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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	1
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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ΕΛΛΗΝΙΚΗ ΚΤΗΝΙΑΤΡΙΚΗ ΕΤΑΙΡΕΙΑ (ΕΚΕ)
HELLENIC VETERINARY MEDICAL SOCIATY (HVMS)

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Περιεχόμενα

History of the Hellenic Veterinary Medical Society

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Research article
Ερευνητικό άρθρο

Effect of different short term synchronization protocols on estrus and fertility in non-pregnant ewes during the breeding season

Ş. Dursun

Aksaray University Faculty of Veterinary Medicine Department of Obstetrics and Gynecology, Aksaray, Türkiye

ABSTRACT. The aim of this study was to investigate the efficacy of different short term synchronization protocols on estrus and pregnancy rates in merino ewes that had not become pregnant after at least three matings during the breeding season. Three different protocols were used as follows: Group I: Ewes (n=30) were inserted intravaginal progesterone sponge (florogestan asetate; 30 mg) for 6 days plus PGF2α (125 µg, i.m.) at the time of sponge removal, Group II: Ewes (40) were treated same as in group 1 plus eCG (250 IU, i.m.) at time of sponge removal, and Group III: Ewes (n=38) were only injected with PGF2α at the same time with the ewes in the other two groups. Estrus was detected by rams (n=4), 24 hours after PGF2α and ewes detected in estrus were mated. Ten rams were used for mating. Pregnancy was diagnosed 60 days after mating by ultrasonography. Estrus, conception and pregnancy rates were: for Group I 100%, 73.3% and 73.3%, for Group II 92.5%, 89.2% and 82.5% and for Group III 81.6%, 100% and 81%. In Group III, pregnancy loss after pregnancy diagnosis was significantly higher (35.4%) compared with the other groups (Group I: 13.6% and Group II: 15%)

Keywords: sheep, season, synchronization, pregnancy, lambing rates

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INTRODUCTION

This study was designed to evaluate fertility of ewes that were not pregnant after at least three matings. The main aim was to reintroduce those ewes to the flock and increase the lambing ratio. In the sheep breeding industry, ewes that do not become pregnant in the breeding season are usually culled off. However, the sheep population has been constantly decreased and good quality ewes with specific characteristics of any breeds, such as those in this study, are especially important. Non-pregnant ewes must be given more chances to become pregnant and stay in the breeding stock.

Estrus synchronization is an important tool in the reproduction management of cow and ewes. Synchronizing the time of breeding and lambing in the flock is highly beneficial for farmers (Gonzalez-Bulnes et al., 2005). Mainly, two different strategies have been developed to synchronize estrus in ewes: prostaglandin based and progestagen based protocols (Gordon, 1975; Timurkan and Yıldız, 2005; Hashim et al., 2013). Short (7 days) and long term (14-day) synchronization protocols are used in sheep and goat. Progesterone applications and eGG at the time of progesterone agent removal or $PGF_{2\alpha}$ administrations 48 hours earlier are usually used (Karaca and Kilboz, 2010). Estrus was detected in 96.7% and in 96.0% of ewes after progesterone application for 14 or 7 days respectively (Tekin et. al., 1992, Cox et al., 2012). Prostaglandin based protocols work through reducing the length of the estrous cycle by regression of the active corpus luteum and progestagen based protocols extend the cycle via administration of exogenous progesterone (Kusina et al., 2000). PGF_{2a} is the main prostaglandin agent used for estrus synchronizations and leads to estrus and ovulation (Menchaca et al., 2004). Progestagens are commonly used in sheep for estrus synchronizations (Killian et al., 1985). Although these strategies have high fertility rates at first service, they also have some weakness in the outcome. According to literature, prostaglandin based protocols leads to lower first services pregnancy rates compared to progestagen based methods due to disrupted follicular dynamics and inconsistency in the ovulation time (Boland et al., 1978; Godfrey et al., 1997; Barret et al., 2002). Progestagens compared to prostaglandins induce low-quality preovulatory follicles leading to poor luteal function and embryo viability (Gonzalez-Bulnes et al., 2005). However, using these protocols in ewes that have not conceived could give another chance for pregnancy and fertility. Furthermore, these methods achieve high estrus and ovulation rates in a short time of period . This study was performed close to the end of the breeding season and inseminating these ewes as soon as possible was of critical importance.

The common practice is to submit ewes in the breeding season to natural mating under controlled conditions following detected estrous. In the flock at which this study was performed, about 5-6% of the ewes did not become pregnant after at least three matings with - rams of proven fertility. These ewes were submitted to a variety of commonly used synchronization protocols (Gordon, 1975; Timurkan and Yıldız, 2005). In the current study, ewes that were not pregnant after at least three matings at natural estrous during the breeding season were submitted to short term synchronization protocols. Progesterone was administered for 6 days and all treatments were performed on the day of the progesterone source removal to reduce the work force and stress (injection, aggregation). Planning the breeding program with lower labor cost and animal care is always desirable (Oyediji et al., 1990; Simonetti et al., 1999). These short term synchronization protocols could also have beneficial effects on regulating the estrous cycle of those ewes that would otherwise be culled off from the flock and submitted to the slaughterhouse.

MATERIAL AND METHODS

Animal Material

This study was performed in the Bahri Dağdaş International Agricultural Research Institute in Konya, Turkey. Anatolian Merino ewes (n=108), housed in this Institute, were used. Mean age of ewes was 5 years and mean body weight was 65 Kg. Breeding season started in early July (the year 2010) and ewes were submitted to mating with rams of proven fertility. The mating date was recorded for each ewe and pregnancy diagnosis was performed by ultrasonography 30-45 days after the mating, if the ewe did not come into estrus again. Non-pregnant ewes were again submitted to mating. This procedure was repeated until each ewe was allowed to mate three times and thereafter still non-pregnant ewes were included in the present study.

Experimental procedure

Three different protocols were used as follow (Fig 1): Group I: Ewes (n=30) were inserted intravaginal progesterone sponge (florogestan acetate, 30 mg; Chronogest® CR/Sünger, İntervet, İstanbul, Türkiye

) for 6 days and injected (i.m.) $PGF_{2\alpha}$ (tiaprost,125 µg; İliren, İntervet, İstanbul, Türkiye) at the time of sponge removal, Group II: Ewes (40) were treated same as in Group I and were also injected (i.m.) with eCG (250 IU; Chronogest/eCG, İntervet, İstanbul, Türkiye) at the time of sponge removal, and Group III: Ewes (n=38) were only injected $PGF_{2\alpha}$ at the same time with the ewes in the other two groups. Estrus was detected twice a day (06:00h and 18:00h, for 4 day) by aproned rams (n=4), 24 hours after $PGF_{2\alpha}$. All ewes detected in estrus were mated. Ten rams of proven fertility were used for mating. Pregnancy was diagnosed 60 days after mating by ultrasonography, as the routine farm management procedure of the latest pregnancy diagnosis of the breeding season, before possible culling.

Statistical analysis

Data were analyzed using z-test ratio comparison with Minitab vertion 16 statistics program. P value < 0.05 was considered to be significant.

Estrus rate was calculated by the number of ewes in estrus / number of ewes in the group x100, Conception rate by the number of pregnant ewes / number of ewes mated in heat x 100, Pregnancy rate by the number of pregnant ewes / number of ewes in the group x 100, Fetal loss by the number of pregnant ewes on day 60 that did not give birth / number of pregnant ewes x100, Lambing ratio by the number of ewes that gave birth / number of ewes in the group x 100, Prolificacy by the number of ewes / number of ewes that gave birth, Twinning rate by the number of ewes with twins / number of ewes in the group x 100.

RESULTS

The results of this study are presented in Table 1. Estrus detection rates, conception and pregnancy rates, lambing rates, number of alive lambs and prolificacy were similar in all three groups. Pregnancy loss after pregnancy diagnosis (d60) was higher ($P < 0.05$) in Group III (35.4%) compared to Group I (13.6%) and Group II (15%). Furthermore, a total of 84 lambs were born from 67 ewes.

Figure1. Experimental model of study. (Sponge=Florogestan acetate 30 mg, PGF= PGF2α 125 µg, eCG=250 IU).

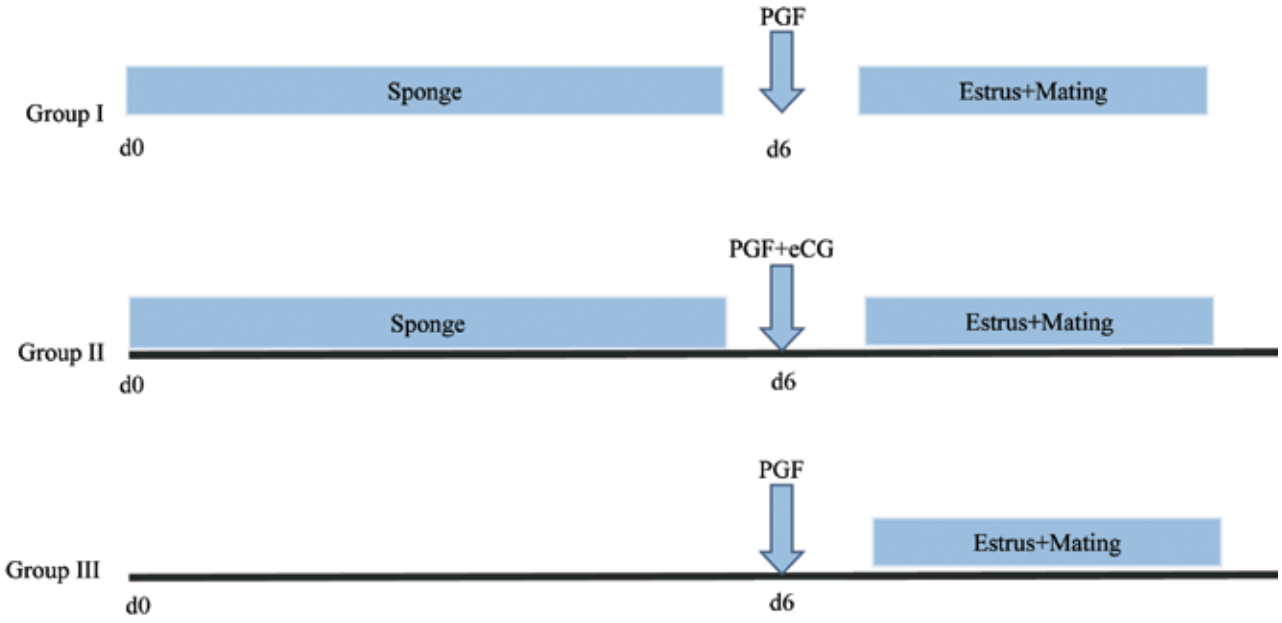

Table 1. Estrus detection, conception and pregnancy rate, lambing ratio, single and twin birth rate, number of alive lambs and prolificacy after short term estrous synchronization protocols.

Group	N	Estrus detection % (n)	Conception % (n)	Pregnancy % (n)	Fetal loss % (n)	Lambing ratio % (n)	Single birth % (n)	Twin birth % (n)	Number of alive lambs (n)	Prolificacy
I	30	100 (30/30)	73.3 (22/30)	73.3 (22/30)	13.6 (3/22)	86.4 (19/22)	84.2 (16/19)	15.8 (3/19)	22	1.16 (22/19)
II	40	92.5 (37/40)	89.2 (33/37)	82.5 (33/40)	15 (5/33)	85 (28/33)	75 (21/28)	25 (7/28)	35	1.25 (35/28)
III	38	81.6 (31/38)	100 (31/31)	81 (31/38)	35.4 * (11/31)	64.6 (20/31)	65 (13/20)	35 (7/20)	27	1.35 (27/20)
Total	108	90 (98/108)	87.8 (86/98)	79.6 (86/108)	22 (19/86)	78 (67/86)			84	

*Significantly different in the column
Group I (n=30): intravaginal progesterone sponge for 6 days plus $PGF_{2\alpha}$ at the time of sponge removal,
Group II (n=40) same as in group 1 plus eCG at the time of sponge removal
Group III (n=38) only $PGF_{2\alpha}$ at the same time with the ewes in the other two groups.

DISCUSSION

In the flock at which this study was performed, the ratio of non-pregnant ewes was about 5-6 % in the breeding season. Since this is a valuable purebred breed, the genetical and economical value of each ewe is important. Therefore, culling off the non-pregnant ewes from the flock was not a desirable option. Thus, short term synchronization protocols were used to remain as many ewes as possible into the flock. Controlled breeding program and hormonal intervention to possibly temporary infertility problem (apart from pathological problems or other situations e.g. inadequate management) might be useful. Two main synchronization strategies are commonly used in sheep reproduction; prostaglandin and progestagen based protocols. In this research institute, these two strategies were previously used during the breeding season on the same breed and pregnancy rates were about 66% (Kirbaş et al.,2012). Pregnancy rate in the present study was higher than that reported previously by Kirbas et al. (2012). Although pregnancy rates were high, the pregnancy loss was an important problem, because it was between 13.6 and 35.4 %. It has been reported that the embryonic loss in ewes after synchronization of estrus and ovulation reaches up to 30% (Wilmut et al., 1986; Nancarrow, 1994); however, multiple pregnancies are common in ewes and loss of an embryo does not cause a loss of pregnancy, since the other embryo continues to grow. In this study, pregnancy diagnosis was performed after day 60 (day 0: mating). Therefore, there is no clue whether non-pregnant ewes had also experienced an embryonic or fetal loss. The pregnancy loss was significantly higher in the group III (only $PGF_{2\alpha}$). Protocols based on $PGF_{2\alpha}$ result in good estrus and ovulation synchronization; however fertility was poor (Menchaca et al., 2004, Quintero-Elisea et al., 2011). Fierro et al. (2011) demonstrated that even double dose of PGF_{2a} 7 days apart created lower progesterone environment leading to low fertility. In the present study, estrus detection rate was about 81% and conception rate was 100% in $PGF_{2\alpha}$ group, which were similar to progesterone based groups. However, lambing rate was numerically lower than progesterone based synchronization protocols (64.6% vs 86.4 and 85%), probably because of higher fetal loss in the PGF group. This loss could be due to long term effect of lower progesterone environment or because preovulatory follicle was not developed properly (Fierro et al., 2011).

The results of this study are similar to earlier reports in different breeds of ewes. Estrus detection rates were between 70 to 95% after progesterone based protocols in different studies (Gonzalez-Bulnes et al., 2005; Koyuncu and Ozis, 2010; Ozyurtlu et al.,2016; Akbaş and Köse, 2017). In this study estrus detection rate was >90%. Furthermore, pregnancy rates on day 60 were 73.3 and 82.5% for the progestagen treated groups. These results clearly showed that ewes in our study had no problems in terms of fertility and they did not become pregnant after three matings in the breeding season possibly because of the management

strategies (early starting season, possible heat stress and vitamin/mineral deficiency). Similarly, ewes synchronized with prostaglandins resulted in even greater pregnancy rate compared to an earlier study in the same farm (81% vs 68% Köse et al., 2016). However, fetal mortality ratio in this group was clearly higher. This is a common problem with synhcronization protocols utilizing prostaglandins, as the embryo quality is somehow lower as shown by Zonturlu et al. (2011). Poor quality embryos after prostaglandin protocol, could explain the pregnancy loss in our study.

Regardless of the synchronization programs used, 86 ewes out of 108 became pregnant (as diagnosed on day 60) in this flock and 67 of them produced a total of 84 lambs. Originally, the aim of this study was to reevaluate ewes that did not become pregnant during the breeding season after at least three matings at natural estrus, in order to minimize the culling of otherwise valuable ewes from the breeding livestock. This has also economic value in terms of reintroducing the ewes back to the flock for the next breeding season and having extra lambs to sell. This aim was mostly achieved, since almost 80% of pregnancy rate (as diagnosed on day 60) was obtained and 78% of the pregnant ewes gave birth to 84 lambs. Instead of culling 108 ewes from the flock, only 22 ewes that did not show estrous were removed from the flock. The most immediate economic benefit, profit (sale of lambs) / total cost of synchronization was 40 folds (67000 TL from lamb sale vs 1600 TL cost for drugs used). In conclusion, valuable ewes could be subjected to short term synchronization protocols at the end of breeding season to prevent the loss of the purebred breeds and to sustain the productivity of the sheep industry. All protocols investigated in this study could be used in farms with similar problems.

CONFLICT OF INTEREST

None declared.

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Research article
Ερευνητικό άρθρο

Remarkable characterization and determination of atypical *E. coli* O157 none producing shiga toxin which can produce cytopathic effect on Vero cell and diarrhea in mice

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ABSTRACT. This study focused on the cytopathic effect of an unusual form of *E. coli* O157:H7 (atypical *E. coli* O157 (a-O157)) on Vero cell and infant mice. Multiplex PCR assay showed that they did not carry the gene for either shiga toxin 1 (stx1) or stx2 and other virulence factors. Increased changes in epithelial cell morphology, inter and intracellular gap junction and invading were assessed using Vero assay. In addition, a-O157infection causes disruptions of intercellular tight junctions, leading to clinical sequelae that include acute diarrhea in mice. Vero cell monolayers were exposed to a-O157influx and disintegrated tight junction of Vero cell line. Infected Vero cell enhance the number of attaching and effacing (A/E) lesions. Collectively, these findings provide in vitro evidence that Vero cell infected by a-O157was shown injury in epithelial cell barrier and induced A/E lesion the same as *E. coli* O157:H7 stx positive. These variants cannot be diagnosed by routine monitoring methods like biochemical assay for *E. coli* O157:H7 stx positive control. Finally, the data suggest that not only typical but also a-O157are an important cause of diarrhea in mice and produce cytopathic effect on Vero cell and may be harmful for food animal and also human.

Keywords: *E. coli* O157, Shiga toxin, Cytopathic effect, Vero cell, Diarrhea, Mice

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INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) O157 cause food-borne illness. It emerged in 1982 as a human pathogen that produces hemorrhagic colitis (HC) and the hemolytic-uremic syndrome (HUS) (Riley et al., 1983; Garcia-Aljaro et al., 2009). Most illness has been associated with eating undercooked contaminated ground beef, swimming in or drinking contaminated water, and eating contaminated vegetables (CCID, 2006). Although the primary reservoir and the main source of human infection are cattle, the bacterium O157 may be found in sheep, deer, goats naturally (Kudva et al., 2004), mice (Mohawk et al., 2010), rat (Zotta et al., 2008) and rabbit (García et al., 2006).

STEC strains have been shown to elaborate potent *phage-encoded* cytotoxins and other virulence factors as well. They divided into *two main groups*, shiga-like toxins (*stx1* and *stx2*) and several variants including three *stx1* subtypes (*stx1a*, *stx1c*, and *stx1d*) and seven *stx2* subtypes (*stx2a,* stx2b, *st x2c*, *stx2d*, *stx2e*, *stx2f*, and *stx2g)* (Garcia-Aljaro et al., 2006; Melton-Celsa et al., 2015). The toxin requires highly specific receptors which bind covalently to sugar residues on *cell-surface* glycoproteins (Paton and Paton, 2010).

All the above mentioned research results demonstrate that 1) toxin and receptor are the main factors for pathogenicity, 2) clinical STEC- O157 isolates lost their toxin genes during sub cultivation and became non-toxigenic (Paton and Paton 2010) and 3) other virulence factors may influence pathogenesis (Whitworth et al., 2008).

Although many studies have shown that stx is necessary for host pathogenesis and cytopathic effect on Vero cell line (Garcia-Aljaro et al., 2009), a non taxonomic pathogroup of *E. coli* O157 strains is affiliated with diarrhea that has no explainable stx genes, but does have the capability of inducing cytopathic effect on the Vero cell. They have also absence of locus of enterocyte effacement (LEE). They so called atypical *E. coli* O157 (a-O157).

Many fecal samples from distress food animals with diarrhea send to our laboratory for bacteriological identification. They are including *culture* on SMAC-CT, *selective and differential media* for isolation of stx-producing *E. coli*. Finally, in contrast to *E. coli* O157 with pale colony, the designated strain had a primrose appearance. In this study, these novel atypical *E. coli* O157 were characterized.

In this study, the cytopathic effects of a-O157 strains that did not harbor stx genes belonging to serotypes isolated from different distress food animals including calves, kids and lamb were evaluated. In particular, the research determines whether stx and LEE negative STEC O157 isolates could be considered virulent and indicates cytopathic effect on Vero cell and is associated with diarrhea in mice.

MATERIAL AND METHODS

Samples

Recto-anal mucosal swap (RAMS) samples from cattle, sheep and goats (ill and healthy animal) were examined for the presence of STEC O157. The RAMS samples were directly streaked onto sorbitol Mac Conkey ager supplemented with appropriate 0.05 mg/L cefixime and 2.5 mg/L *potassium tellurite* (SMAC-CT) (Merck).

Serological test

Various O157 and H Latex agglutination Kit (Mast group, UK) was used for both sorbitol and non sorbitol fermenting colonies.

Bacteria

Strains included in table 1 were grown on 5% sheep blood agar plates (blood agar base, Merck) and incubated overnight (O/N) at 37°C. According to previous study with some modification multiplex variation polymerase chain reaction (MV-PCR) was applied for molecular analysis of *E. coli* isolates (Tahamtan and Namavari, 2014).

DNA extraction and PCR procedure

The isolates cells were grown O/N at 37°C in luria bernati (LB) (Hi-Media) broth with shaking and DNA was extracted using extraction kit (DNP extraction Cina-Gene Company- Iran) for use as a whole-cell template. The 25-µl reaction mixtures included 2.5 U/µl Taq polymerase, 2 mM MgCl2, 0.4 mM deoxynucleoside triphosphates, 5µl 10× buffer, and 2 µl of the DNA whole-cell template. Thermocycler (Eppendorf, Germany) parameters included 95°C (5 min) one cycle and 94°C (30 s), 58°C (45 s), and 72°C (90 s) for 35 cycles, followed by a final extension time for 72°C (10 min). The oligonucleotides used as primers in PCR to detect *E. coli*, *E. coli* O157 and Stx1, Stx2 with variants are shown in table 2. According to PCR analysis a-O157 was applied in the experiment.

Table 1. Bacterial strains isolated from cattle, sheep and goats during routine fecal examination for bacterial identification.

Source	*E. coli* strains	Serotype	PCR results		Vero assay	Mice assay
			Stx1	Stx2	CPE[4]	Diarrhea
1	EDL933	O157:H7	+	+	+++	+
Cattle	RCC-C1387[2]	a-O157:H7[3]	-	-	++	+
Goats	RCC-G1387	a-O157:H7	-	-	++	+
Sheep	RCC-S1387	a-O157:H7	-	-	++	+
TSB	Control	-	-	-	-	-

1: Reference strains kindly provided from Professor David Gally, University of Edinburgh, UK
2: RCC-C (Razi Culture Collection- Cattle), RCC G (goat), and RCC S (sheep)
3: atypical *E. coli* O157
4: Cytopathic effect

Table 2. Oligonucleotide used in this study.

Primer set	Nucleotide sequence (5' – 3')	Size of amplified product (bp)	Gene specificity
Stx1-F Stx1-R	ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG	614	stx1 variants
Stx1OX3	GAA CGA AAT AAT TTA TAT GT CTC ATT AGG TAC AAT TCT	555	stx1OX3
Stx2-1 Stx2-2	CTT CGG TAT CCT ATT CCC GG GGA TGC ATC TCT GGT CAT TG	484	stx2 variants
Stx2-F Stx2-R	CCA TGA CAA CGG ACA GCA GTT CCT GTC AAC TGA GCA GCA CTT TG	779	stx2 variants [not stx2d
Stx2d-1 Stx2d-2	AAG AAG ATA TTT GTA GCG G TAA ACT GCA CTT CAG CAA AT	256	stx2d
Stx2v 1 Stx2v 2	CAT TCA CAG TAA AAG TGG CC GGG TGC CTC CCG GTG AGT TC	385	Stx2vha, stx2vhb, stx2d
3716F 3718R	GCCGCACAACAGCAGGATAAAC TCCGACCCGAAATTCCTTGC	500	T3SS
Stxe a Stxe b	CCT TAA CTA AAA GGA ATA TA CTG GTG GTG TAT GAT TAA TA	230	Stx2e
Intimin f Intimin r	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	881	eae

Tissue culture

Vero cells were used as model epithelia to form monolayers to study the function of all *E. coli* strains. Vero cell lines were acquired from the Razi Institute Cell Bank (Tehran, Iran). They were cultured in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GiBco) and 2% penicillin-streptomycin (Biosera) in an atmosphere containing 5% CO2. Cells were grown in 25-cm2 flasks (Orange) until they were confluent. In addition, monolayers were grown on 6.5- or 12-mm-diameter plates (Corning) at 37°C in the presence of 5% CO2 until they were used.

Vero assay

Twenty-four hours prior to epithelial cells being infected by bacteria, the culture medium was replaced with antibiotic and serum-free medium. Prior to infecting epithelial cells, bacteria were inoculated into 10 ml trypticase soy broth (TSB, Merck) and grown O/N at 37°C with agitation and balanced at a final concentration of 5×10^7 colony forming unit (CFU) ml^{-1} [the minimum CFU of pathogen which has been

shown to have cytopathic effect on the Vero cell after 3 h incubation (Tahamtan et al 2011)]. Serial dilutions of a-O157 (cattle, goats and sheep isolates) were made 1:10 in DMEM and added to each well. *E. coli* O157 EDL 933 stx positive was applied as positive control. Just one well received TSB as negative control. Infected cells then were incubated for up to 72 h at 37°C in 5% CO_2. The viability of cell cultures was checked under the inverted microscope in 3, 6, 9, 12 and 24 h, and up to 72 h of incubation.

Mice assay

Five duplicate groups of germ free Balb/c weaned mice (15 in each group) were chosen. All mice groups were fed orally with serial fourfold concentration of a-O157 (cattle, goats and sheep isolates) and *E. coli* O157 EDL933 (table 1). The remaining one group of mice received TSB as negative control. The infantile mice were followed a week for diarrheal sign and probable mortality rate.

Analysis of data

The results are declared as means - standard *deviations* of the means. Analysis of variance (ANOVA) was utilized to distinguish statistical significant differences ($P<0.05$) among various groups. A two-tailed, paired Student's *t* test was carried out to characterize statistical variation between groups.

RESULTS

Serotype characterization

Serotyping confirmed that the isolates expressed O157 and H7 antigens.

PCR

MV-PCR analysis verified the references strains harbored the stxs genes, but most of the test strains, including the RCCs1387, did not possess any stxs genes (they so called a-O157) (table 1).

Vero cell assay

Supernatants of all lysogens were toxic to Vero cells culture. The incubation of a-O157:H7 (10^7 CFU ml^{-1}) for up to 72 h induced an 80-100% CPE on the Vero cells, the same as positive control. On the other hand, STEC O157 converted to a-O157 that has no stx gene but is able to cause lesions on the cell line (figure 1).

The incubation of Vero cells with a-O157 for 3 h up to 24 h reduced the viability of cell. A trend toward increased cell degeneration is observed with cell exposure duration. Complete syncytial degeneration and round shriveled cells occurred within 72 h after inoculation (figure 1).

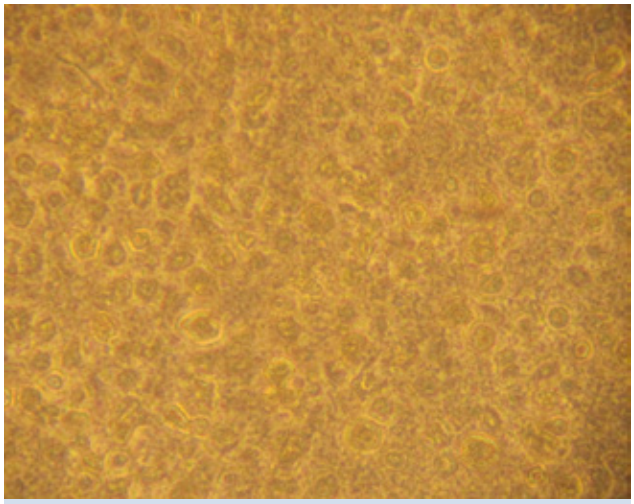

Figure 1. Photographs showing the infection by and the cytopathic effect on monolayer caused by minimum concentrations (10^4 CFU) of a-O157. Mild shrunken round cells are shown.

Logarithmic number of a-O157 demonstrated that viable cell lessened, but no significant differences was observed between lesions caused by various isolates (cattle, sheep and goats) ($p>0.05$). In contrast, a statistically significant increase in the cell degeneration among different exposure time was observed ($p<0.05$). The a-O157 at minimal concentrations (10^4 CFU ml^{-1}) produced only weak syncytial degeneration in the majority of infected Vero cultures when incubated for 3 to 6 h. No significant difference was observed between a-O157 and *E. coli* O157 EDL933 (positive control) ($p<0.05$).

Transmission microscopy of epithelial cells revealed normal cell morphology architecture with intact nuclei. In addition, phase contrast microscopy revealed that there were intact intercellular membrane appositions (Figure 2 a). In contrast, Vero cells infected with a-O157 had inter and intracellular vacuoles (Figure 2 b), and there were separate gaps in the conjunction of intercellular membrane contacts (Figure 2 c) and bacterium invaded cell (Figure 2 d).

Mice assay

a-O157 is able to induce generalized and persistent colonization in mice gastrointestinal (GI) tract the same as positive control. It is localized in the intestine, although it may preliminarily attach to payer's patches before colonization in colon. These strains cause a diversity of clinical complexities ranging from mild

diarrhea, bloody and severe diarrhea, to life threatening illness and finally the mice was dead.

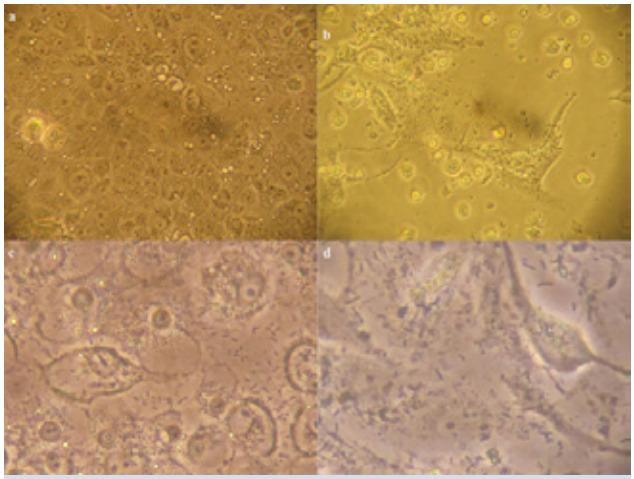

Figure 2. A-Bacterial strains adhere to the surfaces of Vero epithelial cells and demonstrated intact intercellular membrane appositions. B- Inter and intracellular vacuoles were observed after being infected by a-E. coli O157. C- After more exposure to a-E. coli O157 separate gaps in the conjunction of intercellular membrane were observed. D-Finally, the bacterium penetrating into the cells demonstrated cytopathic effect.

DISCUSSION

Several STEC serogroups are important cause of human disease, and the most common of these is O157:H7 (Bielaszewska et al 2007). Although the actual *role* of shiga toxin of *E. coli* O157:H7 in human pathogenesis is well characterized, in addition to these *toxins,* several *other* factors or *toxins may also play* roles *in the pathogenesis.* Furthermore, several genes on the virulence plasmid and on the chromosome have been suggested to have a part in virulence (Gyles 2007).

a-O157 strains were indistinguishable as they exhibited identical biochemical profiles like typical O157:H7 including growth on SMAC agar with no sorbitol fermenting and positive reaction for O and H antiserum tests. According to the genetic profile, a-O157 strains do not harbor stx and other virulence genes. *It has been* suggested that a-*O157* arose from the STEC O157 and the *ancestral E. coli O157*:H7 *has been* maintained during its evolution (Karch et al 2005). Actually, one of the differences was the presence or absence of the genomic segment containing stx, suggesting that a-O157 strains were derived from the O157 strains by the loss of stx in these animals. These STEC strains lost stx gene, giving rise to the a-STEC O157 strains, but kept the toxigenic properties. In fact, STEC O157 converts to a-O157, likewise in microorganisms which are epidemiologically pertaining to or are derivatives of the similar isolate.

Karch and Bielaszewska (2001) also reported sorbitol fermenting (SF) *E. coli* strains serotype O157: H^- in their laboratory that did not contain stx genes. These isolates originated from unrelated patients who suffered from HUS and diarrhea. Random amplified polymorphic DNA PCR analysis showed all isolates belonged to the same genetic cluster.

Moreover, during a family outbreak in Austria, stx-negative SF *E. coli* O157: H^-strains were isolated from patients with negative obligatory bacterial enteric pathogens (Allerberger et al 2000). Before that in Germany, anti O157 serum positive was observed in patients with HUS due to stx-negative SF *E. coli* O157: H^- strains (Schmidt et al 1999).

All the above studies only reported the disease and none of them explained the bacterial strains were isolated. But we have isolated a-O157 strains, examined them, and despite having no toxin gene, they were found to have a detrimental effect on Vero cells and were virulent in mice.

The finding of these *a-O157* pathogens in Vero cell line indicate they may have originated from a *progenitor* of STEC O157. For example, in the transformation of a- O157, the prophage in an integrated phage transformed ca harmless ancestral bacterium into a lethal pathogen (Brussow et al 2004). Feng et al. (2001) findings suggest that isogenic strains (stx negative) are a progeny strain that arose from the parental strain by losing the stxs genes and therefore pathogenicity remained active after losing their stx genes. These may be useful in studying the pathogenesis of stx in O157:H7 infections in humans.

Indeed, whereas cattle have been well established as a major reservoir of STEC O157:H7 (Pierard et al 2012), the origin of stx-negative *E. coli* O157:H7 strains (a-O157), their role in disease, and their pathogenic mechanism are still *not* fully understood. This mention declares that the epidemiology of a-O157:H7 infections may differ from the epidemiology of infections caused by STEC O157:H7.

Pathogenicity of a-O157 isolates is primarily due to its ability to invade and destroy the tissue culture. These are detected by invasion assays using Vero tissue cell culture. However, regardless of these experimental conditions, Vero assay and mouse experiments are not

a good model for explanation of human disease.

a-O157 may internalize but not adhere to Vero cells, and then worsen the tissue culture. Some factors other than stx may exert cytopathic effect on monolayer. The mechanism by which the bacteria *lose* their *stx genes* and kept pathogenicity *has not been* explained. While *these findings suggest that cattle can* excrete very large amounts of *E. coli* O157, the rare isolation of these pathogens from *animals* which are considered as *reservoirs of* atypical O157 *led to the* hypothesis that atypical O157 might be compromised in the human GI tract. Even if this *hypothesis* is confirmed, works are in progress to purify and *further* characterize the influence of a-O157 in human disease. However, the a-O157 strains ability to influence cytopathic effect on Vero cell mediated by unknown process remains. Further study is needed for confirmation of isolates by genome sequencing to determine the phylogenetic relationship with other O157 to detect the heterogeneity within the isolates.

CONCLUSION

Cattle are the main reservoir of *E. coli* O157:H7 but a rare strain of this, a-O157 may not adhere to Vero cells and deteriorate the tissue culture with no Stx genes and might be challenge in human GI tract.

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CONFLICT OF INTEREST

There is no conflict of interest.

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Research article
Ερευνητικό άρθρο

The survivin gene expression in neoplastic hepatocytes from chickens infected with Marek's virus

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ABSTRACT. P53 protein is one of the main proteins in apoptosis pathway. The role of survivin protein in inhibition of P53 in different human cancers has been proved. The expression of survivin can be a main marker in diagnosis and prognosis of different human cancer. Until now, the survivin gene expression in birds infected with Marek's disease was not investigated. In this study, liver tissue samples of chickens infected with Marek's disease virus (MDV) was collected. The identification of MDV was carried out with PCR and histopathologic examination. After identification of MDV, the pathogenicity of infected virus was investigated with specific primers targeted on 132 bp tandem repeat. After this, the survivin gene expression was examined in neoplastic liver samples by Real-Time PCR. The PCR amplification of tumor liver samples showed that all samples were infected with MDV. The result of histopathology and amplification of 132 bp tandem repeat in tumor samples showed that the chickens were infected to pathogenic MDV. Results showed that the survivin gene expression in neoplastic hepatocytes was significantly higher than normal hepatocytes. In conclusion, survivin gene expression can be utilized as a suitable biomarker in diagnosis of Marek's disease in birds. It seems that viral *meq* oncogene in Marek's disease can play role in induction of survivin expression in this disease that it is necessary to be proved.

Keywords: Chicken, Gene expression, Marek's disease, Survivin.

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INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease in chickens that caused by oncogenic herpesvirus (Tulman et al., 2000). This virus replicates in the lymphoid and epithelial tissues (Biggs, 2001). The virus can form lymphoma in different organs and caused anemia and immunosuppression in chickens, especially layers and breeders (Calnek, 2001). The economic importance of this infection is related to mortality, decreased egg production and hatchability in chickens (Schat and Nair, 2013).

The disease firstly described by Joseph Marek, a veterinarian, in a cockerel with nervous sign (Marek, 1907). The virus can induce different and variety forms. The acute classic or visceral, neurological, cutaneous and ocular are common forms of this infection. The prevalent and main form of MD is acute classical form that is characterized by lymphoma in different visceral organs like liver, heart, lungs, spleen, kidney, bursa of Fabricious, proventiculus and gonads (Calnek, 2001).

Survivin, a newly characterized member of the inhibitor of apoptosis proteins (IAP), is a bifunctional protein which regulates cell proliferation and suppresses apoptosis (Altieri and Marchiso, 1999). Survivin is highly expressed during embryonic development and fetal tissues (Altieri, 2015). There is limited information about survivin expression in most normal differentiated tissues (Ambrosini *et al*., 1997). Survivin is also overexpressed in a wide variety of human neoplasms, suggesting that reactivation of the survivin gene frequently occurs in neoplasms (Ambrosini *et al*., 1997).

Currently, survivin protein expression is being used as a prognostic factor in several human neoplasms (Johson and Howerth, 2004). High survivin expression in human neoplasms associated with more aggressive behavior, reduced response to chemotherapeutic agents and decreased survival times, compared with neoplasms that are survivin negative (Altieri, 2015). Manipulation of survivin regulation and expression may leads to the development of new immunotherapy and gene therapy strategies for the treatment of neoplasms (Altieri and Marchiso, 1999). Until today, there is no information regarding survivin gene expression in chicken.

The purpose of this study is to demonstrate the survivin gene expression in chicken's neoplastic tissues and the comparison of the expression rate of survivin in normal and neoplastic tissues.

MATERIALS AND METHODS

After identification of chicken flock with MD, the suspected chickens were transferred to laboratory. The chickens euthanized and necropsied for sampling. The chickens that showed nodular lesions in visceral organs were selected for further examination. For each flock, 3 liver samples with nodular lesions were taken. The sampling was carried out for the detection of MDV and identification of pathogenicity by PCR test, pathological examination and survivin expression by Real-Time PCR. The samples were collected from 5 suspected flocks. Furthermore, liver samples from 5 apparently healthy chickens were collected as negative control.

DNA was extracted from nodular and normal liver tissues using a commercial DNA extraction kit (AccuPrep Genomic DNA Extraction Kit**,** Bioneer co., South Korea).

PCR was carried out to amplify fragments of 314 and 434 bp of the antigen A (specific for MDV-serotype 1) and 132 bp tandem repeat of MDV, respectively. The sequences of the antigen A and 132 bp tandem repeat primers were as following: forward primer: 5′-GAG GTA CCT CAT GGA CGT TCC ACA-3′; reverse primer: 5′-ACA TTC TTT TCG TTG GCG TGG TAT-3′ (Antigen A) (Bechker *et al*., 1992). Forward primer: 5′-TAC TTC CTA TAT ATA GAT TGA GAC GT-3′; reverse primer: 5′-GAG ATC CTC GTA AGG TGT AAT ATA-3′ (132 bp tandem repeat) (Bechker *et al*., 1992). PCR amplification was performed in PCR buffer containing 2 m*M* MgCl2, 200 μ*M* each dNTPs, 1 μ*M* each primer, and 1.0 unit of *Taq* polymerase (Fermentas, Germany) in a 25 μL total reaction volume. The 100 bp DNA ladder plus (Fermentas, St. Leon-Rot, Germany) was used. The amplification was carried out in a thermal cycler (Mastercycler Gradient, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) using the following conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation, annealing, and extension at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec respectively, and a final extension at 72°C for 5 min. The PCR product was then analyzed by electrophoresis in 1% agarose gel and visualized under UV light after staining with ethidium bromide.

In this study live HVT vaccine (FC126 strain belonged to serotype 3, Merial, France) and sterile water

were utilized as positive and negative, respectively.

For analysis of lymphocytic infiltration in liver, the tissue samples were fixed in neutral 10% formaline solution, embedded in paraffin and cut into 4 micrometer thick sections. After deparaffinization, the sections were stained with hematoxylin-eosin (H & E) method (Bancrof and Stevens, 1996).

Total RNA was extracted from normal and neoplastic hepatocytes using a RNeasy kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using a QantiTect Reverse Transcription Kit (Qiagen, Valencia,CA, USA) according to manufacturer's instruction.

Real-time RT-PCR for Survivin gene was performed using specific QuantiTect primer assay (Fowl survivin, Cat no. QT00595896, Qiagen, Valencia, CA, USA) using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA).

The GAPDH was used as housekeeping gene. For the amplification of the GAPDH gene a pair of primers was synthesized by Bioneer (South Korea). The primers' sequences for chicken GAPDH were: Forward primer, 5'-GGTGGTGCTAAGCGTGTTA-3'; reverse primer, 5'-CCCTCCACAATGCCAA-3', resulting in an amplified product of 179 bp (Accession No. X01578) (Li *et al*., 2005).

Quantitative Real-Time RT-PCR was performed in a 20 μL reaction volume containing 10 μL of the SYBR Green PCR master mix (Fermentase, Germany), 1.5 μL of the RT reaction mixture, 0.5 μL each primers and 7.5 μL dH2O using the Applied Biosystems StepOnePlus™ Real-time PCR system (Foster City, CA, USA).

Amplification program included of initial denaturation step at 95°C for 10 minute, flowed by 40 amplification cycles consisting of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and extension at 72°C for 30 s. Real-Time PCR assays were carried out in triplicates and repeated three times. Relative gene expression was calculated using the standard curve method.

All data were analyzed by the statistical package for social sciences (SPSS) version 18.0 software (Chicago, Inc, USA) using one-way analysis of variance (ANOVA) statistical method at $p< 0.05$.

RESULTS

In suspected chickens to MD, a variety of macroscopic lesions was observed in visceral organs where focal nodular and diffuse whitish spots on the liver were featured (Figure 1). In apparently healthy chickens no macroscopic lesions were observed in liver or other organs.

Figure 1. The gross pathological lesions on liver in suspected chicken to MD.

The microscopic section of liver represents focal neoplastic infiltration which it is accompanied with pleomorphic cells. The neoplastic foci are consisting of lymphocyte and lymphoblast infiltration with different size, from small to medium (Figure 2). In apparently healthy chickens with normal liver tissues no microscopic lesions were seen.

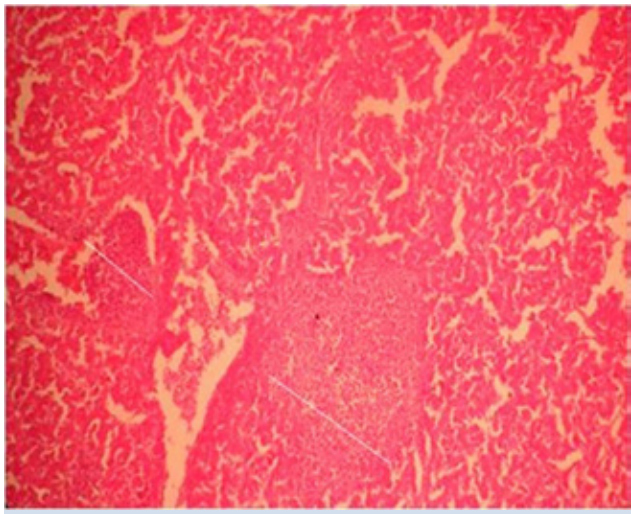

Figure 2. The neoplastic foci in liver of MDV infected chickens (H&E).

