/well of 3% skimmed milk (Sigma) for 24 h at 4°C. After washing once with PBS, the plates were incubated with 100 µl/well controls and test samples at a dilution of 1/80 in the dilution buffer (3% skimmed milk) for 2 h at 37°C. Each serum sample was tested in triplicate. The wells were again washed for four times with PBS and incubated with 100 µL/well Anti-mouse IgG HRP (Sigma USA) diluted to 1:40,000 in the dilution buffer and incubated at 37°C for 2h. Finally, washed for five times with PBS, unbound antibodies were removed and the bound antibodies were detected virtually after 15-20 minutes in the dark using 100µl of 3,3',5,5'-tetramethylbenzidine (TMB), containing hydrogen peroxide (Sigma) as the substrate along with H_2O_2. The reaction was stopped by adding 50µl of HCL 5.8% and absorbance was read in wavelength 450 nm against 630 nm second filters in an ELISA reader (Stat Fax. USA) and ELISA value was obtained (Ndumnego OC et al., 2013). All washing steps were carried out with micro plate washer (STAT FAX, USA).

Linearity

A total of seven serum samples of varying antibody concentrations, S1 through S7, were used for the assessment of linearity. The S1 is the domestic reference serum 'Pool'. Of the S1, the samples S2 through S7 were attained with dilutions of 1/2 to 1/64. The NMS was used for the dilution of all samples. All samples, S1 to S7, were put in three different plates in triplicate. As per protocol, the amount of anti-PA4 in all samples was obtained based on optical density (OD). The curve linearity was obtained from the analysis of the empirically observed log10 amount of antibody in the samples and finally was reported as EU/mL. Through the attained results, the P-value, the correlation coefficient, the y-intercept and the slope of the regression line were calculated (Semenova et al., 2017).

Accuracy

To perform the accuracy test, the samples S1, S3, S5 and S7 were used. In this test, coating was carried

out according to paragraph 2.5; the amount of anti-PA4 was assessed based on OD and reported as EU/mL. The accuracy is expressed as percent recovery and is calculated with the following formula: Mean estimated unitage per mL / assigned unitage per mL × 100.

The calculated concentration from each sample shows the average geometrical estimation based on the S1 which is corrected by the dilution of each sample (Pombo et al., 2004).

Precision

The assessment of repeatability (Intra-plate, Inter-plate, and Intra-day) and intermediate precision between the days and analysts was conducted according to the definitions ICH. Precision was determined using three samples namely S1, S3 and S5 which were in accordance with linearity tests. In this stage, coating was also conducted according to paragraph 2.5. Based on the protocol, the amount of anti-PA4 was measured independently by two analysts in three consecutive days and then the average OD was assessed for each sample. Finally, the results were expressed in EU/mL and the results of OD for each sample were calculated by %CV (SD/mean×100) (Semenova et al., 2012).

Quantification Limit (LOQ)

To perform this test, three samples namely S5, S6 and S7, which indicated a low concentration of the antibody, were used. Coating was conducted according to paragraph 2.5. According to the protocol, the amount of anti-PA4 was measured based on OD in three non-consecutive days within an eight-day period. The average OD was calculated for each sample and at the end, the antibody was reported by EU/mL for each sample. The results of OD were assessed by %CV (SD/mean×100) (Ghosh et al., 2015).

Detection Limit (LOD)

Detection limit (LOD) was obtained using two NMS serums with the dilution of 1/80 and each sample was divided into two different plates in triplicate. Anti-PA4 was measured based on OD according to ELISA protocol in five non-consecutive days in a two-week period. The LOD was estimated by interpolating the mean of all 68 OD values, plus standard deviations. The LOD was calculated by the following formula: Mean (OD) + 3SD; then, it was reported in EU/mL (Ghosh et al., 2015).

Specificity

To perform this test, besides the Pool and NMS serums, other serums namely Foot-and-mouth Disease (FMD), Pasteurella (PAS), Agalactia (AG) and diphtheria pertussis (whooping cough) and tetanus (DTP), which were obtained in previous studies, were used. Three different dilutions (1/80, 1/160 and 1/320) were prepared from each sample using the NMS serum. The serums were put in two separate plates and ELISA value was obtained. Only one dilution (1/80) was used for NMS.

Competitive Inhibition ELISA

To determine the specificity of the measurements performed by ELISA, competitive inhibition ELISA (CI-ELISA) has been developed based on the qualified anti-PA4 IgG ELISA. The CI-ELISA was performed by using following modifications of the standard ELISA procedure. 96-well polystyrene plates (Jet bio, Canada) were coated at 4°C for 24h with 200 ng/well PA4 antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6) per well. The plate was washed once with phosphate buffered saline (PBS). After the blocking with 200 μl skimmed milk %3 at 4°C for 24h and washing once, 100 μl of 1:80 dilution serum and 780 pg to 800ng of PA4 were added and incubated at 37°C for 2h. limiting concentration was determined empirically by the titration of anti-PA4 in standard ELISA. The wells were washed four times with PBS and incubated with HRP anti-mouse immunoglobulin G (Sigma, USA.) at 37ºC for 2h. Finally, wells were washed five times with PBS, 100μ of substrate buffer containing TMB per mL was added, and the mixture was incubated for 15-20 min. In the follow-up step of ELISA, reactions were stopped by adding 50μl of Hcl5.8%, and the optical density (OD) at 450 nm and 630 was determined and ELISA value was obtained. (Ndumnego et al., 2013; Mitic et al., 2016).

Comparing the amount of antibody against PA4 and LF antigens in the vaccinated mice with the mice injected with virulent strains of *B. anthracis*

The two antigens PA4 (200ng) and LF (100ng) were coated in two separate plates to compare the antibody against the LF and PA4 in sera of the vaccinated mice and the mice injected with virulent strains of *B. anthracis*. Tests were performed in duplicate.

RESULTS

Performance Characteristics of the Anti-PA4 IgG ELISA

The assessment of Antibody development pattern proved that all vaccinated mice had anti-PA4 as determined by ELISA, with ELISA values which gradually increased until week 4, then slowly declined (Figure. 1). Antibodies were detectable by 2 to 4 weeks after vaccination (Figure. 1). Sera from the NMS did not develop titers by ELISA (Figure.1).
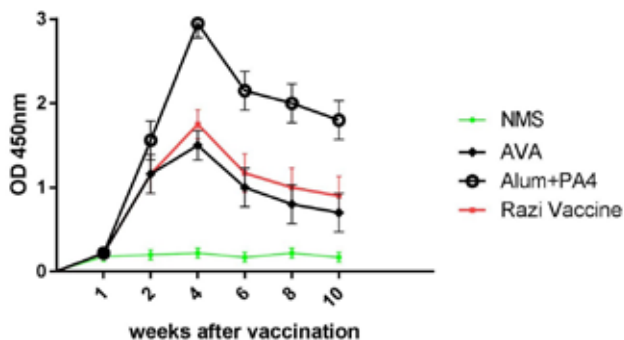
Fig. 1. Antibody levels in vaccinated and NMS mice as determined by ELSA. Open symbols indicate vaccinated mice, and solid symbols indicate NMS mice. Vaccinated mice were inoculated with PA4 antigen plus adjuvants, Razi Vaccine and AVA subcutaneously.

Linearity

After calculating the log10, the obtained OD values from all of the samples, S1 through S7, showed that the curve in the range 14-892 EU/mL was completely linear and the values showed to be as follows: $r^2 = 0.9628$, *P- value* < 0.005 and Y= ax + b (Y= 0.9601x+b) (Figure.2).
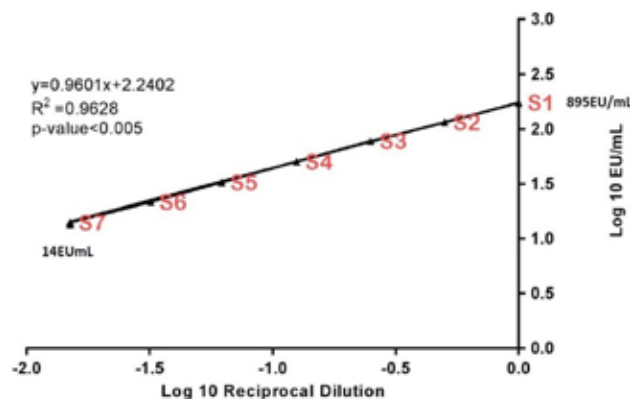
Fig. 2. Linearity curve. EU/mL of seven dilutions of S sera (the pool as domestic reference serum) are plotted against varied dilution (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64). Each point represents the estimated EU / mL of each unit that was tested three times.

Accuracy

The accuracy results in Table-1 are stated in the form of percent recovery. To perform the test, the range between 16-1000 EU/mL was studied and the percent recovery came out to be between 90-117%. The resulted percent fits the validation criteria according to Table 2.

Table 1. Accuracy assessment. The accuracy results are stated in the form of percent recovery.

	Replicates	Target sample (EU/mL)			
		S1 (1000) % Recovery	S3 (250) % Recovery	S5 (62.5) % Recovery	S7 (16) % Recovery
Plate 1	1	94.3	100	82	110
	2	95	101	84	117
	3	112	102	93	120
Plate 2	1	104	100	90	130
	2	110	108	92	120
	3	108	110	90	114
Plate 3	1	93	103	90	130
	2	103	100	103	100
	3	102	103	89	110
Mean		102.36	103	90.33	116.77

Table 2 .Acceptance criteria and characteristics of the anti-PA4 mouse ELISA. To perform the test, the range between 16-1000 EU/mL was studied and the percent recovery came out to be between 90-117%.

Assay characteristic	Acceptance criteria	Results
Prescision-repeatability	%CV < 20	%CV ≤ 13.7
Intra-plate		%CV ≤ 15
Inter-plate		%CV ≤ 12
Intra-day		
Intermediate prescision	%CV ≤ 25	%CV ≤ 18
Days	%CV ≤ 30	%CV ≤ 7
Analysts		
Accuracy	80 - 120% Recovery	90 - 117% Recovery
Limit of detection (LOD)	-	7 EU/mL
Limit of quantitation (LOQ)	-	14 EU/mL
Linearity	-	Working range: 27 - 892 EU/mL $r^2 = 0.96$

Table 3. Specificity. To perform this test, besides the Pool and NMS serums, other serums namely Foot-and-mouth Disease (FMD), Pasteurella (PAS), Agalactia (AG) and diphtheria pertussis (whooping cough) and tetanus (DTP) were used.

Sample Pool	Dilution in well	Elisa values	
		Plate 1	Plate 2
	1:80	99.192	92.419
	1:160	77.943	80.846
	1:320	67.096	61.935
FDM	1:80	1.251	0
	1:160	0	1.221
	1:320	1.114	0
AG	1:80	1.212	0
	1:160	1.231	1.212
	1:320	0	0
PAS	1:80	1.123	1.232
	1:160	0	1.253
	1:320	0	0
DTP	1:80	1.232	1.235
	1:160	1.321	1.123
	1:320	0	1.232
	1:80	0*	0*

*Mean of four Elisa values

Precision, LOD, LOQ

In order to survey the precision, the range between 55-850 EU/mL was calculated by the formula: (S1=850EU/mL, S3=214EU/mL, S5=55EU/mL). For the assessment of precision-repeatability, all OD values were calculated based on %CV and the values obtained as %CV≤ 14 in the intra-plate, %CV≤15 in inter-plate and %CV≤ 12 in the inter-day. All of the results fit the validation criteria which is mentioned in Table 2. To assess the Intermediate-precision, all of the OD values were also calculated based on %CV, with the values standing at Days %CV≤ 18 and Analyst %CV≤ 7, all of which fit the validation criteria. To determine the LOD, we followed the 2.12 paragraph which led to the assessment of 7 EU/mL. The assay of LOQ was conducted between 14-58 EU/mL (S5= 58EU/mL, S6= 27EU/mL, S7= 14EU/mL). All of the OD values were calculated based on %CV, showing

the value of %CV≤ 17 in all Inter-plate and Intra-plate and Intra-day precision, which fits the validation criteria. But, in Inter-day, the value of S7=14EU/mL %CV≤ 26 was obtained, which is not compatible with the criteria. Therefore, although the curve linearity in the 14-895 EU/mL is linear, the lowest amount of anti-PA4 which could be measured by ELISA method is shown to be 27 EU/mL.

Specificity

In Table-3, the increase of ELISA value was only observed in the Pool sample in all three dilutions of 1/80, 1/160, and 1/320 whilst other samples did not reveal any increase. So, the results of this table show that the anti-PA4 is completely specific and does not have any cross-reaction with other antigens. (Table 3)

Comparative analysis of anti-PA4 IgG

A competitive inhibition ELISA (CI-ELISA) was developed based on the qualified anti-PA4 ELISA. The aim of the CI-ELISA was to increase the specificity of the ELISA by reducing the incidence of false positives.

The PA4 was used to prove the ability of binding the antibody to antigen coated to the plate. This diagram shows that Anti-PA4 reacts with PA4 in a liquid-phase, and the PA4 antigen existing in the serum competes with the PA4 antigen coated in the plate on binding with the antibody; this leads to the reduction of ELISA value.

That means, the lower the PA4 in the serum, the more ELISA value will be, and the more it becomes, the less the ELISA value will get. The results of the study shown in Figure. 3 prove this claim.
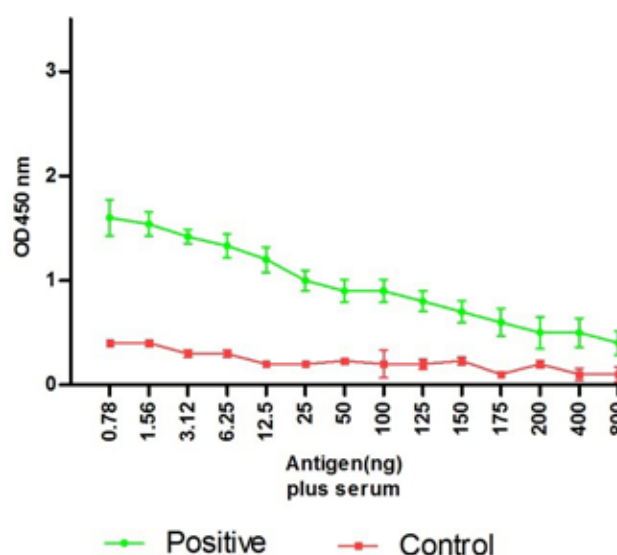
Fig. 3. A competitive inhibition ELISA (CI-ELISA). In the serum sample with the lower amount of PA4 antigen (0.78 ng) and the serum sample with the greater amount of PA4 antigen than the rest of the samples (800 ng), the highest ELISA value and the lowest ELISA value were observed respectively.

Comparing the amount of antibody against PA4 and LF in the vaccinated mice and the mice injected with virulent strain

We show that groups of AVA and Razi anthrax vaccine serums contain antibodies against the PA4 and LF but the Pool serum only has anti-PA4 whilst the NMS and *B. anthracis* 17JB groups lack such antibodies. Therefore, the results indicate that the mice having received the anthrax vaccine produce the antibody against the PA4 and LF after the third week (Table 4). Those injected with the strain 17JB only produce the detectible antibodies against LF after the fifth week. (Table 5),

Table 4. Comparing the amount of antibody against PA4 and LF in vaccine groups. A standard ELISA was performed for Anthrax vaccine adsorbed (AVA), Razi anthrax vaccine, Pool sera, NMS sera against anti PA4 and anti LF antigens. The ELISA value was significantly different between them.

Sample	Dilution in well	Elisa values	
		PA$_4$ coating	LF coating
AVA	1:80	25.403	21.37
Anthrax vaccine RAZI	1:80	28.225	24.193
Pool	1:80	97.983	0
NMS	1:80	0*	1.123*

*Mean of four Elisa values

Table 5. Comparing the amount of antibody against PA4 and LF in 17JB group. In order to detect antibody against PA4 and LF antigens, standard ELISA was performed for two groups of 17JB and NMS in the 3th and 5th weeks after the injection of strain 17JB. The Elisa value (after 5 weeks) was significantly different between them.

Sample	Dilution in well	Elisa value after 3 weeks		Elisa values after 5 weeks	
		PA_4 coating	LF coating	PA_4 coating	LF coating
17JB	1:80	0	1.209	1.151	17.195
NMS	1:80	0*	1.123*	1.021*	1.054*

*Mean of four Elisa values

DISCUSSION

The research conducted on the humoral immune response of the anthrax vaccines show that the role of anti-PA is the main factor of the vaccine's efficacy (Chen L, 2014). Therefore, future studies can look at this parameter more specifically to prove the immunity effects of the anthrax vaccine (Reuveny et al., 2001). The common ELISA kits have been monitored by full structure of PA. However, in an examination, the antibody measured is based solely on an important domain of PA (named PA4). The anti-PA4 can be an effective factor in the monitoring of the humoral immune response of all three existing vaccines against the *B. anthracis* (Flick-Smith et al., 2002).

To obtain the WHO and FDA license for manufacturing such vaccines for humans and animals, researchers need to survey the humoral immunity responses or anti-PA. In this study, we have developed an ELISA method detecting anti-PA4 (Weiss et al., 2007). All of the parameters have been assessed to validate the mentioned methods, and the results of the study show their validity for studying humoral immunity responses. As the study indicates, the coated PA4 antigen in the well plates did not cause any unspecific reaction against other livestock diseases as mentioned in Table 3. Moreover, through other validation tests, this test can be conducted in all laboratories by different researchers and bear the most accurate results. According to Table 4, using this method, the produced antibodies following the injection of anthrax vaccines in mice can be measured and the kinetic answers for future studies can be found. Although the most sensitive animal for such processes is guinea pig, using mice has several other advantages namely the convenience of testing and their abundance which make researchers forgo some of the disadvantages (Pombo et al., 2004).

One of the significant points in the application of this method, according to table 4 and 5, is that we created a model of anthrax disease in the mice via injecting the 17JB strain and measured the produced antibody against PA4 and LF, then compared it with the vaccinated groups. The positive control shows the increase in the antibody against PA4 and LF in the vaccinated groups despite what was shown in the disease groups.

Previous studies show that the amount of anti-PA in animals suffering from anthrax in the form of a skin disease has been increased. Therefore, the method, in which Anti-PA4 did not increase in comparison with the vaccinated group, can be a good option for screening cases suspected with infections from the vaccinated ones (Baillie LW et al., 2010; Ingram et al., 2010). The increase of anti-PA in the disease groups could be due to the presence of antibody against other PA's domains, which needs further studies. In addition, another reason for such diseases can be lack of PA in a pathogenic form (Ingram et al., 2010).

Although the performed studies represented that the levels of antibody increased against LF in the fifth week and this can be the point of difference between negative control and 17JB groups, the disadvantage of the described method is that it is not capable of distinguishing between the negative control group and the 17JB group during the first three weeks.

CONCLUSION

Through the validation tests carried out on this ELISA method, we showed that the detection range of the anti-PA4 can be 27- 892 EU/mL. We also believe that this ELISA method can be adopted in other laboratories in order to assess the immunity responses and differentiation of disease from vaccination after the first month. At the end, we can use this method as one of the alternative potency tests.

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CONFLICT OF INTEREST

None declared.

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Neopterin and Soluble Urokinase Type Plasminogen Activator Receptor as Biomarkers in Dogs with Systemic Inflammatory Response Syndrome

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ABSTRACT. The systemic inflammatory response syndrome (SIRS) is a common cause of mortality in dogs. This study aims to investigate the diagnostic value of markers C-reactive protein (CRP), soluble urokinase-type plasminogen activator receptor (suPAR) and neopterin in dogs with SIRS. The materials of the study consist of 30 dogs with SIRS and 15 healthy dogs that show no clinical symptom, 45 dogs in total. CRP, suPAR and neopterin levels of the serum samples were determined by ELISA using commercial kits. In the study, it was found that CRP, suPAR and neopterin levels of dogs with SIRS were significantly higher (P < 0.001, P < 0.01 and P < 0.01, respectively) than those in the healthy group. The cut-off values for the healthy dogs and dogs with SIRS for CRP, suPAR and neopterin were determined to be 11.20 mg/L, 223.68 ng/L and 3.23 nmol/mL, respectively. At these cut-off values, sensitivity levels were determined as high (92.90%) for CRP, moderate (63.30%) for neopterin, and low (53.30%) for suPAR, and high specificities (93.30%) were determined for all.

In conclusion, it was determined that CRP showed the best performance for the detection of systemic inflammatory response in dogs and neopterin and suPAR could be used as an alternative to CRP. It was also argued that further studies were necessary in order to determine diagnosis and prognosis of diseases.

Keywords: Systemic inflammatory response syndrome, acute phase proteins, dogs

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INTRODUCTION

Systemic inflammatory response syndrome (SIRS) is an immune response against infectious or non-infectious factors (Spapen et al., 2006). Two or more criteria in the findings of body temperature > 39.4 °C or < 37.8 °C, heart rate > 140 beat/minute, respiration rate > 20/minute, leukocyte > 16.0 x 10^9/L or < 6.0 x 10^9/L and > 3% band formation are to be found in order to diagnose SIRS (Torrente et al., 2015). Sepsis is defined as the SIRS developed against infections and is indicated as the most notable cause of death in infectious diseases (Alberti et al., 2003; Martin et al., 2003). Although clinical and diagnostic tests are used in human and veterinary medicine, more sensitive tests are required (Spapen et al., 2006; Nakamura et al., 2008; Mylonakis et al., 2011; Ok et al., 2015; Torrente et al., 2015). Especially in veterinary medicine, the biomarkers that show diagnostic and prognostic values of SIRS or sepsis are not sufficient.

Recent studies state that acute phase proteins can provide diagnostically and prognostically significant information in monitoring SIRS in infectious or non-infectious patients. Acute phase proteins are liver-based glycoproteins formed as a result of acute phase response. Acute phase proteins are called as positive or negative depending on increase (such as C-reactive protein and serum amyloid A) or decrease (such as albumin and transferrin) in their levels during inflammatory states. Acute phase response is a natural defense mechanism that plays a part in limiting the tissue damage and in stimulating recovery after trauma, infection or inflammation (Eckersall and Conner 1988; Tothova et al., 2014). Different researchers reported that C-reactive protein (CRP), which gives non-specific immune responses during the monitoring of infectious diseases, could be used in the diagnosis of infectious diseases. CRP is a liver-based glycoprotein formed as a result of acute phase response. In the studies on veterinary medicine, it was stated that serum CRP, one of type-specific acute phase proteins, may yield significant information in evaluating the clinical presentation of diseases in dogs (Christensen et al., 2014; Ok et al., 2015).

The soluble urokinase-type plasminogen activator receptor (suPAR) is a potential biomarker reported for inflammatory diseases. suPAR is released from neutrophils, endothelial and peripheral mononuclear blood cells and take part in various immune functions such as cell adhesion, migration, differentiation and proliferation. Increased suPAR levels are known as the determinant of the activation of immune and inflammatory systems, and have prognostic values in various diseases (Kofoed et al., 2008; Eugen-Olsen, 2011). Although it was reported that suPAR might have had diagnostic values in sepsis, lung and malignant diseases (Stephens et al., 1997; Wittenhagen et al., 2004; Eugen-Olsen 2011) no study related to dogs with SIRS in veterinary medicine was found in the literature surveys.

Neopterin [2-amino-4-hydroxy-6-(D-erythro-1′, 2′, 3′-trihydroxypropyl)-pteridine] is a low molecular weight molecule belonging to the class of pteridines which biosynthetically derives from guanosinetriphosphate (Murr et al., 2002). Neopterin is synthesized by monocytes and macrophages as a result of stimulating interferon-gamma (IFN- γ) released from active T lymphocytes. Neopterin is the sensitive determinant of cell-mediated immunity (Murr et al., 2002; Cesur et al., 2014). Neopterin release commences 3 days before T cell proliferation reaches the maximum level. Neopterin production increases 1 week before specific antibodies emerge. For this reason, neopterin is shown as an indicator of early inflammation (Millner et al., 1998; Berdowska and Zwirska-Korczala 2001). It was determined in the studies on human medicine that neopterin levels rise in viral infections, autoimmune diseases, inflammatory and malignant diseases (Berdowska and Zwirska-Korczala 2001). It is known that neopterin can be used as an indicator in determining the cellular immune response that plays a part in the pathogenesis of various diseases (Murr et al., 2002; Cesur et al., 2014) however, there are very few studies on this matter in the field of veterinary medicine.

It is important to determine reliable test methods that provide information in evaluating diagnosis and prognosis of patients with SIRS. The purpose of this study is to determine CRP, suPAR and neopterin levels of dogs with SIRS.

MATERIALS AND METHODS

Study design and animals

The animal material of the study consisted of 30 (18 females, 12 males) dogs, which were of different species and gender diagnosed with SIRS by clinical symptoms and haematological findings upon having been brought to the Internal Diseases Clinic of the Faculty of Veterinary Medicine of Sivas Cumhuriyet University for examination and treatment, and 15 (8 females, 7 males) healthy dogs, which showed no disease symptom.

The appetite, body temperature, heart rate, respiration rate, respiration type, capillary refill time, dehydration degree, changes in mucous membrane colour and mental status of dogs were examined. By the evaluation of clinical and haematological findings, the dogs diagnosed with SIRS were considered as the patient group of the study.

The study included dogs with at least two of the criteria below for SIRS diagnosis (Torrente et al., 2015).

Body temperature > 39.4 $^\circ$C or < 37.8 $^\circ$C

Heart rate > 140 beat/minute

Respiration rate > 20/minute

Leucocyte > 16.0 x 10^9/L or < 6.0 x 10^9/L and > 3% band formation.

10 ml blood samples in total from each dogs were taken from Vena cephalica antebrachii into tubes with anticoagulant (potassium EDTA) and no anticoagulant. Haematological parameters (reference value of leucocyte was taken as 6.0-16.0 x 10^9/L) were determined by the automated haematology analyser (BC-2800 Vet haematology analyser Mindray Bio-Medical Electronics Co., Ltd. Nanshan, Shenzhen). Serum samples were obtained by centrifuging for 10 minutes at 3000 rpm the no anticoagulant blood sample in sterile plastic tubes. These samples were stored at -80°C until the biochemical analysis. CRP (Tri-Delta Phase Canine CRP, Tri-Delta Diagnostic, Boonton Township, NJ), suPAR (Canine suPAR ELISA Kit, Yehua Biological Technology Co. Ltd, Shanghai) and neopterin (Canine Neopterin ELISA Kit, Yehua Biological Technology Co. Ltd, Shanghai) levels of the serum samples were determined in the ELISA reader (Thermo Multiskan GO Microplate Spectrophotometer, Waltham, Massachusetts) by using commercial kits by the sandwich enzyme immunoassay method. The intra and inter assay coefficient of variation of CRP was reported by the manufacturer as 6.5-6.9% and 7.8-8.2%, respectively. The assay manufacturer reported that the assay range of suPAR was 5-2000 ng/L, sensitivity 2.43 ng/L, and intra and inter assay precision coefficient of variation <8% and <10%, respectively. The measurement limit of the neopterin assay is 0.2-60 nmol/ml, sensitivity 0.11 nmol/mL, intra and inter assay coefficient of variation <10% and <12%, respectively. The study protocol was approved by the Ethics Committee of Sivas Cumhuriyet University, Turkey (Approval No: 2016/36).

Statistical Analysis

The data were analyzed by using statistical package for the social science-15 (SPSS-15). Analysis of data from 45 dogs randomly selected among patient dogs, as identified by clinical and laboratory methods, and healthy dogs, revealed a statistical significant difference between the groups. The data were presented as mean and standard error of the mean (SEM). The data distribution was evaluated by Kolmogorov-Smirnov test. Comparisons between groups were made by conducting T test for continuous variables with a normal distribution, whilst variables with non-normal distribution were analyzed with the Mann-Whitney U test. Receiver operating characteristic (ROC) curves and the area under these curves were used to assess the diagnostic potential of CRP, suPAR and neopterin levels. ROC analyses were performed for both the dogs with SIRS and the healthy dogs. To assess the diagnostic potential of CRP, suPAR and neopterin levels in the diagnosis of SIRS, the area under curve (AUC) and some cut-off values were analyzed. The results were assessed at a 95% confidence interval and at a significance level of $P < 0.05$.

RESULTS

30 dogs with SIRS clinically inspected in detail and 15 healthy dogs were included in the study. Dogs < 6 months old were not included in the study. Diseases causing SIRS include: gastrointestinal (n = 14), genital system (n = 4), malignant (n = 1), respiratory (n = 7), and dermatological (n = 4) diseases.

Table 1 presents changes in mean values of CRP, suPAR and neopterin levels of both dogs with SIRS and the healthy, and the significance in intergroup differences. In Figure 2-4, distribution of CRP, neopterin and suPAR among dogs with SIRS and healthy are presented. It was determined that the CRP, suPAR and neopterin concentrations of dogs with SIRS were significantly higher ($P < 0.001$, $P < 0.01$, $P < 0.01$, respectively) than those of the dogs in the heathy group (Table 1).

ROC analysis was conducted on the CRP, suPAR and neopterin levels in the healthy animals and the dogs with SIRS. The ROC curves used to assess the diagnostic potential of CRP, suPAR and neopterin levels, are presented in Figure 1 and AUC are shown in Table 2. The ROC analysis was used to establish the cut-off, sensitivity and specificity values. Table 2 shows the cut-off, sensitivity, specificity and AUC values of the CRP, suPAR and neopterin levels of

healthy dogs and dogs with SIRS. The cut-off values for the CRP, suPAR and neopterin levels were determined as 11.20 mg/L, 223.68 mg/L and 3.23 mmol/mL, respectively.

Based on ROC analysis, the area below the reference line is 0.99 for CPR, 0.69 for suPAR and 0.72 for neopterin, and that these biomarkers can offer significant diagnostic information.
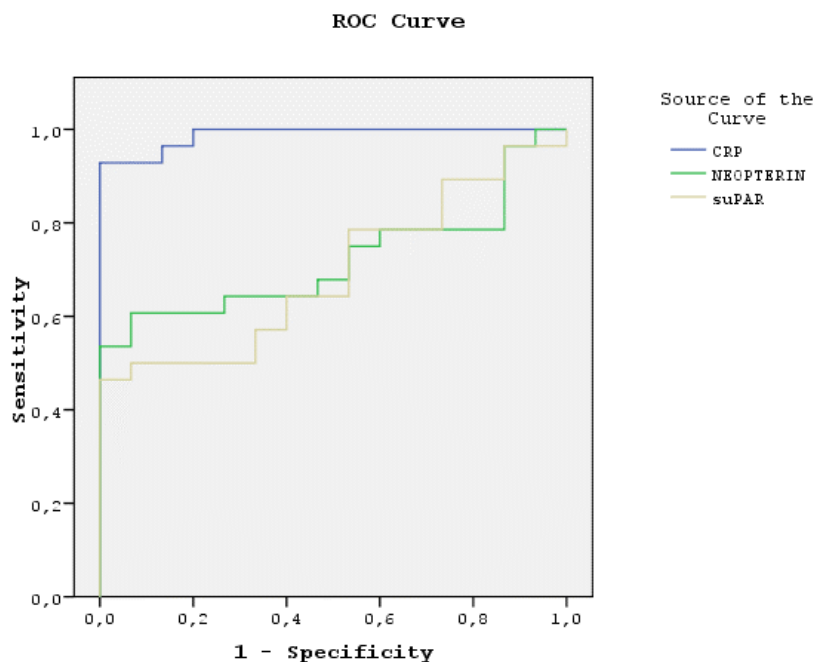
Figure 1. Receiver operating characteristic curve in the diagnosis of dogs with SIRS and healthy of serum levels CRP, suPAR and neopterin
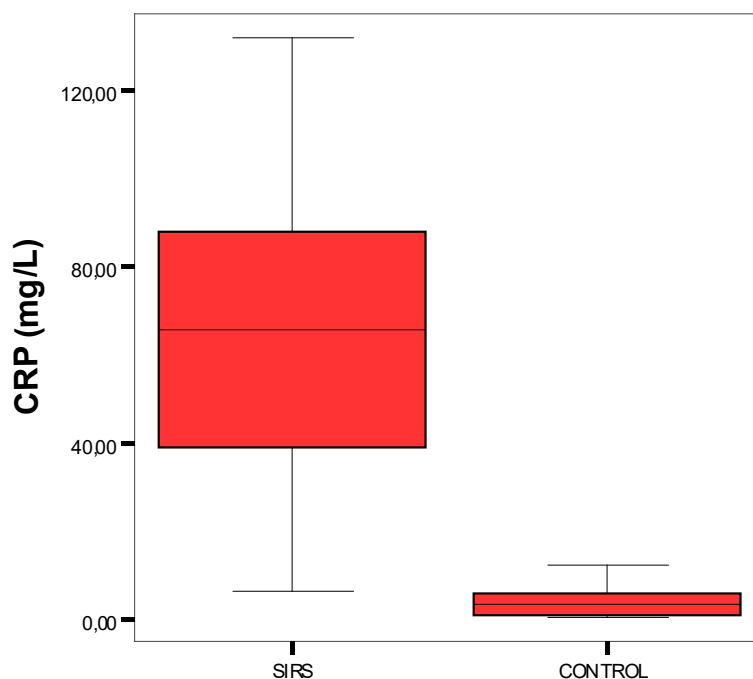
Figure 2. CRP levels in dogs with the systemic inflammatory response syndrome (n=30) and healthy (n=15)
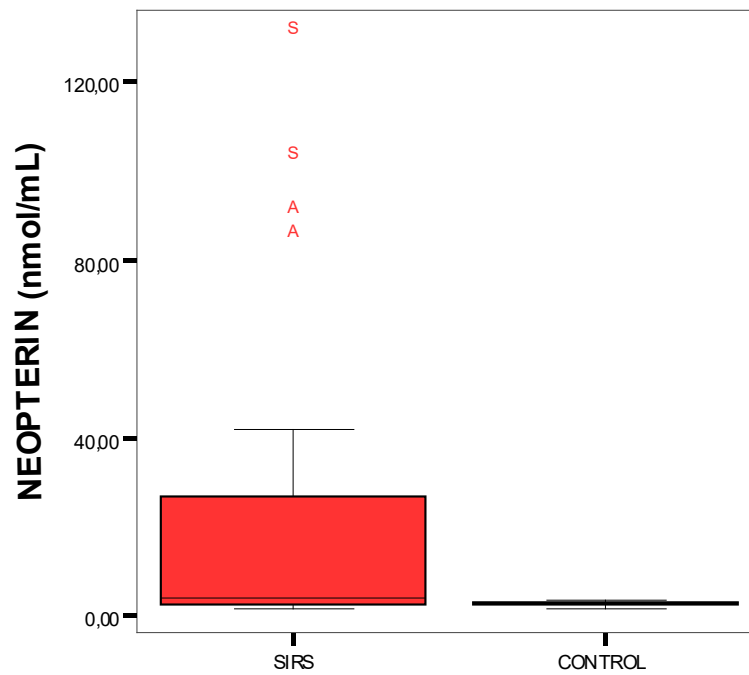
Figure 3. Neopterin levels in dogs with the systemic inflammatory response syndrome (n=30) and healty (n=15)
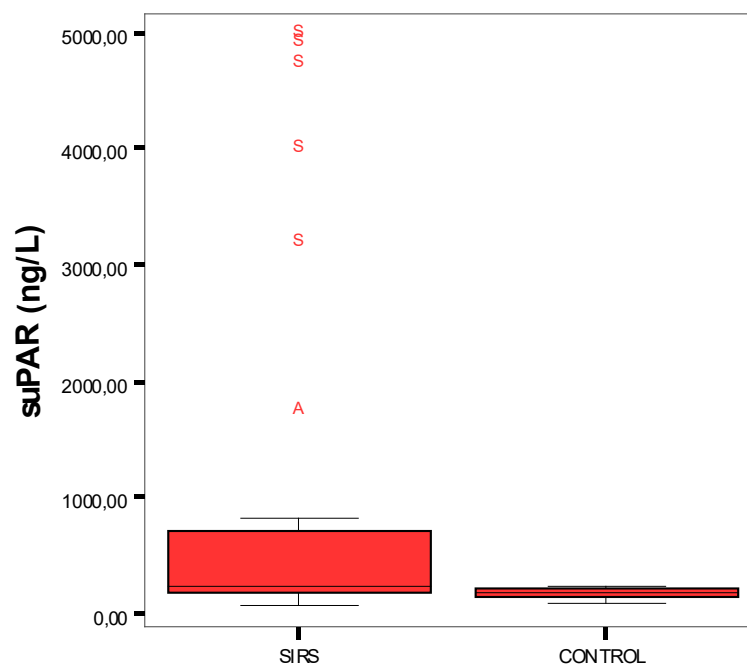
Figure 4. suPAR levels in dogs with the systemic inflammatory response syndrome (n=30) and healthy (n=15)

Table 1. The CRP, suPAR, and neopterin levels of healthy dogs and dogs with SIRS (Mean±SEM)

Parameters	Healthy group	SIRS	P value
CRP (mg/L)	4.30±0.95	66.59±6.57	< 0.001
suPAR (ng/L)	172.12 ±11.78	1001.79±287.60	0.007
Neopterin (nmol/mL)	2.63±0.15	21.08±6.36	0.007

CRP; C-reactive protein, suPAR; soluble urokinase-type plasminogen activator receptor

Table 2. The cut-off, sensitivity, specificity and AUC values of the CRP, suPAR, and neopterin levels of healthy dogs and dogs with SIRS.

Parameters	CRP (mg/L)	suPAR (ng/L)	Neopterin (nmol/mL)
AUC	0.99	0.69	0.72
Cut off	11.20	223.68	3.23
Sensitivity (%)	92.90	53.30	63.30
Specificity (%)	93.30	93.30	93.30
P value	<0.001	0.039	0.018
SEM	0.011	0.079	0.077

AUC; area under the curve, SEM; standard error of mean, CRP; C-reactive protein, suPAR; soluble urokinase-type plasminogen activator receptor

DISCUSSION

SIRS, which develops in various diseases in dogs, requires emergency treatment. Thus, urgent and correct diagnosis is critical. CRP is an acute phase protein released during tissue damage and inflammation. It is synthesized primarily in liver in response to stimulation by cytokines and is defined as major acute phase protein identified in dogs. (Jitpean et al., 2014). Serum CRP level in healthy dogs (Torrente et al., 2015) is < 35 mg/L (when analysis using LifeAssays magnetic permeability-based immunoassay), and it has been reported by various studies that it increases as a response to inflammatory stimulus (Viitanen et al., 2014). Experimental studies have reported that CRP increases before the formation of clinical symptoms and reaches peak levels after 36 hours (Spapen et al., 2006). It has also been reported that CRP increases in diseases such as leptospirosis, babesiosis, parvovirus infections, malignant tumors, pyometra, acute pancreatitis, immune hemolytic anemia, arthritis, glomerulonephritis (Holm et al., 2004; Matijatko et al., 2007; Nakamura et al., 2008; Kocaturk et al., 2010; Tostes Oliveira et al., 2010). In a study on dogs with monocytic ehrlichiosis, it has been found that CRP is an indicator in determining the clinical phase of the diseases, however, is not useful in determining the clinical result (Mylonakis et al., 2011). It was determined that high CRP levels in dogs with pyometra increased the rate of mortality and extended the treatment duration. CRP levels in dogs with no systemic inflammation ranged between 0.07-24.7 mg/L, and that CRP levels in dogs with systemic inflammation ranged between 0.4-907.4 mg/L (Christensen et al., 2014). In this study, it was determined that average CRP levels of dogs with SIRS were significantly higher (P < 0.001) than those of healthy dogs. Besides, the CRP cut-off value was considered to be 11.20 mg/L for the healthy dogs and the dogs with SIRS with sensitivity 92.9%, and specificity 93.3%. In our study, it was observed that CRP has high sensitivity and specificity for the detection of systemic inflammatory response in dogs and can be evaluated as a good biomarker of inflammation in dogs similar to other studies.

Biochemical markers are used to predict the morbidity and mortality as well as the diagnosis of the pathological condition. In veterinary medicine, biochemical parameters have been investigated for diagnostic and prognostic purposes in dogs with SIRS and sepsis (Kocaturk et al., 2010; Torrente et al., 2015; Ok et al., 2015). Inflammatory mediators showing immune activation such as suPAR and neopterin are investigated in order to provide information on clinical diagnosis and prognosis of infectious diseases in human medicine (Murr et al., 2002; Eugen-Olsen 2011; Zeng et al., 2016). suPAR is a marker of fibrinolysis and inflammation that is released from immunologically active cells. suPAR reveals the patho-physiological mechanisms that are active on cell surface (Wittenhagen et al., 2004; Kofoed et al., 2008; Kuleš et al., 2017). In a study comparing dogs with babesiosis and healthy ones, significant increases in suPAR levels were determined and it was stated that it would be useful in the evaluation of the stage of the disease. It was also reported that increased suPAR concentrations may be a reflection of inflammatory response in dogs with babesiosis (Kuleš et al., 2017). In a study on patients with sepsis in intensive care, it was stated that there is an increase in suPAR level throughout acute inflammation (Kofoed et al., 2008). In another study, blood suPAR was shown to be significantly increased in patients with sepsis and stated that it was a valuable parameter to differentiate SIRS from sepsis. They also reported that the use of suPAR and procalcitonin (PCT) combination increases the diagnostic efficacy for sepsis in human medicine (Zeng et al., 2016). In other studies, it was found that suPAR increased significantly in people with SIRS and sepsis, but it

was concluded that the diagnostic value of suPAR in sepsis was insufficient according to classical markers such as PCT and CRP. The reason for this is that the strong induction of PCT and CRP with lipopolysaccharides may explain its benefits in the diagnosis of bacterial infection, whereas suPAR is induced at a lower level with bacterial proteins such as lipopolysaccharide. Considering all these results, suPAR has limited diagnostic value in sepsis compared to classical markers such as PCT and CRP (Kofoed et al., 2007; Koch et al., 2011; Hoenigl et al., 2013; Bilgili and Cinel 2013). The study revealed that the average suPAR levels of dogs with SIRS were significantly higher (P < 0.01) than those of healthy dogs. The cut-off value of suPAR was considered as 223.68 ng/L for healthy dogs and dogs with SIRS, its sensitivity was determined to be low (53.3%), and its specificity as high (93.3%). In our study, it was found that suPAR had low sensitivity compared to CRP, which is a good marker for the detection of inflammation in dogs. The results of our study show that suPAR is insufficient for the diagnosis of systemic inflammatory response in dogs similar to the studies in human medicine.

Neopterin is a critical indicator for cellular immune system activation released by monocytes and macrophages (Hoffmann et al., 2003). It was determined that in intensive care units neopterin levels of patients with sepsis, septic shock, SIRS, or with multiple organ failure increased (Berdowska and Zwirska-Korczala 2001; Alberti et al., 2003; Baydar et al., 2009). It was reported that the neopterin level increased in viral, bacterial, and parasitic infections, apart from sepsis (Berdowska and Zwirska-Korczala 2001; Murr et al., 2002). It was stated that the neopterin levels increased in neonatal calves with septisemic colibacillosis, in relation to IFN-γ as a defense mechanism (Ercan et al., 2016). In a study conducted on 208 intensive care patients in human medicine, the cut-off value of the recommended neopterin for the diagnosis of infection was 18 pg/L (Brunkhorst et al., 1999). In the study, it was found that the serum neopterin levels of dogs with SIRS was higher in comparison to the healthy dogs and that this increase is statistically significant (P < 0.01). The cut-off value of neopterin was considered as 3.23 nmol/mL for SIRS, with sensitivity 63.3%, and specificity 93.3%. In this study, the sensitivity of neopterin was found to be lower than CRP, although higher than suPAR. In human medicine (Brunkhorst et al., 1999), neopterin has been reported to be more sensitive than classical markers of inflammation (such as procalcitonin). On the contrary, in this study, it was observed that systemic inflammation in dogs is less sensitive than CRP, one of the classical biomarkers.

CONCLUSIONS

It was supported by the comparative ROC analysis that CRP has more diagnostic significance than neopterin and suPAR. In the ROC analysis, it was determined that CRP had the highest sensitivity, followed by neopterin and suPAR, respectively, and that CPR, suPAR and neopterin had the same values in terms of specificity. As a result, we determined that CRP showed the best performance for detection of systemic inflammatory response in dogs, and neopterin and suPAR could be used as an alternative to CRP. It is also concluded that further studies are necessary in order to determine diagnosis and prognosis of diseases.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Studies on *Pseudomonas aeruginosa* Infection in Hatcheries and Chicken

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ABSTRACT. The aim of this work was to spot light on the presence of *Pseudomonas aeruginosa* (*P. aeruginosa*) strains in hatcheries and dead in shell embryos. A total of 406 samples representing 200 and 206 swabs from hatcheries environment and yolk sacs of dead in shell embryos were collected from Damietta governorate, Egypt. *P. aeruginosa* was isolated and identified. Some virulent genes (*toxA, psIA* and *fliC*) of *P. aeruginosa* were detected using polymerase chain reaction (PCR). The antimicrobial susceptibility of *P. aeruginosa* was tested *in vitro*. Day and 11 days old broiler chicks were challenged with *P. aeruginosa* to determine the pathogenicity of the isolated strains. The results showed that *P. aeruginosa* was recovered from 16 (8%) out of 200 hatcheries and from 17 (8.25%) out of 206 chicken embryos samples. Isolated strains of *P. aeruginosa* showed presence of *toxA, psIA* and *fliC* virulent genes. *P. aeruginosa* strains were sensitive (100%) to ciprofloxacin, levofloxacin and gentamycin but resistant (100%) to amoxycillin/clavulanic acid, doxycycline and erythromycin. The pathogenicity test of day and 11 days old chicks revealed that *P. aeruginosa* was highly pathogenic induced mortality rates of 72 and 40%, respectively. Septicaemia of internal organs, unabsorbed yolk sacs, pneumonia, greenish exudates in the abdominal cavity**,** liver necrosis and enteritis were the predominant lesions. Histopathological changes supported the previous lesions. In conclusion, *P. aeruginosa* is of great importance pathogen of embryos and newly hatched chicks based on presence of virulent genes as well as *in vivo* pathogenicity study; respectively.

Keywords: Antimicrobials, Chickens, *P. aeruginosa,* Pathogenicity, PCR

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INTRODUCTION

Pseudomonas species are ubiquitous organisms and usually present in soil, humid areas and water (Barnes, 2003). Infection of chickens especially young's with *Pseudomonas aeruginosa* (*P. aeruginosa*) induces septicaemia, mortalities, respiratory manifestations and diarrhea or localized infections in different organs (Walker et al., 2002). High rates of late embryonic deaths and loses of netwly hatched chicks were recorded after infection with *P. aeruginosa* (Fekadu, 2010). Conventional methods for isolation and identification of *P. aeruginosa* faces some difficulties in accurate detection of the bacteria due to misidentification with closely related Gram-negative bacilli (Al-Ahmadi and Roodsari, 2016). So, the use of molecular techniques as polymerase chain reaction (PCR) improves accurate and rapid identification of *P. aeruginosa* (Anuj et al., 2009). *P. aeruginosa* possess different virulent factors inducing toxicity and pathogenicity (Fadhil et al., 2016).

Presence of intrinsic and acquired antibiotic resistance mechanisms creates difficulty of *P. aeruginosa* treatment (Lister *et al*., 2009).

The opportunistic pathogenic nature of *P. aeruginosa*, biofilm formation, induction of chronic infections are factors responsible for high level of multiple drug resistance (Wei and Ma, 2013; Rasamiravaka et al., 2015).

Therefore, this study was designed to identify *P. aeruginosa* strains from hatcheries and dead in shell embryos, detect some virulent genes (*toxA, psIA and fliC*), test the susceptibility of the isolated strains to various antimicrobials *in vitro* and finally examine their pathogenicity in newly hatched chicks.

MATERIALS AND METHODS

Isolation and identification of Pseudomonas isolates

Samples were taken from 8 hatcheries in Damietta governorate, Egypt during the period from March to August, 2018. A total of 406 samples representing 200 swabs from hatcheries environment and 206 swabs from yolk sacs of late dead in shell embryos (Table 1). Samples were collected in sterile plastic containers, kept in ice box and transported as soon as possible to the laboratory. Isolation of Pseudomonas isolates was done according to Quinn *et al*. (2002). The samples were aerobically inoculated into nutrient broth for 24h at 37°C. A loop-full of inoculated broth were streaked onto MacConkey agar and Pseudomonas agar base media and incubated aerobically for 24h at 37°C. The non-lactose fermented colonies were randomly selected and sub-cultured onto nutrient agar plates to observe the pigmentation. The purified colonies were examined for their colonial morphology, pigment production and odour. Identification was carried out through Gram staining, biochemical reactions and sugar fermentation.

Serological identification of *P. aeruginosa* isolates

Serological identification was done in Animal Health Research Institute, Dokki, Egypt using antisera from Denka Seiken Co. Ltd, Tokyo, Japan. It was carried out for detection of somatic antigen "O" using *P. aeruginosa* antisera according to the method of Homma (1980). Agglutination kit contained polyvalent and monovalent antisera. Agglutination was described as positive if it caused a positive slide agglutination reaction.

Molecular detection of *P. aeruginosa* virulent genes

Presence of specific virulent genes of *P. aeruginosa* (*toxA, psIA and fliC*) was examined using PCR. DNA extraction from *P. aeruginosa* isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations are listed in Table (2).

Table 1: The number of the examined samples distributed in Damietta governorate, Egypt

Locality	Number of samples	
	Hatcheries	Chicken embryos
Om El-Reda	25	28
Farskour	25	29
Zarka	25	22
Kafer-Saad	50	58
Cinania	25	15
Kafer El-Batekh	50	54
Total	200	206

Table 2: Virulence target genes, oligonucleotide primers, amplicon sizes and cycling conditions for *P. aeruginosa*

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
toxA	GACAACG CCCTCAG CATCACC AGC CGCTGGC CCATTCG CTCCAGC GCT	396	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 7 min.	Matar et al. (2002)
pslA	TCCCTAC CTCAGCA GCAAGC TGTTGTA GCCGTAG CGTTTCT G	656	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Ghadaksaz et al. (2015)
fliC	TGAACGT GGCTACC AAGAACG TCTGCAG	180	94˚C 5 min.	94˚C 30 sec.	56.2˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	

In vitro-antibiotic susceptibility test of *P. aeruginosa*

In vitro antibiotic susceptibility test of *P. aeruginosa* strains was carried out using disc diffusion method (CLSI, 2015). Culture with 0.5 McFarland density was distributed onto the Müller-Hinton agar. The antibiotic discs were distributed on the agar surfaces and the zones of inhibition were measured after 24 hr.

Pathogenicity test of *P. aeruginosa* in chickens

The experiment was done according to the regulation of Institutional Animal Care and Use Committee (CU-IACUC), Cairo University with Code Number (CU II F 110 18). A total of 80, day-old Cobb chicks was obtained from local hatcheries and 5 birds were subjected for bacteriological examination to confirm absence of *P. aeruginosa*. Chicks were reared on thoroughly cleaned and disinfected semi closed houses and vaccinated using standard protocol for vaccination. Feed and water was given *ad libitum*. Chicks were divided into 3 equal groups, each containing 25 birds. Chicks of group (1) were challenged at day old, while chicks of group (2) were challenged at 11 days old. Chicks in group (3) were kept as blank control negative non challenged group. Each chick in groups (1) and (2) was challenged intramuscularly with 0.2 ml of 24 hr broth culture contained 10^3 viable cell of *P. aeruginosa* /ml (Joh et al., 2005), while those in group (3) were inoculated with sterile phosphate buffer saline. The strain used in challenge is that identified herein. All chickens were kept under observation for 10 days to detect clinical signs, mortalities and post-mortem lesions. Samples were collected from dead birds for re-isolation of *P. aeruginosa*. At the end of the study, samples including; liver, spleen and heart were collected from sacrificed birds for gross lesions, re-isolation of *P. aeruginosa*.

Histopathological examination

Tissue specimens from lung, heart and liver were collected, fixed in 10% neutral buffered formaline, routinely processed by standard paraffin embedding technique, sectioned at 4 micron and finally stained with Hematoxylin and Eosin (H&E) (Bancroft and Gamble, 2002).

RESULTS

Based on cultural, morphological and biochemical characteristics of the isolates, a total of (16, 8%) and (17, 8.25%) *P. aeruginosa* isolates were recovered from 200 hatchery and 206 chicken embryos samples, respectively (Table 3).

Morphologically, *P. aeruginosa* is aerobic β haemolytic colonies on blood agar, on MacConkey agar; it has pale colonies of non-lactose fermenter. The plates

containing characteristic colonies of *P. aeruginosa* (large, irregular, translucent and produced a greenish diffusible pigment and characterized by fruity smell). Microscopically, the organism is Gram-negative short rod. Biochemically, it is positive oxidase, catalase, urea, citrate utilization and gelatin hydrolysis, negative for indole, methyl red and Voges Proskauer. It shows sugar fermentation of glucose, mannose and xylose, but not sucrose, lactose and maltose.

Serological identification of *P. aeruginosa* revealed that all of isolates were belonged to types G and M (Table 3).

Molecular detection of virulent genes of *P. aeruginosa* showed presence of *toA* and *psIA* genes in all strains and *fliC* in almost strains (Figures 1, 2 and 3).

Strains of *P. aeruginosa* showed *in vitro* susceptibility (100%) to ciprofloxacin, levofloxacin and gentamycin and (48.48%) streptomycin but resistant (100%) to amoxycillin/clavulanic acid, doxycycline and erythromycin and (78.78%) nalidixic acid. Intermediate susceptibility was detected to chloramphenicol (75.75%) and colistin sulphate (36.36%) (Table 4).