The 314 bp fragment related to antigen A of MDV was amplified in all liver samples with neoplastic lesions similar to positive control. In positive control, the 132 bp tandem repeat was not amplified while in all clinical samples the 132 bp tandem repeat with band size of 434 bp was amplified. The 314 and 434 bp fragments were not amplified in samples from apparently healthy chickens (Figure 3).

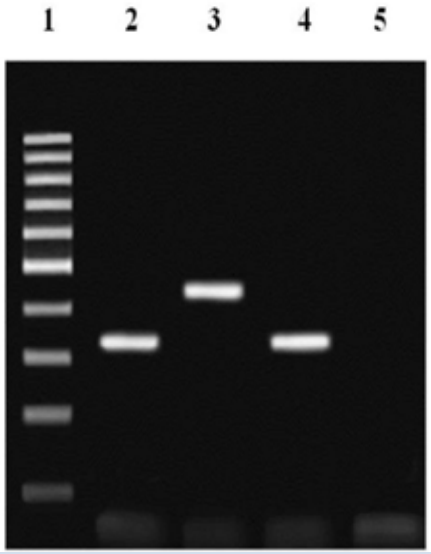

Figure 3. The PCR amplification of the Antigen A and 132bp tandem repeat of MDV in tissue samples from chickens (Lane 1: DNA ladder marker (100 bp) ; lanes 2: positive samples for Antigen A of MDV; Lane 3: positive samples for 132 bp tandem repeat; Lane 4: positive control (HVT Vaccine); and Lane 5: negative control).

The results showed that survivin is expressed in normal and neoplastic hepatocytes in chickens. The expression rate of survivin in normal and neoplastic hepatocytes was 0.113±0.136 and 1.440±0.500, respectively. The comparison of expression rate showed that there is a significant difference in survivin gene expression in normal and neoplastic hepatocytes in apparently healthy and MDV infected chickens (Figure 4).

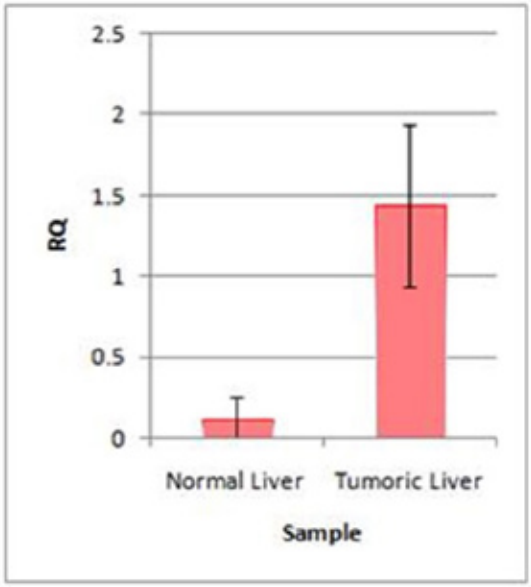

Figure 4. The expression of survivin gene in normal and neoplastic hepatocytes in chicken.

DISCUSSION

Marek's disease is the most important infectious disease in layers and characterized by development of lymphomas in different visceral, epithelial and nervous organs (Schat and Nair, 2013). PCR is the rapid and sensitive test to detect the presence of MDV in clinical samples (Shahzad, *et al*., 2007). Becker *et al* (1992) had designed one pair of primers that it could distinguished the pathogenic and non pathogenic MDV by a PCR assay amplified a 343 bp fragment of 132 bp tandem repeat. Zhu *et al* (1992), Becker *et al* (1992) and Kozdrun *et al* (2001) have declared that the PCR tests can be used for the identification of virulent MDV DNA in pathological samples and to distinguish between the virulent and non-virulent MDV in chickens. Therefore, in this study were utilized two pairs of primers designed on secretory antigen A and on the tandem repeat of 132 bp in order to identify MDV DNA in clinical samples and to differentiate the detected DNA, respectively. PCR and pathology results estimated that the sampling stage has been in stage of tumorigenesis of virus which in this time point, the viral antigen has been detected in tissues with microscopic lesions. Although this study increased knowledge regarding the Marek's disease of birds, it can be also used as a model about human cancer.

P53 protein is one of the most fundamental proteins for apoptosis induction and tumor inhibition (Shaikh and Niranjan, 2015). In normal condition, activation of apoptosis pathway and cell proliferation preserves normal size and performance of tissues (Nachmias *et al*., 2004). Mutation in protein P53 could lead to inactivation of apoptosis pathway, disorder of cell biochemical reactions and abnormal growth of cells (Shaikh and Niranjan, 2015). Survivin has been identified as a member of IAPs family (Johnson and Howerth, 2004). In this study, survivin gene expression in MDV infected hepatocytes in chickens was investigated and evaluated by sensitive and precise method of Real-Time PCR. Survivin plays role in cell proliferation, decrease of apoptosis of tumor cells, resistance to chemotherapy, radiotherapy and recurrence of many human cancers (Alteiri, 2015; Johnson and Howerth, 2004; Lei *et al*., 2010). But until today, it hasn't been investigated in tumor cells of birds. This gene is available in most germinal cells or in differential cells and most of cancers (Altieri, 2015). High expression of survivin in cancer cells indicates its prominent role for apoptosis inhibition during development of tumor. The gene is considerably expressed almost

in all human tumors (Verdecia *et al*., 2000). The cancers that have been reported high expression of this gene include lung, breast, gastric, esophagous, thyroid, colon (Altieri, 2015), larynx, pancreatic (Kato *et al*., 2001), bladder (SWANA et al., 1999), uterine (Saitoh *et al*., 1999), ovarian (Yoshida *et al*., 2001), liver (Ito *et al*., 2000), non-melanoma skin (Grossman *et al*., 1999) cancers. Tamm *et al* (2000) investigated expression of survivin in 60 lines of human cancer cells and observed high expression of survivin for all 60 samples. Furthermore, it has been recently indicated that anti-survivin drugs sensitizes tumor to chemotherapy by induction of apoptosis (Olie *et al*., 2000). Thus, survivin is studied as a suitable antigen for both diagnosis and treatment.

In current study, survivin gene was expressed and compared in liver normal and tumor cells but its expression rate was significantly higher in tumor cells. It seems the high expression of survivin in liver tumor cells in MDV infected chickens could be attributed to the interaction of *meq* oncogenes of Marek's virus with P53 protein. Recently, correlation between *meq* protein of MDV and HSP70 has been proved as an inductor of neoplasm (Zhao et al., 2009) and CDK2 and Rb proteins have been proved as regulators of cell cycle (Altieri, 2015; Shaikh and Niranjan, 2015, Nachmais *et al*., 2004; Zhao *et al*., 2009). Also, it has been shown that *meq* protein could shorten G1 phase of cell cycle (Nair and Kung, 2004). In this regard, some studies indicate that viruses' oncogenes are effective in high expression of survivin. So, already relationship between protein X of hepatitis B virus in human hepatocarcinoma (Marusawa *et al*., 2003), protein Tax type 1 of T-cell leukemia virus (Kawakami *et al*., 2005) and protein E6 of popilloma virus (type 16) in uterine cancer (Borbely *et al*., 2006) with the overexpression of survivin gene has been indicated. Also, it has been shown that oncogene protein LMP2A of Epstein-bar virus can induce the expression of survivin and the prohibition of apoptosis (Hino *et al*., 2008). Therefore, the high survivin expression in liver tumor cells could be due to the activation by *meq* oncogene of MDV.

In this study, has been shown that survivin expression in liver tumor cells has significantly been higher than liver normal cells. Survivin expression in normal cells can be explained by the presence of very small numbers of stem cells (Altieri 2005) in liver tissue that could be used for the preservation of tissue homeostasis. Liver tissue is always regenerating cells to preserve the tissue homeostasis against different chemical materials including drugs and bacterial toxins. Expression of survivin gene in this organ could be associated with the existence of premature and undifferentiated cells at primary step of cell regeneration. In addition, an inhibitor of apoptotic proteins has been observed in some cells of soft tissues. Chen *et al* (2003) and Berendse *et al* (2003) showed reduced gene expression of GLN3 in normal muscle tissue that is because of presence of some myoblast cells with properties of stem cells in muscle tissues. Fan *et al* (2006) presented gene expression of GLN3 in normal tissue and renal tumors that this finding may be as a result of increased proliferation of renal epithelial cells. Thus, according to the low expression of survivin gene in normal liver cell, the high expression of the gene in tumor hepatocytes could be used as a target for immunotherapy in MDV infected birds

In conclusion, the results of this study showed that survivin gene is significantly expressed as an inhibitor of apoptosis gene in tumors with MDV origin in chickens. Immunotherapy of this protein creates a new perspective for the treatment of Marek's disease in valuable birds.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research article
Ερευνητικό άρθρο

Assessment of the efficacy of routine vaccination on the magnitude of Foot and Mouth Disease outbreak in Kafrelsheikh governorate, Delta Region, Egypt

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ABSTRACT. Foot and mouth disease (FMD) is a highly contagious viral disease causes a serious economic impact on livestock production and trading. FMD is an endemic disease in Egypt and a national control program that depends on routine obligatory vaccination of all ruminant species is being followed for disease control. A nation-wide epidemic of FMD was commenced in early 2015 and typical clinical signs of the disease were observed even in vaccinated animals. The morbidity and case fatality rates were high enough to be investigated. In the current study, non-vaccinated and vaccinated animals of different sex and ages were examined to evaluate the efficacy of FMD different vaccines used in Egypt. Clinical, post-mortem and serological examinations were used to confirm the infection, while the molecular investigation was applied to identify the serotype responsible for this epidemic. The incidence rate and the attributable proportion (fraction) of FMD cases which could be avoided by vaccination and vaccine efficacy were calculated. The obtained results confirmed the infection with FMD virus (FMDV) serotype O in both non-vaccinated and vaccinated animals. The incidence of FMD was 86.67% among non-vaccinated animals, while it was ranged from 15% to 31.8% among vaccinated animals according to the type of vaccine used. The attributable fraction was 73.9% and the efficacy of the three used vaccines was 63.3%, 76.92% and 82.25% for Tri-Aphthovac, VSVRI and Meriel vaccines, respectively. In conclusion, vaccination in Egypt is able to minimize the magnitude of outbreaks caused by the same serotype found in the vaccine but was not able to prevent the infection and eliminate the disease. The highest vaccination efficacy was found in Mid-aged animals and male cattle.

Keywords: FMD, Vaccine efficacy, Egypt

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INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals. Foot and Mouth Disease virus (FMDV) virus belongs to genus *Aphthovirus* of family *Picornaviridae* and has 7 distinct serotypes under which many subtypes exist. In Egypt, outbreaks are being reported since 1950 and FMDV serotype O is the most prevalent circulating serotype in most of these outbreaks (Aidaros, 2002; Knowles, et al., 2007). FMDV serotype A was reported in 1953, 1956, 1958, 1967 and in a major outbreak during 2006 (Mackay et al., 1998; Farag et al., 2006; Knowles et al., 2007). In addition, FMDV serotype SAT2 was reported in 1950 and a major outbreak in 2012 (FAO, 2012; Kandeil et al., 2013; Shawky et al., 2013).

The disease has serious economic effects on bovine production and trading because of its transboundary nature of transmission (OIE, 2009). FMD is characterized by fever, lameness, oral lesions and marked salivation. Carrier state usually develops when immunized animals are subjected to infection as well as after the clinical recovery of the diseased animals. Control of FMD depends on the prevention of virus transmission from infected to susceptible animals (in free countries) or by reducing the number of susceptible animals through vaccination (in endemic countries) (Aidaros, 2002). FMD vaccines are usually multivalent because of the limited cross protection between FMDV serotypes (Kandeil et al., 2013). In Egypt, three different FMD vaccines are available; the governmental vaccine prepared by Veterinary Serum and Vaccine Research Institute (VSVRI, Abbasia, Egypt), Tri apthovac vaccine produced by ME VAC company (Cairo-Egypt) and polyvalent Merial vaccine (Merial, France). The two former vaccines contain inactivated A, O and SAT2 serotypes of FMDV. The last vaccine is a commercial vaccine being purchased privately on a wide scale of the farmers, especially large farms to control FMD. This vaccine contains 6 serotypes of FMDV and this is the reason of preference to it by some farmers who believe that this vaccine is more protective and could prevent the deleterious effect of FMD. The first two vaccines are being used in the national control program carried out by the general organization of Veterinary Services (GOVS). This program relies on the vaccination of all ruminant species except camels in Egypt with FMD vaccine twice a year. The vaccination coverage every year does not reach the required figure of target animal population targeted by vaccination due to lack of funding to produce sufficient doses of vaccine every year. This study aimed to assess the vaccination efficacy of the three mentioned vaccines under the field conditions at Kafrelsheikh Governorate (Egypt), describe the main clinical signs in clinically infected animals and identify the circulating FMDV serotype in both non-vaccinated and vaccinated animals in this locality.

MATERIAL AND METHODS

Ethical statement This study was conducted following the ethical protocols and guidelines of the Medical Ethics Committee, Faculty of Medicine, Assiut University, Egypt.

Study area

The current study was conducted in Kafrelsheikh Governorate; an area of 3,437 km² with a very high density of livestock in heart of the Nile Delta. The Governorate consists of 10 districts and 206 villages. Kafrelsheikh is located in the northern part of Egypt, along with the western branch of the Nile and its capital is Kafrelsheikh City (Fig. 1).

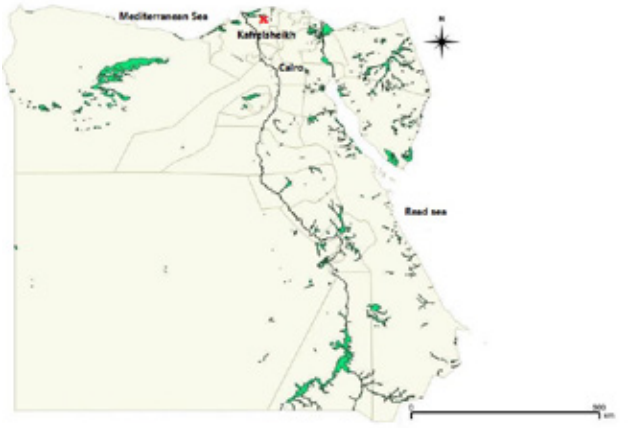

Figure 1. Map shows the area of the study.

Animals

A total of 180 cattle were selected randomly under the field conditions during 2015 FMD outbreak in Kafrelsheikh Governorate. Animals included in the study were divided into two groups, 105 animals were non-vaccinated and 75 animals were vaccinated according to the vaccination program in Egypt (two doses with 6 month interval). These vaccinated animals were vaccinated with one of the three different vaccines commonly used in Egypt; 40 and 22 animals were vaccinated with the vaccine produced by Veterinary Serum and Vaccine Research Institute (VSVRI)

and Tri-Aphthovac vaccine (commercial vaccine produced by the Middle East for Vaccines (ME VAC) company), respectively. These vaccines are being used in the national compulsory vaccination campaigns of the GOVS. Also, 13 animals were vaccinated with the commercial vaccine produced by Meriel.

Clinical examination

The animals were routinely examined for the clinical symptoms of FMD with special attention for the body temperature, mouth cavity, inter-digital space and heart sounds (Jackson and Cockcroft, 2002)

Serological Diagnosis

During the virus replication in the infected animals there are different types of the non-structural proteins (NSPs) are generated and they are a potential target for the immune system. Therefore, those infected animals produce a considerable titer of antibodies (Abs) against NSPs. These Abs are the target of 3ABC ELISA test to differentiate between clinically infected or carrier animals from immunized animals after vaccine application (Brocchi et al., 2006; Clavijo et al., 2004). Serum samples were collected from animals included in the study and tested by SVANOVIR® FMDV 3ABC-Ab ruminant (Boehringer Ingelheim Svanova, Sweden). This ELISA kit differentiates between the antibodies of the active infection and vaccination and positive samples have percent positive positivity (PP) ≥48

Histopathology

Tissue samples from the oral and lingual mucosa were collected from clinically infected animals. Oral, lingual mucosa heart and lung were collected from the dead animals; all these samples were subjected to histopathology (Alexandersen, 2003; Alexandersen and Mowat, 2005).

Molecular Diagnosis

RNA-Extraction

Tissue samples and vesicular fluid were collected from clinically infected animals. Samples transported to the laboratory on ice in viral transport media containing equal volumes of glycerol and phosphate buffered saline (pH 7.2- 7.6). Viral RNA was extracted using the GeneJET RNA Purification Kit (Thermo Fisher SCIENTIFIC - K0731).

Reverse Transcription PCR (RT-PCR)

All samples underwent testing using a two-step RT-PCR. During the first step, Applied Biosystem Kit Cat. No. 4374966 was used for cDNA synthesis. All cDNAs were tested using the P1/P2 universal primers, as well as the serotype-specific primers, respectively (Table 1). Thermal cycling conditions for the universal primers were as follows: 95 ℃ for 5 min, followed by 45 cycles of 94 ℃ for 30 seconds, 48°C for 30 seconds, and 72 ℃ for 1 min. A final extension was performed at 72 ℃ for 10 minutes. The annealing temperature (48 ℃) was modified when serotype-specific primers were used. It was 46 ℃ for serotype O primers, 60 ℃ and 56 ℃ for general SAT and serotype SAT2 primers and 55 ℃ for serotype A primers (EL-Kholy et al., 2007; EL-Shehawy et al., 2011; EL-Khabaz and AL-Hosary, 2017).

Gel Electrophoresis

The PCR products were subjected to 1% agarose gel electrophoresis using ethidium bromide staining and gel documentation system to detect positive bands 216, 402, >700, 880, 863-866 bp which are specific of FMD universal primer, serotype O, SAT, SAT2 and Serotype A, respectively.

Table 1. Different primer used during this study according to EL-Kholy et al., 2007 and EL-Shehawy et al., 2011.

Primer		Sequence (5' to 3')	Length (nt)	Specific band
Universal primer	P1 P2	5'- CCTACCTCCTTCAACTACGG-3' 5'-GAAGGGCCCAGGGTTGGACTC -3'	20 21	216 bp
Serotype O 1D/2B region	PH1 PH2	5'-AGC TTG TAC CAG GGT TTG GC-3' 5'-GCT GCC TAC CTC CTT CAA-3'	20 18	402 bp
Serotype SAT primers	SAT-ID209F FMD-2B208R	5'CCACATACTACTTTTGTGACCTGGA -3' 5'-ACAGCGGCCATGCACGACAG -3'	25 20	≥700 bp
Serotype A primers	PH9 PH10	5'-TAC CAA ATT ACA CAC GGG AA-3' 5'-GAC ATG TCC TCC TGC ATC TG -3'	22 20	863-866 bp

Epidemiological examination

Risk "Cumulative Incidence" was calculated in both non-vaccinated and vaccinated groups according to the following equation

Number of disease onsets/ the number of animals exposed to risk x 100

The attributable fraction which means the expected percentage of reduction in number cases following vaccination and was calculated according to the following equation:

(Number of disease onsets/ the number of animals exposed to risk) x 100

The attributable fraction which means the expected percentage of reduction in number cases following vaccination and was calculated according to the following equation:

((The Risk for exposed group - risk for the unexposed group)/ Risk for exposed group) x100

Vaccine efficacy or Vaccine effectiveness for each vaccine separately was calculated according to the following equation

((Risk among non-vaccinated group − risk among the vaccinated group)/ Risk among the non-vaccinated group)) X 100 (Thrusfield, 2005).

Risk estimates and vaccine efficacy were also estimated for different age groups and sex of the animals used in this study.

RESULTS

Results of clinical, post-mortem and histopathological exanimation

The entire seropositive animal showed the clinical symptoms of the disease which include fever (≥40 C°), excessive salivation, vesicles and erosions on the dorsum of the tongue and hard palate and interdigital space associated with interdigital dermatitis and lameness. Some cases suffered from additional complications like inapetance, detached claws, erosions and ulceration on teats and udder and incurable chronic mastitis. Some cases died suddenly without developing any clinical signs.

Fifty-three animals died due to infection with FMD (12 animals from the vaccinated group and 41 from the non-vaccinated group). All of them showed the clinical signs of the disease. All of these animals were subjected to post-mortem examinations showed congestion of the heart and lung. Histopathological examination revealed the presence of the hydropic degeneration in spinosum cells at covering oral mucosa, this degeneration leads to the appearance of vesicular lesions and subsequently followed by erosive stomatitis. Also, histopathological examination revealed the occurrence of congestion and hemorrhage of the myocardium, lymphocytic interstitial myocarditis, myocardiolysis and extensive myocardiolysis with lymphocytic cell infiltration which is one of the pathognomonic lesions of FMD affections (Fig. 2, 3).

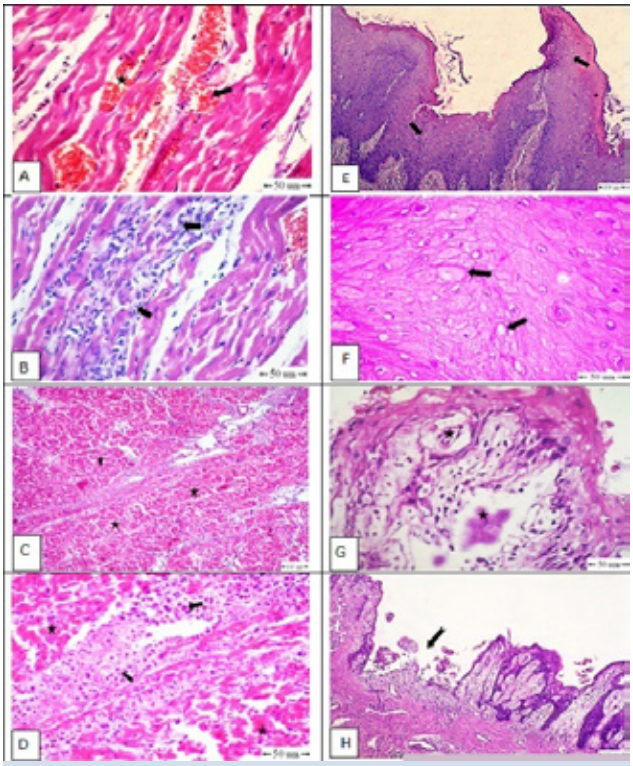

Figure 2. Histopathological finding (I) of heart (A, B, C&D) and oral mucosa (E, F, G &H) from calves infected with FMD. A, Sever congestion (star) and hemorrhage (arrow) in the myocardium (bar=50). B, Lymphocytic interstitial myocarditis (arrow) (bar=50) C, Myocardiolysis (star) (bar=100). D, Extensive myocardiolysis (star) with lymphocytic cell infiltration (arrow) (bar=50). E&F, Hydropic degeneration in spinosum cells at the covering oral mucosa (arrow) (bar=100 &bar=50 respectively). G, Vesicular stomatitis (star) (bar=50). H, Erosive stomatitis (notched arrow) (bar=100).

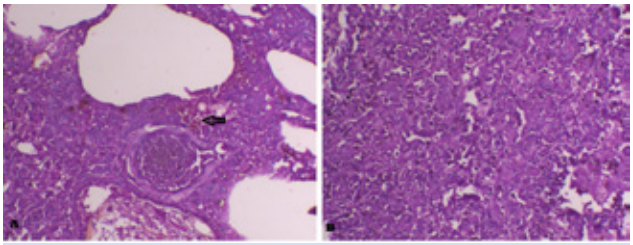

Figure 3. (A) Sever congestion of myocardium and (B) Sever congestion of the lung.

Table 2. Incidence rates in both unvaccinated and vaccinated animals and vaccines efficacy rates.

Non-Vaccinated	Seropositive	Seronegative	Incidence	Vaccinated		Seropositive	Seronegative	Incidence	Efficacy
				Tri-Aphthovac-vaccine	(22)	7	15	31.8 %	63.3%
(105)	91	14	86.67	(VSVRI) Vaccine	(40)	Seropositive	Seronegative	Incidence	Efficacy
						8	32	20 %	76.92 %
				Meriel vaccine	(13)	Seropositive	Seronegative	Incidence	Efficacy
						2	11	15.38 %	82.25 %

Table 3. Incidence rates in both unvaccinated and vaccinated animals and vaccines efficacy rates according to animal's sex and age.

Unvaccinated				**Vaccinated**			
sex	seropositive	seronegative	incidence	seropositive	seronegative	incidence	Vaccine efficacy
Females	52	8	86.7%	12	28	30%	70%
Male	39	6	86.7%	5	30	14.28%	85.71%
Unvaccinated				**Vaccinated**			
Age	seropositive	seronegative	incidence	seropositive	seronegative	incidence	Vaccine efficacy
Blow one year	9	0	100%	12	8	60%	40%
↑1 year:↓5 years	62	14	81.58%	4	50	7.4%	92.6%
↑5 year	20	0	100%	1	0	100%	0%

Results of the serological and epidemiological examination

Within the non-vaccinated group 91 out of 105 animals were seropositive (86.7%), while within the vaccinated group only 17 out of 75 animals were seropositive (22.7%); (7, 8 and 2 seropositive out of 22, 40 and 13 selected animals vaccinated with Tri-Aphthovac, VSVRI and Meriel vaccines, respectively) . All these animals were seropositive against the 3ABC NSPs of the virus (Table 2).

The vaccine efficacy was 63.3%, 76.9% and 82.3% for Tri-Aphthovac, VSVRI and Meriel vaccines, respectively. Attributable fraction for vaccine effectiveness during this epidemic was 73.9%.

The incidence rate and vaccine efficacy were affected by some risk factors like sex and age. In the non-vaccinated group the incidence rate was 86.7% in both sexes and 100% in the young animals below one year and old animals above five years while it was 81.58% in animals in mid-age (above one year and below five years). In the vaccinated group, the incidence was higher in females than in males. The vaccine was more efficient in males than females; 85.71% and 70%, respectively. The incidence rate was higher in young animals and old animals of the vaccinated group and it possibly reflects the low efficacy of the vaccine in these age groups (Table 3).

Results of molecular exanimation

All clinically affected cases were confirmed positive by using both Universal primer (P1/P2) and specific primer for Serotype O 1D/2B region without co-infection with other serotypes (Fig. 4).

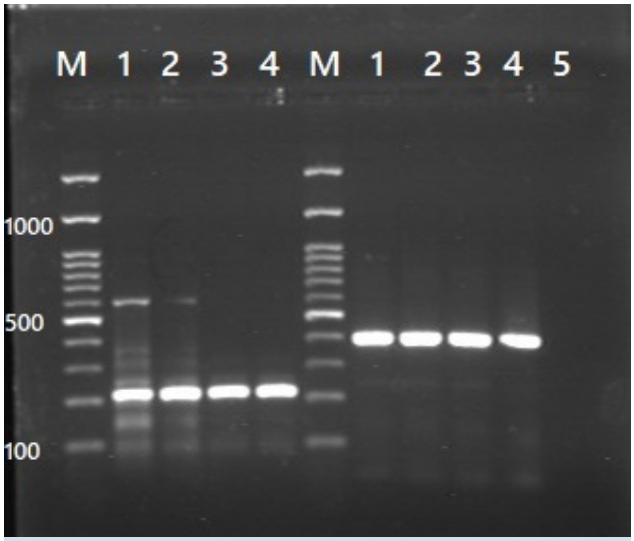

Figure 4. Left, (M) DNA Marker 100bp, Lanes (1:4) positive bands of the universal primer (P1/P2) at 216 bp Right, (M) DNA Marker 100bp Lanes (1:4) positive bands of the specific primer for Serotype O 1D/2B region at 402 bp.

DISCUSSION

Food and mouth disease is an endemic viral disease in Egypt and it remains one of the main obstacles for livestock production. The immunity status of animals is a key factor which influences the clinical outcome of FMD and so, poorly immunized animals are most probably susceptible to this life-threatening disease. On the other hand, animals of intermediate immunity will develop mild symptoms, while those with protective immunity will be asymptomatic. Co-infection with more than one serotype is another important factor influences the clinical severity, the co-infection between serotype O and SAT2 was recorded in previous studies in Assiut Governorate, Upper Egypt (El-Khabaz and Al-Hosary, 2017).

Vaccination is the main preventive measure to protect animals against FMD (Renjun, et al., 2016) particularly in an endemic country of multiple circulating serotypes such as Egypt. On the other hand, all of FMD vaccines provide short-lived immunity and hence it is important to consider the accurate booster vaccinations to prevent the appearance of clinical cases. However, immunization doesn't prevent the development of carrier state and most of the vaccinated animals may have antibody response against the Non-Structural proteins of this virus, particularly against 3ABC, following their exposure to FMDV (Mackay et al., 1998; Parida, 2009). This is maybe possibly the reason for the occurrence of seropositive animals among vaccinated animals observed in the current study.

The obtained results confirmed that the inactivated FMD vaccines provide a protective immunity ranging from 63.3% up to 82.25% according to the type of vaccine against infection with FMDV serotype O which is the only serotype isolated in this study and incriminated in this epidemic. The insufficient vaccine efficacy in this study could result from the short incubation period of the disease which gives the chance for the infection to spread widely before the vaccine protective titer achieved. This theory may explain the appearance of the clinical case and sudden deaths in some vaccinated animals observed in the current study. Because of this scenario, farmers in Egypt think that the vaccine itself is responsible for the development of the clinical cases, particularly in official national vaccination campaign where animals of a village are collected together in one place for vaccination.

The lowest efficacy was observed in case of vaccine prepared by ME VAC company - Middle East, Egypt (Tri apthovac) followed by the vaccine prepared by VSVRI, abbasia, Cairo, Egypt and the highest efficiency was recorded by Merial commercial vaccine (Merial, France). This finding may be attributed to the way of vaccine handling and injection and the persons who carry out the process of vaccination. Vaccination with the former two vaccines are usually applied by paramedical who usually carries out the vaccination process during the obligatory field vaccination campaign and this causes many problems with the vaccination process. Faults that may have direct effects on the vaccine efficacy include the dose, route or preservation as well as lack of experience, training and understanding of the vaccination protocols before and after vaccine administration. On the other hand, commercial vaccine such as Meriel vaccine provides much protection because it is usually applied by a veterinarian rather than paramedical. This agrees with the conclusion of (Fawzy et al., 2017) who declared that vaccination by paramedical and assistant is a weak point in the vaccination process due to faults being followed during the vaccination process.

Some other risk factors have direct effects on the success of vaccination; these factors include animal's age and animal's sex. In the current study, the finding of the animal sex comes in agreement with previous studies that reported that animal sex has no effect on the susceptibility and seropositivity of FMD in non-vaccinated animal (Kibore et al., 2013). On the other hand, some other studies reported a higher incidence rate in female than in males (Hailu et al., 2010). Animal's age plays an important role in the animal susceptibility as in this study the incidence rates were higher in young animals below one year and old animals above five years. This finding may be closely associated with immunity and the ability to produce specific antibodies against this virus (Kibore et al., 2013). On the other hand, the vaccine efficacy was greatly affected by both animal's sex and age. The vaccine was more efficient in males more than females. The incidence rate and efficacy were 14.28% and 85.71% in males and 30% and 70% in females, respectively. This finding may be attributed to some stress factors associated with females like pregnancy and lactation which have direct effects on the ability of immune system to produce the efficient amount of antibodies to protect female animals against this infection. According to the age, the vaccine was more efficient during mid-age (above one year and below five years) where the incidence rate was 7.41% and

the efficiency of vaccination recorded 92.59%. This finding also closely related to the ability of the immune system to produce specific antibodies against this virus. In old and young animals, the immune system is unable to produce enough antibodies (Renjun et al., 2016).

Vaccination against FMD does not provide complete protection against the periodical occurrence of outbreaks in Egypt and the whole vaccination process in the country needs periodical evaluation and updating. On the other hand, active surveillance and molecular epidemiological studies are much required to detect any changes in the virus components and disease epidemiological patterns.

CONCLUSION

This study showed that the vaccination against FMD in Egypt has a protective effect against disease spread and it has a big tendency to minimize the severity of outbreaks caused by the same serotype found in the vaccine. On the other hand, these vaccines were not able to prevent the infection and eliminate the disease. Vaccination is more possible to protect mid-aged animals and males than other age groups and sex. Finally, we concluded that the vaccine being used has to contain the circulating FMDV serotypes in the study area and this could be achieved through continues epidemiological surveys and molecular identification of circulating virus

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Research article
Ερευνητικό άρθρο

Molecular Identification of Vancomycin Resistance and Virulence Genes in Foodborne Enterococci

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ABSTRACT. The study was performed to determine the presence of vancomycin phenotyping genes and some virulence traits in enterococci species. For this purpose, a total of 42 enterococci including 6 vancomycin-resistant and 36 vancomycin-susceptible strains originated from meat/meat products and milk/dairy products were assessed for the *van*A, *van*B and *van*C genes and *agg, esp, gel*E, *ace* and *efa*A virulence genes by using polymerase chain reaction or multiplex polymerase chain reaction. The *van*A gene was found in 12% (n=5) of the strains and *van*C gene in 50% (n=21). From these, three *van*A- (*E. faecalis, E. durans, E. casseliflavus*) and two *van*C-positive (*E. durans*) strains had a minimum inhibitory concentration of > 256 μg/ml as previously determined with the E-test. The strains expressing vancomycin susceptibility originating from ready-to-eat food were found to carry *van*A (n=1) and *van*C (n=5) genes. On the other hand, the *van*B gene was not detected among strains. Moreover, no strain was found to harbor virulence traits studied. Our results indicated that resistant or susceptible enterococci from foods of animal origin can be a possible reservoir for resistance genes and may have a potential role for transfer of genetic elements among enterococci or to other bacteria. Furthermore, to develop epidemiological surveillance systems for foodborne antibiotic resistant pathogens as vancomycin-resistant enterococci and their genes responsible for resistance, primarily *van*A, *van*B, continues to be an essential issue all around the world. The present work provides data for foodborne enterococci isolates harboring *van*A gene from Turkey.

Keywords: enterococcus, food, *van*A, *van*C, vancomycin

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INTRODUCTION

Antibiotic resistance among the microorganisms and emergence of resistance is an ancient phenomenon. Glycopeptide (vancomycin) resistance gene *van*A was detected from 30 000 years old permafrost sample in the Yukon (Canada) and its similarity to modern variants was clearly evidenced. As a result, antibiotic resistance is accepted as a natural phenomenon (D'Costa *et al.*, 2011). In recent times, antibiotic resistant pathogens have become significant public health threat worldwide. The occurrence and spread of vancomycin resistant enterococci (VRE) is another concern because enterococci species are responsible for most of nosocomial infections (Oravcova *et al.*, 2016). In particular, *E. faecalis* and *E. faecium* are the third and fourth prevalent hospital environment acquired pathogens all around the world. Enterococci demonstrate resistance to many different antibiotics, most particularly resistance to glycopeptides. Vancomycin is more important than other antibiotics since it is more frequently used to treat most of Gram-positive bacterial infections. Nine vancomycin resistance genotypes were detected in enterococci (*van*A, *van*B, *van*C, *van*D, *van*E, *van*G, *van*L, *van*M and *van*N) (Werner, 2012). *E. gallinarum* and *E. casseliflavus* strains have *van*C genotype associated with intrinsic vancomycin resistance (Gousia *et al.*, 2015). Strikingly, *van*A, *van*B, *van*G, *van*N and *van*M genotypes are genetically located on plasmid or chromosome and they can be transferred to other species and/or bacteria (Cattoir and Leclercq, 2013). VRE infections, especially caused by high-level resistant enterococci carrying *van*A and/or *van*B genotypes, can only be treated with a few numbers of effective medical agents. Therefore, they are accepted as one of the clinically important antimicrobial resistant pathogens. The *van*A-type vancomycin resistance is very common among the enterococci and the encoding gene has been primarily identified in *E. faecalis*, *E. faecium* and secondly *E. durans*, *E. hirae*, *E. gallinarum*, *E. casseliflavus*, *E. raffinosus*, *E. avium*, *E. mundtii*, *E. cecorum* (Harada *et al.*, 2012).

Together with *E. faecium* and *E. faecalis,* the incidence of other enterococci species isolated from patients display an alarming increase. This is mainly correlated with their increased putative virulence traits and multiple antibiotic resistances (Biswas *et al.*, 2016). The presence of virulence factors in enterococci gives them different roles both as commensal and as pathogen bacteria for human health (Farahani, 2016). Enterococcal virulence factors are divided into two groups; promoting colonization traits such as aggregation substance (*aga*), collagen binding protein (*ace*), endocarditis specific antigen (*efa*A), surface protein (*esp*) and affecting tissues such as cytolysin (*cyl*), gelatinase (*gel*E), hyaluronidase (*hyl*). In addition to these, sex pheromone genes (*cpd*, *cob*, *ccf*, *cad*) work together with other virulence genes help to trigger infection reactions (Chajecka-Wierzchowska *et al.*, 2017). Animal originated food isolates of enterococci may harbor many of above mentioned virulence genes thus, these foods may play important role as potential source for human infections (Yilmaz *et al.*, 2016).

The goals of this study were to investigate the vancomycin resistance profile, to determine the presence of virulence genes in vancomycin-resistant/susceptible enterococci from food of animal origin, and to raise public awareness about the possible health risks.

MATERIALS AND METHODS

Strains

Bacterial strains were from the Food Hygiene and Technology Department collection, Veterinary Faculty. A total of 42 strains consisting of 36 *E. faecium*, 4 *E. avium* and 2 *E. gallinarum* were selected among enterococcci collected from foods of animal origin between September and December 2011 from different cities (Istanbul, Bursa, Yalova, Balikesir) in Marmara Region. API identification and vancomycin/teicoplanin MIC's results (Cetinkaya *et al*., 2013) of the selected strains are summarized in Table 1. Stock cultures were kept frozen (-20ºC) in Brain Heart Infusion broth (Oxoid CM1135, England) containing 20% (v/v) glycerol. The cultures were activated in Brain Heart Infusion broth at 37ºC.

PCR confirmation of strains and Determination of vancomycin resistance and virulence genes

Total DNA from bacterial strains was extracted by using Chelex 100 (Sigma Aldrich, USA). The PCR was processed in a ThermoCycler (Runik, SCM 96G). Each 25 µl reaction mixture consisted of 1 µl template DNA, 1.25 U of Hot Start Taq DNA polymerase (Bioron, Germany), 10 mM of Tris-HCl pH 8.9, 22 mM of KCl, 1.8 mM of $MgCl_2$ (Fermentas, USA), 200 µM of dNTPs (Biolabs, UK) and 0.5 mM of each primers (Sentegen, Turkey). The PCR method was used to confirm previously API identified strains at the genetic level by using species-specific primers (*E. faecium*, *E. faecalis*, *E. durans*, *E. gallinarum*, *E. casseliflavus*, *E. avium*) and to investigate the pres-

ence of *van*A, *van*B and *van*C resistance genes, and the virulence trait genes *agg*, *esp*, *gel*E, *ace* in all strains as well as *efa*A_{fs}, *efa*A_{fm} in *E. faecalis* and *E. faecium*. The primers, their sequences, products sizes and amplification procedures for PCR conditions are presented in Table 2. The PCR products were electrophoresed (Thermo Scientific EC300XL, USA) on 3% agarose gel (Biomax, Dubuque, USA) and visualized (BioRad Gel DocXR+, USA) by ethidium bromide staining.

Table 1. Description of the strains used in this study and the results of screening for vancomycin resistance genes.

No.	Source	API Identification[a]	PCR Identification	Vancomycin MIC's[a] (μg/ml)	Teicoplanin MIC's[a] (μg/ml)	Genes
1	Meatball	*E. gallinarum*	*E. casseliflavus*	> 256	> 256	*van*A
2	Meatball	*E. avium*	*E. durans*	> 256	> 256	*van*A
3	Meatball	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
4	Meatball	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
5	Meatball	*E. avium*	*E. durans*	> 256	> 256	*van*C
6	Meatball	*E. avium*	*E. durans*	> 256	> 256	*van*C
7	Meatball	*E. feacium*	*E. faecalis*	> 256	> 256	not detected
8	Minced meat	*E. faecium*	*E. faecium*	4	≤ 8	*van*A
9	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
10	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
11	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
12	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
13	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
14	Minced meat	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
15	Beef	*E. avium*	*E. faecalis*	> 256	> 256	*van*A
16	Beef	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
17	Beef	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
18	Beef	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
19	Lamb	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
20	Lamb	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
21	Salami	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
22	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
23	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
24	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
25	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
26	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
27	Raw cow's milk	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
28	Raw goat's milk	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
29	Raw goat's milk	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
30	Village cheese	*E. faecium*	*E. faecium*	<4	≤ 8	*van*A
31	Urfa cheese	*E. gallinarum*	*E. gallinarum*	<4	≤ 8	*van*C
32	Kashar cheese	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
33	Cottage cheese	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
34	Mihalic cheese	*E. faecium*	*E. faecalis*	<4	≤ 8	not detected
35	Antep cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
36	Cottage cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
37	Kashar cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
38	Cottage cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
39	White cheese	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
40	Butter	*E. faecium*	*E. faecium*	<4	≤ 8	not detected
41	Butter	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C
42	Butter cream	*E. faecium*	*E. faecium*	<4	≤ 8	*van*C

[a]published in elsewhere (Cetinkaya *et al*., 2013)

Table 2. Oligonucleotide primer sequences and amplification conditions.

Gene	Oligonucleotid sequences (5´-3´)	Product size (bp)	Amplification procedure
fcm	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215	An initial cycle of: 95°C for 4 min, followed by 30 cycles of: 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and final cycle 72°C for 7 min (Jackson *et al*., 2004)
fls	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	
dur	CCTACTGATATTAAGACAGCG TAATCCTAAGATAGGTGTTTG	295	
gal	TTACTTGCTGATTTTGATTCG TGAATTCTTCTTTGAAATCAG	173	
cas	TCCTGAATTAGGTGAAAAAAC GCTAGTTTACCGTCTTTAACG	288	
avi	GCTGCGATTGAAAAATATCCG AAGCCAATGATCGGTGTTTTT	368	
*van*A	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1030	An initial cycle of: 94°C for 5 min, followed by 30 cycles of: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and final cycle 72°C for 10 min (Evers *et al*., 1993)
*van*B	GTGACAAACCGGAGGCGAGGA CCGCCATCCTCCTGCAAAAAA	433	An initial cycle of: 94°C for 5 min, followed by 30 cycles of: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and final cycle 72°C for 10 min (Handwerger *et al*., 1992)
*van*C	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822	An initial cycle of: 94°C for 5 min, followed by 30 cycles of: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and final cycle 72°C for 10 min (Dutka-Malen *et al*., 1995)
esp	TTACCAAGATGGTTCTGTAGGCAC CCAAGTATACTTAGCATCTTTTGG	432	30 cycles of: 94°C for 30 s, 58°C for 30 s, 72°C for 30 s (Shankar *et al*., 1999)
ace	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCG	320	33 cycles of: 94°C for 1 min, 56°C for 1 min, 72°C for 1 min (Mannu *et al*., 2003)
*gel*E	AGTTCATGTCTATTTTCTTCAC CTTCATTATTTACACGTTTG	402	30 cycles of: 94°C for 30 s, 56°C for 30 s, 72°C for 30 s (Mannu *et al*., 2003)
agg	AAGAAAAAGAAGTAGACCAAC AAACGGCAAGACAAGTAAATA	1553	30 cycles of: 94ºC for 30 s, 58ºC for 30 s, 72ºC for 30 s (Eaton and Gasson 2001)
efaA_{fs}	GACAGACCCTCACGAATA AGTTCATCATGCTGTAGTA	705	An initial cycle of: 94ºC for 2 min, 52ºC for 2 min, 72ºC for 2 min, followed by 27 cycles of: 94ºC for 15 s, 52ºC for 15 s, 72ºC for 15 s (Eaton and Gasson 2001)
efaA_{fm}	AACAGATCCGCATGAATA CATTTCATCATCTGATAGTA	735	An initial cycle of: 94ºC for 2 min, 52ºC for 2 min, 72ºC for 2 min, followed by 27 cycles of: 94ºC for 15 s, 52ºC for 15 s, 72ºC for 15 s (Eaton and Gasson 2001)

Table 3. Distribution of vancomycin resistance genes in enterococci (n=42) and their MIC's.

Gene	Numbers of *van*A, *van*B and *van*C positive strains (%)	Strains	Vancomycin/teicoplanin MIC's (μg/ml)
*van*A	5 (11.9%)	*E. casseliflavus* (n=1)	> 256 / > 256
		E. durans (n=1)	> 256 / > 256
		E. faecalis (n=1)	> 256 / > 256
		E. faecium (n=2)	≤ 4 / ≤ 8
*van*B	0		
*van*C	21 (50%)	*E. durans* (n=2)	> 256 / > 256
		E. faecium (n=18)	< 4 / ≤ 8
		E. gallinarum (n=1)	< 4 / ≤ 8
Not detected	16 (38.1%)	*E. faecalis* (n=1)	> 256 / > 256
		E. faecalis (n=1)	< 4 / ≤ 8
		E. faecium (n=14)	< 4 / ≤ 8

RESULTS

PCR identification of tested strains evidenced differences from previous API results. According to PCR, three *E. avium* were identified as *E. durans*; two *E. faecium* as *E. faecalis*, one *E. avium* as *E.faecalis*, and one *E. gallinarum* as *E. casseliflavus* (Table 1).

Among the tested strains *van*A and *van*C genes were found in five (12%) and 21 strains (50%) respectively, meanwhile *van*B gene was not detected. Strains carrying *van*A genes were from cheeses (*E. faecium*), meatballs (*E. durans* and *E. casseliflavus*), minced meat (*E. faecium*) and beef (*E. faecalis*). *Van*C gene was determined in strains derived from five different ready-to-eat dairy products (three cheeses, butter and butter cream). The prevalence of *van*C gene was more common among *E. faecium* (n=18) compared to *E. durans* (n=2) and *E. gallinarum* (n=1). Interestingly, an important percentage (38%) of *E. faecium* (14 strains) and *E. faecalis* (2 strains) did not give any band for these genes.

Data related to the distribution of vancomycin resistance genes in enterococci and their respective minimum inhibitory concentrations (MIC) values for vancomycin/teicoplanin antibiotics is shown in Table 3. Three of five *van*A-positive strains exhibited high MICs to vancomycin/teicoplanin. Among the *van*C gene positive strains, only two *E. durans* had MIC values higher than 256 μg/ml for vancomycin/teicoplanin.

The strains were also screened for the presence some virulence factors such as *agg*, *esp*, *gel*E, *ace* and *efa*A, nonetheless the searched virulence genes were not detected in any tested strain.

DISCUSSION

Different types of acquired vancomycin resistance are known in enterococci, meanwhile the *van*A followed by *van*B are the most prevalent resistance genotype (Werner, 2012). In this study we examined acquired resistance genes including *van*A, *van*B, and *van*C responsible for intrinsic resistance in vancomycin-resistant/susceptible enterococci isolates from animal originated food. The results indicated that strains belonged to *E. faecalis*, *E. faecium*, *E. durans* and *E. casseliflavus* species carried *van*A gene with a prevalence of 11.9% (5 strains). Among these strains an *E. faecium* isolated from ready-to-eat food (village cheese) showed susceptibility to vancomycin (MIC, ≤4 μg/ml).

Several studies from different countries reported the presence of *van*A gene in foodborne enterococci. Lopez *et al*. (2009) reported the prevalence of *van*A gene as 22.6% (two *E. faecium*, three *E. durans* and two *E. hirae*) in 31 VRE isolates. Likewise, Gou-

sia *et al.* (2015) stated that 22 *E. faecium* (15.6%) among 141 enterococci carried *van*A gene. Relatively lower prevalence (2.4% of enterococci from meat and poultry) was reported by Yilmaz *et al*. (2016). Another work revealed the presence of *van*A gene in three vancomycin-susceptible *E. faecalis* isolates and one *E. hirae* isolate (Perin *et al*. 2014). Osman *et al*. (2016) and Harada *et al*. (2012) detected *van*A gene in one *E. faecalis* strain from fish and one *E. cecorum* strain from poultry samples**,** respectively. Contrary results were reported by Kasımoğlu-Doğru *et al.* (2010) and Chajecka-Wierzchowska *et al.* (2016). Contrary results were given by Kasımoğlu-Doğru et al. (2010) and Chajecka-Wierzchowska et al. (2016) suggesting that the strains from food and livestock samples did not harbor *van*A gene. In our study, tested strains had negative results for *van*B gene. These results are similar to those obtained by Kasımoğlu-Doğru *et al.* (2010), Perin *et al.* (2014), Yılmaz *et al.* (2016) and Chajecka-Wierzchowska *et al*. (2016). Nevertheless, Gousia *et al*. (2015) and Lopez *et al*. (2009) reported the presence of *van*B gene in *E. faecium* (1.4%) and *E. faecium* (6.4%) respectively. Another study conducted by Perin et al. (2014) indicated the presence of both *van*A and *van*B genes at seven *E. faecalis* strains.

Many species of enterococci, as stated for *Corynebacterium* spp., *Arcanobacterium haemolyticum* and *Lactococcus* spp. were reported to harbor *van*A ligase gene while *van*B has been primarily determined in *E. faecium* and *E. faecalis*. The difference observed in the dissemination of *van*A and *van*B resistance genes may be attributed to the fact that *van*A gene is mostly located on transposon, a mobile genetic element, in comparison to *van*B gene cluster (Cetinkaya *et al.* 2000). *Van*A gene cluster responds to both vancomycin and teicoplanin resistance but *van*B gene cluster is responsible for resistant to vancomycin but not for teicoplanin (Lefort *et al*., 2004). In our study, strains resistant to vancomycin were also resistant to teicoplanin and they were not carrying *van*B gene.

The presence of *van*C gene in enterococi has been characterized as the intrinsic resistance (Gousia *et al.*, 2015). As seen in Table 1, *van*C gene was found in 10 (eight *E. faecium*, two *E. durans*) meat/meat products and 11 (ten *E. faecium*, one *E. gallinarum*) milk/dairy originated strains. Among the strains carrying *van*C gene, only two *E durans* isolated from meatball samples had vancomycin-resistance with a MIC value of > 256 μg/ml while the others were vancomycin-susceptible (MICs, < 4 μg/ml). Previously, the presence of *van*C1 and *van*C2/3 genes in vancomycin-susceptible *E. faecalis* isolated from broilers in Brazil (De Moura *et al.*, 2013) and *van*C gene in *E. gallinarum* in Canada (Diarra *et al.*, 2010) was published. Chajecka-Wierzchowska *et al.* (2016) also reported the presence of *van*C2/3 genes in *E. casseliflavus* isolates from ready-to-eat meat products but not *van*C1 gene. Moreover, a study in Egypt demonstrated *van*C gene carrying *E. gallinarum* and *E. faecalis* strains from fish samples (Osman *et al.*, 2016). Conversely, lack of *van*C gene in animal originated food was recently reported by Yilmaz *et al*. (2016) in Turkey and by Gousia *et al*. (2015) in Greece.

Enterococci strains carrying virulence factor genes cause more severe infections than the strains lacking these pathogenicity traits. Virulence genes have been frequently observed in *E. faecalis* strains (Chajecka-Wierzchowska *et al.*, 2017). In our study, virulence traits (*agg*, *esp*, *gel*E, *ace* and *efa*A) were not found in any of the tested strains. This can be explained by the limited number of *E. faecalis* tested in the study. In contrast to our results, virulence genes *gel*E, *esp*, *ace*, *asa*1, *efa*A and *hyl* in *E. faecalis*, *E.faecium*, *E. durans* and *E. hirae* isolates from milk and dairy products were observed by Perin *et al*. (2014), Hammad *et al*. (2015) and Gaglio *et al*. (2016). A study performed by Klibi *et al*. (2013) in Tunisia indicated the location of *hyl*, *esp* and *gel*E genes in meat isolates of enterococci species (*E. faecalis*, *E. faecium, E. gallinarum*). Another report revealed the presence of *efa*A, *agg*, *esp*, *gel*E, *cyl*, *cop*, *cpd*, *ccf* genes in *E. faecalis* and *E. faecium* isolated from ready-to eat fermented foods in Turkey (Toğay *et al.*, 2010).

CONCLUSIONS

The presence and prevalence of *van*A and *van*C genes and the absence of *van*B and virulence trait genes in vancomycin-resistant/susceptible enterococci strains were proved. Our findings may be evaluated from two different points: firstly, detection of *van*A gene in VRE strains and particularly in one ready-to-eat food isolate is a matter of interest. These strains can be a part of transmission the high level vancomycin resistance to other strains and/or bacteria. Secondly, the role of food isolates on the spread of pathogenicity genes continues to raise public health concerns. Thus, the lack of any investigated virulence genes in the strains constitutes positive part of the study. Furthermore, monitoring the presence of virulence and *van* genes in different food isolates is essential

to evidence their spreading speed and possible public health risks all over the world.

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CONFLICT OF INTEREST

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

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Research article
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Effect of chitosan coating on the shelf life of ready-to-eat bovine meatballs and the control of *Listeria monocytogenes* growth on their surface during refrigeration storage

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Επίδραση επικάλυψης χιτοζάνης στο χρόνο ζωής προψημένων βόειων κεφτέδων και τον έλεγχο της ανάπτυξης της *Listeria monocytogenes* στην επιφάνεια τους κατά τη διάρκεια συντήρησής τους υπό ψύξη

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ABSTRACT. Edible chitosan coating on the surface of ready-to-eat (RTE) bovine meatballs was evaluated for its effect on their shelf life and the control of *Listeria monocytogenes* at 5 °C. *L. monocytogenes* was inoculated onto the surface of RTE bovine meatballs with and without edible chitosan coating. The samples were stored at 5 °C. Total aerobic viable count (TVC) and the bacterial counts of *L. monocytogenes*, lactic acid bacteria and Enterobacteriaceae were determined on days 0,1,7,14,21 and 28. The sensory characteristics were also evaluated at the same time spots by semi trained panelists. The results of the microbiological analysis depicted that the use of edible chitosan membranes reduced all of the microbial populations that were enumerated, and retarded their growth leading to the conclusion that they can prolong the shelf life of these products by 14 days. Moreover, the population of the inoculated *L. monocytogenes* was about 2 log CFU/g lower in the meatballs coated with chitosan, indicating an inhibitory effect of chitosan in the growth of *L. monocytogenes*. The sensory analysis showed that the samples coated with chitosan were satisfactorily accepted by the panelists even at day 28, in contrast to the samples without chitosan (control samples) which were unacceptable at day 14. These results indicate that edible chitosan coatings represent a potential agent in controlling *L. monocytogenes* on the surface of RTE meatballs as well as other RTE meat products, prolonging their shelf life without affecting their sensory characteristics.

Keywords: Chitosan, *L. monocytogenes*, Ready-To-Eat meatballs, shelf life

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ΠΕΡΙΛΗΨΗ. Η επίδραση εδώδιμων μεμβρανών χιτοζάνης στην επιφάνεια προψημένων (RTE) βόειων κεφτέδων αξιολογήθηκε ως προς το χρόνο ζωής τους και τον έλεγχο της ανάπτυξης της *Listeria monocytogenes* στους 5 °C. *L. monocytogenes* ενοφθαλμίστηκε στην επιφάνεια προψημένων βόειων κεφτέδων με ή χωρίς επικάλυψη χιτοζάνης. Τα δείγματα αποθηκεύτηκαν στους 5 °C και τις ημέρες 0,1,7,14,21 και 28 καταμετρήθηκαν οι μεταβολές των πληθυσμών των ολικών αερόβιων βακτηρίων, της *L. monocytogenes*, των οξυγαλακτικών βακτηρίων και των εντεροβακτηρίων. Τα οργανοληπτικά χαρακτηριστικά των δειγμάτων αξιολογήθηκαν τις ίδιες ημέρες από ομάδα ημιεκπαιδευμένων κριτών. Η ανάλυση των αποτελεσμάτων έδειξε ότι η χρήση εδώδιμων μεμβρανών χιτοζάνης μείωσε όλους τους μικροβιακούς πληθυσμούς που καταμετρήθηκαν και καθυστέρησε την ανάπτυξή τους οδηγώντας στο συμπέρασμα ότι μπορεί να επιμηκύνει το χρόνο ζωής των προϊόντων αυτών κατά 14 ημέρες. Επιπλέον, οι πληθυσμοί της *L. monocytogenes* ήταν περίπου κατά 2 log CFU/g μικρότεροι στους καλυμμένους με χιτοζάνη κεφτέδες. Συνεπώς, η χρήση χιτοζάνης είχε ανασταλτική επίδραση στην ανάπτυξη της *L. monocytogenes*. Η οργανοληπτική αξιολόγηση έδειξε ότι τα εμβαπτισμένα σε χιτοζάνη δείγματα έγιναν αποδεκτά από τους κριτές ακόμα και την ημέρα 28, σε αντίθεση με τα δείγματα χωρίς χιτοζάνη (δείγματα μάρτυρες) τα οποία ήδη από την ημέρα 14 δεν ήταν αποδεκτά. Από τα αποτελέσματα αυτά προκύπτει ότι οι εδώδιμες μεμβράνες χιτοζάνης είναι πιθανό να μπορούν να χρησιμοποιηθούν για τον έλεγχο της ανάπτυξης της *L. monocytogenes* στην επιφάνεια προψημένων κεφτέδων και άλλων προψημένων κρεάτων, επιμηκύνοντας το χρόνο ζωής τους χωρίς να υποβαθμίσουν τα οργανοληπτικά χαρακτηριστικά τους.

Λέξεις ευρετηρίασης*:* Χιτοζάνη, *L. monocytogenes*, προψημένοι κεφτέδες, χρόνος ζωής

INTRODUCTION

RTE products and especially the deli meats, are vulnerable to cross contamination of pathogens due to the multiple steps in their preparation, which can occur from contact with contaminated equipment and the environment (hoppers, slicers, peelers, conveyors, packaging machines etc) (Tompkin, 2002). Moreover, if these products are not processed after their final packaging, and because they are consumed without further cooking, if pathogenic contamination occurs, the chance of illnesses is increased (FSIS-USDA, 2005). Therefore, control of possible pathogens on RTE foods immediately prior to or after packaging is an important control point. Antimicrobial packaging provides a final defense hurdle for inactivation or inhibition of pathogens.

L. monocytogenes is a Gram- positive foodborne pathogen and represents a major food safety concern, especially in RTE foods (FAO, 2004) because of its ability to survive and grow at refrigeration temperatures. *L. monocytogenes* is associated with listeriosis, a disease with high fatality, affecting mainly immunocompromised persons, causing meningitis, and abortions in pregnant women (Beverly, 2004). Deterioration of foods, especially RTE, as well as contamination by pathogens, usually starts on the food surface. Consequently, food surface treatments and packaging after the treatments are of critical importance for food safety and quality (Malhotra et al., 2015). *L. monocytogenes* is a frequent surface contaminant of RTE meat products often occurring during the post-processing phase (Tompkin, 2002).

The increasing demands by the consumers concerning RTE foods with improved freshness and without chemical additives, led to the development of edible films (Kerry et al., 2006) which improve the

quality, the appearance and the safety, and increase the shelf life of RTE foods (Gennadios et al., 1997). Chitosan is one of the substances capable of creating such edible films (Coma, 2008).

Chitosan is a natural, nontoxic, biodegradable biopolymer which derives by the deacetylation of chitin, a main component of the shells of crustaceans such as, shrimp, crab and crawfish (No and Meyers, 2004). Chitosan as well as its oligomers, receive considerable attention due to their antimicrobial, antitumor and hypocholesterolemic abilities (No et al., 2002). Chitosan based edible coating and films, incorporated or not with other antimicrobial agents (essential oils, lauric alginate ester, allyl isothiocynate, nisin, etc) seem to be promising for their application in food preservation (Elsabee and Abdou, 2013; Kerch, 2015; Guo et al., 2017). The inhibitory effect and delay of *L. monocytogenes* growth by chitosan has been reported in several RTE meat products such as servelat sausage (Blom et al., 1997), ham steaks (Ye et al., 2008a) and deli turkey meat (Guo et al., 2014). The mechanism of the antibacterial activity of chitosan is not yet fully understood. However, a widely accepted assumption states that the positively charged chitosan molecules interact with the negatively charged microbial cell membranes, changing the cell permeability, causing a leakage of intracellular constituents and therefore the destruction of the microbial cell (Fang et al., 1994; Sudarshan et al., 1992; Papineau et al., 1991; Young et al., 1982).

The aim of the present study was the evaluation of the effect of chitosan edible coating on the shelf life and the control of *L. monocytogenes* in RTE bovine meatballs stored at 5 °C.

MATERIAL AND METHODS

1. Preparation of *L. monocytogenes* inoculum

L. monocytogenes serotypes Scott A and California (CA) obtained from the collection of the Laboratory of Milk Hygiene and Technology, of the School of Veterinary Medicine of Aristotle University of Thessaloniki, were used during this challenge study. The pure cultures were stored at -80 °C and sub-cultured twice in Tryptone Soy Broth (TSB, BioLab, Budapest, Hungary) at 35 °C for 24h before being used.

2. Preparation of chitosan solution

Chitosan with high molecular weight (>800.000Da, Aldrich Co, Germany) produced by crab shells was used to prepare 1g/100 ml chitosan solution in 1% v/v acetic acid under stirring for 24 hours. The solution was adjusted to a pH of 4 at 25 °C.

3. Preparation and thermal treatment of meatballs

Meatballs were prepared by mixing 800g of minced beef, 200g wheat breadcrumbs, 150g fresh onion, two egg yolks, 10g salt, 5g black pepper and 40ml olive oil. The meatballs were fried in olive oil bath at 180 °C until core temperature reached to 75 °C. They were allowed to dry and cool down to room temperature under a laminar flow before further treatments.

4. Preparation and treatment with chitosan solution of RTE meatballs

One group of the samples were dipped into the chitosan solution for 30 sec and placed on sterile trays under a laminar flow hood for 1h at room temperature 25 °C until the chitosan coating dried (group C). The rest of the samples were not dipped in the chitosan solution (group U-control samples). Overnight *L. monocytogenes* cultures of the two selected serotypes were centrifuged at 1500 rpm for 10 min and the bacterial pellet was resuspended in sterile peptone water and centrifuged again. This process was repeated twice and finally equal volume aliquots of each strain were combined and resuspended in peptone water and was then decimally diluted to 6.60 log CFU/g. An aliquot of 0.1 ml of the mixed culture was inoculated onto each meatball (18±1 g) to reach an initial inoculum of ca 5.30 log CFU/g. The samples remained for 10 minutes under a laminar flow hood in order to air-dry. They were then packaged, per tenth, in disks of expanded polystyrene on special absorbent meat papers, and the dishes were placed in plastic food bags.

Both trays and papers were previously sterilized by UV rays overnight under a laminar flow cabinet. The trays with the samples (inoculated with *L. monocytogenes* and uninoculated for sensory evaluation) were stored in an electronically controlled refrigerator (LBI-150M, Daichan Labtech Co. LTD Korea) at a temperature of 5±0.5 °C. Bacterial counts were determined at days 0, 1, 7, 14, 21 and 28.

5. Microbiological examination

The bacterial counts of the examined bacteria were determined at two samples of each group and the experiment repeated twice. The samples were stored at 5 °C. Total aerobic viable count (TVC) and the bacterial counts of *L. monocytogenes*, lactic acid

bacteria and Enterobacteriaceae were determined on days 0,1,7,14,21 and 28. More specifically, each meatball was mixed in a stomacher bag (Lab Blender 400, A. J. Seward and Co. Ltd., London, UK) for 60 sec with peptone water 0.1% w/v. Serial decimal dilutions were prepared and for the enumeration of *L. monocytogenes*, 0.1 mL of each dilution was streaked onto petri dishes with Agar Listeria Ottavani & Agosti (ALOA agar, LabM, Hal 10, Lancashire, United Kingdom) and incubated at 37 °C for 24-48h.

Total viable counts (TVC), Enterobacteriaceae and lactobacilli were counted on Tryptone Soy agar (Biolab, Budapest, Hungary), Violet Red Bile Glucose agar (VRBG PH EUR agar, BioLab, Budapest, Hungary) and De Man-Rogosa-Sharpe agar (MRS agar, BioLab, Budapest, Hungary) respectively. The counts were expressed as log CFU/g.

6. Sensory evaluation

Two groups of uninoculated meatballs (one group of meatballs with chitosan coating and another without) were stored for sensory evaluation, which was performed by a panel of eight semi-trained panelists at the same selected time spots of microbiological analysis. The sensory characteristics evaluated were color, flavor, taste and texture. Meatballs before consumption were heated in a microwave oven. Overall acceptability of the product (uniniculated meatballs with and without chitosan coating) was based on a 10-point numerical scale and a score of five was the lower limit of acceptability.

7. Statistical analysis

Analyses were based on two separate experiments with each mean Standard Deviation (± SD) being the average of the two determinations. The inhibitory effect of chitosan coating was analyzed using the General Linear Model (GLM) of Analysis of Variance (ANOVA) (STATA version 13, STATA Corp., Texas, USA). Differences at $p \leq 0.05$ were consider to be significant.

RESULTS

The population of TVC between the control samples and the samples coated with chitosan showed no significant difference at day 0 (5.31 log CFU/g) (Table 1). On day 1 the population of TVC in the samples coated with chitosan decreased from 5.31 to 3.97 log CFU/g, while the population of the control samples remained the same. From day 1 until the end of this study, the population of TVC in both sample groups increased. However, the difference between the two groups remained significant and at day 14 the population of TVC of the control samples reached 7.80 log CFU/g and meatballs were microbiologically unacceptable since the predominant flora was not the lactic acid bacteria (5.02 CFU/g) and spoilage is likely to be unacceptable at 10^7-10^8 CFU/g (Health Protection Agency, 2009). Their sensory characteristics were also unacceptable with a score of 3.25 (Table 2) which is very low. At the same time spot (day 14) the TVC population in coated with chitosan samples reached 5.77 log CFU/g. At day 28, the TVC population of the control samples was 8.90 log CFU/g, while in group C it was 6.73 log CFU/g and the sensory score still high (7.5). Therefore, the fact that there was a significant difference between the two groups leads to the conclusion that the use of chitosan has a beneficial effect in the shelf life of this product.

The *L. monocytogenes* counts on the RTE meatballs at day 0 were the same in both groups (5.19 log CFU/g), control and coated with chitosan. On day 1, the counts in the samples treated with chitosan were significantly lower from these in control samples. More specifically, on that day the population of *L. monocytogenes* in the control samples remained 5.19 log CFU/g, while in the treated with chitosan samples it decreased and reached 3.94 log CFU/g. From day 1 until day 28, the population of *L. monocytogenes* in both groups increased, along with their difference reaching its maximum at day 21 (3.16 log CFU/g).

The effect of chitosan in the population of Enterobacteriaceae was different compared to the rest of the bacterial counts (Table 1). On day 0 the Enterobacteriaceae counts of both the control samples and the samples coated with chitosan were similar (1.70 log CFU/g). At day 1 their counts were under the sensitivity of the method. Enterobacteriaceae could not be detected in the meatballs dipped in the chitosan until the end of the experiment, while the population in the control samples increased throughout the experimentation reaching 3.90 and 5.90 log CFU/g at day 14 and 21 respectively.

The lactic acid bacteria counts of the control samples and the samples coated with chitosan solution showed no difference at day 0 (Table 1). However, at day 1 the population in group C was 2.88 log CFU/g, while in group U 3.13 log CFU/g. This difference started decreasing until day 28 when both the control samples and the chitosan treated samples had similar population (6.39 and 6.40 log CFU/g respectively).

Table 1. Changes of the microbial counts (mean±SD) from the surface of RTE meatballs during their preservation at 5 °C for 28 days.

Day	Group of samples	Log CFU/g			
		TVC	*L. monocytogenes*	Enterobacteriaceae	Lactic Acid Bacteria
0	U*	5.31±0.03[a]	5.19±0.17[a]	1.70±0.03[a]	3.13±0.07[a]
	C**	5.31±0.16[a]	5.19±0.12[a]	1.70±0.04[a]	3.13±0.12[a]
1	U	5.31±0.23[a]	5.19±0.15[a]	<1	3.13±0.09[a]
	C	3.97±0.27[a]	3.94±0.21[a]	<1	2.88±0.33[a]
7	U	6.31±0.25[a]	6.07±0.05[a]	<1	5.48±0,28[a]
	C	4.52±0.37[a]	4.67±0.27[a]	<1	4.09±0.07[a]
14	U	7.80±0.42[a]	7.75±0.45[a]	3.90±0.13[a]	5.02±0.01[a]
	C	5.17±0.12[a]	5.02±0.0[a]	<1	4.54±0.21[a]
21	U	8.73±0.31[a]	8.89±0.52[a]	5.90±0.33[a]	5.61±0.14[a]
	C	5.77±0.30[a]	5.73±0.19[a]	<1	5.75±0.11[a]
28	U	8.90±0.43[a]	8.88±0.48[a]	6.13±0.11[a]	6.39±0.22[a]
	C	6.73±0.36[a]	6.72±0.22[a]	<1	6.40±0.28[a]

*Meatballs without chitosan coating
** Meatballs with chitosan coating
[a] Mean values for each microbial index at each day in the same column of different groups are not significantly different ($p>0.05$).

Table 2. Sensory evaluation scores of meatballs with and without chitosan coating during refrigeration storage.

Evaluation day	Evaluation parameter	Score		Overall acceptability	
		Group C*	Group U**	Group C	Group U
0	Taste	9***	10	9.25	9.25
	Color	10	9		
	Flavor	9	9		
	Texture	9	9		
1	Taste	9	9	9.25	9.25
	Color	10	9		
	Flavor	9	10		
	Texture	9	9		
7	Taste	9	8.5	9.25	8.5
	Color	10	7		
	Flavor	9	8		
	Texture	9	8.5		
14	Taste	8	4	8.25	3.75
	Color	9	4		
	Flavor	7	3		
	Texture	9	4		
21	Taste	8			8.25
	Color	9			
	Flavor	7			
	Texture	9			
28	Taste	8			7.5
	Color	7			
	Flavor	7			
	Texture	8			

* Group C=meatballs with chitosan coating
**Group U=meatballs without chitosan coating
***Mean values of the panelists' scores

DISCUSSION

In our study the use of chitosan films led to a reduction of 1.25 log CFU/g of the population of *L. monocytogenes* on the surface of RTE meatballs without preventing totally its growth, but could delay it significantly. The sensory evaluation demonstrated that the shelf life of RTE meatballs during their preservation at 5 °C does not exceed 28 days, while the use of chitosan improved their sensory characteristics.

Beverly et al. (2008) reported that the edible film of chitosan dissolved with acetic or lactic acid at 0.5% (w/v) or 1% (w/v) and stored at 4° C, on RTE roast beef could not prevent the growth of *L. monocytogenes*, but on day 14, *L. monocytogenes* counts were significantly different for all the chitosan-coated samples from the control by 2–3 log CFU/g and remained significantly different on day 28. This could be explained by the decreasing antimicrobial activity of chitosan films due to the decreased availability of amino groups on chitosan (Cargi et al., 2004; Comma et al., 2002). Ye et al. (2008a) reported that chitosan-coated plastic films were not able to control the growth of *L. monocytogenes* on ham steaks stored at 4 °C. A long term antilisterial effect was observed by chitosan film containing 0.001 g/cm^2 sodium lactate (SL) with the counts of *L. monocytogenes* being slightly lower than the initial inoculum. The SL treatment reduced the counts of *L. monocytogenes* from 2.7 to 1.5 log10 CFU/cm^2 during 10 weeks of storage. On week 10 *L. monocytogenes* started to grow, but its count at the end of the 12-week storage was still slightly lower than the initial one. Blom et al. (1997) reported that a mixture of 2.5% SL and 0.25% acetate prevented the growth of *L. monocytogenes* in servelat sausage without affecting the sensory acceptability of the sausage.

Chitosan-coated plastic films with 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL–0.6 mg/cm^2 potassium sorbate (PS) and 2.3 mg/cm^2 SL–500 IU/cm^2 nisin completely inhibited the growth of *L. monocytogenes* on smoked salmon for at least 6 weeks at refrigerated temperature (Ye et al., 2008b).

The antimicrobial activity of chitosan probably originates from its polycationic nature (Kim et al., 2003). The positively charged chitosan molecules interact with the negatively charged microbial cell membranes (Young et al., 1982; Papineau et al., 1991; Sudarshan et al, 1992; Fang et al, 1994). This antimicrobial activity was observed in our study, as the use of chitosan coating in RTE meatballs lead to a delay in the growth of TVC, *L. monocytogenes*, Enterobacteriaceae and lactic acid bacteria, resulting to a prolongation of their shelf life for about 14 days.

Latou et al. (2014) investigated the combined effect of dipping in a chitosan solution (1 g/100 ml) and packaging under modified atmosphere (MAP, 70% CO2, 30% N2) on shelf life extension of refrigerated chicken fillets. TVC were 3.9-4.9 log CFU/g lower on day 6 of storage in treated vs. untreated air-packaged samples with the strongest effect being shown by the combination of chitosan plus MAP. A similar reduction in LAB, Pseudomonads and Enterobacteriaceae was also observed during storage. Based on the microbiological and sensory data, shelf life of air-packaged, chitosan-treated, MAP-treated and chitosan/MAP treated samples was 5, 11, 12 and 14 days, respectively. Guo et al., (2014) investigated the antimicrobial efficacy of the coatings and films against *Listeria innocua* inoculated onto the surface of RTE deli turkey meat. Antimicrobial coatings with 1.94 mg/cm^2 of chitosan and 0.388 mg/cm^2 of LAE (Lauric Arginate Ester) reduced *L. innocua* by ca. 4.5 log CFU/cm^2. Nisin (486 IU/cm^2) showed less effectiveness than LAE (0.388 mg/cm^2) and addition of nisin to the antimicrobial coatings or films containing LAE (0.388 mg/cm^2) did not enhance the total antimicrobial effectiveness.

Kanatt et al. (2013) reported a microbiological shelf-life extension of 10 days using chitosan coating in in RTE cooked meat products (chicken balls, chicken seekh kababs and mutton seekh kababs). Chitosan coating eliminated fecal coliforms, lowered counts of *Staphylococcus* spp. and also retarded lipid oxidation in all the meat products during storage at 0-3 °C. The ability of chitosan coating to reduce microbial load has been reported by other researchers on various products. Darmadji and Izumimoto (1994) reported that chitosan at a concentration of 1 g/100 ml reduced microbial counts by an average of 1-2 log CFU/g in minced beef patties stored at 4 °C for 10 days. Also, addition of chitosan at 1% in fresh pork sausages reduced counts by 0.5-1.5 log CFU/g (Soultos et al., 2008). In other studies, the combined use of rosemary extract or thyme along with chitosan extended the shelf-life of fresh pork sausages and chicken product stored under refrigeration (Georgantelis et al., 2007; Giatrakou et al., 2010). Petrou et al. (2012) reported the extension of the shelf life at 4 °C of a chicken breast meat product treated with chitosan (1.5% w/v) or its combination with oregano oil (0.25 % v/w) by

10 days. Sagoo et al. (2002) demonstrated that total viable counts, yeasts, and molds were reduced by approximately 1-3 log CFU/g on skinless and standard sausages dipped in a 1% chitosan solution before storage at 7 °C for 18 d.

Mohan et al. (2012) reported that the chitosan coating improved the water holding capacity, drip loss and textural properties significantly of double filleted Indian oil sardines compared to untreated samples. The sensory characteristics during chilled storage were acceptable up to 8 and 10 days for 1 and 2% chitosan treated samples respectively, compared to only 5 days for untreated samples.

CONCLUSIONS

The results of microbiological analyses, sensory evaluation and the significant reduction of *L. monocytogenes* on RTE meatballs during chilled storage, indicate that chitosan coatings represent a promising means for the shelf life extension and improvement of microbiological safety of RTE meat products.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

First Report of *Saprolegnia parasitica* from a Marine Species: Gilthead Seabream *(Sparus aurata)* in Brackish Water Conditions

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ABSTRACT. Saprolegniosis is a serious fungal disease that mostly affects freshwater fish species and eggs. It has a cotton wool-like appearance on the body of amphibians, crustaceans and several fish species. Infected gilthead seabream (*Sparus aurata*) were subjected to clinical, microbiological, parasitological and pathological investigation. On the infected skin samples, grey-white cotton-like patches, erosion of the skin and scale affusion were detected. Lesions covered the whole body of *S. aurata* in the advanced stages. Bacterial growth and parasitic symptoms were not observed in microbiological examination. Microscopic examination showed hyphaes carrying cysts that were long and branched. In scanning electron microscopy overviews fungal zoospores were observed. In histopathological observations of sections of skin, erosive-ulcerative dermatitis and mycelium of *Saprolegnia parasitica* were seen in the muscle tissue. Gene sequence-based identification found *Saprolegnia parasitica*. *S. parasitica* has not until now been detected in *S.aurata*. The low salinity of the brackish water is believed to be the predisposing factor of Saprolegniosis in sea bream in this case.

Keywords: Fish disease, fungal disease, *Saprolegnia parasitica*, *Sparus aurata*

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INTRODUCTION

Saprolegniosis is an acute infection and a worldwide mycotic freshwater disease affecting fish. It is widely distributed and all freshwater fish and eggs are susceptible to this infestation (Gaikowski et al., 2003). It is indicated in amphibians, crustaceans and several fish species : (channel catfish (*Ictalurus punctatus*) (Howe et al., 1999); cultured pike (*Sander lucioperca*) (Willoughby, 1985); elver (*Anguilla rostrata*) and suckers (*Catostomus commersonii*) (Roberts, 1989)) and especially in salmonids, tilapia species and carps ((*Oncorhynchus kisutch*) Hatai and Hoshiai, 1993; (*Salmo trutta*) Pickering and Christie, 1990 ; (*Tilapia nilotica*) Zaki et al., 2008; (*Carassius auratus*) Parra-Laca et al., 2015). It has also caused serious production losses (Alderman and Polglase, 1985; Post 1987). In Scottish, Norwegian and Chilean salmon hatcheries and farms in particular, Saprolegnia infections have been reported as causing losses of more than 10% (Langvad, 1994; Phillips et al., 2008; Van den Berg, 2013).

It usually starts with cotton wool-like growth on the head and dorsal fin region then spreads all over the body as focal patches (Abdel-Aziz et al., 2002; Bangyakkun et al., 2003; Osman et al., 2008; Roberts, 2012). In particular, cool and warm water fish eggs are reported to be at risk because their incubation temperatures are generally the same or near the optimum temperature range for zoospore growth (Gaiokowski et al., 2003). Stress factors such as abrasions, poor water quality, malnutrition, overcrowdedness, handling, spawning or any bacterial or parasitic infections lead to the presence of this mycotic disease (Noga, 1993; Pickering, 1994; Hussien et al., 2010). Temperature stress, especially cold temperatures, block immune system activity and reduce defenses against invading disease organisms, causing osmotic stress and mortality (Knights and Lasee, 1996; Hussien et al., 2010). It has been claimed that the final stages of this infection cause impairment of osmoregulation, failure of the respiratory system and, in some cases, of organs (Pickering and Willoughby, 1982; Van den Berg et al., 2013).The oomycete pathogen usually establishes itself focally, invading the stratum spongiosum of the dermis and then extending laterally over the epidermis, eroding it as it spreads. A relatively superficial invasion of the dermis rapidly leads to fluid imbalance and peripheral circulatory failure (shock) due to an inability to maintain blood circulation (Roberts, 2012).

In aquaculture, Saprolegniosis is observed mostly as *Saprolegnia parasitica* in numerous freshwater fish species (Van den Berg et al., 2013). *S.parasitica* was isolated from seabass (Cook and Unwin, 1985, press communication) and meagre (Abou El-Atta and Saleh, 2010) in brackish water conditions. Saprolegnia isolates were found not to develop any sexual stage in in vitro cultures and therefore cannot be identified (Grandes et al., 2000). The difference in radial growth rate, some biochemical characteristics and variation in the esterase isoenzyme pattern are used for the identification of some saprolegnia isolates (Beakes andFord 1983; Hatai et al., 1990; Welsh and McClelland, 1990), but moleculer methods have recently been used to characterise this infection (Van den Berg et al., 2013).

Formalin is the only drug currently approved by FDA for treatment as it has a strong effect and is relatively cheap. It is recommended to use 150-300 mg L^{-1} for the ova and fish (Marking et al., 1994; Taylor Francis et al., 1994). Malachite green was previously used to control Saprolegniosis, but it is now banned because of its carcinogenic, mutagenic and teratogenic characteristics (Gaiokowski et al., 2003). In addition, hydrogen peroxide has been used against Saprolegniosis but is not recognized by the FDA (Barnes et al., 1998; Howe et al., 1999; Gaiokowski et al., 2003).

In the present study, *Saprolegnia parasitica* was isolated from gilthead seabream (*Sparus aurata)*. In general, Saprolegniosis has been reported from fresh water fish species, especially in rainbow trout eggs in Turkey, but in the present water conditions, the fish were in brackish water, which is believed to be the reason for this first observation from sea bream.

MATERIALS AND METHODS

Fish

Infected gilthead seabream *(Sparus aurata)* from the university ponds at Katip Çelebi University Fisheries Research and Training Center which had a fungal infection were studied. A total of 48 fish weighing nearly 100 g were examined during the infestation. Fish were subjected to clinical, microbiological, parasitological and pathological investigation.

Water

During the outbreak, the water parameters of the ponds were monitored. Temperature, salinity, oxygen and pH parameters were determined to be 10 ºC, 3.93‰, 10.07 mg/L and 7.7, respectively.

Microbiological examination

External examination was conducted on the skin, abdomen, fins, scales, and internal organs such as the gills, kidney, spleen, intestine and liver were also investigated. Bacteriological examination was carried out according to Austin and Austin (2007). Bacterial isolates from the kidneys and spleen of infected fish were streaked on Tryptic Soy Agar (TSA, Oxoid) and Tryptic Soy Agar supplemented with 5% defibrinated sheep blood (BTSA) and incubated at 25 °C for 72 hours.

Pure cultures were provided by inoculation of the samples through taking a small tuft of mycellum from the fish skin and grown on Sabouraud Glucose Agar (SGA) and Malt Extract Agar (MEA) at 21 ºC for 3-4 days. Inoculation occured in a Haier Bio-Medical Biological Safety Cabinet. After incubation, unstained and colored examination was conducted with an Olympus BX53 Light Microscope. Colorization of the fungus was conducted with Giemsa staining (Arda, 2006) in order to identificate the fungi samples. Identification was performed according to Dvarak and Atanoesk (1969) both from wet samples from skin ulcers and growth on SGA and MEA (Dvarak and Atanoesk, 1969).

Histopathological examination

For histological examination, infected tissues of the skin with muscles, gills, liver and kidney were fixed in 10% buffered formalin after the necropsy. The tissue was then processed routinely and prepared into paraffin blocks. The blocks of the tissues were cut to 5 μm thickness and stained with Haematoxylin and Eosin (H-E) and periodic acid schiff (PAS) and examined under a light microscope (Culling et al., 1985).

Scanning electron microscopic examination

Samples were sputtered with gold by QUORUM Q150 RES and examined in a Carl Zeiss 300 VP scanning electron microscope in the Central Research Laboratory, Izmir Katip Celebi University.

Molecular Identification

DNA isolation was conducted using the GeneMATRIX Tissue and Bacterial DNA Purification Kit. For PCR amplification of the 5.8S rRNA gene, ITS1 and ITS4 primers were used. Amplified products of the template DNA were sent to the Macrogen direct sequencing service (Macrogen, Holland) for sequence determination. Samples were sequenced in two directions from opposite strands and the results were compared. Sequences were then checked with the BLASTN 2.6.1. database.

RESULTS

Infected fish were found to be stunned while swimming near the surface of water, with a loss of balance and reduced feed intake during the outbreak. The mortality rate was calculated to be 15% in that period. For treatment, formalin (containing approximately 37% formaldehyde by weight) was used to control the infection following a 30 min exposure at 2 ml/L every other day. On the infected skin samples, grey-white cotton-like patches, erosion of the skin and scale affusion were shown (Figure 1). Mycelial growth was detected especially on the head, eyes, gills, fins and body surface. In advanced stages lesions covered the whole body of *S. aurata* (Figure 2). The results of bacteriological and parasitological studies showed that neither pathogenic bacterial growth nor parasites were present in infected fish.

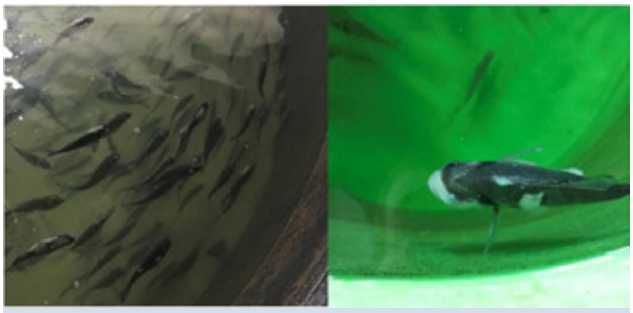

Figure 1. Infected gilthead sea bream (*Sparus aurata)* in unversity ponds.

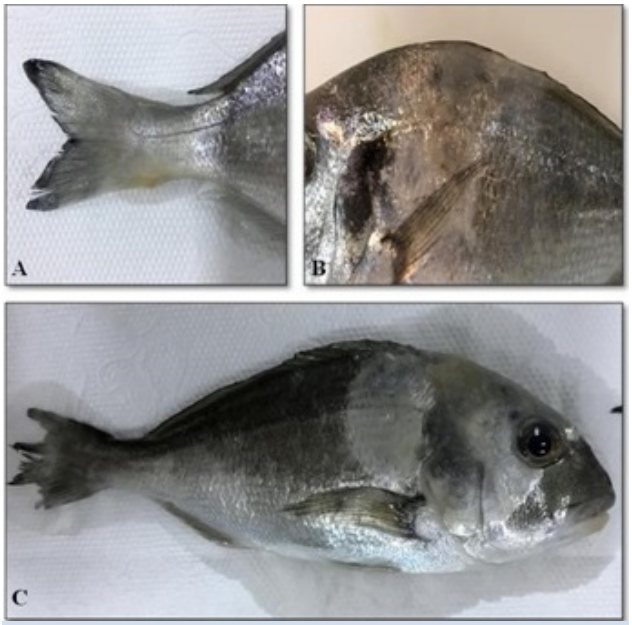

Figure 2. Infected gilthead sea bream (*Sparus aurata*). On the skin, grey-white cotton like *Saprolegnia parasitica* mycelium. A. On the pedincul. B.Dorsolateral. C. Dorsolateral, caudal and on the head localized.

Microbiological results

Isolation of *Saprolegnia parasitica* was conducted on SGA and MEA. In four days complete growth was observed.

Microscopic examination of *Saprolegnia parasitica* from native and colored samples is shown in Figures 3 and 4. The hyphae carrying the cysts are shown to be long and branched. Fungal zoospores from the scanning electron microscopy overviews are displayed (Figure 5).

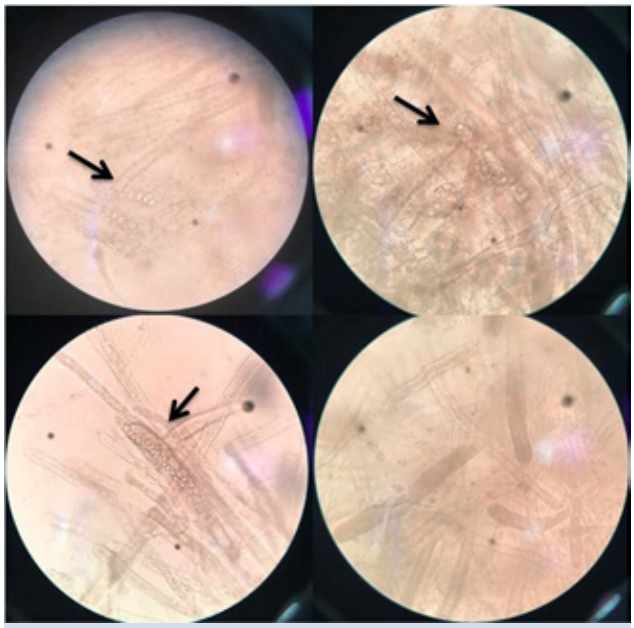

Figure 3. Unstained images *Saprolegnia parasitica* that shows zoospormatogoniums (arrows) (x400).