Table 3: The incidence and the predominant serotypes of *P. aeruginosa* in Damietta governorate, Egypt

Locality	Hatcheries				Chicken embryos			
	Number of samples	Number of positive	% positive	Serotype	Number of samples	Number of positive	% positive	Serotype
Om El-Reda	25	6	24	M	28	5	17.85	G and M
Farskour	25	4	16	M	29	3	10.3	M
Zarka	25	0	0		22	0	0	
Kafer-Saad	50	3	6	M	58	5	8.6	G and M
Cinania	25	0	0		15	0	0	
Kafer El-Batekh	50	3	6	G	54	4	7.4	G
Total	200	16	8		206	17	8.25	
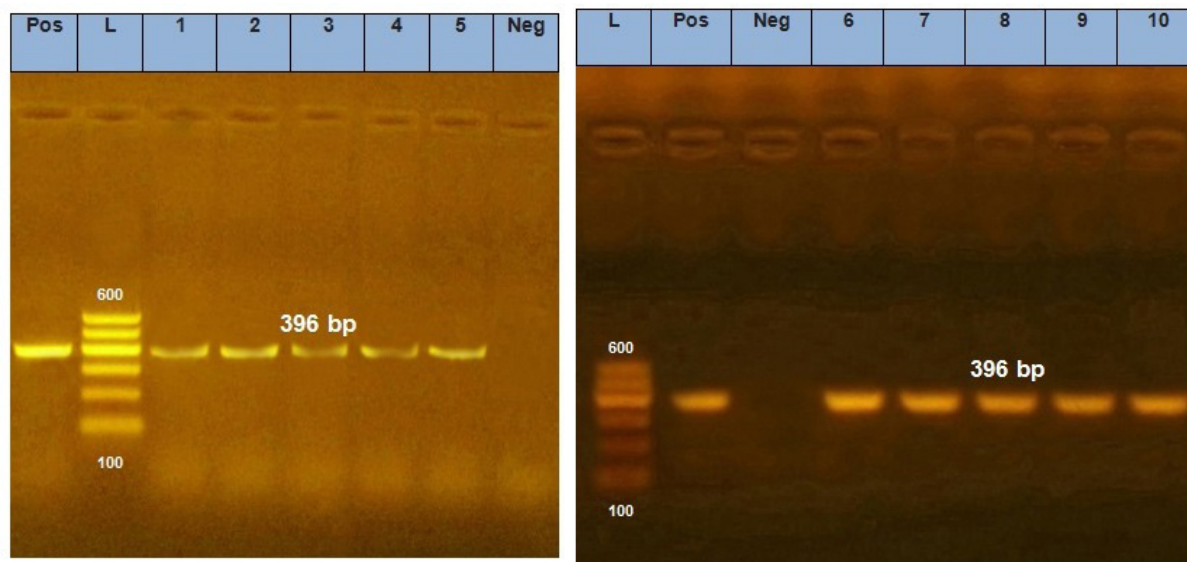
Figure 1: PCR amplification using Pseudomonas genus-specific primers (*toxA* gene), Pos= positive control, L= ladder, lines 1-10 = clinical isolates of *P. aeruginosa*, Neg= negative control
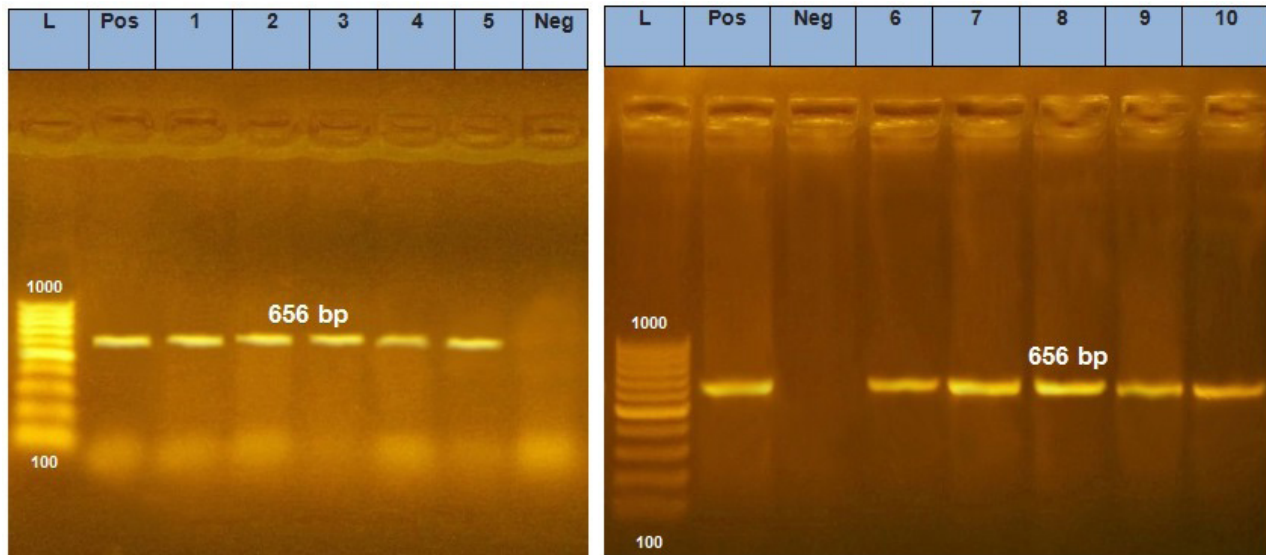
Figure 2: PCR amplification using Pseudomonas genus-specific primers (*psIA* gene), Pos= positive control, L= ladder, lines 1-10 = clinical isolates of *P. aeruginosa*, Neg= negative control
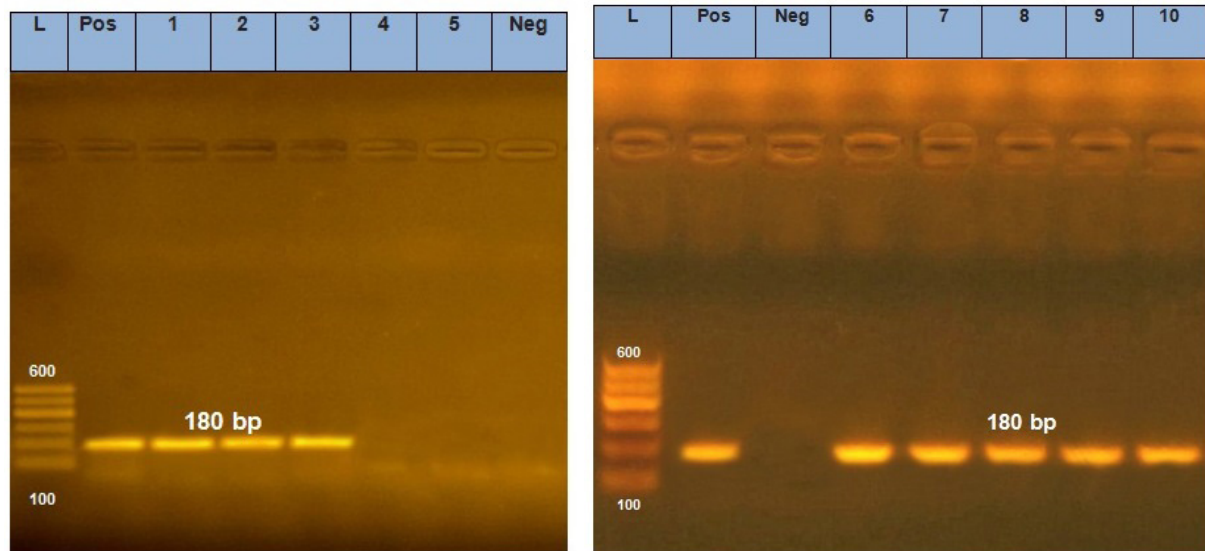
Figure 3: PCR amplification using Pseudomonas genus-specific primers (*filC* gene), Pos= positive control, L= ladder, lines 1-4, 6-10 = clinical isolates of *P. aeruginosa*, Neg= negative control

Table 4: Results of antibiogram test against P. *aeruginosa*

Antibiotic Disc (Code)	Disc content/ μg	Antimicrobial efficacy (%) against 33 strain of *P. aeruginosa*		
		Susceptible	Intermediate susceptibility	Resistant
Amoxycillin/Clavulanic acid (AMC)	30	0 (0%)	0 (0%)	33 (100%)
Chloramphenicol (C)	30	8 (24.2%)	25 (75.75%)	0 (0%)
Ciprofloxacin (CIP)	5	33 (100%)	0 (0%)	0 (0%)
Colistin sulphate (CT)	10	11 (33.3%)	12 (36.36%)	10 (30.3%)
Doxycycline (Do)	30	0 (0%)	0 (0%)	33 (100%)
Erythromycin (E)	15	0 (0%)	0 (0%)	33 (100%)
Gentamycin (CN)	10	33 (100%)	0 (0%)	0 (0%)
Levofloxacin (LEV)	5	33 (100%)	0 (0%)	0 (0%)
Nalidixic acid (NA)	30	0 (0%)	7 (21.2%)	26 (78.78%)
Streptomycin (S)	10	16 (48.48%)	9 (27.27%)	8 (24.2%)

Clinical signs of *P. aeruginosa* challenged chicks were depression, off food, reluctance, sleepy appearance, respiratory signs and greenish diarrhea. Mortalities began on day old *P. aeruginosa* challenged chicks 12-16 hr post challenge (PC) with mortality rate of (18/25, 72%), while appeared on 11 days old chicks 24 hr PC with mortality rate of (10/25, 40%). The post-mortem examination of the dead chick's revealed septicemia, congestion with peticheal haemorrhages on liver, spleen and lung, pericarditis, distention of gall-bladder, unabsorbed yolk sacs, greenish exudates in the abdominal cavity and enteritis. Gross lesions of sacrificed birds showed congestion of the liver, spleen and kidneys, pneumonia, liver necrosis and unabsorbed yolk sacs. *P. aeruginosa* organism was re-isolated from dead and sacrificed birds. No clinical signs or mortalities was recorded on phosphate buffer saline inoculated chicks.

The results of histopathological examination of dead and sacrificed chicks that challenged at one day old are collected in Figure (4). The lung had severe interstitial pneumonia with highly edematous thickening of alveolar wall, highly congested alveolar capillaries as well as mononuclear inflammatory cell infiltration with some heterophiles throughout the interstitial tissue (A). The bronchi and bronchioles had hyperplastic proliferation of their epithelial lining especially goblet cells were had marked hyperplastic proliferation. Mononuclear inflammatory cell infiltration with some heterophiles around bronchi and bronchioles could be detected. Sub-peluraledema could be detected. The heart had marked subepicardial edema with dilatation of sub epicardial blood vessels (B). The liver showed highly congested hepatic sinusoids with disarrangement of hepatic rosettes as well as hepatic cell degeneration and necrosis with infiltration of mononuclear inflammatory cells with some heterophils (C). Wide patches of hepatic cells coagulative necrosis could be detected with infiltration of mononuclear inflammatory cells and some heterophils (D) as well as Kupffer cell activation could be detected.
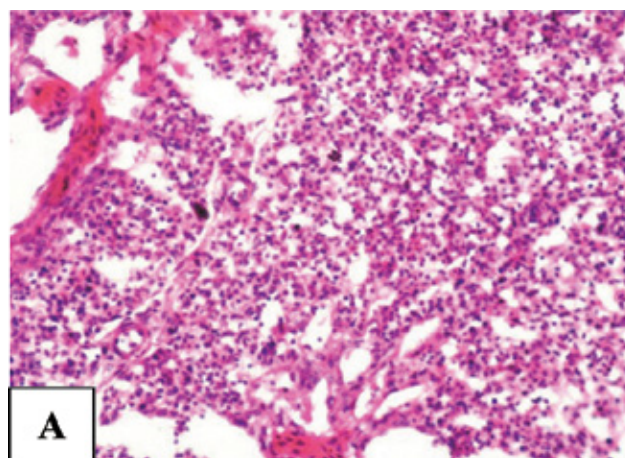
Figure 4 (A): Lung of one-day old chicks showed severe interstitial pneumonia with highly edematous thickening alveolar wall, highly congested alveolar capillaries as well as mononuclear inflammatory cell infiltration with heterophils through the interstitial tissue. (H&E X400).
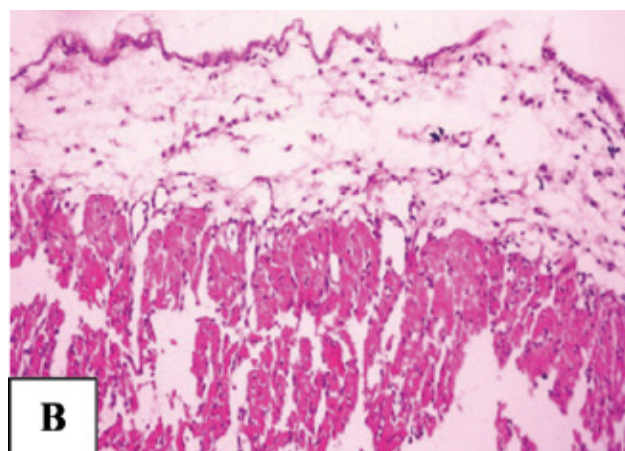
Figure 4 (B): Heart of one-day old chicks showed marked subepicardial edema with dilatation of subepicardial blood vessels. Marked edema could be detected between cardiac muscle fibers accompanied by some degenerative changes of cardimyocytes. (H&E X400).
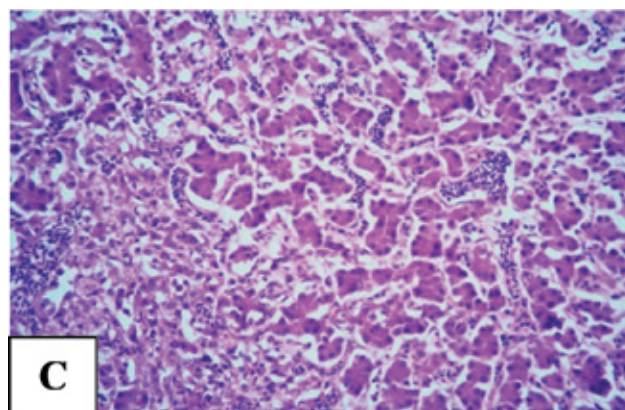
Figure 4 (C): Liver of one-day old chicks showed highly congested hepatic sinusoids with disarrangement of hepatic rosettes as well as patches of hepatic cell necrosis with infiltration of mononuclear inflammatory cells with some heterophils. Kupffer cell activation could be observed. (H&E X400).
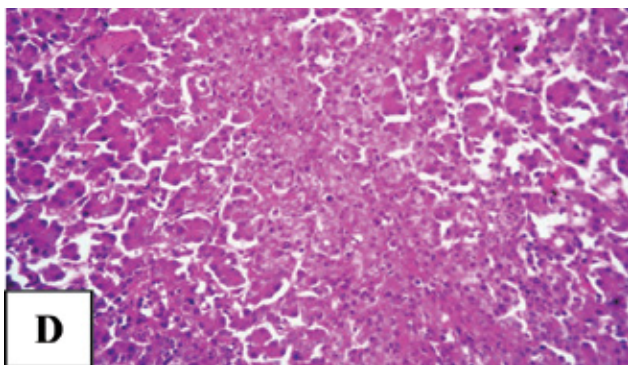
Figure 4 (D): Liver of one-day old chicks showed wide patches of hepatic cells coagulative necrosis could be detected with infiltration of mononuclear inflammatory cells and some heterophils (H&E X400).

The histopathological lesions of dead and sacrificed chicks that challenged at 11 days old are represented in Figure (5). The lungs revealed bronchopneumonia with marked hyperplastic proliferation of its epithelial lining as well as highly hyperplastic proliferated goblet cells (A). The bronchial lumen filled with exudates consisted of desquamated epithelial cells, mononuclear inflammatory cells, heterophils and red blood cells. Marked peribroncheal heterophils and mononuclear cell infiltration could be observed (A). The lung showed patches of consolidated pneumonia and the alveolar lumen was filled with fibrinopurulent exudates (B) with desquamated epithelial cells with necrosis in alveolar wall and heterophils detection in alveolar lumen. Other patches of pulmonary tissue had haemorrhagic pneumonia. Moreover, the lung had pulmonary blood vessel with large red attached thrombus consisted of fibrin, mononuclear cells and red blood cells (C). Other blood vessels had perivascular mononuclear inflammatory cell infiltration with many heterophils and myriads of bacteria. The heart had marked myocarditis, edema and some hemorrhage between cardiomyocytes (D). Zenker's necrosis of most cardiomyocytes could be detected. The wall of most interstitial blood vessels showed degenerative and necrotic changes (D). The liver had dilated hepatic sinusoids with dissociated hepatic rosettes, vacuolar degeneration of many hepatocytes, other hepatocytes had pyknotic nuclei (E). Multiple foci of degenerated and necrotic hepatocytes were seen around central veins which replaced by mononuclear inflammatory cell infiltration and heterophils (E). Kupffer cell activation could be detected. Portal area revealed proliferation of bile duct and highly dilated portal blood vessel. Mononuclear inflammatory cells and hetrophils infiltration could be detected around portal area (F). Other patches of hepatic tissue

had highly dilated portal blood vessel surrounded by dissociated, degenerative, necrotic hepatocytes and marked large haemorrhgic area (G).
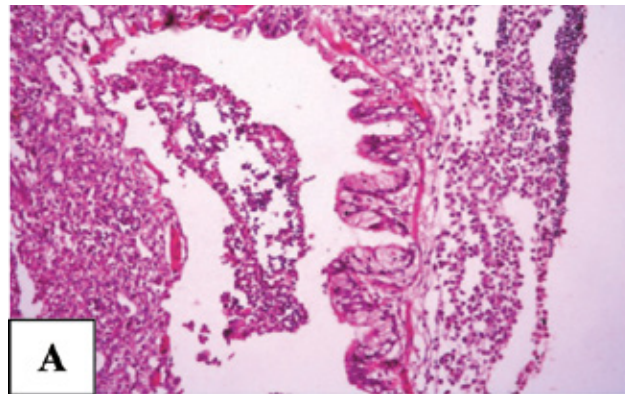
Figure 5 (A): Lung of 11-days old chicks showed bronchus with marked hyperplastic proliferation of its epithelial lining as well as highly hyperplastic proliferated goblet cells. The bronchial lumen had exudates with desquamated epithelial cells, mononuclear inflammatory cells, heterophils and red blood cells. Marked aggregation of peribroncheal heterophils and mononuclear inflammatory cell could be seen. (H&E X400).
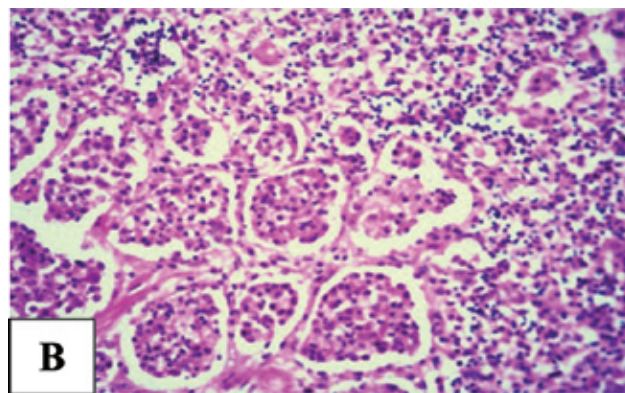
Figure 5 (B): Lung of 11-days old chicks showed patches of consolidated pneumonia, alveolar lumen filled with fibrinopurulent exudates with desquamated epithelial cells, as well as other patches of haemorrhagic pneumonia. (H&E X400).
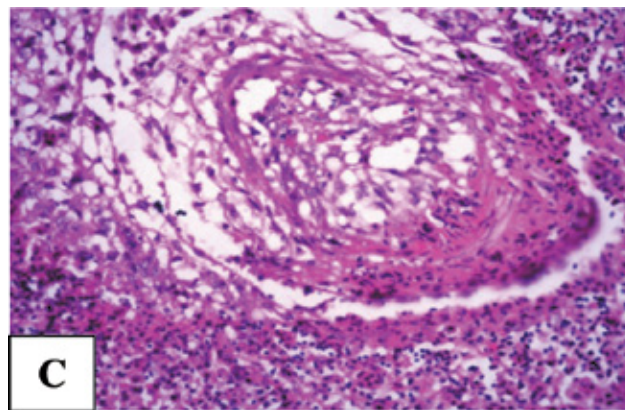
Figure 5 (C): Lung of 11-days old chicks showed pulmonary blood vessel with large red attached thrombus consisted of fibrin, mononuclear cells and red blood cells. Haemorrhgic pneumonia could be seen around thrombosed blood vessel. (H&E X400).
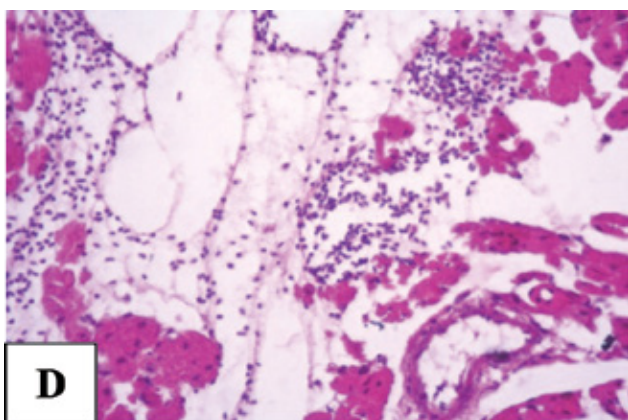
Figure 5 (D): Heart of 11-days old chicks showed marked interstitial edema and some hemorrhage between cardiac muscle fibers. Zenker's necrosis of most myocardial fibers could be seen as well as degenerative and necrotic changes through the wall of most interstitial blood vessels. (H&E X400).
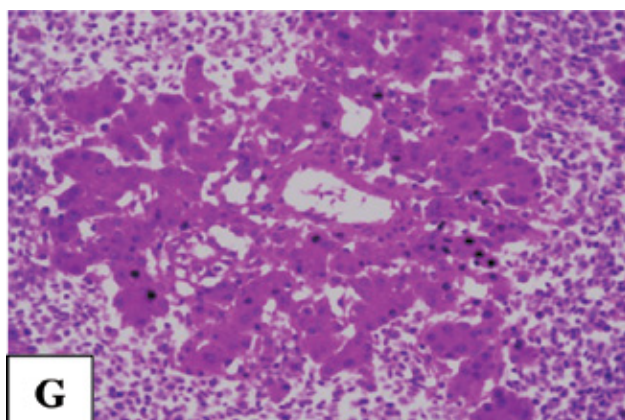
Figure 5 (G): Liver of 11-days old chicks showed highly dilated portal blood vessel, surrounded with degenerative and necrotic hepatocytes. Marked large haemorrhgic areas near necrotic hepatocytes could be seen. (H&E X400).
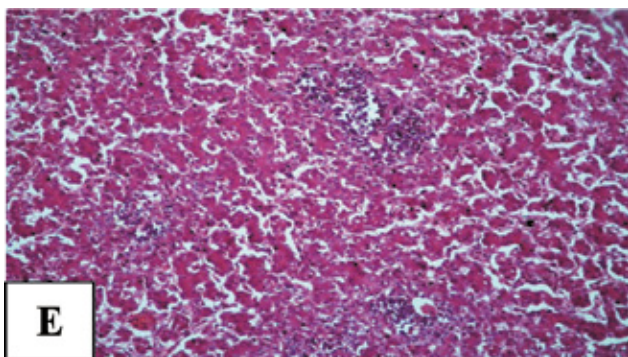
Figure 5 (E): Liver of 11-days old chicks showed dilated hepatic sinusoids with vacuolar degeneration of many hepatocytes, other hepatocytes had pyknotic nuclei. Multiple foci of degenerated and necrotic hepatocytes were seen around central veins which replaced by mononuclear inflammatory cell infiltration and heterophils. Kupffer cell activation could be seen. (H&E X400).
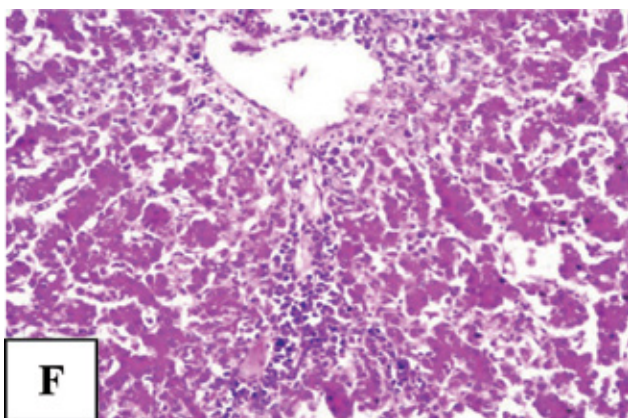
DISCUSSION

In the present investigation, the results of cultural, morphological and biochemical characteristics of *P. aeruginosa* isolates were similar to the findings of Betty et al. (2007) who isolated *P. aeruginosa* with an incidence 8% from 100 chicken suffering from respiratory symptoms. In Egypt, Farghaly et al. (2017) detected presence of *P. aeruginosa* in 42 samples after examination of 480 ones with a percentage of 8.75. Lower isolation percentages were recorded by Hayford (2017) who isolated for *P. aeruginosa* from chickens samples in rates of 1.8 and 2.6%, respectively. Elsayed et al. (2016) isolated *P. aeruginosa* with percentage 22.9% (38 /166) and a high isolation rate from yolk sac of dead-in-shell embryos 26/50 (52%) was observed.

P. aeruginosa was isolated from young chickens with high mortalities and late dead in shell embryos (Fekadu, 2010).

Satish and Priti (2015) succeed in isolation of *P. aeruginosa* from 200 samples of 4- days- old chicks in percentage of 20%; where the isolation rate from healthy birds was 12% and from diseased ones was 30%.

Serological examination of *P. aeruginosa* is very important for epidemiological studies as serotyping facilitates the detection of the prevalent serotypes and locating sources of infection (Nedeljković et al., 2015). Serological identification of the present *P. aeruginosa* isolates were G and M. El-Gohary et al. (2012) demonstrated that the predominant serotypes were A, B, D, F, H, K, L and M.

Due to difficulties and inaccuracy of phenotypic characterization of *P. aeruginosa*, PCR amplification

Figure 5 (F): Liver of 11-days old chicks showed portal area with hyperplastic proliferation of bile duct and highly dilated portal blood vessel. Mononuclear inflammatory cell and hetrophils infiltration could be detected around portal area. Most hepatocytes had degenerative and necrotic changes. (H&E X400).

and sequencing for detection of species specific conserved genes (*toxA, psIA* and *fliC*) in the genome of *P. aeruginosa* can be utilized in its identification and classification. Molecular detection of virulent genes of *P. aeruginosa* in this work showed presence of *toxA* and *psIA* genes in all strains and *fliC* in almost strains. Potential virulence factors secreted by *P. aeruginosa* that are important in its pathogenicity include exotoxin A (*toxA*), which is the most toxic virulence factor detected in this organism (Dong et al., 2015). It inhibits protein biosynthesis, it has a necrotizing activity on tissues causing cell death and contributes to the colonization process (Michalska and Wolf, 2015). Other genes like (*pslA*) is responsible for biofilm formation (Ghadaksaz et al., 2015). Flagellar gene (*fliC*) plays important roles in tissue penetration (Ertugrul et al., 2018).

P. aeruginosa is also known to harbor antibiotic resistant plasmids, integrons and transposons and is able to transfer these genes to other species. Here, isolated strains of *P. aeruginosa* showed *in vitro* susceptibility (100%) to ciprofloxacin, levofloxacin and gentamycin and (48.48%) streptomycin but resistance (100%) to amoxicillin/clavulanic acid, doxycycline and erythromycin and (78.78%) nalidixic acid. Nearly similar finding was observed by Farghaly *et al.* (2017) who demonstrated that 42 *P. aeruginosa* chicken's isolates showed high sensitivity for norfloxacin, ciprofloxacin, and levofloxacin with the percentages of 80.9, 76.2 and 73.8, respectively, 76.2 % to colistin sulfate and gentamicin and 66.7 to streptomycin; however, high resistance (100%) to ampicillin, nalidixic acid and lincomycin followed by trimethoprim sulfamethoxazole with percentage78.6. As well, Elsayed et al. (2016) recorded sensitivity of *P. aeruginosa* strains to ciprofloxacin and gentamycin.

The results of clinical signs and post-mortem lesions of the inoculated chicks were parallel to the results of Mohamed (2004) who found that subcutaneous inoculation of 3 days old chicks with *P. aeruginosa* induced mortality reached 80% with sleepy appearance, closed eyes, sitting on hocks and diarrhea while the gross lesions revealed congestion of all internal organs, peticheal haemorrhages on liver and spleen, pericarditis, pneumonic lungs, swollen of kidneys with deposition of ureats in the ureters, enteritis, enlargement of the gall-bladder and unabsorbed congested yolk sacs. Satish and Priti (2015) found that *P. aeruginosa* induced 100% mortality when inoculated intramuscularly in 7 days old chicks where dead birds showed congested liver with petechial haemorrhages and distended dark gall bladder, congested heart, lungs and kidneys, enlarged yolk sac and haemorrhagic intestines. Walker et al. (2002) and Joh *et al*. (2005) reported that the mortality rate was 95% in chick's inoculated *P. aeruginosa* through yolk where dead birds showed congestion and enlargement of liver, peritoneal fluid in the abdomen, epicarditis, omphalitis and some solidified yolks.

Considering the histopathological examination, severe interstitial pneumonia of one day old chicks led to increase thickening of alveolar wall and capillary membrane and decrease the number of ventilated alveoli resulting in hypoxia. Van Delden (2004) discussed that elastin protein of connective tissues and its degradation which carried out by LasB enzyme which is an important enzyme of elastolytic activity that produced by *P. aeruginosa*. That explains the destruction in alveolar wall and blood vessel wall which led to haemorrhagic pneumonia, also explains the destruction of hepatic sinsoides which led to haemorrhage (wide patches of accumulated red blood cells) in hepatic tissue. Rejmanj et al. (2007) stated that *P. aeruginosa* infection alters the permeability function of the epithelial and endothelial junction and that clarifies pulmonary blood vessels thrombosis. Most histopathological changes of lung, heart and liver were inflammatory in nature that come in agree with Timurkaan et al. (2008) who found inflammatory reaction through heart and lung especially in young birds. Ferro et al. (2004) stated that heterophils functions in 1-14 days old chickens were found inefficient compared to older birds, that inefficiency in function continued until 21 days of age and that was correlated with an increase in susceptibility to bacterial infection. Kupffer cells activation which detected in liver of all infected chicken comes in agree with Katja et al. (2015) who described that Kupffer cells are important cells for filtration of *P. aeruginosa* during systemic infection and that in corporation with heterophils which are essential for bacterial control. Most of liver diseases in chicken were associated with liver failure which was explained by Supertika et al. (2006) who found degeneration and necrosis of hepatic tissue in infected 10 days old chickens. The prominent microscopic lesions of the dead broilers with *P. aeruginosa* were multiple foci of coagulative necrosis with intra lesion bacteria and heterophilic infiltration in the liver and the serosal surface was covered with fibrino-purulent exudates, diffuse fibrinous exudates with bacterial colonization in the epicardium and decreased lymphocytes and moderately increased reticulocytes in the spleen (Joh et al., 2005).

In conclusion, *P. aeruginosa* is of great importance

pathogen as it could be isolated from both hatcheries environment and dead in shell embryos based in detection of virulent genes. *In- vitro* antibiogram is important to detect susceptibility and resistance of *P. aeruginosa* to different antimicrobials. The pathogenicity of *P. aeruginosa* in newly hatched chicks revealed that this organism is pathogenic. Farm management should take strict hygienic measures against possible sources of infection. Further studies are in need to study the effect of this pathogen on Egyptian poultry industry.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Improving market outcomes: A qualitative assessment of the Greek dairy supply chain

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ABSTRACT. This study provides a qualitatative assessment of the overall outcomes of the dairy supply chain in Greece. Based on the framework of the Taskforce for Agricultural Markets, a questionnaire survey with in-depth interviews was conducted to farmers, industries and supermarkets in Greece in order to gain knowledge about trading practices, market transparency, risk management, contracts, access to finance and the role of Producer Organizations. The analysis did not reveal significant unfair trading practices and showed positive prospects for the overall supply chain.

Keywords: supply chain, dairy sector, unfair trading practices, market transparency

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INTRODUCTION

In April 17 2019, the European Commission issued the Directive (EU) 2019/633 (in the remainder of the paper 'Directive') of the European Parliament and of the Council on Unfair Trading Practices (UTP) in business-to-business relationships in the agricultural and food supply chain. The Directive formally introduced important adjustments for the smooth functioning of food supply chains. In particular, within six months after the introduction of the Directive all Member States would have to put into place national legislation regulating UTP and to designate a competent public body to play the role of "enforcement authority" (EA) (Article 4 of the Directive). The EAs would be in charge of inspecting the occurrence of UTPs such as short notice changes in orders; unilateral and retroactive changes in supply agreements; un-agreed returns of unsold products; delayed payments more than 30 days after delivery (Article 3). In addition, EAs would receive complaints from suppliers who are victims of such practices and will investigate them at their own initiative (Articles 5 and 6). In fact, collaboration among EAs across Europe would be expected to address supra-national UTP issues (Article 7). Towards this direction, all Member States should report regularly to the Commission in order to share experience and to provide relevant data (Article 9).

In policy debate, the Directive is the outcome on numerous reports, documents and discussions regarding the consequences of deviations from fair trading practices (Velazquez and Buffaria (2017)). The latest was the Report by the Taskforce for Agricultural Markets (TAM) (2016) in November 2016. The TAM adopted a rather holistic approach that went beyond commenting UTP. Their report proposed an integrated plan of action to improve food market outcomes with interventions in seven specific domains. With this in mind, Velazquez and Buffaria (2017) provided a review of existing literature in order to assess whether the policy framework (before the introduction of the Directive) would be sufficient to ensure a smooth functioning of food supply chains. They proposed that Producer Organizations (POs) could play a very important role, especially in the dairy supply chain, although the UTP Directive could impede the process of strengthening their role if proper adjustments were not provided.

It is not surprising that the EU has focused on UTP, market transparency and, in general, on a holistic approach of food supply chains. All relevant actors (farmers, processors, traders, wholesalers, retailers, consumers) have revealed that frequent and damaging UTP are present in the food supply chain (European Commission, 2018), while 96% of suppliers in the EU food chain claim to have been faced with at least one form of UTP (European Commission, 2014). In fact, the estimated costs of such practices rise to an impressive 30-40 bil € (EESC, 2016), as they can be detected across all links of the food supply chain. Therefore, these issues are important from an economic and social standpoint and affect the overall performance of businesses and actors across the supply chain and therefore EU intervention is required (European Commission, 2018).

The economic repercussions of UTP have been pointed out by numerous authors (see a comprehensive discussion in Falkowski, 2017). However, it is intriguing that the presence of UTP even in a small segment of or in particular supply chains could possibly have 'spillover' effects, thus 'spreading' malpractices throughout markets and supply chains (Menard, 2017). In examining the economic effects of UTP, Sexton (2017) referred to problems related to inefficiency, uncertainty, low innovation and investment. Ambiguous relationships were reported between UTP and innovation and also between UTP and variety of products (Falkowski, 2017), implying that these issues are highly context-related. The European Commission (2014) pointed out the negative effects of UTP on productivity and stressed that markets operating under such practices provide disincentives for actors to enter and operate.

Nonetheless, the problem is not purely economic but also has social dimensions (Menard, 2017). In general, UTP stem from imbalanced distribution of power (Falkowski, 2017), which translates to the relationships among actors. Farmers are the ones most vulnerable to UTP due to their weak bargaining power. According to the Commission *"they are often dependent on bigger downstream partners ... and long production lags and the perishability of many of their products limits their room for manoeuvre"*. It is also widely acknowledged that UTP affect also the relationships between industries and retailers (Sexton, 2017; Falkowski, 2017). Therefore, it is not particularly useful to focus on specific relationships or actors, but it is rather imperative to examine the effects of UTP across the supply chain as a whole, keeping in mind the interdependencies among actors (Cafaggi and Iamiceli, 2017). Despite the importance of trad-

ing malpractices in food markets, available literature on such holistic approaches is disproportional.

The purpose of this study is to contribute to this part of literature by presenting a qualitative study of the dairy supply chain in Greece[1]. In particular, the study provides an overall evaluation of market outcomes alongside with an assessment of the position of each link. The choice of the dairy supply chain is based on three characteristics. First, particular efforts have been made for improving transparency and tackling UTP in this supply chain (for instance, the "Milk package"). Second, dairy farming in Greece has witnessed significant structural changes in the past few years, leading to transformations from which large farms with higher bargaining power emerge. Third, Cooperatives tend to make an innovative appearance and to become strong competitors in processing, which is, however, highly concentrated. These characteristics are explained in detail in subsequent Sections

This study is based on a survey of actors across the supply chain. In particular, selected farmers and representatives of dairy industries and of big supermarket chains were interviewed using a semi-structured questionnaire with open-ended questions. By drawing on their opinions, a qualitative assessment of the overall outcomes of the dairy supply chain was elaborated based on the TAM (2016) framework. The qualitative approach was chosen for two reasons. The first was that until now there are very few integrated assessments of the whole supply chain in Greece. For instance, a survey of manufacturers' opinions regarding trading practices in the Greek food sector was undertaken by Maglaras et al. (2015), but primary producers and retailers were not interviewed. This study aspires to fill in this gap by shedding light on key issues that affect market performance and to propose issues that require further research. The second reason was to address the challenge to assess the suitability of the TAM framework in understanding the dynamics of food supply chains.

The paper is organized as follows: Section 2 presents the seven domains of the TAM (2016) framework, Section 3 provides an indicative picture of the Greek dairy sector and Section 4 describes the methodological framework of the study. Section 5 contains

the empirical results and Section 6 their implications. Section 7 concludes the paper.

THE TASKFORCE FOR AGRICULTURAL MARKETS FRAMEWORK

As mentioned in the Introduction, policy debates regarding UTP and market transparency have been consolidated in the Report of the TAM (2016). The seven domains for intervention proposed by the TAM are briefly outlined below, along with references to other prior related work.

1. Market transparency. The Taskforce proclaimed the need to strengthen market transparency especially for the benefit of the numerous small producers, notably with regard to information on producer and consumer prices, production/supply and consumption/demand trends and patterns. Information asymmetries have been pointed as a factor reducing market transparency, affecting price transmission mechanisms and being the cause of UTP (Falkowski, 2017). A measure to achieve better market information was the establishment of Dashboards at the central EU level.

2. Risk management. The Taskforce endorsed risk management instruments in agricultural production to mitigate the effects of variations in product quality and production volumes as well as price volatility, using the toolkit available by the Common Agricultural Policy (CAP). Risk management is inherently related to innovation and investment, as an external environment bearing low risks or helping actors to manage risks provides higher potential for novelties. The availability of relevant tools affects the decision-making processes and strategies of firms.

3. Futures and other derivative instruments. Such instruments have been used extensively in the grains, oilseed, potato and sugar sectors and, during the last few years, for milk powder, butter and pigmeat. In all cases, they hold the potential to become crucial risk management tools in times of price volatility. Their applicability, however, in the Greek setting is low and no relevant paradigms exist, so this domain was excluded from the analysis.

4. Trading practices in agricultural markets. Unfair Trading Practices have been defined as "…. practices which significantly deviate from good commercial conduct and are contrary to good faith and fair dealing " (European Parliament, 2016). There is a variety of practices that could potentially be cate-

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1 This paper investigates exclusively the dairy cow supply chain. Although sheep and goat milk production is important for the Greek setting, it is not examined in this paper.

gorized as UTP (see Sexton (2017) for a discussion) and the distinction between driving a hard bargain and UTP is sometimes blurry (Taskforce for Agricultural Markets, 2016; Falkowski, 2017). The important economic implications of UTP have led to legislative action as described in Sections 1 and 3 of this paper.

5. Contractualization. This domain referred to strengthening Contract Farming by introducing compulsory contracting among actors as well as the provision of "standard" contract templates to facilitate the parties involved. Sexton (2017) noted that such contracts are used in the USA, but they mainly describe litigation resolution processes rather than the terms of the agreement. A more widespread use of written contracts is an issue that needs to be discussed in improving market outcomes.

6. The CAP and competition law. The TAM specifically urged the Commission to provide sufficient clarifications regarding the institutional framework governing POs. In particular, the TAM calls for additional legislative exceptions to POs in competition law. According to Bijman et al. (2012), in 13 EU-countries POs command more than 50% of the market, therefore their potential expansion could increase even more their market shares, thus opposing to competition laws. Velazquez and Buffaria (2017) underlined that the current upper limit of control of the quantity of a specific product by a PO is not very strict (for instance for milk POs it is 33% of the total national milk production per Cooperative), however

the whole framework of POs under the light of competition law needs to be revised in order to ensure that it should not become a factor hindering their development. In addition, the TAM proposed that specific derogations from Competition Law regarding the milk sector should be maintained.

7. Access to finance. Facilitating farmers' access to finance even by providing guarantees for farmers' loans from local banks through the European Investment Bank. This domain is highly relevant for Greece under the economic crisis, which has deprived farms and businesses from liquidity and has brought serious adverse effects in all sectors (Karanikolas and Martinos, 2012; Ragkos et al., 2015; Ragkos et al., 2016).

THE GREEK DAIRY COW SUPPLY CHAIN

The Greek dairy cow supply chain has witnessed great changes since early 2000s. The number of farms has been decreasing constantly since 2006 (decrease by 53.1% or 6.5% annually), while milk production has remained relatively steady (only 5% reduction from 2000 to 2016) (Table 1). This adjustment denotes the intensification of production, which resulted in an increase in average milk production per farm by 4,8% annually. Indeed, large dairy farms of predominantly entrepreneurial nature emerged - either new or existing which decided to intensify. These farms have better access to information, technical support and innovation and have the possibility to access competitive markets.

Table 1. Structural development of the dairy cow sector in Greece (2006 - 2016)

Year	Farms		Total milk production		Average production	
	Number	Change (%)	(thousand ton)	Change (%)	(ton./farm)	Change (%)
2006-2007	6270		736		117,4	
2007-2008	5627	-10,3	716	-2,7	127,2	8,3
2008-2009	5074	-9,8	699	-2,4	137,8	8,3
2009-2010	4561	-10,1	682	-2,4	149,5	8,5
2010-2011	4259	-6,6	666	-2,3	156,4	4,6
2011-2012	3930	-7,7	642	-3,6	163,4	4,5
2012-2013	3686	-6,2	627	-2,3	170,1	4,1
2013-2014	3558	-3,4	615	-1,9	172,8	1,6
2014-2015	3356	-5,6	609	-0,9	181,5	5,0
2015-2016	3215	-4,2	605	-0,6	188,2	3,7
Total change (2006-2016)	-3055	-48,7	-131	-17,8	70,8	60,3
Annual rate of change (%)	-6,5		-1,95		4,8	

Source: Hellenic Agriculture Organization "Demeter", processed data

The milk processing sector in Greece is oligopolistic, with six companies controlling 70% of the fresh cow milk processing. Recently mergers and takeovers have increased even more concentration in the sector. Multinational dairy companies and large Cooperatives have also penetrated the market (Friesland Campina), while also Greek Cooperatives are becoming increasingly competitive. In the retail sector, the top five retailers account for 56% of the grocery retail market (ICAP, 2013), representing a high degree of concentration, while 80% of retail sales of dairy products are controlled by five supermarkets.

Table 2 presents the actual legislative framework and initiatives for the improvement of the Greek dairy supply chain, organized according to the seven TAM (2016) domains. In addition to the information of Table 2, specific reference should be made to the Rural Development Program of Greece (RDP) 2014-2020, which includes numerous measures aiming to boost the performance of the primary sector in general in terms of better access to finance, risk management and promotion of collective actions (POs). Furthermore, the "Milk package" (Regulation (EU) No 261/2012; OJ L 94, 30.3.2012, p. 38.) is a set of measures that have been proposed at the European level regarding the overall performance of the dairy supply chain. The "Milk package" proclaims important issues, such as the necessity of written contracts, milk delivery reports, Producer Organizations, collective negotiations and other important market transparency issues. Being of pan-European importance, the specific provisions of the "Milk package" are not explicitly presented in Table 2. However, it should be stressed that many of them are crucial to the Greek dairy sector as well, but most of them have not been incorporated in national regulatory framework and only recently action was taken - for instance with Law 4492/2017 (Table 2).

Table 2. Existing framework to improve market outcomes in the Greek dairy supply chain

Domain of intervention	Existing framework/Initiatives in Greece
Market transparency	All milk buyers, including farms which process their own milk, are obliged to access the «ARTEMIS» system (Common Ministerial Decision 1678/111284/2015 (FEK B' 2257/20-10-15) which was repealed by Common Ministerial Decision 838/51008/2019 (FEK B' 964/21-03-19)) and submit electronically the «Monthly Dairy Balance Statements» (Article 3, Common Ministerial Decision 175180/11 (Government Gazette 1721 / 02-082011) (Article 151 of the CMO). The purpose of milk balances is to control the legal use of all types of milk in dairy products as well as to ensure that legal requirements are followed by processors subsidized by national or EU frameworks and the correctness of the claims of the geographical origin of the raw material used in the production of certain dairy products and the handling, disposal and delivery of milk by-products. All businesses involved in buying or processing raw milk (cow, sheep and goat) are obliged to upload data of the milk quantities they process each month. The Hellenic Agriculture Oorganizaton (HAO) 'Demeter' manages data regarding raw milk production and reports monthly volumes of milk processed and corresponding prices. Information from the EU Milk Market Observatory is open to all interested actors (https://ec.europa.eu/agriculture/market-observatory/milk_en)
Risk management	There are no specific policy measures supporting the risk management strategies undertaken by farms and other businesses downstream the supply chain. According to the RDP 2014-2020, Law No 3877/2010 created *".... a national system of protection and insurance of agricultural activity"*. With the same Law a «Directorate for Crisis and Risk Management in the Agricultural Sector» was introduced. However, risk management (agricultural production, income and risks in food chain) is in primitive form in Greece. The insurance of production by the Greek Insurance Organization (ELGA) is compulsory.
Futures/ Derivatives	-

Trading practices	Law 4492/2017 seeks to harmonize the Greek framework with the European, especially when it comes to late payments, in order to ameliorate the performance of supply chains. Under this Law, payments should be done at 60 days from delivery the latest and 30% penalties would be imposed to those who do not comply. Farmers and processors are obliged to announce all data regarding their transaction to designated services. In addition, the Law introduced a new branding scheme for milk, discerning the country where the milk was produced and/or processed and/or packaged, thus reducing problems relating to false labelling. However, the Law did not provide for trade relationships between Supermarkets and dairy industries, which is an issue tackled by the UTP Directive in order to contribute further towards reducing UTP in food supply chains in Greece.
Contractuali-zation	As part of the «Milk package» all Member States have the option to make written contracts between farmers and industries compulsory. This has not happened yet in Greece.
The CAP and competition law	Law 4384/2016 defined the legislative framework for the establishment and operation of Agricultural Cooperatives and Producer Groups. Numerous measures of the RDP 2014-2020 (for instance M01, M02, M07, M16 etc) provide specific opportunities and/or higher support to Cooperatives and Producer Groups. In Greece there are actually no POs of the magnitude described in the «Milk package» (33% of the total national production), while since the total national production stands for 0.45% of the total EU production, neither does the second «milk package» restriction apply (<3.5% of total EU milk production).
Access to finance	One of the most serious problems of the Greek primary sector. It affects farm management and ability to invest. The RDP 2014-2020 provides funding opportunities relating to farm modernization, generational renewal etc. Moreover, the Greek development law 4399/2016 provided aid schemes to support the country's less developed areas and improve competitiveness in high added value sectors. In Greek legislation, however, there is no clear provision for a financial institution geared to financing the agricultural sector.

METHODOLOGICAL FRAMEWORK

Numerous approaches to the assessment of trading practices have been proposed. Rosa et al. (2015) applied an econometric model to analyze the efficiency of the dairy supply chain in Italy and found that decreases in producer prices can be attributed to higher competition rather than information asymmetries. Gorton et al. (2015) also employed a quantitative analysis to study UTPs in food supply chains in the USA and confirmed that trustworthiness was positively related to fair practices. Using a Global Value Chain framework, Lianos and Lombardi (2016) provided a theoretical framework to examine food value chains in contrast to competition law. Sexton (2017) identified three main approaches and four types of methodologies for empirical investigations of trading practices (interpretive methodologies based on interviews; surveys, either online, by phone, or face to face; case studies; modelling).

As this study attempts to provide an assessment of a food supply chain based on the TAM framework, a qualitative approach was chosen in order to acquire rich information to shed light to practices affecting the overall performance and outcomes of the supply chain. Qualitative research can be used to generate knowledge about the perspectives of a phenomenon or a situation and especially in-depth interviews may yield important highlights based on the experience of individuals (Hammarberg et al. 2016). According to Sexton (2017), in-depth knowledge of the relationships among actors in the supply chain could provide explanations of the sources and implications of UTP and demonstrate why such practices are present in some settings and are less important in others. Examples of such approaches in food chain analysis include the work of Broderick et al. (2001), Alonso and O' Neill (2001), Ilbery and Maye (2005) and Connelly et al. (2011).

The study area for the qualitative assessment of the dairy supply chain in this study is the northern part of the country (Thrace, Macedonia and Thessaly) where 80% of Greek cow milk is produced, almost 80% of dairy cows are reared and more than 70% of farms are situated. In-depth interviews were conducted from August to October 2017 using three versions of a carefully designed semi-structured questionnaire. The first was for dairy farmers, the second for industries and the third for Supermarkets. In all versions, questions were open-ended and were categorized in the six domains proposed by the TAM framework (excluding Futures and Derivatives) (Table 2). In more detail, all actors were asked regarding their understanding of

market transparency and the use of information sources; UTP phenomena they had been subject to; use of risk management tools and relevant strategies; access to finance and liquidity (e.g. bank indebtedness and willingness to make loans); use of written contracts and agreements upstream and downstream the supply chain. Furthermore, respondents were asked about their future investment plans but also questions about the profile and development of their businesses. All participants were encouraged to state their opinions for issues not explicitly included in the questionnaire but related to the scope of the study.

Interviews were conducted by at least two enumerators, who noted all responses, and then all notes were systemized and combined. In total, 11 dairy farmers, 8 representatives of dairy industries and two representatives of Supermarket chains were surveyed, all of which are situated in Northern and Central Greece (Thrace, Macedonia and Thessaly). All surveyed dairy farms were family-run; they reared 142 dairy cows on average and yielded 7975 kg milk per cow annually. Among the eight dairy industries, two were Cooperatives and five private companies, while in the eighth the staff and other companies were shareholders. Their common characteristic was that they all processed and distributed fresh cow milk, while for seven of them this was their main activity. Apart from that, each company also produced other dairy products, mainly yoghurt and desserts. Most of these industries also processed sheep and goat milk for cheese, yoghurt or even fresh pasteurized goat milk (two companies). Out of the six private companies, three were family-run and three were S.A.s with numerous shareholders. In addition, all these six companies exported their products, while the two Cooperatives only targeted the Greek market. In the retail sector, the Supermarkets surveyed here were major actors in retail sales in Greece. Since anonymity was guaranteed, no other characteristics of respondents are reported here.

The presentation of the research findings was enriched with original quotations of respondents. This method was preferred to add credibility to the results and to help derive meaningful conclusions from this study. This approach has become standard practice in several similar studies, as the inclusion of whole quotations generally helps readers assess the accuracy of the findings presented and thus adds credibility, while it also enables more in-depth understanding of the importance of the results described (Corden and

Sainsbury, 2006; Corden and Sainsbury, 2007; Anderson, 2010; Bryman, 2016). Examples of previous studies using this technique in order to generate more detailed and in-depth knowledge of respondents' attitudes and opinions include the work of Clark and Gerrig (1990), Austin et al. (2005) and Hammarberg et al. (2016), while no relevant study was found for the dairy sector and for the assessment of the overall value chain performance. Quotations in the Results Section are identified only in terms of the type of respondent (Dairy Farmer (DF); Dairy industry (Ind.); Supermarket (SM)), while Industries are further discerned into Privately owned (Pr), family businesses (Fam) and Cooperatives (Coop).

RESULTS

Market transparency

Milk price was one of the most important factors - if not the most important - affecting the overall performance of the market. For Industries, this was a critical success factor and this was supported further by one respondent who stated that

The development of our firm was largely based on the high milk prices at the time we started (Ind, Coop)

According to most dairy farmers and industries, producer prices changed on a monthly basis and were usually formed according to the distance of the farm from the industry (transportation costs) and, of course, quantity. Milk quality was another important factor, affecting not only the final producer price but also the quality of cooperation between the industry and the farmer. Some dairies tended to set very high quality standards and discard milk of lower quality, while others (especially large ones) processed milk of standard quality but at lower producer prices. Most farmers felt strongly in favour of this strict quality policy and claimed to follow very carefully all the management guidelines provided by industries and veterinarians. For example, one dairy farmer stated that

I am very happy with the Industry, so I do my best to follow their rules. When I do, we both win (DF)

All respondents agreed that in the bargaining process, downstream actors were stronger than the ones upstream. This means that for farmers, Industries were the strong competitor and for the latter *"Supermarkets play the game" (Ind, Pr)*. On the other hand,

even Industries were sometimes in a weak bargaining position and most of the respondents mentioned that the Law 4427/2017 would impact them adversely, as they would have additional responsibilities against farmers, without, however, having any assurance for timely payments from retailers. Quoting a characteristic statement from an Industry respondent

We are the weakest link of the supply chain, as we are pressured from upstream (farmers) for better producer prices and from downstream (supermarkets) for lower retail prices. We cannot shift this pressure to farmers, because they will stop producing and this will be even worse (Ind, Fam.)

Super markets were the most powerful links in the supply chain, they do not leave much room for bargaining and there was total unanimity in this argument. Methods, strategies and practices followed by farmers and dairies to increase their power are presented in other parts of the Results Section and are discussed further in Section 6.

Regarding the formulation of retail prices, a common mechanism was described by Industries with slight variations. The procedure started with Industries proposing a *starting price* (common price list) to all retailers, including Supermarkets. After negotiation, both parties decided a commonly accepted *baseline price*, based on product type and diversity, as well as on their quantity. The difference between *baseline prices* and *starting prices* varied according to the bargaining power of each player. For instance, industry respondents reported a good level of mutual understanding with small retailers (pastry shops etc), but mentioned that bargaining with supermarkets was not flexible because of their power. This was also due to the fact that Supermarkets control much higher proportions of the whole market, compared to Industries, which also demonstrates a lack of horizontal collaboration among Industries, as was explained by one respondent

We work together well in general with other industries, when this is needed, but competition is high and there is very little room for communication. This is one of the reasons why supermarkets define the prices (Ind, Pr)

Nevertheless, differences between *starting prices* and *baseline prices* were lower for high quality products. According to one Industry respondent

While in the beginning super markets did not want to bargain with us and rejected our products, now they come to us and bargain. This is all because of our quality: if your product is good, you will find a decent way to sell it (Ind, Pr)

Supermarket representatives also referred to quality, which they posed in a very central position in their development strategies. It was mentioned that

We are always open to new quality products: we want consumers to connect us with quality and we pay more for that (SM)

In addition, it was ascertained that dairies which fail to provide products of at least acceptable and stable quality in a timely manner were excluded from their shelves.

The prices paid by Supermarkets to industries were usually lower than the agreed *baseline prices* because - in most cases - the contracts also included promotional activities for industries which were translated to lower payments. Such activities (as described by respondents) included: better shelf positioning, separate promotion spot inside the store, television advertisement, in-store promotional activities, supermarket leaflets, availability in more branches of the same chain etc. These promotional activities were somehow "compulsory", in a sense that industries were pushed to choose at least some of them. In other cases, supermarkets agreed to undertake the transportation costs or, in cases of hard bargaining, they agreed to incur part of the marketing costs. Under another type of agreement - explained by one respondent -, the baseline price was fixed, but then, periodically, a "sales price" was set, which brought a reduction of the final payment to the industry. As a result of these practices, Industries got significant lower prices and this was explained specifically by one respondent

When this whole issue with promotional activities started, things were reasonable and price reductions were up to 15%. In the past few years this changed and now price reductions are significantly higher. Supermarkets are far off (Ind, Pr)

Because of this practice, the final price of the product is generally formulated usually 1-3 months after the initial agreement. This method does not allow prices to be revealed to competitors early.

Regarding information sources, there was a considerable divergence between respondents. All indus-

try respondents mentioned that Artemis system was useful and relevant and that they regularly visited EU observatories; however, they expressed different views as to their practical importance. On the other hand, dairy farmers were in general more skeptical about the importance and contribution of Artemis system, while only two of them were aware of the EU Milk Market Observatory.

Risk management

Risk management relative to liquidity and the overall economic environment was discussed with all respondents. Industries did not specifically refer to the availability and/or use of such tools, which could help them overcome financial stress. On the other hand, most farmers had used at least once financial help from an EU-funded measure for investments (e.g. financial support for modernization and investments or the "Young Farmers" measure) and explicitly acknowledged the importance of their decision. However, still this was not a risk management tool but rather a measure to increase economic performance and competitiveness.

During the interviews, the discussion about risk management was expanded to their overall strategies for reducing risks or dealing with it, because this issue was particularly relevant to the overall adverse economic environment. Risk management differed among industry respondents. In general, most of them did not refer to particular strategies and mainly mentioned the ability to foresee market trends, high product quality and stable exports as key aspects to reduce risks. Apart from that, one industry respondent also mentioned the importance of adequate liquidity and of diversification to many products and activities while another one stated that

Only products with added value can support the normal operation of our company. This is why we have turned to yoghurt and ice cream (Ind., Fam.)

One of the topics of this discussion concerned attitudes and practices regarding new investments. All respondents (farms, industry, supermarket) described relatively concrete strategic expansion plans, which all included planning or even actually realizing investments but differed in terms of timing and resources. For farms, common investments included infrastructure (new machinery, expansion of buildings etc), genetic improvement of cows, increases in flock size and novel herd management practices. For dairy industries expansion of infrastructure and

capacity as well as research and development of new products (customized for specific markets) were the main investments planned, because, as one Industry respondent mentioned

Quality products will ensure high bargaining power (Ind, Pr)

Another issue which respondents related to risk management was the number and size of suppliers. Upstream the value chain, industries stated that they preferred to collaborate with a relatively large number of dairy farms of all sizes, in order to avoid dependence on specific suppliers. Industry respondents also affirmed that farm size was not related to the quality of cooperation between them. Some indicative responses are presented below

Medium and/or big farms (2tn/day) are better to work with, because they are more viable due to low production costs and usually, they achieve higher milk quality (Ind, Fam.)

Big farms tend to by unsteady. They change industries very easily, so you cannot rely on them (Ind, Pr.)

It is easy to become highly dependent on a Cooperative and then you lose all your flexibility (Ind, Pr.)

Downstream the supply chain, industries pursued deals with super markets (large chains or local ones) while sales to small retailers only stood for a small part of their total revenues. However, the opposite point was raised regarding the quality of cooperation, which translated to stability and reduced risks for most industries. As one respondent stated

It is sometimes easier to get by with small local retailers: They don't ask too much and they are loyal (Ind, Fam)

Supermarkets were in line with industries, as they tended to develop a large network of suppliers of all types (family, local, nation-wide industries).

Own label products constituted a particular type which was gaining high market shares but also served as a risk management tool for industries. One industry respondent explicitly described the benefits of involvement in this type of production

With these products, we have no unexpected returns. The demand from retailers is regular and the price is fixed, there are not many changes in orders

and supermarkets do not drive a hard bargain. Thus, we operate better. This regularity in orders - production - storage - payments/prices allow us to concentrate on our own brand. Also, these regular payments stand for a significant part of our revenues, which provides us financial stability. (Ind, Pr)

However, own label products could have a negative impact on the overall reputation of the firm, if not enough focus was given on the brand name. This would affect adversely the market demand of the products (branded and private-label) and, consequently, its bargaining position with super markets and retailers. As one Industry respondent explained

Our brand name is our most important asset in bargaining with Supermarkets (Ind, Coop)

Supermarkets also seemed to favor the development of own label markets, as illustrated in the following quotation.

We gain consumers' trust with our own label products and we reduce our reliance on strong labels (SM)

A different pricing policy was described for private label products. These products received a *baseline price* mutually agreed between the Industry and the Supermarket, which was usually explicitly mentioned in the contract and remained steady for a relatively long period. Then, the supermarket covered for a part of this *baseline price* and for the cost of all promotion activities, so that the product finally reached the consumer at a lower price (mark up). In this context, Industries undertook the sole obligation to provide the agreed quantities. Therefore, risk in this type of production lied mainly in the internal processes of the firm and not on external factors, including pricing.

Two issues which are closely related to risk management of dairy industries are logistics and packaging. Having an own transportation and distribution network provided them flexibility to a certain level. All firms had such infrastructure, however the extent of their own distribution network varied significantly. Two types of logistics organization were basically described. The first was adopted by large firms and involved a well-developed own network within a significant radius and external transport only for very long distances. The second type involved a small but well-organized transportation infrastructure for local markets and collaboration with external companies for more remote areas. For this type, written

contracts between dairy industries and transportation companies were rare, however steady cooperation was maintained with specific companies. Concerning packaging, dairy industries involved most commonly worked closely with two or three companies, which provided them with all the necessary materials. Most firms mentioned that Greek local companies of this type were lacking, so they collaborated with nation-scale or foreign companies. None of the respondents mentioned any type of written agreement with this type of suppliers

Trading practices

All respondents referred to the developments of the market and to the leading role that Supermarkets had undertaken and still played. For actors downstream the supply chain, Supermarkets were the strongest link, which puts pressure on them, but the Supermarket respondent also explained that even his company was receiving pressure from multinationals. The following quotations illustrate the diverse opinions of the two types of actors

Supermarkets have been expanding in an irrational way during the past few years: Many new branches, thus high needs for liquidity. The pressure for more funding has been passed on to us (Ind, Pr)

We always try to maintain our shares in markets and it is true that we receive a lot of pressure from multinational suppliers (SM)

Not all industries accepted unsold product returns (in particular one respondent revealed that this was an explicit written term) and also no unsold product returns were accepted for own label (confirmed by at least three respondents). Returned products were usually used for animals (e.g. in swine farms) and one industry used them for biogas production, which covered for 70-80% of their energy needs.