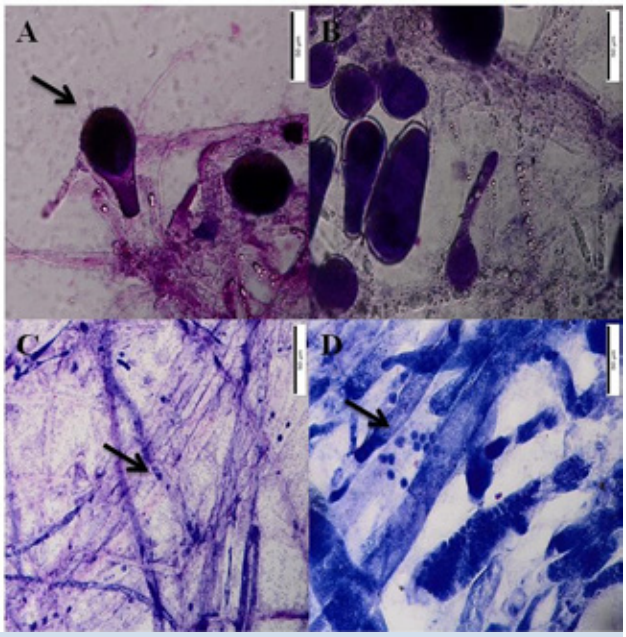

Figure 4. *Saprolegnia parasitica*. A-B. Zoospormatogoniums (arrow). C-D. Primer spormatogoniums (arrows) (Giemsa, x 200).

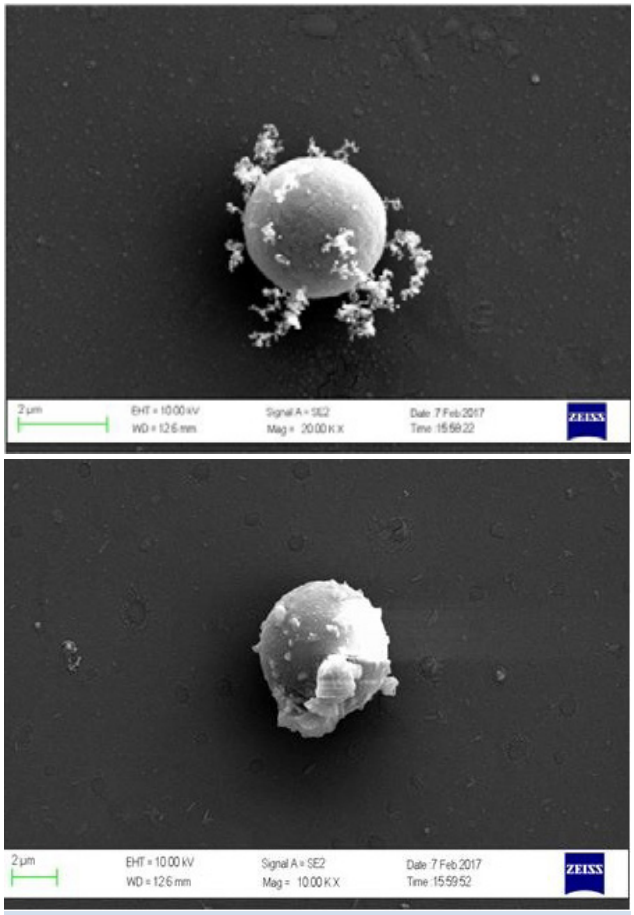

Figure 5. The zoospores of *Saprolegnia parasitica*. SEM. Bar. 2 µm scale.

Histopathological results

Erosive-ulcerative dermatitis was seen in skin sections. In epidermis and hypodermis necrosis, there were regenerations in intact epithelium. Dense filamentous mycelium was detected on the surface. This reacted positively to PAS staining. Mycelium and leukocyte infiltration were observed in dilate flake pockets (Figure 6). Liquefaction necrosis was detected in the muscles of the same area. A small number of mycelia was found among these muscles (Figure 7). Hepatocytes were moderately fatty in the liver, while histopathological findings were not found in other organs.

Molecular Identification Results

The PCR amplification of 18S rRNA gene sequence was checked in the BLASTN 2.6.1 database. The FASTA homology resulted in 100% nucleotide identity between the current isolate and *Saprolegnia parasitica* (accession number AM228725.1)

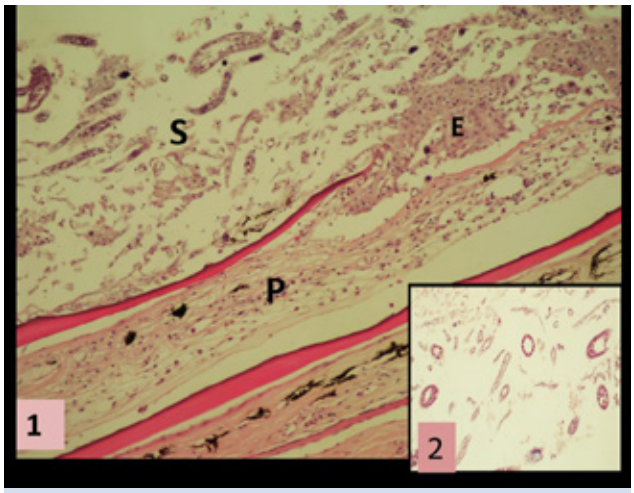

Figure 6. Intensive *Saprolegnia parasitica* hyphas and zoospores on skin surface (S). 1. Dilatation on epidermis and scale pockets (P), cell infiltration. Lateral section of zoosporagonium (H.E. x 200).

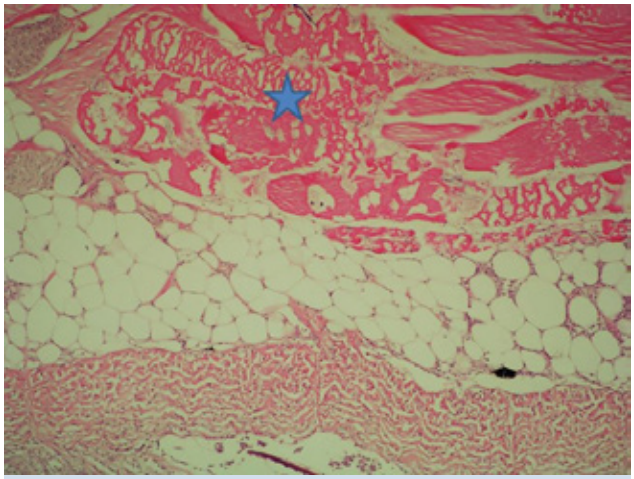

Figure 7. Liquefaction necrosis in subcutaneous muscles .(star).H.E. x100).

DISCUSSION

Saprolegniosis, which is caused by *S. parasitica*, is a worldwide disease that mostly affects freshwater fish and eggs and cause great economic losses to the aquaculture industry (Duboon et al., 2006). Countries like Norway, Ireland, Japan and the UK have reported mass mortalities based on salmon aquaculture (Bruno et al., 2011). Eggs are particularly susceptible to this infestation because of incubation systems that allow close contact and attachment of zoospores, although infertile and dead eggs are also convenient substrate for zoospore colonization (Gaikowski et al., 2003).

Throughout the outbreak, the water parameters of the ponds were observed. Temperature, salinity, oxygen and pH parameters were determined to be 10 ºC, 3.93‰, 10.07 mg/L and 7.7, respectively. Elatta (2013) reported a fungal disease (*Aphanomyces sp*.) in *Sparus aurata* with nearly the same salinity, 3‰. In research on Norwegian salmon hatcheries, Thoen et al (2015) claimed that water temperatures higher than 6 ºC did not result in an increase in the number of Saprolegnia spp. spores, and this was also the case in regard to oxygen pressure and water flow. Chauhan (2014) isolated some fungi species from the pond culture of *Tilapia mossambicus* and identified two of them as *Saprolegnia diclina* and *Saprolegnia parasitica*. During the study, the water temperature was recorded as 16 ± 2 ºC, pH 7.9 ± 1.6 and dissolved oxygen 6.8 ± 3.0 mg/l. These parameters are similar to those found to be suitable for Saprolegniosis reproduction in the current study.

Temperature stress may slow immune system activity and reduce the defense against pathogens (Knights and Lasee, 1996). Temperature shock and poor water quality are also inductors of Saprolegniosis (Yanong, 2003; Gieseker et al., 2006). Howe and Stehly (1998) claimed that during the winter months, fungal infections cause major losses. Bly et al (1992) explained this as the rapid decrease of the water temperature leading to immunosuppression that crosses over with the presence of fungal zoospores. In this study, the Saprolegniosis outbreak occurred after the rapid decrease of water temperature, from 13° C to 10° C, with an increase of turbidity which are also contributing factors along low salinity, in January 2017.

Saprolegniosis has been reported in rainbow trout eggs (Diler, 1992), fry (Kubilay et al., 2008) and adult individuals from different commercial companies in Turkey (Aydın and Küçükgül, 2014). Aydın and Küçükgül (2014) published results showing that in four rainbow trout farms Saprolegniosis was determined in half of these at times in the year when the water temperature was 10-14 ºC. During these periods of time mortality rates were calculated to be 8% and 13.3% as a result of these infestations. In the current study, Saprolegniosis was isolated in a marine species, gilthead sea bream, in nearly the same temperature conditions and caused 15% mortality during the outbreak.

Hussien et al (2010) observed focal greyish-white patches on the skin, fins, gills and head region of *Mugel cephalus* which were caused by the Saprolegniosis infection. Similarly, Das et al (2012) noted visible red or grey patches of filamentous mycelium in Indian major carp fingerlings. Cotton-like patches characteristically reported for Saprolegniosis on macroscopic

examination (Chauhan et al., 2014) were observed on the skin surface of all gilthead sea bream in this study. Localizations were more pronounced in the dorsal and caudal regions, as mentioned above.

Bruno et al (1999) considered Saprolegniosis as a secondary infection arising from bacterial infections, immunosuppression, parasite infestations and poor husbandry. It reduces osmoregulation and often leads to death (Pickering and Willoughby 1988). Kubilay et al (2008) reported Saprolegnia spp. from rainbow trout fry that were infected with *Flavobacterium columnare*. On the contrary, Pottinger and Day (1999) observed no secondary disease problems with the same species. In this study, neither pathogenic bacteria nor parasites were detected in gilthead sea bream during the infestation.

The primary zoosporagonias and mycelia, as described by Mueller (1992), were formed as clusters with PAS stain made from skin-scraping samples. Among these, primer zoospores in the form of free spots were also observed. The predominant clinical and histopathological feature in all cases is the extensive ulcerative lesion, which overlies a penetrating myopathy extending deep into the muscle. It has a greyish-white necrotic superficial covering of degenerating tissue and fungal hyphae (Roberts, 2012; Hussein et al., 2013). Similar findings were observed in this study, but fungal lesions reported in internal organs were not found in sea bass (Chauhan et al., 2014). Lesions and fungi were limited to the skin only. The reason for this is related to the differences between the water and the fish species.

CONCLUSIONS

S. parasitica has not yet been detected in cage farms of *S. aurata*. The reason for the absence of the infestation in cage farms is the high salinity that does not allow any zoospore growth in sea water. Brackish water salinity tends to let both marine and freshwater species exist, as it also does the zoospores of the Saprolegnia species. In this case, the low salinity parameters and the rapid decrease of water temperature caused stress and led to Saprolegniosis in sea bream.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Staphylococcus aureus and bovine mastitis: molecular typing of methicillin-resistance and clinical description of infected quarters

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ABSTRACT. This study targeted *Staphylococcus aureus (S. aureus)*-mastitis of bovine as a possible source of livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA), to describe clinical signs of mastitis associated with MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA). The study area was the Gharbia and Kafrelsheikh governorates, in the central and northern regions of the Egyptian Delta. Clinical examination of animals was performed to detect clinical mastitis (CM) and clinically normal cases were tested by the California mastitis test (CMT) to identify subclinical mastitis (SCM). Accordingly, 38 mastitis cases (68 infected quarters) were detected. Milk samples were collected aseptically and were cultured on Baird Parker agar. Fifty nine Gram-positive cocci-shaped isolates were selected and preserved. In addition, 33 *Staphylococcus* spp. isolates originated from bovine mastitis at the same study area were obtained from Animal Health Research Institute (AHRI). The cocci-shaped Gram-positive bacteria and AHRI *Staphylococcus* spp. isolates were used for molecular identification of *S. aureus* and MRSA. Molecular screening had yielded 17 *S. aureus* isolates, from which five isolates (29.41%) were MRSA and 12 isolates (70.59%) were MSSA. The five MRSA isolates were *mecA* positive, but *mecC* negative. Multilocus sequence typing (MLST) of the five MRSA isolates indicated that all were sequence type 1 (ST1). *S. aureus*-associated cases showed different clinical forms of mastitis, including subclinical, acute, chronic, and gangrenous. However, subclinical mastitis was the only detected form associated with MRSA, which may represent a potential hidden risk for humans. Phenotypic antimicrobial-resistance pattern of MRSA isolates showed resistance to all of the tested β-lactam antimicrobials, with marked

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resistance to tetracycline and gentamycin. Based on our knowledge, this is the first report to identify MRSA ST1 in Egypt. Bovine mastitis could be a source for the dissemination of MRSA to humans and other animals. Additionally, while methicillin-resistance may have no effect on the clinical severity of mastitis, it does affect therapeutic success, particularly when β-lactam antimicrobials are used.

Keywords: *MRSA, Bovine, Mastitis*, *Staphylococcus aureus*

INTRODUCTION

Staphylococci, particularly *S. aureus*, are common pathogens of mastitis in bovines (Haveri et al., 2007), and the *Staphylococcus* spp. associated mastitis is responsible for considerable economic losses (Lammers et al., 2000). *S. aureus* accounts for 25–30% of all intra-mammary infections (IMI) in cows (Poutrel, 1985). Importantly, *S. aureus* IMI results in a 10–25% milk reduction in infected animals (Anderson, 1983). *S. aureus* can induce either CM or SCM, but the subclinical form is more predominant (Anderson, 1983; Lammers et al., 2000; Akineden et al., 2001). While CM has detectable clinical symptoms, SCM has no detectable symptoms and necessitates screening using the CMT (Kasikci et al., 2012). The success in staphylococcal mastitis therapy is dependent on the individual animal, treatment, and pathogen factors (Barkema et al., 2006), and the resistance to antimicrobials is a major factor affecting the cure rates of staphylococcal mastitis (Barkema et al., 2006).

Staphylococci, notably *S. aureus*, have shown resistance to various antimicrobials (Wang et al., 2015), and MRSA strains have gained worldwide attention. MRSA is classified into three categories according to its origin: livestock-associated (LA-MRSA), healthcare-associated (HA-MRSA), and community-associated (CA-MRSA) (Stefani et al., 2012). There is an increasing global interest in LA-MRSA because of its animal and human associated health implications (Graveland et al., 2011). Many sequence types (ST1, ST9, ST97, ST130, ST398, and ST425) of MRSA had been recorded in both cattle and humans (García-Álvarez et al., 2011; Paterson et al., 2012; Spoor et al., 2013; Alba et al., 2015; Cuny et al., 2015). In humans, LA-MRSA strains can colonize tissues, resulting in pneumonia, endocarditis, and other life threatening conditions (Ekkelenkamp et al., 2006; Witte et al., 2007). Molecular epidemiology studies on MRSA in southern Mediterranean countries are limited (Borg et al., 2007). In Egypt, there are very few studies on MRSA strains originating from bovine mastitis (Elhaig and Selim, 2015). Additionally, studies describing the clinical aspects of MRSA- and non-MRSA-associated bovine mastitis are limited. Therefore, this study was intended to screen for MRSA and its sequence types associated with bovine mastitis in Egypt, and to describe the clinical aspects of MRSA- and MSSA-associated bovine mastitis.

MATERIAL AND METHODS

Animals, detection of mastitis and sampling

Study animals were reared in the Gharbia and Kafrelsheikh governorates, in the central and northern regions of the Egyptian Delta. In these areas, the dairy animals are reared in small groups rather than organized farms. Mastitis cases were detected by clinical examination of the animals with special attention to the udder according to Houe et al. (2002), clinically normal cases were tested for SCM by CMT according to Kasikci et al. (2012).

Milk samples were aseptically collected according to Quinn et al. (1994): teats were wiped efficiently with 70% ethyl alcohol, first strip of milk was discarded and a suitable amount of milk (about 5 ml of milk) was collected in a sterile screw-capped Falcon tube. Samples were sent to the laboratory on ice quickly after collection.

Bacteriological examination

Milk samples were centrifuged (1000 *g*/5 min), the supernatant was discarded and sediment was streaked on Baird Parker agar. A 24 h incubation at 37 °C was done according to Silva et al. (2000). Gram-stained Smears of the colonies were examined. The putative *Staphylococcus* species isolates were preserved in glycerol stock at −20 °C.

Animal Health Research Institute (AHRI) *Staphylococcus* species Isolates

In addition to the above mentioned putative isolates, another 33 *Staphylococcus* spp. isolates were obtained from AHRI. Such isolates originated from bovine mastitis cases at the same study area, but clinical data of these isolates were not recorded.

Both of the isolated *Staphylococcus* spp. isolates and the obtained AHRI isolates were used in molecular procedures.

Molecular characterization and typing

For extraction of DNA, Luria-Bertani agar plates were streaked by the isolates and incubated for 24 h at 37 °C. The extraction of DNA was performed by InstaGene matrix (Bio-Rad Laboratories Inc.). The extracted DNA was preserved at −20 °C for use in PCR assays. Primer sequences of *S. aureus*, MRSA and MLST are shown in Table 1.

A PCR assay targeting a 359-bp region of the *S. aureus* thermonuclease (*nuc*) gene was used to detect *S. aureus* as described by Sasaki et al. (2010) with a few modifications. Briefly, a 25-µl reaction was prepared containing 5 µL of DNA, 0.2 mM dNTPs, 1× buffer, 0.5 U of AmpliTaq Gold (Applied Biosystems), and primers (each of 20 pmol). The thermal cycler conditions consisted of 95 °C /10 min, 35 cycles (95 °C/30 s, 56 °C/35 s, and 72 °C/1 min), followed by 72 °C/10 min.

MRSA was identified by PCR targeting 147- and 138-bp regions of *mecA* and *mecC* ($mecA_{LGA251}$) as described by Zhang et al. (2005) and Stegger et al. (2012), respectively, with a few modifications. The 25 µL reaction consisted of 5 µL of DNA, 0.2 mM dNTPs, 1× buffer, 0.5 U of AmpliTaq Gold (Applied Biosystems), and primers (20 pmol of each). Mixtures of *mecA* and *mecC* were initially heated at 94 °C for 4 min/15 min, followed by 35/30 cycles of 94 °C/30 s, 52 /59 °C for 30 s/1 min, and 72 °C for 45 s/1 min, respectively. A final extension was conducted at 72 °C/7 min.

Multi-locus sequence typing of MRSA isolates was performed using seven housekeeping genes according to Enright et al. (2000) and allelic profiles were obtained from MLST web site (http://saureus.beta.mlst.net/).

Table 1. Primers of *S. Aureus*, MRSA and MLST.

Primer	Gene	Sequence (5'-3')	Size (bp)	References
au-F3	*Nuc*	TCGCTTGCTATGATT GTGG	359	Sasaki et al., 2010
au-nucR		GCCAATGTTCTACCA TAGC		
***MecA*147-F**	*MecA*	GTG AAG ATA TAC CAA GTG ATT	147	Zhang et al., 2005
***MecA*147-R**		ATG CGC TAT AGA TTG AAA GGA T		
***mecA*$_{LGA251}$MultiFP**	*mecA*$_{LGA251}$	GAAAAAAAGGCTTAGAACGCCTC	138	Stegger et al., 2011
***mecA*$_{LGA251}$MultiRP**		GAAGATCTTTTCCGTTTTCAGC		
***arcC*-Up**	*Arc*	TTGATTCACCAGCGCGTATTGTC	456	Enright et al., 2000
***arcC*-Dn**		AGGTATCTGCTTCAATCAGCG		
***aroE*-Up**	*aroE*	ATCGGAAATCCTATTTCACATTC	456	
***aroE*-Dn**		GGTGTTGTATTAATAACGATATC		
***glpF*-Up**	*GlpF*	CTAGGAACTGCAATCTTAATCC	465	
***glpF*-Dn**		TGGTAAAATCGCATGTCCAATTC		
***gmk*-Up**	*Gmk*	ATCGTTTTATCGGGACCATC	429	
***gmk*-Dn**		TCATTAACTACAACGTAATCGTA		
***pta*-Up**	*Pta*	GTTAAAATCGTATTACCTGAAGG	474	
***pta*-Dn**		GACCCTTTTGTTGAAAAGCTTAA		
***tpi*-Up**	*Tpi*	TCGTTCATTCTGAACGTCGTGAA	402	
***tpi*-Dn**		TTTGCACCTTCTAACAATTGTAC		
***yqiL*-Up**	*YqiL*	CAGCATACAGGACACCTATTGGC	516	
***yqi*L-Dn**		CGTTGAGGAATCGATACTGGAAC		

Antimicrobial susceptibility testing

All *S. aureus* isolates were examined for their susceptibility to ampicillin, tetracycline, ciprofloxacin, gentamicin, sulfamethoxazole and trimethoprim, teicoplanin, ceftriaxone, amoxicillin and clavulanic acid, oxacillin, and cefoxitin. The antibiotic disc diffusion guidelines of The Clinical and Laboratory Standards Institute (2005) were followed.

RESULTS

Clinical and descriptive aspects of mastitis cases

A total of 38 mastitis cases (68 affected quarters) were detected. Twenty five cases had single diseased quarter, while 13 cases had multiple affected quarters. Different clinical forms of mastitis were noticed as shown in Figure 1.

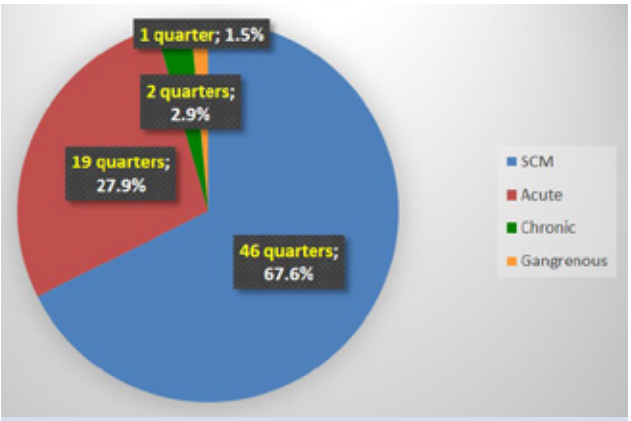

Figure 1.

Subclinical mastitis presented no obvious symptoms except of reduced amount of milk from the affected quarters, the physical characters of milk were normal. On the other hand, acute mastitis showed hotness, pain and enlargement of the affected quarter. Systemic reaction appeared as fever, congested mucous membranes, reduced appetite, increased heart and respiratory rates were recorded in a few cases of acute mastitis. The physical characters of milk were changed; yellowish semi-transparent offensive odor milk were the most common detected changes. Chronic affected quarters were fibrous discharging scanty amount of watery milk, while gangrenous quarter was of blackish discoloration, cold temperature and discharging bloody milk.

Identification of *S. aureus*

Gram-positive cocci-shaped bacteria were isolated from 36/38 mastitis cases (94.7%) and 49/68 infected quarters (72.1%). Based on Baird Parker agar growth and Gram-stained smears, 59 gram-positive cocci-shaped isolates were selected. Molecular screening for *S. aureus* yielded seven isolates from seven individual cases (four subclinical cases, one acute, one chronic and one gangrenous case). In addition, molecular screening of the AHRI isolates resulted in an additional 10 isolates of *S. aureus*. Consequently, 17 *S. aureus* isolates were used in further phenotypic and genotypic investigations.

Antimicrobial susceptibility of *S. aureus* isolated strains

A marked resistance of *S. aureus* to teicoplanin and ampicillin was observed. In addition, oxacillin-resistance was evident in five *S. aureus* isolates. *S. aureus* phenotypic and genotypic profiles are listed in Table 2.

Molecular screening/typing of MRSA

Out of the 17 *S. aureus* strains, 12 isolates (70.59%) were MSSA and five (two originated from the clinical cases and three AHRI isolates) strains contained *mecA* (29.41%) and were identified as MRSA ST1. However, *mecC* was absent in all of the *S. aureus* isolates.

Clinical nature of MRSA-associated bovine mastitis

The two cases (AHRI isolates had no clinical data) which were infected by MRSA ST1 had showed SCM, which may indicate that MRSA ST1 may be unable to induce severe mastitis cases.

DISCUSSION

LA-MRSA strains are reported to induce endocarditis, pneumonia, soft tissue and skin conditions in humans (Ekkelenkamp et al., 2006; Witte et al., 2007). Interestingly, the current study identified a high percentage of MRSA ST1 (29.41%) amongst the *S. aureus* strains isolated from mastitis in bovines. However, larger scale investigations are important to assess LA-MRSA of bovine mastitis origin and its potential risk for humans in Egypt. MRSA ST1 is a wide spread LA-MRSA lineage with a broad host range including humans (Alba et al., 2015). Although it is commonly isolated from pigs, recent studies had recorded MRSA ST1 associated with cattle and dairy farming in some countries such as Italy and Hungary (Juhász-Kaszanyitzky et al., 2007; Alba et al., 2015). Moreover, studies had showed high genetic similarity (90-100%) between human and cattle associated MRSA ST1 and confirmed complete ability of the later to colonize and infect humans (Juhász-Kaszanyitzky et al., 2007; Alba et al., 2015).

Table 2. Phenotypic and genotypic profiles of *S. Aureus* isolates.

Isolates	Phenotypic resistance		Genotypic
	β-lactams	Other antimicrobials	resistance
Sa28	AMP, FOX	TEC	
Sa69	AMP, AMC, FOX, CRO, OXA	TET, TEC	*mecA*
Sa70	AMP, AMC, FOX, CRO	TET, GEN, TEC	*mecA*
Sa101	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	*mecA*
Sa104	FOX	TEC	
Sa107	AMP	TET, GEN,TEC	
Sa119	AMP	GEN, TEC	
Sa120	AMP	-	
Sa131	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	*mecA*
Sa135	AMP, FOX	TET, TEC	
Sa136	-	TET, TEC	
Sa137	AMP, AMC, FOX, CRO, OXA	GEN, TEC	*mecA*
Sa140	AMP	-	
Sa144	AMP, AMC, FOX, CRO, OXA	TET, GEN, TEC	
Sa146	AMP, FOX	-	
Sa158	AMP, AMC	-	
Sa164	AMP	-	

TET, tetracycline; CIP, ciprofloxacin; SXT, sulfamethoxazole and trimethoprim; GEN, gentamicin; AMP, ampicillin; AMC, amoxicillin and clavulanic acid; FOX, cefoxitin; TEC, teicoplanin; CRO, ceftriaxone; OXA, oxacillin.

The affection of a single quarter, in spite of lack of hygienic milking procedures, in most of the detected mastitis cases indicated the low contagiousness of the involved pathogens. This was confirmed by PCR which showed that most of Staphylococcus isolates were not *S. aureus*. In addition, SCM was more prevalent than CM, which agrees with a recent study in Egypt (Elhaig & Selim, 2015), and highlights the importance and widespread nature of SCM.

Staphylococcus aureus was associated with different clinical forms of mastitis. The resulting clinical form of *S. aureus* mastitis is a multifactorial process which is influenced by host's immunity and virulence determinants of *S. aureus* isolates (Haveri et al., 2007). On the other hand, the current study showed that MRSA ST1 was associated only with SCM. This is because most LA-MRSA isolates lack many mastitis-associated virulence factors such as toxic shock syndrome toxin 1, hemolysins, and enterotoxins (Monecke et al., 2007; Walther et al., 2009). Despite this, the involvement of LA-MRSA (ST398) in a few CM cases has been reported previously (Vanderhaeghen et al., 2010).

Antimicrobial susceptibility of methicillin resistant and sensitive *Staphylococcus aureus* isolates revealed prominent differences. The MRSA isolates showed resistance to most of the tested antimicrobials (Table 2). The resistance of MRSA to oxacillin and other β-lactam antimicrobials can be attributed to the existence of *mecA*, which codes for a penicillin binding protein with low affinity for all β-lactams (Hartman & Tomasz, 1984).

In conclusion, SCM is more prevalent than CM, *S. aureus* associated bovine mastitis was of variable clinical nature (subclinical, acute, chronic and gangrenous), bovine mastitis is a source of LA-MRSA ST1 which is a health risk for humans and source of infection of animals. Additionally, MRSA may be unable to induce severe mastitis, but it will affect therapeutic success, particularly when β-lactams are used for treatment.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

The effects of royal jelly on oxidative stress and toxicity in tissues induced by malathion, an organophosphate insecticide

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ABSTRACT. Royal jelly is a bee product frequently used in pharmaceutical, food and cosmetic industries due to its biological activities. The present study aimed to determine the effects of royal jelly on malathion-induced toxicity and biochemical changes. The rats that were used as experimental animals in the study were divided into 6 groups. Control group rats were administered nothing, while carrier chemicals (1% DMSO) were administered to sham group rats. Malathion group (MAL) rats were injected with 0.8 g/kg malathion in DMSO subcutaneously. Saline solution that included 100 mg/kg royal jelly was administered with gavage to the rats in the royal jelly group (RJ). 100 mg/kg royal jelly was administered to RJ+MAL group rats via gavage 1 hour before the injection of 0.8 g/kg malathion. 100 mg/kg royal jelly was administered to MAL+RJ group rats via gavage 1 hour after the injection of 0.8 g/kg malathion. After the experimental process (24 hours), blood samples were taken from the rats in each group under anesthesia (ketamine+xylazine). MDA, NO, GSH, GPx (glutathione peroxidase), CAT, SOD and AChE activities were determined in blood, liver, kidney and brain tissues. It was found that erythrocyte, liver, kidney and brain MDA (malondialdehyde) concentrations in MAL groups were statistically significantly higher when compared to the other groups ($p<0.05$). It was observed that GSH (glutathione) concentrations increased in the brain, while they decreased in erythrocyte, liver and kidney in the MAL group when compared to the control and sham groups. CAT (catalase) concentration significantly decreased in erythrocyte, liver, kidney and brain tissues in the MAL group when compared to the control and sham groups ($p<0.05$). SOD (superoxide dismutase) concentration in the MAL group decreased significantly ($p<0.05$) when compared to other groups, while SOD concentration increased significantly in the therapy and prevention groups ($p<0.05$) when compared to the others. It was found that serum acetylcholinesterase (AChE) concentration was significantly lower in the MAL group when compared to sham and control groups ($p<0.05$). Thus, it was concluded that malathion led to lipid peroxidation and oxidative stress in MDA and NO (nitric oxide) levels and toxicity in AChE activities. It was also determined that royal jelly could be effective against oxidative damage and toxicity. The findings suggested that the antioxidant effect of royal jelly could support the treatment of malathion, which is one of the insecticides that contain organophosphate and could lead to oxidative stress. It is considered that the prophylactic characteristics of royal jelly was more effective on malathion toxicity when compared to therapatic properties.

Keywords: antioxidant, malathion, organophosphate, oxidative stress, royal jelly

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INTRODUCTION

Royal jelly is a product secreted by the mandible and the pharyngeal gland of 5-15 days old worker bees to feed the young larvae (Khazaei et al., 2018). As soon as it is secreted, royal jelly is used directly to feed the larvae or the queen bee. The royal jelly is the primary nutrient for the queen bee, and it alters the morphology, longevity, and behavior of the queen bee during growth and development periods (Bucekova et al., 2017). Dry weight of the royal jelly includes sugar, protein, fat and amino acids. It also includes trace quantities of vitamins and minerals. Royal jelly contains all amino acids that are essential to humans. The most important amino acids among these are aspartic acid and glutamic acid. Among the enzymes found in the composition of royal jelly, glucose oxidase, phosphatase and cholinesterase are the most important. The most important fatty acid is 10-hydroxy-delta- (2) -decanoic acid (10-HDA), while the most important amino acids are glutamic acid and aspartic acid. It was suggested that the biological effects of the royal jelly were due to its royalactin, apicin and 10-hydroxy-delta-(2)-decanoic acid (10-HDA) content (Kim et al. 2010; Bincoletto et al., 2005). Several studies reported anti-tumor (Tamura et al., 1987), anti-diabetic and immune system stimulator (Heidrick et al. 1984), antiallergic (Okamoto et al., 2003), antibacterial and antiviral (Fontana et al., 2004), and anti-inflammatory (Majtan et al., 2010) properties of royal jelly.

Insecticides are the largest group of pesticides that are used to control insects which damage the agricultural crops. They affect the nervous system or destroy the biomolecules via reactive oxygen species. Since the nervous systems of the insects are highly developed and resemble those of mammals, the toxic effects of the insecticides and the organs they target are similar across the species (Costa et al., 2008). The insecticide dose, the intake method, the rate of metabolization, and the rate of exposure are significant. Globally, a large number of individuals die due to pesticide poisoning every year, and the majority of these cases are induced by insecticides (Jeyaratnam, 1990). Organophosphate compounds are not well soluble in water, however they are well soluble in oils and organic solvents. These insecticides are hydrolyzed in 2-4 weeks at the sites of administration or in solution form. Malathion [O,O-dimethyl-S-(1,2-dicarbethoxy-ethyl) phosphonodithioate], also known as Carbophos, Maldison and Mercaptothion, is an organophosphate insecticide with low toxicity in mammals and used for insect control worldwide (Tós-Luty et al., 2003; Choudhary et al., 2008). Malathion is converted to more toxic malaoxon by cytochrome P450 enzymes in liver. Malaoxon is an inhibitor of the acetylcholinesterase enzyme (Aker et al., 2008). Carboxylesterase enzyme works faster in mammals when compared to insects, thus both malathion and malaoxon breaks down faster in mammals. Due to its high activity in humans, its toxic effects are scarcely observed. In insects, since this enzyme works slower, malaoxon accumulation leads to neural contractions and death (Timur et al., 2003).

Free oxygen radicals are molecules that can easily exchange electrons with other molecules due to the absence of electrons. Aerobic organisms are exposed to reactive oxygen species induced by molecular oxygen during their lifespan. The degradation of oxidant-antioxidant balance favoring the free radicals leads to various metabolic problems (Gutteridge and Mitchell, 1999). Certain studies demonstrated that antioxidant administration could reduce malathion induced oxidative stress (Kalender et al., 2010; Sodhi et al., 2008; John et al., 2001).

The present study was conducted to investigate oxidative stress that occurs due to organophosphate insecticides and malathion induced toxicity and the protective and therapeutic effects of royal jelly known to be effective against oxidative stress in rats. For this purpose, blood, liver, kidney and brain tissue MDA and NO concentrations, antioxidant levels (GSH, CAT, GPx, SOD) and AchE activities were investigated.

MATERIAL AND METHOD

Animals and Experimental Protocol

Ethics committee approval was obtained from Afyon Kocatepe University Animal Ethics Board Committee (AKÜHADYEK-156-12). Forty-two 180-220 g Wistar-Albino rats were procured from Afyon Kocatepe University Experimental Animal Research and Application Center. The rats were kept in an environment with ideal light and temperature conditions (12 hours light/12 hours darkness) in polypropylene cages. The rats were fed ad libitum with standard mice food and water. The number of animals (n=7) was determined based on the requirements for a secure statistical analysis (Aksoy and Aslan, 2017). The rats were divided into 6 groups. No substances were administered/injeced to the control group rats during the experiments. Injections were administered to the

sham group using DMSO (1%) solution subcutaneously on the loose skin behind the neck. MAL group rats were injected with 0.8 g/kg malathion in 1% DMSO subcutaneously (Moore et al. 2011; Aksoy et al., 2017). Royal jelly (RJ) group rats were administered 100 mg/kg/day royal jelly (Aksoy and Aslan, 2017). Royal jelly was dissolved in equal volumes of saline solution. MAL+ RJ group rats were first administered 100 mg/kg royal jelly in saline solution orally. After 1 hour, 0.8 g/kg malathion in 1% DMSO was injected subcutaneously. RJ+ MAL group was initially injected with 0.8 g/kg malathion in 1% DMSO subcutaneously. After 1 hour, 100 mg/kg royal jelly dissolved in saline solution was administered orally. All groups were fed on a regular basis. All rats were sacrificed, and blood samples were collected on the 24th hour of the study. Anesthesia was performed by intramuscular injection with ketamine+xylazine. Blood was collected and allowed to clot. Serum was separated at 3500 rpm for 15 minutes at 4°C, then used for the determination of serum AchE concentrations. Tissues were homogenized (1.40; w/v) in 0.1 M of phosphate buffer (pH 7.4) that contained 1mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 18000×g for 15 min at 4°C to yield a clear supernatant fraction that was used for MDA, NO, GSH and CAT analyses. The erythrocytes were prepared with 5 min centrifuge at 3000 rpm and with 0.9% NaCl for 4000 rpm, after which the supernatants were discarded. Erythrocytes were hemolyzed with cold distilled water, and SOD and GPx values were measured.

Biochemical Analysis

MDA concentration was determined by the Jain and Ohkawa method (Jain et al., 1989; Ohkawa et al., 1979), which is based on the measurement of the absorbance of the colored complex induced by TCA-TBA reaction at high temperatures at 535 nm. GSH concentration was determined by the method based on DTNB reduction (Beutler et al., 1963). CAT enzyme activity was measured by Aebi method (Aebi, 1974), based on the measurement of the reduction of the absorbance of H_2O_2, broken down by the enzyme at 240 nm. NOx levels were determined with the Griess method, where the absorbance of VCl_3 (Vanadium(III) chloride) and NEDD (N-1-napthylethylenediamine dihydrochloride) formation with the addition of Somogyi reactive in the deproteinized tissues at 546 nm (Miranda et al., 2001). Serum AChE activity was measured with quantitative sandwich EIA kits. In the measurement of SOD enzyme activity, the reaction catalyzed by xanthine oxidase from xanthine produces uric acid and superoxide radicals. The produced superoxide radical reacts with 2-(4-iodophenyl)-3-(4-nitro-phenyl-5-phenyltetrazolium chloride to form a red colored formazan compound. An assay kit was used to determine the SOD activity by the inhibition degree of this reaction. GSH was reduced by glutathione reductase in the presence of NADPH, during which NADPH (Nicotinamide adenine dinucleotide phosphate) is to $NADP^+$ (Oxidized nicotinamide adenine dinucleotide phosphate). The change in absorbance due to the decrease in reduced NADPH was measured spectrophotometrically at 340 nm and GPx enzyme activity was determined using the assay kit.

Statistical Analysis

The data were expressed as the mean±standard deviation (SD) values. Statistical comparisons were conducted using ANOVA with Duncan post-hoc tests. Differences within $p<0.05$ were considered significant. The SPSS (12.0 Chicago, IL) for Windows software was used for statistical analyses.

RESULTS AND DISCUSSION

Since OP (organophosphate) compounds are widely used, they lead to serious toxic events. The toxic effects of OP compounds occur by inhibition of the acetylcholinesterase enzyme. Another toxic effect of OP insecticide is through the production of reactive oxygen species (ROS) and facilitation of their adverse effects on biomolecules that are important for the organisms. It was emphasized that OPs are one of the causes of oxidative stress. It was demonstrated that OP compounds lead to intensive ROS production and oxidative destruction in biomolecules that are significant for the metabolism (Altuntas et al., 2003). In a previous study, it was demonstrated that malathion administration resulted in cytotoxic and genotoxic effects in bone marrow and liver cells. In the same study, it was stated that RJ was a potent antioxidant against cytotoxic and genotoxic effects on bone marrow and liver cells (Abd El-Monem, 2011). The present study was designed to determine the antioxidative effects of royal jelly on malathion induced toxicity/oxidative stress in various tissues.

Lipids are among the biomolecule groups that are destroyed by reactive oxygen species. Lipid peroxidation is among the most harmful reactions to metabolism since it proceeds as a self-sustaining chain

reaction and is irreversible. The most important product formed during the last stage of lipid peroxidation is MDA. It directly damages other cell components directly by destroying the membrane structure and indirectly by producing reactive aldehydes. These reactions lead to oxidative degradation in cellular membrane and severe tissue damage. MDA is frequently used to determine oxidative damage (Gaweł et., al 2004). The erythrocyte and liver, kidney and brain tissue MDA concentrations are presented in Table 1 for all groups. It was observed that there was a statistically significant difference between MDA levels of all MAL administered tissues and that of the other groups ($p < 0.05$). The MAL group erythrocyte MDA concentration was statistically different when compared to the control and sham groups. This demonstrated that OPs led to lipid peroxidation and played a role in oxidative stress. The high MDA in malathion administered group indicated lipid peroxidation. Possamai et al. (2007) analyzed oxidative damages in different tissues induced by acute and sub-chronic malathion exposure. It was observed that the present study provided a better biomarker of acute and sub-chronic malathion oxidative stress, and in particular demonstrated that lipoperoxidation was involved in OPs toxicity. The fact that MDA concentration was lower in royal jelly administered groups when compared to MAL groups ($p < 0.05$) demonstrated that royal jelly reduced the damage induced by free radicals. It is suggested that it could be effective against oxidative stress by lowering the MDA when used as a preventive or therapeutic agent.

Table 1. Erythrocyte, liver, kidney and brain tissue MDA levels in malathion toxicity.

Group	Erytrocyte MDA (nmol/gHb)	Liver MDA (nmol/g protein)	Kidney MDA (nmol/g protein)	Brain MDA (nmol/g protein)
Control	30.59±1.87^{a}	56.40±3.80^{a}	40.17±4.73^{b}	37.26±2.98^{a}
Sham	34.23±4.53^{abc}	68.74±2.64^{b}	46.70±4.14^{c}	90.38±19.78^{c}
MAL	39.43±2.08^{d}	72.71±2.59^{c}	60.50±5.06^{d}	122.10±7.89^{d}
RJ	32.65±4.55^{ab}	58.57±1.49^{a}	34.55±3.55^{a}	37.20±4.09^{a}
RJ + MAL	37.10±2.80^{bcd}	68.31±3.47^{b}	33.99±2.66^{a}	57.14±3.47^{b}
MAL + RJ	37.43±2.75^{cd}	67.15±3.14^{b}	32.35±4.18^{a}	51.57±9.71^{b}

Values are mean ± standard deviations (SD); n=7. a,b,c,d Different letters in the same column represent statistically significant differences ($P<0.05$). MAL, Malathion; RJ, Royal Jelly; MDA, Malondialdehyde; Hb, Hemoglobin.

NO, a free radical species produced by certain mammal cell types due to unpaired electrons, is synthesized mainly by Nitric Oxide Synthase (NOS) activity. NOS produces citrulline and NO from the L-arginine amino acid using the oxygen (Stuehr, 2004). As seen in Table 2, erythrocyte NO concentrations statistically significantly increased in malathion group when compared to the control ($p<0.05$). This suggested that malathion led to oxidative damage in erythrocytes. There was a statistically significant difference between liver NO concentration in the malathion group and control and sham groups ($p < 0.05$). This finding demonstrated that malathion led to oxidative damage in liver. When kidney and brain NO concentrations were examined, it was determined that there was no statistical difference between control, sham, and malathion groups. Although there was no statistically significant difference between liver NO concentrations in RJ + MAL and MAL + RJ groups, NO concentrations were lower in other groups when compared to the malathion group. No statistically significant differences were observed between kidney and brain NO concentrations in sham, control, malathion, RJ, RJ+MAL and MAL+RJ groups. A previous study investigated the effects of caffeic acid phenethyl ester (CAPE) and ellagic acid (EA) on nitric oxide (NO) activities in lung, liver and kidney tissues of rats exposed to acute malathion toxicity. NO levels significantly increased, however CAPE and EA decreased ($p < 0.05$) NO levels due to severe tissue damage induced by MAL (Alp et al., 2011).

Table 2. Erythrocyte, liver, kidney and brain tissue NO levels in malathion toxicity.

Group	Erytrocyte NO µmol (NOx)/g Hb	Liver NO µmol(NOx)/g protein	Kidney NO µmol(NOx)/g protein	Brain NO µmol(NOx)/g protein
Control	102.87±6.48^{a}	95.43±7.31^{a}	75.54±5.68^{ab}	326.17±13.50^{ab}
Sham	114.52±12.05^{ab}	105.44±2.88^{b}	59.18±4.22^{a}	335.19±19.33^{a}
MAL	126.56±13.22^{b}	124.58±4.45^{d}	68.96±7.76^{ab}	330.30±30.80^{ab}
RJ	115.86±11.59^{ab}	115.28±8.59^{c}	87.90±28.60^{b}	322.74±33.67^{b}
RJ + MAL	110.92±8.29^{a}	98.67±5.91^{ab}	79.73±4.70^{ab}	343.18±26.55^{a}
MAL + RJ	108.55±11.53^{a}	95.53±2.12^{a}	86.39±20.23^{b}	330.03±20.13^{ab}

Values are mean ± standard deviations (SD); n=7. a,b Different letters in the same column represent statistically significant differences (P<0.05). MAL, Malathion; RJ, Royal Jelly; NO, Nitric Oxide; Hb, Hemoglobin.

GSH is a tripeptide synthesized in liver. It is found at high levels in several tissues, especially in liver tissues. GSH is among the significant antioxidant cellular molecules. It reacts with free radicals and peroxides to protect the cell against oxidative damage. The cellular GSH concentration has a great impact on the antioxidant system. GSH can react with superoxide and hydroxyl radicals, thus directly exhibiting free radical scavenging properties. In particular, brain tissue is highly susceptible to lipid peroxidation due to its low GSH content, antioxidant defenses, and high polyunsaturated fatty acid content. (Barón and Muriel; 1999). As seen in Table 3, there was a statistically significant difference between the erythrocyte, liver, kidney and brain GSH levels in malathion group when compared to sham and control groups (p <0.05). The erythrocyte, liver and kidney GSH levels were lower in MAL group when compared to control and sham groups. When brain GSH levels were examined, it was determined that there was a statistically significant increase (p <0.05) in the MAL group when compared to the control. Thus, it can be suggested that malathion administration increased toxicity in erythrocyte, liver and kidney tissues and GSH was utilized as an antioxidant defense agent. Salem et al. (2015) examined the biochemical changes that were induced by the administration of curcumin, royal jelly, and both against the oxidative stress induced by carbon tetrachloride (CCl4) in liver. It was demonstrated that curcumin, royal jelly and curcumin + royal jelly administration had significant hepatoprotective effects, preventing ROS scavenging activity and oxidative stress. The statistically significant differences (p <0.05) between erythrocyte and tissue GSH concentrations in both RJ+MAL and MAL+RJ groups when compared to MAL group suggested that royal jelly might act as protective and therapeutic agent through GSH, a significant part of the antioxidant system in malathion toxicity.

Table 3. Erythrocyte, liver, kidney and brain tissue GSH levels in malathion toxicity.

Group	Erytrocyte GSH (nmol/gHb)	Liver GSH (µmol/g protein)	Kidney GSH (µmol/g protein)	Brain GSH (µmol/g protein)
Control	7.27±0.23^{c}	406.41±6.16^{d}	465.33±71.01^{c}	728.24±20.64^{d}
Sham	6.77±0.44^{b}	384.75±6.75^{b}	346.00±45.70^{ab}	623.70±62.35^{ab}
MAL	5.86±0.35^{a}	341.35±6.75^{a}	283.31±66.38^{a}	737.28±33.43^{d}
RJ	7.37±0.23^{c}	340.42±6.53^{a}	361.46±40.30^{b}	693.11±10.63^{cd}
RJ + MAL	7.16±0.23^{c}	392.87±2.99^{c}	370.00±16.47^{b}	597.38±42.62^{a}
MAL + RJ	6.55±0.25^{b}	392.75±3.25^{c}	474.10±34.60^{c}	668.30±43.16^{bc}

Values are mean ± standard deviations (SD); n=7. a,b,c,d Different letters in the same column represent statistically significant differences (P<0.05). MAL, Malathion; RJ, Royal Jelly; GSH, Glutathione; Hb, Hemoglobin.

CAT is a hemoprotein that converts hydrogen peroxide into water and oxygen molecules. The amount of hydrogen peroxide in the medium is more active than catalase enzyme. When the hydrogen peroxide content is low, GPx steps in and removes H_2O_2 from the medium (Gechev et al., 2002). Table 4 demonstrated that erythrocyte and liver, kidney and brain tissue CAT concentrations in the MAL group were statistically significantly lower when compared to the control group ($p < 0.05$). It was suggested that CAT concentration decreased due to participation of catalase enzyme in antioxidative defense against oxidative damage induced by malathion administration to erythrocyte and the tissues. It was found that RJ + MAL group erythrocyte and kidney and brain tissue CAT levels were statistically higher when compared to those of the MAL group. This suggested that royal jelly may be protective and supportive in MAL induced nephropathy and neuropathy, especially in kidney and brain tissues. In a study conducted with royal jelly, cytoprotective effects of nicotine on human umbilical vainn endothelial cell (HUVECs) toxicity and catalase peroxidative functions were investigated. As a result of that study, it was concluded that hydrogen peroxide production during nicotine toxicity and vacuole-like structure formation would counteract the effect of nicotine through the RJ catalase activity (Supabphol and Supabphol, 2013).

Table 4. Erythrocyte, liver, kidney and brain tissue CAT levels in malathion toxicity.

Group	Erytrocyte CAT (k/gHb)	Liver CAT (k/g protein)	Kidney CAT (k/g protein)	Brain CAT (k/g protein)
Control	3.36±0.97^{cd}	26.75±4.30^{b}	1.76±0.62^{b}	3.64±0.35^{d}
Sham	2.06±0.29^{ab}	21.10±1.56^{a}	1.53±0.30^{c}	2.27±0.27^{b}
MAL	1.88±0.41^{a}	16.90±3.77^{a}	1.27±0.32^{a}	1.70±0.45^{a}
RJ	3.87±0.55^{d}	20.37±3.57^{a}	1.89±0.35^{b}	3.20±0.17^{c}
RJ + MAL	2.92±0.68^{bc}	17.05±2.95^{a}	1.67±0.32^{c}	2.89±0.21^{c}
MAL + RJ	2.74±0.68^{abc}	19.27±3.43^{a}	1.42±0.28^{c}	2.13±0.20^{b}

Values are mean ± standard deviations (SD); n=7. a,b,c,d Different letters in the same column represent statistically significant differences ($P<0.05$). MAL, Malathion; RJ, Royal Jelly; CAT, Catalase; Hb, Hemoglobin.

AchE activity is used as biomarker in determination of organophosphate insecticide contamination. OP compounds lead to toxic effects in humans and animals by inhibiting the acetylcholinesterase enzyme. To exhibit toxic action, OP should be oxidized to "P = O" or oxon metabolites. If it contains sulfur atoms (P = S), it does not exhibit inhibitory properties. The oxon intra-metabolites that are formed as a result of activation are hydrolyzed by the enzymes. These enzymes are present in mammal cells, however most insects lack these enzymes. Thus, insects are more susceptible to OP insecticides. Studies on organophosphate pesticides demonstrated that AChE inhibition varies based on the dose and duration of the administration, while inhibition rate varies based on the species and tissue. A 20% inhibition in acetylcholinesterase activity is an indication of the impact of organophosphorus pesticides. An inhibition of 50% or higher indicates a life-threatening condition (Worek et al., 1997). In an experimental study conducted to investigate the effect of malathion toxicity on acetylcholinesterase activity in mice, mature mice were exposed to malathion at different doses at different times. It was found that malathion inhibited acetylcholinesterase activity in liver (Wankhade et al., 2009). It was observed that the acetylcholineesterase activity in the MAL group was statistically significantly lower when compared to all other groups ($p < 0.05$) as demonstrated in Table 5. This suggested that malathion administration inhibited the enzyme. Furthermore, it is noteworthythat there was no statistically significant difference between the enzyme activities in control group and the therapeutic group.

SOD- is a metalloenzyme that protects the organism against toxic reactive oxygen derivatives by catalyzing the conversion of the superoxide radical to hydrogen peroxide and molecular oxygen. The physiological function of SOD is to protect oxygen-metab-

olizing cells against the adverse effects of superoxide free radicals such as lipid peroxidation (Culotta et al., 2006). As seen in Table 5, it was found that erythrocyte SOD activity in malathion group was statistically significantly lower when compared to all groups ($p < 0.05$). It was also found that SOD enzyme activities in protective and therapeutic groups were statistically significantly higher when compared to the control group. The SOD activity findings reflected the presence of malathion induced toxicity and the defense by royal jelly due to its SOD content. Cihan et al. (2013) conducted a study to investigate the effects of bee spring on oxidative damage due to gamma-radiation in liver and lung tissues. In all exposed rats, lung and liver MDA concentrations were higher and GSH-Px, CAT and SOD were lower when compared to the control($p<0.001$). It was observed that the application of royal jelly led to a significant decrease in oxidative stress parameters and an increase in antioxidant concentrations.

GPx- is a selenoenzyme that is responsible for the removal of cellular hydroperoxides and prevents cellular damage. The tissues with the highest enzyme activity are erythrocytes and liver tissues. GPx is the most important enzyme that protects lipids against peroxidation at the intracellular level. Therefore, this enzyme, located in the cellular cytosolic compartment, protects the cellular structure and functions (Cheeseman and Slater, 1993). As seen in Table 5, it was observed that the erythrocyte GPx activity in the malathion group was statistically significantly lower when compared to the control group ($p < 0.05$). The decrease observed in the MAL group indicated that the GPx enzyme was responsible for preventing/inhibiting lipid peroxidation and hydroperoxides. The GPx activities in therapeutic and RJ groups were not statistically significantly different when compared to the control group ($p>0.05$). It was observed that royal jelly supported GPx activity in malathion induced toxicity.

Table 5. Serum acetylcholinesterase, erytrocyte superoxide dismutase and glutathione peroxidase enzyme activities.

Group	Serum AChE (U/L)	Erytrocyte SOD (U/g Hb)	Erytrocyte GPx (U/g Hb)
Control	165.59±17.31^{c}	1029.17±106.48^{c}	60.96±8.86^{c}
Sham	145.48±23.44^{bc}	904.82±159.66^{b}	55.67±7.51^{abc}
MAL	94.54±7.38^{a}	722.03±51.24^{a}	45.77±8.01^{ab}
RJ	135.01±21.90^{b}	1144.44±70.15^{c}	65.30±10.95^{c}
RJ + MAL	127.72±11.14^{b}	1974.74±67.98^{d}	43.60±4.78^{a}
MAL + RJ	168.76±19.00^{c}	2010.83±42.87^{d}	57.15±11.46^{bc}

Values are mean ± standard deviations (SD); n=7. a,b,c,d Different letters in the same column represent statistically significant differences ($P<0.05$). MAL, Malathion; RJ, Royal Jelly; AChE, acetylcholine esterase; SOD, superoxide dismutase; GPx, glutathione peroxidase; Hb, Hemoglobin.

CONCLUSION

In conclusion, organophosphate toxicity affects several individuals, especially as a result of the contamination of nutrients. In the present study, it was observed via acetylcholinesterase concentration that malathion led to toxicity. Blood and tissue MDA levels demonstrated that malathion administration induced lipid peroxidation and NO levels demonstrated free radical formation in blood and liver tissues. The protective/preventive effects of royal jelly against lipid peroxidation were also observed. GSH concentrations in liver and kidney tissue, CAT concentrations in brain and kidney tissues and erythrocyte SOD and GPx concentrations demonstrated that royal jelly supported the antioxidant defense against oxidative damage in these tissues. It can be suggested that royal jelly has particularly preventative effects against toxicity.

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CONFLICT OF INTEREST

The authors declare no conflict of interes

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Research article
Ερευνητικό άρθρο

Physicochemical, Technological and Sensory Properties of Chicken Meatballs Processed with Dietary Fibers

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ABSTRACT. In this study, raw chicken meatball samples were incorporated with apple, lemon and pea fibers at different concentrations (0, 4, 8 and 12%). Their physicochemical properties were evaluated at different refrigerated storage time spots (1st, 5th and 10th days) while the fried samples were investigated with respect to their color, technological and sensory properties. The results revealed that the physicochemical properties of raw samples were significantly (P <0.05) affected by fiber type and concentration within the storage periods while color, technological and sensory properties of the fried samples were also significantly changed (P <0.05) according to the fiber type and concentration. Thiobarbutiric acid reactive substance values of raw samples incorporated with the fibers were observed to be lower than those of the control samples at the end of the storage period, indicating that fiber addition could delay lipid oxidation increasing their storage stability. Fiber addition affected the brightness (L^* values), redness (a^* values) and yellowness (b^* values) of both the raw and fried samples. Regarding technological properties of the fried samples, fiber addition generally increased (P <0.05) frying yield, and moisture retention values up to 4%, followed by a decrease at further concentrations. Meatball diameter decreased by addition at level of 4% for all fiber types, but further increase in the fiber concentration did not decrease these values. The maximum fat retention was observed in the fried samples incorporated with the apple and lemon fibers at 12 % concentration. Sensory properties were affected by fiber concentration up to 8%, which constituted the highest tolerated concentration. As a result, fiber addition positively affected the physicochemical and technological properties of the meatballs, but this affect was strongly related to the fiber type and its concentration.

Keywords: chicken meatball, apple fiber, lemon fiber, pea fiber, physicochemical and technological properties

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INTRODUCTION

Recently, consumers in developed societies have been more interested in healthier diets. Food manufacturers have increased their efforts to contribute to limit health problems such as diabetes, obesity, and cardiovascular diseases by producing low-fat and low-calorie chicken products (Tabarestani and Tehrani, 2014). High-fat chicken products are also related to the enzymatic (proteolytic deterioration), oxidative, textural problems during storage period, leading to changes in essential fatty acid and vitamin composition, decreasing the nutritional values of such products as well as deteriorating their sensory properties. These problems cause financial losses for producers and increase consumers' health risks. Thus, many studies have been conducted to improve the quality and stability of these foods (Khalil, 2000; Mc-Carthy et al., 2001; Castro et al., 2011; Ibrahim et al., 2011).

Addition of dietary fibers is one of the strategies to overcome the aforementioned problems Dietary fibers contribute healthier diet because a high-fiber diet normalizes bowel movements, maintain bowel health, lowers cholesterol levels and control blood sugar levels, and helps in achieving healthy body weight. Therefore, the trend for daily diets with high levels of dietary fiber is the incentive to produce such products (Sarıçoban et al., 2010). Dietary fibers are also more attractive than other materials such as thickeners and binders like starches, gums and whey protein concentrate, etc. since they have lower cost and are more available than the others. Fibers are known to remarkably develop technological properties of meat products due to their high water holding capacity, which reveals their possibility to be also used in the production of low-fat chicken meatballs. This appears to be good strategy to improve textural and sensory properties and to increase the shelf life of the chicken meatballs by reducing the metabolites resulting from spoilage. Accordingly, it was reported that the fibers could be used to prevent deterioration of meat and chicken products, increasing the quality of the final products (Talukder and Sharma, 2010; Elleuch et al., 2011; Pinero et al., 2008; Petracci et al., 2013).

In spite of the aforementioned beneficial health effects of the dietary fibers, their usage in meat formulations, is depending on their functionality and interaction with other ingredients in the formula. This limits their usage in some aspects. Therefore, the possible interactions of the dietary fibers with main components of meat products should be determined in order for them to play a key role on the functionality of final products. The present work was undertaken to determine the effect of fiber type (apple, lemon and pea) and concentration (0, 4, 8 and 12%) on physicochemical properties of raw chicken meatballs at different refrigeration storage periods (1st, 5th and 10th days) as well as on color, technological and sensory properties of fried chicken meatballs.

MATERIALS AND METHODS

Materials

Apple fiber (moisture–9.3%, total dietary fiber–70%, ash–1.40%, crude protein–6.8 %, crude fat–2.1 %, water holding capacity 4 mL/g) and lemon fiber (moisture–5.6%, total dietary fiber–90%, ash–1.60%, crude protein–2.2 %, crude fat–0.4 %, water holding capacity 6 mL/g) were purchased from Herbafood Co. (Herbafood Ingredients GmbH, Germany). Pea fiber (moisture–5.99%, total dietary fiber–70%, ash–4.30%, crude protein–7.2 %, crude fat–2.6 %, water holding capacity 3.7 mL/g) was obtained from Roquette Co. (France). Chicken breast meat used in meatball production was obtained from a local seller in Adiyaman, Turkey. Corn oil used as a frying medium was procured from Yudum Co. (Balıkesir, Turkey). A mini fryer (Arzum, 246, Turkey) was used for frying operations.

Methods

Preparation of chicken meatballs

Chicken breast meats were kept and transferred at -18 °C in plastic bags to laboratory. Before use in the meatball production, they were thawed at 4 °C and minced using a grinder machine (Tefal, Le Hachoir 1500, France). For preparation of the experimental batches, 4750 g ground meat was mixed with 100 g corn oil, 75 g salt, 50 g black pepper and 25 g curry and kneaded for 15 min to obtain a homogeneous raw meatball mixture. Then, the dough was allocated into three groups and each of them was separately added with dietary fibers (apple, lemon and pea) at four different concentrations (0% (Control), 4%, 8% and 12%, based on 100 g of the dough). Each sample was re-kneaded and shaped into meatballs by rounding with hand at equal diameters (approx. 20 g weight and 30 mm diameter for each sample, measured by a digital caliper). Then, all the meatball samples were separated into two groups as raw and cooked meatballs. Forty meatballs were produced for each treatment. The physicochemical analyses (pH, Thio-

barbutiric Acid Reactive Substance, L^*, a^* and b^*) were conducted for the raw meatball samples placed in polystyrene foam dishes, wrapped with stretch film and stored at 4 °C and examined at certain storage days (1st, 5th and 10th days). The color (L^*, a^* and b^*), technological (frying yield, reduction in diameter, moisture retention and fat retention), and sensory (appearance, odor, taste and texture) analyses were performed for the chicken meatball samples fried in 1 L of corn oil at 180 °C for 5 min.

Determination of physicochemical analyses

The pH values of the samples were measured by using pH meter (WTW 315 i set model, Weilhem, Germany) after homogenization, as outlined (AOAC, 2002). The extent of oxidative rancidity (Thiobarbutiric Acid Reactive Substance, TBARS) was determined as described by Tarladgis et al. (1969). The absorbance was read at 538 nm (UV-160 A, UV-Visible Recording Spectrophotometer, Shimadzu, Tokyo, Japan) against a reagent blank. The TBARS numbers were expressed as mg of malon-dialdehyde (MDA/kg) equivalents. Color measurements were conducted using a Minolta Chroma Meter CR-400 (Konica Minolta, Inc., Osaka, Japan) with illuminant D65, 2° observer, Diffuse/O mode, 8-mm aperture of the instrument for illumination and 8mm for measurement. Prior to the measurements, a white reference tile (L^*= 97·10, a^*= –4·88, b^*=7·04) was employed to calibrate the instrument. The meatball samples were subjected to air for at least 20 min at 25 °C before the measurements. For each meatball sample, three locations were measured in terms of L^* (brightness), a^*(±red–green) and b^*(±yellow–blue) color values and the measurements were averaged and recorded (Hunt et al., 1991).

Determination of technological properties

Frying yield

Frying yield of the meatball samples were calculated using the equation (Eq. (1)) employed by Murphy et al (1975) and Tekin et al., 2010):

$$\text{Frying yield \%} = \frac{\text{fried meatball weight}}{\text{raw meatball weight}} \times 100 \quad (1)$$

Reduction in diameter

The reduction in meatball diameter before and after frying was estimated by a digital caliper (Mitutoyo, Japan) using the following equation (Eq. (2)):

$$\text{Reduction in diameter \%} = \frac{\text{raw meatball diameter} - \text{fried meatball diameter}}{\text{raw meatball diameter}} \times 100$$

$$\text{Reduction in diameter \%} = \frac{\text{raw meatball diameter} - \text{cooked meatball diameter}}{\text{raw meatball diameter}} \times 100 \quad (2)$$

Moisture retention

The amounts of moisture retained in the fried meatballs per 100 g sample can be indicated by moisture retention values. Moisture of raw and fried meatball samples was determined by oven air method, as outlined (AOAC, 2002). Moisture retention values were calculated according to the following equation (Eq. (3)) (Soltanizadeh and Ghiasi-Esfehani, 2015):

$$\text{Moisture retention \%} = \frac{\text{moisture in fried meatball \%}}{\text{moisture in raw meatball \%}} \times \text{frying yield} \quad (3)$$

Fat retention

Fat retention values were calculated according to the Eq. (4) (Tekin et al., 2010; Soltanizadeh and Ghiasi-Esfehani, 2015):

$$\text{Fat retention \%} = \frac{\text{(fried weight x fat in fried meatball \%)}}{\text{(raw weight x fat in raw meatball \%)}} \times 100 \quad (4)$$

Sensory analysis

The acceptability of sensory profile of the fried meatballs was evaluated by semi-trained 10 panellists of age between 20 and 40. Each panellist was served with 3 samples. Fried chicken meatballs were served in a random order to the ten panellists. The panellists were also served with water and cracker biscuits between the assessments to allow them to rinse properly and neutralize carryover flavours. Panellists were enabled to sit in the different locations separated from frying and preparation room. Panellists evaluated the coded samples to reduce bias. The sensory properties were evaluated using a hedonic scale for the appearance, odor, taste, and texture. The values in the scale indicated the following reactions: 1: dislike extremely to 9: like extremely (Gokalp et al., 1999).

Table 1. Effect of fiber type and concentration on physicochemical values of raw meatballs at different storage periods.

		1st day				5th day				10th day			
		Fiber concentration				Fiber concentration				Fiber concentration			
	Fiber type	**Control (0%)**	**4%**	**8%**	**12%**	**Control (0%)**	**4%**	**8%**	**12%**	**Control (0%)**	**4%**	**8%**	**12%**
pH	Apple	6.07^{aAY}	5.82^{bBX}	5.55^{bCX}	5.25^{bDX}	5.91^{aAZ}	5.67^{aBY}	5.41^{bCZ}	5.14^{bDX}	6.51^{aAX}	5.92^{bBX}	5.49^{bCY}	5.17^{bDX}
	Lemon	6.07^{aAY}	5.76^{bBX}	5.38^{cCX}	5.13^{cDX}	5.91^{aAZ}	5.71^{aAX}	5.27^{cBX}	4.92^{cCY}	6.51^{aAX}	5.99^{bBX}	5.36^{bCX}	4.91^{cDY}
	Pea	6.07^{aBY}	6.09^{aBY}	6.14^{aABX}	6.19^{aAX}	5.91^{aBZ}	6.01^{aABY}	6.09^{aAX}	6.12^{aAXY}	6.51^{aAX}	6.58^{aAX}	6.09^{aBX}	6.02^{aBY}
TBARS (mg/kg)	Apple	0.085^{aBZ}	0.070^{cBZ}	0.095^{bBY}	0.195^{aAZ}	0.345^{aAY}	0.300^{aAY}	0.365^{aAY}	0.395^{aAY}	1.565^{aAX}	0.850^{abBX}	0.865^{aBX}	0.845^{aBX}
	Lemon	0.085^{aCZ}	0.120^{bBZ}	0.165^{aAY}	0.130^{aBY}	0.345^{aAY}	0.400^{aAY}	0.325^{aAXY}	0.285^{aAY}	1.565^{aAX}	0.765^{bBCX}	0.650^{aCX}	0.995^{aBX}
	Pea	0.085^{aCZ}	0.275^{aAY}	0.205^{aBZ}	0.185^{aBY}	0.345^{aAY}	0.325^{aAY}	0.315^{aAY}	0.350^{aAY}	1.565^{aAX}	1.020^{aBCX}	1.140^{aBX}	0.810^{aCX}
*L**	Apple	45.94^{aAX}	40.65^{bBX}	38.29^{bBCX}	36.62^{bCX}	44.93^{aAXY}	38.35^{cBY}	37.25^{bBCX}	35.90^{bCX}	42.33^{aAY}	37.56^{cBY}	34.81^{bCY}	34.66^{bCX}
	Lemon	45.94^{aBX}	45.47^{aBX}	46.16^{aABX}	48.59^{aAX}	44.93^{aAXY}	44.20^{bBY}	44.62^{aBXY}	48.52^{aAX}	42.33^{aBY}	40.01^{bCZ}	41.63^{aBCY}	47.29^{aAY}
	Pea	45.94^{aBX}	46.39^{aBX}	47.95^{aABX}	50.81^{aAX}	44.93^{aAXY}	45.41^{aBX}	45.57^{aBXY}	48.93^{aAXY}	42.33^{aBY}	42.46^{aBY}	44.43^{aBY}	47.01^{aAY}
*a**	Apple	1.50^{aCX}	4.26^{aBX}	5.25^{aABX}	5.78^{aAX}	0.23^{aDY}	2.60^{aCY}	4.11^{aBY}	4.56^{aAY}	1.32^{aCX}	2.43^{aBY}	3.99^{aAY}	4.30^{aAY}
	Lemon	1.50^{aAX}	1.17^{bABX}	1.05^{cABX}	0.77^{bBX}	0.23^{aAY}	0.01^{bABY}	-0.08^{bABY}	-0.60^{cBY}	1.32^{aAX}	0.13^{cBY}	-0.38^{cBCY}	-0.75^{cCY}
	Pea	1.50^{aAX}	1.32^{bAX}	1.53^{bAX}	0.73^{bBX}	0.23^{aAY}	-0.29^{bBY}	0.09^{bAZ}	0.18^{bAY}	1.32^{aAX}	1.07^{bABX}	0.74^{bBCY}	0.38^{bCXY}
*b**	Apple	16.74^{aAX}	15.18^{bBX}	13.96^{aCX}	13.55^{bCX}	15.24^{aAX}	14.04^{bABY}	13.83^{bABX}	13.08^{bBXY}	14.89^{aAX}	13.30^{cABZ}	12.42^{bBY}	12.50^{bBY}
	Lemon	16.74^{aAX}	17.85^{aAX}	16.81^{aAX}	17.33^{abAX}	15.24^{aCX}	17.54^{aBCX}	17.95^{aBX}	20.56^{aAX}	14.89^{aCX}	15.25^{bBCY}	16.85^{aBX}	20.06^{aAX}
	Pea	16.74^{aCX}	18.09^{aBCX}	19.23^{aABX}	20.52^{aAX}	15.24^{aCX}	17.67^{aBX}	18.31^{aBXY}	20.38^{aAX}	14.89^{aCX}	16.45^{aBCX}	17.46^{aABY}	18.94^{aAX}

[a-c] Within each column, different superscript lowercase letters show differences between the fiber types within each concentration ($p< 0.05$). [A-D] Within each row, different superscript uppercase letters show differences between the fiber concentrations within each storage period ($p < 0.05$). [X-Z] Within each row, different superscript uppercase letters show differences between the storage periods with respect to same fiber type and concentration ($p < 0.05$).

Statistical analysis

The experimental procedure was repeated twice with three replications. Data were subjected to statistical analysis using JMP version 9.0.2 (SAS Institute, Inc., Cary, USA). Least Significant Differences (LSD) test was used to determine if the effects of factors on the studied parameters were significant ($p < 0.05$).

RESULTS AND DISCUSSIONS

Effect of fiber type and concentration on physicochemical properties of raw meatballs

Table 1 shows the effect of fiber type and concentration on physicochemical values of raw meatballs at different storage periods. As can be seen, pH values were significantly ($P <0.05$) influenced by fiber addition to raw meatball samples, decreasing by addition and apple and lemon fibers, but increasing by addition of pea fiber at the 1st and 5th storage day. These results could be expected due to malic and citric acid contents of apple and lemon, respectively. On the other hand, these effects were strongly fiber-concentration dependent, implying that pH values were more changed by further increase in the fiber concentration. At the end of the 10th storage day, pH values of the all raw samples were observed to decrease. In the literature different results were reported. Sanchez-Zapata et al. (2010) determined the pH value of burgers processed with tiger nut fiber in the range of 6.16-6.20 and observed that the fiber addition did not affect the pH values of pork burgers.

TBARS values, an indicator of oxidation stability of a food product, were observed to increase by storage time (Table 1). This can be expected because lipid oxidation increases by the storage time. At the 5th storage day, fiber addition did have any remarkable effect on the lipid oxidation of the raw samples, while apple, lemon or pea fiber addition significantly ($P <0.05$) limited the lipid oxidation of the raw samples at the 10th day of storage time, as revealed by the lower TBARS values observed in the raw meatball samples processed with the dietary fibers. When the dietary fibers were compared with each other in

terms of their performance to limit lipid oxidation in the raw samples, it can be stated that all the fiber types had almost similar effects, but at 4% concentration, lemon and apple fibers retarded lipid oxidation more effective than did pie fiber. Similar results were reported in the literature. Cava et al. (2012) observed that tomato fiber and beef root fibers addition reduced the lipid oxidation in chicken products, reporting that oxidation was fiber concentration-dependent. On the other hand, they determined the TBARS values in the range of 2.03-3.82 mg/kg at 10th day of storage (4^{o}C). In addition, higher TBARS values were reported by Schormuller (1969) at the end of the storage period. In our study, lower TBARS values were determined, revealing that the studied fibers could successfully retard lipid oxidation. Also, the TBARS values of the raw meatballs in the end of the storage were determined at the levels of consumption that were given between 0.7 to 1 mg/kg by Gokalp et al (1999).

The color properties of the raw meatballs were expressed as L^* (brightness), a^* (redness) and b^* (yellowness) in this study. The results are also presented in Table 1 where it can be seen that apple fiber addition decreased (P <0.05) the brightness of the raw meatball samples while lemon and pea fiber addition generally increased the brightness (P <0.05) at all the storage periods. These effects were concentration-dependent. Accordingly, lemon and pea fiber addition resulted in brighter raw product in almost all concentrations at all test time spots. An inverse trend was observed in the redness values. In other words, apple fiber addition increased (P <0.05) the redness of the raw meatball samples while lemon and pea fiber addition decreased (P <0.05) at all the test time spots. Similarly, these effects were also concentration-dependent. Accordingly, apple fiber addition resulted in redder raw product during storage. Regarding yellowness of the raw meatball samples, the same phenomenon observed in the L^* values; namely, apple fiber addition decreased (P <0.05) the yellowness of the raw meatball samples, while lemon and pea fiber addition generally increased (P <0.05) during storage. These effects were also concentration-dependent. Accordingly, lemon and pea fiber addition resulted in yellower raw product in almost all concentrations at all tst time spots during storage. Similar results were observed by Aleson-Carbonel et al. (2005) who determined that the inclusion of fiber from citrus changed color values of beef burgers.

Effect of fiber type and concentration on color and technological properties of fried meatballs

Table 2 shows the effect of fiber type and concentration on color properties of fried meatball samples. As can be seen, a different phenomenon was observed in the fried meatball samples in terms of the color values. Apple fiber addition resulted in darker, greener and more bluish (P <0.05) product than did lemon and pea fibers and this effect was prominent at increasing concentration levels. In other words, lemon and pea fiber addition resulted in brighter, redder and yellower product than did apple fiber addition; however, this effect was more prominent when the meatballs were processed with pea fiber. Similar results were reported by Allesson- Carbonell et al. (2005) and Sanchez-Zapata et al. (2010) for beef and pork burgers, respectively.

Table 2. Effect of fiber type and concentration on color properties of fried meatballs.

		Fiber concentration			
	Fiber type	**Control (0%)**	**4%**	**8%**	**12%**
L^*	Apple	41.21^{aA}	29.70^{bB}	25.58^{cC}	22.54^{cC}
	Lemon	41.21^{aAB}	45.27^{aA}	42.52^{bAB}	39.97^{bB}
	Pea	41.21^{aC}	43.95^{aB}	46.10^{aB}	49.87^{aA}
a^*	Apple	7.21^{aB}	11.38^{aA}	8.26^{cB}	5.66^{cC}
	Lemon	7.21^{aC}	8.61^{bB}	9.66^{aA}	9.32^{bA}
	Pea	7.21^{aC}	9.47^{bB}	11.45^{aA}	11.85^{aA}
b^*	Apple	19.10^{aA}	11.73^{bB}	7.37^{cC}	5.17^{cD}
	Lemon	19.10^{aAB}	20.96^{aA}	19.87^{bAB}	18.65^{bB}
	Pea	19.10^{aC}	20.75^{aB}	22.23^{aB}	24.03^{aA}

$^{a-c}$ Within each column, different superscript lowercase letters show differences between the fiber types within each concentration (p < 0.05). $^{A-D}$ Within each row, different superscript uppercase letters show differences between the concentrations within each fiber (p < 0.05).

Table 3. Effect of fiber type and concentration on technological properties of fried meatballs.

Technological properties	Fiber type	Fiber concentration			
		Control (0%)	4%	8%	12%
Frying yield (%)	Apple	86.04^{aB}	90.59^{aA}	83.49^{bC}	79.09^{bD}
	Lemon	86.04^{aA}	83.45^{bA}	77.15^{cB}	78.81^{bB}
	Pea	86.04^{aC}	90.03^{aA}	87.69^{aB}	86.27^{aBC}
Reduction in diameter (%)	Apple	6.30^{aA}	-1.90^{aB}	-0.25^{abB}	1.57^{aAB}
	Lemon	6.30^{aA}	-1.68^{aB}	3.20^{aAB}	1.61^{aAB}
	Pea	6.30^{aA}	-2.52^{aB}	-2.30^{bB}	-2.87^{aB}
Moisture retention (%)	Apple	74.00^{aB}	81.21^{aA}	71.11^{bB}	62.86^{bC}
	Lemon	74.00^{aA}	72.63^{bA}	61.34^{cB}	56.29^{cC}
	Pea	74.00^{aB}	80.59^{aA}	78.44^{aA}	74.20^{aB}
Fat retention (%)	Apple	3.58^{aAB}	3.37^{aB}	3.31^{bB}	4.74^{bA}
	Lemon	3.58^{aB}	3.05^{aB}	4.18^{aB}	8.26^{aA}
	Pea	3.58^{aA}	3.28^{aAB}	2.59^{bBC}	2.36^{cC}

$^{a-c}$ Within each column, different superscript lowercase letters show differences between the fiber types within each concentration ($p < 0.05$). $^{A-C}$ Within each row, different superscript uppercase letters show differences between the concentrations within each fiber ($p < 0.05$).

Effect of fiber type and concentration on technological properties of fried meatballs can be seen in Table 3. As seen, frying yield of the meatball samples increased (P <0.05) with the addition of apple and pea fibers at 4%. This was attributed to the ability of apple and pea fibers to keep the moisture and fat in the matrix. The mechanism responsible for moisture and fat retention was suggested to be affiliated with the swelling of the fibers, which would enable them to absorb some fat and interact with the protein in ground chicken to form a matrix. This phenomenon finally was hypothesized to hinder the coalescence and migration of fat out of the fried meatballs (Anderson and Berry, 2001). As a result, the apple and pea fibers could be said to have high fat retention ability, reducing the cooking loss and so increasing the frying yield. However, further increase resulted in a decrease in the frying yield of the meatball samples. This could be ascribed to hard and friable structure caused by the fact that the fiber addition in higher concentrations gave rise to softer structure, finally leading to loss of fat and moisture. It should be also pointed out here that lemon fiber addition did not increase the frying yield in spite of its high total fiber content (90 %). This could have been due to the lowest pH values of the raw meatball samples processed with lemon fiber (Table 1). As can be seen from the table, the raw meatball samples processed with lemon fiber had generally the lowest pH values, which caused pH of the samples to approach the isoelectric point of proteins where moisture retention ability of the chicken proteins is almost close to zero.

The effect of fiber type and concentration on reduction in diameter of the meatball samples can be seen from Table 3. As can be clearly seen, the fiber addition significantly (P <0.05) decreased the reduction in diameter values. This result could also be expected due to the fact that fibers have capability to entrap fat and water, which led to a decrement in the reduction in diameter of the meatball samples in this study (Tekin et al., 2010). However, this effect was prominent at 4 % concentration and further increase in the fiber concentration did not change these values. This implicated that the fiber addition decreased reduction in meatball diameter but further increase in fiber concentration (8 and 12 %) did not significantly change the diameters of meatballs. It is interesting to report here that pea fiber addition even increased the diameter of meatballs (Table 3). Similar results have been previously reported for wheat, cellulose, oat, inulin and carrot fibers (Kılıncceker, 2017; Kılınççeker and Kurt, 2018).

Moisture retention values were observed to be significantly (P <0.05) affected by fiber type and concentration (Table 3). Apple and pea fibers increased the moisture retention values. This could be similarly affiliated with the effect of these fibers to increase water retention ability of meatballs; namely, this result

could be due the capability of the fibers to keep the moisture in the matrix (Tekin et al., 2010). On the other hand, an inverse trend was also observed at their further concentrations. Namely, after 8 % concentration, these fibers could not hold moisture; furthermore at 12 % concentration, these fibers started to release the moisture that they could hold at 4 % concentration. Same phenomenon could was not observed in the meatball samples processed with lemon fiber. Increase in the level of lemon fiber resulted in decrease of the moisture retention values. This could also be attributed to the aforementioned explanation that the raw meatball samples processed with lemon fiber had generally the lowest pH values, leading pH of the meatball samples to approach the isoelectric point of proteins at which moisture retention ability of the chicken proteins is almost close to zero.

Table 3 also presents the effect of fiber type and concentration on the fat retention values of fried meatball samples. The fat retention of meatballs increased (P <0.05) with apple and lemon fiber addition. It was reported that fat retention is a complex phenomenon which is probably the result of several chemical and physical mechanisms. In these mechanisms, proteins are thought to be perfect fat binders since they have double-functions in regards of fat interactions in which non-polar side chains of proteins furnish sites for lipid–protein interactions and interfacial film formation. Moreover, myofibrillar proteins gelation, which forms three-dimensional matrix, hold fat (Zayas, 1997; Anderson and Berry, 2001). In addition, fibers possess some fat-holding properties (Sosulski and Cadden, 1982). Accordingly, in our study, further increase in the apple and lemon fibers also increased the fat retention of the patty samples (Table 3). This was due to the dominant impact of these fibers to entrap fat. The maximum fat retention could be achieved by addition of lemon fiber at 12 % concentration. Briefly, it could be concluded that the effects of apple and especially lemon fibers were strongly dependent on the fiber level. Regarding the effect of pea fiber, the fat retention values were observed to decrease by increase in the pea fiber concentration, which reveals that pea fiber was not an affective fiber source in increasing the fat retention in the chicken meatballs.

The sensory properties of fried chicken meatballs

Sensory scores allocated for each sensory characteristic are shown in Table 4. The sensory results from the present study revealed that fiber addition did not significantly influence the sensory scores for appearance, color, odor, taste and texture. However, further increase in fiber concentration caused a decrease in these scores. In general, the panelists gave a lower score to the chicken meatballs processed with the fibers in the higher concentration (at 12%). Therefore, addition of these fibers into the chicken meatballs should be kept under 12 %.

Table 4. Effect of fiber type and concentration on sensory properties of fried meatballs.

		Fiber concentration			
Sensory properties	**Fiber type**	**Control (0%)**	**4%**	**8%**	**12%**
Appearance	Apple	4.95^{aA}	4.65^{aAB}	5.20^{bA}	2.60^{bB}
	Lemon	4.95^{aB}	5.70^{aAB}	6.40^{aA}	4.60^{bB}
	Pea	4.95^{aA}	5.90^{aA}	6.45^{aA}	7.35^{aA}
Odor	Apple	5.70^{aA}	5.50^{aA}	5.50^{bA}	4.00^{bB}
	Lemon	5.70^{aA}	6.00^{aA}	5.70^{bA}	5.45^{aA}
	Pea	5.70^{aA}	6.30^{aA}	6.05^{aA}	6.35^{aA}
Taste	Apple	6.25^{aA}	6.00^{aA}	5.70^{bA}	2.85^{bB}
	Lemon	6.25^{aA}	6.10^{aA}	6.05^{abA}	4.75^{abB}
	Pea	6.25^{aA}	6.70^{aA}	6.55^{aA}	6.20^{aA}
Texture	Apple	6.45^{aA}	6.15^{aA}	6.25^{aA}	3.15^{bB}
	Lemon	6.45^{aA}	6.05^{aAB}	5.85^{aAB}	4.65^{abB}
	Pea	6.45^{aA}	7.00^{aA}	6.45^{aA}	6.15^{aA}

$^{a-c}$ Within each column, different superscript lowercase letters show differences between the fiber types within each concentration (p < 0.05). $^{A-C}$ Within each row, different superscript uppercase letters show differences between the concentrations within each fiber (p < 0.05).

CONCLUSION

Apple and pea fibers exhibited good performance increasing the frying yield, reducing the diameter, moisture and fat of chicken meatballs, while lemon fiber had the best performance for increasing the fat retention. In addition, these fibers did not negatively affect the sensory properties of the fried chicken meatballs at the concentrations 4 and 8%. Therefore, these fibers might be a promising ingredient for the development of low-fat meat products with improved cooking properties at high-temperature processing as well as for production of healthier products with high fiber content. However, effect of fiber was concentration dependent and this should be taken into consideration in applications in the meat industry. Therefore, the results of this study may be useful for meat industry which aims to augment the product yield for meatballs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research article
Ερευνητικό άρθρο

Evaluation of Abnormal Limb Conformation in Jumping Thoroughbred Horses

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ABSTRACT. Subjective, objective and photographic image using software Auto CAD programme were used for evaluation of limb conformation in 51 jumping thoroughbred horses. The abnormal limb conformations were included: standing under in front (58%), steep shoulder (55%), carpus valgus (45%), calf knee (31%), standing under behind (31%), short pelvis (31%) and straight hocks (16%). Linear and angular limb measurements showed standing under in front had increased elbow joint lateral angle, and decreased fore fetlock joint lateral angle. Steep shoulder displayed a significant increase ($P< 0.05$) in shoulder joint lateral angle and significant decrease in forearm front length. calf knee horses had significant decrease in the lateral angle of carpus joint. Short pelvis had a significant decrease in lateral length of pelvis and significant increase in croup angle and stifle joint lateral angle. Straight hocks showed the lateral length of both pelvis and gaskin showed a significant decrease and significant increase in the stifle joint lateral angle. Therefore, the current use of linear and angular measurements in relationships to abnormal limb conformation in the present study will allow for estimation of the future performance and soundness in jumping thoroughbred horses. In addition can be considered for selection athletic horse with less risk of lameness.

Keywords: abnormal conformation, limb, jumper, horses, Auto CAD program me.

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INTRODUCTION

Conformation, simply defined as "the relationship of form to function", is an indicator of performance and orthopedic health of athletic horses (McIlwraith et al., 2003). Many conformational faults were thought to play a role in musculoskeletal problems predisposition (Globe, 1992). The biomechanical loading of horse limbs and the risk of musculoskeletal injuries were attributed to conformation and the position of the limbs during stance phase of the stride (Dyson, 2018a). Good conformation is not synonymous with success and on the other hand, faulty conformation is not unsoundness, but it is a warning sign (Ross and McIlwraith, 2011). Poor conformation can produce abnormal strain on particular part of the body or limbs resulting in stretching or fracture and consequently lameness (Hawcroft, 1993).

Evaluation of horse conformation is carried out through subjective (Stashak, 1987) and objective methods (Johnston, 1996; Barrey, 1999, Clayton and Schamhardt, 2001). The subjective evaluation of conformation is the traditional and still the primary used method for evaluating equine conformation. However, it varies greatly between judges because it is based mainly on experience or opinion and very little on research (Magnusson and Thafvelin,1985). Both linear measurements and linear scoring system are the most popular methods to judge conformation in horses. Duensing et al. (2016) and Padilha et al. (2017) affirmed that the use of linear and angular measurements allowed an efficient selection of durable and competitive sport horses and could used as performance indices useful in a study of a bread specific conformation of athletic horses.