Late payments

One industry respondent discussed the issue of late payments from Supermarkets to dairy industries. He mentioned that the whole issue started from the dairies themselves, initially as a tool for market penetration. Quoting his own words:

Some small local industries proposed to super markets to pay them later instead of lowering the price of products. They considered late payments better than lower payments. Little by little, this practice became popular (Ind, Pr)

It was confirmed by industries and supermarket respondents that Supermarkets pay dairy industries within 90-150 days from delivery. However, it should be stressed that one supermarket denied that this is a form of UTP, as the time of payments was always agreed by both sides, not in written though. It was mentioned that only one Supermarket chain currently makes monthly payments, but mainly for private label products. However, it was described that in some cases supermarkets provided a kind of an advance payment. The Supermarket respondent elaborated extensively on his opinion of UTP and explained that his firm avoided such practices, because in the long run they have negative effects on the market and of the business. In a specific part of this discussion he mentioned that

Unilateral changes do not favor any link of the supply chain. The new Law will not be of any use if the market itself does not decide to abolish UTP (SM)

Industries did not report late payments for farmers. On average payments were actually made within 30-60 days after delivery. According to the industries, whenever there was a contractual agreement, payments were always made according to the contract: one part was deposited in the farmer's bank account and the rest was paid by cheque. Farmers confirmed that late payments were actually very rare - especially compared to 1-2 years ago - and that especially in cases of trustworthy relationships with family-run industries, late payments were treated with understanding. Some farmers also mentioned that industries provided them deposits in times of increased need for liquidity and retained the amount from future milk sales. A dairy farmer mentioned that

They have helped me in times of need, so I should also be understanding when are in too deep (DF)

Contracts with farmers

There are cases of written contracts, which were explicitly described by few farmers and two dairy industries. One dairy farmer - owner of one of the largest farms in the survey - described a type of written contract by means of which the industry provided technical advice, veterinary support and drugs and retained the value of these services from monthly milk payments. Apart from this example, however, it was common not to make written contracts because prices changed monthly while milk production by farms remained relatively stable throughout the year, unlike sheep and goat milk (especially from September to November). In addition, the survey revealed that most farmers were risk-averse and preferred long standing collaboration based on mutual understanding and trustworthy relationships. This was also confirmed by all Industry respondents. The following quotations illustrate this point of view.

Contracts with farmers are not necessary. We maintain long-standing cooperation with most of the farms, while also new farms who join us, they do so based on trustworthy relationships. (Ind., Fam.)

I trust my industry, but even if I didn't, I 'm not sure that a contract would help me a lot (DF)

Cooperatives operated under a different rationale. Although their management resembled private firms, they primarily worked with their members, with which, obviously, there was no need for a further contractual agreement.

CAP and competition

It has already been discussed that Greek cooperatives are away from the maximum thresholds set by European legislation concerning competition. When it comes to farms, this is also the case as most Greek dairy farms are not of a large size that would render them capable of affecting market prices (Table 1).

Access to finance

As was expected, many respondents made specific reference to the overall fiscal environment. Although some industry respondents made particular references to taxation and social security payment rates, none of them revealed relocation to neighboring countries as an option that they would consider. One respondent from a Supermarket chain assessed access to finance as the most important issue in the operation of the firm, although he did not report such problems. He also confirmed that Supermarkets provided deposits to dairies in order to improve their liquidity "*...especially during the first period of capital controls*".

Liquidity was pointed out one of the key issues for survival, performance and expansion. Especially Cooperatives found it more difficult to use existing funding tools from banks, so specific adjustments were needed. Nonetheless, most businesses managed to survive and even thrive during the crisis. Liquidity did not seem to be a serious constraint, as is highlighted in the quotation below. The fact that all of them

planned some type of investment and/or expansion confirms this allegation made by respondents.

There was never a problem with liquidity, even during the crisis. The crisis only reduced our high growth rates (Ind, Fam)

Liquidity and financing issues were also raised by farmers, although to a less degree. Two farmers claimed not to have suffered from the crisis in this respect. In fact, financing issues were mentioned mostly in terms of low prices rather than because of irregular cash flows, although in the past there were periods of late payments. Bank loans were scarce and none of the respondents mentioned high indebtedness to banks.

Most industry respondents referred to the recent bankruptcy of a major Supermarket chain. All of those who raised this issue agreed that it caused a major turbulence in the market, not only at the actual period of the bankruptcy but also before, and that the consequences are still evident. In fact, this was one of the sources of reduced liquidity for industries for one period. As explained by one Industry respondent

Things are not the same in the market after this bankruptcy. Supermarkets started to ask us to accept more in-store promotional activities and the situation became more suffocating for all of us (Ind, Pr)

DISCUSSION

Table 3 summarizes the findings of the survey. The Table shows the main issues actually affecting the outcomes of the Greek dairy supply chain. In what follows some additional points are discussed and compared to previous findings.

The fact that retailers are the strongest link in the supply chain has also been pointed out by other authors. Maglaras et al. (2015) noted that this was a global phenomenon, attributable, *inter alia*, to high retail market concentration and closer outlook on consumer preferences as well as to the increasing market shares of own brands. Lianos and Lombardi (2015) proclaimed the need for specific legislative adjustments to address this issue, while Sexton (2017) provided a list of specific cases where retailers were found to exerce UTP. This fact was partially due to information asymmetries, which compromised market transparency. Indeed, although industry respondents stated constant use of Milk Price Observatories, dairy farmers did not know about their existence and only used the

Artemis system to input milk production data. Therefore, actors did not share a relatively equal level of information. This implied that there was considerable room for improvements and that the implementation of the Directive could play an important role towards this direction, for instance by facilitating the exchange of information and experience between States and by collecting and publishing complaints about UTP.

The survey showed that the use of written contracts was not widespread. Written contracts was avoided by risk-taking farmers who sought to profit from temporal increases in prices and grasp market opportunities, while risk-averse farmers preferred to build their collaboration upon trust. These findings were in line with Gorton et al. (2015), who found that trustworthy relationships were affected by (i) the number of commercial buyers, (ii) the ease of farmers' costs of switching among buyers, (iii) the size of the supplier, and (iv) the supplier's membership in a marketing cooperative.

The issue of dependence (also highlighted by other authors e.g. Maglaras et al. (2015); Dobson (2005); Sexton (2017)) was raised by survey respondents. Farmers and industries tried to reduce their dependence on their buyers (industries and retailers respectively). One type of this strategy was the improvement of quality. Although Sexton (2017) argued that quality reduction was a relatively common way to reduce costs under UTP, this was not supported by the qualitative data of this survey. In fact, most industries stated the opposite i.e. that they sought to increase quality in order to improve their bargaining position. Farmers were in line with this and also wanted to increase their quality. Another type of this strategy was innovation and investment, for which the survey revealed high potential, contrary to what would be expected under UTP (e.g. Sexton, 2017). Dairy farmers tended to increase their size and to produce more in order to increase their bargaining power. The R&D departments of industries launched new products in order to increase their bargaining power against supermarkets and to diversify towards covering the demands of international markets. This type of behavior was also pointed out by Inderst and Wey (2007) in their elaboration of a theoretical framework to explain retailers' market power. Hence, the overall operation of the Greek dairy supply chain did not hinder product innovation and market development. Quality, innovation and investment in food supply chains were context-related and should be treated as such.

Table 3. Overall assessment of the Greek dairy cow sector based on respondents' views (TAM (2016) framework)

Market transparency	Prices are formulated based on numerous factors, including strict quality standards. Final product prices are formulated after a few months. Information asymmetries.
Risk management	Industries did not declare the use of risk management tools, while for farmers the use of CAP funding has been used extensively, still not as a risk management tool. Regarding risk management practices and strategies, «Own label» products help the firm to operate in a smoother way. Regular payments provide financial stability. Dairies seek to combine branded, private label and exportable products to minimize risks. Specialization on products with high added value (yoghurt and ice-cream) and new products. Most investments during the crisis were made with own funds (see also (7)). Various strategies in terms of choice of packaging suppliers and logistics. Risk-averse farmers tend to sell to the same dairy for long periods, while others (especially big) prefer to change dairies for higher prices. Dairies in general prefer to sign deals with large supermarkets as a risk-aversion mechanism.
Trading practices in agricultural markets	Supermarkets are the strongest link and "set the rules" in negotiations. Unilateral changes only during negotiations. Payments from industries to farmers are usually not late. There are delayed payments from supermarkets to industries (2-5 months), which, nevertheless, were initiated by some dairy industries. Own label products gain market shares, without causing explicit problems to actors in the supply chain. In-store promotional activities decrease the final price paid to industries and this is going to be tackled by the Directive.
Contractua-lization	Written contracts are not usual either for farmers/industries or for industries/suppliers. Even when contracts are signed with farmers, prices are not fixed (they change monthly according to market trends, quantity and quality). Trustworthy relationships are important. Agreements between supermarkets and industries are written and formal and include a basic level of information about promotional activities, product process, returns of perished products. No contracts with small retailers
The CAP and competition law	Issues here are not highly pertinent to the Greek dairy cow sector, however a facilitation of the process for access to finance by POs would be beneficial. In general, the provision of the «Milk package» are not implemented in the Greek setting and there is much room for improvements towards this direction.
Access to finance	Adequate liquidity would boost even more the operation and performance of industries. The crisis and late payments reduce it. For farmers, liquidity is not a serious issue and they plan to make investments mostly with own funds. Cooperatives find it more difficult to use existing funding tools from banks. Industries and Supermarkets make easement payments to their respective suppliers.

Own label products have been identified as a potential source of UTP (Maglaras et al., 2015) but in this study this was not confirmed. None of the respondents expressed negative assessments of the impact of these products on their performance. On the other hand, two industry respondents claimed that these products help them to balance their activities and reduce risks, while Supermarkets were also in favour of promoting such products.

Regarding POs, the survey revealed an alternative operation model. Although it is acknowledged that larger POs are able to provide better services to members and contribute to higher producer prices, not only locally and for their members, but also for other settings ((Velazquez and Buffaria, 2017; Bijman et al, 2012), the institutional framework in Europe hinders the development of large Cooperatives, as they oppose the Competition Law lacking relevant derogations. In Greece, dairy Cooperatives are now expanding and it is interesting to see how this process will evolve in the future.

CONCLUSIONS

This study constitutes a first endeavor to implement the TAM (2016) framework in assessing the market outcomes of the dairy cow supply chain in Greece. Using qualitative survey data, the supply chain was evaluated in six out of the seven domains, as futures were not applicable to the context of the study. The assessment of the views of farmers, industries and supermarkets showed that the TAM (2016) framework is a useful tool to collect qualitative market information and to assess outcomes and performance for the supply chain as a whole and not only for specific actors, links or segments. Since the qualitative analysis did not reveal any important UTP or other practices that are particularly harmful to the overall performance of the Greek dairy supply chain, the sector showed positive prospects. Indeed, as Sexton (2017) pointed out, loosely concentrated market structures entail worse results for farmers that highly concentrated markets, the current developments in the structure of the sector show a transition towards a more efficient organization, maybe as a result of

modernization. However, a good level of horizontal collaboration among Greek dairy industries might be what is actually missing from the sector, as this lack of cooperation was identified as a cause of late payments and potentially a source of UTP between Supermarkets and dairy industries. From the primary production point of view, the provisions of the "Milk package" could be important in improving market outcomes in Greece in general.

One of the main conclusions of this study is that there is ample room for further research, both in the dairy sector and in the field of trading practices and improvement of market outcomes in general. In fact, the main limitation of this study is that it only com-

prises a relatively small number of farmers and super-markets and a part of dairy industries. Nonetheless, the in-depth interviews have raised important issues to be investigated and analyzed in the future. More qualitative studies of trading relationships are neces-sary to understand supply chain dynamics and these should be complemented by quantitative assessments of their true repercussions. This is in line with Sexton (2017) who also pointed out a gap in the analysis of UTPs in food supply chains. One more specific issue to be addressed is the role of consumers in maintain-ing or combatting UTPs and the effects of the latter to their consumption patterns. Falkowski (2017) provid-ed a discussion of available methods and approaches in this domain, which remains relatively unexplored.

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The Effect of Capsaicin on IGF-I and IGF-IR Expression in Ovarian Granulosa Cells

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ABSTRACT. Capsaicin (trans-8-methyl-N-vanillyl-6-noneadamide) is a pungent ingredient in red peppers from the Capsicum family. Insulin-like growth factor-I (IGF-I) is expressed in granulosa cells and has an important role in ovarian development. However, there are no data about the IGF-I expression in ovarian granulosa cells after capsaicin treatment. The aim of this study was to investigate the expression of IGF-I and its receptor (insulin-like growth factor-I receptor [IGF-IR]) in primary rat ovarian granulosa cells after low and high doses of capsaicin treatment. For this, granulosa cells were isolated and cultured from ovaries of 30-day-old female Sprague-Dawley rats. Granulosa cell plates were divided into four groups as cell control (C), vehicle control (V), and 50 μM and 150 μM capsaicin groups. In experimental groups, granulosa cells were exposed to capsaicin for 24 hours and immunocytochemistry was performed afterwards using anti-IGF-I and anti-IGF-IR antibodies. Both IGF-I and IGF-IR expressions were found to be significantly increased in parallel to the capsaicin doses. Elevated levels of IGF-I may be a risk factor for ovarian development. Because of the crucial role of IGF-I in ovary development, capsaicin treatment can be effective on follicular development and/or disorders characterized by high IGF-I levels.

Keywords: ovary, capsaicin, IGF-I, IGF-IR

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INTRODUCTION

Insulin-like growth factor-I (IGF-I) is a basic peptide comprised of 70 amino acids and promotes differentiation of various cell types with its anti-apoptotic activity and anabolic effect (Daughaday and Rotwein 1989). In addition to ubiquitous distribution in various tissues, the ovary is a major site of IGF-I production in mammalians (Daughaday and Rotwein 1989). IGF-I is highly expressed in the ovary and its mRNA is concentrated in all stages of developing follicles' granulosa cells (Kadakia *et al.,* 2001). IGF-I and its receptor (IGF-IR) are important factors that regulate ovarian cells' proliferation and differentiation as well as follicular development and ovulation (Armstrong and Webb 1997; Zhou *et al.*, 2013; Baumgarten *et al.,* 2017). IGF-I enhances FSH-stimulated estrogen and progesterone production by increasing steroid biosynthetic enzyme activities and induce LH receptors (Davoren and Hsueh 1884; el-Roeiy *et al.,* 1993). In vitro studies demonstrated that IGF-I stimulates granulosa cell proliferation in the ovary of rat (Adashi *et al.,* 1985), human (Wood *et al.,* 1993), pig (Xia *et al.,* 1994), sheep (Campbell *et al.,* 1995) and cow (Armstrong *et al.,* 1996; Stubbs *et al.,* 2013). IGF-I null mice are infertile with an arrest at the preantral follicle stage similar to FSHβ- and FSHR-deficient ovaries (Baker *et al.,* 2000). In addition, IGF-IR stimulates the development, transformation and differentiation of cells (Baserga 1995 and 2000; Chen and Sharon 2013). Previous studies showed that removal of the cell membrane IGF-IR by the abolition of the IGF-IR gene, suppression of cell expression or inhibition of function could lead to cell transformation (Baserga 1995; Baumgarten *et al.*, 2017).

Capsaicin (CAP) is the pungent ingredient in hot chili peppers of the family Capsicum. It is widely consumed as food additive and topical analgesic (Surh and Lee 1996; Arora *et al.*, 2011). Besides, CAP is currently being utilized for therapeutic treatment of various clinical conditions such as pain relief, rheumatoid arthritis, diabetic neuropathy, obesity, cardiovascular and gastrointestinal conditions (Josse *et al.*, 2010; Sharma *et al.*, 2013). CAP excites sensory neurons by binding to its receptor (TRPV1- capsaicin-sensitive receptor transient receptor potential, vanilloid type 1), localize on primary afferent neurons (Wardle *et al.*, 1997; Nagy *et al.,* 2004; Nakagawa and Hiura 2006). CAP-sensitive sensory neurons are nociceptive neurons that are known to activate ligand-gated, nonselective cation channels such as CGRP, substance P (SP) and neurokinin A (Jessell *et al.,* 1978; Saria *et al.,* 1987). Some researchers have suggested that CAP-sensitive sensory nerves could play a role in regulating the fertility and follicle development in females (Traurig *et al.,* 1984; Pintado *et al.,* 2003). Little is known about the effects of CAP on the female reproductive system. Pintado *et al.* (2003) treated female rats neonatally with a high dose of CAP (50 mg/kg) and found that rats exhibited an apparently normal courtship behavior but a lower reproductive success and litter size compared with control animals. On the contrary, low dose CAP protected the follicles from apoptosis and atresia, and stimulated follicular development (Zik *et al.,* 2010). Ozer *et al*. (2005) fed laying hens with a diet containing red hot pepper and demonstrated that follicular development was stimulated and laying performance was improved.

Previous studies have identified a link between the insulin family of trophic factors and TRPV1, showing that IGF-I sensitize TRPV1 receptors (van Buren *et al*, 2005). But to our knowledge, the mechanism of the effect of capsaicin in connection with IGF-I and IGF-IR in rat ovarian granulosa cells has not been reported. The objectives of this *in vitro* study, thus, were to examine (1) the expression of IGF-I and IGF-IR in rat ovarian granulosa cells, (2) effect of low and high dose CAP treatment on the expression of IGF-I and IGF-IR in rat ovarian granulosa cells.

MATERIALS-METHODS

Animals

Female Sprague-Dawley rats (30 days old) obtained from the Bursa Uludag University Experimental Animals Breeding and Research Center were used throughout the experiments. All procedures were performed after the approval of the Bursa Uludag University Animal Research Local Ethics Committee (Approval No. 2011-09/03). Handling and euthanasia of rats were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. The animals were housed five per cage at 20-24°C with a 60-70% humidity and 12/12 h light/dark cycle, and fed ad libitum. Euthanasia was performed by cervical dislocation after ether inhalation and the ovaries were harvested for culturing.

In Vitro Culture and Treatment

Granulosa Cell Culture

Granulosa cells were prepared as described previously with some modification (Uzumcu and Lin 1994; Zachow and Uzumcu 2006). Briefly, ovaries from the animals were rinsed in cold Hanks' Balanced

Salt Solution (HBSS) medium (PAN-Biotech GmbH; P04-34500; Germany) supplemented with 1% 1,000 U/ml penicillin G, 10 mg/ml streptomycin sulfate (Bio-Ind; 03-031-1B; CT, USA). Afterwards, the ovaries were cleaned of all connective tissues and fat and were moved into Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium (Gibco™;11039-021) supplemented with 10% fetal bovine serum (FBS) (Bio-Ind; 04-007-1A) and 0.1% 1,000 U/ml penicillin G, 10 mg/ml streptomycin sulfate. Ovaries were punctured using a non-enzymatic needle puncture method with 27-gauge needle to extrude granulosa cells and the extract was then filtered through a 70 µm filter. The cell suspension was centrifuged at 200 g for 5 minutes, resuspended in a culture containing DMEM/F-12 medium supplemented with 10% FBS, and 0.1%, 1,000 U/ml penicillin G, 10 mg/ml streptomycin sulfate and then plated (Uzumcu and Lin 1994; Zachow and Uzumcu 2006).

The following day (day 0), the media were replaced with fresh media. Four groups were assigned: (1) cell control group (C), (2) media containing vehicle solution (0.01% DMSO; Ambresco; N182) for vehicle control (V), (3) low dose (50 µM) and (4) high dose (150 µM) of CAP (Sigma-Aldrich; M2028) diluted in vehicle solution. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. Twenty-four hours after treatment, the experiments were terminated.

Immunocytochemistry

Granulosa cells were grown on coverslips in 24-well plates. The cells on the coverslips were washed three times in PBS. The cells were then fixed in 4% paraformaldehyde at room temperature for 15 min and permeabilized with 0.1% Triton X-100 for 10 min. The cells were blocked (Vector Lab., MP7401) for 20 min and incubated with anti-IGF-I (1:200), and anti-IGF-IR (1:250) antibodies at 4°C overnight and with the secondary antibody (Vector Lab.; MP7401) for 30 min. Cells were then treated with 3,3'-diaminobenzidine (DAB) for 5 min, counterstained with Harris Hematoxylin for 2 min, and the slides were then examined under Nikon Eclipse 80i microscope at a magnification of x400. Five microscopic areas were randomly counted and the percent value of stained cells was calculated for each experiment groups by both investigators. The average of two readings was taken.

Immunoflorescence

The cells were blocked with 5% BSA (in PBS) for 1 h followed by incubation with IGF-I (1:200) and IGF-IR (1:250) antibodies at 4°C overnight. Cells were then incubated with bovine anti-rabbit IgG-FITC (Santa Cruz; sc2365) secondary antibody (1:200) for 1 h in a dark room. Then coverslips were mounted and cells were visualized under Nikon Eclipse 80i microscope.

Statistical Analysis

The data were analyzed using the IBM SPSS Statistics 22. The normality of the data were determined by the Shapiro-Wilk test. Statistical significance between the groups was analyzed by the Kruskal-Wallis Test, followed by Mann-Whitney U posthoc test. Bonferroni correction was applied in order to control of alpha (α/k=0,008). All experimental data are expressed as mean±SD of three separate experiments, each carried out in replicate. A value of p≤0.05 was taken as statistically significant.

RESULTS

Granulosa cells in well plates were examined 24 h after CAP addition. Regular epithelioid structure and compact cell-cell interaction were observed in low dose CAP (50 µM) and control groups (Figure 1A). However, in the high dose group (150 µM), the structure of granulosa cells appeared deformed and cell-cell interactions were disrupted (Figure 1B).

The expression of IGF-I and IGF-IR was observed in the cytoplasm of granulosa cells ; the intensity was more prominent in the perinuclear area (Figures 2, 3). Rat ovarian granulosa cells with/without CAP addition were able to release the IGF-I and IGF-IR (Figures 2-4). Dose of CAP (50 and 150 µM) had a significant (p≤ 0.05) influence on IGF-I and IGF-IR expression. Highest number of cells expressing both IGF-I and IGF-IR was found after CAP treatment at the highest dose when compared to control and vehicle groups (p≤ 0.05) (Figure 4).

There was no statistical significance between the control and the vehicle group (p>0.05) regarding IGF-I immunoreactive cells, while CAP treated groups had significantly higher immunoreactive cells compared to the control groups (p≤ 0.05) (Figures 2, 4). Also, there is statistical significance between the groups administered dose 50 µM and 150 µM of CAP (p≤ 0.05) (Figures 2, 4). When IGF-IR results were evaluated, there was no significant difference between the control groups, but the difference was significant between the control and CAP groups difference of CAP groups. (p≤ 0.05) (Figures 3, 4). The number of granulosa cells expressing IGF-IR was more than IGF-I positive cells in all groups (Figure 4).
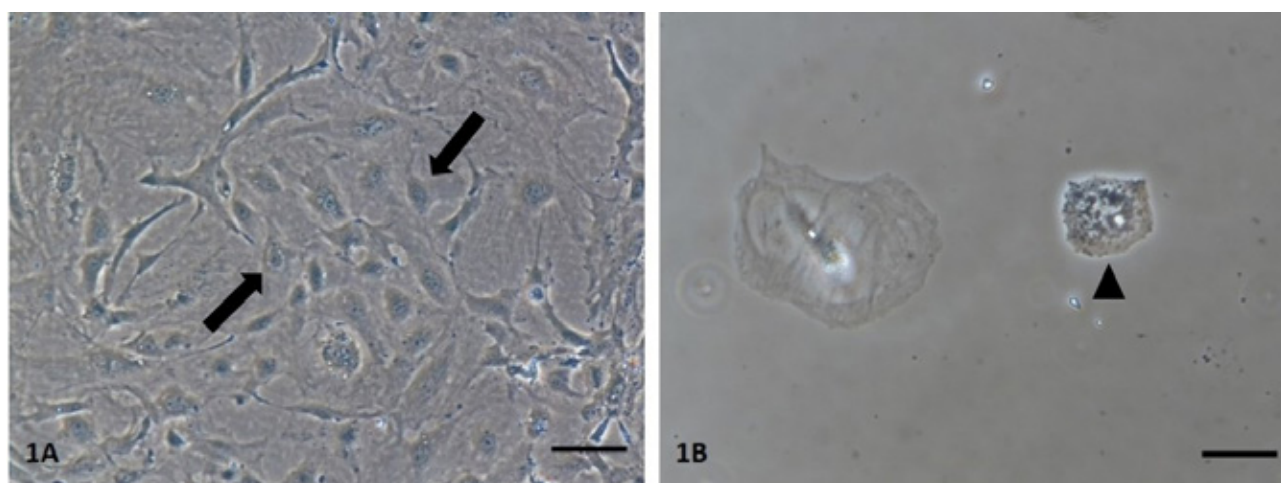
Figure 1. Morphological structure of ovarian granulosa cells A. Healthy ovarian granulosa cells (arrow) after 24 h, 50 µM CAP treatment, (Bar: 100µM), B. Apoptotic ovarian granulosa cells (arrowhead) after 24 h, 150 µM CAP treatment, (Bar: 100µM).
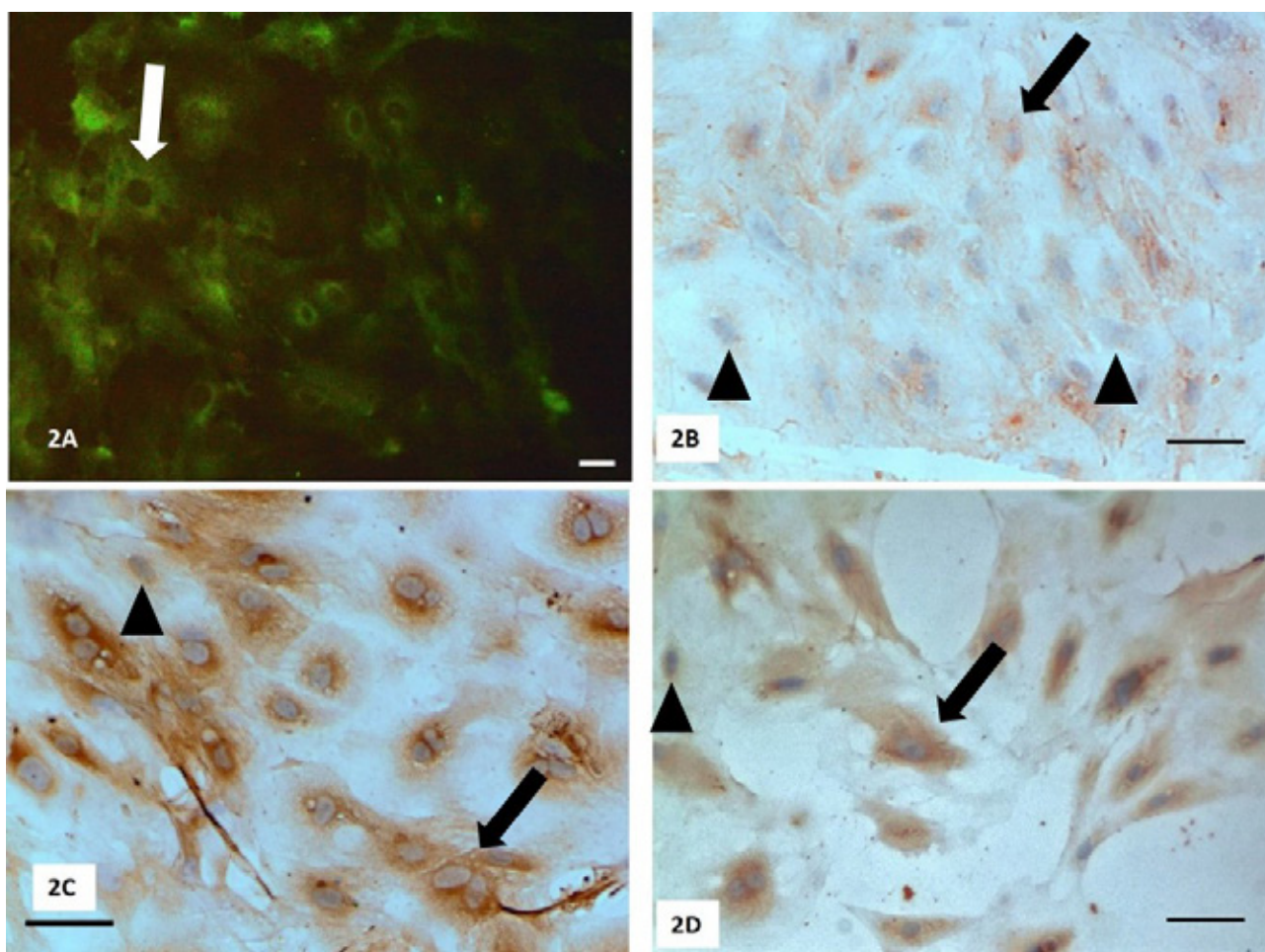
Figure 2. A. Positive (arrow) IGF-I expression in ovarian granulosa cells; 24 h, 50 µM CAP treatment (IF method) (Bar: 25µM). B. Positive (arrow) and negative (arrowhead) IGF-I expression in ovarian granulosa cells; control group (ICC method), (Bar: 50µM), C. Positive (arrow) and negative (arrowhead) IGF-I expression in ovarian granulosa cells; 24h, 50 µM CAP treatment (ICC method), (Bar: 100µM), D. Positive (arrow) and negative (arrowhead) IGF-I expression in ovarian granulosa cells; 24h, 150 µM CAP treatment (ICC method), (Bar: 50µM).
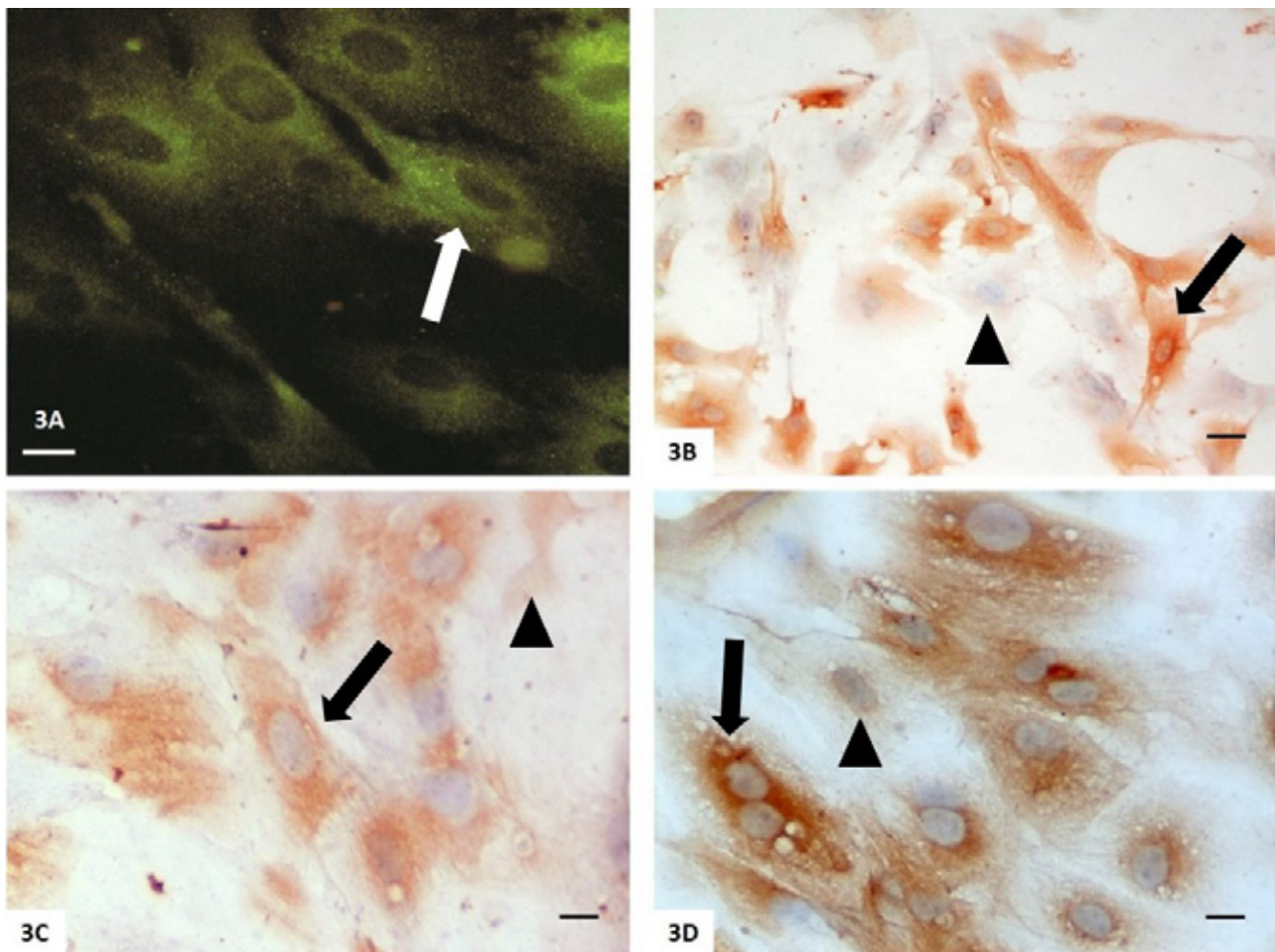
Figure 3. A. Positive (arrow) IGF-IR expression in ovarian granulosa cells; 24 h, 50 µM CAP treatment (IF method) (Bar: 25µM), B. Positive (arrow) and negative (arrowhead) IGF-IR expression in ovarian granulosa cells; control group (ICC method), (Bar: 25µM), C. Positive (arrow) and negative (arrowhead) IGF-IR expression in ovarian granulosa cells; 24h, 50 µM CAP treatment (ICC method), (Bar: 25µM), D. Positive (arrow) and negative (arrowhead) IGF-IR expression in ovarian granulosa cells; 24h, 150 µM CAP treatment (ICC method), (Bar:25 µM).
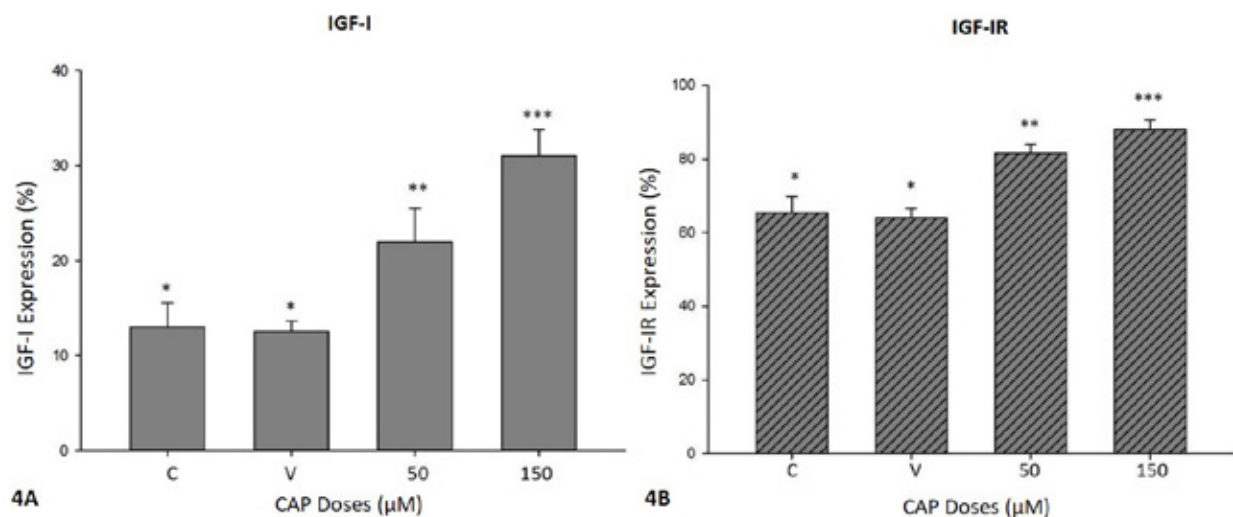
Figure 4. A. IGF-I expression, B. IGF-IR expression in ovarian granulosa cells.
*, **, ***; difference between groups, $p \leq 0.05$. C, cell control ; V, Vehicle control.

DISCUSSION

Many growth factors have been reported to participate in ovary physiology (Sirotkin 2011). IGF-I is one of them that amplifies gonadotropin action in granulosa and theca interstitial cells by acting on the IGF-IR (Maestro *et al.,* 1997). Stimulating effect of IGF-I on granulosa cell proliferation has been shown in many in vitro studies (Adashi *et al.,* 1985; Campbell *et al.*, 1995; Armstrong *et al.,* 1996; Surh and Lee 1996). IGF-I stimulates initiation of preantral follicles development by revealing mRNA and protein levels. The presence of IGF-IR was demonstrated in the same study (Stubbs *et al.,* 2013). Baumgarten *et al.* (2017) reported that IGF-I and IGF-IR expression were found in granulosa cells of follicles from the primary to the antral stage and expression of IGF-IR in granulosa cells is essential for reproduction and lack of IGF-IR leads to apoptosis in granulosa cells. IGF-I gene expression in cell type-spesific in ovary, 10-fold greater abundance of IGF-I transcripts in granulosa cells as compared with theca-interstitial cells (Hernandez *et al.,* 1989). IGF-I staining localization and intensity in granulosa cells in our study also support previous data.

Effect of capsaicin on various type of cells is influenced by the administered dose (Pintado *et al.,* 2003; Ozer *et al.,* 2005; Zhang *et al.,* 2008; Zik *et al.,* 2010; Alatriste *et al.,* 2013). Many researchers have suggested that high dose of CAP affects steroidogenesis by creating degeneration in the sensory nerves of the hypothalamus-pituitary-ovarian pathway (Moran *et al.,* 2003; Pintado *et al.,* 2003; Alatriste *et al.,* 2013). Moran *et al.* (2003) injected CAP (50 mg/kg, sc) at birth and in 3 day-old rats which resulted in a significant delay of puberty and first vaginal estrus, as well as lower preantral and antral follicles. The studies about the effect of the low dose of CAP are limited (Ozer *et al.,* 2005; Zik *et al.*, 2010). Zik *et al.* (2010) observed that low dose of CAP inhibits apoptosis and stimulates follicular development and proliferation of the granulosa cells. In addition, Ozer *et. al.* (2005) determined that low dose of red hot pepper (10 g/kg) added in diet of laying henscaused a significant increase in ovarian weight, follicle number and earlier onset of puberty compared to the control group.

In our previous studies, we found that low doses of CAP (10, 50 and 100 µM) increased cell proliferation in granulosa cells, but high doses (150 and 200 µM) induced apoptosis (Guler and Zik 2018). However, there are no studies on the expression of IGF-I and IGF-IR after CAP treatment on ovarian granulosa cells.

Many researchers have observed that IGF levels increase by applying toxic substances to ovarian cells (Holloway *et al.,* 2007; Cansu *et al.,* 2008; Ozden-Akkaya *et al.*, 2017). Holloway *et al.,* (2007) administered dichlorodiphenylchloroethylene (DDT), a pesticide that can negatively affect ovarian function, and investigated IGF expression in vivo in rat ovaries and in vitro primary culture of human granulosa cells. They observed that IGF-I expression was increased in parallel to the increasing concentration of DDT. Ozden-Akkaya and *et al.*, (2017) injected methoxychlor to fetal and neonatal rats and they found that IGF-I expression increased in granulosa cells. On the other hand, the increase in IGF-I expression in interstitial cells was directly related to the polycystic ovary syndrome (Schildkraut *et al.,* 1996; King *et al.*, 2013). To our knowledge, the studies have been conducted on the expression of IGF-I and IGF-IR after CAP treatment on ovarian granulosa cells have not been reported. In some studies, the relationship between IGF-I and TRPV1 and IGF-IR and TRPV1 have also been demonstrated in different tissues and cells (Caprodossi *et al.,* 2011; Li *et al.,* 2013; Lilja *et al.,* 2007). Li and *et al.* (2013) showed that IGF-I expression increased in tibia bone marrow and it is responsible for the up-regulation of TRPV1 expression and function in the peripheral nerves. In another study IGF-I and insulin enhance TRPV1 protein expression in neublastoma cell line (Lilja *et al,* 2007).

In our experiments, the cell culture lasted 24 h with two different doses of CAP. Our results showed that the higher dose of CAP increased number of cells expressing IGF-I and IGF-IR. The expression results of IGF-I in other studies is consistent with our results (Halloway *et al.,* 2007; Cansu *et al.,* 2007; Ozden-Akkaya *et al.,* 2017). Several researchers observed co-existence of suppressed IGF-I expression and increased IGF-IR expression (Ozden-Akkaya *et al.,* 2017). But in our study, number of IGF-I and IGF-IR immunopositive granulosa cells increased with increasing doses of CAP.

Our results show that IGF-I and IGF-IR expressions were CAP dose-dependent. As the application dose of CAP increased, IGF-I and IGF-IR expressions increased in parallel, but morphological deformation was observed in cells.

CONCLUSION

In conclusion, this study demonstrates for the first time that CAP, a widespread food additive, can increase the expression of the essential ovarian growth factor, IGF and its receptor IGF-IR. High dose of CAP may be a risk factor and result in adverse reproductive outcomes. Our results are expected to lead to a focus of future in vivo studies about ovarian infertility in connection with IGF and CAP treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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Clinical signs, aetiology and outcome in 30 cats with recurrent seizures (2002-2018)

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ABSTRACT. The purpose of the current study was to describe seizure types, aetiology, treatment response and outcome in 30cats with recurrent seizures. This retrospective case series study included the medical records of client-owned cats, admitted to the Clinic of Companion Animals for seizure disorders, from 2002 to 2018. Recorded data included history, seizure type, physical and neurological examination findings, blood pressure measurements, complete blood counts, serum biochemistry profile and urinalysis, tentative/final diagnosis, treatment, hospitalization and long-term outcome. A total of 298 feline neurology cases were examined during the study period. Of those, 38 cases were admitted for seizure disorders and 30 met the inclusion criteria. Median age on admission was 38,2 months (3,1 years) (range 2 months- 14 years old).Seizure types, as per owner description, were generalized (26 cats), focal with secondary generalization (3 cats), focal (2 cats) and complex (1 cat). Cluster seizures appeared at least once in 18/30 and status epilepticus in 7/30. Different seizure types were also recorded in individual cases. Fifteen cats (15/30) were hospitalized at least once and the duration of hospitalization ranged from 1-10 days. In most cases, in which diagnosis was established, epilepsy wassecondary (25/30), due to previous head trauma(11/30),metabolic (1/30), or inflammatory (4/30) disease, arterial hypertension (3/30), toxicosis (3/30) and intracranial neoplasia (2/30).Congenital hydrocephalus was detected in 1 cat. Tentative diagnosis could not be established in 5cats; however differential diagnosis included inflammatory or neoplastic encephalopathies.Antiepileptic drug monotherapy (phenobarbital or levetiracetam) was sufficient to control the seizures in 14 cases, while administration of combination therapy with 2 or more antiepileptic drugs was required in 5 cases (phenobarbital, levetiracetam, gabapentin). Successful control was achieved in 11/30 animals for 1-5 years. Seven cats required intensive care at least once due to status epilepticus. Until today, eighteen (18/30) cats are still alive and 11 died or were euthanized.Although in most cases epilepsy was secondary, seizure control was adequate and quality of life (QoL) was improved with antiepileptic drugs when metabolic, inflammatory and neoplastic encephalopathies were excluded.

Keywords: levetiracetam, phenobarbital, post-traumatic seizures, status epilepticus

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INTRODUCTION

Seizures can be primary (idiopathic) or secondary in cats (Bailey and Dewey, 2009). The major categories of secondary disorders in cats include vascular events, encephalitis, neurotoxicoses, head trauma, metabolic disease, neoplasia, congenital and degenerative conditions (Kline, 1998; Bailey and Dewey, 2009). Whether a seizure event is epileptic can only be suspected based on clinical, laboratory, and neuroimaging findings as electroencephalography diagnostic techniques have not yet been developed to contribute diagnostically in veterinary medicine (Pakozdy et al., 2014).

Antiepileptic drugs (AED) administered in cats, include phenobarbital (PB), levetiracetam (LEV), and zonisamide (ZSD) (Bailey and Dewey, 2009; Ukai et al., 2018). The effectiveness of treatment is evaluated by a more than 50% reduction in seizures, thus marking a successful dosage regimen. On the contrary, a reduction of less than 50% or an increase in the number of seizures is indicative of progressive disease, refractory seizures, poor client compliance or inadequate dosing (Bailey and Dewey, 2009).

Comparisons among previous feline epilepsy case series and reviews can be difficult due to different diagnostic methodology and lack of standardized definitions. Follow-up stend to be brief and large case series referring to the full spectrum of seizure disorders in the cat are lacking.

The aim of this retrospective study was to report the seizure types, suspected aetiology, treatment response and long-term outcome in selected feline cases with recurrent seizures.

MATERIALS AND METHODS

In this retrospective study, case records were reviewed from January 2002 to December 2018. From a total of 298 feline neurological cases, 38 cases were admitted for seizure disorders and 30 met the inclusion criteria of the study. These included recurrent episodes of seizure activity, complete records and a minimum of 6-month-follow up period. Epidemiological data and history included breed, age, gender, age of seizure onset, duration of seizure and frequency of seizure activity, previous or concurrent neurological deficits, seizure type (including the presence of cluster seizures or status epilepticus), treatment protocol, antiepileptic drugs with associated side effects and long-term outcome. Physical/ neurological examination, clinicopathological (complete blood counts, serum biochemistry, urinalysis) and diagnostic imaging findings, blood pressure measurements were also recorded. Cerebrospinal fluid analysis, serological tests for *Toxoplasma gondii* and feline *Coronavirus* infection were recorded when available. Owners were contacted via telephone when serial re-examinations were not recorded, in order to complete follow-up data (current treatment and status of the cat, frequency of seizures, concurrent neurological or other disorders). Long-term outcome (survival time) started on admission and lasted until the end of the follow-up period for the survived cases and until death for those cats that died. In survived cases, excluding hospitalized animals, follow up period ranged from 6 months to 12 months.

RESULTS

Feline population of the study

The study population consisted of 23 domestic short-haired, 5 Persian/ Persian crossbred and 2 Siamese cats. Seventeen (17/30) cats were male (11/30 intact, 6/30 castrated) and 13/30 were female (8/30 intact, 5/30 spayed). Median body weight was 4,3 kg for 16/30 cats. Eight cats (8/30) were obese (>4 kg), and 6/30 were <2kg (kittens and cats with normal body condition).

In 13 cats seizure onset was reported to happen prior to 6 months of age, however the animals were admitted at 1-4 years of age (10/30). Age of seizure onset ranged from 2 weeks to 14 years. All cats were admitted for seizure disorders however the frequency varied among the cats. Fourteen cases were admitted due to the appearance of cluster seizures, eleven cats for increasing seizure frequency ranging from once a month to once a week and four cats for having a first seizure episode.

Seizure activity

Seizures were the only neurological disorder in 23/30 cases. The recorded pre-ictal signs (aura) included behavioral changes. The ictus or actual seizure event was characterized by a variety of abnormal behavioral patterns depending on whether the seizure was focal, generalized or complex. The seizure type included generalized (26 cats), focal with secondary generalization (3 cats), focal (2 cats), and complex (1 cat) seizures. Generalized seizures included tonic-clonic muscle spasms in 20 cats and tonic muscle spasms in 6 cats. Involuntary urination or defecation during seizures was detected in 6 cases. Salivation was apparent in 4 cases, as observed by the owner. Duration of seizure episodes was recorded in 20 animals, and varied from approximately 1 minute (18

cats) to 2-3 minutes (3 cats). Different seizure types were also recorded in individual cases. Complex seizures presented as behavioral changes, rapid running and facial twitching.Aggression developed or continued (post-ictus) after the seizure activity in 8/30 cats. Additional neurological signs werecircling in 4 cats, cognitive dysfunction in 3 cats, intention tremors in 1 cat, compulsive behavior (aimless pacing) in 1 cat. Cluster seizures developed at least once in 18/30. Status epilepticus was present in 7/30 cases.

Seizure etiology

Due to financial constraints, most of the owners declined advanced diagnostic imaging investigation (CT/MRI). Therefore, tentative diagnosis was based on history, age of seizure onset, clinical course of the disorder (development of clinical signs), clinical and neurological examination, presence or absence of concurrent neurological signs, response toantiepileptic drugs (AED), long-term outcome and necropsy (where available). Diagnosis of metabolic encephalopathy(hepatic encephalopathy) was based on clinicopathological evaluation. Age on seizure onset ranged from 2.5 months to 180 months (median 71.5 months).

Advanced diagnostic imaging was performed in 8/30 cats, one of which was diagnosed with congenital hydrocephalus using ultrasonography, necropsy in 4/30, funduscopy in 5/30, thyroid profile (T4, fT4, TSH) in 2/30, serological test for *Toxoplasma gondii* or feline Coronavirus infection in 3/30.

Historical evidence and clinical examination presumed the cause of seizures in 17/30 cats. Elevencats had a history of head trauma,threecatshad increased serial blood pressure measurements and there were evidence of toxicity in three cases (application of a canine permethrin ectoparasitic product in 2 cats and organophosphate toxicity in 1 cat).

In most cases, in which diagnosis was established, epilepsy was secondary (25/30),presumed due to previous head trauma (11/30), metabolic (1/30), inflammatory (4/30),toxicosis (3/30), intracranial neoplasia (2/30).Congenital hydrocephalus was detected in 1 cat. In those 4 cats with encephalitis, serological tests for FeLV/ FIV infection were negative. From the four cats diagnosed with inflammatory disease, 3 had feline infectious peritonitis (FIP) encephalitis, two died during hospitalization and the other was lost to follow-up. The fourth cat had toxoplasmosis. Tentative diagnosis could be established in 5 cats and the differential diagnosis included secondary encephalopathies. Differential diagnosis in these 5 cats included inflammatory or neoplastic encephalopathies. From these 5 cats, in one cat, primary encephalopathy was suspected.

Treatment, hospitalization and survival time

Therapy using one AED was effective in 14 cases (phenobarbital(PB)9/14, or levetiracetam(LEV)5/14). Therapy using more than one AED was necessary in 5 cases (4 cats PB+LEV, 1 cat PB+LEV+gabapentin). LEV withdrawal seizures (owner compliance) were observed in 2 cats after a missed dose of LEV. Nineteen cats received life-long antiepileptic medication.

In our study, 14 cats were treated with PB;alone (monotherapy, 9/14) or in combination with other AEDs. Of those 9 cats with PB monotherapy, seizure frequency was reduced in 3, while 2 animals had poor seizure response and 4 were lost tofollow up. Four cats underwent LEV monotherapy with adequate seizure control while one hadpoor response. Three cats had good seizure control with PB+LEV combination therapy but one cat was not controlled with the same drug regimen. One cat underwentPB+LEV+Gabapentin therapy but had poor long term seizure control. That cat was previously diagnosed with congenital hydrocephalus.

Fifteen cats required hospitalization (with or without intensive care) at least once (duration range 1-10 days). Seven from those 15 cats required intensive care due to status epilepticus. Five cats died during hospitalization. Euthanasia was elected in 2 cats due to poor control of status epilepticus. Four cats (not the abovementioned) died due to a concurrent disease (3 chronic renal disease, 1 diabetes mellitus).Eighteen cats were alive when the follow-up call was performed and the survival time ranged from 1-12 years. One cat was lost to follow-up.

Successful control (>50% reduction in seizure activity) was achieved in 11catswith a marked reduction in aggression and improved quality of life (QoL). Eleven cats (11/30) remained controlled for at least 1-5 years. Poor response was noted in 6 cats due to owner non-compliance (3), underlying disease (2), or both (1). Phenobarbital levels were monitored when therapy response was poor in 3 cats.

There were other neurological disorders in both cats with controlled seizures or not.In those cats with poor seizure control, aggression was noted (4 cats). Additional neurological sigs included cognitive dysfunction (3 cats), compulsive circling (2 cats), spas-

tic tetraparesis (1 cat), spastic tetraplegia (2 cats), right-sided, absent menace response reflex (1 cat) and bilateral absent menace response reflex (1 cat), ataxia in all 4 limbs (2 cats), blindness (2 cats) and altered behavior (episodes of pointless running, hissing at shadows) (1 cat).

DISCUSSION

Secondary epilepsy is reported to be more frequent than primary epilepsy in cats (Pakozdy et al., 2010). This was also observed in the current study, in which a diagnosis of secondary epilepsy was established in 25 cats and was speculated in 5 cats. Presumed post-traumatic epilepsy was the predominant diagnosis in our feline population, while previous studies report intracranial neoplasia as the main cause of seizures(Pakozdy et al., 2010). Data from history were adequate to establish diagnosis as post-traumatic epilepsy because either the owners had witnessed the accident, or the cats were found with external injuries suggesting head trauma.

Antiepileptic drug therapy is recommended when seizures occur post-trauma (Bailey and Dewey, 2009). Eleven cats developed seizures post-traumatically due to head trauma and required antiepileptic medication for controlling them. It has been reported that cats with medical history of mild to moderate head trauma had ≤ 5.6% probability of developing post-traumatic seizures and owners did not observe any seizure activity during the follow-up period (Grohmann et al., 2012). In another study, it was speculated that seizures could appear immediate after head trauma or delayed and thus the owners should be informed about the potential need for antiepileptic drug either at the time of the trauma or in the future; the prognosis was good (Kline, 1998).

Despite the small number of cases diagnosed with FIP encephalitis, the prognosis is known to be poor, as it remains a fatal disease despite the various therapeutic modalities that have been proposed (Gunn-Moore and Reed, 2011). In particular, the occurrence of seizures in cats with FIP infection indicates extensive brain damage; therefore it is an unfavourable predictive sign (Timmann et al., 2007).The third case, diagnosed with toxoplasmosis, was still alive 6 months after the first referral. Although the seizures were apparently there, their frequency had been decreased. The prognosis for toxoplasmosis is guarded, due to the potential of recurrence of the neurological disorders (Phofl and Dewey, 2005; Gunn-Moore and Reed, 2011).In our case, prognosisseems to be good in the

6-month-period that we set as follow-up period.

Seizures may result from a wide variety of extra-cranial causes, including toxins, drugs and metabolic disease (O' Brien, 1998). In this study, seizures due to metabolic causes were less frequent (1 case due to hepatic encephalopathy). In previous studies (Barnes et al., 2004; Rusbridge, 2005), hepatic encephalopathy was the predominant diagnosis in the metabolic group of diseases in comparison to others that indicate infrequent seizure occurrence due to hepatic encephalopathy (Kline, 1998). A more recent study indicated a higher percentage of feline seizure cases due to metabolic/toxic causes (Schriefl et al., 2008).Seizures due to toxicosis appeared in three cats (1 cat was exposed to organophosphates and 2 on permethrin product) in the current study. Thus, in many cats presented with seizures, a careful history of drug use and potential of toxin exposure is essential (O' Brien, 1998).

Vascular diseases include hypertensive encephalopathies and ischemic encepthalopathies(Pakozdy et al., 2014). Hypertensive encephalopathies can be the result of chronic renal disease, hyperthyroidism and hypertrophic myocardiopathy (Kline, 1998). In our study, there were 3 cases with hypertension that clinically affected the brain and appeared seizures. All the three cats had a primary disease (2 cats with chronic renal disease and 1 cat with hyperthyroidism), that caused seizures through hypertension.

Two cats were diagnosed with intracranial neoplasia. One of the tumors was histologically confirmed as meningioma but there was no histological examination of the second's cat tumor.Meningiomas are one of the most commonly observed intracranial neoplasms, appearing with seizures (Tokem et al., 2006).

A tentative diagnosis could only be reached in 5 cases and included secondary epilepsy and differential diagnosis included inflammatory or neoplastic encephalopathy. Final diagnosis was not established because specific diagnostic testing (advanced imaging and cerebrospinal fluid analysis) were not pursued. Thus, these 5 cases whose routine clinicopathological investigation was unremarkable were evaluated for the response to treatment through follow-up. In many cases of feline epilepsy, an underlying cause of seizures was suspected but never proven ante-mortem (Kline, 1998). These include previous post-traumatic, post-inflammatory and post-ischemic lesions that were quiescent and non-progressive (Kline, 1998).

Cats were 1-4 years old on admission,although seizures had started earlier (<6 months old in 13/30 cats).Age of seizure onset was between 1 and 3 years in 8/30 cases. The multifactorial aetiologyof seizures in cats of all age groups indicated that advanced diagnostics were necessary even when clinipathological investigation was normal (Bailey and Dewey, 2009).

Regarding the type of seizures, cats appear to have generalized seizures more frequently than focal, in the current study. This finding was comparable to those of other studies indicating the generalized seizures as the most common type of seizure that appear in cats with secondary epilepsy (Barnes et al., 2004; Finnerty et al., 2014). The type of seizure did not indicate a specific diagnosis(Tomek et al., 2006). Primary, seizures are complex focal with or without secondary generalization (Parent and Quesnel, 1996; Kline, 1998; Rusbridge, 2005; Schreifl et al., 2008). This study indicated that cranial trauma can be a leading cause of seizures in cats.

The age of seizure onset range from 2.5 months to 15 years in the present study. The median age of seizure onset for the study population was 71.5 months (5.9 years). This finding was in parallel with previously published work in which the seizure onset ranges from 6 months to 18 years (Barnes et al., 2004), 3 months to 13 years (Quesnel et al., 1997) and 4 months to 20 years (Pakozdy et al., 2010). A possible explanation for such wideage variability of seizure onset was the underlying aetiology that included cats with secondary epilepsy and idiopathic epilepsy in all studies mentioned. In one study, the onset of seizures in the study population was noticed before 1 year of age but there was no indication of specific underlying cause (Schwartz- Porsche and Kaiser, 1989). The age of seizure onset was associated with the underlying aetiology indicating that cats with idiopathic epilepsy appeared epileptic seizures in a younger age than cats with secondary epilepsy.