Limbs are essential for the functionality of the horse and conformation faults are strongly predisposed to limb pathology and lameness. Various conformation faults such as standing over at the knees, standing under at the knees, bench knees, cow hocks, and sickle hocks are reported to cause bog spavin, bone spavin, curb and many other affections (Pretorius, 2003). Therefore, the aim of this study was to evaluate objectively the fore and hind limbs conformation faults in relationship to lengths and angles measurements in jumping thoroughbred horses in Egypt.

MATERIAL AND METHODS

This study was carried out on 51 jumping thoroughbred horses (38 geldings and 13 females) bred in Armed Forces Equestrian Club, Egypt. Their average age was 8 years (5-15 years) and weighted 450-600 kg. All horses received the same management procedures, same training courses, and almost joined the same number of jumping courses/year.

Subjective evaluation of body conformation was carried out according to Stashak (1987). Objective method of evaluation of lengths and angles were performed according to Holmstrom *et al.* (1990), Magnusson and Thafvelin (1990); Anderson and McIlwraith (2004) and Robert *et al.* (2013). All horses were photographed standing on a flat, horizontal solid surface and were haltered. Reference points were established on the skin of the horse according Anderson and McIlwraith (2004a).Two views were taken for each horse; lateral (from the left side) and front photographs using Samsung PL80 28-mm digital camera 5X, 12 Megapixel. These photographs were digitally analyzed using computer aided image analysis; AutoCAD 2013 v19 program, a commercial software application for 2D and 3D computer-aided design, developed by Autodesk, Inc., available since 1982, California, USA. Finally, lengths and angles were measured for each horse using a measuring tape and goniometer used for calibration and scaling the measurements taken on the photos by AutoCAD 2013 v19 program.

Statistical analysis:

Descriptive statistical analysis for lengths and angles was done by IBM®SPSS® statistics v 20 program (IBM Corporation, 2009, New York, USA). All data were presented as mean and standard Deviation (SD) values. Kolmogorov–Smirnov test was conducted and $P > 0.05$ so the distribution was normal. Independent samples t-test was used to compare the normal and abnormal lengths and angles. A p value <0.05 was considered significant.

RESULTS

Seven varieties of abnormal limb conformation were recorded in the examined horses. These abnormal conformations included standing under in front (Fig.2), steep shoulder, carpus valgus (Fig3) and calf knee (Fig.4) in the forelimb and standing under behind (5) short pelvis (Fig.6) and straight hocks in the hind limb. The abnormal horse conformation showed characteristic variable lengths and angles of the limbs compared to the normal horses as shown in (Tables 1 & 2).

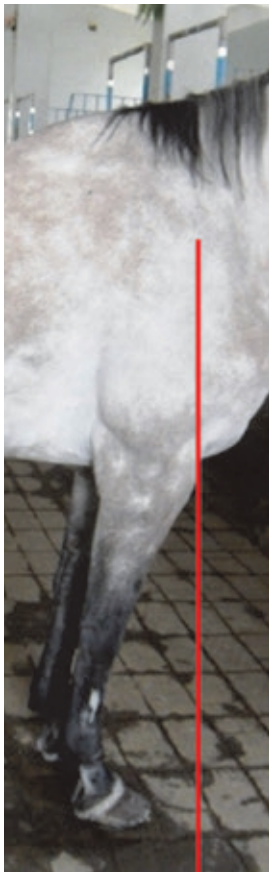

Figure 1. Thoroughbred jumper horse showing standing under in front the fore limb under the body when the horse is viewed from the side.

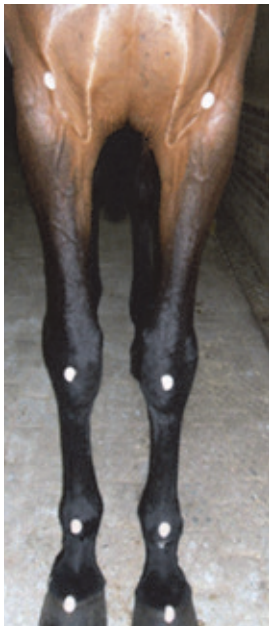

Figure 2. Thoroughbred jumper horse with carpal joint is directed medially from the front (Capus Valgus) and base narrow.

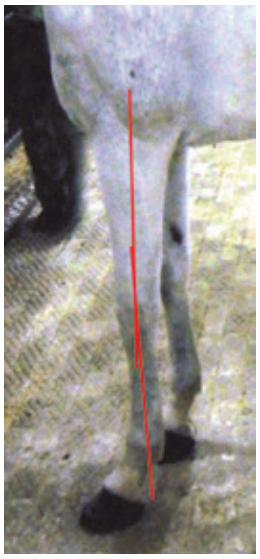

Figure 3. Thoroughbred jumper horse showing the carpal joint is directed backward from the lateral view (Calf knee).

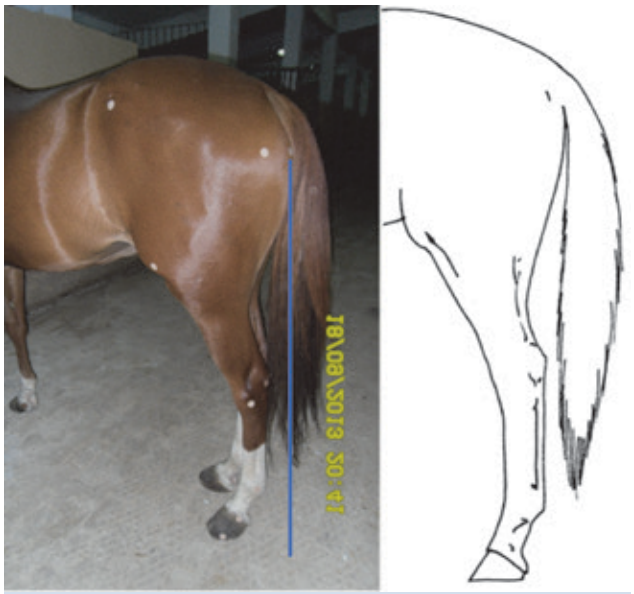

Figure 4. Thoroughbred jumper horse (lateral view): the hind limb is placed too far forward. The perpendicular line drawn from the point of the buttock (tuber ischii) would strike the ground slightly far behind the limb.

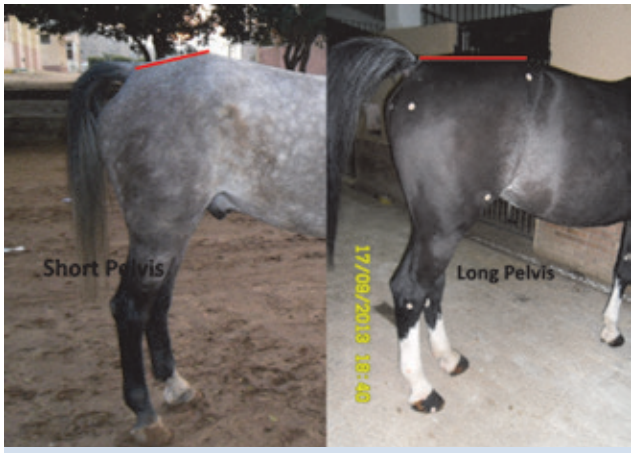

Figure 5. Thoroughbred jumper horse showing short and long pelvis.

The forelimbs abnormal conformations (Table 1) were included standing under in front (58%) of the total examined animals. The measured lateral lengths of the arm and hind cannon showed significant (P<0.05) increase. Lateral angle of elbow joint had significant (P<0.05) increase and the fetlock joint angle displayed significant (P<0.05) decrease in comparison with normal horses.

Steep shoulder represented (55%) of examined horses and displayed a significant (P<0.05) increase in shoulder joint lateral angle. Fore arm front length revealed a significant (P<0.05) decrease in comparison with normal horses. *The* findings results of carpus valgus represented (45%) of the total examined horses and the carpal joint angles had significant (P<0.05) decrease. The lateral and front lengths of fore arm showed a significant (P<0.05 increase in comparison with normal horses.

Calf knee abnormal conformation was recorded in 16 horses (31%) of the total examined horses and had a significant (P<0.05) decrease in the lateral angle of carpal joints compared to normal horses (Fig. 4).

The abnormal hind limbs conformation recorded (Table2) were Standing under behind and represented (31%) of the total examined animals. Thigh lateral length and stifle joint lateral angle showed a significant (P<0.05) increase compared and Gaskin length showed a significant decrease in comparison with normal horses. Short pelvis represented (31%) of the total horses examined had a significant (P<0.05) decrease in lateral pelvis length compared to normal horses. Short pelvis displayed a significant (P<0.05) increase in croup angle and stifle joint lateral anglein comparison with normal horses**.**

Straight hock was found in (16%) of the total examined animals. The lateral length of both pelvis and gaskin showed a significant (P<0.05) decrease and the stifle joint angle displayed a significant (P<0.05) increase compared to normal horses.

Table 1. Lengths and angles of normal and abnormal forelimbs conformations in the examined jumping thoroughbred horses.

Trait	**Lengths (cm)**			**Angles (°)**		
	Variable	**Normal**	**Abnormal**	**variable**	**Normal**	**Abnormal**
Standing under in front	Shoulder	69±3.5	70±5.3	Shoulder	98±3.8	99±3.4
	Arm	33±2.6	35.8±2.7*	Elbow	136±4.3	139±3.7*
	Fore arm	45±2.3	46±2.5	Fore fetlock	143±3.8	140±5.6*
	Fore cannon	27.5±2.7	28.7±2.1			
	Hind cannon	34.85±2.6	37.85±3.5			
Steep shoulder	Shoulder	69±5.8	69.92±4.19	Shoulder	97±3.8	100±3*
	Arm	34±2.8	34.89±2.8	Elbow	137±5	139.4±3.5
	Fore arm	48.3±2.9	46.5±2.5*			
Carpus valgus	Fore arm (lateral view)	46±2.5	48±2.9*	Right dorsal carpal	177.8±1.6	174±2*
	Fore arm (front view)	44.7±2.4	46±2.5*	Left dorsal carpal	178.5±1	174±1.3*
	Fore cannon	28.98±2.3	28.15±2.5			
Calf knee	Fore arm	47.8±2.9	49±3.2	Lateral carpal	178.78±1	176.38±2.2*
	Fore cannon	27±2.3	28.8±2.4			

*Significant at P< 0.05.

Table 2. Lengths and angles of normal and abnormal hind limb conformations in the examined jumping thoroughbred horses.

Trait	Lengths (cm)			Angles (°)		
	variable	Normal	Abnormal	variable	Normal	Abnormal
Standing under behind	Pelvis	53±4.7	51±4.4	Croup	143±3.8	145±5.8
	Thigh	47±4.8	51±4.2*	Hip	85±5.8	89±8.4
	Gaskin	56±3.4	51±4.4*	Stifle	105.33±7	119±11.6*
	Hind cannon	36.5±3.1	36±3.1	Tarsal	147±4.9	148±4.4
	Pelvis	54±4	48±4*	Hind fetlock	151±8	151±7
Straight hocks				Hip	85.9±5.7	90.5
	Thigh	47±5	47±5.3	Stifle	106±7	117.8±11.6*
	Gaskin	56±3	48.9±4.9*	Tarsal	148°±3.2°	150.7±4.7
	Hind cannon	35.8±2	37±3.6	Hind fetlock	151±8.4	156±6.3
Short pelvis	Pelvis	54± 4	49 ± 4.1*	Croup	143° ± 5°	145° ± 5.7*
				Hip	89.6°± 9°	87.5°± 5.9°
				Stifle	110°±9.3°	118°±13*
				Tarsal	147°± 3.9°	149°± 5.9°
				Hind fetlock	150°± 6.6°	156°± 7°

*Significant at P< 0.05.

DISCUSSION

In the current study, 7 abnormal conformations of fore and hind limbs in jumping thoroughbred horses were recorded and categorized. Each conformation was standardized objectively and the corresponding lengths and angles of each were evaluated.

Standing under in front represented 58% of the total examined horses. In contrast, HÖlmstrom et al,. (1990) reported the incidence of standing under in front in Swedish Warmblood horses as 5.6%. This difference could be attributed to the difference between the examined breeds in both studies. Thus, the horse's breed may play a role in the development of this abnormal conformation. Meanwhile, this high incidence ranks the standing under in front as the most common abnormal conformation in jumping thoroughbreds horses. This finding agreed with Marks (2000) who reported that many of the outstanding jumpers were extremely camped under.

Stashak (1987) and Marks (2000) reported that steep shoulder was more common in jumping horses. In this respect, Marks (2000) observed that long upright scapula with shoulder joint angle of about 105° and laid-back withers provides the vertical propulsion from the front legs that is necessary for jumping big fences. Interestingly, there were no significant differences in shoulder length between steep and normal shoulder in the present study. This finding disagrees with the previous findings (Anderson et al., 2004b). Moreover, statistical analysis of the present results revealed a significant decrease (P< 0.05) in fore arm length in horses with steep shoulder compared to horses with sloping shoulder. This could explain the role of fore arm length in the sloping and steep shoulders.

The elbow joint angle in steep shoulder in jumping thoroughbred horses was 140°. This agrees the finding reported previously (Stashak, 1987). Additionally, HÖlmstrom et al., (1990) found that a more flexed elbow with a horizontal scapula results in a longer stance duration, improved gait quality and collection in the fore limbs during jumping.

Carpus valgus with mean carpus joint angle of 186°±2° was observed in 45% of the examined horses. However, HÖlmstrom *et al.* (1990) reported the 'narrow at knees' with front carpal angles >180° in 11.8% of Swedish Warmblood trotters (HÖlmstrom et al. 1995 and Anderson et al. 2004). This great difference in the incidence of carpus valgus in thoroughbred jumping horses and Swedish Warmblood trotters suggesting again the role of horse's breed in development of this abnormal limb conformation. In addition, Weller et al., (2006) reported that carpus valgus of 5° is normal and doesn't stop horses from pursuing their racing career. Furthermore, the lateral and front fore arm length had significant increase (P< 0.05) in the examined thoroughbred jumping horses which play an important role in jumping horses.

Jumping thoroughbred horses had' calf knee' with mean carpus joint lateral angle of 176.38°± 2.2° in 31% of examined horses. However, HÖlmstrom *et al.* (1990) reported this abnormal conformation in 18.7% of elite show jumpers of Swedish Warmblood breed. Therefore, the horse's breed could be playing a role in the development of this abnormal conformation. Furthermore, Anderson et al., (2004) stated that carpus joint angle < 180° considered calf knee and played a major role in carpal fractures and joint disease due to the additional stress on knee joints (Thomas, 2005). In contrast, Marks (2000) and Weller et al., (2006) considered the carpal joint angle of 186° ± 3° 'normal' in National Hunt racehorses and much less of concern for jumpers and dressage horses.

Standing under behind was found in 31% of the population. In contrast, HÖlmstrom et al, (1990) found this abnormal conformation in 4.3% of show jumpers of Swedish Warmblood horses. These different incidences could be attributed to the horse's breed. The affected horses had significant increase (P< 0.05) in thigh length and stifle joint angle. Similar findings were reported by (HÖlmstrom et al., 1990) who concluded that the femur is the most individual conformational detail in sport horses. They added that a long and forwardly sloping femur allowed the horse to keep its balance more easily by placing the hind limbs more under the horse, closer to the center of gravity. On other hand, Magnusson and Thafvelin (1990) concluded that the better performing horses had straighter stifle angles. In the present study, gaskin length was found to be smaller in horses with 'standing under behind'. Furthermore, the most interesting observation in the present study is that pelvis, thigh and gaskin lengths had equal length in horses suffering from standing under behind.

Short pelvis was reported in 31% of the examined horses. The affected horses had pelvis length < 49± 4.1 cm, associated with decreased croup angle and increased stifle joint angle in comparison with normal horses. Short pelvis provides less length of muscular attachments to the thigh and gaskin that diminishes the engine power in jumping events. Flat croup helps the horses to go faster by encouraging long and flowing strides (Senna et al, 2015, Thomas, 2005 and Keegan, 2005).

Straight hocks were found in 16% of the population. It is worth mentioning that stifle joint angle showed significant increase (P< 0.05) while pelvis and gaskin lengths showed a significant decrease (P< 0.05) in the affected horses. However, all joints of the hind limb of the affected horses showed marked increase in their measurements compared to normal horses. While the pelvis and gaskin lengths decreased, the hock joint tended to be straighter in the examined thoroughbred jumping horses. Moreover, Dyson et al. (2018b) observed increased tarsal joint angles was associated with proximal suspensory desmopathy, oscillation of the hock during stance, rotation of the distal aspect of the limb and the foot placed further under the trunk. Furthermore, Straight hock conformation may lead to thoroughpin (Thomas, 2005) upward fixation of patella, bone and bog spavin, hoof bruises and quarter cracks (Dyson 1995) and Mostafa et al. (2014 a&b).

In conclusion, horses standing under in front have an increased elbow joint lateral angle, and decreased fore fetlock joint lateral angle. Horses with a steep shoulder have a decreased forearm front length, meanwhile horses with carpus valgus show an increased length of forearm. Horses with calf knee display a decreased lateral angle of carpus joint; however, horses with short pelvis

have increased croup and stifle joint lateral angles. Therefore, the current use of linear and angular measurements in relationships to abnormal limb conformation in the present study will allow for estimation of the future performance and soundness in jumping thoroughbred horses. In addition can be considered for selection athletic horse with less risk of lameness.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the Publication of this article.

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Research article
Ερευνητικό άρθρο

Red wine polyphenols modulate bone loss in the ovariectomized rat model of postmenopausal osteoporosis

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ABSTRACT. The aim of this study was to examine the effect of Red Wine Polyphenols (RWPs) extract on bone mineral density (BMD) in the ovariectomized (OVX) rat model. Thirty-five 10-month-old Wistar rats were separated into controls (CTRL), OVX and OVX plus RWPs in their drinking water (dose, 50 mg/kg body weight per day), starting immediately after OVX for 6 months. Body and uterine weight, BMD of the tibia at baseline, 3 and 6 months post-OVX, and 3-pointing bending of the femur, were examined. Statistical comparison of the total tibia BMD within groups during the study period showed a significant reduction in the OVX and OVX+RWPs groups both from baseline to 3 and 6 months and from 3 to 6 months, whereas in the CTRL group, there was no significant change. For the proximal tibial metaphysis, comparison of BMD percentage changes from baseline to 3 months and 6 months and from 3 to 6 months revealed highly statistical differences between OVX and OVX+RWPs groups (P = 0.000). OVX induced a significant reduction of biomechanical parameters as expected; the 3-point bending test showed that the maximum force before fracture, energy absorption and fracture stress significantly increased in the OVX group treated with RWPs compared with the nontreated OVX rats (P = 0.048, P = 0.002 and P = 0.003, respectively). Dietary intake of RWPs for 6 months significantly prevented trabecular bone loss and improved bone strength in estrogen-deficient ovariectomized rats.

Keywords: Rats, Osteoporosis, Polyphenols, Bone mineral density, Three point bending

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ΠΕΡΙΛΗΨΗ. Σκοπός αυτής της μελέτης ήταν να εξεταστεί η επίδραση του εκχυλίσματος πολυφαινολών του κόκκινου κρασιού (RWPs) στην οστική πυκνότητα (BMD) σε μοντέλο ωοθηκεκτομημένων επίμυων. Τριάντα πέντε Wistar επίμυες, ηλικίας 10 μηνών, χωρίστηκαν στις εξής ομάδες: ομάδα ελέγχου (CTRL), ωοθηκεκτομής (OVX) και την ομάδα ωοθηκεκτομής που έλαβε το εκχύλισμα RWPs (OVX+RWPs) σε συγκέντρωση 50 mg/kg σωματικού βάρους ανά ημέρα, ξεκινώντας αμέσως μετά από την ωοθηκεκτομή για 6 μήνες. Προσδιορίστηκε η οστική πυκνότητα πριν από οποιαδήποτε παρέμβαση, στους 3 και 6 μήνες. Οι επίμυες ευθανατώθηκαν και τα μηριαία οστά εξετάστηκαν με την δοκιμασία κάμψης τριών σημείων. Η σύγκριση των τιμών οστικής πυκνότητας ολόκληρης της κνήμης μεταξύ των ομάδων, ανέδειξε στατιστικά σημαντική μείωση στις ομάδες OVX και OVX+RWPs τόσο από την αρχική μέτρηση στους 3 και 6 μήνες, όσο και από τους 3 στους 6 μήνες, ενώ στην ομάδα CTRL δεν υπήρξε σημαντική αλλαγή. Η ποσοστιαία μεταβολή της οστικής πυκνότητας στην εγγύς μετάφυση της κνήμης από την αρχική μέτρηση στους 3 και 6 μήνες και από τους 3 στους 6 μήνες, αποκάλυψε στατιστικά σημαντικές διαφορές μεταξύ των ομάδων OVX και OVX + RWPs (P = 0.000). Η ωοθηκεκτομή προκάλεσε σημαντική μείωση των εμβιομηχανικών παραμέτρων όπως αναμενόταν, η δοκιμασία κάμψης τριών σημείων έδειξε ότι η μέγιστη φόρτιση πριν από τη θραύση, η απορρόφηση ενέργειας και η τάση θραύσης αυξήθηκε στατιστικά σημαντικά στην ομάδα OVX που υποβλήθηκε σε θεραπεία με RWPs σε σύγκριση με τους OVX επιμύες (P = 0.048, P = 0.002 και P = 0.003 αντίστοιχα). Η διατροφική πρόσληψη RWPs για 6 μήνες απέτρεψε την ταχεία οστική απώλεια και βελτίωσε την αντοχή των οστών σε ωοθηκεκτομημένους επίμυες.

Λέξεις ευρετηρίασης: Επίμυες, Οστεοπόρωση, Πολυφαινόλες, Οστική πυκνότητα, Κάμψη τριών σημείων

INTRODUCTION

Osteoporosis is a severe condition of low bone mineral density (BMD). Patients with osteoporosis have a high fracture hazard, leading to physical impairment, significant pain and even untimely death (Lloret et al., 2016; Thulkar et al., 2016; Weisz, 2017). Preventive treatments include maintaining or increasing bone mineral density (BMD) when its loss is induced by decreasing levels of sex steroid levels with age in both males and females (Wang et al., 2017). Hormone replacement therapy (HRT) has been established as the method of choice to delay bone turnover and BMD loss, at all skeletal sites in early and late postmenopausal females (Cao et al., 2014; Ran et al., 2017). HRT significantly decelerates fracture rate while raising the risk of venous thromboembolism, coronary heart disease and stroke, thus, poor compliance has emerged (L'Hermite, 2017; Miller et al., 2016; Oster et al., 1982).

Polyphenols have gained growing interest within the last years, due to their antioxidant function and mild estrogenic effect (De França et al., 2013). Antioxidants curtail inflammation by anti-inflammatory signaling, and regulate osteoblastogenesis, osteoclastogenesis and osteoimmunological activity.

A few studies have displayed that grape extracts have been promising in preserving or enhancing bone calcium retention (Yahara et al., 2005; Zhang et al., 2014); some have shown benefits by reducing bone turnover, but the effect of grape consumption on bone health has not been fully determined (Hohman & Weaver, 2015; Yahara et al., 2005). So far, there is no evidence backing the protective effect of red grape consumption on bone density, despite their antioxidant composition. The objective of this study was to examine the effect of Red Wine Polyphenols (RWPs) extract on bone mineral density and strength in the ovariectomized rat model of postmenopausal osteoporosis.

MATERIAL AND METHODS

Laboratory animals

Thirty-five ten-month-old virgin female Wistar rats were acquired from the laboratory animal center of the Hellenic Pasteur Institute (Athens, Greece). They were housed in the registered animal house of the Laboratory for Research of the Musculoskeletal System of the School of Medicine, National & Kapodistrian University of Athens, Greece. The experimental protocol was approved by the General Directorate of Veterinary Services with the authorization code 262/13-1-2011, consistent with Greek legislation (Presidential Decree 160/1991, in compliance to the Directive 86/609/EEC in force at the time).

The rats' body weight ranged from 260 to 330 g. They were housed by three to four in conventional open-top cages (transparent polycarbonate 45 x 30 x 20 cm; IFFA) with shredded wood bedding. The animals were maintained on a 12:12 h light/dark photoperiod, 55 - 65% relative humidity, 19 - 22^{0}C temperature and 15 air changes per hour.

Experimental groups

The rats were acclimatized to the laboratory environment 1 month before commencing any examination or treatment. After the adjustment period, using a randomization procedure stratified according to body weight, the 11-month-old animals were assigned to three groups (Figure 1). Eleven of the 35 rats consisted the sham-operated non-ovariectomized control group (CTRL, n=11). Twenty four rats underwent bilateral ovariectomy by the ventral approach; of these, 13 rats consisted the ovariectomized group without treatment (OVX, n=13) and the other 11 rats were treated with RWPs extract (OVX+RWPs, n=11). A sample size of rats was derived from a sample size calculation using data from our previous studies that investigated the protective effect of dietary interventions to ovariectomy-induced osteoporosis in rats (Dontas et al., 2011; Dontas et al., 2006).

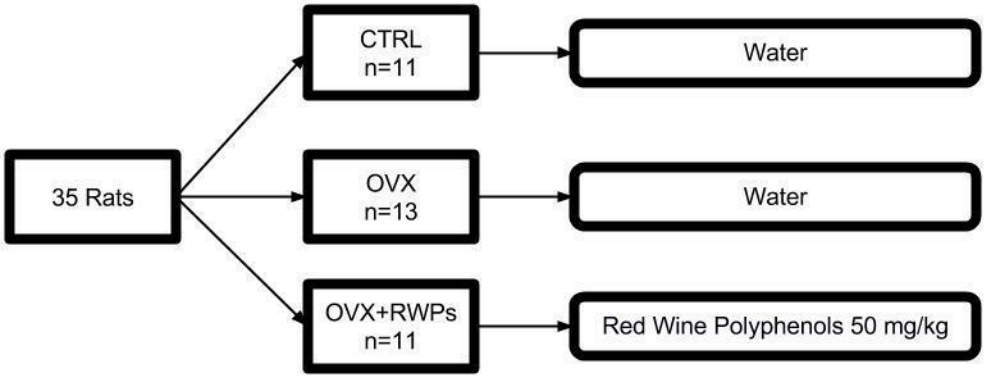


Figure 1. Study design.

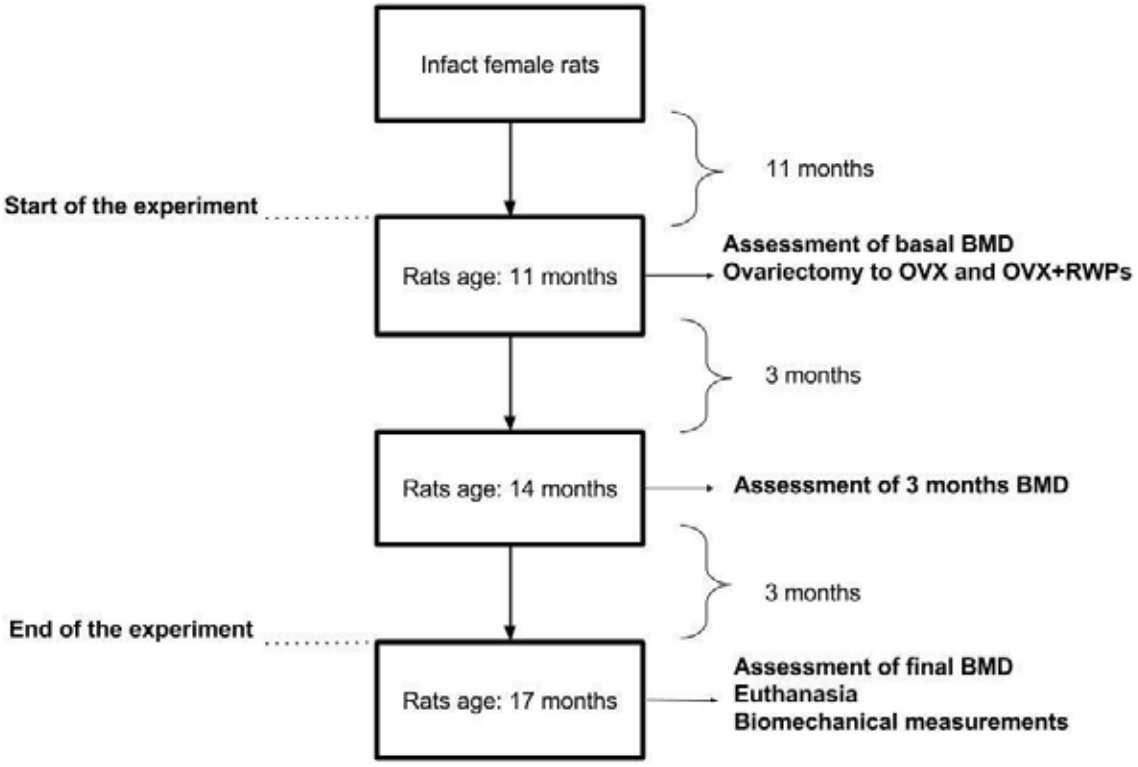


Figure 2. Flow-chart of experimental protocol.

Body weight and food intake

After ovariectomy, all rats had *ad libitum* access to a pelleted chow of soybean-free/low phytoestrogen rat diet (pellet, type 4RF21 GLP, Mucedola, Settimo Milanese, Italy). This diet was used to prevent confounding from residual isoflavones (Pawlowski et al., 2014). Rats were fed according to the mean food intake of the CTRL group, in order to avoid ovariectomy-induced obesity and obesity-related bone density changes. Adjustments of food intake took place twice weekly.

Body weight was recorded weekly throughout the 6-month study period; mean values±SD for final weights are presented in Table 1.

Table 1. Body weight, fat and uterus weight.

Group	CTRL[a] Mean (SD)	OVX[b] Mean (SD)	OVX+RWPs[c] Mean (SD)	P Anova
Body Weight, g	330.90 (33.33)[b,c]	377.07 (35,83)[a]	372.90 (17.43)[a]	**0.001**
Fat, g	22.94 (5.19)[b]	29.72 (7.74)[a]	29.75 (5.67)	**0.023**
Uterus, g	0.41 (0.09)[b,c]	0.14 (0.03)[a]	0.21 (0.29)[a]	**0.002**

Note: superscript letters (a, b, c) indicates significant difference ($P < 0.05$) after Bonferroni correction.
Groups: a: Control (CTRL), **b:** Ovariectomy (OVX), **c:** Ovariectomy treated with RWPs extract (OVX+RWPs).

Extract

Administration

CTRL and OVX rats had *ad libitum* access to filtered tap water in drinking bottles; RWPs extract was administered to the RWPs group through their drinking water (dose 50 mg/kg body weight/day), immediately after ovariectomy. Treatment was continued for 6 months. Water and extract ingestion was documented twice a week in all rat cages. The bottles with RWPs were shaken twice a day to prevent the gathering of precipitate.

Analysis

Vitis vinifera species comprise numerous varieties and Muscat is one of the oldest and most widespread in the world. In the present study, the red wine was obtained from the Muscat Hamburg (MHW) variety and used for the preparation of an extract rich in polyphenols using macroporus resin (XAD-4) technology.

The MHW was provided by the Tyrnavos Cooperative Winery & Distillery, Thessaly region, Greece. After concentration under reduced pressure, the dry residue was calculated at 2.54 g per 100 ml of wine. In laboratory scale, two different procedures were used for the recovery of the extract enriched in MHW polyphenols (MHWPE). Column mode: A 40 × 3 cm glass column packed with 50 mL of resin XAD-4 was used. MHW (400 ml) was passed through the column at a flow rate of 2 mL/ min. Total duration of the adsorption step was 200 min. and desorption duration 45 min. The temperature was 20°C throughout all the experimental procedures. After reaching adsorptive equilibration, the resin column was first washed with H_2O and then desorbed with 100 ml of EtOH 95°. After desorption, the alcoholic solution with a high content of polyphenolic compounds was condensed to remove EtOH. Finally, the crude extract was obtained by drying at 40 °C to achieve a constant weight (3.11 g). Therefore, the percentage of recovered polyphenolic fraction (MHWPECM) was calculated at 0.78 g/100 ml MHW.

Bath mode: 400 ml of MHW and 50 mL of XAD-4 were placed in a flask of 250 ml and stirred for 12 hours in room temperature. After filtration, the resin was washed with water and the polyphenolic fraction

recovered using 100 ml of EtOH 95°. Finally, 1.93 g of crude extract (MHWPEBM) was obtained after removal of solvent in a yield of 0.48 g/100 ml MHW.

The polyphenolic content in fractions obtained from MHW using column and bath mode (MHWPECM and MHWPEBM) was calculated by Folin-Ciocalteau and found to be 92.5 and 88.6 mg GAE/g of extract, respectively. These results showed that column mode was more effective for the recovery of polyphenolics from MHW and this procedure was used for the preparation of MHWPE in pilot scale.

For pilot scale processes, a column of 150 × 38 cm filled with 125 L of XAD-4 resin was used. MHW (300 L) was fed into the resin through a 0.5 in. diameter PVC pipe using a Wilden diaphragmatic pump. The flow rate was 2500 mL/min (150 L/h) and the total duration of the adsorption-desorption process was 180 min. Finally, EtOH (150 L) was evaporated under vacuum in a QVF glass 200 L evaporator and consequently in a Buchi 20 L rotary evaporator. The dry extract (MHWPECM 2.25 Kg) was used for the *in vivo* evaluation of its activity on osteoporosis. The MHWPECM dry extract was finally diluted with distilled water to obtain 0.6 g/L concentration and kept at 4 °C.

Ovariectomy

All animals except the CTRL group underwent ovariectomy. Ovariectomy was performed when the rats were 11 months old, using the technique defined by Waynforth (Waynforth & Flecknell, 1992). They were anesthetized by an intramuscular injection of dexmedetomidine (0.25 mg/kg) and ketamine hydrochloride (50 mg.kg), administered analgesia (carprofen 4 mg/kg) and chemoprophylaxis (enrofloxacin 10 mg/kg) subcutaneously, laparotomized via a ventral midline incision using aseptic procedures and their ovaries were removed bilaterally. The incision was sutured by single interrupted sutures in layers.

Bone densitometry

Prior to surgery, baseline bone mineral density (BMD) of all rats were obtained. BMD measurements of the entire and proximal tibia were also carried out 3 and 6 months post-OVX; their values were estimated using the technique defined by Patsaki et al (2016) and Dontas et al (2006; 2011).

Euthanasia, specimen collection and examination

The animals were anesthetized (3 mg/kg xylazine, 100 mg/kg ketamine hydrochloride, intramuscular) and euthanised after exsanguination from the abdominal aorta. At necropsy, the animals' abdominal cavity was examined for any abnormality and in the OVX groups for successful ovariectomy. The uteri were carefully removed free of surrounding tissues and their wet weight was immediately measured. The abdominal and perirenal fat tissue was also removed and immediately weighed. The femurs were dissected from the animals, cleaned of soft tissue and stored at -20°C to determine bone mechanical properties.

Bone biomechanical testing

Prior to biomechanical testing, the frozen femurs were thawed for 1 hour before the test and were rehydrated in physiologic saline. The femurs' length was measured with a micrometer and the middle of the diaphysis was marked. The femur was placed in the MTS INSIGHT 1kN testing frame (MTS, Eden Prairie) on two rounded supports at a distance of 20 mm from each other. The tests were quasi-static under displacement control mode at a rate of 1 mm/min. The force was applied to the middle of the diaphysis, until fracture. During loading, force and deflection were recorded. For the latter the RTSS_HR Video Extensometer (Limess, Krefeld, Germany) and the TestWorks 4 Software (WR Medical Electronics CO, Maplewood, USA) were used. From the force-deflection curve, femoral biomechanical parameters were determined, including maximum force (F_{max}), stiffness, energy absorption (W_{abs}).

In order to calculate also the fracture stress, σ_{max}, characterizing the material independently of the specimen's geometry, the characteristics of the bones' cross section had to be determined. In this context, the proximal one-half portion of the broken femur was embedded into specially designed plastic cups containing dental resin. The bone-dental resin complex was polished using LaboPol 5 (Struers), so as to trace the full cross-sectional shape. After deburring using Image-Pro Plus Processing Software, the photography session followed using LEICA MZ6 modular stereomicroscope. Picture processing was done with the stereoscope using the Solid Works software, enabling determination of: the cross section's geometrical centre, the loading axis and its eccentricity, the cross section's area, the inner and outer radii, the mean and minimum thickness of the cross section, the second moments of area with respect to the principal axes, the neutral axis and the critical point of the section. Using these characteristics the bones' fracture stress

(σ_{max}) was determined by applying the Bernoulli-Euler technical bending theory.

Statistical analysis

Data are stated as mean ± standard deviation (S.D.) for continuous parameters and as proportions for categorical variables. The Kolmogorov-Smirnov test was used for normality analysis of the variables. The comparison of variables at separate time points took place with the use of the one way analysis of variance (ANOVA) model. The repeated Measures ANOVA model was used for the comparison of dissimilar time quantity of variables for each assembly individually. Pairwise multiple comparisons were conducted with Tukey critical difference.

To indicate the trend in the first 6 months of treatment, the mean percentage changes after 3 and 6 months respectively were estimated. Comparison of the percentage change from baseline during the observation period between three groups was analyzed with the one way ANOVA model. Pairwise comparisons were performed using the Bonferroni, Kruskal-Wallis and Mann-Whitney tests in case of abuse of normalcy. Pearson or Spearman's correlation coefficients were used to examine the relation among quantitative variables. All tests were two-sided, statistical significance set at $P < 0.05$. All analyses were conducted with SPSS vr 17.00 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Ill., USA).

RESULTS

Body weight and uterus weight

Administration of the plant extract did not present any adverse effect on the treated animals as observed by monitoring their body weights, fluid and food consumption.

Comparison of the animals' mean body weights at the end of the experiment showed a significant weight gain of OVX and OVX+RWPs animals. This resulted in a significant difference between OVX and CTRL ($P = 0.002$), as well as between OVX+RWPs and CTRL ($P = 0.008$), whereas there was no difference between OVX and OVX+RWPs animals.

The uteri of OVX animals were macroscopically visibly atrophic, and their mean uterine weight was significantly lower in contrast to CTRL ($P = 0.002$). The mean uterine weight of OVX+RWPs animals was identical to that of OVX and significantly lower in comparison with the CTRL ($P = 0.03$).

Comparison of the animals mean fat values at the end of the experiment showed a significant weight gain of OVX ($P = 0.044$) groups in comparison with the CTRL group (Table 1).

Table 2. Comparison of absolute values and mean percentage changes (in g/cm^2) of bone mineral density (BMD) of the total tibia among groups measured at baseline, 3 and 6 months post-ovariectomy

Total Tibia BMD								
Group	Baseline Mean (SD) (g/cm^2)	3 months* Mean (SD) (g/cm^2)	6 months** Mean (SD) (g/cm^2)	% change from baseline to 3 months* Mean (SD)	% change from baseline to 6 months** Mean (SD)	P*** Baseline vs 3 months	P*** Baseline vs 6 months	P*** 3 months vs 6 months
Control	0.213 (0.011)	0.215 (0.012)	0.217 (0.011)	1.04 (2.88)	1.68 (3.45)	0.816	0.471	0.975
OVX	0.213 (0.009)	0.200 (0.006)	0.190 (0.007)	-6.02 (2.65)	-10.73 (3.72)	**0.000**	**0.000**	**0.000**
OVX+RWPs	0.213 (0.012)	0.204 (0.013)	0.192 (0.009)	-4.06 (2.90)	-9.59 (4.97)	**0.002**	**0.000**	**0.007**

Groups: Control (CTRL), Ovariectomy (OVX), Ovariectomy treated with RWPs extract (OVX+RWPs).
* All pairwise comparisons between groups are statistically significant $P < 0.05$ except OVX vs. OVX+RWPs group.
** All pairwise comparisons between groups are statistically significant $P < 0.05$ except OVX vs. OVX+RWPs group.
*** Time effect comparisons after Bonferroni correction.

Table 3. Comparison of absolute values and mean percentage changes (in g/cm^2) of bone mineral density (BMD) of the proximal tibia among groups measured at baseline, 3 and 6 months post-ovariectomy.

Proximal Tibia BMD								
Group	Baseline Mean (SD) (g/cm^2)	3 months* Mean (SD) (g/cm^2)	6 months** Mean (SD) (g/cm^2)	% change from baseline to 3 months* Mean (SD)	% change from baseline to 6 months** Mean (SD)	P*** Baseline vs 3 months	P*** Baseline vs 6 months	P*** 3 months vs 6 months
Control	0.331 (0.022)	0.351 (0.018)	0.357 (0.017)	6.25 (3.39)	8.11 (3.13)	**0.000**	**0.003**	0.198
OVX	0.330 (0.023)	0.258 (0.031)	0.224 (0.024)	-21.82 (7.03)	-32.13 (5.91)	**0.000**	**0.000**	**0.000**
OVX+RWPs	0.351 (0.025)	0.318 (0.022)	0.285 (0.023)	-9.49 (4.91)	-18.57 (7.80)	**0.000**	**0.000**	**0.001**

Groups: Control (CTRL). Ovariectomy (OVX). Ovariectomy treated with RWPs extract (OVX+RWPs).
* All pairwise comparisons between groups are statistically significant P < 0.05.
** All pairwise comparisons between groups are statistically significant P < 0.05.
*** Time effect comparisons after Bonferroni correction.

Bone densitometry and biomechanical testing

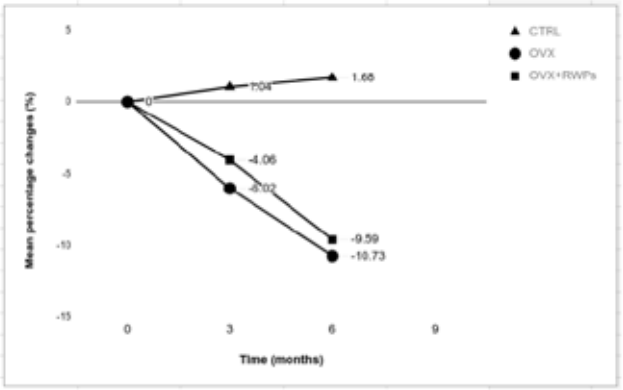
Comparison of absolute values of BMD of the total tibia of the three groups, during the study, are shown in Table 2. Values are in grams per centimeters squared.

At baseline, statistical comparison of the absolute values of BMD of both sites examined within groups showed no statistical difference. At 3 and 6 months, the total tibia BMD of the CTRL rats had a significantly higher mean value than OVX and OVX+RWPs rats (P < 0.005).

Statistical comparison of the total tibia BMD within groups during the experimental period, showed a significant reduction in the OVX and OVX+RWPs groups both from baseline to 3 and 6 months and from 3 to 6 months, whereas in the CTRL group, there was no statistical significance. From the data in Table 3, it is apparent that the comparison of absolute values of BMD of the proximal tibia, during the experimental period, showed significant differences between the three groups. The BMD absolute values of the proximal tibial metaphysis within groups were slightly increased in the control group, whereas in both OVX and OVX+RWPs groups they decreased significantly from baseline to 3 months, from baseline to 6 months and from 3 to 6 months, respectively.

Treated rats (OVX+RWPs) had significantly higher BMD values than OVX rats with level of significance P < 0.05 for the proximal tibia at 3 and 6 months. These findings suggest that that RWPs intake prevented ovariectomy-induced bone loss.

Comparison of the percentage changes of the total tibia at 3 and 6 months post-ovariectomy between CTRL and OVX and between CTRL and OVX+RWPs were significant, however between the OVX and OVX+RWPs groups they were nonsignificant (Fig. 3).

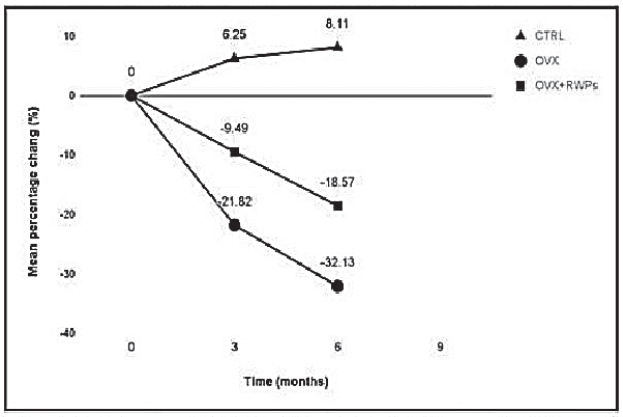


Groups: Control (CTRL), Ovariectomy (OVX), Ovariectomy treated with RWPs extract (OVX+RWPs).

Figure 3. Comparison of mean percentage changes (in g/cm^2) of bone mineral density (BMD) of the total tibia among groups measured at baseline, 3 and 6 months post-ovariectomy.

For the proximal tibial metaphysis, pairwise comparison revealed highly statistical significant differences (Mann-Whitney test) between all groups at 3 and 6 months (Fig. 4). At 3 and 6 months, the dif-

ference between OVX and OVX+RWPs was highly significant (P = 0.000), indicating a highly protective effect on the treated animals.



Groups: Control (CTRL), Ovariectomy (OVX), Ovariectomy treated with RWPs extract (OVX+RWPs).

Figure 4. Comparison of mean percentage changes (in g/cm^2) of bone mineral density (BMD) of the proximal tibia among groups measured at baseline, 3 and 6 months post-ovariectomy.

Table 4 presents the results obtained from the biomechanical parameters of the three groups. Three-point-bending (3PB) revealed that the F_{max} was significantly lower in the OVX group in contrast to CTRL (P = 0.042) and OVX+RWPs groups (P = 0.048). It is noteworthy that the Fmax in the OVX+RWPs animals was identical to CTRL rats. W_{abs} was significantly lower in the OVX group as compared with CTRL (P = 0.004) and OVX+RWPs group (P = 0.002). It is remarkable that the W_{abs} in the OVX+RWPs group was similar to CTRL group. σ_{max} was significantly greater in the treated group compared with OVX group (P = 0.003). Additionally, comparison of the σ_{max} mean values between OVX and CTRL was significant (P = 0.048), however between the CTRL and OVX+RWPs group, it was nonsignificant.

Table 4. Comparison of biomechanical parameters among three study groups.

Group	CTRL[a] Mean (SD)	OVX[b] Mean (SD)	OVX+RWPs[c] Mean (SD)	P Anova
F_{max} (N)	118.90 (19.00)[b]	100.90 (16.60)[a,c]	118.50 (16.49)[b]	**0.022**
W_{abs} (mJ)	19.25 (2.91)[b]	12.95 (2.09)[a,c]	19.60 (6.58)[b]	**0.001**
σ_{max} (MPa)	184.29 (17.64)[b]	155.28 (12.71)[a,c]	198.01 (46.04)[b]	**0.003**

Note: superscript letters (a, b, c) indicates significant difference (P < 0.05) after Bonferroni correction.
Groups: a: Control (CTRL), **b:** Ovariectomy (OVX), **c:** Ovariectomy treated with RWPs extract (OVX+RWPs).

DISCUSSION

Hormone replacement therapy (HRT) has been used for prevention and treatment of postmenopausal osteoporosis but may cause serious side-effects (Ferguson, 2004). The results from the Women's Health Initiative Study that HRT significantly augments the risk of breast cancer, ovarian cancer, heart attack, stroke, thromboembolism and Alzheimer's disease (Canalis et al., 1988; Termine & Wong, 1998), increased women's non-compliance to HRT.

Polyphenols have been tested for their antiosteoporotic activity, because of their estrogenic and antioxidant effects. Some studies have displayed that grape extracts have been promising in preserving or enhancing bone density (Hohman & Weaver, 2015; Yahara et al., 2005). We investigated the effect of RWPs extract administration as an alternative treatment to the long-term HRT commonly used by osteoporotic women.

The body weight of the ovariectomized rats increased steadily throughout the study even though all animals had the same daily food intake. These results are consistent with those of other studies which showed that OVX rats had significantly higher body weight compared to CTRL rats, due to fat deposition caused by estrogen deficiency (Patsaki et al., 2016). As also indicated by Dontas et al (2006; 2011), the

uteri of our OVX animals were macroscopically visibly atrophic, and their mean uterine weight was significantly lower in contrast to CTRL (P = 0.002).

Although the mechanism of beneficial health effects of RWPs remains unclear, several studies strongly suggest that they do not act via estrogen receptor binding (Agarwal et al., 2000; Eng et al., 2003; Sharma et al., 2004). Similarly, in this study RWPs extract administration was not able to modulate postmenopausal adiposity.

Cortical bone loss markedly increases activation of Haversian remodeling in humans (Lelovas, et al., 2008). However, the rat cortical compartment displays a low level of Haversian remodeling, in spite of increased endosteal osteoblasts and it is considered impractical to use cortical bones for bone loss studies (Jee & Yao, 2001). Data available from studies of our group revealed that the effects of OVX on BMD of cortical compartments of bone are lower than in trabecular bone (Patsaki et al., 2016; Dontas et al., 2011; Dontas et al., 2006). Statistical comparison of the total tibia BMD within groups during the study period, showed a significant reduction in the OVX and OVX-+RWPs groups both from baseline to 3 and 6 months and from 3 to 6 months, whereas in the CTRL group, there was no statistical significance. Cortical bone, which has a longer remodelling period than trabecular bone (Jee & Yao, 2001), may need a longer experimental study to show potential beneficial effects of a treatment.

In the post-OVX period, a deterioration in trabecular bone density clearly shows rapid quantitative loss of bone, similar to the increased risk of fragility fractures in human postmenopausal osteoporosis, due to decline in endogenous estrogen production. The severe decrease of trabecular BMD in the metaphyseal sites after OVX were similar to the previous reports (Jee & Yao, 2001). Statistical comparison of the proximal tibia BMD absolute values, during the experimental period, showed a significant increase in the treated rats than OVX rats at 3 and 6 months. Pairwise comparison of BMD percentage changes from baseline to 3 months and 6 months and from 3 to 6 months, revealed highly statistical differences between OVX and OVX+RWPs .

The bone density results of the present study, demonstrating bone quantity, are in accordance with the bone quality investigation regarding maximum load, energy absorption and maximum stress, conducted by *ex vivo* 3PB of the femur. OVX induced a significant reduction of biomechanical parameters as expected, whereas the OVX group treated with RWPs showed a significant increase in comparison with the nontreated OVX. It is noteworthy that the Fmax, W_{abs} and σ_{max} in the OVX+RWPs animals was similar to CTRL rats. Similar studies with grape-enriched diet–fed rats, demonstrated improved bone strength of the femora (Hohman & Weaver, 2015; Yahara et al., 2005). Bone strength is the most important variable determined as it directly expresses the resistance of the whole bone to fracture, incorporating both its elastic and plastic behavior. Particularly in the clinical setting, bone strength is the ultimate goal of anti-osteoporotic treatments.

CONCLUSIONS

In conclusion, dietary intake of Red Wine extract for 6 months produced a remarkable effect on prevention of osteopenia and bone strength, in estrogen deficient ovariectomized rats, without hypertrophic effect on the uterus. RWPs could be considered as an attractive natural alternative anti-osteoporotic herbal treatment due to the additive beneficial action of its different substances, such as polyphenols. More experimental studies are needed to clarify the precise mechanism of action of RWPs, as well as a prospective clinical study in postmenopausal women with bone loss, before clinical recommendations can be made.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Research article
Ερευνητικό άρθρο

Effects of ruminal pH and subacute ruminal acidosis on milk yield and composition of Holstein cows in different stages of lactation

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Επίδραση του pH του περιεχομένου της μεγάλης κοιλίας και της υποξείας δυσπεπτικής οξέωσης στα ποσοτικά και ποιοτικά χαρακτηριστικά του γάλακτος αγελάδων Holstein σε διαφορετικά στάδια της γαλακτικής περιόδου

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ABSTRACT. Subacute ruminal acidosis (SARA; implying a rumen fluid pH between 5.5 and 5.0), is one of the most important metabolic diseases of dairy cows. In this study, the effect of SARA and rumen fluid pH on milk yield and composition was assessed in dairy cows under field conditions, with repeated measurements in the same cows, at different stages of lactation. Rumenocentesis was performed in 83 Holstein cows of a commercial herd at 30, 90, and 150 days in milk (DIM). Rumen fluid pH was measured on-site using a portable pH-meter. Milk yield was also recorded at the same days. Milk samples were analyzed for fat, protein, lactose and total solids content. For the statistical analysis, mixed linear regression models were used. Prevalence of SARA was 48.2%, 53.8% and 65.3% at 30, 90 and 150 DIM, respectively. There was a significant negative effect of SARA and decreased rumen fluid pH on milk fat content; SARA was associated with a decrease of milk fat content by 0.22%, while a one-unit increase of rumen fluid pH, even within the normal range, was associated with a 0.28% increase of milk fat content and 0.44% increase of milk total solids content. There was no effect of SARA on milk yield or protein, lactose and total solids content. In conclusion, under field conditions, SARA and decreased rumen fluid pH reduce milk fat content.

Keywords: Subacute ruminal acidosis, dairy cow, milk composition, milk fat.

ΠΕΡΙΛΗΨΗ. Η υποξεία δυσπεπτική οξέωση (ΥΔΟ) αποτελεί μία από τις πλέον σημαντικές μεταβολικές νόσους των γαλακτοπαραγωγών αγελάδων. Ορίζεται ως η πτώση του pH της μεγάλης κοιλίας (ΜΚ) κάτω από 5,5 (έως 5,0) και ο κύριος τρόπος διάγνωσής της στην κλινική πράξη μέχρι σήμερα είναι η παρακέντηση της ΜΚ. Η ΥΔΟ επηρεάζει την ομαλή παραγωγή λιπαρών οξέων στη ΜΚ και, μεταξύ άλλων, προκαλεί μείωση της λιποπεριεκτικότητας και της παραγόμενης ποσότητας γάλακτος. Κύριος σκοπός της έρευνας ήταν η διερεύνηση της σχέσης της ΥΔΟ και του pH του περιεχομένου της ΜΚ με την ποσότητα και την ποιότητα του γάλακτος, σε διαφορετικά στάδια της γαλακτικής περιόδου, υπό συνθήκες εκτροφής. Χρησιμοποιήθηκαν 83 αγελάδες φυλής Holstein μιας εμπορικής εκτροφής, από τις οποίες ελήφθησαν δείγματα περιεχομένου της ΜΚ με παρακέντηση για τον προσδιορισμό του pH, τις ημέρες 30, 90 και 150 της γαλακτικής περιόδου. Η εκτροφή διέθετε αυτόματη ατομική γαλακτομέτρηση και τις ημέρες των παρακεντήσεων γινόταν δειγματοληψία γάλακτος για προσδιορισμό της χημικής του σύνθεσης (περιεκτικότητα σε λίπος, πρωτεΐνες, λακτόζη και ολικά στερεά). Η ανάλυση των δεδομένων έγινε με μια σειρά γραμμικών μοντέλων μικτών επιδράσεων. Ο επιπολασμός της ΥΔΟ ήταν 48,2%, 53,8% και 65,3% τις ημέρες 30, 90 και 150 της γαλακτικής περιόδου, αντίστοιχα. Βρέθηκε ότι η ΥΔΟ σχετίζεται με μείωση της λιποπεριεκτικότητας του γάλακτος κατά 0,22%. Η αύξηση του pH της ΜΚ κατά 1 μονάδα, ακόμη κι εντός των φυσιολογικών ορίων, σχετίστηκε με αύξηση της λιποπεριεκτικότητας του γάλακτος κατά 0,28% και της συγκέντρωσης των ολικών στερεών του γάλακτος κατά 0,44%. Η ημερήσια γαλακτοπαραγωγή, δεν επηρεάστηκε από την ΥΔΟ, καθώς και η συγκέντρωση της πρωτεΐνης, της λακτόζης και των ολικών στερεών του γάλακτος.

Λέξεις ευρετηρίασης: Υποξεία δυσπεπτική οξέωση, αγελάδες γαλακτοπαραγωγής, χημική σύνθεση γάλακτος, λιποπεριεκτικότητα

INTRODUCTION

Subacute ruminal acidosis (SARA) is an issue of major concern in dairy cattle, with significant physiological and economic impacts (Nocek, 1997, Kleen et al., 2003, Enemark, 2008). It is commonly observed in intensive farming systems (Krause and Oetzel, 2006) and is characterized by rumen fluid pH between 5.0 and 5.5, without any characteristic clinical signs (Kleen et al., 2003). This pH reduction is caused by the excessive accumulation of short chain fatty acids (SCFA), associated with the ingestion of diets rich in readily fermentable carbohydrates and their slow absorption by the rumen wall (Kleen et al., 2003). Given the lack of characteristic clinical signs, a definite diagnosis of SARA in clinical practice is only established by measuring the rumen fluid pH, either at a specific time-point after feeding (rumen fluid collection by stomach tubing or, more credibly, by rumenocentesis (Duffield et al., 2004) or continuously (using rumen boluses and pH monitoring systems) (Villot et al, 2017).

Consequences of SARA in dairy cows may include a decrease in milk yield and in milk fat and protein content, due to changes in rumen fermentation and fatty acids' profile (Stone, 1999; Plaizier et al., 2009). Low ruminal pH alters rumen bacteria populations and fermentation patterns, favouring the production of specific long chain fatty acids (LCFA), which, after absorption, inhibit milk fat synthesis in the udder (Kennely et al., 1999).

Studies regarding the effects of SARA on milk yield and composition (Stone, 1999; Fairfield et al.,

2007; Gozho et al., 2007), as well as on rumen fluid fatty acids content (Kennely et al., 1999; Murphy et al., 2000) have produced controversial results. Moreover, these studies deal with experimentally induced SARA, mostly in early lactation cows. Considering the importance of SARA, it is questionable the lack of relevant field studies in the available literature.

Therefore, the objective here was to assess the effect of ruminal pH and SARA on milk yield and composition in cows of a commercial herd, repeatedly, in three different time points during lactation.

MATERIALS AND METHODS

This study was conducted following the approval of the ethics and research committee of the Faculty of Veterinary Medicine, Aristotle University of Thessaloniki. The farmer gave informed consent for the cows to be included in the study and the sampling procedures. For the purposes of the study, the farm was visited three times weekly for 15 consecutive months, for sample collection and clinical examinations.

Animals and Management

A total of 83 lactating Holstein cows (44 primiparous and 39 multiparous) from a commercial dairy farm, located in the region of Thessaloniki, Greece, were included in the study. Farm selection was based on historical data about SARA prevalence (farm #11, Kitkas et al., 2013). Dry cows were housed in a bedded pack shed as a single group (no far-off/close-up groups) and lactating ones in a two-row free-stall barn, again as a single group. The feed bunk for lactating cows was equipped with headlocks (65 cm center to center) and its length was 50 m, which was appropriate for 77 instead of 83 cows.

Lactating cows were offered a total mixed ration set to meet the National Research Council's recommendations (NRC, 2001) regarding net energy and metabolizable protein requirements, according to milk production level (Table 1). Dry cows' ration is presented in Table 1. Transition from the dry cow ration to the lactation one was abrupt (no close-up and far-off group).

Cows were milked twice daily. Daily milk yield (DMY) was automatically recorded for each individual cow using an automatic milk yield recording system (AfiFarm Herd Management Software®, Afimilk Ltd., Kibbutz Afikim, Israel).

Table 1. Composition of the diets fed to the cows of the study.

Ingredients	Lactation period ration (kg, as fed)	Dry period ration (kg, as fed)
Corn sillage	30.00	14.00
Alfalfa hay	3.50	--
Wheat straw	1.00	5.00
Corn grain	5.00	--
Wheat bran	2.00	1.00
Soybean meal	3.50	1.50
Mineral/vitamin supplement	0.30	0.15
Calcium carbonate	0.10	0.10
Sodium bicarbonate	0.18	--

Body Condition Scoring and Clinical Examination

Body condition score (BCS) was recorded for all cows at 30, 90 and 150 days in milk (DIM), always by the first author, using a five-point scale with increments of 0.25 (Ferguson et al., 1994). Clinical examination was performed on all cows routinely at the above time points and every time the farmer reported a sudden milk drop or clinical illness.

Rumen Fluid Sampling and Analyses

Rumen fluid was sampled via rumenocentesis, at the predetermined time-points (30, 90 and 150 DIM). The puncture site was selected and prepared as described by Garrett et al. (1999). The cows were restrained without sedation. Local anaesthesia was performed prior to each rumenocentesis, injecting 4 mL of 2% Xylocaine (AstraZeneka, Athens, Greece), at the puncture site (2 mL subcutaneously and 2 mL intramuscularly). Afterwards, a 16-G and 13 cm long stainless-steel needle (H. Hauptner & Richard Herberholz GmbH & Co. KG, Solingen, Germany) was used to aspirate 2 to 3 mL of rumen fluid, within 20 sec, into a 5 mL disposable plastic syringe. Rumenocenteses were consistently performed between 12:00-14:00, in order to be within the suggested time-frame of 5-8 hours after the morning feeding.

All cows were monitored for 10 days after each rumenocentesis for the presence of complications like peritonitis, hematoma or abscess formation at the puncture site. Minor complications were recorded only in 3 cases; namely, a small abscess (<3 cm) in

two cows and a larger one (approximately 10 cm) in one cow, all after the 1st rumenocentesis (at 30 DIM). All abscesses resolved spontaneously within two weeks, whereas during this period DMY of the three cows was not affected. From the 83 cows that were initially enrolled, 8 were culled before the end of the study (5 of them before 90 DIM and 3 between 90 and 150 DIM), due to mastitis and/or lameness. Therefore, 236 rumenocenteses were totally performed.

Rumen fluid pH was measured on-site immediately after collection, in room conditions, using a portable pH meter (Horiba, B-213, Kyoto, Japan). The pH cut-off value to define SARA was set at 5.5 (Garrett et al., 1999).

Milk Sampling and Analyses

At the days of rumenocenteses, milk samples were collected from each individual cow at the morning milking using standard sampling protocols, following the recommendations of the International Committee for Animal Recording (ICAR 2016); the samples were maintained at 4°C during transportation to the laboratory. Milk composition (fat, protein, lactose and total solids content) was determined within 24 hours after sample collection, by infrared analysis (FTIR interferometer), using a Milkoscan FT6000 Analyzer (Foss Electric, Denmark).

Statistical Analysis

Data were analyzed using SPSS 21. Initially, the differences regarding milk yield and composition between SARA-positive and SARA-negative cows and among sampling occasions (30, 90 and 150 DIM) were assessed using a one-way ANOVA analysis. For the comparisons among sampling occasions, Bonferroni test was used as *post hoc* test. The differences regarding SARA prevalence among the three sampling occasions were assessed using the χ^2 test.

Afterwards, a series of mixed linear regression models were built to assess the effects of i) SARA and ii) rumen fluid pH on a) daily milk yield and b) milk composition (fat, protein, lactose and total solids content).

The model used to quantify these effects for the g_{th} sampling occasion, of the h_{th} cow (DMY_{gh}), is described as below (Model 1):

$$Y_{gh} = \mu + SARA_{gh} + P_h + G_h + \beta_1 \cdot L + \beta_2 \cdot S + \gamma_h + \delta_h + e_{gh} \text{ (Model 1)}$$

Where:

Y_{gh}= Milk yield, milk composition, μ= intercept, $SARA_{gh}$ = fixed effect of SARA status (2 levels, 0 = no SARA, 1 = SARA), P_h = fixed effect of parity number (2 levels, 1st and ≥ 2nd parity), G_h = fixed effect of days in milk (3 levels; 30, 90 and 150 DIM), β_1= fixed effect of the regression coefficient of the milk lactose or protein or fat content (L) (for the models estimating the effect of SARA on milk yield, milk fat and milk protein content, respectively); for the estimation of the effect of SARA on milk lactose content the specific fixed effect (β_1) was excluded from the model, β_2= fixed effect of the regression coefficient of BCS (S) γ_h= repeated variation of the h_{th} cow, δ_i = random variation of the h_{th} cow and e_{gh} = residual error.

The fixed effect of SARA status was replaced by the fixed effect of the regression coefficient of the rumen fluid pH in all of the aforementioned models, which otherwise were built using the same explanatory variables and setting up, in order to calculate the effects of rumen fluid pH on i) DMY and ii) milk composition.

Among first order autoregressive (ARH1), compound symmetry (CS) and unstructure (UN), the covariance structure with the lowest Akaike's information criteria (AIC) was included, in case a significant improvement of the model was observed ($P<0.05$). The assumptions of homoscedasticity, normal distribution and linearity for the models were checked by visually assessing the plots of standardized residuals against standardized predicted values and histograms, as well as the probability-probability and quantile-quantile plots of standardized residuals.

RESULTS

One hundred and thirty-one SARA cases were recorded throughout the study; 85 in primiparous and 46 in multiparous cows. Prevalence of SARA was 48.2%, 53.8% and 65.3% at 30, 90 and 150 DIM, respectively; the difference in SARA prevalence between 30 and 150 DIM was significant ($P<0.05$). Sixteen cows were SARA-positive (13 primiparous and 3 multiparous) and 13 were SARA-negative (3 primiparous and 10 multiparous) in all 3 sampling occasions. Statistics of all measured parameters (ruminal pH, BCS, DMY and milk composition) for SARA-positive and SARA-negative cows, in each sampling occasion, and partial comparisons between and among them are summarized in Tables 2 and 3.

Rumen fluid pH was significantly lower (mean difference of 0.65) for SARA-positive compared to SARA-negative cows (P<0.001) in all three samplings; interestingly, there were no significant differences either among SARA-positive or among SARA-negative ones at the different sampling days. There was no effect of either SARA status or sampling day on BCS (Table 2).

Mean DMY as well as mean fat, protein, lactose and total solids content were 26.17 L, 3.57%, 2.96%, 4.73% and 11.61%, respectively. Figure 1 presents the box-plots of the rumen pH, milk composition and BCS during the 3 sampling occasions.

Milk yield was not affected by SARA status (Table 3). Milk fat content was significantly lower (P<0.05) in SARA-positive cows at DIM 30; a tendency for lower fat content for SARA-positive cows was again evident at DIM 150 (3.43 vs. 3.92%, P=0.053). Protein content was significantly higher (P<0.05) at DIM 150 for both SARA-positive and SARA-negative cows compared to DIM 30 and DIM 90. Fat to protein ratio was significantly lower for SARA-positive cows (P<0.05) at DIM 30 and 150. Lactose content of SARA-negative cows was higher (P<0.05) at DIM 30 than at DIM 150.

The use of linear regression models also showed that SARA did not affect DMY, milk protein and total solids concentrations. On the contrary, SARA significantly reduced milk fat content by 0.22% (P<0.05) and fat to protein ratio by 0.08 (P<0.05) (Table 4).

When rumen fluid pH was used into the models as a continuous variable (Table 4), no significant effects on DMY, milk protein, and milk lactose content were observed. A one-unit increase of ruminal pH was associated with 0.28% increase of milk fat content (P<0.05), 0.09 increase of fat to protein ratio (P<0.05) and 0.44% increase of milk total solids content (P<0.05).

Table 2. Descriptive statistics of ruminal pH and Body Condition Score (BCS) by days-in-milk (DIM) in SARA-positive and SARA-negative cows.

		Ruminal pH	BCS
Sampling		Mean (±SD)	Mean (±SD)
30 DIM	Cows with SARA n=40	5.26±0.16[a]	2.79±0.31
	Cows without SARA n=43	5.87±0.33[b]	2.83±0.38
	Total cows n=83	5.58±0.40	2.81±0.35
90 DIM	Cows with SARA n=42	5.19±0.19[a]	2.72±0.25
	Cows without SARA n=36	5.86±0.35[b]	2.82±0.36
	Total cows n=78	5.50±0.43	2.77±0.31
150 DIM	Cows with SARA n=49	5.20±0.18[a]	2.82±0.33
	Cows without SARA n=26	5.80±0.32[b]	2.74±0.36
	Total cows n=75	5.41±0.37	2.79±0.34

[a - b] For each sampling occasion separately, means within a row with different superscripts differ (P < 0.05)

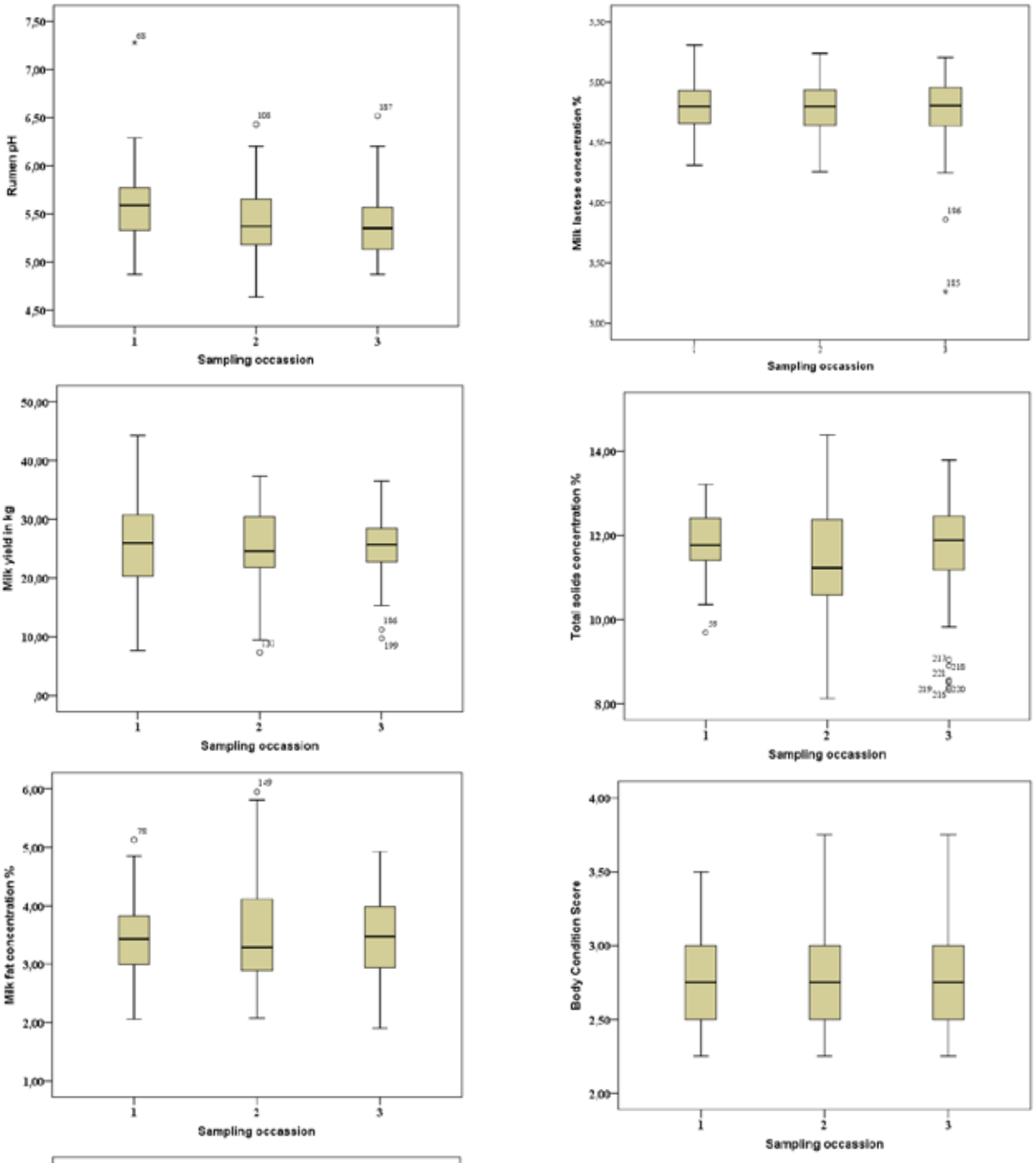

Figure 1. Box-plots of rumen fluid's pH, milk yield, milk composition and body condition score during the first, second and third sampling occasions (30, 90 and 150 days-in-milk, respectively).

Table 3. Descriptive statistics of daily milk yield (DMY) and milk composition by days-in-milk (DIM) in SARA-positive and SARA-negative cows.

Sampling		DMY (kg)	Fat content %	Protein content %	Fat:Protein ratio	Lactose content %	Total solids content %
		Mean (±SD)	Mean (±SD)	Mean (±SD)	Mean (±SD)	Mean (±SD)	Mean (±SD)
30 DIM	Cows with SARA n=40	26.60±9.58	3.30±0.66^{a}	2.90±0.27^{x}	1.15±0.26^{a}	4.79±0.22	11.67±0.68
	Cows without SARA n=43	27.13±6.77	3.65±0.65^{b}	2.86±0.22^{t}	1.28±0.22^{b}	4.73±0.20^{t}	11.91±0.79
	Total cows n=83	26.87±8.22	3.49±0.67	2.88±0.25	1.22±0.25	4.76±0.21	11.80±0.74
90 DIM	Cows with SARA n=42	25.50±7.18	3.44±1.52	2.89±0.21^{x}	1.20±0.57	4.75±0.0.26	11.21±1.87
	Cows without SARA n=36	25.68±6.13	3.81±0.84	2.94±0.24^{t}	1.30±0.28	4.71±0.27^{t}	11.59±11.31
	Total cows n=78	25.58±6.67	3.61±1.26	2.91±0.23	1.25±0.46	4.73±0.26	11.38±1.64
150 DIM	Cows with SARA n=49	25.95±6.34	3.43±0.86	3.11±0.25^{y}	1.10±0.27^{a}	4.76±0.27	11.55±1.28
	Cows without SARA n=26	26.23±4.87	3.92±1.32	3.07±0.32^{r}	1.27±0.32^{b}	4.61±0.44^{r}	11.80±1.27
	Total cows n=75	26.05±5.83	3.60±1.06	3.09±0.28	1.16±0.30	4.71±0.35	11.64±1.27

$^{a-b}$ Means referring to cows with SARA and cows without SARA, for each sampling occasion separately, with different superscripts differ ($P < 0.05$)
$^{x-y}$ Means referring to cows with SARA during the three sampling occasions with different superscripts differ ($P < 0.05$)
$^{t-r}$ Means referring to cows without SARA during the three sampling occasions with different superscripts differ ($P < 0.05$)

Table 4. Significant effects of subacute ruminal acidosis (SARA) and rumen fluid's pH on milk composition.

					95 % CI	
	Parameter	*B*	SE	P-value	Lower	Upper
SARA	Fat concentration (%)	-0.22	0.101	0.028	-0.42	-0.02
	Fat:protein ratio	-0.08	0.034	0.024	-0.14	-0.01
pH	Fat content (%)	0.28	0.124	0.025	0.03	0.53
	Fat:protein ratio	0.09	0.043	0.033	0.01	0.18
	Total solids content (%)	0.44	0.173	0.012	0.10	0.78

DISCUSSION

As asserted in the Introduction, the novelty of this study is that SARA was not experimentally induced; instead, this was conducted on a commercial dairy herd (field conditions) and each cow was sampled at three different time points at the first half of lactation.

Dairy cows are prone to SARA in early- and mid-lactation (Kleen et al, 2003); however, the majority of studies on SARA prevalence were conducted between 10 and 90 DIM. Prevalence of reported SARA cases can range significantly. Overall, among cows studied it was found to be 11% (O'Grady et al.,

2008), 13.8% (Kleen et al., 2009), 14.0% (Stefańska et al., 2016), 15.7% (Kitkas et al., 2013), 20.0% (Kleen et al., 2013), 20.1% (Oetzel et al., 1999), and 27.6% (Tajik et al., 2009). The notion it that there was a considerable variation among herds, ranging from 0.0% to 38.0% (Kleen et al., 2009). SARA-positive cows were more than 40.0% of the herd in about one third of herds examined by Garrett et al. (1997), more than 33.0% of the total in 30.0% of those examined by Morgante et al. (2007) and more than 25.0%, again, in one third of the herds examined by Kitkas et al. (2013).

The particularly high SARA prevalence recorded in the present study was rather expected, since the main selection criterion was the known history of high SARA prevalence on this specific farm (Kitkas et al., 2013). An adequate number of SARA-positive cows was necessary to detect statistically significant differences in the examined variables and this farm fitted well to the SARA-positive herd profile. Prevalence was high in early- and mid-lactation (48.2%, 53.8% and 65.3% at 30, 90 and 150 DIM, respectively). Relevant prevalence was lower in other studies, which included more farms. Garrett et al. (1997) reported SARA prevalence of 19.0% vs. 26.0%, Kleen (2004) 11.0% vs. 18.0% and Tajik et al. (2009) 29.3% vs. 26.4%, in early- and mid-lactation cows, respectively. In the above studies the differences in SARA prevalence at the various stages of lactation can be attributed to different management practices across herds in different regions and countries over time. The latter was not the case in the present study that refers to a single farm.

Prevalence of SARA at DIM 150 (mid-lactation) was significantly higher than at DIM 30 (early lactation). Generally, the over-accumulation of SCFA that causes SARA in early lactation results from their low absorption rate, associated with the short length of rumen papillae and the low number of bacteria capable of utilizing them, due to inappropriate transition management (Kleen et al., 2003), while in mid-lactation the over-accumulation of SCFA results from the high intake of low buffering capacity (low in effective fiber), high energy rations (Plaizier et al., 2009). Indeed, the inadequate transition management was the culprit for high SARA prevalence for this particular farm in early lactation (DIM 30), but for mid-lactation cows (DIM 90 and DIM 150) feed bunk management (inadequate bunk space for the 83 cows) and not the ration was the plausible cause. Moreover, continuous bouts of acidosis make it more difficult for cows to restore normal ruminal pH (Dohme et al., 2008), which might explain the increased prevalence of SARA as lactation progressed.

The above could also partly explain why 16 cows were SARA-positive throughout the study. An alternative explanation for that and for the fact that 13 cows were SARA-negative at all samplings is that some cows might be genetically susceptible while others resistant to SARA. This could be a research challenge for the future.

Regarding parity, almost twice as much cases of SARA were recorded in primiparous than in multiparous cows. This finding is in agreement with Enemark et al. (2004) and Krause and Oetzel (2005), who stated that primiparous cows are more prone to the disease. Access to feed bunk for primiparous is difficult in the presence of older cows, due to competitive interactions, resulting in the consumption of large meals in short time periods (Oetzel, 2003). This was certainly the case on this farm, where feed bunk length was inadequate.

There was no effect of SARA status or rumen fluid pH on milk yield in this study. Stone (1999) found an increase of 2.7 kg in daily milk yield when corn meal replaced high-moisture corn in a commercial herd; actually, this is the only field study with high yielding cows assessed in the literature. In other cases, where milk production was negatively affected by SARA, cows were either low producing (Bipin et al., 2016) or rumen cannulated ones under experimental settings (Enjalbert et al., 2008; Malekkhahi et al., 2016; Xu et al., 2016). However, a negative effect of SARA on milk production is not always observed (Gozho et al., 2007; Danscher et al., 2015) under the same conditions. Stage of lactation may affect the outcome of such comparisons; during early lactation, cows may compensate for a negative energy balance by mobilizing fat reserves. In this case, BCS loss is usually greater for SARA-positive cows (Kleen et al., 2003) but not always (Tajik et al., 2009); this was not observed in our study, either. Comparisons regarding yield traits should account for the effect of genotype. Higher genetic merit cows which are SARA-positive due to higher DM intake (Enemark, 2008; Kleen et al., 2013; Plaizier et al., 2009), produce less milk than their genetic potential dictates and thus, no difference is detected when they are compared with SARA-negative cows. This could explain the present results for DMY; unfortunately, breeding values for milk yield

were not available and, therefore, could not be included in the statistical model. Previous lactation records could be paired but, in our case, as SARA was a permanent problem in this herd (Kitkas et al., 2013), records were not considered representative of true genetic potential; moreover, most SARA cases were observed in primiparous cows.

While the one-way analysis of variance showed a significant negative effect of SARA on milk fat content only at DIM 30, the use of a mixed linear regression model that besides SARA or pH included parity, DIM, BCS and protein content as independent variables, clearly showed a significant effect of both on milk fat content. Stone (1999) reported a reduction of milk fat of 0.30%; the difference in favor of SARA-negative cows was similar (0.22%) in this study. Other researchers have also found a decrease in milk fat content (Enjalbert et al., 2008; Danscher et al., 2015; Bipin et al., 2016; Malekkhahi et al., 2016; Xu et al., 2016) but, again, most were experimental studies using a small number of cows. Individual test-day milk fat records of mid-lactation cows have been proposed as a herd-level screening tool (Enemark, 2008). However, milk fat depression is not a consistent finding. Keunen et al. (2002) and Gozho et al. (2007) found no effect under experimental conditions; neither did Tajik et al. (2009) who sampled a small number of cows under field conditions. To our knowledge, the present study is the only one reporting a significant negative effect of SARA and low ruminal pH on milk fat content, using linear regression models.

There was no effect of SARA status or rumen fluid pH on milk protein content in this study. This is in contrast with Stone (1999) who showed a drop of 0.10%, as well as with the results of Keunen et al. (2002) and Xu et al. (2016); on the other hand, Li et al. (2012) found an increase in milk protein content. Our result is in agreement with most other researchers (Gozho et al., 2007; Enjalbert et al., 2008; Tajik et al., 2009; Danscher et al., 2015; Malekkhahi et al., 2016), who found no statistically significant differences. Either microbial protein yield is not significantly affected or differences are impossible to be detected because the number of cows used is too small.

Both the one-way analysis of variance and the use of mixed linear models detected statistically significant differences in milk fat to protein ratio in this study. This ratio is not commonly reported in the aforementioned studies but, as in most of them milk fat content decreases in SARA-positive cows while milk protein does not, it could be assumed that this is generally the case. The milk fat to protein ratio is not considered useful in investigating SARA-induced milk fat depression cases; besides problems related to analytical procedures, reasons mentioned include different physiologic processes of milk fat and protein synthesis and lack of scientific documentation (Oetzel, 2007). However, a ratio is just a number (fraction) and whatever the reason, when the numerator decreases while the denominator does not, the ratio decreases as well. Nevertheless, there are no published peer-review data so far dealing with the effect of SARA or ruminal pH on this ratio. In light of our findings, more research is warranted on this issue, in our quest to come up with an inexpensive (monthly DHI records), non-invasive method to screen herds for SARA.

Effect of SARA status or rumen pH on milk lactose content is not reported in the literature. There was no such effect in this study. There was no effect of SARA status on milk total solids content either, but a one-unit increase of rumen fluid pH would increase total solids content by 0.44%; fat would represent about 64% of the total. This has obvious economic benefits.

CONCLUSIONS

The present study is the first that was conducted under field conditions, with repeated measurements from the same animals, and denotes a clear negative effect of SARA and low rumen fluid pH on milk fat content. Milk yield and protein content were not affected. The present study also demonstrated a reduction of the milk fat:protein ratio, a number not often mentioned in relative studies but an inexpensive measure for practitioners to suspect SARA and pursue further examination. The fact that a significant number of cows were consistently SARA-positive or SARA-negative throughout the study, under the same conditions, should be further investigated.

CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

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Research article
Ερευνητικό άρθρο

Methylsulfonylmethane alone or in combination with thiocolchicoside modulate autoimmune disease in rats with adjuvant arthritis

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ABSTRACT. Many active anti-arthritic natural substances are revealed into the past years. The aim of study was to assess the combined efficacy of methylsulfonylmethane (MSM) with thiocolchicoside (Th) (compound MTh) and MSM alone against adjuvant-induced arthritis (AA) in rats. In the first experiment 30 rats and in the second - 21 rats were randomly divided into 6 groups: I gr. - AA + MSM (77 mg/kg), II and V gr. - AA + diclofenac (DF), III and VI gr. - the control AA groups without treatment, IV gr. - MTh (38 mg/kg). DF (1 mg/kg) in both experiments was used as a reference drug. 6 rats were as the healthy control group. AA rats were treated from day 0 to 17. All preparations were suspended in 0.5 ml of 1% starch gel and injected orally 5 days a week. Body weight and joint swelling were monitored 3 times a week. Development of polyarthritis, blood indices, pro-/antioxidant activity and pro-inflammatory cytokines in blood serum, and histopathology of the liver and paw were assessed at the end of experiment. MSM significantly decreased joint swelling on days 3 and 13. MTh in twice lower dose more markedly suppressed joint swelling and also significantly reduced the changes in soft periarticular tissues, synovium and cartilage as compared to the control AA group. Both preparations alleviated infiltration with inflammatory cells and synovial proliferation, as well as protected cartilage destruction and decreased pannus formation. MSM and MTh improved the blood indices and insignificantly suppressed IL-17. They markedly decreased the level of malondialdehyde (MDA). Some anti-oxidant activity of preparations was also confirmed. No toxic effects on the liver were revealed. MSM and MTh attenuated the development of AA in rats. Combination therapy was more effective than single MSM and required the twice lower doses to receive the beneficial anti-arthritic effect. Both preparations could be the potential preventive or therapeutic candidates for the treatment of autoimmune processes in combination with other drugs.

Keywords: methylsulfonylmethane, thiocolchicoside, adjuvant arthritis, rat

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disorder characterized by severe pain, inflammation of synovial joints with neovascularization, pannus formation and destruction of cartilage. Activated rheumatoid synovial fibroblasts (RASFs) actively participate in RA synovitis (Huber et al., 2006) and aggressively proliferate to form a pannus, which produce inflammatory mediators (Han et al., 2003). Defective apoptosis of RASFs is an important mechanism contributing to synovial hyperplasia in RA (Pattacini et al., 2010; Xiao et al., 2012).

The conventional drug treatment of RA consists from analgesic, non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), corticosteroids to biologics. However, a common effect of long-term therapy is the development of resistance to treatment and an increased occurrence of adverse effects. In the past several years, there has been a vast increase in a number of complementary drugs purported to have joint-protesting and the antioxidant and free radicals scavenging properties (Kripa et al., 2011; Lopez, 2012; Ezaki et al., 2013), because many studies have validated a role of oxidative stress in RA (Filipin et al., 2008; Kundu et al., 2012; Stamp et al., 2012; Biniecka et al., 2011).

Agents that we investigated are methylsulfonylmethane (MSM) and MSM in combination with Thiocolchicoside (Th) (compound with code name MTh).

MSM is a non-toxic, natural compound widely used as a dietary supplement for its beneficial effects against various diseases, especially arthritis to reduce arthritic and rheumatic pain (Kim et al., 2006; Ameye and Chee, 2006) and (often in combination with glucosamine and chondroitin) to treat or prevent osteoarthritis (Gregory et al., 2008). Previous studies demonstrated that MSM possessed antioxidant and anti-inflammatory properties (Kim et al., 2009; Maranon et al., 2008; Nakhostin-Roohi et al., 2011; Amirshahrokhi et al., 2013) by increasing antioxidant enzyme levels and reducing malondialdehide (MDA), myeloperoxidase (MPO) and TNF-α (Amirshahrokhi et al., 2013). Its administration protects the development of type II collagen-induced arthritis in mice by modifying the immune responses (Hasegawa et al., 2004). MSM also shows anti-apoptotic effects because dysregulation of apoptosis has been linked with various complications such as autoimmune, inflammatory and other diseases (Karabay et al., 2014).

Thiocolchicoside (Th) is a semi-synthetic sulfur derivative of colchicoside, a naturally occurring glucoside derived from the plant *Gloriosa superba* (Umarkar et al., 2011). It has been used clinically as a muscle relaxant, anti-inflammatory, and analgesic drug (Umarkar et al., 2011; Soonawalla and Joshi, 2008; Kumar et al., 2014; Lahoti, 2012) for the treatment of orthopedic, traumatic and rheumatological disorders (Sandouk et al., 1994). Th significantly suppressed enhanced osteoclastogenesis (Reuter et al., 2012), which is an important pathological feature in many age-associated bone diseases including RA and osteoarthritis (Phan et al., 2004; Reuter et al., 2012). The phytoconstituents that present in the *Gloriosa superba* show analgesic, anti-inflammatory and wound healing action in rats (John et al., 2010; Joshi et al., 2010). Colchicine, an analog of colchicoside, is also a commonly used drug, especially for rheumatic diseases (Efe et al., 2011).