Phenobarbital (PB) is the current drug of choice in cats with multiple seizure episodes (Berg et al., 2006; Dewey, 2006;Thomas and Dewey, 2008; Finnerty et al., 2014). There are several studies supporting the efficacy of PB of seizure control in epileptic cats (Finnerty et al., 2014); however in our study the number of cats with adequate control of seizures using PB as monotherapy was equal to those cats that are not well controlled. Moreover equal number of cats that underwent PB monotherapy was lost during follow-up period. Probably, whether the number of lost cases was smaller and the study population with

PB monotherapy was larger, they could have a different impact on PB efficacy.Adequate seizure control using LEV montherapyor an adjunctive therapy was superior to those cats that did not. The finding of our study was in parallel with another previous study in which levetiracetam was used as an adjunctive anticonvulsant therapy in cats (Bailey et al., 2008). There was a marked reduction in seizure frequency (>50% reduction) and in some cases there were no seizures after levetiracetam initiation (Bailey et al., 2008).In 3 cases, the owners were not consistent with the appropriate administration of levetiracetam (every 8 hours/day) leading to deterioration of the patients.

Although LEV was proved to be superior PB (either as monotherapy or as an adjunctive therapy), regarding seizure control, the study population was small for such a speculation.

In the current study, most cats can improve their quality of life (QoL) and reduce aggression through a successful control (>50% reduction of seizures). The median survival time for seizure cats and the successful control was 1-5 years in our study. Thus, outcome in cats with secondary epilepsy was long in the current study. This finding was in contrast with another previous study in which survival time was shorter in cats with symptomatic epilepsy; however in the same study survival time was longer in cats with probable symptomatic epilepsy (Barnes et al., 2004) indicating that the cause and the degree of the brain damage in secondary epilepsy can influence survival time. Euthanasia was elected in 2 cats, in the current study,-due to poor prognosis. This finding is in parallel with another study in which euthanasia was elected soon after a diagnosis was established due to poor prognosis in cats(Barnes et al., 2004),or due to unacceptable seizure frequency in a study with epileptic dogs (Berendt et al., 2007).

Both cats and dogs had a poor prognosis when status epilepticus appear (Bateman and Parent, 1999;Schriefl et al., 2008). Despite the providing data, there was no evidence supporting this hypothesis in our study. More than half of the study population (18/30), appearing cluster seizures, had a good control of seizures. This finding was in parallel with another study performed in epileptic dogs in which the type of seizures was not associated with the survival time (Berendt et al., 2007). Through this finding, it can be assumed that cluster seizures are treatable and not all cats die during hospitalization or soon after that, depending on seizure aetiology. For instance, most cats in our study popu-

lation were post-traumatic cases, with a stable damage in the brain tissue; things could be different if our study population was consisted of cats with neoplastic or inflammatory encephalopathies, which both cause progressive damage to the brain tissue.

Due to the nature of the study, some data were lacking. Advanced imaging tests (CT and MRI) were available in few cases. Cerebrospinal fluid analysis was not performed however it can only diagnose inflammatory encephalopathies and necropsy was performed in few cases. Therefore, the final diagnosis was based mainly on history, follow-up and AED efficacy. Cat population involved in the current study was not a representative population of seizure cats in this area. A possible explanation for this hypothesis is the nature of the clinic, which is a second-opinion clinic and many cases may had been lost before their admission. The sample size was small; therefore it is difficult to draw accurate conclusions. The aim of this study was to offer a general viewpoint on feline cases admitted to a veterinary clinic in the particular geographic location.

CONCLUSIONS

The most common cause was post-traumatic epilepsy in this study. The type of seizures did not indicate their aetiology. Cluster seizures appeared frequently and they were not necessarily an indicator of poor prognosis.

Reduced aggression and improved quality of life were noted following antiepileptic drug administration. Survival time in feline seizure patients in this study was apparently longer than time reported on previous studies.

Although final diagnosis was not available, seizure control was adequate and quality of life (QoL) was improved with AED when metabolic, inflammatory and neoplastic encephalopathies were excluded.

CONFLICT OF INTEREST

None of the authors had a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Health Risks associated with residual pesticide levels in fish reared in purified wastewater from slaughterhouse

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ABSTRACT. The main objective of the present research was to determine the concentrations of the selected pesticides in muscle, liver and skin of common carp. Fish were sampled in two different seasons from fish pond which received previously treated slaughterhouse wastewater. Pesticides including etridiazole, chloroneb, trifluralin, propachlor, chlorothalonil, hexa-chlorocyclopentadiene, atrazine, simazine, alachlor, metribuzin, metolachlor, DCPA, cyanazine, chlorobenzilate, endrin aldehyde, cis permethrin and trans permethrin were determined by using a GS-MS method. Many of pesticides were not determined or determined in low concentrations. Propachlor was found in muscle, skin and liver. The recommended acceptable daily intake was higher in comparison with the estimated daily intake for examined pesticides via fish reared in treated slaughterhouse wastewater. It is very important to maintain the safety of the fresh fish produced in wastewater in order to ensure food safety and avoid health problems in humans.

Keywords: common carp; food safety; environmental protection; integrated aquaculture system, propachlor, risk assessment

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INTRODUCTION

Fish meat is highly acceptable protein source worldwide. Moreover, fish represents an excellent source of fatty acids in healthy nutrition (Ljubojević et al., 2015). From another point of view, consumers are more and more oriented to the safety issues due to the potential presence of different environmental contaminants in fish meat. Consumption of fish contaminated with pesticides may lead to poisoning of consumers (Aamir et al., 2016). Pesticides are widely used in agriculture (Hladik et al., 2016). It is highly important to understand the nature, bioaccumulation and distribution of pesticides in aquatic environment and also the routes of contamination in order to control and prevent outbreaks of poisoning associated with fish consumption. Pesticides are present in aquatic environment and can be transferred into fish and enter into the food chain (Guo et al., 2008). Due to the fact that pesticides are highly soluble in fats, they accumulate in fish tissue (El-Shahawi et al., 2010). According to Cao et al. (2007) pesticides can remain in soil and water for years. There is increasing trend in the use of wastewater in fish production, particularly in developing countries. The fish rearing in waste water may represents a significant risk for public health (Kim and Aga, 2007). Fish may represent a bioindicator of environmental contamination and the health risk arising from fish produced in wastewater filled fish ponds is questionable. Thus, the safety of fish reared in wastewater filled ponds could be a serious concern for public health. There are several studies regarding the level of pesticides in common carp, mainly organochlorine pesticides in different parts of the world (Svobodová et al., 2003; Hu et al., 2010; Ondarza et al., 2010; Pelić et al., 2019), but no available data exists about the level of other pesticides in fish produced in treated slaughterhouse wastewater in scientific literature. It should be highlighted that such systems may be long-term solution for aquaculture but also for slaughterhouses worldwide. The main objective of the present research was to determine the concentrations of the selected pesticides in muscle, liver and skin of common carp reared in fish pond which received previously treated slaughterhouse wastewater. For that purpose, common carp meat, liver and skin were examined using GS-MS, for 17 pesticides (etridiazole, chloroneb, trifluralin, propachlor, chlorothalonil, hexa- chlorocyclopentadiene, atrazine, simazine, alachlor, metribuzin, metolachlor, DCPA, cyanazine, chlorobenzilate, endrin aldehyde, cis permethrin and trans permethrin) in order to evaluate the slaughterhouse wastewater treatment systems for their efficiency as well as the safety of produced fish meat.

MATERIAL AND METHODS

An earthen pond with an area of 1 ha and average depth of 1.3 m was built in the Pećinci village (N 44°54′19″, E 19°57′35″), Srem District, the Republic of Serbia. Purified water from slaughterhouse was used for filling the pond. Continuous aeration was provided. Two year old fingerlings of common carp were stocked at density 2500 individuals/ha. Industrially produced completed feed mixture and locally available ingredients were used as fish feed and feed was provided manually twice per day. Seven individuals of common carp were harvested from the pond in spring (April) and in autumn (October). All samples were dissected, dorsal muscles without skin, liver and skin were homogenised and prepared for analyses. All chemicals and reagents used in this experiment were of analytical grade with high purity. Preparation of sample which was based on extraction with acetonitrile (CAN), (Sigma-Aldrich, St. Louis, USA) in the presence of anhydrous magnesium sulfate ($MgSO_4$) and anhydrous sodium acetate (CH_3COONa) Merck, Darmstadt, Germany) was performed as described by Kartalović et al. (2016). The gas-mass chromatography was Agilent 7890B/5977A MSD and operating conditions were as follow: fused silica column [30 m x 0.25 μm film of HP-5M - thickness]; injection temperature was set at 280°C using the splitless mode and the volume injected was 4 μL. The column temperature was programmed as follows; held at 50°C for 0.4 min; 50-195 °C at 25 °C per min, held 1.5 min; 195-265 at 8 °C per min and maintained at 315 °C for 1.25 minutes on 20 °C per min, MSD temperature was 280 °C. Calculation of precision, accuracy, linearity, LOD and LOQ (Table 1) was performed by MassHunter Software (Agilent Technologies, Santa Clara, CA, USA). The quantification was based on external calibrations curves prepared from the standard solution of each of the pesticides.

Data analysis was performed using Excel (Microsoft Excel, 2007) to determine the descriptive statistic parameters.

Table 1. Method performance data obtained in blank common carp samples, spiked with 50 µg/kg (n=20)

Pesticides	LOD (µg/kg)	LOQ (µg/kg)	Precision (%)	Linearity (r²)	Recovery (%)	RSD (%)
Etridiazole	1.5	4.9	4.8	1.00	97.4	6.2
Chloroneb	0.9	2.1	7.8	1.00	98.1	7.9
Trifluralin	0.4	1.3	0.8	1.00	99.3	9.2
Propachlor	0.3	1	9	1.00	99.6	8.3
Chlorothalonil	0.4	1.2	5.4	1.00	98.1	12.6
Hexa-chlorocyclopentadiene	1.2	4.1	3.4	1.00	97.3	3.8
Atrazine	0.4	1.2	4.4	1.00	90.4	8.1
Simazine	0.5	1.6	3.2	1.00	94.2	4.2
Alachlor	1.1	3.6	4.5	1.00	91.8	8.6
Metribuzin	0.8	2.6	8.3	1.00	89.3	8.9
Metolachlor	1.2	4.4	3.1	1.00	96.6	3.7
DCPA	1.3	4.8	3.8	1.00	95.4	3.9
Cyanazine	0.8	2.9	8.1	1.00	93.4	11.2
Chlorobenzilate	1.1	3.7	4.7	1.00	91.6	10.3
Endrin aldehyde	1.4	4.8	3.5	1.00	95.2	8.3
Cis permethrin	1.2	4.2	3.8	1.00	96.4	3.4
Trans permethrin	1.4	4.7	11.7	1.00	112.3	12.3

LOD, Limits of detection; LOQ, Limits of quantification; RSD, relative standard deviation

Table 2. Concentrations (µg/kg) of pesticides in meat, skin and liver of common carp (n = 7) in spring

Pesticide	Muscle tissue			Skin			Liver		
	Frequency (%)	Range	X±SD	Frequency (%)	Range	X±SD	Frequency (%)	Range	X±SD
Propachlor	100	11.4-11.5	11.4±0.05	100	20.40- 21.32	20.83±0.38	100	11.3-11.5	11.4±0.1
Atrazine	14.29	<lod-26.88	/	0	<lod	/	0	<lod	/
Endrin aldehyde	71.43	<lod-38.25	37.30±0.53	0	<lod	/	0	<lod	/

* All other analysed pesticides were below LOD.

Table 3. Concentrations (µg/kg) of pesticides in meat, skin and liver of common carp (n = 7) in autumn

OC Pesticide	Muscle tissue			Skin			Liver		
	Frequency (%)	Range	X±SD	Frequency (%)	Range	X±SD	Frequency (%)	Range	X±SD
Chloroneb	0	<lod	/	28.57	<lod-22.31	13.40±12.61	0	<lod	/
Propachlor	100	20.46-20.59	20.53±0.06	100	20.46-34.15	24.49±6.59	100	20.47-20.74	20.58±0.12
Atrazine	0	<lod	/	28.57	<lod-115.83	102.51±18.84	0	<lod	/
Simazine	14.29	<lod-45.28	/	28.57	<lod-91.99	85.27±9.51	0	<lod	/
Alachlor	0	<lod	/	42.86	<lod -90.78	78.53±21.13	0	<lod	/
Metribuzin	0	<lod	/	28.57	<lod-126.74	126.67±0.1	0	<lod	/
Metolachlor	0	<lod	/	28.57	<lod-44.09	44.11±0.2	0	<lod	/
DCPA	0	<lod	/	14.29	<lod-28.47		0	<lod	/
Cyanazine	0	<lod	/	28.57	<lod-404.85	385.8±26.92	0	<lod	/
Chlorobenzilate	0	<lod	/	14.29	<lod-138.31		0	<lod	/
Endrin aldehyde	0	<lod	/	14.29	<lod-111.13	/	0	<lod	/

* All other analysed pesticides were below LOD.

RESULTS

Table 1 shows method performance data for 17 selected pesticides. The results of the study are shown in Table 2 (spring) and Table 3 (autumn). Generally, the concentrations of pesticides were low in all analysed samples in both seasons. A number of pesticides were not detected neither in muscle tissue, and neither in liver and skin samples. It includes the following: etridiazole, trifluralin, chlorothalonil, hexa- chlorocyclopentadiene, cis permethrin and trans permethrin. In autumn concentrations of propachlor were two fold higher compared to concentrations obtained in spring and were 20.53 ± 0.06 µg/kg in muscle tissue, in skin concentrations were in range 20.46 - 34.15 µg/kg and in liver was 20.58 ± 0.12 µg/kg. Atrazine was above the limit of detection in two samples of skin and simazine in one sample of muscle tissue and two samples of skin. Alachlor was found in three samples of skin and metribuzin in two. Metachlor, DCPA and cyanazine were found only in skin samples. Chlorobenzilate was found only in one sample of skin, while endrin aldehyde was present only in one sample of skin in amount of 111.13 µg/kg.

DISCUSSION

Concentrations of pesticides were generally comparable or lower in comparison with previous results reported in studies of common carp and *Cyprinidae* species worldwide (Svobodová et al., 2003; Darko et al., 2008). Etridiazole, chlorothalonil, atrazine, simazine, metribuzin, metolachlor, DCPA and chlorobenzilat are registered for use in Serbia. All of those pesticides except etridiazole and chlorothalonil were detected in low levels in skin of common carp. Pesticides such as atrazine, simazine, trifluralin, alachlor are listed as priority substances of the Directive 2008/105/EC (EEC, 2008) and the Directive 2013/39/EC (EEC, 2013) and three of those pesticides were detected in the present study. A number of pesticides not registered (chloroneb) or banned in Serbia were also found (mainly propachlor, alachlor and endrin aldehyde) and their presence could be attributed to illegal use. The pesticides reported in this study are not often examined so the data regarding their content in fish including common carp are scarce. Papadakis et al. (2015) reported the detection of etridiazole, metolachlor, atrazine, and alachlor (maximum concentrations of 0.026, 0.688, 1.465 and 1.098 µg/L, respectively, in the surface waters of Lake Vistonis Basin, Greece. Kong et al. (2008) highlighted that pesticides were used widely in China, and that pesticides like at- razine, simazine, and alachlor have a harmful effects on endocrine system. All the mentioned pesticides were detected but only in skin and in relatively low concentrations in common carp in our study. Inadequate regulation and poor agriculture management are the main reasons for the fact that banned pesticides are still in illegal usage. Among pesticides determined in the common carp samples of muscle, liver and skin residual concentration of propachlor was dominant in all samples in both season. The determined concentrations were the lowest in liver, then in muscle, while the highest levels were measured in skin. Ondarza et al. (2010) also reported that the pesticide levels were higher in muscle then in liver. Since the skin is a kind of protective barrier, it may be the reason for the highest concentrations of pesticides detected in it. Thomas et al. (2012) examined levels of pesticides in muscles of farmed common carp in France and also found that the levels of pesticides were low. Also, Padula et al. (2008) didn't detect any pesticide residues in wild and farmed southern bluefin tuna (*Thunnus maccoyii*). Kong et al. (2008) evaluated the municipal sewage treatment system in China for their pollutant removal efficiency. They found that concentrations of alachlor, acetochlor, atrazine were 0.074 - 0.021 µg/l, 0.160 - 0.096 µg/l and 0.238 - 0.184 µg/l, respectively, and the total removal efficiency of atrazine was poorest through the sewage treatment systems. Our results showed that the wastewater treatment systems in slaughterhouse were efficient in removing pesticides, and consequently that the discharged effluent is not harmful for the aquatic environment. Furthermore, Khalil and Hussein (1997) reported that the primary and secondary treated waste effluents were successfully used to growth the Nile tilapia. The presented results showed that common carp could be successfully reared in treated slaughterhouse wastewater with regard to pesticides contamination. Janković et al. (2012) reported that estimated weekly intake of common carp in Serbia is 29.4 g. The risk related to pesticides via consumption of examined fish for human of body weight of 70 kg is compared with acceptable daily intake (ADI) of selected pesticides recommended by various organization (Table 4). The estimated daily intake (EDI) was significantly lower than ADI for each pesticide, even in the worst case scenario. Having that in mind, it can be concluded that this intake would not pose a health hazard in human populations.

Table 4. Estimated daily intakes (EDI) of pesticides through common carp muscle by human (average body wt 70 kg) in Serbia

Pesticide	Average concentration (µg/g wet wt)	EDI (µg/kg body wt/day)	ADI (µg/kg body wt/day)	Reference for ADI
Propachlor	0.02	0.0012	0.013 (mg/kg bw/day)	US EPA (1998)
Atrazine	0.03	0.0018	5 µg/kg	WHO (1990)
Simazine	0.05	0.003	5 mg/kg bw per day	US EPA (1987)
Endrin aldehyde	0.038	0.0023	0.2	US EPA (1989)

EDI, Estimated daily intake; ADI, acceptable daily intake

CONCLUSIONS

Pesticide levels in all tissues of examined fish were below the permitted levels. The pesticides included in the present study are not usually included in the monitoring studies in Serbia but also in different states worldwide. However, the obtained results revealed that several of those pesticides could pose a significant human health hazard and also an environmental hazard. This should be taken into account by the authority so that those pesticides should be included in some new monitoring schemes. The knowledge and proper management of pesticides associated with the fish consumption are of considerable medical, economic and environmental importance. Continuous monitoring, including appropriate testing of fish meat in order to determine the presence of pesticides is necessary. It is important due to the fact that fish is an important food source but also is an important indicator of environmental pollution. The obtained results provide important data for the risk assessment of pesticides from fish reared in integrated systems.

However, the further research are necessary for developing risk management tools for integrated aquaculture production systems.

CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Effects of Safranal on Tissue Oxidative Stress in Sub-Chronic Thinner-Addicted Rats

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ABSTRACT. The present study was carried out to determine the effects of sub-chronic thinner addiction on the oxidant-antioxidant balance and oxidative stress on certain tissues and the possible protective effect of safranal against thinner toxication in rats. Adult male Wistar albino rats were randomly divided into four groups of 10 animals each as follows: control (C), safranal (S), thinner (T) and thinner+safranal (T+S). The control group received 1cc saline by gastric gavage. Safranal was administered to S and T+S groups by using gastric gavage at a dose of 100 mg/kg/day and volume of 0.1 mL/kg/day. Thinner inhalation was applied to T and T+S groups in a container with NaOH tablets twice a day. Levels of malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (NO_x) metabolites, total antioxidant capacity (TAS) and total oxidant capacity (TOS) were determined in liver, lung, brain, kidney and testis tissues of the rats. In the T+S group, it was observed that the MDA levels significantly decreased in all tissues, except the kidney, in comparison to the thinner inhalation group (p = 0.000). When the NOx levels of the T+S group were compared with the levels of the T group, it was concluded that there existed a statistically significant decrease in the NOx levels in all tissues (p = 0.000).

In T+S group, it was observed that safranal either eliminated or mitigated oxidative stress that developed in tissues through decreasing MDA and TOS levels and increasing GSH and TAS levels and caused significant decreases in NO_x levels in all tissues. As a result, it was determined that safranal, although not uniform for all tissue types, had a protective potential against the damaging effects of oxidative stress caused by sub-chronic thinner inhalation.

KeyWords: Thinner inhalation, oxidative stress, safranal, tissue

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INTRODUCTION

Thinner is an organic solvent which is commonly used for industrial applications and currently has an increasing improper use as a pleasure inducing and narcotic substance. Thinner is a mixture of toluene, benzene, acetone, methanol, hexane and other substances (Nedzvetskii et al., 2012). It was indicated that the thinner used in Turkey was composed of 63% of toluene, 13% of acetone, 10% of isobutyl acetate, 7.5% of isobutane and 6.5% of butylene glycol and its harmful effects were mostly associated with toluene, where the remaining substances activated these harmful effects (Cobanoglu et al., 2007).

Reactive oxygen (ROS) and nitrogen (RNS) derivatives occur in the organism due to exposure to environmental and chemical agents and the interaction of such derivatives with macromolecules leads to oxidative stress (Mattia et al., 2003). Similar to various internal and external toxic agents, the toxic effects of thinner also emerge through free radicals (Mattia et al. 1991, Martínez-Alfaro et al., 2011).

Long-term use of volatile organic solvents has been shown to cause permanent damage to the lung and liver (Marjot and McLeod, 1989; Al-Alousi, 1989). Mattia et al. (1991; 1993a,b) reported in vivo and in vitro studies that ROS formation is accelerated and lipid peroxidation is increased in brain, liver, kidney and lung tissues with the effect of intraperitoneal administration of toluene. Al-Alousi (1989) emphasized that pulmonary edema is the cause of death due to volatile substances. When the results of many studies are considered, it has been observed that there are many damages as a result of using thinner in industrial area and for narcotic use. Studies show that MDA levels increase and GSH levels decrease due to degradation of lipid peroxidation due to oxidative stress of organic solvents (Ulakoglu et al. 1998b, Dillioglugil et al. 2005, El-Nabi Kamel and Shehata 2008).

In recent years, research in phytotherapy focused on the effects of the components in plant extracts on various diseases, rather than the effect of plant extracts. Safranal is one of the components of saffron extract. Saffron is an herb with antioxidant effects, included in medical literature and is acknowledged to have an increasing significance due to its therapeutic effects (Moghaddasi, 2010; Assimopoulou et al., 2015).The radical scavenging activity of safranal results with antidiabetic, antioxidant, anticancer and hypotensive properties, thus various studies concluded that safranal could be used in industries such as food, pharmaceuticals and cosmetics (Assimopoulou et al., 2005, Imenshahidi et al., 2010, Hazman ve Bozkurt, 2015). Karafakioglu et al. (2017) reported that safranal (200 mg / kg) treatment may have a protective effect against cisplatin-induced nephrotoxicity and oxidative stress in rats. Alayunt et al.(2019) observed that particularly high-dose (100 mg/kg) administration of safranal to rats promoted the antioxidant system and be inflammatory against TNF-α and IL-6 cytokines on damage induced by CCl_4.

The main aim of our study is to investigate to what extent thinner, which is a mixture of organic solvent, affects brain, lung, liver, kidney, testis via respiratory system and to investigate the effect of safranal on this condition. The results of the study may contribute to the development of new approaches both in the protection of worker health on an industrial scale and in the treatment of addicts using volatile substances.

MATERIALS AND METHODS

Chemicals

Safranal (Cat no:Sigma-W338907), Total oxidant (TOS) and antioxidant status (TAS) commercial kits (Rell Assay:RL0024, RL0017) and the other chemical used in the study were purchased from Sigma -Aldrich (Sigma Aldrich Chemical Co. St. Louis, Missouri, ABD).

Animals

In the present study , 40 Wistar albino male rats (weighing ~250-300 g) were used. The animals were housed under standard conditions of temperature ($23 \pm 2°C$), humidity, and dark-light cycle (lights on from 6:00 am to 6:00 pm).. Throughout the study, the rats were maintained on standard rat feed and tap water *ad libitum*. They were fed twice daily at 0900 and 1900. Before starting the thinner experiments, rats were subjected to preexperiments in order to prevent possible toxication severity complications. All the animals were carefully monitored and maintained in accordance with the ethical recommendation of the University of Afyon Kocatepe Animal Ethics Committee The permission was gained on 7 May 2015 with the number 49533702/46.

Experimental procedure

A total of 40 rats were included in the study. They were divided into 4 groups as follows: Control Group (C) (n=10), Sub-chronic Thinner Inhalation Group (T) (n=10), Sub-chronic Thinner Inhalation + Sa-

franal Group (*T+S*) (n=10) and Safranal Group (S) (n=10). Control group (C); comprised 10 healthy rats. The rats in the control group were administered 1 cc of physiological saline daily by gastric gavage for 8 weeks. Rats were fed with standard rat feed for 8 weeks and their weights were recorded regularly during the experiments. Sub-chronic Thinner Inhalation Group (T); rats were placed in a specially designed cage size 100x60x40 cm with air ventilation that allowed inhalation of controlled amounts of thinner (5ml). This application was repeated twice a day for 8 weeks and was terminated when 50% of the rats lost their reflexes to stand (About 5-6 min) (Konuk et al., 2012). Sub-chronic Thinner Inhalation+Safranal Group (*T+S*); treated as T group and additionally a dose of 100 mg/kg of safranal dissolved in 1 cc of physiological saline solution was administered via gastric gavage for 8 weeks. Commercial safranal (Sigma-w338907) was used in the study. Safranal Group (S); a dose of 100 mg/kg of safranal dissolved in 1 cc of physiological saline solution was administered via gastric gavage for 8 weeks. The dose of safranal used in this study was based on previous studies (Hosseinzadeh et al., 2013; Hazman ve Bozkurt, 2015; Hazman and Ovalı, 2015).

Sample preparation

At the end of the experimental period, the rats were anesthetized and sacrificed by cervical dislocation. Rats were anesthetized with 10 mg/kg-im xylazine and 50 mg/kg-im ketamine injection. All rats were sacrificed on the 56th day (8 weeks) of the experiment and tissue samples (brain, lung, liver, kidney, testis) were collected for examination of clinical biochemical analysis. Tissue samples (0.5 g) were placed in homogenizer and were added 5 mL of phosphate buffer solution (PBS) (pH: 7.4) and were homogenized by means of the homogenizer and the sonicator. The homogenates were centrifuged at 15000xrpm and +4°C for 10 minutes, and the supernatants were stored at -80 °C in deep freeze pending analysis.

Measurement of parameters

In the obtained supernatants, the Malondialdehyde (MDA) levels were determined according to Ohkawa et al. (1979), the reduced glutathione (GSH) analyzes were conducted based on Beutler (1963) and the amounts of Nitric oxide (NOx) were determined via the "Vanadium chloride (III) - Gries Reaction" method of Miranda et al. (2001). Total oxidant (TOS) and antioxidant status (TAS) measurements were performed via Eliza Reader (Biotek, ELx800) using commercial kits based on the method developed by Erel (2004, 2005). The amount of toluene in the blood was measured and the level of inhalation in T and T + S groups were determined. The toluene levels in the rats' blood were determined using the GS-MS in Acıbadem Labmed Central Laboratory (Istanbul, Turkey).

Statistical analysis

ANCOVA method was used for statistical analysis and the difference between the groups was tested using the Bonferroni test. The live weight averages of the groups at the zeroth and eighth week were chosen as the covariate. Data were analyzed using the SPSS® 24 package program. The results were expressed as "mean±standard error" (X±SE) and $p < 0.05$ was considered as the significance value.

RESULTS

Blood toluene levels

Blood toluene levels of rats that inhaled sub-chronic thinner are presented in Table 1. No statistically significant difference was determined for inhaled toluene levels in T and T+S groups.

MDA levels measured in tissue samples

The changes in MDA, which is an indicator of from lipid peroxidation, in the liver, lung, kidney, brain and testis tissue samples retrieved from the four experiment groups constituted in the present study, are presented in Table 2. Comparing the MDA levels of the T group with that of the control group levels it could be observed that a significant increase of MDA levels were determined in liver, lung, testis and kidney tissues ($p=0.000$).

GSH levels measured in tissue samples

The GSH levels in groups are presented in Table 3. It was observed that GSH levels in liver, kidney brain, and testis tissues was a significant decreased between T group and that of the control groups (Respectively $p=0.013$; $p=0.000$; $p= 0.015$; $p= 0.007$)

TAS and TOS levels measured in tissue samples

Changes of TAS and TOS levels in all tissues in groups are presented in Table 4 and Table 5, respectively. Comparing the TAS levels of the T group with that of the control group levels it was determined that TAS levels only significantly decreased in liver and kidney tissues ($p=0.000$). It was identified that liv-

er and brain TOS levels only significantly increased between the T group and that of the control group (p=0.000; p=0.001). It was observed that the liver and kidney tissues TAS levels significantly increased between T+S group and that the T group (p =0.000). It was concluded that liver tissue TOS levels significantly decreased between the T+S group and the T group (p =0.000).

NO_x levels measured in tissue samples

The changes in the NO_x levels obtained from all tissues of the experiment groups are presented in Table 6. It was observed that the NO_x level significantly increased only in the brain tissue between T group and the control group (p=0.000). When the NO_x levels of the T+S group were compared with the levels of the T group, it was concluded that there existed a statistically significant decrease in the NO_x levels in all tissues (p =0.000).

Table 1. Blood toluene levels of rats in T and T+S groups

Groups	C $\overline{x}\pm SE$	S $\overline{x}\pm SE$	T $\overline{x}\pm SE$	T+S $\overline{x}\pm SE$	p
Toluene (ng/ml)	-	-	1,525±0,071	1,481±0,071	0,730

C: Control Group; T : Sub-chronic Thinner Inhalation Group; T+S: Sub-chronic Thinner Inhalation + Safranal Group; S: Safranal Group; X ± SE: mean ± standard error; p<0.05

Table 2. MDA levels measured in tissue samples

MDA (nmol/g tissue)	C $\overline{x}\pm SE$	S $\overline{x}\pm SE$	T $\overline{x}\pm SE$	T+S $\overline{x}\pm SE$	p
LIVER	34,82±1,60[bc]	36,71±1,24 [ab]	41,81±1,18 [a]	29,38±1,46 [c]	0,000
LUNG	19,95±0,94[b]	19,83±0,73[b]	24,27±0,69[a]	18,32±0,86[b]	0,000
KIDNEY	24,18±1,81[b]	27,32±1,39[ab]	32,83±1,33 [a]	27,82±1,65 [ab]	0,000
BRAIN	27,32±0,88[a]	22,72±0,68 [b]	28,35±0,65 [a]	17,09±0,79 [c]	0,000
TESTIS	20,28±2,05 [b]	19,58±1,58 [b]	29,54±1,51 [a]	17,97±1,87 [b]	0,000

MDA: Malondialdehyde; C: Control Group; T : Sub-chronic Thinner Inhalation Group; T+S: Sub-chronic Thinner Inhalation + Safranal Group; S: Safranal Group; X ± SE: mean ± standard error; a,b,c: different letters on the same line indicate a significant difference p<0.05

Table 3. GSH levels measured in tissue samples

GSH (nmol/g tissue)	C $\overline{x}\pm SE$	S $\overline{x}\pm SE$	T $\overline{x}\pm SE$	T+S $\overline{x}\pm SE$	p
LIVER	19,93±1,05 [a]	19,25±0,81[ab]	16,47±0,78 [b]	18,69±0,96[ab]	0,013
LUNG	16,46±0,70[ab]	16,71±0,54 [a]	17,37±0,52 [a]	13,64±0,64[b]	0,000
KIDNEY	15,99±0,98 [ab]	17,39±0,76[a]	12,91±0,72 [c]	14,09±0,89[bc]	0,000
BRAIN	14,70±0,75 [a]	14,36±0,58 [a]	12,41±0,55[b]	13,14±0,69[ab]	0,015
TESTIS	18,8±1,6 [a]	18,71±1,22 [ab]	13,76±1,16 [b]	18,60±1,44[ab]	0,007

GSH: reduced glutathione; C: Control Group; T : Sub-chronic Thinner Inhalation Group; T+S: Sub-chronic Thinner Inhalation + Safranal Group; S: Safranal Group; X ± SE: mean ± standard error; a,b,c: different letters on the same line indicate a significant difference p<0.05

Table 4. TAS levels measured in tissue samples

TAS (mmol Trolox Equiv./L)	C $\overline{x}\pm SE$	S $\overline{x}\pm SE$	T $\overline{x}\pm SE$	T+S $\overline{x}\pm SE$	p
LIVER	0,89±0,07 [a]	0,39±0,05 [b]	0,38±0,05 [b]	0,80±0,06 [a]	0,000
LUNG	0,28±0,09	0,29±0,07	0,32±0,07	0,49±0,09	0,213
KIDNEY	0,56±0,07[a]	0,45±0,06 [a]	0,15±0,06 [b]	0,49±0,07 [a]	0,000
BRAIN	0,35±0,07	0,22±0,05	0,32±0,05	0,35±0,06	0,158
TESTIS	0,43±0,05	0,33±0,04	0,30±0,03	0,38±0,04	0,060

TAS: Total antioxidant status; C: Control Group; T : Sub-chronic Thinner Inhalation Group; T+S:Sub-chronic Thinner Inhalation + Safranal Group; S: Safranal Group; X ± SE: mean ± standard error; a,b,c: different letters on the same line indicate a significant difference p<0.05

Table 5. TOS levels measured in tissue samples

TOS (μmol H₂O₂Equiv./L)	C x̄±SE	S x̄±SE	T x̄±SE	T+S x̄±SE	p
LIVER	18,24±2,03[b]	28,82±1,56[a]	35,32±1,49[a]	22,51±1,85[b]	0,000
LUNG	13,24±1,89	16,17±1,46	16,75±1,39	13,26±1,73	0,129
KIDNEY	12,64±2,31	12,62±1,78	16,10±1,70	12,56±2,11	0,361
BRAIN	7,46±1,98[b]	13,14±1,53[ab]	17,26±1,46[a]	15,44±1,81[ab]	0,001
TESTIS	11,74±1,25	11,74±0,97	13,77±0,92	10,91±1,14	0,193

TOS: Total oxidant status; C: Control Group; T : Sub-chronic Thinner Inhalation Group; T+S: Sub-chronic Thinner Inhalation + Safranal Group; S: Safranal Group; X ± SE: mean ± standard error; a,b,c: different letters on the same line indicate a significant difference p<0.05

Table 6. NOₓ levels measured in tissue samples

NOₓ (μmol/g tissue)	C x̄±SE	S x̄±SE	T x̄±SE	T+S x̄±SE	p
LIVER	7,23±1,314[ab]	12,09±1,02[a]	9,32±0,97[a]	3,00±1,19[b]	0,000
LUNG	4,32±0,89[ab]	6,86±0,69[a]	6,54±0,66[a]	2,85±0,82[b]	0,000
KIDNEY	5,09±0,90[ab]	8,63±0,69[a]	6,99±0,67[a]	2,29±0,82[b]	0,000
BRAIN	3,32±1,01[b]	7,71±0,78[a]	6,98±0,74[a]	3,62±0,92[b]	0,000
TESTIS	5,64±0,95[ab]	5,87±0,73[a]	6,69±0,69[a]	2,81±0,86[b]	0,006

NOx: Nitric oxide; C: Control Group; T : Sub-chronic Thinner Inhalation Group; T+S: Sub-chronic Thinner Inhalation + Safranal Group; S: Safranal Group; X ± SE: mean ± standard error; a,b,c: different letters on the same line indicate a significant difference p<0.05

DISCUSSION

In the present study, it was observed that rats did not exhibit any escape reflexes during the application in the first two weeks of the inhalation, however, rats became more aggressive and exhibited escaping movements after the third week, their urination and excretion increased, and the uncontrolled rotational movements and standing reflexes deteriorated. Such observations obtained during the present study supported the previous findings reported in the literature suggesting that thinner acted as a neurotoxic agent and there existed evidence of intoxication in the central nervous system due to thinner inhalation (Bölükbaşı, 2005). It was also observed that the rats in the T+S group exhibited calmer behaviors contrary to the aggressive behaviors detected in the thinner inhalation group. Such finding could be interpreted as the mitigating or suppressing effect of safranal in case of the intoxication due to thinner inhalation. However, such finding should be supported through research conducted at molecular level. The difference between the blood toluene levels of the rats in the T and T + S groups indicates that the groups inhaled the same level of thinner.

Hosseinzadeh et al. (2013) suggest that the oral administration of safranal, in subacute toxicity test did not induce any toxic effects in many organs. Safranal at doses of 100 mg/kg by oral rout for 8 weeks did not cause any death in rats. However, an increase in the movement of animals was observed in the first 5 minutes after the application. This might be related to the irritant nature of most essential oils.

Higher doses and longer durations of thinner inhalation increases the monooxygenases based on CYP-450 and therefore results in ROS (Backes, 1993).. It is acknowledged that the toxic effects of organic solvents on cellular damage occurs due to the initiation of lipid peroxidation by free oxygen radicals such as superoxide anion ferryl and hydroxyl ions or more generally by the ROS (Mattia et al., 1991, 1993; Ahmed-Choudhury et al.,1998). Konuk et al. (2012) reported that sub-chronic thinner inhalation in rats increased free radical production. Additionally, exposure to sub-chronic toluene was reported not only to increase ROS production measures, but also to significantly affect various antioxidants (Kodavanti et al. 2015).

Given that the membrane phospholipids are the main target of free radicals, concentrations of MDA, one of the end products of lipid peroxidation (LP), were reported to be directly related to the severity of damage (Aleksandrovskii et al., 1988). In the pres-

ent study, once the MDA levels of the thinner group were compared with the MDA levels of the control group, it was observed that the MDA levels significantly increased in liver, lung, testis and kidney tissues, however, no significant increase was detected for the brain tissues despite the relative increase. Such findings were found to be consistent with the previous studies (Ulakoglu et al., 1998a, 1998b; Karaözler et al., 2002; Halifeoğlu et al., 2000). It was reported that in vivo and in vitro exposure to thinner or toluene accelerated the ROS formation in the brain, liver, kidney and lungs of rats and increased lipid (Mattia et al., 1991;1993; Halifeoğlu et al., 2000; Baydas et al., 2005). In parallel with these results, it is possible to state that sub-chronic thinner inhalation increased lipid peroxidation in tissues in the present study. It was reported that saffron and its components exhibited hydroxyl and DPPH radical scavenging activity (Hosseinzadeh et al., 2009; Assimopoulou et al., 2013). The literature review yielded no studies that focused on investigating the effects of safranal on thinner toxicity. In studies conducted through the use of safranal as an antioxidant molecule, it was stated that the increased MDA levels were decreased due to the antioxidative properties of safranal (Hosseinzadeh and Sadeghnia 2005; Mehdizadeh et al., 2013; Sadeghnia et al., 2013; Samarghandian et al., 2014, 2015). In the present study, the tissue MDA levels in T+S groups were found to be close to the levels of the control group and such finding indicated that safranal had a protective effect against the damaging effects of oxidation reactions in the organs and had an antioxidant potential in preventing lipid peroxidation, however such potentials did not exhibit uniform intensities for all tissue types.

GSH protects cells from oxidative damage through a direct interaction with the free radicals of the sulfhydryl group or through acting as a cofactor (Valko et al., 2006). In the present study, once the GSH levels of the tissue samples of the five organs of the T group were compared to those of the control group, it was determined that the GSH levels in the liver, testis, brain and kidney tissues were statistically significantly lower. On the other hand, there existed a statistically insignificant increase in the lung tissues, contrary to the findings in literature. Similar studies that focused on the effects of thinner on GSH levels reported that GSH levels decreased due to thinner inhalation (Baydas et al., 2003,2005; Ulakoğlu et al.,1998a; El-Nabı Kamel and Shehata 2008; Dillioglugil et al., 2005; Ahmadizadeh et al., 2014). In conclusion, the

decreasing GSH levels in all tissues except the lung were considered to be due to the free oxygen radicals such as superoxide anions, ferryl and hydroxyl ions formed by the catabolism of thinner metabolites such as benzyl alcohol and benzaldehyde, which increased due to thinner inhalation. Hazman and Bozkurt (2015) and Samarghandian et al. (2014) reported that safranal increased the GSH levels in diabetic rats and suppressed the oxidative stress based on diabetes. In the present study, it was observed that the GSH levels decreased in the liver, testis, brain and kidney tissues of the T group were back to the control levels in the T+S group. Therefore, the authors considered that the excessive amounts of free radicals due to thinner inhalation were neutralized by safranal and the oxidative stress levels were decreased or safranal increased the biosynthesis GSH.

It was reported that the antioxidant/oxidant status in the body was more simply evaluated by TAS and TOS measurements and gave an insight about the sensitive balance between the in vivo oxidants and antioxidants as a result of synergistic interactions (Ghiselli et al., 2000; Erel, 2004; Erel, 2005; Kayar et al., 2015). However, once the antioxidant/oxidant balance was deteriorated in favor of oxidants and oxidative stress occurred, a significant negative correlation could be observed between the TAS and TOS levels (Erel, 2005). Considering the TAS and TOS levels for all tissues, TAS levels were determined to numerically increase in the T+S group when compared to the T group, and the TOS levels were determined to decrease. Once the data from the Safranal group were compared to the data of the control group, the expected TAS-TOS negative correlation could not be observed. On the other hand, similar studies that focused on the effects of thinner on TAS and TOS levels indicated that the decrease in total antioxidant capacity levels due to thinner inhalation was a result of the oxidative stress (Bayil et al., 2008; Konuk et al.,2012 Hazman and Bozkurt (2015) suggested that safranal increased the TAS levels and decreased the TOS levels especially in diabetic rats, hence, acted exactly similar to an antioxidant. The present study also states that safranal has a protective effect against the oxidative damage due to thinner inhalation.

NO is a free radical that is defined as a biological signal molecule and plays an important role in both physiological and pathophysiological processes (Velayutham. and Zweier, 2013). In the presence of stressors, it could be produced by the catalytic ac-

tion of inducible NO-synthase (iNOS) and could be at higher concentrations. NO could lead to damage to proteins, lipids and the DNA through direct or superoxide reaction and thus lead to the formation of a highly reactive peroxide anion (Rahal et al., 2014). In the present study, the increased NO_x levels in the T groups in comparison to the control group indicated the presence of thinner metabolites and their activated release. While Konuk et al. (2012) determined that nitric oxide metabolites in rats were higher in T groups when compared to the control, Maniscalco et al. (2004) concluded that NO_x levels increased in the blood of shoe and leather workers, breathing organic solvents such as toluene, xylene and methyl-ethyl ketone. Once the NO_x levels of the S group were compared to those of the control group, a numeric increase was observed for all tissues. Farahmand et al. (2013) stated that safranal could be effective as a hormetin and in cases of mild oxidative damage that causes the activation of the antioxidative enzymes. Several studies that investigated the anti-inflammatory and antioxidant potentials of safranal reported a decrease in NO_x level due to safranal administration (Samarghandian et al., 2014; Bukhari et al., 2015; Hazman and Bozkurt 2015). In the present study, decreased NO_x levels in the T+S group indicated that safranal decreased the thinner-based stressors.

CONCLUSIONS

In conclusion, it was determined that the oxidative stress in liver, lung, brain, testis and kidney tissues developed due to thinner inhalation was eliminated or mitigated due to safranal administration, either by de-

creasing the NO_x, MDA and TOS levels or by increasing the GSH and TAS levels. Safranal was considered to have important antioxidant potentials in protecting the studied tissues against the harmful effects of oxidative stress. However, it was evident that safranal did not provide a uniform effect for every tissue. Although safranal appears to be a biological antioxidant, there exists scarce evidence of its effect as a direct chemical antioxidant. However, in the present study, the comparison between the data of the safranal group and the control group indicated close values for NO_x and TOS levels of the T group and the expected TAS-TOS correlation was not achieved. Such results could indicate the significance of safranal dose in medical applications. Therefore, it was considered that safranal, effective as a hormetin, could also act indirectly as an antioxidant. Within the scope of these outcomes, it becomes essential to consider not to exceed the specific limit values of solvent inhalation in several industries. It is as well considered significant that applying treatment methods that include safranal for the rehabilitation of volatile substance addicts could contribute to the development of new treatment modulations.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Comparison of Protective Effects of Melatonin and Amifostine on Radiation-Induced Renal Oxidative Stress in Rats

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ABSTRACT. In this study, we aimed to compare the protective effects of melatonin and amifostine on radiation-induced oxidative stress. Fifty female Wistar rats (3-4 months old, weighing 200±25 g) were divided into five groups (with ten rats each) and treated as follows: control (Cont), radiotherapy alone (RT), radiotherapy + amifostine (RT+AMI), radiotherapy + melatonin (RT+MEL), radiotherapy + amifostine + melatonin (RT+AMI+MEL). Rats were irradiated individually with a single dose of 8 Gy and amifostine (200 mg/kg) and melatonin (10 mg/kg) was administered to rats 30 minutes before irradiation. At the end of this follow-up period (72 hours) the rats were sacrificed. Spectrophotometric Analysis has been performed to kidney tissue samples. As a result of statistical comparison between groups after RT, total antioxidant capacity (TAC) decreased, total oxidant status (TOS) and oxidative stress index (OSI) increased, although the statistically significant change was only for OSI (p = 0.030). Addition of AMI or MEL to RT increased TAC and OSI significantly (p = 0.000), but there was no additive effect for TAC and OSI when both drugs were given together (p = 1.000, p = 0.172, respectively). In terms of TOS, statistically significant increasing was only for AMI (p = 0.000). There was protective effect when both drugs were given together against on Radiation-Induced Renal Oxidative Stress.

Keywords: melatonin, amifostine, radiation-induced renal oxidative stress, rats

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INTRODUCTION

After exposure to radiation, free radicals occur in cells within milliseconds. The subsequent alterations in intracellular processes following irradiation are due to the initial oxidative damage caused by these free radicals. The physiological signs of these radiation-induced alterations have been suggested to contribute to adaptive responses, bystander effects, cytotoxicity, radiosensitization, genomic instability, inflammation, and fibrosis. While most of the molecular changes associated with the initial production of free radicals at the time of irradiation are known, the contribution of metabolic processes to biological outcomes following exposure to radiation have been identified recently (Spitz, Azzam, Li, & Gius, 2004). Superoxide anion (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) are the reactive oxygen species (ROS) that form after the radiolysis of water (Kaushal, Chandrashekar, & Juncos, 2019; Zhang et al., 2019). There are some protective enzymes in cells such as superoxide dismutase, catalase, and glutathione peroxidase that are increased in radiation-induced oxidative stress (Bhosle, Huilgol, & Mishra, 2005; Musa, Shabeeb, & Alhilfi, 2019). In this context, O_2^- can be eliminated by Superoxide dismutases (SOD), and Glutathione peroxidase (GSH-Px) is the major antioxidant enzyme responsible for hydrogene proxide (H_2O_2) detoxification (Sun, Chen, Li, & Ge, 1998). Amifostine (WR-2721), as an organic thiophosphate, is the only FDA-approved radioprotectant agent used in radiotherapy and capable of ROS scavenging (Cakmak, Severcan, Zorlu, & Severcan, 2016). Melatonin (N-acetyl-5-methoxytryptamine), a hormone majorly secreted in the pineal gland, has abilities to scavenge free radicals as well as antioxidant effects by stimulating antioxidant enzymes (Musa, Shabeeb, & Alhilfi, 2019).

In this study, we aimed to compare the protective effects of melatonin and amifostine on radiation-induced oxidative stress. No functional endpoints were tested.

MATERIALS AND METHODS

Study design and animals

All experiments were conducted adhering to the guidelines of the institutional animal ethics committee (TUHDYEK-2012/18). This work was supported by the Scientific Research Projects Coordination Unit of Trakya University (Project Number: TUBAP:2012/104). As a result of the power analysis performed for the study design, it was planned to recruit 10 rats to each group for %95 reliability. Inclusion cirteria: Weight between 170 g and 230 g, female rats, survival after treatment. Exclusion criteria: The weight less than 150 g or greater than 280 g, male rats, the dead rats after treatment. Fifty female Wistar rats, 3-4 months old, weighing 200 ± 25 g, maintained under standard temperature and humidity conditions, were used in the study. The animals had free access to sterile water and food and were housed in a polypropylene cage containing sterile paddy husk for bedding throughout the experiment. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. The animals were divided into five groups (with ten rats each) and treated as follows:

- Group 1: control (Cont);

- Group 2: radiotherapy alone (RT);

- Group 3: radiotherapy + amifostine (RT+AMI);

- Group 4: radiotherapy + melatonin (RT+MEL);

- Group 5: radiotherapy + amifostine + melatonin (RT+AMI+MEL).

Animals in the RT group were treated with 0.9% saline solution (SS) 30 minutes before irradiation. Amifostine was administered to the rats in the RT+AMI and RT+AMI+MEL groups 30 minutes before irradiation. Animals in the RT+AMI and RT+AMI+MEL groups received amifostine (200 mg/kg, ER-KIM Ilac, Istanbul, Turkey) by intraperitoneal injection before irradiation (Cosar et al., 2012). Melatonin was administered to the rats in the RT+MEL and RT+AMI+MEL groups 30 minutes before irradiation. Animals in the RT+MEL and RT+AMI+MEL groups received melatonin (10 mg/kg, Sigma Chemical Co, St. Louis, USA) by intraperitoneal injection before irradiation (Sener, Jahovic, Tosun, Atasoy, & Yegen, 2003). All experimental procedures were performed on anesthetized rats. Anesthesia was performed via intramuscular ketamine (100 mg/kg, Pfizer Ilac, Istanbul, Turkey) and xylazine (3.9 mg/kg, Interhas A.S., Istanbul, Turkey) during irradiation. The follow-up period was 72 hours in all groups. At the end of this follow-up period, after all rats were anesthetized, the rats were sacrificed using cervical dislocation method (Cosar et al., 2012). After irradiation, the animals were closely monitored until they recovered

from anesthesia. No animals died due to irradiation or medication.

Irradiation

Rats were anesthetized and fixed on their blocks across a blue Styrofoam (Med-Tec, Orange City, IA, USA) treatment couch in prone position. RT, RT+A-MI, RT+MEL and RT+AMI+MEL groups were irradiated individually with a single dose of 8 Gy using a 60Co treatment unit (Cirus, cis-Bio Int., Gif-sur-Yvette, France). Dose rate was 1.15 Gy/min.

Spectrophotometric Analysis

Kidney tissue samples were excised, weighed, and immediately stored at -50ºC for later spectrophotometric analyses. The kidney tissues cleaned with 1.15% ice-cold KCl, minced, and then homogenized in five volumes (w/v) of the same solution. The homogenates were spun at 14000 rpm for 30 min at +4ºC, and assays were performed on the resultant supernatant. The protein concentration of the tissue was measured using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The total antioxidant capacity (TAC) of supernatant fractions was evaluated using a novel automated and colorimetric measurement method developed by Erel et al. (Erel, 2004). Hydroxyl radicals, the most potent biological radicals, are produced in this method. In the assay, the ferrous ion solution present in Reagent 1 is mixed with hydrogen peroxide, which is present in Reagent 2. The subsequently produced radicals, such as brown-colored dianisidine radical cations produced by the hydroxyl radicals, are also potent radicals. Using this method, the antioxidative effect of the sample is measured against the potent-free radical reactions initiated by the produced hydroxyl radicals. The assay has excellent precision values lower than 3%. TAC results are expressed as µmole Trolox equivalent/mg protein. The total oxidant status (TOS) of supernatant fractions was evaluated using a novel automated and colorimetric measurement method developed by Erel (Erel, 2005). Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is increased by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of µmole H_2O_2 equivalent/mg protein (Ozturk et al., 2011). The ratio of TOS to TAC was regarded as the oxidative stress index (OSI). The units for kidney tissue TOS and TAC values were converted to µmole H_2O_2 Equiv./gram protein. The kidney tissue OSI value was calculated as follows: OSI = ((TOS, µmole H_2O_2 Equiv./gram protein)/(TAC, µmole H_2O_2 Equiv./gram protein) × 100) (Aycicek, Erel, & Kocyigit, 2005).

Statistical Analysis

All statistical evaluations were made using the SPSS 19 package program. One way analysis of variance and Mann-Whitney U test (post hoc with Bonferoni correction) were used to determine differences between the groups. All parameters were given as mean ± SD. Significance was considered at $p < 0.05$.

RESULTS

All the animals were included in the analysis. After radiotherapy, although TAC decreased, TOS and OSI increased, although only the change in OSI was statistically significant ($p=0.03$). Addition of amifostine or melatonin to radiotherapy increased TAC significantly ($p=0.00$), but there was no additive effect for TAC when both drugs were given together. In terms of TOS, there was an increase, but this was statistically significant only for amifostine. Results for OSI were similar to TAC results (Table 1 and table 2).

Table 1. Spectrophotometric analysis results

Group		TAC (µmole H_2O_2 Equiv./gram protein)	TOS (µmole H_2O_2 Equiv./gram protein)	OSI = ((TOC, µmole H_2O_2 Equiv./gram protein)/ (TAC, µmole H_2O_2 Equiv./gram protein) × 100)
1	Control	3.58±0.45	0.99±0.34	0.02±0.009
2	RT	2.98±0.67	2.11±0.91	0.07±0.030 A*
3	RT+AMI	4.16±0.21 E***	6.7±1.53 E***	0.15±0.030 E***
4	RT+MEL	4.50±0.62 F***	3.02±1.27	0.07±0.039
5	RT+AMI+MEL	2.89±0.48	2.92±0.71	0.10±0.30

Data are given as mean±SD. * p< 0.05, ** p<0.01, ***p<0.001. Group Comparisons: A: 1 and 2, B: 1 and 3, C: 1 and 4, D: 1 and 5, E: 2 and 3, F: 2 and 4, G: 2 and 5. Abbreviations: AMI, Amifostine; MEL, Melathonin; RT, Radiotherapy; TAC, Total Antioxidant Capacity; TOS, Total Oxidant Status; OSI, Oxidative Stress Index

Table 2. The statistical comparison of the groups for TAC, TOS and OSI

	Groups	1	2	3	4
TAC	2	.131			
	3	.147	**.000**		
	4	**.002**	**.000**	1.000	
	5	.050	1.000	**.000**	**,000**
TOS	2	.208			
	3	.000	.000		
	4	**.001**	.581	**.000**	
	5	**.002**	.888	**.000**	1,000
OSI	2	**.030**			
	3	.000	.000		
	4	**.029**	1.000	**.000**	
	5	**.000**	.172	**.001**	.178

Abbreviations: AMI, Amifostine; MEL, Melathonin; RT, Radiotherapy; TAC, Total Antioxidant Capacity; TOS, Total Oxidant Status; OSI, Oxidative Stress Index , Group 1: control (Cont), Group 2: radiotherapy alone (RT), Group 3: radiotherapy + amifostine (RT+AMI), Group 4: radiotherapy + melatonin (RT+MEL), Group 5: radiotherapy + amifostine + melatonin (RT+AMI+MEL),

DISCUSSION

The main finding of this study is melatonin and amifostine may have some protective effect on radiation-induced renal oxidative stress. Radiation is an important inducer of oxidative stress, and chronic oxidative stress after total body irradiation is thought to be the cause of radiation nephropathy in rats (Ozbek, 2012). In our study, whole body irradiation of rats provoked oxidative stress in the kidney, identified by elevated levels of TOS and decreased levels of TAC compared to their respective values in control rats. The decrease in antioxidants might result from their increased utilization to neutralize the excess of free radicals, as well as their release to the blood stream resulting from radiation-induced cell membrane damage (Saada Helen & Azab Khaled, 2001). In addition, protein oxidation may contribute to the partial inactivation of enzymes (Kregel & Zhang, 2007).

The major enzymatic antioxidants are SOD, catalase, and GSH-Px. SOD is generally thought to act as a bulk scavenger of superoxide radicals. H_2O_2 that is produced by the action of SOD or the action of oxidases, such as xanthine oxidase, is reduced to water by catalase and GSH-Px. Catalase exists as a tetramer composed of 4 identical monomers, each of which contains a heme group at the active site. Degradation of H_2O_2 is accomplished via catalase. Catalase also binds NADPH as a reducing equivalent to prevent oxidative inactivation of the enzyme by H_2O_2 as it is reduced to water (Kirkman, Rolfo, Ferraris, & Gaetani, 1999; Zelko, Mariani, & Folz, 2002). Enzymes in the redox cycle responsible for the reduction of H_2O_2 and lipid hydroperoxides (generated as a result of membrane lipid peroxidation) include GSH-Px. GSH-Px reduces H_2O_2 and lipid peroxides to their corresponding alcohols (Flohe, Gunzler, & Schock, 1973).

The role of melatonin as a protector against ionizing radiation has been investigated in different studies. These results suggest that the radioprotective effect of melatonin is not species-specific and acts in a similar way in different biological systems (Blickenstaff, Brandstadter, Reddy, & Witt, 1994; Vijayalaxmi, Reiter, & Meltz, 1995; Vijayalaxmi, Reiter, Sewerynek, et al., 1995). Melatonin is a remarkably efficient oxygen radical scavenger. In vitro, melatonin is five-fold better at neutralizing hydroxyl radicals than glutathione and twofold more effective at inactivating peroxyl radicals than vitamin E (Baldwin & Barrett, 1998; Haghi-Aminjan et al., 2018). Also, melatonin's protective effects in some subcellular compartments may be due to its indirect antioxidative actions such as stimulation of enzymes that either promote the synthesis of other antioxidants or metabolize reactive species to non-radical products (El-Sokkary, Omar, Hassanein, Cuzzocrea, & Reiter, 2002; Hara et al., 1997; Kotler, Rodriguez, Sainz, Antolin, & Menendez-Pelaez, 1998). Histopathological studies have shown that melatonin has a protective effect against radiation-induced nephrotoxicity (Kucuktulu et al., 2012; Ozen et al., 2013).

Amifostine is an organic thiophosphate ester prodrug and must be activated by alkaline phosphatase to be converted into an active sulfhydryl compound

(Kaldir et al., 2008; Levi et al., 2002). It has been shown that amifostine can ameliorate functional renal damage in rat kidneys (Caloglu et al., 2009; Rolleman et al., 2007). Amifostine is a broad-spectrum cytoprotective agent and selectively protects all normal tissues without decreasing the response of neoplastic tissues to the cytotoxic effects of radiation therapy. Normal tissues have higher alkaline phosphatase activity in the plasma membrane, higher interstitial pH, and better vascularity when compared with tumor cells (Hensley et al., 2009). It has been known from previous study that the sublethal dose of whole body ionizing radiation had a protective effect against amifostine damage to brain tissue (Cakmak et al., 2009). The protective effect of amifostine against renal oxidative stress has been shown (Jacevic et al., 2018; Stankiewicz & Skrzydlewska, 2003).

In our study, addition of amifostine or melatonin to radiotherapy increased TAC significantly. There was no statistically significant difference in TAC between amifostine and melatonin administration, and there was no additive effect on TAC and TOS when both drugs were given together. In terms of TOS, there was an increase, but this was statistically significant only for amifostine administration. An interesting finding of this study is that mean OSI levels in Group 3 almost two-fold higher in Group 2. This may be due to an increase in total oxidant capacity in kidney tissue. And finally, there was no significant protective effect of both drugs on OSI.

CONCLUSIONS

In conclusion, we suggest that melatonin and amifostine have some protective effects on radiation-induced renal oxidative stress.. For this reason, we think that this study is very important for the literature. Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted. Strengths and limitations of the study should be discussed as well.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Effect of nebulized formoterol, ipratropium bromide, and furosemide in combination with fluticasone propionate on arterial blood gases of premature calves with respiratory distress syndrome

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ABSTRACT. The purpose of this study was to assess the clinical effect of nebulized formoterol (FM), ipratropium bromide (IB) and furosemide (FS) combined with fluticasone propionate (FP) on lung function in premature calves with Respiratory Distress Syndrome (RDS). Thirty-six premature calves with RDS were randomly assigned to six different treatment groups (D1 to D6). All groups received the standard treatment, including oxygen and support treatment. Calves in D1 received only the standard treatment. The following combinations of nebulized drugs were used for the other groups: D2: FP, D3: FP+FM; D4: FP+IB; D5: FP+FS and D6: FP+IB+FM+FS. The treatment period (72 h) involved the application of FM (15 µg totally/12 h), IB (2 µg/kg/12 h), FS (1 mg/kg/12 h) and FP (15 µg/kg/12 h) for five minutes. A significant increase over time in blood pH, partial pressure of oxygen (PaO_2), oxygen saturation ($SatO_2$) and a decrease in partial pressure of carbon dioxide ($PaCO_2$) and lactate were detected in all groups that received nebulized treatment; while in the D1, a significant change was observed only for $PaCO_2$. Calves in D6 had the highest PaO_2 and lowest $PaCO_2$ values amongst all groups at the end of treatment. No statistical difference was observed between the Nebulization Groups (NG). Nebulized FM, IB and FS with FP combination in premature calves with RDS, in addition to the standard treatment showed a significant curative effect on lung function.

Keywords: arterial blood gases, nebulized drugs, respiratory, treatment, premature calves

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INTRODUCTION

Premature calves often suffer from life-threatening disorders due to incomplete development of organs, such as lungs, (Bleul, 2009) resulting in a decreased gas exchange capacity due to decreased lung volume and capillary surface area. An insufficient exchange of gas results in hypoxemia and long-lasting oxygen requirements (Ok and Birdane, 2000; Aydoğdu et al., 2016). In premature infants, surfactant deficiency and inflammation-associated fibrosis have been reported to play an important role in increasing the respiratory distress syndrome (RDS) risk (Murch et al., 1996).

The availability of nebulized drugs alleviates the clinical signs of RDS and improves pulmonary function (Genicot et al., 1994; Yildiz and Ok, 2017). Fluticasone propionate (FP) is one of the most commonly used inhaled steroids to reduce inflammation in pulmonary disorders and for treatment of asthma symptoms. It is also extensively used for treating respiratory problems in horses (Robinson et al., 2009) and in premature calves with RDS (Yildiz and Ok, 2017). Diuretics, such as furosemide (FS), decrease interstitial oedema and pulmonary vascular resistance and facilitate gas exchange; thereby reduce oxygen consumption (Bancalari et al., 2005). Inhaled FS has been considered to improve lung function in premature calves with RDS (Yildiz and Ok, 2017). Beta-2 adrenergic or anticholinergic aerosols are useful in treating bronchial constriction in preterm infants and they have been known to relieve from acute respiratory distress (Jarjour et al., 2006). Formoterol (FM) and ipratropium bromide (IB) are long-acting β-2 adrenergic agonists, which exert their action by relaxing the smooth muscles of the respiratory tract. The onset of action of FM is rapid and has been shown to exert long-lasting bronchodilation in children with bronchial asthma (Faulds and Hollingshead, 1991). Leemans et al., (2009) found that inhaled salbutamol and IB were effective in the treatment of respiratory disorders in cats.

Therefore, the hypothesis of this study was whether the administration of nebulized FP combined with FM, IB and FS, additionally to oxygen therapy and standard clinical care, significantly improves the condition of premature calves with RDS, just as reported in human, feline and equine neonates.

MATERIALS AND METHODS

Animals: Thirty-six premature calves of different breeds (Holstein, n=27; Simmental, n=5 and Brown Swiss, n=4) diagnosed with RDS were admitted to the Animal Hospital of the Faculty of Veterinary Medicine of Selcuk University, within 2-12 h of birth. A number from 1 to 6 was randomly assigned to these premature calves using a random number generator, as follows: D1 (n=6), D2 (n=6), D3 (n=6), D4 (n=6), D5 (n=6) and D6 (n=6). D1 was considered the standart treatment group whereas D2, D3, D4, D5 and D6 the nebulized treatment groups. Inclusion criteria were the gestational age (230 to 260 days) and RDS following a clinical examination (Yildiz and Ok, 2017). RDS criteria for premature calves included: hypoxemia ($PaO_2 < 60$ mm Hg in arterial blood); hypercapnia ($PaCO_2 > 45$ mm Hg in arterial blood); tachypnea (respiratory rate; RR $\geq$ 45 breaths/min) and expiration accentuated by an abdominal lift (Bleul et al., 2008; Yildiz and Ok, 2017). Peripheral oxygen saturation (SpO_2) was measured using a patient monitor device (Compact, Econet, Germany) and calves were clinically examined during the study. Calves having RDS as a result of other underlying diseases such as aspiration pneumonia or trauma were excluded from the study. Approval of the study was obtained from institutional ethics board of the Veterinary Faculty of Selcuk University (no: 2014/05).

Sample collection and blood analysis

Sodium heparin-containing plastic insulin syringes were used for the collection of arterial blood samples anaerobically from the *arteria auricularis*, as described in a previous study (Yildiz and Ok, 2017). Arterial blood pH, PaO_2, $PaCO_2$, $SatO_2$, lactate and base excess (BE) were analysed within 15 min at 0, 24, 48 and 72 h using a blood gas analyser (GEM Premier 3000, Instrumentation Laboratory, Lexington). The 0 h 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 combination of the drugs.

Standard treatment and oxygen therapy protocol for premature calves

Standard treatment, including oxygen therapy, supportive treatment and clinical care, was provided to each calf in all groups. Supportive treatment and clinical care protocols are described by Yildiz and Ok (2017) and oxygen therapy via mask by Bleul et al. (2008).

Nebulizer treatment protocol for premature calves

While calves in the D2 to D6 received nebulizer drugs for 5 min, calves in the control group received only 2.5 mL saline solution (every 12 h for 72 h) for 5 min via a nebulizer machine (NebuTech, GmbH, Germany). The nebulizer drug combinations were administered as follows: D2: FP; D3: FP + FM; D4: FP + IB; D5: FP + FS and D6: FP + FM + IB + FS.

Brand names of the drugs and dosages enrolled in the study as follows; FM *(VENTOFOR, Liconsa A.S, Spania)*, 15 µg totally, BID, 3 days; IB *(ATROVENt, Boehringer Ingelheim, Istanbul, Turkey)*, 2 µg / kg, BID, 3 days, FS *(DIURIL, Vetaş,* İstanbul*, Turkey)*, 1 mg/kg, BID, 3 days, FP *(FLIXOTIDE, GlaxoSmithKline, Australia)*, 15 µg/kg, BID, 3 days. Each drugs were diluted with 2.5 mL saline solution and were administered for 5 min using a nebulizer machine (Fig 1).

Figure 1. Nebulized drug admistration to premature calves.

Statistical analysis

Normality of data was assessed with the Kolmogorov-Smirnov test. To evaluate differences in results among the groups One-way ANOVA (Duncan's posthoc multiple comparison test) were used and the Kruskal-Wallis test was used with non-normally distributed data. The level of significance was set at $P < 0.05$. Data were analysed with a statistical software package (IBM SPSS, version 14.01 for Windows, SPSS Inc, Chicago).

RESULTS

In this study, 88.8% of premature calves with RDS (32/36) responded well to the therapy. Out of the 32 calved that survived, 28 belonged to the nebulized treatment groups (D2 to D6). Four premature calves with RDS died (D1, n=2; D4, n=1 and D6, n=1).

Generally, the symptoms observed during the clinical examination of premature calves were: apnea or tachypnea, abdominal lift, weakness, cyanosis, hypothermia, wheezing and grunting sounds, lack or absence of sucking reflex, depression and increased capillary refill time . Symptoms such as tachycardia due to bronchodilator applications and hyperglycaemia due to corticosteroids were not observed. There were no differences in gestational age and admitted time to clinic after birth among the experimental groups. All data are presented as mean and standard error of the mean (mean ± SEM).

Blood gas findings

The parameters of arterial blood gas in premature calves with RDS are presented in Table 1.

In D2 to D6, there are marked increases in values including pH, pO_2, $SatO_2$ and decreases in pCO_2 and lactate levels. These values with their significance was presented separately in our group results.

Group D1: No significant differences were observed in blood gas parameters during the treatment. The gestational age at birth was 246 ± 3.79 days and admitted time to clinic after birth was 7.33 ± 1.45 h of the group.

Group D2: Significant increases was seen in values of blood pH, BE and $SatO_2$ at 24, 48 and 72 h during the treatment. Additionally, a decrease in lactate and $PaCO_2$ were obtained at 24, 48 and 72 h in this group (Table 1). The birth age was mean 248 ± 3.39 day and admitted time to clinic after birth was mean 7.17 ± 1.19 hour of the group.

Group D3: A significant ($P < 0.05$) increase in the blood pH, BE and $SatO_2$ values was obtained at the 24, 48 and 72 h of the treatment. A decrease in lactate and $PaCO_2$ value at 24, 48 and 72 h was also seen in these calves. A significant ($P < 0.05$) increase in PaO_2 value at 24, 48 and 72 h was found (Table 1). The birth age was mean 247 ± 3.93 day and admitted time to clinic after birth was mean 5.83 ± 1.16 hour of the group.

Group D4: A significant ($P < 0.05$) increase in blood pH, PaO_2 and $SatO_2$ values with a decrease in lactate and $PaCO_2$ values was seen in the calves at 24, 48, and 72 h of treatment. The birth age was mean 243 ± 2.70 day and admitted time to clinic after birth was mean 6.67 ± 1.26 hour of the group.

Group D5: A significant ($P < 0.05$) increase in

blood pH, BE and $SatO_2$ values during 24, 48 and 72 h of the treatment was seen. In addition, a decrease in lactate values during 48 and 72 h and $PaCO_2$ values at the 72 h was found in this group. No significant difference was observed in PaO_2 value of premature calves with RDS (Table 1). The birth age was mean 244 ± 3.52 day and admitted time to clinic after birth was mean 6.33 ± 1.45 hour of the group.

Group D6: A significant ($P < 0.05$) increase in

blood pH, BE and $SatO_2$ values during the 24, 48 and 72 h and while there were no significant difference between 0 h and 24 h, a significant increase in PaO_2 value between the 24, 48 and 72 h was seen in this group. A decrease in lactate value during the 48 and 72 h and $PaCO_2$ value during the 24, 48 and 72 h were seen in this group (Table 1). The birth age was mean 246 ± 4.16 day and admitted time to clinic after birth was mean 5.83 ± 1.92 hour of the group.

Table 1. Arterial blood gas values in the all premature calves with RDS in the present study (Mean±SEM)

Parameters	Groups	0. hour	24. hour	48. hour	72. hour
pH	D1	7.11±0.11 [b]	7.26±0.09 [aB]	7.31±0.10 [a]	7.38±0.12 [a]
	D2	7.29±0.04 [b]	7.45±0.03 [aA]	7.47±0.04 [a]	7.47±0.02 [a]
	D3	7.31±0.02 [b]	7.44±0.02 [aA]	7.46±0.01 [a]	7.48±0.01 [a]
	D4	7.24±0.08 [b]	7.44±0.02 [aA]	7.47±0.03 [a]	7.47±0.01 [a]
	D5	7.09±0.1 [b]	7.41±0.03 [aA]	7.43±0.03 [a]	7.48±0.02 [a]
	D6	7.30±0.04 [c]	7.41±0.01 [bA]	7.46±0.01 [ab]	7.50±0.01 [a]
$PaCO_2$ mmHg	D1	61.6±7.48	55.3±8.29 [A]	53.8±6.58 [A]	50.6±7.77
	D2	54.3±5.38 [a]	39.3±1.61 [bB]	39.0±3.86 [bB]	42.3±2.74 [b]
	D3	50.6±3.9 [a]	42.1±2.23 [bB]	39.0±1.93 [bB]	39.8±1.62 [b]
	D4	57.3±6.98 [a]	44.0±2.34b [AB]	42.2±1.83 [bB]	42.4±2.77 [b]
	D5	58.1±7.35 [a]	45.6±3.01 [abAB]	46.0±3.06 [abAB]	42.1±3.15 [b]
	D6	49.1±0.79 [a]	41.5±1.52 [bB]	39.0±1.95 [bB]	37.8±0.80 [b]
PaO_2 mmHg	D1	33.5±5.14	36.3±5.67	39.6±7.2	43.5±7.32 [B]
	D2	37.6±4.62	50.6±4.93	52.8±5.06	50.3±4.72 [AB]
	D3	36.3±1.91 [c]	45.5±4.01 [b]	54.5±3.06 [a]	55.3±1.76 [aAB]
	D4	33.8±4.16 [b]	46.8±5.9 [ab]	45.8±7.08 [ab]	56.6±5.26 [aAB]
	D5	38.0±5.67	39.8±6.11	43.3±5.71	48.5±5.57 [AB]
	D6	36.5±3.84 [c]	44.0±2.91 [c]	55.4±2.38 [b]	60.2±3.17 [aA]
BE	D1	-9.75±4.62 [B]	-3.30±4.33 [B]	0.27±4.88	4.10±6.04
	D2	-1.48±1.41 [bAB]	3.40±1.47 [aA]	4.13±1.52 [a]	6.95±1.19 [a]
	D3	-1.2±1.22 [bAB]	4.20±1.53 [aA]	4.18±0.96 [a]	6.07±1.91 [a]
	D4	5.52±2.50 [A]	5.17±1.12 [A]	6.52±1.84	6.70±1.98
	D5	-5.4±3.92 [bB]	3.40±1.41 [aA]	5.18±2.21 [a]	6.75±1.77 [a]
	D6	-3.6±2.33 [bAB]	1.18±0.61 [aAB]	3.88±1.44 [a]	5.90±1.33 [a]
SatO2 %	D1	41.3±11.7	54.0±11.8 [B]	60.1±13.64 [B]	69.1±12.5 [B]
	D2	61.3±10.4	83.5±6.45 [A]	84.8±6.64 [A]	85.0±4.63 [AB]
	D3	62.6±3.28 [b]	83.0±5.01 [aA]	89.1±1.62 [aA]	90.5±0.72 [aA]
	D4	52.3±10.2 [b]	80.1±4.77 [aA]	78.0±8.79 [aAB]	89.8±2.03 [aA]
	D5	38.0±13.1 [b]	68.1±7.89 [aAB]	75.1±5.63 [aAB]	83.5±4.16 [aAB]
	D6	59.0±7.47 [b]	76.0±5.54 [aAB]	89.8±1.16 [aA]	90.2±1.56 [aA]
Lactate mmol/L	D1	7.95±2.55 [AB]	5.65±1.96 [AB]	4.88±2.08	4.03±2.23
	D2	3.67±0.47 [aB]	1.55±0.41 [bC]	1.30±0.31 [b]	0.75±0.07 [b]
	D3	4.23±0.61 [aAB]	1.47±0.10 [bC]	1.18±0.28 [b]	0.78±0.10 [b]
	D4	5.72±0.89 [aAB]	2.82±0.46 [bBC]	1.44±0.19 [ab]	0.92±0.15 [b]
	D5	8.9±2.27 [aA]	4.08±1.19 [abABC]	3.48±2.03 [b]	2.58±1.16 [b]
	D6	7.95±1.07 [aAB]	6.38±1.32 [aA]	2.94±0.79 [b]	2.62±1.05 [b]

Different letters in the same row (a, b), in the same column (A, B) are statistically significant ($P < 0,05$), pH: concentration of hydrogen ions, $PaCO_2$: partial pressure of arterial carbon dioxide, PaO_2 partial pressure of arterial oxygen, $SatO_2$%: oxygen saturation, SEM: Standart error of mean.

pH: The pH value was significantly (P < 0.05) increased in all groups at 24 h. However, on comparing with D1, it was found that the nebulizer groups showed a statistically higher (P < 0.05) pH value at the 24 h. Yet, no statistical difference was observed between the nebulizer groups (Table 1).

PaCO₂: The values of $PaCO_2$ showed a statistical decrease (P < 0.05) at 24 and 48 h in D3, D4 and D6 groups as compared to D1 group. D6 group showed the lowest mean value of $PaCO_2$ amongst all groups at the end of the treatment. However, no statistical difference was observed between the nebulizer groups (Table 1).

PaO₂: On comparing D1 with nebulizer treatment groups, it was seen that all the nebulizer treatment groups except D6 did not show any statistical differences at the 72 h. D6 showed the highest value (P < 0.05) of PaO_2 amongst all groups at 72 h (Table 1). Yet, no statistical difference was seen amongst the nebulizer groups (Table 1).

BE: On comparing D1 group with nebulizer treatment groups, D2, D3, D4 and D5 showed statistical differences (P < 0.05) at the 24 h. But no statistical difference was determined amongst the nebulizer groups (Table 1).

SatO₂: On comparing D1 group with nebulizer treatment groups, statistical differences (P < 0.05) were seen in D2, D3, D4 at the 24 h, in D2, D3, D6 at the 48 h, and in D3, D4, D6 at the 72 h. However, no statistical difference was observed amongst the nebulizer groups (Table 1).

Lactate: On comparing D1 group with nebulizer treatment groups, a statistically significant difference was observed in lactate value at 24 h in D2, D3 and D6. Though there was no statistical difference between the groups at the end of the treatment, the highest mean value of lactate was found in D1 (Table 1).

Amongst the calves that did not survive, high values of lactate (> 8.5 mmol/L) and $PaCO_2$ (> 75 mmHg) and low levels of PaO_2 (< 40 mmHg) and $SatO_2$ (< 52%) were observed before death. The clinical symptoms of the non-survive premature calves were continuous abdominal respiration, high RR (> 70/min), absence of sucking reflex, abdominal distension related with feeding and low body temperature (34-36.5 °C). Parameters used for monitoring and clinical observations, including rectal temperature respiratory rate and peripheral oxygen saturation (SpO_2) are presented in Table 2.

Table 2. Monitoring and clinical parameters of the premature calves with RDS in the present study (Mean±SEM)

Paramaters	Groups	0. hour	24. hour	48. hour	72. hour
Temperature (°C)	D1	35.5±0.75[bAB]	38.3±0.41[aAB]	38.3±0.34[aAB]	38.4±0.20[a]
	D2	36.1±1.01[bAB]	38.3±0.14[aAB]	38.6±0.35[aAB]	38.7±0.23[a]
	D3	37.7±0.50[A]	38.7±0.21[A]	38.5±0.16[AB]	38.3±0.25
	D4	36.0±1.32[b]	38.0±0.30[abAB]	38.8±0.20[aA]	38.7±0.30[a]
	D5	34.6±1.03[bB]	37.6±0.42[aAB]	38.1±0.14[aAB]	38.6±0.29[a]
	D6	36.2±0.78[bAB]	37.4±0.62[abB]	38.0±0.15[aB]	38.5±0.15[a]
Respiratory rate (minute)	D1	51.3±9.22	64.6±13.8	58.3±13.6	56.0±14.9
	D2	47.5±10.3	58.8±12.3	65.6±15.3	35.0±5.26
	D3	49.6±6.4	45.2±9.33	44.8±4.59	37.2±2.58
	D4	54.3±7.51	52.3±7.47	44.4±7.55	37.5±5.66
	D5	49.8±6.73	58.0±13.2	50.3±7.23	35.0±5.99
	D6	68.0±8.10[a]	49.5±7.51[b]	44.0±7.23[b]	36.0±4.47[b]
SpO₂ (%)	D1	74.0±6.87[b]	85.0±5.97[ab]	89.5±4.97[abAB]	87.5±1.76[a]
	D2	79.4±8.40	91.6±2.48	95.3±1.76[A]	95.5±1.44
	D3	83.1±3.15[b]	92.6±1.15[a]	94.8±0.83[aA]	93.5±0.99[a]
	D4	75.1±4.19[b]	85.3±2.85[a]	88.8±0.86[aAB]	90.6±3.11[a]
	D5	70.5±4.92[b]	85.8±4.16[a]	84.3±3.49[aB]	90.3±3.12[a]
	D6	83.5±3.38	87.5±1.54	88.8±1.59[AB]	90.8±3.26

Different letters in the same row (a, b), in the same column (A, B) are statistically significant (P < 0,05), SpO_2 (%): Peripheral oxygen saturation, SEM: Standart error of mean.

DISCUSSION

Although numerous medical treatment strategies in human medicine, such as antenatal steroids, surfactant replacement, nitric oxide administration, and mechanic ventilation strategies (Sahni and Phelps, 2011), there has been no shown economically useful intervention to treat the RDS of premature calves, except for a study (Yildiz and Ok, 2017) to date.

A reliable method for the evaluation of pulmonary function (Bleul et al., 2007) is to determine arterial blood pCO_2 and pO_2. PaO_2 of less than 60 mmHg is considered to be indicative of RDS in infants (Verder et al., 1999). In healthy calves after birth, PaO_2 levels were showed to vary between 47 to 58 mmHg. In the calves with RDS one after birth, PaO_2 levels were found to change between 29 ± 12 and 38 ± 8.8 (Bleul, 2009). Yildiz and Ok (2017) reported that the mean value of PaO_2 levels in the premature calves with RDS at admitted to the clinic was found to be between 28 ± 3.5 and 36 ± 5.4 mmHg. In present study, the value of PaO_2 at 0 h was found to be between 33 ± 5.1 and 38 ± 5.6 mmHg (Table 1) and these animals posed difficulty in respiration (Table 2), which can be considered pathognomonic for all treatment groups. The mean value of PaO_2 was observed to be above 50 mmHg at the 72 h in all nebulizer groups, except D5. In addition, the mean respiratory rate reverted back to the normal level (< 45 RR/min) in the nebulizer treatment groups (Table 2). The mean value of PaO_2 in D6 increased to mean 60 ± 3.1 mmHg, and that of RR/min decreased to 36 ± 4.4 at the 72 h (Table 1-2). However, only the mean value of PaO_2 in D6 reached above the specified critical value (60 mmHg) (Bleul, 2009). This is indicating that nebulized treatment has a positive effect in pulmonary function. The level of PaO_2 in D1 at the 72 h was found to be 43.5 ± 7.32 mmHg (Table 1), while the mean respiratory rate continued to be 56 RR/min (Table 2). The level of PaO_2 below 45 mmHg is likely to be pathognomonic for RDS with significant clinical signs such as high respiratory rate (> 45 RR/min) (Bleul, 2009; Yildiz and Ok, 2017). Oxygen therapy is recommended for use in any newborn where $SatO_2$ is less than 90% or PaO_2 is less than 60 mmHg. The low levels of arterial $SatO_2$ (50.6 ± 7.7) in D1 indicate that the treatment without nebulized drugs may be insufficient to raise the level of $SatO_2$ up to the recommended values (Palmer, 2005; Yildiz and Ok, 2017). On the other hand, a combination of inhaler FP along with FM, IB and FS in addition to the standard treatment increases the value of $SatO_2$ adequately to the recommended levels (Palmer, 2005; Yildiz and Ok, 2017) in premature calves with RDS. Inefficient vital capacity of premature calves impairs the elimination of carbon dioxide from lungs, increasing its level in blood resulting in respiratory acidosis (Bleul et al., 2007) and the increase in the level of $PaCO_2$ above 45 mmHg indicates to RDS (Bleul et al., 2008). Yildiz and Ok (2017) reported that the levels of $PaCO_2$ (50 to 74 mmHg) in premature calves with RDS significantly decreased by the inhaler therapy. In our study, the levels of $PaCO_2$ were determined that higher than 49 mmHg in all premature groups at 0 h (Table 1). The $PaCO_2$ values decreased in all groups during the treatment period (Table 1). The results of this study are consistent with the previous studies (Bleul et al., 2007; Yildiz and Ok, 2017). The studies (Bleul, 2009; Güzelbekteş et al., 2012; Yildiz and Ok, 2017) suggested that blood pCO2 and lactate levels are important biomarkers for the detection of tissue hypoxia in premature calves. The concentration of plasma lactate greater than 4 mmol/L is a predictor for death within 24 h in cattle with pneumonia (Coghe et al., 2000). In a study with premature calves (Yildiz and Ok, 2017) has been reported that the mean lactate levels before the treatment were between 3.9 and 8.4 mmol/L and they also reported that in premature calves which were treated with nebulized drug combination with oxygen decreased the lactate to the normal level (< 2 mmol/L) however, in the premature calves that were treated only with oxygen therapy, the mean lactate level did not fall below the critical level (4 mmol/L). In the present study, mean lactate concentration at 0 h ranged between 3.67 and 7.95 mmol/L for all groups (Table 1). A significant ($P < 0.05$) decrease in the blood lactate levels below the critical value was seen at the 72 h in the nebulizer treatment groups (Table 1). However, the lactate levels of premature calves who did not receive nebulized treatment (D1 group) showed no significant decrease during the course of the treatment and did not fall below the critical level even at the 72 h (Table 1). Yildiz and Ok (2017) reported that high lactate (> 10 mmol/l) and $PaCO_2$ (> 74 mmHg) levels may be an indicator for high risk of death in premature calves with RDS. And also, in premature calves with RDS, Yildiz et al. (2017) reported that the cut-off values for lactate and pCO_2 were 7.5 mmol/L and 63.5 mmHg, respectively and positive correlation was found between mortality and increasing lactate and pCO_2 levels. In this study, premature calves which have lactate level higher than 8.5 mmol/L were non-survived like previous studies

(Yildiz et al., 2017; Yildiz and Ok, 2017). Premature calves had high CO_2 and low base excess levels along with decreased pH at admission to hospital (Yildiz et al., 2017). Yildiz and Ok (2017) found that the pH values of arterial blood in premature calves that received nebulized treatment were within the normal range (7.35-7.45) at the 72 h of the study except in those premature calves who did not receive nebulized treatment (pH < 7.35). In D1, the pH value remained below normal, possibly due to the continued CO_2 retention in blood (Bleul et al., 2007), since the mean value of $PaCO_2$ was found to be more than 50 mmHg at the 24 and 48 h of the treatment (Table 1).

The findings of this study show that nebulized therapy is beneficial for pulmonary functions in premature calves. These effects were most likely associated with nebulized FP combination with FM, IB, and FS. FP is known to reduce the release of inflammatory mediators in the lungs resulting from anoxic conditions (Robinson et al., 2009). FM and IB, the inhaled bronchodilators, have been reported to be effective in improving lung function and decreasing the respiratory symptoms (Duvivier et al., 1999; Chhabra et al., 2006) and also preventing neutrophilic infiltration and pulmonary oedema (Zhang et al., 2010). Inhaler diuretics like furosemide improve pulmonary compliance (Broadstone et al., 1991).

CONCLUSIONS

In conclusion, despite numerous medical advances, no single intervention will prevent or treat the RDS of premature calves. The use of nebulized drugs provides short-term improvement in lung mechanics, still these results do not support to use of these drugs instead of surfactant. But the drug combinations can be used in veterinary practice due to their ease of applicability and low cost for to reduce the serious damage due to the RDS in the first few days. The therapeutic effect of nebulized FP combination along with FM, IB, and FS on pulmonary function is a promising treatment for premature calves with RDS.

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CONFLICT OF INTEREST

None declared.

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A study of *Neospora caninum* antibody seroprevalence ın dairy cows in Turkey

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ABSTRACT. *Neospora caninum* is a intracellular protozoan parasite and is one of the major causes of repeated abortions, foetal malformations, pre-term deliveries, stillbirth and possible loss of milk yield in livestock. The presence of specific antibodies against *N. caninum* in the blood serum of dairy cows is investigated in the present study. A total of 184 blood serum samples of dairy cows were examined in Bursa province in the Marmara Region. *N. caninum* antibodies were measured using an indirect enzyme-linked immunosorbent assay (ELISA) (The Svanovir Neospora-Ab ELISA). From the total sample, antibodies to *N. caninum* were detected in 62 of the 184 examined cows (33.3%) and neurological findings were seen in a calf.

Keywords: Neospora caninum, ELISA, dairy cows, abort, Turkey, Bursa.

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INTRODUCTION

Many factors affect livestock production and productivity. The economic performance of dairy activity is directly related to the reproductive rates of the herd (Lamy et al., 2012). *Neospora caninum* is considered one of the major causes of repeated abortions in livestock. Neosporosis is a parasitic disease caused by *N. caninum*, a cyst-forming coccidian species and an obligate intracellular parasite. *N. caninum* is considered one of the major causes of repeated abortions, foetal malformations, pre-term deliveries, stillbirth and possible loss of milk yield in livestock, thus generating severe economic losses (Bartova et al., 2015; Dubey and Lindsay, 1996; 1-5). Dogs were the first discovered definitive hosts of this parasite. Other natural definitive hosts are coyotes, dingo and grey wolves (Dubey and Schares, 2011-12). The transmission of parasite is both vertical and horizontal. But the vertical transmission is more frequent than the horizontal. Cows may remain infected with *N. caninum* for their whole life and transmit the infeciton over several generations (Davison et al., 1999-31). The pregnant cow may show no outward signs but when the parasite passes to the unborn calf it can cause abortion. And also come calves survive the gestation period and born neurological signs or still born, others may look healthy but are infected with the parasite.

Serological prevalences of *N. caninum* in cattle worldwide have been reported by Dubey and Schares, 2011-12. In Europe, seroprevalence of *N. caninum* were detected 1%, in Germany, 15.2% in Greece, 0.7% in Norway, 55.9% in Romania, 22.5% in Spain, 20.1% in Slovakia, 2.8% in Sweden, 10.77% in Turkey and 12.9% in the United Kingdom (Dubey and Schares, 2011-12). During the 16 year period 2000-2016, several studies focusing on the detection of *N. caninum* antibodies in dogs and cattle in Turkey (Bıyıkoglu et al., 2003; Vural et al., 2006; Ocal et al., 2014; Adanır et al., 2015; Karatepe and Karatepe, 2016).

The aim of the present study was to determine the positivity to *N. caninum* and the influence of the infeciton on the occurrence of abortions and mothers of calves with neurological signs in dairy farm in Bursa in Turkey.

MATERIALS AND METHODS

Cattle of various ages, breeding systems and localities in Bursa, Turkey. Bursa is located near the Sea of Marmara, right opposite of Istanbul and in the Cities place category with the gps **coordinates** of 40° 11' 35.8728'' N and 29° 4' 27.1272'' E. Blood samples were collected from 186 adult dairy cows (Holstein), aged 3-5 years in Bursa province, located in northwestern Turkey. Blood samples were collected through vein puncture from each animalin tubes without anticoagulant, and to obtain the serum, blood samples were centrifuged. The serum samples were stored at -20°C until analysis. *N. caninum* antibodies were measured using an indirect enzyme-linked immunosorbent assay (ELISA) (The Svanovir Neospora-Ab ELISA). Abort and embryonic death records of positive animals were examined retrospectively.

RESULTS

From the total sample, antibodies to *N. caninum* were detected in 62 of the 184 examined cows (33.3%, 95% CI 17.2%-49.4%). Retrospective records of 62 animals with positive titration of *N. caninum* were examined (table 1,2). In the clinical course of the calf with neurological signs, it was observed that the suction reflex was strong and there was a spastic paralysis in the hind legs starting from the pelvic muscles. In the hind limbs, there was a hyperextension which was not corrected by hand and the head was slightly tilted to the left and there were tremors. No pathology was found in the general clinical course of its mother. As a result of clinical and serological findings, congenital neosporosis was diagnosed in the calf. The calf died as a result of progressive paralysis within 13 days. The operational veterinarian was asked to send the brain, lung and heart tissues from the necropsy for histopathological evaluation. However, histopathological evaluation was not performed due to deterioration.

Table 1: Result of 2nd and 3rd pregnancies of 49* dairy cattle which developed abortion or early embryonic death at the 1st pregnancies

	A	B	C	Total number of dairy cattle
1st pregnancy	25	24	13	62
2nd pregnancy	5	6	38	49*
3. pregnancy	0	2	47	49*

A: Number of early embryonic death **B:** Number of abortions formed in 4-6 months of pregnancy **C:** Number of normal pregnancy

Table 2: Results of 2nd, 3rd and 4th pregnancy of 13* cows with no problems in first pregnancy

	A	B	C	Total number of dairy cattle
1st pregnancy	25	24	**13***	62
2nd pregnancy	3	3	**7**	**13***
3rd pregnancy	0	4	3	7**
4th pregnancy	0	2	5***	7**

A: Number of early embryonic death **B:** Number of abortions formed in 4-6 months of pregnancy **C:** Number of normal pregnancy **D:** Number of calves with neurological signs
**Number of cattle with no problems in 1st and 2nd pregnancy
***Neurological findings were found in the calf of one of the cows

DISCUSSION

Neosporosis has aroused an immense interest mainly due to repeated abortions in cows and a negative effect on their breeding economy. The majority of *N. caninum* infections appear to have a chronic course, probably causing life-long persistence of the parasite in tissues of infected animals (Dubey, 1996; Ricardo Vilas Boas, 2015). Abortion, occurring during the middle of gestation, is the primary clinical sign of the infection in cattle. There was a significant association between seropositivity for *N. caninum* and occurrences of abortions among dairy cows, thus reinforcing the role of *N. caninum* as a major cause of reproductive disorders (Dubey et al 2006; bruhn, 2013; ghalmi, 2012). It is important to note that the reproductive problems described on 36 farms (57.14%) were occurrences of abortions, while on 26 (41,26%), they consisted of birth of weak calves, among which some evolving to death. Abortion is the main clinical manifestation of bovine neosporosis in dairy cattle, and depending on the stage of pregnancy, this can lead to abortion or the birth of a healthy but chronically infected calf. Most abortions occur in the early second trimester, but they may ocur throughout gestation (Dubey et al., 2006). Bursa province, where the study is conducted, is important for dairy cattle farms. Therefore, abortions are economically important. In this study, serological screening confirmed the influence of *N. caninum* on the occurence of abortions in selected dairy herd and abortion were occured between 5th and 8th months of gestation.

Transplacental transmission during pregnancy can occur in pregnant cows infected with *N. caninum*. Congenitally infected fetus birth or abortion is closely related to the immunity of pregnant cow, placenta and fetus (Klauck et al., 2016). Some studies regarding neosporosis have shown that fetal infection decreases with the rise of the number of gestations, and consequently with the animal's age, mainly be-cause animals acquire immunity against the parasite, so, the animals may not show high levels of antibody against *N. caninum*. This may be thought to be related to increased immunity (Romero et al., 2002; Dijkstra et al., 2003; Williams et al., 2009). Abort is the most common site reflection. However, congenitally infected calves with low calving syndrome have low chance of survival and may have neural pathological clinical implications. Sometimes, as a result of congenital encephalomyelitis, calves with paresis and dysphoria may occur in the hind legs and neurological reflections may occur after weeks of birth (Dubey and Schares, 2011;Klauck et al., 2016). In this study, there was a calf with neurological signs. Our results were parellel to the other studies andsuggest that abortion and / or early embryonic deaths in animals infected with *N. caninum* are much higher in heifers, and that subsequent pregnancies may end up in some infected animals normally.

During the past decade, *N. caninum* infection has emerged as an important reproductive disease in cattle throughout the world. Surveys in several countries from three continents have identified *N. caninum* infection as the major diagnosed cause of bovine abortion (anderson et al). In Europe, antibodies against *N. caninum* have been detected in 2.8-60 % cattle. There are considerable differences among countries, within countries, and between regions, with the highest in Turkey- 60 % (Kul et al.,2009) and the lowest in Sweden- 2.8% (Loobuyck et al.,2009). In Turkey, Aktas M et al (2005) found that out of 513 cattle in the Eastern Anatolia Region, 36 (7.01 %) were found to be seropositive by cELISA. In another study, in the Marmara Region, rate of seropositivity was detected to be 8,4% (32/366) (Oncel et al.,2003; Bıyıkoglu et al.,2003). In the present study, *N. caninum* antibodies were found in 33.3% cows post-abortion in the Marmara Region, Bursa province. This solution was showed that *N. caninum* infection is one of the major

causes of repeated abortions ın Turkey. The results of the above studies carried out in different regions of Turkey is in line with this results (Vural et al., 2006; Kaya et al., 2011; Celik et al., 2013; Ocal et al., 2014; Karatepe and Karatepe, 2016).

CONCLUSION

In conclusion, both results obtained in this study and the other results in Turkey, as in many countries in Europe have also revealed the presence of serolog-ically *N. caninum* in cattle in Turkey. The risk of *N. caninum* seems to be important for dairy cows with cases of abortion and prevention should be increased by checked *N. caninum* regularly in the future studies. In addition, more detailed research on the pathogenesis and consequences of vertical transmission is required.

CONFLICT OF INTEREST
None declared.

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Genetic characterization of *Lactococcus garvieae* isolated from farmed rainbow trout by random amplified polymorphic DNA-PCR in Iran

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ABSTRACT. Lactococcosis is one of the main bacterial infections of fish around the world. *Lactococcus garvieae* has been a major cause of rainbow trout losses in freshwater farming. This study aimed to genotype and determine the variability of *L. garvieae* isolated from infected farmed rainbow trout in Iran by the RAPD-PCR method. Bacterial samples were collected from 12 farms located in the western part of Iran and suspected to carry Lactococcus infection. Two hundred bacterial cultures containing cocci shaped bacteria were cultured in Trypticase soy agar (TSA) and blood agar mediums. All bacterial cultures were tested by conventional microbiological and biochemical tests, and PCR assay to identify *L. garvieae* by 16S rDNA genes. The RAPD-PCR method was used to determine the genetic pattern of all isolates. The sample strain pattern of the isolates was analyzed in the NTSYS program. According to a similarity coefficient index of 70%, all *L. garvieae* isolates were separated into two groups with four RAPD profile types. The highest and the lowest genetic pairwise similarity among the isolates were 98% and 54%, respectively. The results of the present study revealed that RAPD-PCR is an applicable method to describe the genetic diversity of different strains of *L .garvieae* among farmed fish.

Keywords: Lactococcus garvieae; Genetic characterization; RAPD-PCR; Rainbow trout

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INTRODUCTION

Lactococcus garvieae is a causative agent of lactococcosis that affects different types of fish and crustaceans in freshwater and marine cultures around the world (Vendrell *et al*., 2006). This pathogen was first isolated from some saltwater fish species in Japan that was initially called *Enterococcus seriolicida* (Kusuda *et al*., 1991). Evidence shows that the outbreak of lactococcosis increases with rising water temperatures from about 18 °C and could be controlled when the temperature drops to 13°C (Gibello *et al.*, 2016). In addition to fish and other animals (Villani *et al*., 2001), some infections associated with *L. garvieae* have also been reported in humans (Elliot *et al*., 1991; Li *et al*., 2008; Chan *et al*., 2011). The handling and eating of raw fish are important routes leading to human infections and have resulted in extending the zoonotic status of the pathogen (Gibello *et al*., 2016). This bacterium is also considered an emerging pathogen in both veterinary and human medicine (Meyburgh, 2017). *L. garvieae* can be identified by several laboratory methods from conventional microbiological tests to different kinds of molecular tests such as PCR, pulsed-field gel electrophoresis, ribotyping, random amplified polymorphic DNA (RAPD) PCR, sau-PCR and amplified fragment length polymorphism methods (Altun *et al.*, 2013). The multilocus sequence typing test (MLST) has also been used to improve knowledge of the evolutionary history and the genomic complexity of *L. garvieae* (Ferrario *et al*., 2013). In epidemiological studies of lactococcosis outbreaks, the RAPD-PCR method can be carried out as a potential test for identifying genetic variation of the causative agent. It is made applicable by using a single optional primer in a PCR reaction, resulting in the amplification of many discrete DNA products (Altun *et al*., 2013; Ravelo *et al*., 2003). *L. garvieae* strains are divided into three epidemiological groups according to genotypic variations determined by RAPD-DNA technique (Ravelo *et al*., 2003).

Lactococcosis is known as a significant infectious disease of farmed fish all over the world. In recent years, it has been one of the main causes of economic losses among rainbow trout farms in Iran. The first outbreak of Streptococcosis was reported in 2005 (Soltani *et al*., 2005) and then other researchers studied different subjects relating to the isolation, identification, pathogenicity and molecular evaluation of some streptococcal agents (Sharifiyazdi *et al*., 2010; Soltani *et al*., 2008; Fadaeifard *et al.*, 2012). The aim of the present study was genotyping of forty-three *L.*

garvieae isolates obtained from rainbow trout farms in Iran. In this study, the RAPD-PCR method was used to determine genetic variability and relationship among isolates from different geographic areas of this country.

MATERIALS AND METHODS

Sampling and bacterial isolation

Samples were obtained from 12 rainbow trout farms of Iran during 2016-2018. These farms are located in the western part of this country (Fig 1). They had been chosen based on primary evaluations of suspected fish to clinical signs of Streptococcosis/ Lactococcosis disease. Samples were obtained by the stratified cluster sampling method. The target farms were chosen from four high trout-producing regions in Iran. In each region, three farms and from each farm five specimens were randomly collected. The study was done according to the research project with the approval certificate number of EC/0181. Sterile swabs were obtained from the kidney, spleen, and liver of each fish inoculated aseptically on blood agar (with 5% sheep red blood cells) and Trypticase soy agar (TSA, Merck) media and subsequently incubated aerobically at 22°C for 24 to 48 hours. Gram stain and cellular morphologies were examined at 1000×. All gram-positive cocci shaped colonies were chosen for further tests.

Figure 1. Location and geographical distribution of rainbow trout farms sampled in Iran

Phenotypic and biochemical characterization

An overnight culture of the pure colony was subjected to the morphological, physiological and biochemical tests recommended by Austin & Austin (2012) which are described in Table 1. The condition of bacterial growth was determined in the four different ranges of temperatures (15°C, 20°C, 37°C, and 40°C) and pH (5-9.5) by culturing in nutrient broth

(NB). The hemolytic reaction of the isolates was examined on nutrient agar containing 5% sheep red blood cells. Acid production was checked by the carbohydrate fermentation procedure using the inoculation of the isolates into nutrient broth containing the respective carbohydrates such as glucose, lactose, and sucrose.

Molecular diagnosis

The isolates were studied using the PCR method as a molecular diagnostic test. Genomic DNA was extracted from pure colonies in the TSB using a genomic DNA purification kit (Fermentas, Lithuania) according to the manufacturer's instructions. PCR reaction was performed with *L. garvieae* specific primer pairs (pLG-1:5′-CATAACAATGAGAATCGC-3′) and (pLG-2: 5′- GCACCCTCGCGGGTTG-3′) for genetic confirmation of isolates as described by Zlotkin *et al*. (1998). All isolates were identified with an amplification product of 1100 bp. The PCR assay was carried out in a total volume of 25 μl reaction mixture containing 2 μl of template DNA, with a 1 μM of each primer, 2 mM $MgCl_2$, 200 μM of each dNTP, 1 U of Taq DNA polymerase (Fermentas, Lithuania), and 2.5 μl of PCR buffer 10X. The amplification was performed in a thermal cycler (Flex Cycler, Germany) with an initial denaturation step at 94°C for 3 min, 35 cycles of a denaturation step at 94°C for 60s, primer annealing at 55°C for 60s and extension at 72°C for 90s. The final extension was performed in 10 min at 72°C. *L. garvieae* strain (CB10, Iranian strain) isolated from clinical samples of lactococcosis in Iran (Soltani *et al*., 2008) was chosen as a positive control and *Streptococcus iniae* (ATCC 29178) was used as the negative control. The PCR products were detected by running the amplification mixture in 2% agarose gel with 1X Tris-acetate-EDTA buffer and stained with ethidium bromide (0.5μg ml $^{-1}$) after the run.

Genotyping by the RAPD method

The RAPD-PCR test was used to identify genetic variation in 43 *L. garvieae* isolates which had already been confirmed by the PCR test. Amplification reactions were carried out in a 50 μl volume of 50mM KCl, 10 mM Tris- HCl (pH:8.3), 3 mM $MgCl_2$, 250 μM each dNTP, 1.5mM of primers, 4 units Taq polymerase enzyme and a 3 μl template DNA. In this reaction, M13 primer (5'-GAGGGTGGCGGTTCT-3') was used and the program was performed as previously described by Altun *et al*. (2013). The amplification program was run on initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 43°C for 40s and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min. The amplified product was run on a 1.5% agarose gel electrophoresis.

Data analysis

The RAPD test was performed three times on each isolate to ensure the accuracy of the results. After determining PCR products on the gel, they were sorted into: without any DNA (0) and with DNA (1). Thereafter they were analyzed with numerical taxonomy and a multivariate analysis system (NTSYS) software, version 2.2. Jacquard's similarity coefficient (JSC), Dice Similarity Coefficient (DSC) and a simple matching coefficient (SMC) were calculated to utilize the factors in defining a genetic similarity percentage among all isolates.

RESULTS

Physiological and Biochemical tests

Obtained results were compared with the reference strain of *L. garvieae*. Results presented in Table.1 showed that many of the isolates were gram-positive cocci, catalase and oxidase negative, alfa and beta-hemolytic on blood agar (5% sheep's blood), non-motile, grow in a pH range of 5 to 9.5, and a temperature range of 15-40 °C, O/F positive, show no reaction in Indole, have no growth in urea and ornithine and have a fermentative reaction in glucose and lactose. The listed characteristics are highly similar to those found in comparable researches.

Table 1. Morphological and biochemical characteristic of isolated *Lactococcus garvieae* and comparison with published data

	Present study	Austin and Austin, 2012	Soltani *et al*, 2008
Gram stain	Ovoid cocci	Ovoid cocci	Cocci
Morphology	+	+	+
Hemolysis	a/ß	a/ß	a/ß
Catalase	-	-	-
Oxidase	-	-	-
Motility	-	-	-
O/F	+/+	+/+	+/+
Indole	-	?	-
Glucose	+	+	+
Lactose	+	+	+/-
Sucrose	?	?	V
Ornithine	-	-	-
Urea	-	?	-
Growth at:			
pH (5-9.5)	+	+	+
Temperature (15-40°C)	+	+	+

V = variable results, ? = Not defined.

PCR assay

The PCR assay resulted in the amplification of the 1100 bp band (16S rDNA) for all cocci-shaped bacteria which had been previously confirmed by conventional laboratory tests. From 200 samples obtained from infected rainbow trout, 43 isolates were identified as *L. garvieae*. Amplified bands of *L. garvieae* isolates are shown in Fig 2.
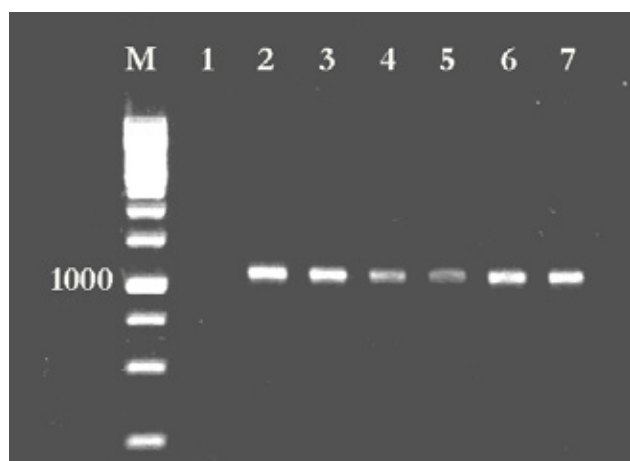
Figure 2. Amplification products from infected rainbow trout using PCR assay for the detection of *L. garvieae* (1,100 bp). Lane M, 1kb DNA ladder; lane 1, negative control; lane 2, positive control; lanes 3-7; test samples.

RAPD- PCR test

RAPD- PCR results showed the patterns obtained in different bands ranging from 220 to 2500 bp in size (Fig 3). Firstly, they were scored as 0 (with a band) and 1 (without a band) and then all obtained data were analyzed by NTSYS software. According to three different coefficients of data (SMC= 0.777, DSC= 0.688, and JSC=0.771), a Simple Match Coefficient at the value of 77% for the highest rate was used for calculating a genetic similarity percentage and for drawing the dendrogram. There was a 100% similarity only between isolates 35 and 39. The next highest genetic similarity was shown between isolates 41 and 43 (98 %) and the lowest was shown between isolates 1 and 22 (54%). In the dendrogram that was drawn for 43 isolates (Fig 4) with a 71% similarity coefficient, they were clustered into two groups A and B. Group A also divided into two subgroups A1 (isolate 1) and A2 (isolate 2). Furthermore, group B was divided into two subgroups, B1 (including 40 isolates) and B2 (isolate 9). It was found that using the 85% similarity coefficient, the number of subgroups rose, reaching 20 subgroups. Also, using the 93% similarity coefficient isolates clustered to 31 subgroups.
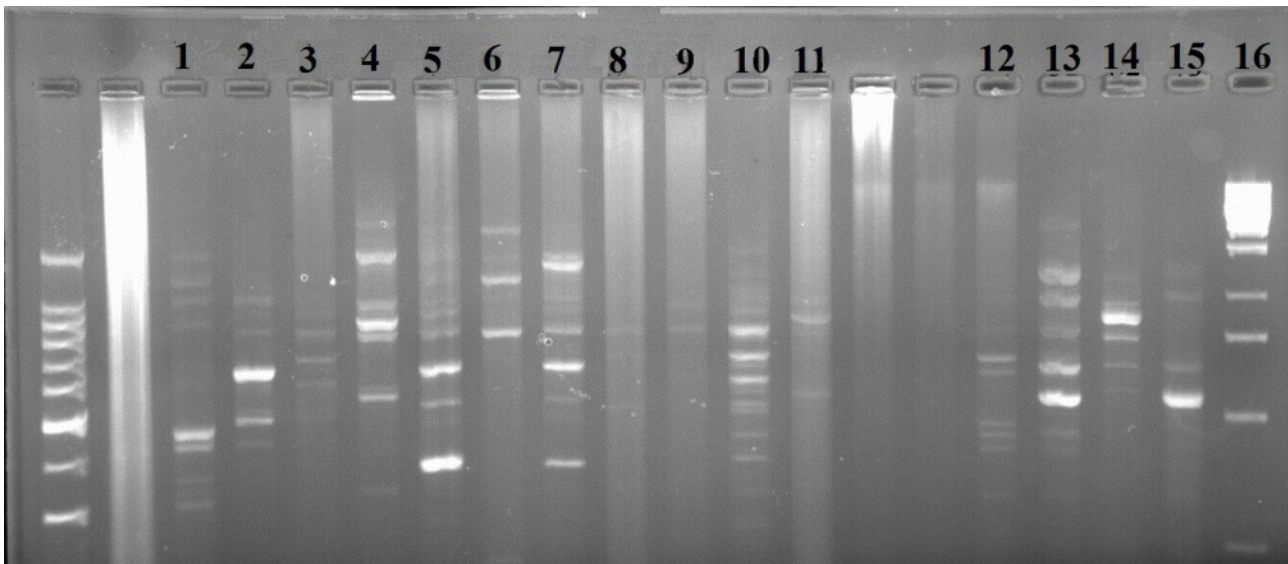
Figure 3. Agarose gel electrophoresis of RAPD-PCR products of *Lactococcus garvieae* isolates. A (lanes 1-16) that show different patterns of bands ranging from 220 to 2,500 bp in size, First lane on the left: 100bp DNA ladder and last lane on the right: 1kb DNA Ladder.
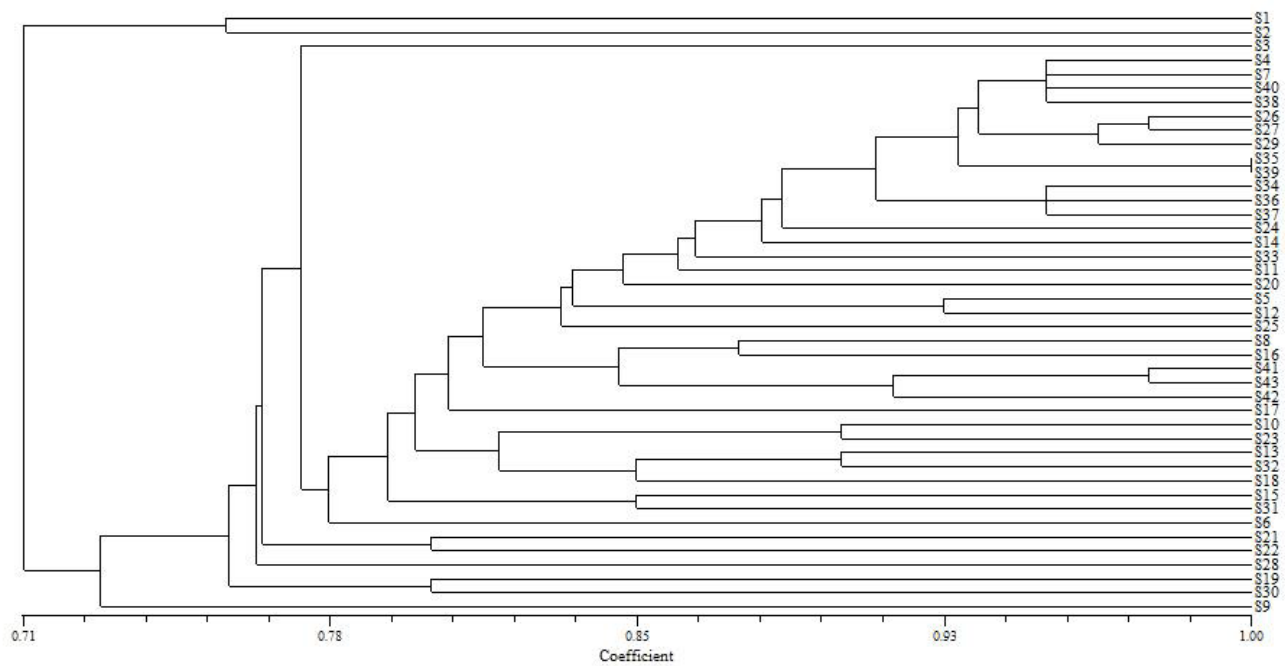
Figure 4. A dendrogram is drawn by using a Simple Match Algorithm with a Cofting coefficient of 0.777 for forty- three *L. garvieae* isolates.

DISCUSSION

Lactococcus garvieae has been isolated from a wide range of fish, dairy animals and human samples (Vendrell *et al*., 2006; Fortina *et al*., 2007; Li *et al*., 2008). The pathogen has been recently associated with an increasing number of Lactococcosis infections in various kinds of fish particularly in farmed salmonid (Ravelo *et al*., 2003; Brunt and Austin, 2005). In recent years, it has also been the most significant cause of trout losses in Iran. There have been phenotypic typing and serotyping methods to identify and characterize the streptococcal isolates, but molecular assays such as classical ribotyping, PFGE, RAPD, and DNA sequencing techniques have been chosen as easy and fast techniques to detect and genotype *L. garvieae* strains (Magarinos *et al*., 2000; Altun et al., 2004; Ravelo et al., 2003). Among different mentioned methods, RAPD has been introduced as a reproducible test with a high differentiating rate, which has proved to be appropriate for the epidemiological analysis of a variety of bacteria, especially fish pathogens (Welsh *et al*., 1990; Ravelo *et al*., 2003; Foschino *et al*., 2008).

In the present study, specimens were collected from clinically infected rainbow trout in different geographical areas of Iran. Out of 200 isolates which were obtained from bacteriological tests, 43 isolates were identified as *L. garvieae* by PCR test and consequently characterized by RAPD-PCR assay with a universal M13 primer. Finally, the simple match coefficient was used to determine the genetic similarity of all isolates. Except for the 100% similarity observed between isolates 35 and 39, the lowest and highest percentages of similarity were found in the range of 54% and 98% among isolates. According to a dendrogram which has been drawn with a 71% similarity level, all isolates are placed into two groups A and B, whereas at an 85% similarity level, the number of branches has increased to 21 profiles (RAPD-types), which indicates a high genetic diversity among the studied isolates. Others have also studied the genetic characterization of this pathogen obtained from various sources of fish and animals with those different results previously being published. Our results displayed a close genetic relationship between isolates included in group B2 and obtained from just two sampling regions in Iran showing the highest genetic similarity between isolates in comparison with other regions. Pathogens can be distributed or transmitted by asymptomatic carriers or contaminated eggs into susceptible populations. Contaminated water and feed and also the presence of some leeches species in the aquaculture environments could have facilitated the *L. garvieae* dissemination (Woo and Bruno, 2011; Vendrell et al., 2006).

Foschino *et al*. (2008) used two universal M13 and P5 primers for genotyping of 81 *L. garvieae* strains from fish and dairy products using the RAPD-PCR method. M13 and P5 primers have the ability to differentiate 52 and 27 genotypes, respectively. All isolates were divided into 5 groups using the 52 RAPD types. They also showed that *L. garvieae* strains isolated from dairy samples were generally not related to those obtained from lactococcosis outbreaks in fish. The RAPD -PCR assay was used by Ravelo *et al*. (2003) to determine the genetic similarity of *L. garvieae* strains isolated from different geographic areas of the world. By applying two P5 and P6 primers, isolates were divided into three genogroups according to an analysis of 90%, 80% and75% similarity among different profiles. These isolates were isolated from rainbow trout and yellow-tail. Altun *et al*. (2013) evaluated RAPD PCR analysis for genotyping 12 *L. garvieae* isolates from Turkey, England and, Spain. After genotyping all isolates using ERIC2 primer, they were placed in three clusters according to a similarity coefficient index of 70%, and it was found that 66.6 % of isolates were related to the LG1 genotype.

Our results indicate that *L. garvieae* can originate from different sources with various genetic profiles. The M13 primer has been a highly productive potential primer to genotype *L. garvieae* isolates and the results agree with Foschino *et al*. (2008) and Altun et al. (2013). Bacterial samples were collected from different regions of Iran and this can increase the possibility of genetic variability of isolates derived from various sources of cultured freshwater and marine fish, cultured prawns, animals and food (Meyburgh *et al*., 2017; Wang *et al*., 2007; Tsai *et al*., 2012). On the other hand, trout farming is a fast-growing industry in Iran that it can increase distribution of the causative agent of lactococcosis in all aquaculture-related environment, as well as other places, have access to fish farms, and consequently raising the exposure of other animals to get the lactococcus infections and tend to enhances the pathogenesis and outbreak of the disease. According to Vela *et al*. (2000) phenotypic and genotypic characterization of *L. garvieae* isolates from some European countries originated from different sources of fish, animals, and human showed a low genetic similarity between them. That suggested

diverse infection sources for the different lactococcosis outbreaks. RAPD-PCR assay has been used as a highly robust test in genome scanning by genetic qualification and comparison among species and isolates (Belkum and Meis, 1994). This method enables the detection of genetic relationships amongst bacterial fish pathogens with a high differentiating rate (Magarinos *et al*., 2000; Foschino *et al*., 2008; Findik *et al*., 2011).

CONCLUSION

High genetic diversity among all isolates indicates the necessity of studying cultured fish in different ways and according to their sources. This shows that the pathogen has an expanded genetic variation which plays a significant role in the prevalence of lactococcosis in the aquaculture industry. Knowledge of the geographical distribution of isolates and ge-netic diversity data of this pathogen will help us to design new products such as vaccines based on the DNA components of bacteria and continue to deliver practical ideas to control the disease. The finding of the present study suggests that RAPD-PCR can be a simple and efficient method to map the distribution of the pathogen.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Carcass characteristics and meat quality of broilers fed on earthworm (*Lumbricus rubellus*) meal

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ABSTRACT. The aim of the research was to evaluate the carcass characteristics and meat quality from chickens fed on diets in which fish meal was substituted with raw earthworms (*Lumbricus rubellus*) or earthworm meal. In the trial which lasted 42-days, 100 one-day-old *Hybro* broilers were divided into the control and three experimental groups. The control group was fed on standard broiler feed, the first (E-I) and the second experimental groups (E-II) were fed a diet in which 50% or 100% of fish meal was substituted with earthworm meal, respectively, whilst the third group (E-III) consumed feed without fish meal, but was given raw chopped earthworms *ad libitum* from day 1 to day 42. The replacement of fish meal with fresh earthworms resulted in significantly lower carcass weights in the E-III group in comparison with the control group (p<0.05). The differences in drumstick, thigh and breast meat share relative to the carcass mass were not significant (p>0.05). The lowest fat content in thigh and breast meat was in the group in which fish meal was replaced with earthworm meal. The lightness (L*) of thigh and breast meat was highest in the broilers fed fresh earthworms *ad libitum.* No significant differences in pH value were detected between the experimental groups (p>0.05). The most consumer acceptable were drumstick samples from E-II group and the least acceptable samples from the control group. Earthworm meal may be considered an adequate substitute for fish meal in broiler chickens' diet since it does not impair the production performance, carcass yield and meat quality.

Keywords: broilers, earthworm meal, carcass, meat quality, test panel

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INTRODUCTION

Besides reducing environmental pollution and production of quality fertiliser - biohumus, treatment of manure with various earthworm species provides significant quantities of a newly emerging feed i.e. worms (Ncobela and Chimonyo, 2015; Jacob, 2015). The production and demand for poultry meat is supposed to continue growing worldwide. This increase in meat production will require huge quantities of protein feed such as fish and soybean meals, which are expensive. For these reasons, there is a continuous research on the introduction of alternative protein feeds, which will help to solve the problem of deficient high-value proteins for animal nutrition (Khan et al., 2016; Djordjević et al., 2008; Radulović et al., 2018; Tiroesele and Moreki, 2012). Nowadays, with the existing methods of extraction and meal preparation 25-50% of proteins can be obtained from earthworm meal. The meal produced from red Californian earthworms (*Eisenia fetida*) is a tasty feed characterised by a high protein content (60-70%) of favourable amino acid composition that can replace fish and soybean meals in every phase of broiler production (Istiqomah et al., 2009). No negative effects on production performance in broiler chickens fed earthworm meal (obtained from *Perionyx excavatus*), as well as no detrimental impact on their health and food conversion rate were observed in previous studies (Vu and Quang, 2010).

Nutrition can significantly influence the poultry meat quality. The nutritive value of meat it is primarily determined by the content and composition of proteins and fats. Physical parameters for meat assessment are its colour, pH and water binding capacity. Colour is one of the first characteristics noted by the consumers, especially in the fillet products, and is also an indicator of meat quality (Fanatico et al., 2007). Factors determining sensory quality have the major influence on the consumer acceptability and are decisive in meat consumption. Parameters which determine carcass quality are carcass yield, breast meat yield, and the meat fat content (Araujo et al., 2004; Suchy et al., 2002). Nutrition is a factor which is most frequently used to improve the desired nutritional, sensory and technological quality of meat (Džinić et al., 2012).

Research has shown that red earthworms *(Lumbricus rubellus)* either dried or fresh, can successfully be used as an alternative protein feed component in broiler chickens fattening (Vu and Quang, 2010).

However, to our knowledge not much is known about the influence of these feed components on carcass parameters and broiler meat quality. Thus, our research targeted their influence on carcass parameters (processed carcass yield and relative weight of main body parts - thighs, drumsticks and breasts), meat quality (basic chemical composition of red and white meats), physical characteristics of thigh and breast meat (pH and colour) and sensory characteristics of broiler drumsticks. The present study was part of a bigger research project and the data on the effects on the production performance in broilers, their health and food conversion have been reported earlier (Janković at al., 2015).

MATERIAL AND METHODS

Birds, menagement and experimental diets

The study was performed on a total of 100 one-days-old *Hybro G* female chickens (mean initial body weight 44.90±3.86 g). The chickens were equally divided into a control and three experimental groups. Throughout the experiment the preventive measures, housing and nursing were adjusted to the floor rearing system of *Hybro G* broilers. Feed and water were provided *ad libitum*. The ambient conditions were identical for all the groups and in agreement with the recommended technological standards for *Hybro G* broilers. Research protocols concerning animal handling were in accordance with the guidelines proposed by the Ethical Committee of the University of Belgrade, Faculty of Veterinary Medicine, and with the EU Directive 2010/63/EU for animal experiments.

Broiler feed

The control group (C) was fed with standard feed mixture (NRC, 1994). In the first (E-I) and second (E-II) experimental groups 50% and 100% of fish meal (FM) was replaced with earthworm meal respectively, whereas the third experimental group (E-III) was fed on diets with no fish meal, but was given fresh chopped earthworms from day 1 to day 42 of the experiment *ad libitum*. The composition of the diets in each experimental phase is given in Table 1.

Table 1. Composition of broiler diets in each experimental phase [%]

| Ingredients [%] | Experimental phase[1] | | | | | | | | | | | |
| | Days 1-21 | | | | Days 22-35 | | | | Days 36-42 | | | |
	C	E-I	E-II	E-III	C	E-I	E-II	E-III	C*	E-I*	E-II*	E-III*
Maize	55.7	53.8	51.9	60.7	62.4	61.2	60.0	65.4	66.0	66.0	66.0	66.0
Soybean meal 44%	26.0	26.0	26.0	26.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0
Sunflower meal 33%	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Dicalcium phosphate	1.0	1.2	1.6	1.6	1.0	1.1	1.2	1.2	1.1	1.1	1.1	1.1
[2]Limestone	0.6	0.8	0.9	0.6	0.8	0.8	0.8	0.8	1.1	1.1	1.1	1.1
Brewers Yeast	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	2.0	2.0	2.0	2.0
Fish meal	5.0	2.5	-	-	3.0	1.5	-	-	-	-	-	-
Earthworm meal	-	4.0	8.0	-	-	2.5	5.0	-	-	-	-	-
Fresh earthworm (ad libitum)	-	-	-	FE	-	-	-	FE	-	-	-	FE
Soybeane oil	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Salt	0.2	0.2	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
[3]VMP	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total	100	100	100	100	100	100	100	100	100	100	100	100

[1]Experimental phases: I experimental phase (days 1-21); II experimental phase (days 22-35); III experimental phase (days 36-42); C - Control group; E-I - first experimental group; E-II-second experimental group; E-III - third experimental group;

*- In the final phase of fattening (36th - 42nd day), all experimental groups and the control group were fed on the same mixture: fish and worm meal were excluded from diet in the experimental group1 and 2, respectively; the experimental group 3 was fed on the same mixture, but was given fresh chopped worms during the entire fattening period. This is why all three groups have same numerical values of chemical content of the mixture.

[2]Limestone - Calcium carbonate;

[3]VMP - Vitamin mineral premix

[4]FE - Fresh earthworm

The earthworms (*Lumbricus rubellus)* used in the experiment were obtained by vermicomposting cattle manure ("Lumbri bio agrar", Leštane, Belgrade, Serbia). They were washed with water to remove dirt from the surface and afterwards kept in cold water (at 14°C) for 24 hours to free them from faecal mud (Edwards, 1985). The earthworms were dried in an oven at 50[0]C for 10 hours following the method described by Istiqomah et al. (2009). After drying, the worms were weighed and ground with a hammer milling machine into powder. The mixtures intended for feeding the experimental animals were mixed (25 min.) in the Vrieco-Nauta conical screw mixer with a capacity of 50 kg (Hosokawa Micron Group, Osaka, Japan). Broilers were fed on starter feed from day 1 to 21, grower from day 22 to 35, and finisher from day 36 to 42 (all groups).

Chemical analysis of feedstuff

Standard methods of AOAC (2006) were followed for measuring dry matter, fat, cellulose, proteins and crude ash. Calcium was determined by colorimetric method SRPS ISO 6490-1:2001 (2001) and phosphorus contents by method SRPS ISO 6491:2002 (2002). The concentrations of lysine and methionine, as well as the dietary gross energy (MJ/kg) were calculated according to Sauvant at al. (2004).

Slaughtering parameters of poultry meat

The broilers were transported to a slaughterhouse at the end of the experiment, on day 42. Having been measured individually, the birds were electrically stunned, sacrificed by manual exsanguination, plucked and eviscerated. The measurement of body weight before slaughter, and carcass mass after slaughtering was done by the usual procedure for an industrial slaughter house. Carcass yield was calculated from the ratio between the mass of chilled carcass and body mass before slaughter. Chilled carcasses were cut in accordance with legislations, Rules on the quality of poultry meat (Sl. paper SFRJ 1/81, 51/88) (1988) into main commercial cuts (thighs, drumsticks and breasts) and measured on automatic scales with a precision of ±0.05 g.

Chemical analysis of the meat

In order to assess the meat quality certain chemical analyses were performed. By means of random sampling 10 carcasses were chosen from each group and from these 7 random samples of following tissues were taken: breasts, thighs and drumsticks. The preparation of these samples was done according to AOAC procedure (2006). The chemical analyses of meat samples (breast and thighs) were performed after 24 hours of storage at 4^0C following standard procedures: water content was measured by means of loss of mass while drying homogenised samples at $105\pm1°$C until constant mass was achieved; fat content was determined by the Soxhlet method i.e. extracted from samples extracted with petrol ether and dried at $105\pm1°$C to constant mass; proteins were quantified by the method described by Kjeldahl; ash was determined by sample incineration at 550°C until constant mass.

Physical characteristics of the meat

To assess the meat quality certain physical and sensory analyses were carried out. Ten carcasses randomly chosen from each group were used as a source of 7 samples of thigh and breast muscles taken at random. The detection of meat colour was done 24h post mortem using the colorimeter Konica Minolta CR-400 Chroma Meter (Ramsey, NJ, USA) calibrated on white panel (L*=93.30; a*=0.32 and 1.8; b*=0.33). Measurement area Φ 8.0 mm, illumination D_{65}, viewing angle 0°. The colour was measured in terms of the CIE system (Commission International de L'éclairage, 1976), where L* value defines lightness, a* redness and b* yellowness in meat samples prepared according to Warriss (2000). The colour of each sample resulted from three consecutive measurements and is expressed as the averages for each of the parameters assessed.

The pH values were measured 24h after slaughtering and cooling of carcasses with a pH meter "Testo 205" (Testo Ltd., Hampshire, UK) by direct insertion of the probe into the thigh and breast muscles (with $\pm$ 0.01 accuracy). Before and during its use the pH meter was calibrated with standard phosphate buffers (pH of the calibration buffer was 7.00 and 4.00 at 20°C). The mean of two pH values measured at the same point was considered to be the result (Korkeala et al.,1986).

Sensory evaluation of broiler meat samples

The sensory evaluation of broiler meat was performed by trained analysts. The panel consisted of twelve assessors who were chosen according to the defined standard, which is identical to the international one ISO 8586:2012 (2012). The means of preparation of all the samples were identical. The skin was removed from the drumstick meat before thermal treatment. Thermal processing was performed on an electric grill and lasted for about 20 minutes, until the temperature of 80^0C was achieved in the core of each piece of meat. After the roasting the samples were presented to the assessors on identical plastic saucers. They were asked to inspect the smell and taste, and rank the samples so that the first place would take the most acceptable, followed by less acceptable and the least acceptable one. The differences in the acceptability of broiler meat were detected with Rang test ISO 8587/2006 (2006). The significance of the differences in the acceptability at levels 95% and 99% was determined having in mind the number of samples, number of rankings and the difference in the sums of ranks between the samples.

Statistical analysis

Data were statistically processed and analysed by the Graph Pad Prism 5.0 software. Study results were statistically analysed and presented using mean value and standard deviation. The one-way analysis of variance (ANOVA) was applied and followed by Tukey HSD-test.

RESULTS

The chemical composition of fresh earthworms and dehydrated earthworm meal is presented in Table 2. These results represent the mean values of the chemical analyses of the earthworms sampled at regular intervals.

The chemical compositions of complete broiler feeds in the experiment are given in Table 3. The results showed that they fully satisfied the animals' requirements NRC (1994) as well as those of the experiment.

The average body mass before slaughter was lowest in E-II broilers (Figure 1), the group fed on the diet without fish meal which was completely replaced with earthworm meal, whilst the highest was in the control. However, the differences between the groups were not significant (p>0.05). Carcass weight was lowest in E-III group and the highest in the control, and differed significantly (p<0.05). Carcass yield broiler was lowest in E-III group and the highest in the E-II group but the differences between the groups were not significant.

Table 2. Chemical composition of fish meal, fresh worms and earthworm meal [100% DM]

Chemical composition	Fish meal	Fresh worms	Redworm meal
Moisture, %	10.00	84.76	11.44
Ash, %	19.57	1.32	9.20
Crude Protein, %	67.39	6.89	41.42
Crude Fat, %	7.17	2.25	9.20
Crude Fibre, %	0.65	0.55	1.77
NFE[1], %	5.22	4.14	25.00
Ca, %	7.28	0.20	1.46
P, %	3.48	0.14	0.80
Lysine, %	5.11	0.51	3.33
Methionine, %	1.96	0.14	0.96

[1] NFE - nitrogen free extract;

Table 3. Chemical composition of feed mixture for broilers per experimental phase [%]

Chemical composition [%]	Feed mixture I (01-21 days)				Feed mixture II (22-35 days)				Feed mixture III (36-42 days for all groups)			
	C	E-I	E-II	E-III	C	E-I	E-II	E-III	C*	E-I*	E-II*	E-III*
Moisture	11.11	11.13	11.15	11.36	11.33	11.34	11.36	11.48	11.50	11.50	11.50	11.50
Ash	5.40	5.68	5.60	4.56	5.13	5.27	5.41	4.62	4.94	4.94	4.94	4.94
Crude protein	22.28	22.28	22.27	19.58	19.38	19.41	19.45	17.76	17.32	17.32	17.32	17.32
Crude fat	5.47	5.60	5.73	5.34	5.56	5.64	5.72	5.48	5.48	5.48	5.48	5.48
Crude fibre	4.02	4.03	4.05	4.09	3.85	3.87	3.87	3.90	3.88	3.88	3.88	3.88
NFE	51.71	51.26	50.82	55.05	54.74	54.46	54.17	56.75	56.86	56.86	56.86	56.86
Ca	0.98	1.00	1.00	0.65	0.90	0.88	0.87	0.7	0.82	0.82	0.82	0.82
P	0.81	0.80	0.80	0.66	0.74	0.74	0.75	0.65	0.66	0.66	0.66	0.66
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
ME (MJ/kg)	12.79	12.61	12.43	12.96	13.03	12.93	12.82	13.10	13.09	13.09	13.09	13.09

C - Control group; E-I - first experimental group; E-II-second experimental group; E-III - third experimental group
*- In the final phase of fattening (36th - 42nd day), all experimental groups and the control group were fed on the same mixture: fish and worm meal were excluded from diet in the experimental group1 and 2, respectively; the experimental group 3 was fed on the same mixture, but was given fresh chopped worms during the entire fattening period. This is why all three groups have same numerical values of chemical content of the mixture.
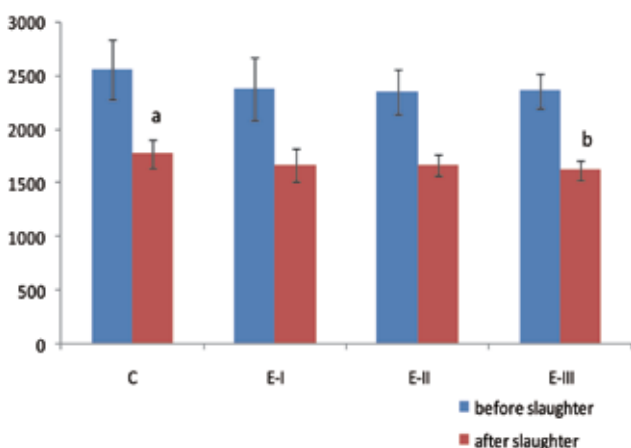
Figure 1. Broiler body mass (before slaughter) and carcass mass (g); Value expressed as $X \pm Sd$; [a,b,c,d] - small letters indicate significant difference (p<0.05)

Figure 2. Mass of whole legs (thigh and drumstick) (g); Value expressed as $X \pm Sd$; [a,b,c,d] - small letters indicate significant difference (p<0.05)

The average mass of thighs and drumsticks (Figure 2) was the lowest in broilers of the E-III group and the highest in the control, and differed significantly (p<0.05).

The mass share of the thigh and drumstick was lowest in broilers of the E-III group and the highest in E-I group. Nevertheless, the differences between the groups were insignificant (p>0.05).
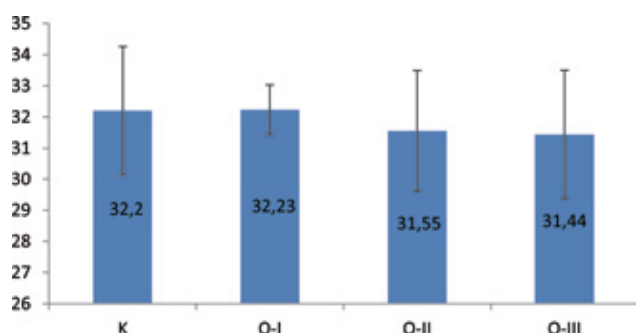
Figure 3. Mass share of breast meat in the carcass mass (%); Value expressed as X ± Sd

The average mass of breast meat (Figure 4) was lowest in the E-III group and the highest in the control, but the difference was not assessed as significant (p>0.05).
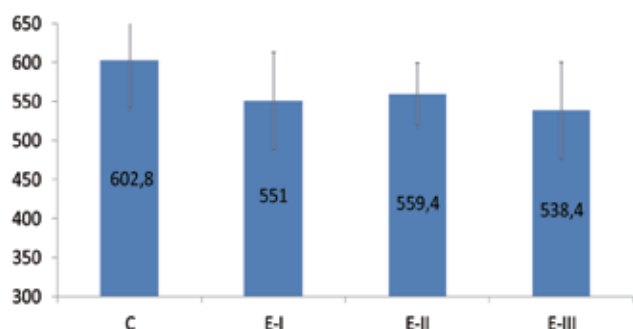
Figure 4. Mass breast meat (g); Value expressed as X ± Sd

In Figure 5 it can be seen that the relative mass share of breast meat in the carcass was highest in broilers of E-II group and the least in E-III. However, the difference between them was not significant (p>0.05).
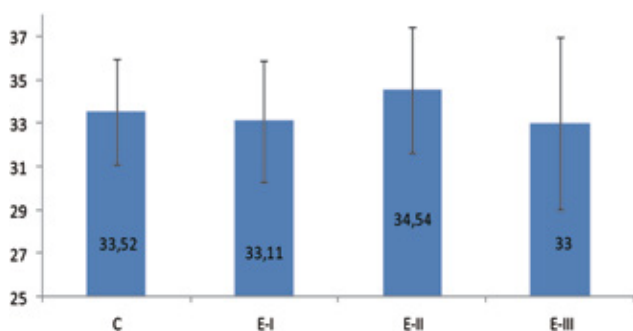
Figure 5. Relative average weight of breast meat (%)

The results of chemical analyses of the meat are presented in Table 4.

From the results of the analysis of the thigh meat it is noticeable that the mean water content in the broilers of E-I group was significantly lower than in the other groups (p<0.01). The lowest average fat con-

tent in thigh meat was detected in samples taken from E-II group whilst the highest was in E-I group. All the intergroup differences in fat content were significant (p<0.01), with the exception of the one between the control and E-I (p>0.05). Protein levels were significantly higher (p<0.01) in E-I group in comparison to the control. A significant difference in protein content was recorded for groups E-I and E-III (p<0.05). Different diets did not influence the concentrations of minerals in thigh meat (p>0.05).

According to the results of chemical analysis, in breast muscles (Table 4) the water content was highest in E-I group significantly higher than in all the others experimental groups (p<0.01). The average fat content was lowest in E-II group. Differences in fat content between the groups were significant (p<0.01). Proteins were detected in lowest concentrations in the breast meat in group E-I, highest in breast meat in E-III group. The differences in protein content were significant (p<0.01) between the control and E-I on one side and E-I other groups on the other side. Ash content was highest in meat E-I group, significant differences were observed between the control and E-1 group (p<0.05), as well as between groups E-I and E-III.

The results of instrumental assessment of the colour of thigh meat (Table 5) showed that its lightness (L*) was significantly higher (p<0.01) in the group which was given feed without fish meal, but had access to fresh earthworms *ad libitum* in comparison to all other groups. The lightness in the control was significantly higher (p<0.01) in comparison with E-I and E-II. Redness (a*) was significantly more intensive in the group in which fish meal was completely replaced with earthworm meal in comparison with the others groups. The yellowness (b*) in E-III was significantly higher (p<0.01) in comparison with the control, E-I and E-II groups. Moreover, in the control yellowness was significantly higher (p<0.01) in comparison with E-II.

Breast meat was lightest in group E-III and darkest in E-II (Table 5). Significant differences (p<0.01) were detected between all the groups tested. The redness of breast meat in group E-III was significantly higher (p<0.01) in comparison with all other groups. The yellowness in the control and E-III was significantly higher (p<0.01) in comparison with the two other groups.

The results measurements of pH in thigh and

drumstick meat (Table 5) were found to be with no significant differences between groups (p>0.05). The results of electrochemical measurements of pH in breast meat (Table 5) were found to be without differences between the groups (p>0.05).

In Table 6 the differences in the assessment of acceptability of drumstick meat samples can be seen. Ranking meat samples resulted in the notion that the most acceptable were those from broilers of the E-II group, significantly more acceptable than any other. The samples from the control group were assessed as the least acceptable by the panel of jurors.

Table 4. Chemical composition* of thigh and breast meat (%)

Chemical composition	C	E-I	E-II	E-III
Thigh meat				
Water	72.29 ± 0.38^x	70.79 ± 0.32^y	71.95 ± 0.13^x	72.29 ± 0.21^x
Fat	6.74 ± 0.27^x	$6.80\pm0.12^{a, x}$	6.24 ± 0.14^y	6.54 ± 0.13^c
Protein	$20.10\pm0.50^{a, x}$	21.38 ± 0.24^a	20.91 ± 0.38^c	20.58 ± 0.75^a
Ash	1.03 ± 0.00	1.03 ± 0.00	1.02 ± 0.00	1.03 ± 0.00
Breast meat				
Water	$74.36\pm0.38^{a,x}$	75.38 ± 0.23^y	$74.82\pm0.21^{b,x}$	74.18 ± 0.30^y
Fat	1.88 ± 0.06^x	1.34 ± 0.05^x	1.31 ± 0.04^x	1.89 ± 0.07^y
Protein	22.50 ± 0.33^x	21.82 ± 0.18^y	22.45 ± 0.20^x	22.51 ± 0.30^x
Ash	1.42 ± 0.02^a	1.45 ± 0.03^b	1.42 ± 0.01	1.42 ± 0.02^a

*$X \pm Sd$ results are presented as mean ± standard deviation (SD); small letters indicate significant difference: a, b, c, d (p<0.05); x,y,z,w (p<0.01) within the same row

Table 5. pH and colour values of thing and breast meat

	C	E-I	E-II	E-III
Thigh meat				
pH	6.45 ± 0.03	6.45 ± 0.03	6.45 ± 0.02	6.46 ± 0.03
L*	50.43 ± 0.24^a	49.52 ± 0.20^b	49.15 ± 0.24^b	52.15 ± 0.41^c
a*	6.92 ± 0.11^A	6.76 ± 0.11^a	$7.15\pm 0.19^{B,b}$	6.89 ± 0.12^a
b*	5.87 ± 0.12^a	$5.74\pm0.13^{a,b}$	5.58 ± 0.14^b	6.14 ± 0.15^c
Breast meat				
pH	5.85 ± 0.04	5.85 ± 0.03	5.86 ± 0.03	5.86 ± 0.03
L*	47.30 ± 0.29^a	46.36 ± 0.26^b	45.39 ± 0.29^c	48.08 ± 0.24^d
a*	4.69 ± 0.08^a	4.33 ± 0.12^b	$4.51\pm0.16^{a,b}$	4.89 ± 0.12^c
b*	8.13 ± 0.16^a	7.43 ± 0.13^b	7.25 ± 0.20^b	8.16 ± 0.17^a

L* = lightness; a*=redness; b*=yellowness; values in the same row marked with different upper case (A,B,C,D) or lower case letters (a,b,c,d) differ significantly (p<0.05 and p<0.01, respectively)

Table 6. Significance of differences between acceptability assessment of broiler **drumstick meat samples**

Groups	E- III	E-II	E-I	C
Sum of rank	27	48	30	25
C	2^{ns}	23**	5^{ns}	-
E-I	2^{ns}	18*	-	
E-II	21**	-		
E-III	-			

ns=not significant (p>0.05); *p<0.05; **p<0.01

DISCUSSION

Chemical analysis of feed

The protein content (41.42%) in the earthworm meal is lower in comparison to some literature data, which ranged between 46.57% and 63.0% (Khan et al., 2016; Jacob, 2015), but also more than reported by Mahmoud et al. (2015), who claimed that the content was 38.87%. Similarly, the amino acid content was somewhat lower compared to relevant published data, but still satisfactory. The results of the absolute content of amino acids are in agreement with the levels obtained by other authors (Sogbesan and Ugwumba, 2008). Fat contents were higher than the ones reported in literature according to Dynes (2003). Due to high fat contents in comparison with other feed components, earthworms are a rich source of energy with regard to dry matter. The ash content in earthworms is in agreement with some previously published data obtained by Sogbesan and Ugwumba (2008). The results of chemical analyses of feed consumed by broiler chickens in this research confirmed that it satisfied the norms required by the management guide for *Hybro* broilers and regulations NRC.

Slaughtering parameters of broiler meat

The analysis revealed that various diets did not affect significantly the average broiler body mass measured immediately before slaughter. These results are in agreement with the report of Bahadori et al. (2015), who, having studied the nutritive value of earthworms, found no significant differences in the body mass between the control and experimental groups. By contrast, Ton et al. (2009) claimed that the mean body mass of the broilers fed on diets containing earthworms (*Perionyx excavatus*) was higher than in the control.

Lower mass of chilled carcasses in comparison to the control was registered in the group which consumed feed with chopped raw earthworms instead of fishmeal. Resnawati (2002) conducted the research in order to clarify the effect of earthworm meal in the diet on carcass parameters of broiler chickens and observed no significant differences between the treatments, on that occasion he concluded that earthworm meal could be used up to 15 percent in broiler diets with no impact on carcass parameters or feed conversion. Results by Mahmoud et al. (2015) indicated that good results of broiler performance and carcass characteristics were obtained when 3.5% of earthworm meal was included in the broiler diet.

There were no significant differences in the carcasses yield of the broilers in the experiment. However, the lowest carcass yield was in the third experimental group i.e. 1.18% lower than in the control. The second group achieved 2.58% and the first one 0.92% higher carcass yields than the control. Ton et al. (2009) suggested that feeding with various quantities (1%, 1.5% and 2% on a dry matter basis) of red worms (*Perionyx excavatus*) resulted in higher yield of carcass.

Significantly lower weight of thighs and drumsticks in comparison to the control were detected in the group fed on feed in which the whole content of fish meal was replaced with chopped fresh earthworms. The mass share of thigh and drumstick meat in the carcass mass was lowest in the third group and the highest in the first one (32.23%). These data on the relative weight of whole legs are in accordance with those published by Bogosavljević-Bošković et al. (2010).

Breast meat is the carcass component with the highest economic value. The average chicken breast mass was lowest in the third experimental group and highest in the control, but they did not differ significantly. The yield of breast meat results from the satisfactory quality of feed which was used in the experiment: had there been a shortage of limiting amino acids in broiler feed, the primary indicator would have been a poor breast meat yield because the most intensive protein synthesis takes place in breast muscles (Araujo et al., 2004). However, the highest share of breast meat in chilled carcasses was detected in the second experimental group (34.54%) and the lowest in the third group (33.00%), but the differences were insignificant. Ton et al. (2009) reported about relative weights of deboned breast meat from 25.26% to 27.75% in broilers fed earthworms (1%, 1.5% and 2% on a dry matter basis). Having considered the slaughtering parameters for the experiment as a whole one may conclude that broilers fed on diets in which the fish meal was replaced with earthworm meal achieved satisfactory production results. There were no significant differences in carcass yield between the chicken groups. In addition, the relative weight of more valuable main commercial cuts were highly satisfactory, especially the one of breast meat.

Chemical composition of thigh and breast meat

Besides carcass yield, chemical composition is a main criterion for broiler meat quality assessment. The

analysis of thigh meat revealed that its average water content ranged from 70.79% to 72.29% and that there were some highly significant differences between the experimental groups. The percentage of water in thigh meat measured in the current research was lower than reported previously by Ristić et al. (2008).

The intergroup difference in fat content was significant. The lowest average level in thigh meat (6.24%) was measured in the group consuming the feed with completely substituted fish meal with earthworm meal and the highest (6.80%) in chicken fed on partially substituted diet. Fat content in thigh meat in the experimental groups was lower than claimed for *Hybro* broilers (8.9%-9.3%) by other researchers (Suchy et al., 2002; Ristić et al., 2008). These lower fat percentages in poultry meat are of utmost importance for the consumers, which may be concluded from increasing demands of the market for lean meat.

The average protein levels in thigh meat ranged from 20.10% to 21.38%, and varied significantly between groups. The highest level was detected in the meat of chickens which were fed on diets with 50% of fish meal and 50% of earthworm meal. The protein content in thigh meat obtained in the research is somewhat higher than for *Hybro G* broilers (18.3%-19.1%) claimed by Bogosavljević-Bošković et al. (2010) and Suchy et al. (2002). The ash percentages in thigh meat were roughly the same in all chickens and similar to previously published data obtained by other authors (Bogosavljević-Bošković et al., 2010; Suchy et al., 2002).

The percentage of water in breast meat varied from 74.18% to 75.38%, with significant differences between the chicken groups. These results correspond exactly to those obtained by Ristić et al. (2008), who measured a water content of 75%. The average fat content in breast meat varied significantly, and ranged from 1.31% in the second experimental group to 1.89% in the third one. These results are much lower than previously published data (2.1 to 2.5%) claimed by Suchy et al. (2002). Partial or complete replacement of fish meal with earthworm meal or raw earthworms leads to significantly lower percentages of fat in broiler breast meat. This is of outstanding nutritional importance since low fat content, especially in white meat, is one of the reasons for the increasing demand for poultry meat. The average protein levels in breast meat ranged from 21.82% to 22.51%, with significant differences between groups. Having analysed the results obtained in the current experiment, one

can conclude that the complete replacement of fish meal with earthworm meal or raw earthworms in the broiler diet exerts highly positive effects on protein contents in breast meat. These protein percentages in breast meat correspond to data for *Hybro G* broilers (22.5% to 22.7%) claimed by Suchy et al. (2002), but are somewhat lower (22.5% to 23.7%) than those presented by Bogosavljević-Bošković et al. (2010). Ash content varied slightly from 1.42% to 1.45%, which is higher than the one (1.1%) published by Ristić et al. (2013).

Physical characteristics of chicken meat

The results of the measurements of meat colour in thigh samples revealed significant differences in colour. Highest L* and b* values were detected in E-III group, which consumed the feed without fish meal but had chopped worms *ad libitum*. Thigh meat was reddest in E-II group. The lowest L* value, indicating the darkest meat, was in the broiler group which was fed on the feed with completely replaced fish meal with earthworm meal.

Breast meat of the chickens in E-III had significantly higher L* and b* values than those in other groups. The lowest was the L* value in broiler meat from E-II group. Qiao et al. (2002) have classified chicken breast muscle into three groups according to its colour: lighter-than-normal or light (L*>53), normal (48<L*<51), and darker-than-normal or dark (L*<46). Comparison of our results with these criteria leads to the conclusion that breast muscles of all broiler groups had normal colour and differed only slightly and were all visually acceptable.

Measurements of the electrochemical reaction of thigh meat failed to detect significant differences between inspected meat samples. These results are comparable with the data on the average pH (which is claimed to be 6.4) of fresh poultry meat (Ristić and Damme, 2013). The shelf-life of breast meat, its smell, colour, water adsorption during marination, water-holding capacity and loss during thermal processing depend mainly on pH. The results showed that there were no significant differences in pH values in breast meat between the groups, which is in accordance with the claims of Ton et al. (2009).

By means of sensory assessment it was discovered that the most acceptable were the drumsticks and breast meat originating from broilers fed on the diet without any fish meal, which was completely substi-

tuted with earthworm meal, and the least acceptable those from the control group and E-III group. The differences in the acceptability were based on the complete impression about the samples assessed - the colour, smell, juiciness, tenderness, texture, taste, aftertaste, fullness of flavour - on all traits percepti-ble by senses, rather than on one characteristic only (Baltić, 1994; Font-I-Furnols and Guerrero, 2014).

Conclusion is that earthworm meal may be considered an adequate substitute for fish meal in broiler chickens' diet since it does not impair the production performance, carcass yield and meat quality.

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Relationship between body condition score, blood metabolites and oxidative stress in transition period and reproductive performance of dairy cows

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ABSTRACT. The aim of the current study was to investigate the relationship between prepartum body condition score (BCS), blood metabolites (Glucose, beta-hydroxybutyrate; BHB), oxidative stress (Malondialdehyde; MDA, Gluta-thione peroxidase; GSH-Px) in transition period and some reproductive parameters in Holstein dairy cows. Fifty cows were divided into two groups [low-BCS = 2.75-3.0 (n=25); adequate-BCS = 3.25-3.75 (n=25)] according to BCS on the 21st day before expected calving. BCS was also recorded at 0 and +21 days after calving. Blood samples collected at -21, 0 and +21 days from calving for MDA and GSH-Px analysis as well as at -21, +14 and +21 days of postpartum for glucose and BHB. Data on reproductive parameters obtained from farm records were also collected. MDA concentrations were higher in group of low-BCS compared to group of adequate-BCS at -21 and 0 d related to calving (p<0.05). GSH-Px activity was lower in group of low-BCS than adequate one during the transition period (p<0.05). Low-BCS group also showed higher concentrations of BHB at +14 and +21 days after calving. During the transition period, low-BCS group had lower glucose concentrations compared to adequate one (p<0.05). Adequate-BCS group of cows showed shorter intervals of calving to first estrus (p<0.01) and calving to conception (p<0.01). In conclusion, cows with lower BCS at the prepartum period had worse metabolic and oxidative balance during the transition period. This situation also was associated with worse reproductive performance in cows.

Keywords: body condition score, cow, fertility, oxidative stress, metabolites,transition period

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INTRODUCTION

Transition period has been defined as the most critical period in relation to health status and reproductive performance of dairy cows. Transition dairy cows undergo dramatic changes including metabolic, physiological, nutritional and oxidant/antioxidant balance. This process can result in a period of negative energy balance and loss of BCS when rations do not meet the energy demand for a dairy cow at this reproductive phase (Konvicna et al., 2015, Barletta et al., 2017).

The body condition score is a simple, practical and reliable method for evaluating the energy and fat reserves in dairy cows (Roche et al., 2007, Barletta et al., 2017). BCS is associated with dry matter intake, milk yield, cow immun response and reproductive performance. Many authors agree that controlling BCS of high yielding dairy cows could minimize the effect of negative energy balance which is one of the most important reasons of poor reproductive performance (Stefanska et al., 2016). It is also well known that low BCS and high BCS loss are associated with increased incidence of anestrus and anovulatory cycles, and reduced conception rate (Ferguson et al., 1994, Barletta et al., 2017). Barletta et al. (2017) noted that the BCS at 21 d before calving was more informative for BCS loss or gain than the BCS near calving.

Oxidative stress occurs in cows when energy demand, non-esterified fatty acids (NEFA) and lipid peroxidation concurrently increase in this period (Gheise et al., 2017). Oxidative stress characterised by an increased production of free radicals and decreased antioxidant defense is the result of an imbalance between oxidant and antioxidant mechanisms (Konvicna et al., 2015). Oxidants damage to macromolecules of cells and lead to disruption of normal metabolism and physiology. These alterations in cells lead to metabolic disorders and diseases in dairy cows (Pilarczyk et al., 2012).

In humans, oxidative stress is associated with high body mass index and body weight loss (Vincent and Taylor, 2006). The oxidative stress especially as a result of high BCS in dairy cows has been also observed (Bernabucci et al., 2005).

Few studies concerning the relationship between BCS and; metabolic or oxidative balance in dairy cows are available (Laubenthal et al., 2017). However, the reproductive performance of cows has not been studied in these studies. The aim of the current study was to investigate the hypothesis that there is a relationship between prepartum body condition score (BCS), blood metabolites [Glucose, β-hydroxybutyrate (BHB)], oxidative stress [malondialdehyde (MDA), glutathione peroxidase (GSH-Px)] in transition period and reproductive parameters (calving to estrus interval, calving to conception interval and insemination index) in dairy cows.

MATERIALS AND METHODS

The current study was carried out in a large commercial dairy farm consisting of 1000 lactating dairy cows located in Western Thrace. All cows were housed in the same building under the same environmental and nutritional conditions. All animals were fed a mixed ration containing grass, corn silage and commercial concentrate twice a day ad libitum. The ration for dry and lactating cows contains 16% and 19% of crude protein, respectively.

A total 50 pregnant Holstein cows (2-4 parturitions) were selected according to their BCS on the 21st day before expected calving date. The animals were divided according to BCS (Gheise et al. 2017) into two equal groups: low-BCS (2.75-3.0, n=25) and adequate-BCS (3.25=3.75, n=25). The BCS was recorded by the same person using a scale of 1-5 with 0.25 increments as described by Ferguson et al. (1994). And BCS was recorded again at 0 and +21 days after calving. Twin calvings, stillbirths and dystocia were excluded from the study.

For monitoring the oxidative stress, blood samples were collected from the coccygeal vein into tubes with K_2EDTA (for MDA analysis) and into tubes with heparin (for GSH-Px analysis) at -21, 0 and +21 days from calving. The tubes with K_2EDTA were centrifuged at 1419 × g at 4 °C for 20 min. and plasma were stored at -18 °C until analysed. Plasma MDA concentrations (μmol/l) were analysed by the method of Konvicna et al. (2015). Hemolysates were quickly stored at -20 °C following the processed for preperaton of hemolysate and enzyme activities were estimated with the method described by Sharma et al. (2011).

Blood samples were also taken from the coccygeal vein into tubes without anticoagulant at -21, +14 and +21 days from calving for glucose and BHB analysis. The concentrations of glucose (mg/dL) and β-HBA (mmol/L) were measured using commercial kits (WAKO®, USA, Cat No. 439-90901 and Cat No. 417-73501, respectively) according to the manufacturer's instructions. Blood samples were collected before morning feeding in the same environmental conditions. After blood collections, serum were separated by centrifugation for 10 min at 3,000 g and stored at -20°C until assayed.

Reproductive performance of the two groups was assessed based on the calving-to-estrus interval, calving-to-conception interval, and insemination index.

Before performing the statistical analysis, data were examined with Shapiro-Wilk test for normality and Levene test for homogeneity of variances as parametric test assumptions. Descriptive statistics for each variable were calculated and presented as "Mean ± Standart Error of Mean". Student t-test was used to evaluate the difference between groups for the lactation, and reproductive variables. To test the differences in BCS, MDA, GSH-Px, glucose and BHB parameters between groups, General Linear Models with the repeated measures design were used. When a significant difference was revealed, any significant terms were compared by Simple effect analysis with Bonferroni adjustment. P<0.05 was considered as significant in all analyses. SPSS® for Windows 14.1 (Licence No:9869264) was used in the analysis of the data.

RESULTS

Mean values of the body condition score in low-BCS and adequate-BCS cows at -21, 0 and +21 days from calving were showed in Table 1. In both groups of cows, the mean BCS loss during transition period was determined as 0.25-0.75 unit. Mean BCS at -21 and 0 days from calving were significantly higher in group of adequate-BCS than group of low-BCS (p<0.05; Table 1).

MDA concentrations increased from -21 day to 0 days (p<0.05) in both groups of cows. MDA concentrations were markedly higher in cows with low-BCS compared to adequate-BCS at -21 and 0 days from calving (p<0.05; Table 1). However, no significant difference was observed between the two groups at +21 days from calving (p>0.05).

In both groups of cows, GSH-Px activity decreased from -21 days to 0 days, and it increased from 0 to +21 days (p<0.05). Cows with low-BCS had lower GSH-Px activity than adequate-BCS cows in all sampling dates (p<0.05; Table 1).

Glucose concentrations significantly varied between sampling dates, with low-BCS cows showing lower glucose concentrations (p<0.05; Table 2), in all dates.

No significant differences were observed between two groups in blood BHB concentrations at -21 days from calving (p>0.05). However, BHB concentrations were higher in cows with low-BCS than adequate-BCS at +14 and +21 days from calving (p<0.05; Table 2).

Table 1. The values of the body condition score, Malondialdehyde (MDA, μmol/l) concentrations and Glutathione peroxidase (GSH-Px, units/mg Hb) activity in low-BCS (n=25) and adequate-BCS (n=25) cows at different sampling dates (mean±SEM).

Parameters	Days from calving			P value		
	-21	0	+21	Group	Time	Group*Time
BCS						
Adequate-BCS	$3.40\pm0.02^{a,A}$	$3.47\pm0.04^{a,A}$	$2.90\pm0.03^{b,A}$	<0.001	<0.001	<0.001
Low-BCS	$2.91\pm0.03^{b,B}$	$3.16\pm0.05^{a,B}$	$2.82\pm0.04^{b,A}$			
MDA						
Adequate-BCS	$3.35\pm0.04^{b,B}$	$3.50\pm0.04^{a,B}$	$3.55\pm0.02^{a,A}$	0.003	0.001	0.074
Low-BCS	$3.51\pm0.08^{b,A}$	$3.69\pm0.06^{a,A}$	$3.55\pm0.03^{b,A}$			
GSH-Px						
Adequate-BCS	$2.85\pm0.08^{a,A}$	$1.69\pm0.03^{c,A}$	$2.12\pm0.07^{b,A}$	0.001	<0.001	0.150
Low-BCS	$2.42\pm0.10^{a,B}$	$1.46\pm0.04^{c,B}$	$1.89\pm0.05^{b,B}$			

Mean values within a row (a-b) or a column (A-B) with different superscript letters differ significantly.

Table 2. Serum glucose (mg/dL) and BHB (mmol/L) concentration of low-BCS (n=25) and adequate-BCS (n=25) at different sampling dates (mean±SEM).

Parameters	Days from calving			P value		
	-21	+14	+21	Group	Time	Group*Time
Glucose						
Adequate-BCS	$58.76\pm0.76^{a,A}$	$53.15\pm1.05^{b,A}$	$56.78\pm0.66^{a,A}$	<0.001	<0.001	0.019
Low-BCS	$49.63\pm2.18^{ab,B}$	$47.47\pm1.08^{b,B}$	$53.47\pm0.77^{a,B}$			
BHB						
Adequate-BCS	$0.14\pm0.01^{b,A}$	$0.41\pm0.06^{a,B}$	$0.16\pm0.01^{b,B}$	0.016	<0.001	0.109
Low-BCS	$0.16\pm0.01^{c,A}$	$0.69\pm0.09^{a,A}$	$0.27\pm0.05^{b,A}$			

Mean values within a row (a-b) or a column (A-B) with different superscripts differ significantly.

Table 3. Reproductive parameters of low-BCS (n=25) and adequate-BCS (n=25) dairy cows.

Fertility parameters	Groups		
	Adequate-BCS	Low-BCS	p
Calving to estrus interval	40.22±0.2	48.47±0.3	<0.001
Calving to conception interval	112.76±4.42	144.79±10.05	<0.05
Insemination index	2.10±0.12	3.01±0.25	<0.05

Table 3 shows the reproductive parameters of cows with low-BCS and adequate-BCS. The calving-to-estrus intervals (p<0.001) and calving-to-conception intervals (p<0.05) were quite long in cows with low-BCS (Table 3); cows with low-BCS had also higher insemination index (p<0.05), compared to cows with adequate-BCS.

DISCUSSION

Results obtained from the current study showed that cows with low-BCS at prepartum period had higher oxidative stress, lower antioxidant activity, higher BHB and lower glucose levels during the transition period. All these metabolic and oxidative imbalances in cows with low-BCS manifest as poor fertility.

During the transition period, body fat reserves are mobilized to meet the cow's energy requirements. Adipose tissue plays an important role in the maintenance of metabolic homeostasis (Alharthi et al., 2018). The mobilization of body fat reserves results in body condition score loss and increased concentrations of NEFA in blood. The intensified process of NEFA oxidation in the liver results in increased production of reactive oxygen species (ROS) and oxidative stress during the transition period (Folnozic et al., 2015, Turk et al., 2015).

Oxidative stress and antioxidant status depend on the stage of lactation, nutrition, disease, season and environmental stresses such as heat stress (Pilarczyk et al., 2012, Turk et al., 2015, Çolakoğlu et al., 2017). Additionally, obesity is related to oxidative stress, increased oxidant and decreased antioxidant activity in humans (Roh et al., 2017). Visceral fat in human is a source of several pro-inflamatory cytokines. The enhanced expression of these cytokines in obese patients can induce a proinflammatory environment and facilitate oxidative damage (Furukawa et al., 2004). Similar relationships have been observed in animals such as dog and cows (Bernabucci et al., 2005, Pasquini et al., 2013). A correlation between high body mass index, body weight loss and oxidant stress is also known in humans (Vincent and Taylor, 2006), cows (Bernabucci et al., 2005, Laubenthal et al., 2017) and dogs (Pasqui-

ni et al., 2013). BCS is associated with lipid mobilization and the imbalance of oxidative status in transition cows (Bernabucci et al., 2005). Increasing BCS was associated with enhanced oxidative stress (Laubenthal et al., 2017). However, previous results on the relation between BCS and oxidant markers are contradictory. Gheise et al. (2017) reported that the concentration of plasma MDA was affected by pre-calving BCS in dairy cows. Bernabucci et al. (2005) reported that cows with high BCS (>3.0) before calving and with more BCS losses had higher reactive oxygen metabolites (ROM) in the circulation. In contrast, Alharthi et al. (2018) used cows with higher BCS than our study and indicated that BCS prior to parturition had no effect on the concentration of ROM. O'Boyle et al. (2006) have not reported any significant differences in total lipid hydroperoxide levels between high BCS (≥3.5) and normal BCS (2.5-2.7) in mild lactation cows. In the present study, we evaluated adequate and low-BCS cows and blood MDA levels were significantly higher in low-BCS cows compared to adequate-BCS cows. These discrepancies may be related to oxidative stress markers, lactation time and different BCS points used in groups of the studies. In addition, the results of the study presented here are in agreement with previous studies (Bernabucci et al., 2005, Çolakoğlu et al., 2017, Alharthi et al., 2018) showing that the MDA levels were higher at calving and early lactation periods compared to prepartum period. During the calving time and early lactation, increased energy and oxygen requirements may lead to increased ROS production. In addition to this metabolic and oxidative imbalance, the low BCS further adversely affect cow's general health.

ROS are neutralised by the antioxidant system such as GSH-Px, SOD (Festila et al., 2012). In a previous study (Alharthi et al., 2018), cows with low-BCS (≤3.25) had higher expression levels of enzyme SOD1 than cows with high BCS (≥3.75). Bernabucci et al. (2005) reported that cows with high BCS (>3.0) before calving and those with more BCS losses had lower SOD activity in circulation. Gheise et al. (2017) reported that plasma SOD and GSH-Px activities were affected by pre-calving BCS and that high BCS cows had lower plasma SOD and GSH-Px activities than

cows with medium BCS. GSH-Px enzyme is an indicator of oxidative stress (Festila et al., 2012). O'Boyle et al. (2006) have also found that cows with high BCS (≥3.5) had lower total antioxidant potential than cows with normal BCS (2.5-2.7). However, authors have found any significant differences in GSH-Px activities between cows with normal BCS and high BCS in mild lactation (O'Boyle et al., 2006). The present study results showed low GSH-Px activity in cows with low-BCS during the transition period. Additionally, higher GSH-Px activity, in both groups, was found at prepartum period than after calving, similar to reports by Colakoglu et al. (2017) and Festila et al. (2012). As in MDA concentrations, the discrepancies in relative GSH-Px activity may be related to sampling time and BCS point used in groups of the studies. Our findings may suggest that low-BCS cows are more exposed to oxidative stress during the transition period, which manifested as higher MDA and lower GSH-Px activity.

Serum BHB and NEFA during the transition period are energy metabolites and changes of NEFA and BHB concentrations can be used as an indicators of energy balance (Chapel et al., 2017). BHB concentrations are influenced by BCS. Some authors reported that cows with high pre-calving BCS had higher plasma BHB concentrations that could be associated with the higher fat mobilizations (Gheise et al., 2017). Walsh et al. (2007) also demonstrated that cows with high BCS had higher levels of BHB at 15 days pp. Bernabucci et al. (2005) found that cows with high BCS have higher BHB levels but they did not find any significant differences in BHB levels between cows with low and medium BCS. However, other studies (Folnozic et al., 2015) have described that BHB concentrations were numerically higher in cows with adipose BCS than in cows with optimal BCS, but the differences were not significant. In the present study, we evaluated low and adequate-BCS cows and found that low-BCS cows had higher BHB concentrations at pp 14 and 21 days. Findings obtained from this study may also suggest that not only high BCS but also low-BCS can cause high BHB levels. Moreover, it was suggested that BCS was associated with postpartum BHB levels.

Glucose, as a primary metabolic source of energy, is essential for the fetal growth and milk production. Folnozic et al. (2015) observed that adipose cows had higher glucose concentration than cows with optimal body condition on the day of calving. However, other authors (Bernabucci et al., 2005) have not found any significant differences in glucose concentrations among cows with low (<2.5), medium (2.6-3.0) and high BCS (>3.0). Delfino et al. (2018) have not also found any significant differences in glucose concentrations between high (>3.5) and low BCS (≤3.5). Gheise et al. (2017) have not found any significant differences in glucose concentrations between medium and high BCS. In our present study, the adequate-BCS cows showed a significantly lower mean glucose concentration during the transition period, compared to low-BCS. It could be suggested that low and adequate-BCS cows have different energy requirements and energy utilization during the transition period.

Roche et al., (2007) have reported that reproductive performance was affected by BCS. In contrast, some authors reported that pre-calving BCS was not significantly associated with reproductive performance (Buckley et al., 2003). Godara et al. (2016) found that postpartum interval to estrous was significantly affected by pre-calving BCS. Chapel et al. (2017) have shown that cows with BCS>3.75 had worse reproductive performance than optimal BCS (3.5-3.75). Additionally, BHB concentrations are related to decreased reproductive performance (Walsh et al., 2007). Ovarian activity and the immune system are also adversely affected by the lower concentrations of glucose and higher concentrations of BHB (Lucy et al., 2014). Leroy et al. (2006) further suggested direct toxic effects of BHB on ova maturation. The elevation of free radicals as well as BCS and BHB levels have a negative effect on all body systems including the reproductive performance (Megahed et al., 2008). Higher oxidant levels in serum and follicular fluid was associated with infertility in women (Ozkaya and Nazıroglu, 2010). In addition, it has been reported that oxidative stress is negatively correlated with ovarian oestradiol-17β concentrations (Appasamy et al., 2008) and this situation may result in ovarian disfunction. The results of the present study were in accordance with these studies. We found that the calving-to-estrus interval, the calving-to-conception interval and the insemination index (rate) were higher in low-BCS cows that exhibited high levels of MDA and BHB, low levels of GSH-Px and glucose.

CONCLUSION

It is known that either high or low-BCS affects cow health, oxidative balance, and fertility. Our findings suggest that low-BCS cows had worse oxidative status, metabolic profiles and lower reproductive performance than adequate-BCS cows. Accordingly, different feeding strategies such as antioxidant sup-

plements should be utilized for low-BCS cows to improve their metabolic and oxidative status.

CONFLICTING OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Effect of ghrelin peripheral administration on growth performance, carcass quality, and selected serum parameters in broiler chickens

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ABSTRACT. The aim of present study was to investigate the short-term and long-term effect of the peripheral administration of ghrelin on the growth performance (feed intake, weight gain, and feed conversion ratio), carcass quality, and selected serum biochemical (glucose, total cholesterol, triglyceride, and total protein) and hormonal (T_3, T_4, and corticostrone) indices in broiler chickens. 240 one-day-old broiler chickens were selected, and allocated into three treatment groups (control and two experimental groups). On day-21 of the rearing period, ghrelin was peripherally administrated to three experimental groups. The control group contained birds without any administration of peptide or solution, groups G50 and G100; included birds with Ip-injection of 50 and 100 (ng/100g BW) ghrelin peptide, respectively. The peripheral administration of exogenous ghrelin did not affect feed intake, body weight gain (BWG), feed conversion ratio (FCR) and carcass characteristics in broiler chickens. In short-term samples taken 12h after ghrelin infusion, the glucose level was increased in ghrelin-treated groups (162 and 151 mg/dl in G50 and G100 compared with 117 mg/dl in control; P< 0.01) and there were significant declines for TC, triglyceride, and TP in the ghrelin-treated groups (G50 and G100) compared with the control. In addition, long-term glucose level has a greater value in G50 and G100 (182 and 200.66 mg/dl) compared with control (133.60 mg/dl) group (P< 0.01). A significant decline was also observed for TC and triglyceride content in the ghrelin-treated groups (P<0.05). There was no significant difference among groups for TP in short-term and long-term samples. There was a significant increase for T4 in ghrelin-treated groups (G50 and G100) compared with the control (4.55 and 4.57 ng/ml vs 4.20 ng/ml respectively; P< 0.05) in long-term samples. In conclusion, the peripheral administration of ghrelin in broiler chickens, during the commercial rearing period did not affect the overall growth performance, carcass quality and feed conversion ratio. The infusion of exogenous ghrelin may increase the levels of serum glucose, decrease total cholesterol and triglyceride, and T_4 levels are increased in the long-term (and not in the short-term or 12h after administration).

Keywords: Commercial growth performance, ghrelin, metabolism, regulatory peptide, broiler chicken.

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INTRODUCTION

Ghrelin is one of most discussed regulatory peptide in the recent two decades (Kojima et al., 1999; Benso et al., 2013; Pradhan et al., 2013). Due to its multifaceted properties, ghrelin is the one of multifunctional peptides with strong effects on endocrine axes (Kluge et al., 2010; Spencer et al., 2012), oxidative system, appetite (Inui et al., 2004; Klok et al., 2007), and growth performance (Ukkola and Pöykkö., 2002; Dimaraki and Jaffe, 2006), although not limited to these functions. Ghrelin has general (same) and specific functions in various species of animals (mammalian and non-mammalian), especially in birds (Kaiya et al., 2009, 2013; Tachibana and Tsutsui., 2016). In addition, the peptide structure of ghrelin can be different in animal species (Kojima et al., 2008).

The identification of chicken ghrelin by Kaiya et al. (2002) resulted in several scientific debates, due to the different effects of ghrelin on feed intake in avian species (Saito et al., 2002; Kaiya et al., 2013; Tachibana and Tsutsui., 2016). Ghrelin gene expression, density of ghrelin immunopositive cells, and the peripheral ghrelin level in chickens vary according to age (Yu et al., 2016) and feed additives (Poorghasemi et al., 2018). Ghrelin is known to serve as biological signal of energy utilization and is involved in energy homeostasis in broiler chickens (Song et al., 2018).

Administration of ghrelin with peripheral and central methods in chicken is evident in numerous studies (Geelissen et al., 2006; Oclon and Pietras., 2011; Zendehdel and Hassanpour., 2014). it generally reduces feed intake (hypophagia) in broiler chickens, however, in none of these studies, the performance and carcass quality of chicken were investigated. In a study with *in ovo* (pre-hatching) administration of ghrelin in chicken, a significant decrease in post-hatching feed intake was observed (Lotfi et al., 2013). However, after a post-hatch treatment the results were different (Kaiya et al., (2007), since plasma ghrelin levels in laying hens were not affected by its administration. Also in other study, the plasma level of ghrelin was not correlated with the laying-performance of layer-type birds (Höhne et al., 2017).

On the other hand, the effects of peripheral ghrelin administration in poultry species is not completely similar; for example in domestic geese (*Anser anser domesticus*) results in an increase of feed intake during the growing period (Aghdam Shahryar and Lotfi., 2015), a finding that is in contrast with that of Oclon´ and Pietras, (2011) and Lotfi et al., (2013) in broiler chickens.

Interestingly, the administration of ghrelin antagonist ([D-Lys3]-GHRP-6), decreased feed intake, and increased plasma T_4 in chicken (Aghdam Shahryar and Lotfi., 2016). Taking into consideration the different effects of ghrelin in birds, it seems that several mechanisms involved in ghrelin activity, and this peptide affects endocrine-axes, such as hypothalamo-pituitary-adrenal and hypothalamus-pituitary-thyroid axis (Oclon´ and Pietras, 2011; Kluge et al., 2010).

In literature, the effects of ghrelin on hormonal parameters of poultry species are relatively similar, and there is no considerable difference among poultry species. In detail, the administration of ghrelin causes temperate insulin decreases in geese (hyperglycemic effect; Aghdam Shahryar et al., 2014), and in newly-hatched broiler chicks (Lotfi et al., 2011), and it may cause an increase of thyroid hormones levels in poultry species, especially in chickens and domesticated turkey (Aghdam Shahryar and Lotfi., 2013; 2017).

In overall, although several studies have been conducted examining the effects of ghrelin in poultry species, the effect of peripheral administration of exogenous ghrelin on broilers' growth performance parameters such as feed intake and carcass quality have not been fully assessed. It seems that acknowledge of the effect of ghrelin on broilers' performance is necessary for completing ghrelin "puzzle" in poultry. Aim of the present study was therefore to investigate the short-term and long-term effects of peripheral administration of ghrelin on growth performance (feed intake, weight gain, and feed conversion ratio), carcass quality, and selected serum biochemical (glucose, total cholesterol, triglyceride, and total protein) and hormonal (T_3, T_4, and corticostrone) indices in broiler chickens.

MATERIALS AND METHODS

The experiment was conducted at Poultry Farm of Islamic Azad University, Shabestar Branch (North West of Iran) in summer 2016.

Grouping, Feeding and Housing

In the present study, 240 one-day-old male broiler chicks (Ross 308) were selected, and assigned into 3 treatment groups (one control and two experimental

groups). The treatment included 5 replicates and there were 16 chickens per replicate. A completely randomized design (CRD) was used in this experiment. Diets included three formulas (Table 1), in accordance to NRC (1994). The diets and fresh water offered *ad libitum* to birds. The ambient temperature was gradually decreased from 32 °C on day 1 to 24 °C on day 20 and was then kept constant. The lighting program was provided as 23h L:1 h D throughout the study.

Table 1. Ingredients and nutrient specifications of experimental diets

Item	Starter	Grower	Finisher
Ingredient, %	1-10 d	11-24 d	25-42 d
Corn (cp =8.5%)	53.82	54.50	56.08
Soybean meal (cp= 44%)	40.00	39.00	37.00
Soybean oil	1.80	2.80	3.50
DCP	1.45	1.18	1.22
Oyster meal	1.70	1.47	1.31
Vitamin - mineral premix[1]	0.50	0.50	0.50
DL-Methionine	0.32	0.21	0.14
Lysine	0.16	0.09	-
Salt	0.25	0.25	0.25
Compositions (calculated)			
ME *(kcal/kg)*	2854	2950	3000
Crude Protein%	21.36	21	21
Ca %	0.99	0.86	0.70
P available %	0.49	0.425	0.414
Met + Cys %	1.02	0.89	0.80
Lysine %	1.34	1.19	1.13

[1]Vitamin and mineral premix provided per kilogram of diet: vitamin A, 12.000 IU; cholecalciferol 1.500 IU; vitamin E, 30 mg; vitamin K3, 5 mg; vitamin B1, 3 mg; vitamin B2, 6 mg; vitamin B6, 5 mg; vitamin B12, 30 μg; Ca-D- panthotenate, 10 mg; folic acid, 0.75 mg; D-biotin, 0.08 mg; Mn, 80 mg; Zn, 60 mg; Fe, 40 mg; Cu, 5 mg; Se, 0.15 mg; Co, 0.1 mg; I, 0.4 mg

On day-21 of the experimental rearing period, one group was considered as the control while the two other groups served as the experimental groups and they were subjected to ghrelin Ip-Injection (Sigma Aldrich, USA).

The experimental groups were characterized as follows:

- Group 1 (control): Birds without any administration of peptide or solution.

- Group 2 (G50): Ip-injection of 50 ng/100g body weight ghrelin peptide on day-21.

- Group 3 (G100): Ip-injection of 100 ng/100g

body weight ghrelin peptide on day-21.

Injection procedure

Lyophilized ghrelin powder (rat) was purchased from Sigma Aldrich Company (G8903, USA). Similar peptide with chicken ghrelin was used in the present experiment, in accordance with Saito et al. (2002b). The powder was solved in distilled water according to the manufacturer's instructions. The infused solution (0.5 ml) was administered to each chicken. Injections were performed using 30g needle on day 21. The procedure was carried out in a sterile environment and in a special injection chamber, according to animal ethics (Reg. no.7884).

At the end of day 21 (12 h after injection), blood samples (9 samples/group) were collected from all groups for the examination and assessment of the initial effect of the injected solution. The rearing period continued up to day 42 under commercial rearing conditions, which were similar to those in the commercial broiler farms.

Data collection and analysis

Feed intake, BWG (body weight gain) and FCR (feed conversion rate) were recorded for three weeks, namely from day 21 up to day 42. On day 42 (end of the rearing period), after measuring the weight of chickens, additional blood samples were collected (from the vein of the wing). Blood samples were analyzed to determine the serum thyroid hormones (T_3 and T_4) and the following biochemical indices: glucose, total cholesterol (TC), triglyceride, total protein (TP). Measurements were performed by using a biochemical auto-analyzer with Elisa kits of Pars Azmoon Company (Biochemical kits, Pars Azmoon Co. Tehran, Iran) for checking biochemical parameters. Also, Roche testing kits (12017709122, Roche Ltd., Basel, Switzerland) and Chicken CORT ELISA Kit (MBS701668, MyBiosource, Inc., San Diego, CA) were used for thyroid hormones, and corticosterone, respectively.

Carcass traits

On the 42 day of the study, 5 birds from each replicate (pen) were randomly selected and slaughtered by decapitation. After removal of skin and feather, carcass, breast and thigh muscles, liver, and abdominal fat were weighed individually. Yields were expressed as the percentage of live BW.

Statistical analysis

Statistical analysis of the data was conducted using the GLM procedure by SAS Statistical Analysis Software (SAS Inst. Inc., Cary, NC, 2000). Significant differences among the experimental and control groups were detected by ANOVA (analysis of variance) and Tukey test. The probability value was set at P< 0.05 for checking the statistical significant differences among groups. The applied statistical model is the following:

$$Y_{ij} = \mu + T_i + E_{ij}$$

Where,

Y_{ij}: all dependent variable

μ: overall mean

T_i: the effect of ghrelin levels (i = 1, 2, 3)

E_{ij}: the random effect of residual

RESULTS

Growth performance and carcass characteristics

The performance of broiler chickens with respect to feed intake, BWG, and FCR is presented in Table 2. The results obtained in this study indicate that the peripheral administration of exogenous ghrelin caused minor reductions (*ns*) in feed intake and in BWG (*ns*) during the rearing period. Hence, there was no significant change in FCR, due to ghrelin administration.

In other words, the administration of two different doses of ghrelin did not cause any effect on the performance of broiler chickens, as illustrated in Table 2.

In Table 3, no significant difference between the control and ghrelin-treated groups for live weight, carcass yield, and relative weight of breast muscle, abdominal fat, and liver (*ns*) was also observed and there was only a significant decrease in thigh percentage of carcass in the ghrelin-treated groups (P<0.01).

Blood biochemical parameters

In Table 4 (short-term effect), the glucose level was increased in the ghrelin-treated groups (G50 and G100) compared with the control (162 and 151 mg/dl vs 117 mg/dl, respectively; P< 0.01) and a significant decrease for TC, and triglyceride in the ghrelin-treated groups (G50 and G100) compared with the control. Triglyceride levels were lower in G100 group. In long-term effect (table 5), the glucose level was at a greater level in G50 and G100 as compared with the control (182 and 200.66 mg/dl vs 133.60 mg/dl, respectively; P< 0.01), in accordance with the findings of short-term effects. A significant decline was observed for TC and triglyceride in the ghrelin- treated groups (P<0.05). There was no significant change among groups for TP.

Blood hormonal parameters

In Table 6 (short-term effect), there was no significant differences among groups. A trend of lower T3 and corticosterone and higher T_4 levels existed in ghrelin administered groups. In Table 7, (long-term effect), there was a significant increase for T_4 in the ghrelin-treated groups (G50 and G100), compared with the control (4.57 ng/ml vs 4.20 ng/ml; P< 0.05). There was no significant difference among groups for T_3 and corticostrone.

Table 2. Performance of male broiler chick subjected to IP-injection of ghrelin

Treatment	Injection dosage (ng/100g BW)	Growing period d 1-21			Finishing period d 21-42		
		Feed intake (g)	BWG[1] (g)	FCR[2]	Feed intake (g)	BWG (g)	FCR
Intact (control)	0	890.25	552.50	1.61	1471.10	775.30	1.82
G50	50	845.50	544.20	1.55	1389.40	768.10	1.81
G100	100	840.20	553.40	1.52	1361.30	770.30	1.76
P-value		0.4129	0.3351	0.0857	0.2341	0.1412	0.0905
SEM		21.29	15.14	0.04	20.16	12.20	0.03

[1] BWG: body weight gain.
[2] FCR: feed conversion ratio.

Table 3. Carcass traits [1] of male broiler chick subjected to IP-injection of ghrelin (% of live weight)

Treatments	Injection dosage (ng/100g BW)	Live weight (g)	Carcass yield (%)	Breast muscle (%)	Thigh (%)	Abdominal fat (%)	Liver (%)
Intact (control)	0	1903	69.29	25.10	25.30[a]	1.88	2.99
G50	50	1855	67.04	23.90	23.98[c]	2.01	2.65
G100	100	1955	69.83	24.27	24.67[b]	1.95	2.65
P-value		0.2156	0.4017	0.0942	0.004	0.5283	0.0578
SEM		35.22	1.43	0.323	0.11	0.07	0.09

[a-c] Values in the same row not sharing a common superscript differ significantly (P< 0.05).
[1] Carcass traits are presented with percent of carcass weight.
Sample number per organ = 5

Table 4. Serum biochemical indices in male broiler chick subjected to IP-injection of ghrelin in day 21(12 h after ghrelin administration)

Treatment	Injection dosage (ng/100g BW)	Glucose (mg/dL)	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Total protein (g/dL)
(control)	0	117.00[b]	191.00[a]	78.30[a]	5.03[a]
G50	50	162.00[a]	156.33[b]	85.33[a]	4.30[b]
G100	100	151.00[a]	157.31[b]	61.00[b]	4.40[b]
P-value		0.005	0.0014	0.0022	0.0106
SEM		6.17	4.03	2.80	0.12

[a-b] Values in the same row not sharing a common superscript differ significantly (P< 0.05).

Table 5. Serum biochemical indices in male broiler chick subjected to IP-injection of ghrelin in day 42

Treatment	Injection dosage (ng/100g BW)	Glucose (mg/dL)	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Total protein (g/dL)
(control)	0	133.60[c]	192.31[a]	79.62[a]	5.10
G50	50	182.00[b]	137.65[c]	69.31[b]	5.00
G100	100	200.66[a]	168.33[b]	69.66[b]	4.80
P-value		0.001	0.02	0.0313	0.0893
SEM		3.73	4.06	2.08	0.14

[a-c] Values in the same row not sharing a common superscript differ significantly (P< 0.05).

Table 6. Some of serum hormones indices in male broiler chick subjected to IP-injection of ghrelin in day 21(12 h after ghrelin administration)

Treatment	Injection dosage (ng/100g BW)	T3 (ng/mL)	T4 (ng/mL)	Corticosterone (ng/mL)
(control)	0	2.16	4.10	4.83
G50	50	1.73	4.63	3.96
G100	100	1.80	4.53	4.43
P-value		0.0544	0.0673	0.076
SEM		0.16	0.21	0.32

Table 7. Some of serum hormones indices in male broiler chick subjected to IP-injection of ghrelin in day 42.

Treatment	Injection dosage (ng/100g BW)	T3 (ng/mL)	T4 (ng/mL)	Corticosterone (ng/mL)
(control)	0	1.70	4.20[b]	5.93
G50	50	1.53	4.55[a]	4.80
G100	100	1.66	4.57[a]	4.83
P-value		0.1517	0.0250	0.081
SEM		0.05	0.19	0.43

[a-c] Values in the same row not sharing a common superscript differ significantly (P< 0.05).

DISCUSSION

As already pointed out, ghrelin is mainly considered as a "hunger signal" in chicken, similar to its function in mammalian systems (Kaiya et al., 2007, 2013). This function is referred to the endogenous ghrelin (Kaiya et al., 2002, 2007) and is possibly not connected with the peripherally administered exogenous ghrelin that was applied in the present study. The administration of exogenous ghrelin has different effects on appetite or feed intake in chickens (Stimulation/No effect/Inhibition) as reviewed by Kaiya et al., (2013). Tachibana et al., (2011) reported that feed intake is not affected by the administration of ghrelin in neonatal broiler chicks, whereas Buyse et al. (2009) found that peripherally injected ghrelin inhibited feed intake. In the present study (table 2), feed intake was not affected by ghrelin-administration, and there was no significant effect on the growth performance or feed conversion ratio. Therefore, the present findings are in accordance with the results of Tachibana et al. (2011), who stated that the administration of ghrelin did not affect feed intake. In a recent study conducted by Höhne et al. (2017) on laying hens and also in a study of our research team in geese (Aghdam Shahryar and Lotfi, 2015), peripheral ghrelin administration did not affect energy demands or feed intake at whole growing period or at least finishing rearing-period. It appears that in agreement with Kaiya et al., (2013), only central-/ or endogenous- ghrelin may have a significant effect on appetite (feed intake) regulation in birds, and not exogenous or peripherally-administered ghrelin, especially in finishing rearing-period of chickens. Conversely, exogenous -(or administered) ghrelin may play a potential role in appetite regulation, only in the growing period (before d 21). In the studies of Buyse et al. (2009) and Lotfi et al. (2013) in neonatal chickens, the infusion of exogenous ghrelin significantly affected subsequent feed intake and metabolism.

These findings indicate that the endogenous ghrelin system may be completely developed in a later period and is not affected by peripherally-administered excessive ghrelin. This hypothesis is in agreement with the findings of Höhne et al. (2017), who found that plasma ghrelin has no significant effects on energy status and performance of layer-type chickens.

As indicated, there were significant short-term increases for glucose, and decreases in TC, triglyceride, and TP levels in groups subjected to ghrelin-administration (table 4). On the other hand, TP level did not differ among experimental groups on day 42 (ns; table 5).

The infusion of ghrelin that is one of the key regulatory peptides in glucose metabolism (Vestergaard., et al., 2008; Delhanty and Van der lely., 2011; Dezaki., 2013) may cause notable elevation in serum glucose levels.

Lotfi et al. (2013) showed a hyperglycemic effect of *in ovo*-administered ghrelin in newly-hatched chicks. It seems that the up-regulation of plasma glucose level due to ghrelin infusion is a physiological pathway to obtain energy for hemostasis in birds (Geelissen et al., 2006; Lotfi et al., 2013; Aghdam Shahryar et al., 2014). Therefore, the findings of the present study for glucose level in both short- and long-term samples is in agreement with the regulatory effect of ghrelin on glucose metabolism. On the other hand, reduction in the levels of lipidemic indices (TC and triglyceride) in the present study (table 4 and 5) revealed the lipolytic effect of ghrelin, which was reported in newly-hatched chicks by Buyse (2009). In this regard, molecular studies showed that the infusion of exogenous ghrelin may stimulate the mRNA expression of lipolytic genes in birds (Furuse et al., 2001; Geelissen et al., 2006; Buyse et al., 2009; Ocelon and Pietras., 2011; Kaiya et al., 2013). Ghrelin may trigger lipolysis to obtain energy and glucose for homeostasis in broiler chickens, as suggested by Briggs and Andrews (2011).

In accordance with the findings of the present study, T4 as a main thyroid hormone and as a hormone which plays a key role in metabolism (Kim, 2010) increased significantly in the groups treated with ghrelin as a long-term result (table 7), whereas the thyroid hormones did not differ in the short-term samples (table 6). It has been reported that the administration of ghrelin might cause an increase in fT_4 and there was a direct effect of ghrelin on the activity of the thyroid gland (Benso et al. 2013). Our previous study on newly-hatched chickens showed that an *in ovo* administration of ghrelin caused an increase in T_4 (Aghdam Shahryar and Lotfi., 2013) and in another study on domesticated turkey (Aghdam Shahryar and Lotfi., 2017), the peripheral-administration of ghrelin caused a significant increase in the T4 long-term levels. Also, similar results were reported in ruminants by Khazali (2005). A possible mechanism for this stimulatory effect on the level of T_4 could be correlated to the direct effect of ghrelin on the hypothalamus-pituitary-thyroid (HPT) axis (Kluge et al., 2010).

CONCLUSION

In conclusion, the peripheral administration of ghrelin in broiler chicken, during the commercial rearing period did not affect the overall growth performance, carcass quality and feed conversion ratio. However, it may increase the levels of serum glucose, and decrease the total cholesterol and triglyceride levels. In serum hormones, T_4 increases in the long-term (and not in the short-term). It seems that ghrelin affected and stimulated the hypothalamic - pituitary - thyroid axis and subsequently increased the levels of T_4. This mechanism indicated that exogenous ghrelin might increase metabolic rate and regulate lipogenesis in birds, whereas it is insufficient to affect feed intake or the total growth performance. These findings indicate that the endogenous ghrelin system may have been developed in an earlier period and it is not affected by the peripheral-administration of excessive ghrelin. Also, the possible effect of ghrelin on appetite/ feed intake in chicken, may only be considerable in the earlier period (growing period: d 0-21) as reported in the related literature. In a comparative term- it can be suggested that ghrelin has some similar and some various physiological effects in difference species of poultry, also been mentioned in the reviews by Honda et al., (2017) and Kaiya et al., (2013). Also, with attention to the temperate or weak metabolic effect of peripherally-administered exogenous ghrelin in mature chickens (finishing period), the administration of exogenous ghrelin cannot be useful during commercial rearing, in terms of performance. Further studies are warranted for the full elucidation of ghrelin activities.

CONFLICT OF INTEREST
None declared.

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Effects of standard diets from different sources on growth and some organ parameters of rats

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ABSTRACT. This study aims to determine the effects of open and closed formulated standard diets supplied from different sources on growth performance and internal organ development of laboratory rats. Five-week-old 32 Wistar rats were used. A special control group diet was produced in accordance with the criteria determined by the National Research Council (NRC) (1995). Three different most preferred commercial open and closed-formula diets produced by international and local companies were used as trial groups' diets. The experiment was carried out for 12 weeks. Weekly feed consumption, body weight change, internal organ weight, intestinal organ weigths and lengths, intestinal villi heigth and crypt depth were measured in groups. The body weight values of the control group and the first group fed with open-formula diet were found at the highest level (P <0.05). The control group diet had a positive effect on small intestine villi heigth and crypt depth (P <0.05). The nutrient contents and energy values of the diets of experimental groups were determined as different from the commercial firm notifications. As a result of the research, it is concluded that the diets prepared with open-formula give more reliable results in the growth performance and development of internal organs of Wistar rats.

Keywords: Diet, growth performance, internal organ, open formula, wistar.

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INTRODUCTION

The number of studies using live animal is increasing day by day in order to get the most realistic results in many areas, especially in health and science (Genc, 2017). Alive animal model is the best model that can best reveal the cell, tissue, organ and system integrity. However, the fact that alive animal model is accepted as an appropriate model depends on the health status of the animals. The selection of healthy animals and the monitoring of their health status during the research are also dependent on many external factors and one of them is nutrition (Faith and Hessler, 2006). Inappropriate changes in feeding dynamics of laboratory animals cause physiological and behaviorally rapid and evident reactions, and may also prevent the healthy conduct of research. Thus, the result of the experiments may also be affected by this situation. That is why, alive animals used in research are subject to internationally accepted feeding procedures and fed (Barnard et al., 2009) with the well known and appropriate diets.

Commercial diets are collected under three main headings based on the principle of nutrition of laboratory animals, production methodology and reporting of nutrient content: open, closed and fixed-formulas. All nutrients and energy values contained in diets with open-formula are presented in numerical form, while other diets do not provide this information (Barnard et al., 2009). Open-formula diet manufacturers can prepare dietary formula to meet different requirements of the researchers' needs. On the other hand, because their content are not indicated barely, other commercial formulations, impose a limitation on the use of diets of these formulations in order not to adversely affect animal health and research outcome. Due to the heat sterilization process that is still widespread in the production of laboratory animal diet, protein levels are kept high during production because of high levels of nutrient loss (Barszcz et al., 2014; Bielohuby et al., 2010; Schaafsma, 2005). Ten to fifteen percent of the energy taken from a normal diet comes from proteins. However, in the case of high-protein diet, this rate increases to 20-30% (Plantenga et al., 2009). High protein with carbohydrates also increases the activity of glucagon-like peptide-1 (GLP-1) which provides insulin release (Lejeune et al., 2006; Plantenga et al., 2009) thus, the feeling of satiety increases. The same effect is explained (Veldhorst et al., 2008; Plantenga et el., 2009) primarily by the increase in oxygen consumption and body temperature, formation of lack of oxygen, and consequently in the feeling of saturation. As the type and amount of nutrient contents of diets to be used in the study may affect the metabolism, developmental levels, hormonal patterns and behaviors of animals; it is important that, these parameters are known by the researchers in advance and can be changed.

In some countries, pellet feed production mostly address the ruminant and poultry sector. Due to commercial and economic production practices, production of at least 1-2 tons per factory in each batch can not meet the need for different types of research and dietary requirements of 20-25 kg in laboratory animal trials (Genc, 2017). In such a case, the researchers are making improper manipulations in diets with "standard" characteristics to form different dietary groups. The variety of feed materials used in the production of laboratory animal diets may create differences in nutrient composition. Even if the nutrient values of the diets are provided at an appropriate level, the differences in macro and micro nutrient levels may affect the animal health and performance (Andreoli et al., 2016; Nagano et al., 2016; Ronis et al., 2016; Genç, 2017). The diets produced with closed-formula -those are not reporting of micro-nutrients and raw materials or not conforming to the standards- appear to be a greater risk to influence the result of the research. Yet, there are studies reporting that micro-nutrients may have an effect on the fetus during DNA methylation stage, especially with inadequate or unbalanced intake in the fetal period (Mc Kay et al., 2012; Vanhees et al., 2014).

The aim of this study was to determine the nutritional profile and nutritional effects of standard mice and rat diets produced with open and closed formula obtained from three different sources in an *in vivo* trial.

MATERIALS AND METHODS

All procedures that were used on animals in this research had been approved by Ondokuz Mayis University Animal Experimentation Ethics Committee with Ethics committee acceptance number: 2017-40.

Data on the number of animals used to form the research groups is given in Table 1. In this study, 5-week-old 32 Wistar rats consuming group feeds with their mothers since the birth to the end of weaning period (0-5 weeks) were used.

After the weaning (5th week), 4 male and 4 female rats were selected randomly from each mother rat and feeding was continued with 1 control and 3 experimental groups with 8 animals per group. Mothers and other offspring were excluded from the study after 5th

week. The study was carried out until the rats reached to the 12 weeks old by taking into consideration the age and body weight of adult rats. Animals of both sexes were used to eliminate the gender factor. After the formation of the groups, male and female animals were fed their own group diets in different cages.

Before the study, each mother was fed with group feed for 10 days in advance. Number of mother (n), litter (n) and litter size (n), female and male rat ratio (n/n) of the groups between 0-5th and 6th-12th weeks of study and G-power analysis values prior to forming groups were given in Table 1. The number of animals used was determined with the G-Power test by reference to a previously conducted similar study (Idoko et al., 2015).

Diets in all groups were preferred to identify diets produced as standard mice and rat feeds. The control group has an open-formula and was prepared by a commercial company in accordance with the characteristics reported in the NRC (1995). The control group diet ration contains soybean meal, bonkalite (wheat), corn, rice bran, calcium carbonate, molasses, soybean oil, dicalcium phosphate, salt (NaCl), vitamin-mineral premix, probiotic (*Saccharomyces cerevisiae*), and mycotoxin binder. The diet of the first experimental group was formed by a globally preferred open-formula commercial diet consist of wheat, dehulled extracted toasted soya, wheatfeed, barley, dehulled cooked soya, soya oil, calcium carbonate, dicalcium phosphate, salt (NaCl), and vitamin-mineral premix supplement. The diet of the 2nd group was formed with a closed-formula based on cereals, oilseed residues, bonkalit, calcium carbonate, saccharose, alfa alfa meal, pellet binder, choline, salt, antioxidant and vitamin-mineral premix and sold by an international company. The 3rd group's diet is a closed-formula product of a company that sells in the local market and based on soybean meal, wheat, corn and vitamin-mineral premix. The feedstuffs of the experimental groups were given as declared by the companies. The nutrient contents of all diets are given in Table 2.

The animals were born in Ondokuz Mayıs University Experimental Animal Research and Application Center and were housed in standard plexiglass cages at 20 - 23 °C heat, 50-60% humidity and 12 hours light and 12 hours dark conditions. Wood chips were used as bedding material in all groups during the study. Throughout the research, water and diet were provided as *ad-libitum*.

Nutrient analysis

The nutrient analysis of the control and experimental groups' diets used in the study was carried out at the Ondokuz Mayıs University Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases Laboratories. All groups consumed dry matter (DM), nutrient levels (crude ash (CA), crude protein (CP), crude cellulose CC)) determined according to Weende (AOAC 2012), while the ether extract (EE) level was determined by Soxhelet extraction device according to the method reported by Keskin (1975). Metabolic energy (ME) value was determined according to the formula reported by Yalçın (2011).

ME, MJ/kg DM = (0,01465 x crude protein) + (0,03558 x ether ectract) + (0,01465 x Nitrogen - free substance)

The nutrient analysis values were compared with the companies' own statements (Table 2).

Evaluation of performance and internal organ parameters

Weekly measures of individual body weights of animals were performed with A&D Weighing GF 3000 Balance (0.01g readability). The daily intakes of the group's diet were carried out by group weighing weekly. Body weight gain and feed consumption were calculated from the difference of weight value from the previous week. In the postmortem period, the weights of the internal organs (heart, liver, spleen, stomach and intestines) and the length of the digestive tract sections were evaluated with A&D Weighing GF 3000 Balance (0.01g readability) and tape measure respectively.

Histopathological examination

At the end of the study, all rats were euthanized with appropriate anesthetic (Ketamine 150 mg/kg + Xylazine 30 mg/kg body weight) and intestinal organs were taken in accordance with proper asepsis and antiseptic rules. The intestines taken under appropriate conditions were fixed for 12-24 hours in the 10% formaldehyde solution and were passed through the routine histological tissue follow-up procedures and embedded in paraffin. Section in 5µ thickness taken from organs which were dried in drying oven and Crossmon's (1937) tripple staining method was applied in order to see the normal histological structures of the intestines. Heigth of intestinal villi and depth of crypts were measured. Preparations were photographed with The Nikon digital-sight imaging system and the Nikon 50I research microscope.

Table 1. Number of mother (n), litter (n) and litter size (n), female and male rat ratio (n/n) of the groups between 0-5th and 6th-12th weeks of study and g-power analysis values prior to forming groups.

	0-5 weeks			6-12 weeks
Mother (Group)	**Litter**	**Litter size**	**Female/Male**	**Female/Male**
Control	1	9	5/4	4/4
1	1	11	6/5	4/4
2	1	12	4/8	4/4
3	1	9	4/5	4/4

g-power: $\alpha=0.05$; $1-\beta=\%80$; $n^0=8$ (Estimation of approximately unit number in study).

Table 2. Dry matter (DM) (%), crude ash (CA) (%), crude protein (CP) (%), ether extract (EE) (%), crude cellulose (CC) (%), and metabolic energy (kcal/kg ME) values of diets.

Diet	DM		CA		CP		EE		CC		ME	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Control	92.0	92.0	8.0	8.0	24.0	24.0	3.0	3.0	4.0	4.0	2920	2920
1	92.0	93.0	7.0	6.9	20.0	19.1	2.0	4.7	4.0	3.8	2906	2921
2	92.0	92.5	6.0	6.1	20.0	19.2	4.0	4.1	7.0	6.1	2937	3226
3	92.0	93.0	7.0	7.2	19.0	24.0	2.0	4.7	6.0	13.0	2830	2600

A: Nutrient values reported by the diet companies. B: Nutrient values found in the feed analysis.

Statistical Analysis

In this study, the conformity of the data to normal distribution was determined by Kolmogorov Smirnov test. After the weaning of the control and experimental groups, the body weight values from 6 to 12 weeks old age, feed consumption, weight and length of some organs and the histological values of organs were analyzed by using variance analysis. TUKEY HSD test was used for the importance control of the difference between the groups.

RESULTS

Nutrient analysis and Metabolic Energy values

The nutrient contents of the diets and the results of the analysis of energy values (A) are given in Table 2 and compared with the declaration of the companies (B). The percentage values of nutrients and ME values reported by the companies in the diets of the research groups were found different from the results of the analyzes. The difference in CP value was acceptable in the 1st and 2nd trial groups, but was high in the 3rd trial group. Ether extract percentages were found higher than the values reported in all experimental groups. In the 1st and 2nd trial groups, it was found that the values of CC were close to the reported values but in the 3rd trial group, the values reported were more than doubled. Metabolic energy values were found close to those reported in the first group diet with an open formula, higher than those reported in the second group diet and much lower than those reported in the third group.

Performance Parameters

The weekly amount of feed consumption after weaning (6th-12th weeks) is given in Table 3. The highest feed consumption was observed in the animals fed with the 3rd group diet and the lowest feed consumption was observed in the animals fed with the 2nd group diet.

Table 3. Weekly feed consumption (FC) (g) values of groups (n = 8) from 6th to 12th week.

	Groups			
Week	**Control**	**Group 1**	**Group 2**	**Group 3**
6	1195	1115	980	865
7	1256	1195	1007	901
8	1300	1213	1098	1265
9	1348	1299	1176	1365
10	1400	1365	1256	1456
11	1489	1402	1398	1501
12	1545	1490	1450	1605

Table 4. The weekly body weight (BW) values of the control (n=8) and experimental groups (n=8) from 6th to 12th week (Mean ± SE).

Week	Control	Group 1	Group 2	Group 3	P value
		Groups			
6	120.87±3.22	95.12±9.61	97.87±2.83	92.25±4.19	0.005
7	150.87±8.37	114.12±11.07	120.12±3.07	107.75±4.67	0.002
8	179.12±12.39	133.50±16.06	133.00±3.04	117.37±5.80	0.002
9	195.87±14.94	159.62±16.00	136.25±3.73	129.25±8.73	0.002
10	207.00±16.13	180.50±20.94	142.62±5.29	145.75±9.50	0.10
11	217.37±17.89	204.75±23.69	150.00±9.77	165.12±10.49	0.022
12	230.12±18.88	228.75±26.49	168.75±9.16	183.00±14.43	0.50

Table 5. Internal organ weight (g) and length (cm) values (Mean ± SE) in groups (n=8)

Internal organs	Control	Group 1	Group 2	Group 3	P value
		Groups' Weight (g)			
Heart	0.78±0.05	0.84±0.04	0.66±0.03	0.69±0.02	0.016
Liver	10.38±1.01	9.97±0.82	7.74±0.46	7.50±0.44	0.014
Lien	0.58±0.03	0.59±0.04	0.43±0.02	0.44±0.02	0.002
Stomach	1.30±0.04	1.47±0.08	1.21±0.05	1.41±0.05	0.026
Small intestine	8.72±0.33	7.63±0.13	7.56±0.14	7.06±0.32	0.001
Large intestine	3.03±0.14	2.66±0.12	2.92±0.10	2.96±0.13	0.230
		Length (cm)			
Small intestine	105.93±1.83	107.00±2.91	100.27±1.97	100.75±1.99	0.085
Large intestine	20.31±0.74	20.62±0.95	20.62±0.99	21.37±0.30	0.812

Table 6. Small intestine crypts (SC) depth (μm), small intestine villi (SV) heigth (μm) and large intestine crypt (LC) depth (μm) values (Mean ± SE) of the groups (n = 8).

Crypt & villus	Control	Group 1	Group 2	Group 3	P value
		Groups			
SC	84.11±2.79	69.70±4.46	78.66±1.56	81.85±4.13	0.032
SV	634.29±48.80	434.08±31.73	350.95±26.60	478.36±12.81	0.000
LC	154.42±12.32	138.85±5.95	164.31±13.74	170.55±6.39	0.164

The weekly body weight changes of the groups after the weaning are given in Table 4. At the end of the experiment, it was observed that the body weight values were significant (P<0,05) at the 6th, 7th, 8th, 9th and 11th weeks, and the difference between the control group and the 2nd group at the 12th week was statistically significant (P<0,05). In groups, hierarchy fights and related stress symptoms were observed at the 8th and the 10th weeks. However, no serious injuries and deaths were observed in the groups.

Internal organ weight and length values are given in Table 5. Heart, liver, spleen, stomach and small intestine weights were significantly different between groups (P<0.05), while large intestine weight and length of both intestines did not make a significant difference between groups (P>0.05).

Histopathological Findings

At the end of the experiment, the small intestine crypt (SC), large intestine crypt (LC) depths and small intestine villi (SV) heigh of the groups were measured and given in Table 6. Images of intestinal villi and crypts of control and experimental groups given in Figure 1. Small intestine villi heigh and crypt depth were found significant (P<0,05) and among the groups, the highest villus heigh and crypt depth were found in the control group. Crypts lenghts of the large intestine did not make a significant difference between the groups (P>0.05). No pathological finding was observed in the internal organs and intestines in any group.
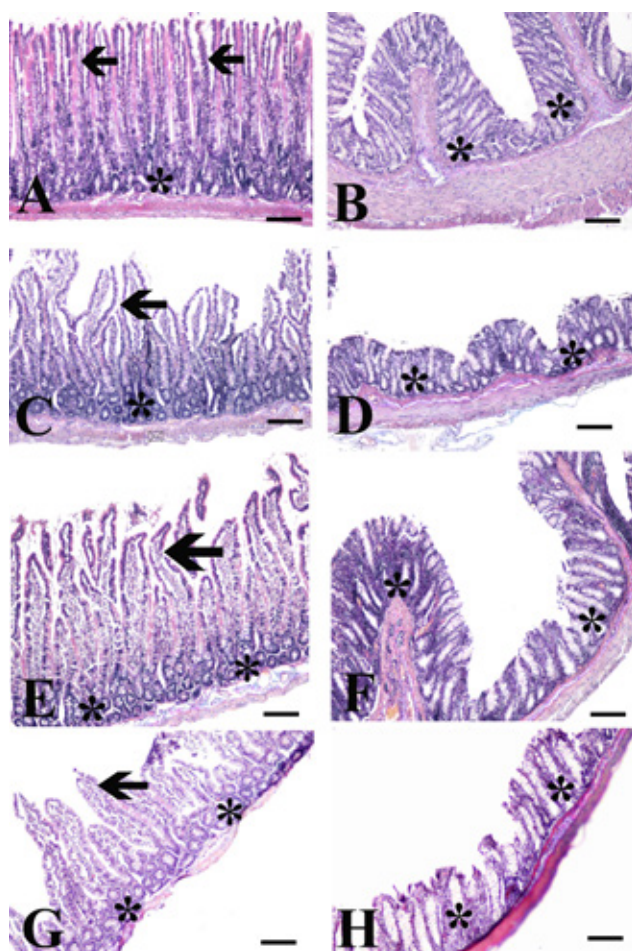
Figure 1. Images of intestinal villi and crypts of control and experimental groups.
A: Control group small intestine; B: Control group large intestine; C: 1. group small intestine; D: 1. group large intestine; E: 2. group small intestine; F: 2. group large intestine; G: 3. group small intestine; H: 3. group large intestine, Crossmon's Trichrome Stain. *: crypt, arrow: Villus intestinalis, Bar: 100μm.

DISCUSSION

The standard mouse-rat diets, obtained from three commercial firms, have different levels of nutrient content (Table 2). The use of different raw materials in the diets as a source of energy and protein naturally give rise to such a result. In addition, the given percentages of nutrient contents and metabolic energy values of the research diets by the companies were found different from our research analyzes. This finding is in agreement with the results obtained from Barnard et al. (2009)'s research, in which different standard diets were formed using different raw materials. If the rations where natural raw materials are not purified, the plants in the same harvest period have significant potential (P<0.05) in nutrient values in different batches and have the potential to affect the physiological parameters of animals (Thigpen et al.,

2004; Thigpen et al., 2007). Although the crude protein content in research diets is within the legal limits according to NRC (1995), the fact that the diets characteristics stability should be considered in the studies to ensure the repeatability of the study.

During the period between 6th and 12th weeks when individual weighing was made, feed loss was higher due to the natural feeding behavior observed in rodents. Due to the continuous extension of the incissive teeth of rodents, their diets are made of hard feeds in the form of pellets and are important in terms of dental health. However, in the conventional feeding method, feed residues are produced which are not consumed during the gnawing of pellet feed and these residues are taken together with the litter and feed consumption among the groups does not show homogeneity. For these reasons, feed consumption value is not a sufficient parameter in the evaluation of nutritional performance of rodent laboratory animals. Thus, feed consumption rate values were not evaluated in this study.

The body weight gain was at the highest level in the control group for each week, but only the 10th week was statistically insignificant (P>0.05). The difference on body weight gain between the control and the 2nd group at the 12th week was statistically significant (P<0.05). The animals in the 1st group fed with the open-formula diet showed that the body weight increase was mathematically closer to the control group than the other groups. Differences in performance values may be caused by unspecified raw material sources in the diets of the manufacturing company with closed-formula or lack of macro-micro nutrients. In a study, Pichon et al. (2008) investigated the effects of different protein sources on dietary consumption and body weight gain. It was shown that body weight gain and dietary consumption were significantly (P<0.05) lower in rats fed with a diet containing beta lactoglobulin than those fed with dietary alpha lactoalbumin. Faipoux et al. (2008) showed that protein-related saturation sensation is provided with vagal feedback. Because of the similarity (Plantenga et al., 2009) of saturation between the groups in case of dietary nutrition with a high level protein or amino acid source, the effect of other parameters in the diet may be covered. Bowen et al. (2006a; 2006b) in their research on the effects of high-carbohydrate diets showed that there was no difference in dietary consumption between the groups fed with high casein and wheat protein. Yürük (2014) pointed out that, the metabolism of active substances used in research should be well known and reported that, feed consumption of animals fed with

diets using fructose as a source of energy is similar to rats fed with other standard diets.

It is reported (Garlick et al., 1991; Tome, 2004) that, the rats can distinguish between the taste of some essential amino acids and their dietary consumption varies depending on the presence of these amino acids. The presence of such a detection mechanism also emphasizes the physiological importance of providing a sufficient amount of protein synthesis (Plantenga et al., 2009) and its effect on feed consumption.

Diets should be fed to animals after being analyzed and reported for antinutritional substance contents. Most of the diets with closed-formula have no antinutritional substance report. The phytoestrogen levels, which are one of these substances, may differ between the raw material of the plant during the same harvest period. Phytoestrogens can be effective on feed and water consumption, anxiety behaviors, stress, insulin, leptin and thyroid hormone levels. (Lephart et al., 2004; Torre et al., 2008).

The effects of protein and fat imbalance on growth performance can also be affected by gender factor. It has been reported in a study (Bellinger et al., 2005) that low protein intake causes less fat consumption in female offspring but no such effect has been observed in males. Krishnakumari et al. (1979) reported that the rats consuming the diets prepared by using different raw materials consume more delicious ingredients. It is reported (Kasaoka et al., 2004) that high levels of histidine in diets may reduce dietary consumption.

Studies on the effects of standard diets on internal organ weights, lengths and development of intestinal villi and crypt are very limited. In our study, it was observed that the liver and small intestine weights were highest in the control group (P<0.05), and the heart, spleen and stomach weights were highest in the 1st group (P<0.05). According to Bailey et al. (2004) liver weight value is the most proportional among the internal organs to the body weight value. Findings in our study are in compliance with this information that the highest liver weight is in the control group with the highest body weight value. Small intestines in digestive system are the highest (P<0.05) in control group. These results may be explained by the fact that they consume the control diet, which is prepared under control due to the right and balanced requirement of nutrients. Belobrajdic et al. (2014) reported that there was no significant effect on internal organ weights (P>0.05) in rats consuming open-formula diets containing different protein sources. The different results and notifications between experimental studies can be caused by the fact that animals are of different strains and genders. Physiological and anatomical individual differences in outbred strains such as Wistar are quite common. Therefore, it can be said that inbred strains are more suitable for performance studies.

In our study, the small intestine crypt depth and villus height were found the highest (P<0.05) in the group fed with the open-formula control group diet contains 24% CP content. This data is in agreement with the declaration of Pelizzon (2016) on the effects of open-formula diets on the morphology of the intestinal system in rats and mice. In a study (Syme, 1982) performed with rats consuming isoenergetic rations, especially after 4th week, higher protein level effected the intestinal structer, villus heigth and crypt depth improvement better. Same results reported by King et al.(1983) in a study performed by rats consumed diet contains different levels of aminopeptidase and isomaltase. The effects of dietary crude protein levels on villus heigth and crypt depth values are explained by the increase in enterocyte activity due to the density of amino acids. (Syme, 1982; King et al. 1983).

Although intestine villi heigth and crypt depth values showed statistically significant (P<0.05) differences, there was no significant difference (P>0.05) between intestinal lengths. These parameters can be evaluated with a longer research period.

CONCLUSION

As a result, it was concluded that open formula diets had a positive effect on body weight gain, small intestine villi heigth, crypt depth and internal organ weight in rats during the developmental period compared to the diets produced with closed formula. Based on the data obtained, it may be suggested that the laboratory animal diets to be used in the research should be selected from the open formula products whose nutrient content is specified. Although open formula diets are more costly, it can be said that, they are more advantageous than closed formula diets because they give more positive results on animal development. In addition, it may be recommended to conduct a longer term research using other inbred species.

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CONFLICT OF INTEREST

None declared.

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Genetic improvement of indigenous Greek sheep and goat breeds

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ABSTRACT. The objective of the study was twofold: (i) to review the genetics of production and resilience traits of indigenous Greek small ruminant breeds as well as the evolution of national breeding programs, and (ii) explore innovative and feasible approaches to overcome the challenges and constraints towards improving these breeds and enhancing the sustainability of the small ruminant sector. Previous studies on the genetic basis of production and resilience traits of indigenous breeds revealed high improvement potential. However, the lack of follow-up action has failed to produce applicable results. Thus, implementation of scientific findings in existing breeding programs for these breeds is extremely limited. The latter has contributed to the overall poor success of such programs. Furthermore, due to the fact that most farmers do not comprehend the strategic importance of genetic improvement and lack motivation for self-funding, breeding programs have mostly relied on European or government funding; the latter has resulted in intermittent implementation. Therefore, most programs failed to improve performance of indigenous Greek breeds, many of which were consequently replaced by foreign breeds of higher productivity and documented merit. In order to facilitate the design of breeding schemes and overcome the challenges towards improvement of indigenous breeds, an integrative approach is necessary. The latter should be based on identification of specific breeding objectives, reflecting the priorities and needs of the sector, as well as the capacity of the indigenous populations. Scientific advances exemplified by genomic selection and novel reproductive technologies will enable faster and more effective genetic improvement. The overall approach is expected to enhance the competitiveness of indigenous Greek small ruminant breeds and the sustainability of the sector.

Keywords: genetic improvement, sheep, goats, indigenous breeds, Greece

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INTRODUCTION

Dairy sheep and goat farming is economically important in Mediterranean countries. It also has ecological and sociological impact, as it utilizes harsh and agriculturally low output areas thereby underpinning the sustainability of rural populations (Carta et al., 2009). Dairy small ruminant farming systems are quite diverse and mostly rely on natural resources for grazing. Under these systems, local breeds are considered the most suitable, as they are well-adapted to the local environment (Barillet, 2007; Gelasakis et al., 2010; 2017).

In Greece, small ruminant production dominates the livestock sector. In 2017, the national flock comprised about 8.6 million sheep and 3.8 million goats. With this population size, the Greek national goat herd was the largest and the national sheep flock was ranked fourth among EU-28 countries (Eurostat, 2017a). Small ruminants in Greece are mainly dairy and their milk is mostly processed into cheese and other dairy products (Gelasakis et al., 2010). In 2017, milk production was almost 800,000 tons, which represented about 57% of the total milk produced in Greece (Eurostat, 2017b). Based on the latest available data, annual sheep cheese production was 125,000 tons, rendering Greece the top sheep-cheese producing country worldwide; whereas, goat cheese production (40,000 tons) was the fourth largest globally (FAO, 2014). Meat production, although considered as a secondary activity, is also highly ranked among the EU-28 countries. Specifically in 2017, small ruminant meat production was ranked fourth (71,500 tons) representing 9.3% of the total EU-28 production (Eurostat, 2017a).

Greek small ruminant flocks are usually reared under semi-intensive or semi-extensive farming systems with a trend towards intensification (Gelasakis et al., 2012a; Gelasakis et al., 2017). Adaptation to more intensive farming relies on achieving high ewe and doe productivity. Despite the fact that indigenous breeds share a diverse genetic background and have considerable improvement potential (Hatziminaoglu et al., 1990), national genetic improvement programs have failed to achieve sufficient genetic gain, resulting in unprecedented import rates of foreign improved dairy breeds. In 2016, almost 435,000 sheep (increased by 33% compared to 2014) and 3,500 goats (decreased by 50%) were imported in Greece from EU countries, ranking Greece second and fifth regarding import rates in EU, respectively (European Commission, 2014; 2016).

Replacement of indigenous Greek breeds and uncontrolled crossbreeding with imported sheep and goats has caused the loss of invaluable autochthonous genetic material. Hence, population of most indigenous breeds has decreased dramatically, while even commonly used breeds are constantly losing ground. The objective of the study was twofold: (i) to review the genetics of production and resilience traits of indigenous Greek small ruminant breeds as well as the evolution of national breeding programs, and (ii) explore innovative and feasible approaches to overcome the challenges and constraints towards improving these breeds and enhancing the sustainability of the small ruminant sector.

GENETICS OF PRODUCTION AND RESILIENCE TRAITS

Dairy-related traits

Research outcomes form the pillar of designing successful breeding programs. Indigenous Greek small ruminant breeds first gained scientific interest in the 1980s. The early studies focused on estimating heritability and genetic correlations of dairy-related traits (*i.e.* milk yield, udder traits) and lamb output of Chios sheep, using pedigree information and phenotypic data (Mavrogenis, 1982; Mavrogenis et al., 1988). Subsequently, genetic studies included more traits (*i.e.* milk content, somatic cell count, litter size, body weight) and sheep breeds (*i.e.* Boutsko, Sfakia and Lesvos). These studies showed that improvement of important traits was possible through selection (Kominakis et al., 1998; Ligda et al., 2000; Mavrogenis and Papachristoforou, 2000; Kominakis et al., 2002; Ligda et al., 2003; Nikolaou et al., 2004). Milk yield and composition were found to be antagonistically correlated indicating that selection for one trait would adversely affect the other (Volanis et al., 2002). Respective studies on indigenous Greek goats are limited. A study on Skopelos dairy goats showed that their milk yield is likely to improve through selection (Kominakis et al., 2000). All the above traits are important for dairy small ruminant production. However, inclusion of multiple traits in the same breeding scheme needs further investigation, especially when traits are genetically correlated.

Following the initial genetic studies based on the assumption of polygenic inheritance, Chatziplis et al. (2012) suggested that major genes are likely to be involved in the inheritance of milk traits of Chios sheep.

The same research team studied the acetyl-coenzyme A acyltransferase 2 (ACAA2) gene of Chios sheep and found a single nucleotide polymorphism (SNP) associated with milk yield (Orford et al., 2012). This SNP explained most of the milk yield variation in the same flocks even when analyzed along with 15 microsatellite markers; the latter were located close to Quantitative Trait Loci (QTL) that were found to affect milk yield in other sheep breeds (Chatziplis et al., 2013). Saridaki et al. (2017), performed a Genome-wide Association Study (GWAS) for total lactation milk yield on records of approximately 500 Frizarta ewes and found significant associations (only at chromosome level) with three SNPs located on chromosomes 5 and 23. Further analysis revealed a QTL related to milk yield, which was located close to the SNP found on chromosome 23. Another study on the same dataset, based on haplotype block regression analyses, identified 9 SNPs (4 of which were located in genomic areas associated with milk traits in other breeds) and 11 candidate genes that may be associated with milk yield and composition of Frizarta ewes (Saridaki et al., 2019). Interestingly, none of the SNPs reported in the first study of Saridaki et al. (2017), were verified in the second, after the alteration of the analysis protocol.

Another recent study, on the prolactin gene of Chios sheep, revealed a SNP positively associated with fat content and negatively with milk yield (Miltiadou et al., 2017). Polymorphisms in the caprine gene encoding a_{s1} casein (CSN1S1), known to be associated with goat milk traits (Moioli et al., 2007), have been studied on Skopelos dairy goats. Findings indicated that two out of the three most common genotypes were associated with higher protein and fat content (Arsenos et al., 2014; Kalamaki et al., 2014). Nevertheless, in all the above studies, sample size was quite small ranging from 230 to 318 animals; in order to use SNPs located in genes of interest as markers of selection for milk traits, the estimated effects need to be validated in bigger population samples.

Improvement of udder traits became crucial after the introduction of machine milking in dairy sheep and goat production. Machine milking efficiency depends on udder morphology, among other parameters (Marnet and McKusick, 2001). Many direct and indirect measurements have been used to describe udder morphology of small ruminants and improve their milkability (Fernandez et al., 1995; De la Fuente et al., 1996; Casu et al., 2006). However, the appropriate

udder traits for selection may differ among breeds and breed-specific studies are necessary to design genetic improvement programs. Gelasakis et al. (2012b) described the udder morphology of Chios sheep using both direct and indirect measurements, taking into account relationships with milking efficiency. Strong correlations among traits were observed indicating that udder improvement could be based on selection for only indirect linear traits, which can be assessed faster at a lower cost. A study on Frizarta sheep reported low to moderate heritability estimates (0.05 - 0.21) for indirect linear udder traits (Kominakis et al., 2013). Studies on a larger scale are necessary in order to make safe assumptions for selection of udder traits in Greek sheep.

Disease resistance

Response to infectious pathogens and susceptibility to disease are traits exhibiting considerable host genetic variation (Bishop, 2015). Studies of genetic variation underlying disease resistance in small ruminants have showed that selection for resistance to certain diseases is possible (Bishop and Morris, 2007). Respective studies on indigenous Greek sheep and goats have focused on scrapie, mastitis, footrot and nematode resistance.

Scrapie resistance

Resistance to classical scrapie has been associated with polymorphisms at codons 136 (alanine/valine), 154 (arginine/histidine) and 171 (glutamine/histidine/arginine) of the ovine *PRNP* gene in many sheep breeds (Bossers et al., 1996; Hunter et al., 1996). The aforementioned polymorphisms are most commonly combined into five haplotypes; namely ARR, ARH, ARQ, AHQ and VRQ. Combinations of the above haplotypes result in many genotypes, which are classified into five risk groups, according to the National Scrapie Plan (Warner et al., 2006). More than 25 *PRNP* genotypes have been detected in indigenous Greek sheep indicating high levels of genetic variation. Genotypes of risk groups 1 and 2, which are less susceptible to classical scrapie, have been reported in largely ranging frequencies (4.31 - 40% and 6.7 - 71.4% for risk groups 1 and 2, respectively) in both purebred and crossbred Greek sheep (Billinis et al., 2004; Ekateriniadou et al., 2007a; 2007b; Boukouvala et al., 2018). No significant associations of *PRNP* genotypes with milk production and reproduction traits have been found in Chios sheep, indicating that selection for scrapie resistance is not expected to ad-

versely affect these traits (Psifidi et al., 2011). Based on the latter findings, a selective breeding program has been implemented in Chios sheep aiming to increase the frequency of the resistant allele ARR.

In goats, more than 50 *PRNP* polymorphisms have been reported. Alleles 222K, 146S/D and 211Q in the caprine *PRNP* gene have been strongly associated with resistance to disease (Ricci et al., 2017). However, diverse frequencies of these alleles have been observed among and within breeds and countries. Thus, breed-specific studies are necessary for the development of selection schemes in each country (Ricci et al., 2017; Vouraki et al., 2018). Studies of both healthy and affected Greek goats have reported mutations that lead to amino acid substitutions in over 20 codons of the caprine *PRNP* gene, including the ones that have been associated with scrapie resistance (222K, 146S/D, 211Q) (Billinis et al., 2002; Bouzalas et al., 2010; Fragkiadaki et al., 2011; Kanata et al., 2014). However, the latter studies involved mostly small samples (n = 51 - 436 goats) of crossbred and undefined goat populations in Greece. Vouraki et al. (2018) reported frequencies of approximately 6% for the resistant allele 222K estimated over a larger population sample (n = 551 goats) of two indigenous Greek breeds (Eghoria and Skopelos). A follow-up study on the same dataset found no associations of allele 222K with dairy traits (Vouraki et al., 2019). Thus, selection for the resistant allele 222K is not expected to have adverse effects on dairy goat traits and could be the basis for the development of a breeding program to enhance scrapie resistance in the two breeds.

Mastitis resistance

Mastitis resistance in small ruminants is commonly assessed using milk somatic cell count (SCC); increased values of SCC indicate udder inflammation. SCC in milk is a heritable trait. Furthermore, recent studies have revealed SNPs associated with mastitis resistance, which render genomic selection feasible (Bishop, 2015). Heritability estimates of SCC in Chios sheep were moderate (0.17) during the first weeks of lactation (Bramis et al., 2014). Further studies on the same data set reported SNPs associated with SCC and other mastitis traits (*i.e.* incidence of clinical mastitis, Total Viable Count - TVC, California Mastitis Test) and identified 24 Quantitative Trait Loci (QTLs), 19 of which had been previously reported in other unrelated dairy sheep breeds. Based on the above, 14 candidate genes were detected as most likely related to mastitis resistance in Chios sheep (Banos

et al., 2017). Detection of the same QTLs in unrelated dairy sheep breeds indicates that implementation of mutual genomic selection programs for the enhancement of mastitis resistance might be effective across breeds. However, further studies are necessary prior to implementation of such programs. Relative studies on other indigenous Greek sheep and goat breeds are largely missing.

Footrot resistance

Polymorphisms located within Class I and II regions of the ovine major histocompatibility complex (MHC) have been associated with resistance to footrot (Escayg et al., 1997). Gelasakis et al. (2013) investigated the genetic profile of *DQA2* gene (located in Class II region of the MHC) of Chios sheep and detected 20 alleles, indicating the highly polymorphic nature of the locus. Allele 1101 in that gene was associated with susceptibility to footrot. The latter finding is in agreement with earlier studies on other sheep breeds; however, the overall allelic frequencies were highly divergent. In general, such associations are inconsistent across breeds and populations rendering genetic selection for resistance to footrot complicated. GWAS, which enable the detection of multiple markers associated with ovine footrot, are considered a more efficient approach towards comprehending the genetic architecture of the disease (Raadsma et al., 2013; Bishop, 2015).

Nematode resistance

Nematode resistance in sheep has been associated with genes located in Class I to III regions of the MHC. Such associations are usually assessed using Fecal Egg Counts (FEC) (Dukkipati et al., 2006). *DRB1* gene (located in Class II region of the MHC) was investigated in Greek sheep and 39 alleles were reported. Genotypes heterozygous for three of the latter alleles were associated with lower FEC (Spetsarias et al., 2016). Despite the variation observed in *DRB1* gene, further studies involving other genes are needed in order to incorporate the above findings into a breeding scheme. Furthermore, studies on nematode resistance based on FEC need to be interpreted with caution; since FEC are not distinguished among nematode species, inferences are not necessarily applicable for all nematode infections (Dukkipati et al., 2006).

Genetic structure and diversity

Characterization of genetic structure and diver-

sity within and between breeds is an important step towards revealing the genetic architecture of animal traits. Studies aiming to characterize indigenous Greek sheep breeds and assess genetic distances between them have been based on polymorphisms of blood proteins (Rogdakis et al., 1995; Koutsouli and Rogdakis, 2002) and different types of markers such as microsatellite loci (Bizelis et al., 2007; Koutsouli et al., 2007; Ligda et al., 2009; Mastranestasis et al., 2015; Loukovitis et al., 2016), Random Amplified Polymorphic DNA (RAPD) (Mastranestasis et al., 2011) and SNPs (Pariset et al., 2011; Kominakis et al., 2017a; Michailidou et al., 2018). Respective studies on indigenous Greek goats are limited and used either microsatellite markers (Cañon et al., 2006) or SNPs (Pariset et al., 2009).

Most of the above studies involved populations lacking pedigrees and small sample sizes per breed (30 - 100 animals). Only Mastranestasis et al. (2015) and Kominakis et al. (2017a) used relatively large sample sizes (350 Lesvos sheep and 503 Frizarta ewes, respectively) and published follow-up studies. The latter searched for possible associations of phenotypic traits (*i.e.* body traits, milk yield, litter size) with markers detected in the initial studies (Mastranestasis et al., 2016; Kominakis et al., 2017b; Saridaki et al., 2019).

Genetic studies of indigenous Greek sheep and goats indicate high genetic variability and, therefore, improvement potential of production and fitness traits. However, a notable lack of continuity between research activity and practical implementation is observed. Research objectives are altered on the basis of scientific trends resulting in intermittent studies and no further efforts are made towards the application of research outcomes. Consequently, implementation of scientific findings in breeding programs is extremely restricted.

BREEDING PROGRAMS

Introduction of breeding programs

Breeding programs of indigenous Greek small ruminant breeds were first introduced in the late 1970s. Since then, such programs of variable duration, some of which are still ongoing, have been implemented in 15 sheep and two goat breeds. The overall aim has been either improvement of production traits or, in case of rare breeds, conservation and diversity management. The only breeding objective has been milk yield except for the cases of Chios and Frizarta sheep.

Despite the improvement potential of indigenous Greek sheep and goats, breeding programs were not always successful, mostly because implementation was commonly interrupted for time periods varying from months to years. The vast majority of farmers do not comprehend the strategic importance and the long-term benefits of genetic improvement and thus, they are reluctant to invest in it. Reliance of breeding programs exclusively on European and/or government financial support has caused lack of consistency; animal recording paused due to shortage of other sources of financing, whenever funding was pending.

Chios sheep

Chios sheep are highly producing (average commercial lactation milk yield: 324 kg) and present a considerable amount of variance of dairy traits (*i.e.* milk yield, lactation length) indicating high improvement potential (Basdagianni et al., 2018). Hence, Chios sheep are among the most commonly reared indigenous sheep in Greece and the most extensively studied. Breeding objectives for Chios sheep include milk yield and resistance to scrapie. Selection for the latter has been based on genotyping rams for the associated gene, following the relevant study of Psifidi et al. (2011). Although scientific studies on several Chios sheep traits have been published, only the two aforementioned traits have been included in the breeding scheme. Since 2004, average commercial lactation milk yield has increased by ca. 20 kg (Basdagianni, 2006). However, full potential of the breed has not been reached and improvement rates are quite low given that the breeding program is ongoing for more than 15 years. The absence of systematic use of sire referencing schemes and artificial insemination (AI) across the breeding population is a major drawback in the genetic improvement program and contributes to the overall limited improvement of the traits included in the breeding goal.

Frizarta sheep

Frizarta is another relatively highly producing indigenous Greek sheep breed with average commercial lactation milk yield of *ca.* 250 kg and average fat and protein contents of 6.2% and 5.5%, which are favorable for cheese production (Saridaki et al., 2019). Frizarta sheep have been subject to many studies. However, breeding objectives include only milk yield and quality (fat and protein content). Udder traits are recorded once a year in the beginning of lactation and are candidate traits for selection towards improv-

ing machine milkability (Agricultural and Livestock Union of Western Greece, n.d.). In recently published GWAS on milk and body size traits of Frizarta sheep, the idea of implementing genomic selection has been introduced (Kominakis et al., 2017b; Saridaki et al., 2019). A promising attribute of the breeding program of Frizarta sheep is the extensive implementation of AI across the breeding population.

Despite the few examples of efficiency, most breeding programs have failed to reach their full potential. Poor program management and lack of motivation for self-funding by farmers significantly affected the outcome. Thus, foreign improved breeds of higher productivity have replaced many indigenous small ruminant populations. The latter have decreased dramatically and some indigenous Greek breeds are now facing endangerment or even extinction issues.

TAKING THE NEXT STEP: AN INTEGRATIVE APPROACH

Tackling the challenges towards improvement of indigenous Greek small ruminant breeds requires collaboration of all stakeholders and experts involved in animal production, in an integrative holistic approach. This approach can be broken down into three levels; (i) setting appropriate breeding objectives, (ii) design of breeding schemes based on applied research outcomes, and (iii) practical implementation of breeding programs.

Determination of breeding objectives

In order to set genetic improvement objectives, specifying the needs of dairy industry comes first. Industry is mainly focused on processing milk of small ruminants into cheese (Moatsou and Govaris, 2011). Cheese yield is affected by milk quality traits and coagulation properties, which are characterized by genetic variation indicating improvement potential (Vacca et al., 2018). Milk hygiene traits are also important for dairy industry. According to the EU regulation, milk with increased SCC and TVC is inappropriate for human consumption (European Parliament and Council, 2004). Udder health is commonly compromised due to poor adaptation to machine milking (Gelasakis et al., 2015). Hence, udder conformation improvement for better milkability is also crucial.

Farmers, on the other hand, prioritize milk yield because it is their main source of income (Pulina et al., 2018). Unfortunately, Greek farms producing milk of higher quality are not rewarded; thus, a lack

of motivation for improvement is observed. Animal resilience is also critical for farms as it limits animal replacement needs and enhances their welfare. Animals more resistant to diseases and well adapted to rearing conditions are necessary to maintain a sustainable production (Theodoridis et al., 2018; Rose et al., 2019).

Breeding program design

Integration of the aforementioned multiple objectives in selection schemes requires careful design. Investigation of the genetic background of traits and possible intercorrelations is crucial. Respective studies on milk, udder and resilience traits of indigenous Greek small ruminant breeds are largely missing. Therefore, scientific research should focus on the above and engage in delivering results that are applicable to breeding schemes. To this purpose, genomics is a promising approach as it addresses issues of complex heritability (Hayes et al., 2013). Vast amounts of genomic data are available through high-density genotyping, making it possible to detect genomic regions associated with phenotypic traits of interest (Zhang et al., 2012). Increasing the accuracy of early-life selection is another key advantage of genomic technologies (Hayes et al., 2009; Pryce and Daetwyler, 2012). Furthermore, inclusion of environmental and climate data in genomic studies may simplify investigation of complex resilience traits (Lv et al., 2014).

Although initially expensive, genomic technologies have become affordable, making their implementation on a larger scale easier (Mrode et al., 2018). Advances in imputation techniques enable genomic strategies based on an optimal mixture of strategically targeted genotyping with high-density arrays combined with large-scale genotyping with inexpensive low-density arrays (Aliloo et al., 2018). Therefore, genomic selection is a feasible solution for breeding schemes of multiple objectives.

Practical implementation of breeding programs

Implementation of breeding programs may be based on a three-tier pyramid structure that ensures efficiency and facilitates dissemination of genetic improvement (Figure 1). To this purpose, after the initial screening of the population, a nucleus of animals of high improvement potential can be formed. These animals should be reared in an elite group of well-monitored flocks warranting high welfare standards and sufficient rearing conditions; under such practices animals are more likely to reach full potential. Perfor-

mance recording and genome-wide genotyping of the animals of the nucleus should be thoroughly implemented. Thus, the nucleus will produce sires of high breeding value, which will be used for breeding purposes in the rest of the purebred farms. The main role of the second tier, where a bigger number of flocks are involved, is to generate more animals with the desired genotypes of the top tier population. Animals of the second tier, that are proved to be of high breeding value, can also be used as sires or dams to enhance genetic diversity in the top tier when necessary. Thus, animal performance in second tier flocks is recorded, yet to a smaller extent than in the nucleus, and in some flocks low-density genotyping is also implemented. Finally, flocks involved in the third tier do not record animal performance and rely on the first two tiers for sires and dams of higher breeding value. Thus, flocks at the bottom of the structure benefit from genetic improvement achieved in the first two tiers, although it would take a longer time for them to improve. Such programs have been successfully implemented mainly in poultry and swine, and more recently in dairy ruminants (Harris & Newman, 1994).

Prerequisite for the successful implementation of genetic improvement programs is securing pedigree accuracy, which can be compromised when matings are done by natural service. On the contrary, use of AI ensures closer monitoring, resulting in more accurate paternity records. Furthermore, AI achieves more efficient use of valuable genetic material and faster collection of progeny performance data, allowing for earlier evaluation of sires (Baldassare and Karatzas, 2004). The latter can be further facilitated through combining AI and genomic selection. Studies on French Lacaune and Manech sheep have showed that incorporation of genomic prediction based on parent performance assists accurate evaluation of candidate sires in a younger age. Such practices increase annual genetic gain without inflating cost (Buisson et al., 2014). Therefore, in the frame of a well-designed breeding scheme, introduction of AI and genomic selection in the nucleus and expansion of its use across the whole population accelerates genetic improvement.

The suggested approach is expected to improve indigenous Greek small ruminant breeds, given that the initial population is sufficiently large and presents improvement potential. Efficient collaboration among all stakeholders involved is expected to provide feedback that can be used for re-evaluation of the scheme, integration of novel technologies and approaches, and inclusion of new breeding objectives.
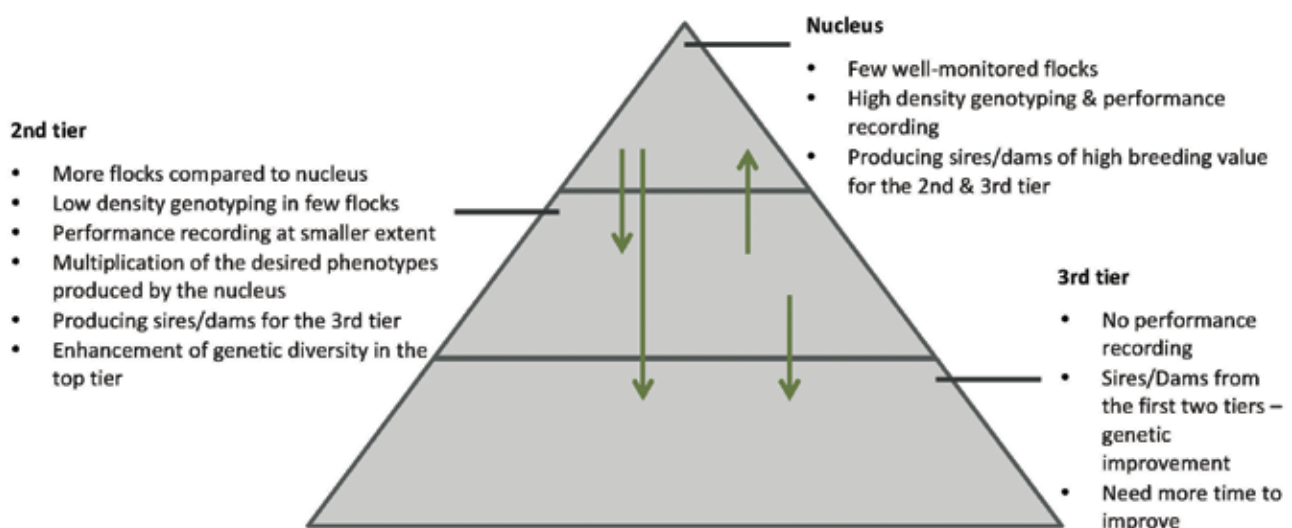

Figure 1: Three-tier pyramid structure that facilitates dissemination of genetic improvement in breeding programs (green arrows indicating supply of sires/dams among tiers)

CONCLUSIONS

Indigenous Greek small ruminant breeds exhibit substantial genetic diversity, which indicates high improvement potential. The latter is supported by scientific publications on production and fitness traits. However, no follow-up action has been taken to systematically incorporate research outcomes into breeding programs. Furthermore, implementation initiatives of such programs have been interrupted more than once due to lack of funding and failed to achieve substantial improvement.

Setting appropriate breeding objectives is the first key step to designing sophisticated breeding schemes that take into account the needs of the sector, market demands and the capacity and potential of indigenous small ruminant populations. Consistent population monitoring and accurate record keeping are essential. Integration of scientific advances, especially in genomics and reproductive technologies, is expected to underpin the successful implementation of breeding programs and acceleration of genetic improvement. Most importantly, commitment and good communication among all stakeholders, including farmers, industry and scientists, is necessary to ensure efficiency of such programs. Implementation of breeding programs incorporating all the above features is expected to improve the competitiveness of indigenous Greek small ruminant breeds and enhance the sustainability of the sector.

CONFLICT OF INTEREST

None declared.

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Effect of environmental heat stress on *Kıvırcık* ram sperm parameters

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ABSTRACT. The relationship between environmental conditions and reproduction has been in sight of research. Therefore, there has been a growing interest in effect of climate change, global warming and subsequently environmental heat stress on gametes in last decades. Thus, the present study was designed to investigate the effect of environmental heat stress on *Kıvırcık* ram sperm parameters. In this study, semen samples *(n=*72) were collected from six mature rams during a 12-month study period. The temperature and relative humidity of animal box were controlled daily. The mean monthly temperature humidity index (THI) values were calculated to determine heat stress and control periods. According to the mean monthly THI values, the 36 of 72 ejaculates, which were collected between May and October, were classified as heat stress period samples. The others (*n=*36), which were collected between November and April, were considered as control period samples. The heat stress period sperm samples had lower sperm concentration, motility, viability, membrane integrity and higher abnormal sperm rate compared to control period sperm samples (P < 0.05). Semen volume, sperm DNA integrity and the ability of sperm to undergo acrosome reaction were detected similar between the heat stress and control periods. In conclusion, environmental heat stress was found deleterious for some sperm parameters in *Kıvırcık* rams.

Keywords: Heat stress; Ram sperm; Membrane integrity; DNA integrity; Acrosome reaction.

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INTRODUCTION

Stress is revealed by the inability of a living organism to cope with environmental conditions (Dobson and Smith, 2000). Living organisms are influenced by various environmental stress factors such as heat. In last decades, the effect of environmental heat stress on reproduction of wild and domestic animals has received greater attention because of global climate change and global warming. Heat stress essentially causes over production of reactive oxygen species (ROS) and induces oxidative stress (Slimen et al., 2014). The exaggerated production of ROS impairs the physiology and functions of different type of cells by damaging lipids, proteins, polysaccharides and DNA (Cross et al., 1987). Although controlled production of ROS plays important role in sperm functions, uncontrolled production of ROS causes sperm pathology (De Lamirande et al., 1997). Thus, different techniques have been developed to investigate the effect of heat stress on male fertility [e.g., insulation of the entire scrotum or neck of the scrotum (Arman et al., 2006; Brito et al., 2003; Kastelic et al., 1996), immersion of the scrotum in a hot water bath (Banks et al., 2005; Rockett et al., 2001), surgically induced cryptorchidism (Yin et al., 1997) and artificially heated animal boxes (Meyerhoeffer et al., 1985) or naturally hot places (Al-Ghetaa, 2012; Nichi et al., 2006). Previous studies have shown that heat stress affects testicular and epididymal sperm cells (Banks et al., 2005; Karabinus et al., 1997; Sailer et al., 1997) furthermore the spermatocytes and early spermatids are the highly vulnerable germ cells against to heat stress (Rockett et al., 2001; Setchell, 2006). Artificially heating of testes decreases sperm DNA integrity (Banks et al., 2005), semen concentration, sperm motility and fertilizing capacity of sperm in rodents (Jannes et al., 1998). Moreover, similar induction of heat stress increases apoptosis in the germ cells and alters the expression of some genes in the testis (Rockett et al., 2001) and epididymis (Li et al., 2008).

In farm animals, artificially local heating of testes decreases sperm motility (Arman et al., 2006), damages sperm plasma and acrosome membranes (Hamilton et al., 2016) as well as chromatin of the epididymal and testicular sperm cells (Karabinus et al., 1997). The environmental heat stress increases abnormal sperm rate in bulls (Nichi et al., 2006) and elevates dead and abnormal sperm rate in sheep (Al-Ghetaa, 2012). Previous studies indicated that sperm quality gradually returns normal levels within 8 weeks in bulls (Meyerhoeffer et al., 1985) and within 47 days in rams after heat stress (Hamilton et al., 2016). Mostly the previous studies related to the heat stress focused on effect of artificially local heating of testes on sperm parameters. Moreover, the studies based on environmental heat stress were generally investigated the effect of heat stress on basic sperm parameters (motility, viability and morphological abnormalities). Thus, the present study was designed to provide further information about the effect of environmental heat stress on *Kıvırcık* ram sperm parameters including sperm plasma membrane, DNA integrity and ability of sperm to undergo acrosome reaction.

MATERIALS AND METHODS

Animal care and management

Six mature *Kıvırcık* rams were used in this study. The *Kıvırcık* is common breed of the region where the present study was conducted. The animals were housed in an animal box at the Department of Reproduction and Artificial Insemination, Aydın Adnan Menderes University, Turkey. The animals were submitted to uniform nutritional conditions in 15 months. The first 3 months were considered an adaptation period in which the animals adjust to the new feeding and housing system. Permission for use of the experimental rams was obtained from the ethical committee of our university (2012/44).

Calculation of temperature humidity index

The study was conducted in Aydın (approximately 37° north, 27° east and 64 m above sea level) Turkey, where temperatures can reach 45°C under the sun in summer months. The temperature and relative humidity of animal box was controlled daily. The daily temperature humidity index (THI) was calculated with the formula that was previously reported by Marai et al. (2007). In the formula below, T and RH represent respectively temperature (°C) and relative humidity (%).

$$THI = T - (0.31 - 0.0031 \text{ x } RH) \text{ x } (T - 14.4)$$

Determination of heat stress and control periods

The mean monthly THI values were used to distinguish heat stress and control periods and to determine the level of heat stress (Table 2) according to the previously defined heat stress categories for sheep by Marai et al. (2007) (Table 1).

Table 1. Definition of heat stress categories according to THI values by Marai et al. (2007).

THI values	Heat stress category
THI < 22.2	Absence of heat stress
22.2 ≤ THI < 23.3	Moderate heat stress
23.3 ≤ THI < 25.6	Severe heat stress
THI ≥ 25.6	Extreme severe heat stress

Table 2. Definition of heat stress categories in heat stress and control periods according to mean monthly THI values.

Periods	Months	Mean THI ± SEM.	Heat stress category
Heat stress period	May	22.3 ± 0.32	Moderate heat stress
	June	24.6 ± 0.27	Severe heat stress
	July	27.1 ± 0.19	Extreme severe heat stress
	August	28.1 ± 0.21	Extreme severe heat stress
	September	22.6 ± 0.22	Moderate heat stress
	October	17.9 ± 0.38	Absence of heat stress (Prolonged effect of heat stress)
Control period	November	15.6 ± 0.27	Absence of heat stress
	December	10.2 ± 0.44	Absence of heat stress
	January	13.0 ± 0.34	Absence of heat stress
	February	13.6 ± 0.29	Absence of heat stress
	March	15.0 ± 0.27	Absence of heat stress
	April	18.2 ± 0.29	Absence of heat stress

Semen collection and evaluation

Semen was collected from six mature *Kıvırcık* rams using an electro-ejaculator at 15-day intervals throughout the study period (12 months). A total of 72 ejaculates (12 ejaculates per ram) were evaluated immediately after collection. Semen volumes and concentrations were determined using a micropipette and a haemocytometer respectively. Motility parameter was evaluated subjectively under a phase contrast microscope at 200× magnification (TMS Nikon, Tokyo, Japan), equipped with a heated stage adjusted to 37 °C using an 8 μl sample of diluted semen [1:5 with Tris, citric acid, glucose (TCG) solution including 300 mM Tris, 95 mM citric acid monohydrate and 28 mM glucose, pH 7.0]. Sperm viability was determined using the eosin-nigrosin (EN) staining procedure. Briefly, diluted sperm samples (5 μl) were stained with EN stain (1% eosin, 3% sodium citrate and 3% nigrosin in distilled water) and thin, uniform smears were made. After air dried, 200 sperm from each sample were observed for live (unstained) or dead (stained) sperm heads under bright field microscopy at 400× magnification. The morphologically abnormal sperm rate was detected with a wet-mount slide method. Briefly, 10 μl semen samples were added in 1 ml of Hancock's solution (Hancock, 1952). A total of 200 sperm from each sample were counted for morphological abnormalities under a phase-contrast microscope at ×1000 magnification.

Assessment of sperm plasma membrane integrity

Sperm plasma membrane integrity was evaluated with hypo-osmotic swelling test in combination with an EN staining procedure (Aksoy et al., 2008). Briefly, 25 μl sperm samples were mixed to 475 μl of fructose solution (100 mOsm/L). The mixed samples were incubated in 35°C water bath for 15 min. Following incubation, sperm samples were stained with EN stain and sperm plasma membrane integrity was assessed with under bright field microscope at 400× magnification. A total of 200 sperm from each sample were scored for numbers of live-intact and total intact (respectively, unstained heads of sperm with coiled tails and stained and unstained heads of sperm with coiled tails).

Assessment of sperm DNA integrity

Sperm DNA integrity was analyzed with comet assay procedure as reported previously (Küçük et al., 2014). Briefly, the slides were covered with 1% normal-melting-point agarose in phosphate buffered saline (PBS). A 10 μl aliquot of diluted sperm extended

in 190 µl 0.75% low-melting-point agarose in PBS final concentration of 2 x 10⁴ sperm was added to each slide, covered with a coverslip and cooled for 10 min at 4°C to solidify. After solidification, coverslip were removed gently. The slides were immersed in an alkaline lysis solution (1 M Tris–HCl, 1% N-lauroyl-sarcosine, 0.5 M EDTA, 0.3 M mercaptoethanol pH >10) at 4°C overnight for the lysis process. The slides were then washed with distilled water to remove lysis solution. The slides were placed in the alkaline electrophoresis solution including 1 mM EDTA and 300 mM NaOH for equilibration (20 min) and electrophoresis (50 V for 40 min). Subsequently, the slides were washed with neutralizing solution of 0.4 M Tris (pH 7) and then fixed with an alcohol series (50%, 70% and 90%). The nucleus of sperm cells were stained with DAPI (40,6-diamidino-2-phenyindole, dilactate) and covered with coverslips. The images of one-hundred randomly chosen sperm nucleus have intact or non-intact chromatin were immediately taken under fluorescent microscope (QuickCam Pro). The images were evaluated with Image J software (version 1.47v; NIH. USA) (Pic. 1) to determine DNA fragmented sperm rate.

Assessment of the ability of sperm to undergo acrosome reaction

Acrosome reacted sperm rate was determined with a Coomassie Blue G-250 staining procedure (Ahmad et al., 2013) in fresh semen, at 0 min after 1 h incubation period, 20, 40 and 60 min after lysophosphatidylcholine (LPC) treatment, respectively. In fresh semen, a 15 µl sperm sample was directly fixed. Another 200 µl portion of fresh sperm was diluted in 2 ml TCG and centrifugation was applied twice at 800× g for 5 min to remove seminal plasma. The remaining sperm pellets were diluted in 1 ml TCG and incubated in a water bath at 35°C for 1 h to induce capacitation. Following incubation, the sperm samples were treated with LPC, an acrosome reaction-inducing agent, as described previously (Ahmad et al., 2013). Briefly, 980 µl sperm sample was supplemented with 20 µl of LPC from a stock solution (10 mg/ml in TCG). The final LPC concentration was 200 µg/ml. The sperm samples were treated with LPC at 35°C for 20, 40 and 60 min. A total of 200 spermatozoa from each sample were observed with bright field microscopy at 400× magnification to detect whether they had visible acrosome (intact) or had invisible acrosome (reacted).

Statistical analyses

All the results collected from present study were analyzed with a Statistical Package (Minitab version 17 Statistical Software). Descriptive statistics were used to determine the mean monthly temperature and humidity index. Two-sample *t*-test was applied to compare the sperm parameters (semen volume, concentration, motility, percentage of live, abnormal, live-intact, total-intact, DNA damaged and acrosome reacted sperm rates) between the heat stress and control periods. A probability level of P < 0.05 was used to define statistical significance. All results were presented as mean ± SEM.

RESULTS

Estimation of heat stress and control periods

The mean monthly THI values and severity of heat stress are presented in Table 2. The five months (from May to September), when THI values higher than 22.2 (Marai et al., 2007), were classified as heat stress period. Since detrimental effects of heat stress disappear gradually within approximately one spermatogenesis cycle (47 days) in rams (Hamilton et al., 2016) October was also added in heat stress period (May to October). Remaining six months (from November to April) when the THI values were lower than 22.2, were classified as control period (Table 2).

Evaluation of semen parameters

There was no difference (P > 0.05) in semen volume between the heat stress and control periods (Figure 1). Sperm concentration was lower (P < 0.05) in heat stress period compared to the control period (Figure 2). Moreover, heat stress decreased (P < 0.05) sperm motility, live sperm rate and increased abnormal sperm rate (Figure 3). Heat stress also decreased sperm plasma membrane integrity. Total-intact sperm rate was lower (P < 0.05) in the heat stress period compared to the control period (Figure 3). Live-intact sperm rate was lower in the heat stress period compared to control period but statistical difference was P = 0.06. DNA damaged sperm rates were found similar in the heat stress and control periods (Figure 3). LPC induced acrosome reacted sperm rate were also found similar in both periods (Figure 4).
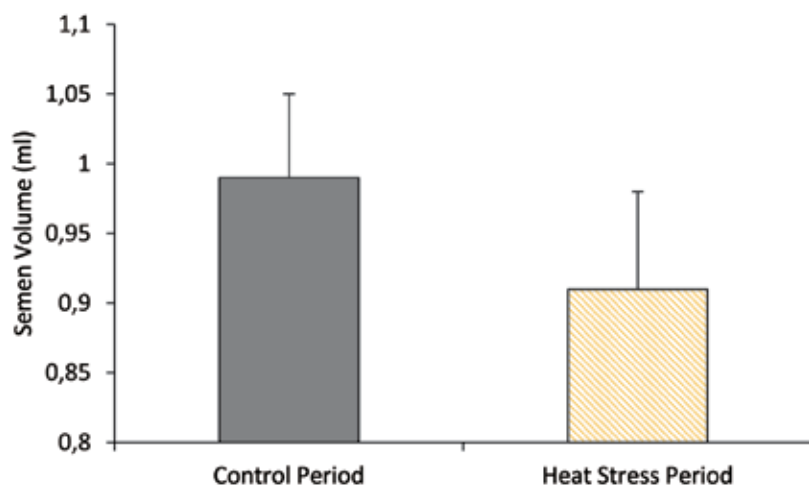
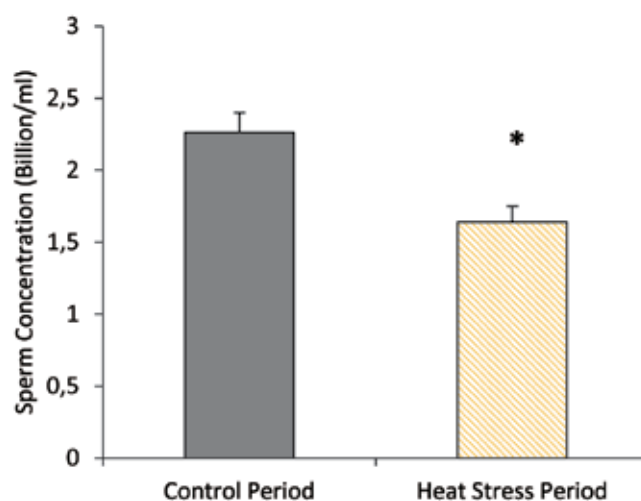**Figure 1.** The effect of heat stress on semen volume in rams (P > 0.05).

Figure 2. Effect of heat stress on sperm concentration in rams. Asterisks indicate P < 0.05.
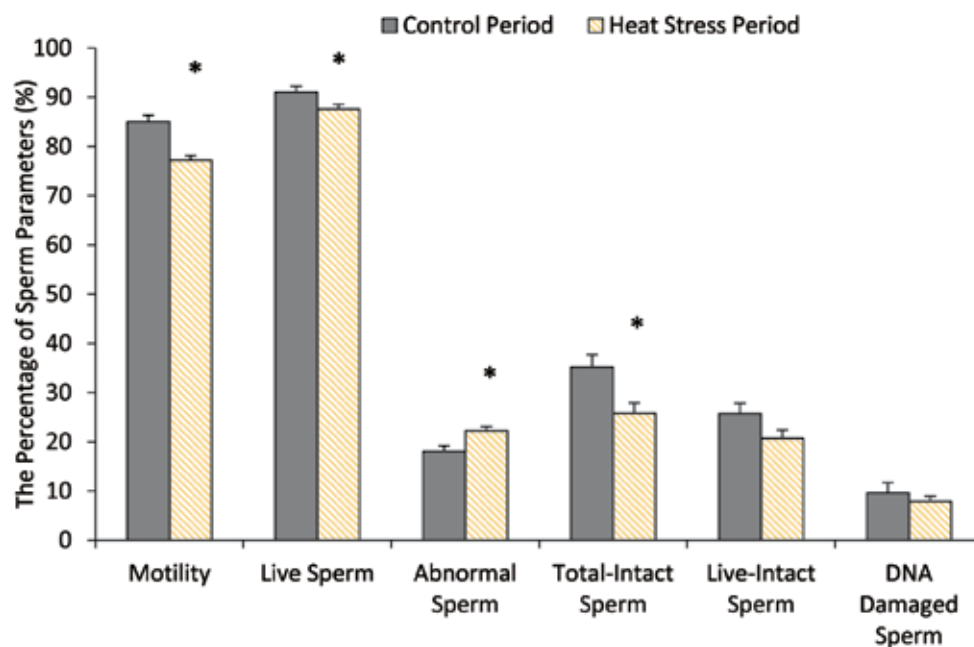

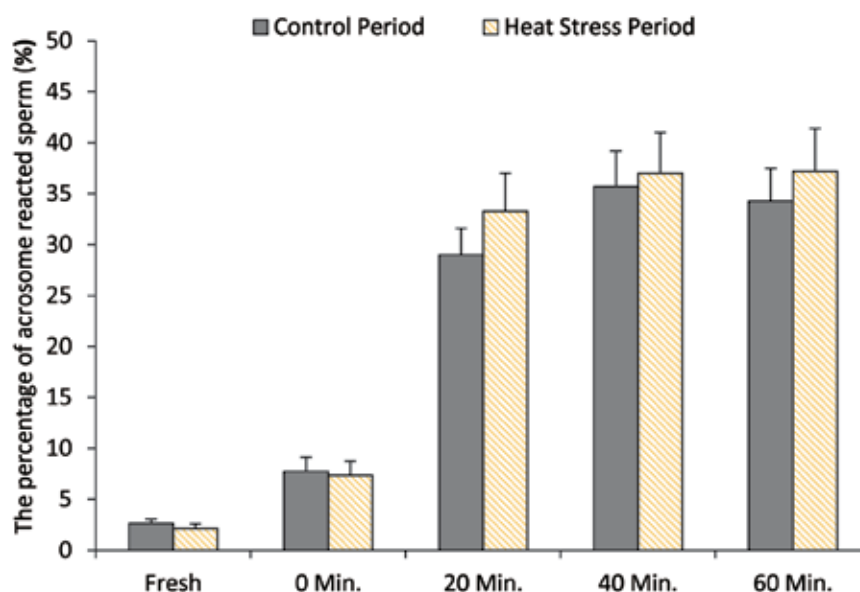**Figure 3.** Effect of heat stress on in vitro sperm parameters in rams. Asterisks indicate P < 0.05.

Figure 4. Effect of heat stress on acrosome reacted sperm rates in fresh semen and at 0 min (after 1 h incubation), 20, 40 and 60 min after LPC treatment, respectively (P > 0.05).

DISCUSSION

The testicular temperature is lower than body temperature in most livestock and this is necessary for successful spermatogenesis (Banks et al., 2005). The optimum scrotal temperature for spermatogenesis is maintained by specialized tissues such as muscles, sweat glands and pampiniform plexus (Setchell, 2006; Setchell and Breed, 2006; Blazquez et al., 1988). However, high environmental temperature can cause an increase in scrotal temperature, production of reactive oxygen species (ROS) and trigger oxidative stress in the testes (Nichi et al., 2006). Several antioxidants and various types of heat shock proteins have important roles in preventing the detrimental effects of heat stress on sperm (Rockett et al., 2001; Nichi et al., 2006; Hamilton et al., 2016). Despite such defense mechanisms, heat stress may adversely affect sperm quality in most mammals.

In the present study, semen volume was similar in heat stress and control periods. However, sperm concentration was lower during heat stress period compared to the control period (P < 0.05) probably as a result of heat stress induced spermatogenic failure (Jannes et al., 1998) and induction of apoptosis in germ cells especially in spermatocytes (Rockett et al., 2001). Heat stress also decreased sperm motility, viability and morphology (P < 0.05). Previously it has been reported that heat stress can damage seminiferous epithelium (Rockett et al., 2001) and sertoli cells (Hassanpour et al., 2015). Moreover, heat stress af-

fects the epithelium of cauda epididymis, alters ion concentrations, protein composition of epididymal fluid and inhibits the special ability of the cauda to store and prolong the life of sperm cells (Bedford, 1991). Therefore, such unfavorable testicular and epididymal environment might account for the lower motility, viable and morphologically normal sperm rate during heat stress period (Fig. 3).

Even though there were some studies focused on effect of environmental heat stress on some sperm parameters in livestock, the present study is unique because it investigates effect of environmental heat stress on ability of sperm to undergo acrosome reaction, sperm plasma membrane and DNA integrity in rams. The sperm plasma membrane is particularly rich in polyunsaturated fatty acids and highly vulnerable against lipid peroxidation, which is triggered by over production of ROS and oxidative stress (Vernet et al., 2004). Additionally, compared to other species ram sperm plasma membrane is more susceptible to lipid peroxidation due to its relatively high polyunsaturated/saturated fatty acid content and low proportions of cholesterol/phospholipids content in the plasma membrane (Darin-Bennett and White, 1977). The present study indicated that heat stress damaged plasma membrane integrity of ram sperm is in agreement with the previous results of Hamilton et al. (2016). The higher sperm plasma membrane disorders in heat stress group might be resulted from lipid peroxidation that induced by over production of ROS during heat

stress period.

Although Hamilton et al. (2016) indicated that insulation of testes increased acrosome damaged sperm rate in rams, Maya-Soriano et al. (2015) reported that environmental heat stress did not altered acrosome damaged sperm rate in rabbits. Maya-Soriano et al. (2015) explained the similar acrosome damaged sperm rates between environmental heat stress and control group as a result of adaptation of rabbits to the extended heat stress conditions. Previous studies also reported that immersion of the testes in 40 to 42°C water bath increased the DNA damaged sperm rate compared to control in mice (Banks et al., 2005; Sailer et al.,1997). In the present study, DNA damaged and acrosome reacted sperm rates were found similar during the heat stress and control periods. The similarity of DNA damaged and acrosome reacted sperm rates in heat stress and control periods might be related to the adaptation of Kıvırcık rams to the extended heat stress condition. Furthermore, unfavorable effect of heat stress on sperm DNA and acrosomal integrity might be limited by specialized tissues to maintain optimal scrotal temperature such as musculus cremaster, scrotal sweat glands in present experimental conditions. In our study, musculus cremaster and scrotal sweat glands were more effective to maintain optimal scrotal temperature compared to previous studies utilizing artificial local heating of testes. Artificially local heating of testis with insulation material or hot water completely block functions of these defense systems. Additionally, ram sperm nuclei includes only Protamine I (Balhorn, 1982) which is maximally cross linked by disulphide bridges, and more stable than mouse sperm nuclei including Protamine I and II (Motoishi et al., 1996). On the other hand, when the lower sperm concentration during heat stress period considered as evidence in present study, similar DNA damaged sperm rates during heat stress and control period might be related to the elimination of DNA damaged germ cells (spermatocytes and early spermatids) by apoptotic defense mechanisms during heat stress period.

CONCLUSION

Environmental heat stress unfavorably influenced sperm concentration, motility, viability, morphology and plasma membrane integrity in Kıvırcık rams. However, semen volume, sperm DNA integrity and the ability of sperm to undergo acrosome reaction were not altered under the present environmental heat stress conditions.

CONFLICT OF INTEREST

None of the authors has any conflict of interest to declare. All the authors are aware of submission and agreed to be listed as co-author.

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Enzootic bovine leukosis accompanied by splenomegaly in an 8-month-old calf

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ABSTRACT. In this report, an 8-month-old calf (crossbred, Holstein × Japanese Black) developed fever and accompanied abomasum displacement. Blood chemical test showed remarkably high values of white blood cell count and heteromorphic lymphocytes. In pathological appraisal, enlarged splenomegaly and swelling of the lymph nodes were observed. Histopathological examination revealed invasion of tumor cells derived from B1 cells into systemic lymph nodes, liver and spleen. The provirus loads of bovine leukemia virus (BLV) was 1,439 copies per 10 ng DNA by using real time PCR. In conclusion, this case was diagnosed as bovine leukemia caused by BLV infection with a huge splenomegaly.

Keywords: BLV virus, juvenile, splenomegaly.

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CASE HISTORY

Bovine leukemia is classified into two types: sporadic bovine leukosis (SBL) and enzootic bovine leukosis (EBL) (Kettmann, 1994). SBL is a generalized lymphadenopathy in calves, including a thymic form which is developed in 6-month to 2-year-old cattle and a cutaneous form occurred in 1- to 3-year-old cattle. EBL is a contagious disease caused by infection with bovine leukemia virus (BLV) which is belonging to the genus Deltaretrovirus, subfamily Orthoretroviridae, and family Retroviridae (Sagata et. al., 1985). Most BLV-infected cattle are asymptomatic infections (Aleukemia: AL) (Rodríguez et. al., 2011), but 20 to 30% of infected cattle show persistent lymphocytosis (PL) with polyclonal B cell hyperproliferation. Furthermore, 2 to 3% of them develop a B-cell leukemia, malignant lymphosarcoma formed in the lymph nodes, leading cause of death (Schwartz 1994). The incidence of EBL is observed more frequently in adult cattle over the age of 3 years than young calf. (Gutiérrez et. al., 2014). However, we report a suspicious case of EBL in 8-month-old calf which showed remarkable splenomegaly in this report. Our aim is description and thorough investigation of the clinical case.

An 8-month-old calf (castrated male, crossbreed, Holstein × Japanese Black) was examined because of decreased appetite and cough. The calf showed several symptoms such as abdominal pain, dehydration, fever (39.9 °C), and high respiration rate (60 breaths per min). In addition, it had been treated with enrofloxacin (Baytril®, Bayer, 0.025mL/kg, subcutaneously, SID) in order to control a suspected pulmonary inflammation. The serum hematological parameters provided a high leukocyte count (600,500 per 1 μL blood, reference interval (RI) : 4,900-12,000 per 1μL blood) and a percentage of heteromorphic lymphocytes was 90.5%. Moreover, lactate dehydrogenase (LD) and gamma-glutamyl transpeptidase (γ- GTP), the serum biochemical parameters, also showed high values (LD: 4,203 U/L, RI : 697-1,445 U/L, γ- GTP: 138 U/L, RI : 15-39 U/L).

After the calf was sedated with xylazine (Seractal®, Bayer, 0.3mg/kg, intramuscularly, SID) and euthanized after anesthesia with pentobarbital (Somnopentyl, Kyoritsu Pharmaceutical, 2mg/kg, intravenously) compassionately because of poor prognosis, the pathological autopsy was conducted, and we found a remarkable splenomegaly (93 × 36 cm, the weight of about 14 kg, Fig.1). Each of the lymph nodes (bilateral lateral cervical, lower ilium and mesentery) was enlarged and its cleavage surface showed grayish white.

Figure.1: A huge splenomegaly (93 × 36 cm, the weight of about 14 kg). Necrosis and bleeding were observed.

The collected tissue samples were fixed with 10% neutral buffered formalin solution, embedded in paraffin, cut into thin sections and stained with hematoxylin and eosin (HE). As histological findings, tumor cells which showed mantle zone histological patterns and nodular or diffuse histological patterns were observed in each lymph node and spleen (Fig.2). Extracapsular invasion was rarely seen in lymph nodes. Additionally, the tumor cells were also observed in blood vessels of liver (Fig.3), and high magnification view of that area showed a monotonous proliferation of tumor cells (Fig.4). The tumor cells had round, oval or slightly irregular nuclei with inconspicuous nucleoli and cytoplasm which was moderately abundant.
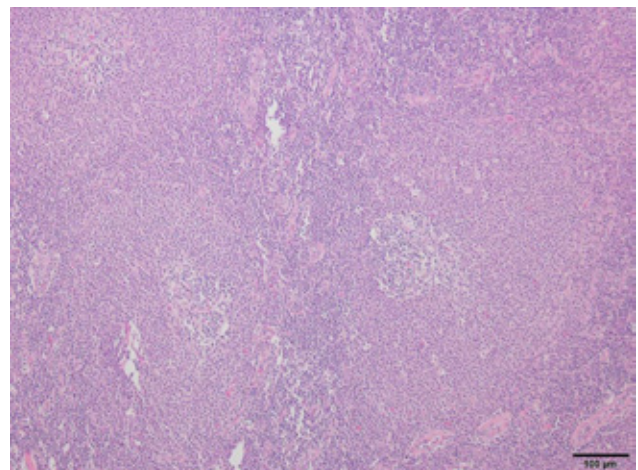

Figure.2: Histological appearance of iliac lymph node. Tumor cells which showed mantle zone histological patterns were observed. Hematoxylin and eosin (HE) staining.
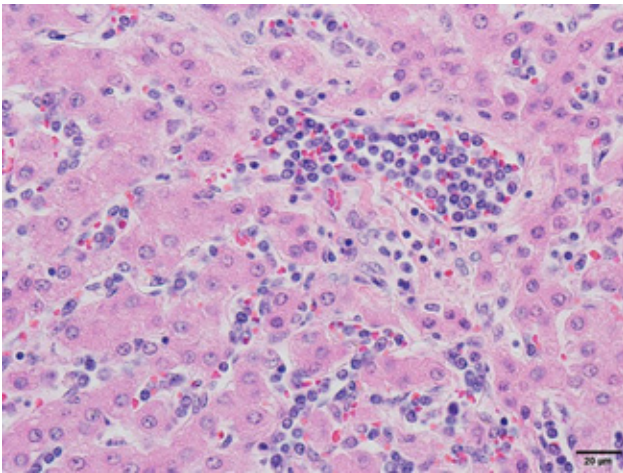
Figure.3: Histological appearance of liver tissue. Tumor cells were observed in blood vessels and sinusoid. HE staining.
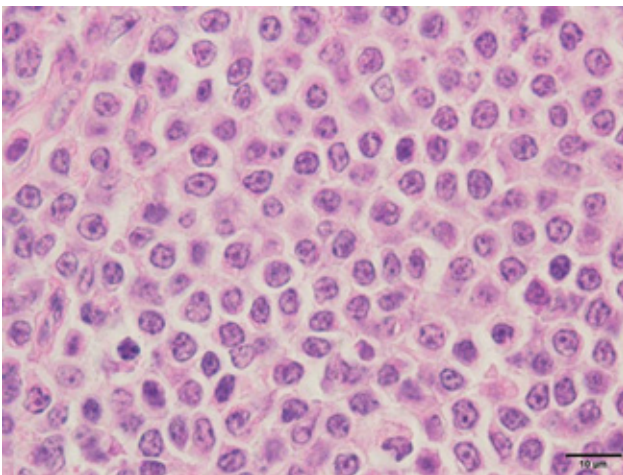

Figure.4: Histological appearance of iliac lymph node. A monotonous proliferation of tumor cells were observed. There were round, oval or slightly irregular nuclei with inconspicuous nucleoli and cytoplasm which was moderately abundant. HE staining.

Immunohistochemical examination was carried out using paraffin section of the tissue by polymer-based detection method according to the following procedures: Endogenous peroxidase activity was quenched by a solution of 0.3% hydrogen peroxide in methanol, after which antigen retrieval of the sections were performed by microwave heating. Antibodies targeting human CD20 (Thermo Fisher Scientific, Waltham, MA), human CD5 (Thermo Fisher Scientific), human CD3 (Agilent Technologies Japan, Tokyo, Japan) and TdT (Nichirei Biosciences, Tokyo, Japan) were used as primary antibodies. As results, the tumor cells expressed CD20 and CD5, but not CD3 and TdT (Fig.5).

To confirm the expression levels of cell surface markers in more detail, flow cytometry analysis was conducted using peripheral blood mononuclear cells prepared by density-gradient centrifugation. Cells were stained with following antibodies according to the method previously described (Nishimori et. al., 2017): bovine CD5 (Washington State University Monoclonal Antibody Center, Pullman WA; CAC-T105A), bovine IgM (Bio-Rad Laboratories, Hercules, CA; IL-A30), bovine WC4 (Bio-Rad Laboratories; CC55), bovine CD21 (Washington State University Monoclonal Antibody Center; GB25A), bovine CD3 (Washington State University Monoclonal Antibody Center; MM1A), bovine CD4 (Bio-Rad Laboratories; CC30) and bovine CD8 (Bio-Rad Laboratories; CC63). Alexa Fluor 647-conjugated goat anti-Mouse IgG (H+L) antibody (Thermo Fisher Scientific) was used as a secondary antibody, and for IgM staining, antibody labeling was conducted by Zenon Alexa Fluor 488 mouse IgG1 labeling kit (Thermo Fisher Scientific). The results clearly indicated the single cell population expressing CD5, IgM and CD21, but not expressing other markers (Fig.6).

To quantify BLV provirus loads in peripheral blood, genomic DNA was extracted by using an automatic nucleic acid extractor (magLEAD12gC, Precision System Science, Chiba, Japan) and BLV real time PCR was performed with Probe / Primer / Positive control for bovine leukemia virus detection (TaKaRa Bio, Otsu, Japan) (Soumura et. al., 2007). The result showed a quite high value of BLV provirus loads, 1,439 copies per 10 ng DNA, suggesting an interaction between tumor development and BLV infection.

The DNA extract was also used for B-cell clonality analysis, nested PCR targeting a variable region of immunoglobulin heavy chain, according to the method previously described (Nishimori et. al., 2017). As a result, we confirmed a single DNA band of the amplicon against the target region demonstrating monoclonal proliferation of B cells (Fig.7).
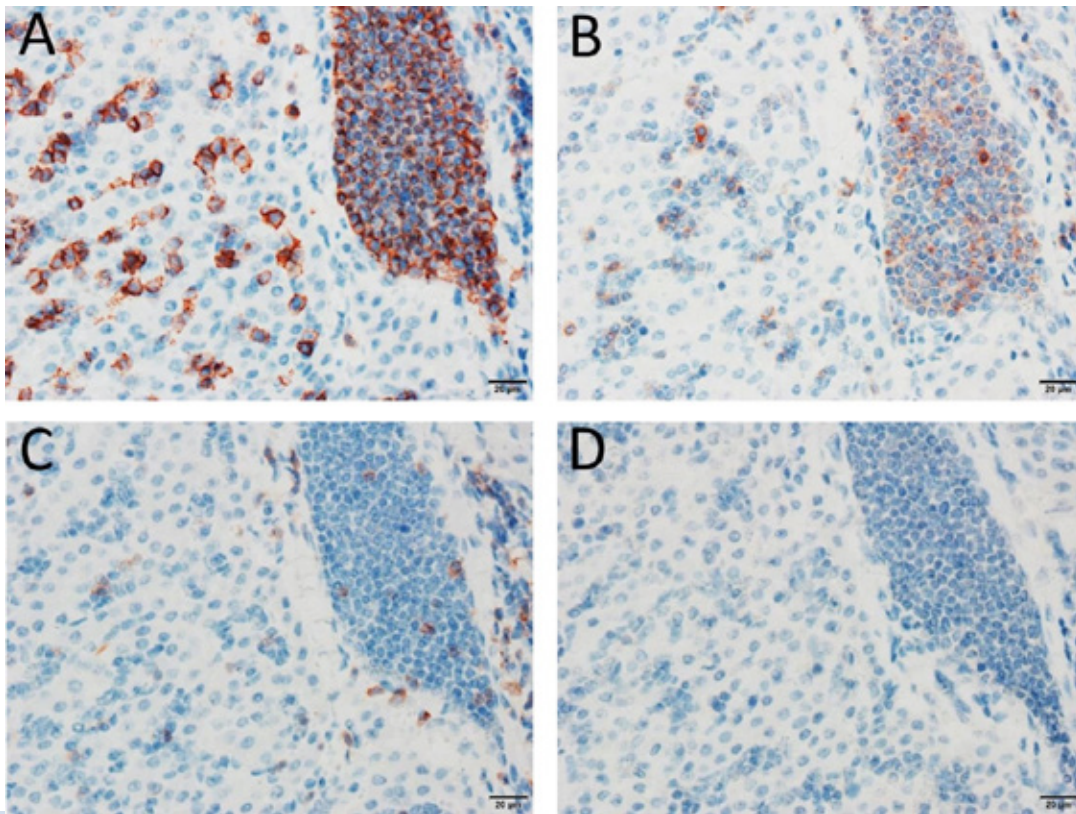
Figure.5: Immunohistochemical examination of tumor cells in liver tissue. Tumor cells were CD20 positive (A), CD5 positive (B), CD3 negative (C), TdT negative (D).
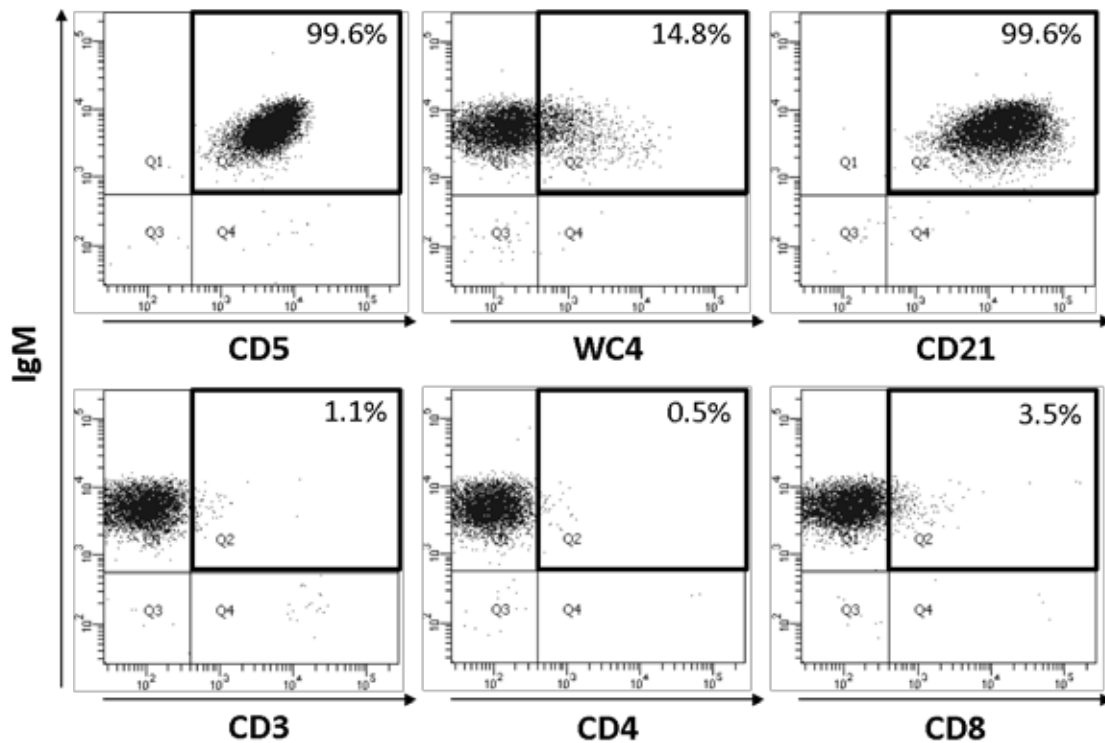

Figure.6: Flow cytometric analysis of peripheral blood mononuclear cells. Cells were first stained with anti-CD5 monoclonal antibody (mAb), anti-WC4 mAb, anti-CD21 mAb, anti-CD3 mAb, anti-CD4 mAb and anti-CD8 mAb, followed by Alexa Fluor 647-conjugated secondary antibody. Then cells were stained with Alexa Fluor 488-labeled anti-IgM mAb for double staining. The numbers in the figure indicate the percentages of double positive cells, and peripheral blood mononuclear cells showed CD5 positive, IgM positive and CD21 positive.
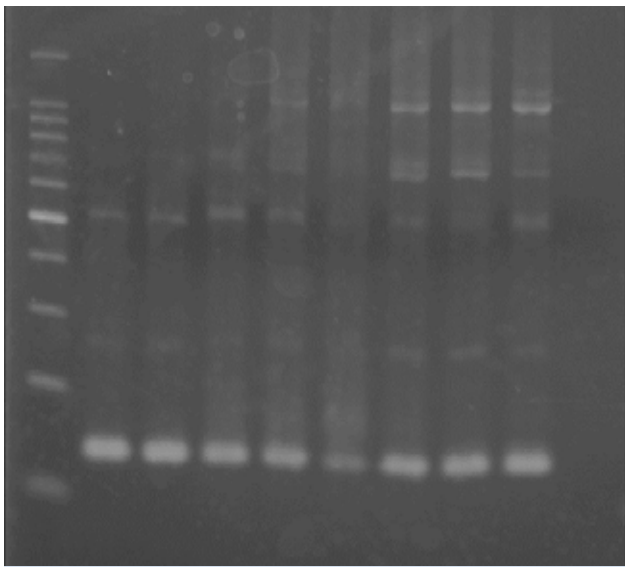
Figure.7: A single band patterns for the target region demonstrating monoclonal proliferation of B cells. Lane 1, white blood cells; lane 2, peripheral blood mononuclear cells; lane 3, a superficial cervical lymph node; lane 4, an iliac lymph node; lane 5, a mesenteric lymph node; lane 6, a mediastinal lymph node; lane 7, a pancreatico-duodenal lymph node; lane 8, spleen; N, negative control. The arrow indicates PCR amplification of the target region.

DISCUSSION

In previous studies, it was reported that the infection rates of BLV in Japan had increased from around 5% in the 1980s to 35.2% on a nationwide survey conducted in 2009-2011 (Murakami et. al., 2013). Thus, farmers suffer economic losses due to development of bovine lymphoma causing not only death of cattle, but also large costs for treatment and diagnosis of the infected animals (Da et. al., 1993). Early detection of cattle infected with BLV is effective for preventing BLV-free cattle from the infection and reducing economic losses. Moreover, an epidemiological study also suggests an importance of controlling BLV infection; it demonstrates that the incidence of other infectious diseases increases in BLV infected cattle compared with uninfected cattle (Emanuelson et. al., 1992).

Currently, little is known about the occurrence of EBL in young cattle under 1 year of age, and only a few reports investigate about that (Oguma et. al., 2017). Moreover, complication of splenomegaly is infrequent in common EBL cases. So, we consider that our report will contribute to accumulation of knowledge on bovine leukemia in young cattle and will be helpful for the diagnosis in the field. In this report, it was not able to investigate whether mother-to-child transmission of BLV had occurred or not because the dam of the case animal had already slaughtered. However, according to previous reports, almost all calves that are infected with

BLV immediately after birth are likely to develop into PL (Agresti et. al., 1993), and probability of BLV transmission in utero is around 4% to 18% (Ferrer et. al., 1975). Additionally, in the case of calves whose mother showed high provirus loads, more than 40% of the infants have been confirmed BLV positive whereas BLV positive rates were only 9.4% in the calves born from mothers with low provirus loads (Mekata et. al., 2015). In this case, we consider that BLV infection could be induced by vertical transmission via colostrum or placenta rather than by horizontal transmission via insects because the calf showed quite high value of white blood cell count and BLV provirus loads. Moreover, we concluded that the tumor cells in this case were derived from B1 cells, which is consistent with a previous report investigating BLV-associated lymphomas in cattle (Vernau et. al., 1997). Additionally, a single DNA band of the amplicon against the target region demonstrated monoclonal proliferation of B cells (Fig.7).

Because of the entire results we presented above, this case was diagnosed as EBL, which is clonal expansion of tumor cells derived from B1-cell lineage due to BLV infection, in spite of the young age of the animal. This finding is different from a widely accepted belief that EBL occurs in mainly adult cattle.

It is known that lymphoid tumor in adult lymphoma usually has a predilection for heart, abomasum, uterus, kidney, and spinal cord (Burton et. al., 2010). However, tumor invasion into those organs was not confirmed in this case, but instead, invasion into liver, spleen and lymph nodes was observed. In particular, the splenomegaly was one of the most important finding because it is infrequent in common EBL cases. There are some reports about the incidence of splenomegaly in cattle induced by Anaplasma marginale infection (Jaswal et. al., 2015), hemophagocytic histiocytic sarcoma (Matsuda et. al., 2010), and Trypanosoma vivax infection (Fatihu et. al., 2008). In this case, the parasites listed above were not observed in histological examination. Bovine leukemia is grouped into malignant lymphoma, and in our case, tumor cells would grow in the spleen, which caused enlarged splenomegaly.

There is still limited information about the onset of EBL in juvenile cattle. Further study on BLV infection and its dynamics during young stage would be necessary to elucidate its detailed mechanism.

CONFLICT OF INTEREST
None declared.

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ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