We investigated the effects of MSM and MTh, because to our knowledge, there is no evidence to confirm this issue in detail by using the combination of MSM and Th (compound MTh) by using adjuvant-induced arthritis (AA) in rats which is an experimental model of RA and widely used for testing and developing anti-arthritic and anti-inflammatory agents. Treatment with diclofenac (DF), a standard prescription drug used to treat RA, was used as reference for purposes of comparison.

MATERIALS AND METHODS

Reagents

Complete Freund's adjuvant (CFA), 10% formalin, spirit-formol, hematoxylin, eosin, picrofuxin, toluidine blue, methyl-green-pyronin-y, acetic acid, trichloracetic acid, orthophosphoric acid, thiobarbituric acid, nitric acid, ferrous sulfate, ascorbic acid, ammonium molybdate, hydrogen peroxide were purchased from Sigma-Aldrich Chemie and Fluca Chemie GmbH (Germany), ketamidor from Richer Pharma AG (Wels, Austria), sedaxylan from Eurovet Animal Health B.V (Holland), and diclofenac (DF) - from Glaxo Wellcome. The enzyme-linked immunosorbent assay (ELISA) kits for determination of IL-6 and IL-17 were produced by Abcam®.

Animals

57 adult male albino rats of 9-10 weeks old, weighing between 180 and 200 g were obtained from the breeding unit of the Department of Biomodels

of the State Research Institute Centre for Innovative Medicine, and used in two experiments of the present study. The animals were divided into 6 experimental groups (n = 7-10 per group) and one healthy animal group (n = 6), housed in plastic cages and maintained in a temperature-controlled (22 ± 2°C) environment with a relative humidity of 60% and a 12-h light/dark cycle. The rats were allowed one week to adapt to their environment before initiation of the experiments. They were given standard commercial rodent pellets and water was freely available for the duration of the study. Animal care and handling throughout the experimental procedures in this study was in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. All experimental protocols in this study were approved by the Lithuanian Laboratory Animal Use Ethical Committee for Animal Experiments under the State Food and Veterinary Service.

Induction, treatment and assessment of adjuvant arthritis

Adjuvant arthritis (AA) was induced in 51 rats by a single intra-dermal injection of complete Freund's adjuvant (CFA 0.1 ml) into the foot pad of left hind paw (Murunikara et al., 2014).

Concentrations of compounds and DF sufficient to provide anti-inflammatory effects were selected based upon values reported in the literature and our preliminary studies. In the first experiment 30 rats with AA were divided into 3 groups (10 animals per group), as follows: Group I - Rats received MSM (77 mg/kg), Group II - Rats received DF (1 mg/kg), Group III - Rats without treatment served as the control group. In the second experiment 21 animal was divided into 3 groups (7 rats per group): Group IV - Rats received MTh (38 mg/kg; Th dose in this preparation - 0.25 mg/kg), Group V - DF (1 mg/kg), Group VI - Rats without treatment served as the control group. AA rats in both experiments were medicated from day 0 to 17. All preparations were suspended in 0.5 ml of 1% starch gel and injected orally 5 days a week. Rats of the control groups received the same volume of the starch gel. Six healthy animals (Group VII) served as healthy control. Body weight and joint swelling were monitored three times a week. The anti-arthritic effect of MSM, MTh or DF was evaluated by measuring the paw volume plethysmometrically by using plethysmometer (PVP 1001; Kent Scientific Corporation). The inhibition percentage of investigated indices was calculated for each animal group in comparison with the control AA group. Duration of experiment was 17 days.

At the end of experimental period all animals were killed by decapitation and then blood was collected for the examination of ESR and leukocytes by using blood analyzer Picoscale (Hungary). Blood serum was collected from each animal to isolate serum. The blood was centrifuged at 3000*g* for 10 min. and serum drawn and stored at -20°C. The internal organs were removed and weighted. The liver and the injected paws were used for the histopathological examination.

Histopathological examination

The liver and injected paws from AA and healthy rats were excised followed by routine fixation, decalcification, and paraffin embedding. Paraffin bees wax tissue blocks were prepared for sectioning at 5 micron thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained with hematoxylin-eosin, picrofuxin, toluidine blue, methyl-green-pyronin-y and safranin O for histopathological examination through the light microscope. Histological assessment of changes in the liver, soft periarticular tissues, synovium and cartilage was performed. During the processing and analysis of the liver and joints, the pathologist was "blinded" to the animal groups and the drug treatments. Each parameter was scored on a 0 to 3 point scale, where 0 means the absence of changes, 0.5 - traces of changes, 1 - minimal changes, 2 - moderate changes, 3 - heavy changes.

Determination of lipid peroxide level and catalase (CAT) activity in blood serum

Lipid peroxidation assessed as malondialdehide (MDA) levels in the blood serum, expressed as nmol/ml, were determined by the thiobarbituric acid reaction at 532 nm spectrophotometrically by the method of Gavrilov and co-workers (1987). Catalase (CAT) activity, expressed in nmol/L/min, was measured at 410 nm as described by Koroliuk and co-authors (1988).

Measurement of cytokines levels

Cytokines like IL-6 and IL-17 were measured in the serum of the control and experimental animals by using respective enzyme-linked immunosorbent assay (ELISA) kits specific for rats (ab119536-IL-17 Rat ELISA Kit and ab119548-IL-6 Rat ELISA Kit) according to procedure recommended by manufacturer's instructions (abcam®; UK). Each sample was assayed in duplicate. The concentrations of cytokines were determined with the help of a standard curve.

Statistical analysis

Statistical analysis was done by one-way analysis of variance ANOVA using PRISM Software (GraphPad Software, San Diego, CA, USA) and Student's t-test. The nonparametric Mann-Whitney U test was used to evaluate the histological changes. All data were expressed as the mean ± SEM and considered to be statistically significant at P values smaller than or equal to 0.05.

RESULTS

Effect of MSM and MTh on the body and organ's weight

The initial body weight of rats did not differ among the groups and slightly increased during the experiment in all investigated groups although no significant differences between the groups were observed (data not shown).

Significant increase of the liver, kidney and spleen weight and decrease of the thymus weight were observed in rats with AA compared with the healthy animals (Table 1).

In the first experiment rats treated with MSM (group I) or DF (group II) showed only a significant decrease of the relative kidney weight (MSM - $P < 0.05$; DF - $P < 0.001$) in comparison with the animals of the control group III. The relative weights of the liver were the same as in the healthy rats, but the spleen weight was significantly higher and the thymus weight was lower in both treated groups than those in the group of healthy animals.

In the second experiment rats treated with MTh (group IV) showed a significantly lower the relative weight of the liver ($P < 0.02$) and markedly higher weight of the thymus ($P < 0.01$) than those in the control VI group. The same effect was found after the treatment of AA with DF (liver - $P < 0.01$; thymus - $P < 0.001$).

Histopathological changes in the liver

There was no histopathological alteration and the normal histological structure of hepatic parenchyma was recorded in the liver sections of healthy animals (data not shown).

The effect of MSM on the liver of rats with AA was similar to the effect of diclofenac (Table 2). Both preparations MSM and DF significantly decreased inflammatory infiltration of hepatic stroma with lymphocytes by 50.6% ($P < 0.01$) and 56.8% ($P < 0.01$) and the general inflammatory reaction by 38.7% ($P < 0.05$) and 48.1% ($P < 0.05$) respectively in comparison with the control AA group. Preparation MTh, diminished alteration of parenchyma by 28.8% ($P < 0.02$), infiltration of hepatic stroma with lymphocytes by 80.9% ($P < 0.02$), macrophages by 79% ($P < 0.05$) and general inflammatory reaction by 64.2% ($P < 0.05$). The penetration of inflammatory cells into the lobule decreased by 88.3% compared to the control AA group ($P < 0.05$). The effect of MTh on the liver was similar to the effect of DF except infiltration with macrophages, where this preparation didn't show the significant decrease. So, after the treatment with MSM and MTh no toxic effects on the liver were observed, and they improved histopathological changes in this organ caused by AA. It should be noted that the effect of MTh was more significant than that of MSM although it was used in twice lower dose.

Table 1. Effect of MSM and MTh on relative organ's weight

Experiment	Groups	Liver (g/kg^{-1})	Kidney (g/kg^{-1})	Spleen (g/kg^{-1})	Thymus (g/kg^{-1})
First	IAA + MSM	3.24±0.11	0.88±0.03 *	$^{+}$0.45±0.03	$^{+}$0.124±0.010
	II AA + DF	2.95±0.07 *	0.86±0.02 *	$^{+}$0.40±0.01 *	0.166±0.016 *
	IIIAA control	$^{+}$3.46±0.13	$^{+}$0.96±0.02	$^{+}$0.51±0.03	$^{+}$0.110±0.010
Second	IVAA+ MTh	3.45±0.22 *	$^{+}$0.91±0.02	$^{+}$0.64±0.07	$^{+}$0.120±0.009 *
	V AA + DF	$^{+}$3.50±0.13 *	0.88±0.02	$^{+}$0.58±0.05	$^{+}$0.130±0.009 *
	VIAA control	$^{+}$4.39±0.24	$^{+}$1.00±0.05	$^{+}$0.88±0.10	$^{+}$0.080±0.008
Healthy rats		3.05±0.10	0.84±0.02	0.25±0.01	0.170±0.016

Note: Adjuvant arthritis (AA) was induced by a single injection of 0.1 ml of complete Freund's adjuvant (CFA) into the left hind paw. I group was treated prophylactically (since AA inducing day) with 77 mg/kg of methylsulfonylmethane (MSM) and IV group with 38 mg/kg of MTh (MSM and thiocolchicoside (Th) complex). II and V groups in both experiments were treated with 1 mg/kg of diclofenac (DF). Preparations were suspended in the 1% starch gel and injected orally 5 times a week in a volume of 0.5 ml. Controls AA groups (III and VI gr.) received the same volume of the starch gel. * The differences are significant in comparison with the control group. + The differences are significant in comparison with the healthy animal's group.

Table 2. Effects of MSM and MTh on the liver in AA rats

Index		Groups					
		First experiment			Second experiment		
		I AA+MSM	II AA+DF	III AA control	IV AA+MTh	V AA+DF	VI AA control
Alteration of parenchyma		0.75±0.08	0.65±0.11	0.88±0.15	1.21±0.10 *	0.71±0.10 *	1.70±0.12
Hypervolemia of *V. centralis*		0.55±0.09	0.60±0.10	0.63±0.16	1.36±0.21	0.79±0.15	0.80±0.30
Inflammatory infiltration of hepatic stroma	Lymphocytes	0.40±0.10 *	0.35±0.11 *	0.81±0.09	0.21±0.10 *	0.14±0.09 *	1.10±0.29
	Granulocytes	0.10±0.06	0	0.13±0.08	0.21±0.15	0	0.30±0.20
	Macrophages	0.90±0.12	0.70±0.18	0.88±0.16	0.21±0.15 *	0.29±0.15	1.00±0.31
	General	0.65±0.13 *	0.55±0.17 *	1.06±0.11	0.43±0.17 *	0.29±0.15 *	1.20±0.30
	Penetration into the lobule	0.75±0.13	0.45±0.20	0.56±0.17	0.14±0.14 *	0.14±0.14 *	1.20±0.37

Note: Adjuvant arthritis (AA) was induced by a single injection of 0.1 ml complete Freund's adjuvant (CFA) into the left hind paw. I group (n = 10) was treated prophylactically (since AA inducing day) with 77 mg/kg of methylsulfonylmethane (MSM) and IV group (n =7) with 38 mg/kg of MTh ((MSM and thiocolchicoside (Th) complex). II (n = 10) and V (n = 7) groups in both experiments were treated with 1 mg/kg of diclofenac (DF). Preparations were suspended into the starch gel and injected orally 5 times a week in a volume of 0.5 ml. Controls AA groups (III and VI gr.) received the same volume of the starch gel. Duration of experiment is 17 days. Hepatic tissue was fixed in spirit-formol (1:9), embedded in paraffin, and 5 μm-thick histological sections of the tissue were stained with haematoxylin-eosin (for visualization of inflammation and inflammatory cell infiltration and necrosis of hepatocytes). The histological assessment of changes in the liver was performed in a blinded manner by pathologist. Each parameter was scored on a 0 to 3 point scale, where 0 means the absence of changes, 0.5 - traces of changes, 1 - minimal changes, 2 - moderate changes, 3 - heavy changes. * The differences are significant in comparison with the control group.

Hematological parameters

There was a significant increase in ESR and leukocyte's count of arthritic rats in both experiments when compared with the healthy rats (Fig. 1A). MSM and MTh significantly reduced ESR by 48.7% ($P < 0.01$) and 41.2% ($P < 0.001$), and leukocyte's count by 41.2% ($P < 0.001$) and 24.6% ($P < 0.02$) respectively compared with due control groups.

Effect of MSM and MTh on AA induced alterations in lipid peroxidation and antioxidant enzyme CAT activity

AA (Groups III and VI) induced serum lipid peroxidation reflected by a significant increase in MDA level compared with the healthy control (Fig. 1B). This was also accompanied by a significant 39.9% decrease in CAT activity in the first experiment and by 44.7% in the second experiment. Treatment with MSM (Group II) caused a significant 44.8% ($P < 0.0001$) reduction in MDA level as a 20.3% elevation in CAT activity compared to the control AA group III. It should be noted that the effect of MSM and DF on MDA and CAT activity was similar.

Although the administration of MTh for the treatment of AA (Group IV) didn't induce a significant change in CAT activity (it insignificantly increased by 27.2% and the differences between the treated and the control group was near to significant - $t = 1.86$), it markedly ($P < 0.001$) decreased MDA level in the blood serum by 58% in comparison with the control AA group VI.

Effect of MSM and MTh on pro-inflammatory cytokines in blood serum of rats with AA

The cytokines in the serum were detected on day 17 after immunization with CFA. We couldn't to detect IL-6 in the blood serum of healthy, tested and the control animals. MSM and MTh suppressed IL-17 level by 22.58% and 27.9% respectively but they didn't induce the significant changes compared to the control AA groups (Fig. 1C).

Effect of AA treatment with MSM and MTh on the joint swelling

Effect of both preparations on joint swelling was not more effective than DF (Fig. 2). MSM significantly decreased joint swelling only on days 3 ($P < 0.001$) and 13 ($P < 0.05$), and at the end of experiment this suppression reached 19.6% in comparison with the control group III (Fig. 2A). MTh suppressed joint swelling till day 10 ($P < 0.05$-0.002) and on day 15 ($P < 0.02$) (Fig. 2B). So, preparation MTh was significantly active on all the days in the acute phase of AA.

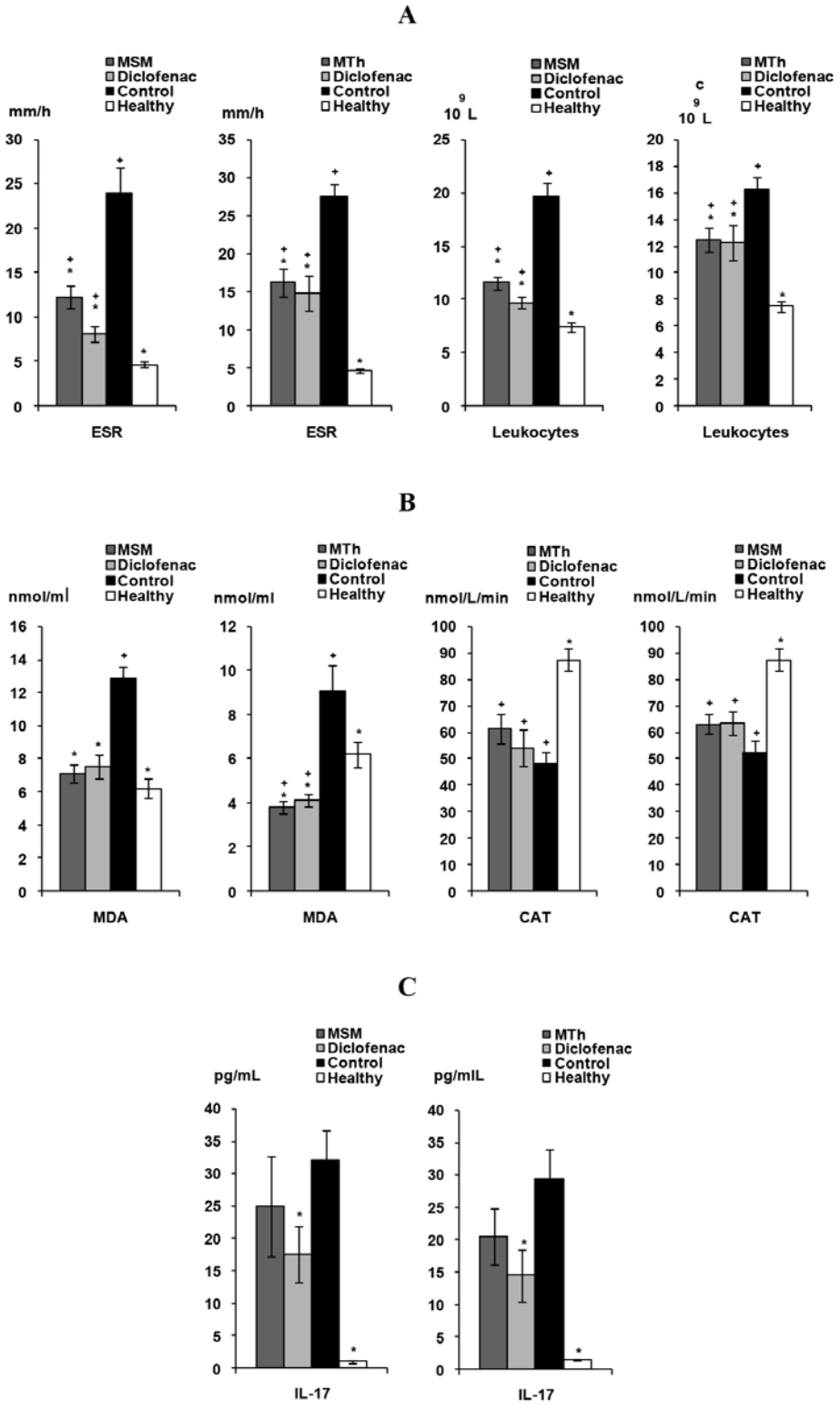

Figure 1. Blood indices (A), pro-/antioxidant activities (B) and level of interleukin-17 (C) in rats with adjuvant arthritis treated with preparations MSM and MTh.
Adjuvant arthritis (AA) was induced by a single injection of 0.1 ml complete Freund's adjuvant (CFA) into the left hind paw. I group (n = 10) was treated prophylactically (since AA inducing day) with 77 mg/kg of methylsulfonylmethane (MSM) and IV group (n =7) with 38 mg/kg of MTh ((MSM and thiocolchicoside (Th) complex). II (n = 10) and V (n = 7) groups in both experiments were treated with 1 mg/kg of diclofenac (DF). Preparations were suspended into the starch gel and injected orally 5 times a week in a volume of 0.5 ml. Controls AA groups (III and VI gr.) received the same volume of the starch gel. * Differences are statistically significant in comparison with the control group. + Differences are statistically significant in comparison with healthy rats.

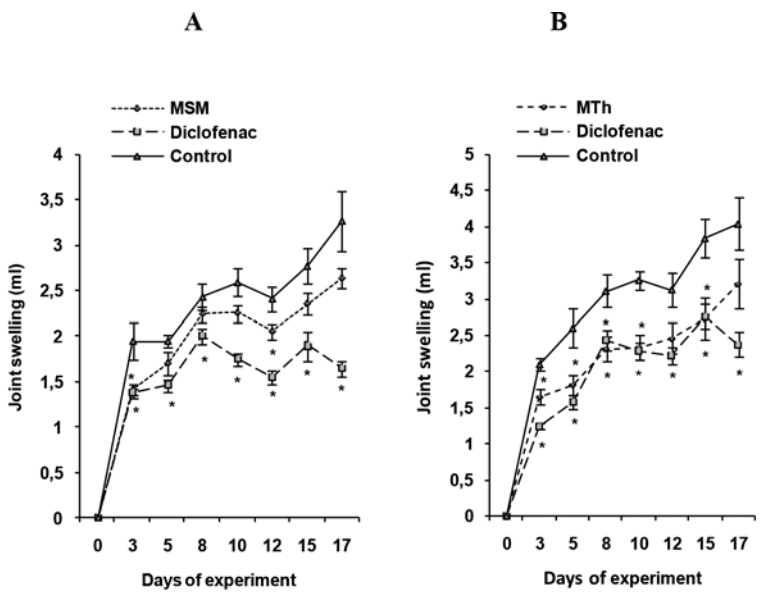

Figure 2. Joint swelling of rats with adjuvant arthritis treated with preparations MSM (A) and MTh (B).
Adjuvant arthritis (AA) was induced by a single injection of 0.1 ml complete Freund's adjuvant (CFA) into the left hind paw. I group (n = 10) was treated prophylactically (since AA inducing day) with 77 mg/kg of methylsulfonylmethane (MSM) and IV group (n =7) with 38 mg/kg of MTh ((MSM and thiocolchicoside (Th) complex). II (n = 10) and V (n = 7) groups in both experiments were treated with 1 mg/kg of diclofenac (DF). Preparations were suspended into the starch gel and injected orally 5 times a week in a volume of 0.5 ml. Controls AA groups (III and VI gr.) received the same volume of the starch gel. * Differences are statistically significant in comparison with the control group.

Effect of MSM and MTh treatment on histological findings in the joints

There were no abnormalities in the joints of healthy rats (data not shown). CFA administered control groups showed thinning of cartilage, erosions, pannus formation, extensive infiltration of inflammatory cells in the soft periarticular tissues and synovium.

MSM and MTh decreased inflammatory infiltration in the soft periarticular tissues (Table 3). Significantly lower infiltration with macrophages ($P < 0.02$) was observed after the treatment with MSM and leukocytes ($P < 0.0001$) - with MTh. Both preparations decreased general inflammatory reaction: MSM by 36.6% ($P < 0.001$) and MTh by 21.3% ($P < 0.0001$) compared with the control arthritic groups. Suppression of edema and angiomatosis was also observed, although this supression was some lower than by using diclofenac. MSM markedly suppressed edema and angiomatosis by 49% ($P < 0.001$) and 46.6% ($P < 0.002$) and MTh by 33% ($P < 0.001$) and 31% ($P < 0.01$) respectively in comparison with the control AA groups.

The highest pathological changes in the synovium were found in the control AA groups. Both investigated preparations MSM and MTh significantly suppressed synovium villy proliferation. Proliferation decreased by 38.6% in MSM group ($P < 0.001$) and by 29.6% in MTh group ($P < 0.02$). Significant differences among the treated and the control AA groups were observed after evaluation of synovium edema, where marked decrease of it by 42.7% *vs* control was found in the group that received MSM ($P < 0.05$) and by 46.5% ($P < 0.001$) in the group treated with MTh. Preparation MSM significantly decreased synovium inflammatory infiltration with macrophages by 73.3% and it was the same as the action of DF ($P < 0.001$), and preparation MTh - infiltration with leukocytes by 51.9% ($P < 0.05$). General inflammatory reaction in synovium was lower by 37.5% ($P < 0.02$) than in the control AA group after the treatment with MSM and by 35.6% ($P < 0.01$) in animals treated with MTh. Differently than MSM, preparation MTh significantly diminished angiomatosis in synovium tissue by 48.5% ($P < 0.01$).

Table 3. Histopathological changes in the joints after the treatment of AA with MSM and MTh

Tissue	Index		First experiment			Second experiment		
			Groups			Groups		
			I AA+MSM	II AA+DF	III AA control	IV AA+MTh	V AA+DF	VI AA control
Soft periarticular tissues	Inflammatory infiltration	Lymphocytes	1.05±0.09	0.90±0.19	1.19±0.26	1.14±0.14	0.71±0.10 *	1.50±0.18
		Leukocytes	1.10±0.23	0.60±0.18 *	1.63±0.29	2.36±0.09 *	1.21±0.21 *	3.00±0.00
		Macrophages	0.60±0.10 *	0.90±0.12	1.00±0.09	1.14±0.18	0.86±0.09 *	1.67±0.17
		General	1.35±0.11 *	1.35±0.08 *	2.13±0.08	2.36±0.09 *	1.36±0.09 *	3.00±0.00
	Edema		1.05±0.09 *	0.65±0.11 *	2.06±0.11	1.79±0.10 *	0.71±0.15 *	2.67±0.17
	Angiomatosis		0.70±0.08 *	0.65±0.08 *	1.31±0.13	1.78±0.15 *	0.57±0.07 *	2.58±0.20
Synovium	Proliferation		1.00±0.10 *	0.80±0.08 *	1.63±0.21	1.64±0.18 *	0.86±0.14 *	2.33±0.17
	Edema		0.75±0.13 *	0.25±0.11 *	1.31±0.09	1.07±0.20	0.36±0.14 *	2.00±0.00
	Inflammatory infiltration	Lymphocytes	0.55±0.12	0.35±0.13 *	0.94±0.20	1.14±0.09	0.36±0.09	0.83±0.21
		Leukocytes	0.75±0.13	0.25±0.11 *	1.13±0.23	1.00±0.27 *	0.36±0.21 *	2.08±0.35
		Macrophages	0.20±0.08 *	0.20±0.08 *	0.75±0.09	1.07±0.13	0.50±0.11 *	1.17±0.21
		General	0.90±0.12 *	0.55±0.12 *	1.44±0.15	1.50±0.15 *	0.71±0.15 *	2.33±0.17
	Angiomatosis		0.75±0.08	0.40±0.10 *	1.25±0.23	1.07±0.20 *	0.57±0.17 *	2.08±0.20
Cartilage		Erosium	0.80±0.20 *	0.65±0.15 *	2.00±0.00	2.00±0.00	2.00±0.00	2.00±0.00
		Usura	0.30±0.13 *	0.20±0.11 *	1.06±0.27	1.28±0.18	0.21±0.10 *	1.50±0.22
		Fissura	0	0	0	0.64±0.18	0	0.50±0.34
	Pannus		0.35±0.11 *	0.05±0.05 *	1.00±0.27	1.57±0.20 *	0.43±0.23 *	2.33±0.21
	Thinning of cartilage		0.10±0.06	0.05±0.05	0.38±0.16	0.43±0.17 *	0.07±0.07 *	1.33±0.36

Note: Adjuvant arthritis (AA) was induced by a single injection of 0.1 ml complete Freund's adjuvant (CFA) into the left hind paw. I group (n = 10) was treated prophylactically (since AA inducing day) with 77 mg/kg of methylsulfonylmethane (MSM) and IV group (n =7) with 38 mg/kg of MTh (MSM and thiocolchicoside (Th) complex). II (n = 10) and V (n = 7) groups in both experiments were treated with 1 mg/kg of diclofenac (DF). Preparations were suspended into the starch gel and injected orally 5 times a week in a volume of 0.5 ml. Controls AA groups (III and VI gr.) received the same volume of the starch gel. * The differences are significant in comparison with the control group.

Alone MSM in dose of 77 mg/kg showed more pronounced effect on cartilage than combined preparation MTh in twice lower dose. There were no fissures in all the tested groups of animals in the first experiment. Erosium ($P < 0.001$) and usures ($P < 0.05$) significantly decreased in MSM group. Both preparations MSM and MTh significantly suppressed pannus formation compared with the control groups: MSM induced 65% suppression ($P < 0.05$) and MTh - 32.6% suppression ($P < 0.05$). Traces of cartilage thinning was observed only in two animals treated with MSM, but no significant changes were observed in comparison with the control AA group. MTh significantly decreased thinning of cartilage by 67.7% ($P < 0.05$), although this effect was lesser than effect of DF.

DISCUSSION

RA is presently one of the most predominant diseases worldwide. However, unfortunately, there has been no specific cure for RA. Because current treatments for arthritis result in unwanted side effects and tend to be expensive, natural products devoid of such disadvantages offer a novel opportunity. Through this study, we have demonstrated that the supplementation of compounds such as MSM and MTh, could play a role in protecting against biochemical, immunological and histological alterations in AA rats. The results of our study suggest that the prophylactic use of MSM and its combination with Th (compound MTh) in twice lower dose may have a significant effect on slowing disease progression although this therapeutic effect was not stronger than the effect of DF. MTh and

to a lesser extent MSM, were found to inhibit joint swelling and the treatment with both preparations was also associated with decreased histological changes in joints. The main pathological changes of RA are that leukocytes infiltrate into articular cavity, cause recurrent synovitis (Cooles and Isaacs, 2011) and that invasive pannus forms to damage cartilage, bone and surrounding tissue. In our study, compound MTh significantly decreased infiltration with leukocytes in the soft periarticular tissues and synovium.

It is known that MSM is one of the most popular dietary supplements, which is expected to relieve inflammation in arthritis. Recently, it has been shown that orally pretreatment of rats with MSM (400 mg/kg) exhibits a hepato-protective effect against carbon tetrachloride induced acute liver injury by suppressing pro-inflammatory cytokine release and apoptotic Bax/Bcl-2 ratio and increasing antioxidant enzyme levels (Kamel and Morsy, 2013). MSM is used as singly or in combination with other nutrients in dietary supplements for OA because sulfur is needed for formation of connective tissue.

In our study we combined MSM with Th (complex MTh). The latter has been used clinically as a muscle relaxant, anti-inflammatory, and analgesic drug (Umarkar et al., 2011; Soonawalla and Joshi, 2008; Kumar et al., 2014; Lahoti, 2012) for the treatment of orthopedic, traumatic and rheumatologic disorders (Sandouk et al., 1994). We indicated a successful anti-arthritic activity of this combined preparation. It is interesting that MTh in twice lower dose than MSM alone exerted inhibitory effect on the development of AA.

No particular behavior, clinical or physiological signs were observed in animals treated with both compounds, suggesting that used preparation's doses are probably not toxic *in vivo*. It was confirmed also by histological examination of the liver, where significantly lower alterations of hepatic parenchyma and stroma were found in the treated groups.

Increase in leukocyte count has been suggested to be one of the characteristic diagnoses of arthritis. In our present study, arthritic animals showed elevated leukocyte level. MSM and MTh significantly decreased leukocytes, revealing their beneficial role against arthritis. ESR which significantly increased in arthritic control groups has been remarkably decreased after the treatment with both preparations and standard drug DF, thus justifying significant role of preparations in arthritic conditions.

ROS in arthritis is not surprising since oxidative stress or ROS serve as mediators of tissue damage (Jayadevi et al., 2013). Antioxidant enzymes are present in biological systems to protect the tissue from oxidative injury (Sabina and Rasool, 2008). Our results demonstrated that the activity of CAT was decreased in AA animals, which may have been due to increased production of free radicals. The decrease in anti-oxidant enzyme activity correlated with increased lipid peroxidation quantified by measurement of the MDA. It is well known that MDA is a terminal product of lipid peroxidation and so the content of MDA can be used to estimate the extent of lipid peroxidation (Huang et al., 2012; Messarah et al., 2010). Elevated levels of MDA have been reported in the serum, liver and synovial fluid in patients with RA (Sarban et al., 2005). The increased levels of MDA may be due to its release from neutrophils and monocytes during inflammation. It appeared that the antioxidant defense was compromised in the AA and other kinds of arthritis as evidenced by increased MDA concentrations and decreased antioxidant enzyme CAT activity (Huang et al., 2012). In our study biochemical assessments showed attenuated lipid peroxidation and increased CAT activity after the treatment with preparations MSM and MTh as compared to the control AA groups. Taken together, our results prove some antioxidant action of these compounds. May be higher doses of MSM and MTh in our experiments could be used to achieve more pronounced antioxidant activity.

Data on therapeutic effects of MSM and MTh led us to assay the ability of these preparations to prevent AA development in rats. These findings are consistent with those reported by other authors (Huang et al., 2012; Kamel and El Morsy, 2013), who also showed an ability of MSM or Th to reduce inflammation and to enhance antioxidant enzyme activity and induction of direct scavenging of free radicals.

It is known that pro-inflammatory cytokines such as TNF-α, IL-6, IL-17 play an important role in RA. Cytokines observed to be induced in the early phases following exposure to CFA are TNF-α, IL-12, IL-6, IFN-γ and several chemokine's (Billiau and Matthys, 2001). IL-6 is cytokine likely to be induced by CFA that may be relevant to induction of autoimmune diseases. But as state Billiau and Matthys (2001), direct *in vivo* demonstration of IL-6 production following injection of CFA is not available, that also showed our investigations.

Another cytokine IL-17, a major product of T cells, plays the important role as an upstream mediator of RA pathogenesis. It promotes inflammation via enhancing the production of IL-1β, TNF-α and IL-6 (Zhang et al., 2013; Shi et al., 2015). In our study, concentration of IL-17 significantly increased in the blood serum of rats with AA compared to the healthy control, and its reduction was demonstrated in response to MSM and MTh. Although no significant differences were found in the serum concentrations of IL-17, both preparations had a tendency to reduce this cytokine level compared with the control AA groups.

Data discussed above suggest that the anti-arthritic effect of investigated compounds could occur through the inhibition of oxidative stress in AA and increased antioxidant activity after the treatment.

CONCLUSIONS

This study has demonstrated that the oral administration of MSM or its combination with Th (compound MTh) attenuates the development of AA in rats. Compounds had an inhibitory effect on joint swelling in AA model induced by CFA injection. This correlated with attenuation of histological changes in injected joints, decreased level of IL-17, together with significantly lower levels of MDA and increased CAT activity. These therapeutic benefits were comparable with those achieved by treating with the clinically available drug, diclofenac. MSM alone and in combination with Th (compound MTh) can be the safe and well tolerated dietary supplements that are two important parameters for the promising drug candidates for joint diseases therapy. These are the first data to indicate successful anti-arthritic activity of combined MTh preparation. Findings provide clues that investigated compounds may be the promising agents for preventing and treating of autoimmune diseases in combination with other drugs.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Research article
Ερευνητικό άρθρο

Investigation of some gastric *Helicobacter* species in saliva and dental plaque of stray cats by cultural and PCR methods

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ABSTRACT. The purpose of this study was to explore the presence of gastric *Helicobacter* species in the oral cavity of stray cats in the Kars region. Saliva and dental plaque samples collected from 100 stray cats were evaluated by culture and PCR methods in terms of gastric *Helicobacter* species. For culture, samples were plated on 5% defibrinated-horse blood and 5% defibrinated-sheep blood enriched selective agar plates supplemented with Vancomycin (6 µg/ml), Polymyxine B (2.500 IU/l), Trimethoprim (20 µg/ml) and Amphotericin B (2.5 µg/ml). Molecular methods were also included to study by using the PCR targeting amplification of the 16S rRNA gene sequence for *Helicobacter* genus and *urease B* gene sequence for each *Helicobacter* species. As the results of cultural examination, *Helicobacter* spp. were isolated from 10 (10%) cats (10 saliva and 5 dental plaque samples) and these were further identified as *H. heilmannii* by PCR. Direct analysis of samples by genus-specific PCR revealed that a total of 70 (50 saliva and 20 dental plaques) samples from 65 cats were positive in terms of *Helicobacter* DNA. As the results of species - specific PCR analysis of these samples 34 (48.57%) (24 saliva and 10 dental plaque samples) were identified as *H. heilmannii*, while the remaining 36 (51.42%) were found to be negative in terms of related species (*H. heilmannii, H. pylori* and *H. felis*). It has been concluded that these bacteria, identified in the oral cavity of the cats, may play a role in transmission of infection to humans.

Keywords: stray cat, saliva, dental plaque, Gastric Helicobacter, *Helicobacter heilmannii*, isolation, PCR

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INTRODUCTION

Gastric *Helicobacter* spp. are microaerophilic, non-spore forming, Gram negative, helical bacteria which present in gastrointestinal system of humans and various animals (cat, dog, pig etc.) and can cause severe inflammatory changes in humans (Clyne et al. 1995; Wroblewski et al. 2010). Although the most common type of infection in humans is caused by *H. pylori*, the other Gastric *Helicobacter* species (*H. felis, H. heilmannii, H. bizzozeronii*) may lead to gastric diseases in a similar manner and *H. heilmannii*, which is commonly colonized in the stomach of cat and dogs, has zoonotic attribute (Joosten et al. 2016). It is known that *H. pylori* causes peptic ulcer, gastritis and gastric cancer in humans as well as non-digestive diseases (Ishaq and Nunn 2015). *H. pylori* infection is known to be the most common human infection worldwide with an approximately 50% of the world's populations are infected (Go 2002). The role of these agents in gastrointestinal disease in dogs and cats is uncertain. However, the presence of gastric Helicobacter species was reported in canines with and without clinical complaints (Handt et al. 1994; Jalava et al. 2001). In addition to stomach settlement, the presence of the Gastric Helicobacter in oral cavity, which has oral - oral, fecal - oral and gastric - oral transmission routes, also plays an important role in the infectious cycle. Invasive and non-invasive methods are used for the diagnosis of Gastric Helicobacter (Logan and Walker 2001) and of which culture is known as gold standard. However, the difficulties in growth conditions and being as rare cultivable bacteria (Glupczynski 1998) have led to the researchers to use of alternative diagnostic methods such as PCR which has higher sensitivity than culture based methods (Rahman et al. 2013). In such studies conducted in stomach biopsy, saliva, dental plaque and stool samples, 16S rRNA gene - based PCR for *Helicobacter* spp. (Ghil et al. 2009) and *urease B* gene - based PCR for species identification are used (Neiger et al. 1998).

The aim of this study was to investigate the presence of *H. heilmannii*, *H. felis* and *H. pylori* in oral cavity of stray cats with normal clinical appearance in Kars, Turkey. Saliva and dental plaque samples were used and subjected through culture and PCR methods.

MATERIALS AND METHODS

Animal material and sampling

A hundred stray cats with normal clinical appearance and behaviour were randomly selected from free-ranging area in Kars, Turkey and included to this study. The study was conducted with the approval of the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK - 2014/053). Saliva and dental plaque samples were taken in duplicate from the cats that were caught by a trap and restricted for a while. Saliva samples were collected by swabbing buccal mucosa with sterile cotton swaps and transferred into tubes containing 0.5 ml sterile saline. Dental plaque samples were removed from the tooth surfaces with steril periodontal curette and placed in 0.5 ml sterile saline. The samples were transferred in short time (approximately in 2 hours) to the Microbiology laboratories of the Veterinary Faculty of the Kafkas University, under cold conditions (2-8 ◦C). One copy of samples were subjected to culture processes immediately after the transfer and the others were kept at -20 ◦C till the PCR analysis.

Cultural analysis

For the isolation of Gastric *Helicobacter* species, swab samples were inoculated onto the 5% defibrinated - horse blood chocolate agar and 5% defibrinated - sheep blood agar plates which were supplemented with 2.5 μg/ml Amphotericin B (Sigma A2942), 20 μg/ml Trimethoprim (Sigma T7883), 6 μg/ml Vancomycin (Sigma 1404-93-9) and 2.500 IU/l Polymyxin B (Sigma P4932) (Norris et al. 1999). The plates were incubated at 37°C under microaerobic condition with using a kit (Anaerocult C, Merck 1.16275) for 3 to 7 days. Bacteria were identified as *Helicobacter* spp. by considering the microscopic morphology under light microscope and strong positive urease, catalase, oxidase activities (Bento-Miranda and Figueiredo 2014).

Genus and species-specific PCR analysis

PCR was used for confirmation of the isolates and for direct molecular analysis of the samples (saliva and dental plaque) tested. For this purpose, genus - specific PCR (Ghil et al. 2009) targeting the 16S rRNA gene of *Helicobacter* and species - specific PCR (Neiger et al. 1998) targeting the *urease B* gene of *Helicobacter* species including *H. pylori*, *H. felis* and *H. heilmannii* were applied (Table 1). DNA extraction from the isolates and samples was carried out with a commercially available kit in accordance with the instructions of the manufacturer (QIAamp DNA Mini Kit, 51306). A total of 25 μl PCR reaction was prepared for both PCR with the following contents: 200 mM deoxyribonucleoside triphosphates mixture (Sigma D7295), ×1 PCR buffer (Sigma P2192), 25 pmol of each primer (Biomers, Germany), 0.5 U Taq polymerase (Sigma D6677), and

2.5 μl template DNA. Thermal condition of genus - specific PCR was adjusted as one cycle initial denaturation at 94°C for 2.5 min, 40 cycles amplification consisting of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, extension at 72°C for 1 min and one cycle final extension at 72°C for 15 min. A species - specific PCR analysis with thermal condition was applied as one cycle initial step consisting of denaturation at 94°C for 3 min, at 57°C for 2 min and at 72°C for 3 min, followed by 30 cycles amplification consisting of denaturation at 94°C for 30 sec, primer annealing at 57°C for 30 sec, extension at 72°C for 1 min and a final extension cycle at 72°C for 5 min. PCR reactions were carried out in the presence of positive controls obtained from characterized *Helicobacter* species of Kafkas University, Veterinary Faculty strain collection and negative control using RNase - DNase free water. A horizontal gel electrophoresis technique containing 1.5% agarose was used to visualize the amplified PCR products reported in Table 1.

RESULTS

Culture results

Following the incubation of samples on the aforementioned mediums, the oxidase, catalase and urease positive, Gram - negative helical shaped bacilli with S-type, transparent, white-grey colonies were evaluated as *Helicobacter* spp. From the 100 saliva samples tested, 10 (10%) were positive and from the 100 dental plaque samples tested 5 (5%) were positive for *Helicobacter* spp. culture. Consequently, from the 100 cats tested, 10 (10%) presented positive for *Helicobacter* spp. culture, of which 5 had positive culture from both saliva and dental plaque samples. According to the genus and species-specific PCR of these isolates all were identified as *H. heilmannii* by yielding 400 bp and 580 bp amplified product, respectively (Figs. 1, 2 and Table 2).

Genus and species - specific PCR results

Sequences obtained from PCR products were identified as *Helicobacter* spp. origin. Of the 100 saliva samples 50 (50%) were positive for Helicobacter and of the 100 dental plaque samples, 20 (20%) were positive (Fig. 1). Overall, of the 100 cats, 65 (65%) were positive for *Helicobacter* spp. of which 5 had positive PCR results in both saliva and dental plaque samples. Nineteen (38%) of the 50 PCR positive for Helicobacter saliva samples, and 10 (50%) of the 20 positive dental plaque samples were confirmed to be of *H. heilmannii* origin. Overall, of the 100 cats tested, 29 (29%) were positive for *H. heilmannii* (Fig. 2, Table 2). Neither culture method nor PCR were able to detect other Gastric Helicobacter species, *H. felis* and/or *H. pylori*, in any of the samples.

Table 1: Genus and species - specific primers used for PCR of Helicobacter identification

Primer sequences		Band size (bp)	Gene region
Genus - specific primers			
Helicobacter F	5' - GCT ACG ATC C - 3'	400	16S rRNA
Helicobacter R	5' - GAT TTT ACC CCT ACA - 3'		
Species - specific primers			
H. pylori F	5' - GGA ATT CCA GAT CTA AAA AGA TTA GCA GAA AAG - 3'	1.707	Urease B
H. pylori R	5' - GGA ATT CGT CGA CCT AGA AAA TGC TAA GTT G - 3'		
H. felis F	5' - ATG AAA CTA ACG CCT AAA GAA CTA G - 3'	1.150	
H. felis R	5' - GGA GAG ATA AAG TGA ATA TGC GT - 3'		
H. heilmannii F	5' - GGG CGA TAA AGT GCG CTT G - 3'	580	
H. heilmannii R	5' - CGT GTC AAT GAG AGC AGG - 3'		

Table 2: The overall results of culture and PCR of *Helicobacter* species from samples

Sample	The number of sample	Cultural method		Direct PCR analysis		
		Positive samples	Identified agent†	Genus - specific PCR	Species -specific PCR	Identified agent
Saliva	100	10	*H. heilmannii*	50	19	*H. heilmannii*
Dental plaque	100	5		20	10	
Total	200	15 (10)‡		70 (65)‡	34 (29)‡	

† The isolates were also confirmed by PCRs.
‡ The values in the brackets stand for the number of cats.

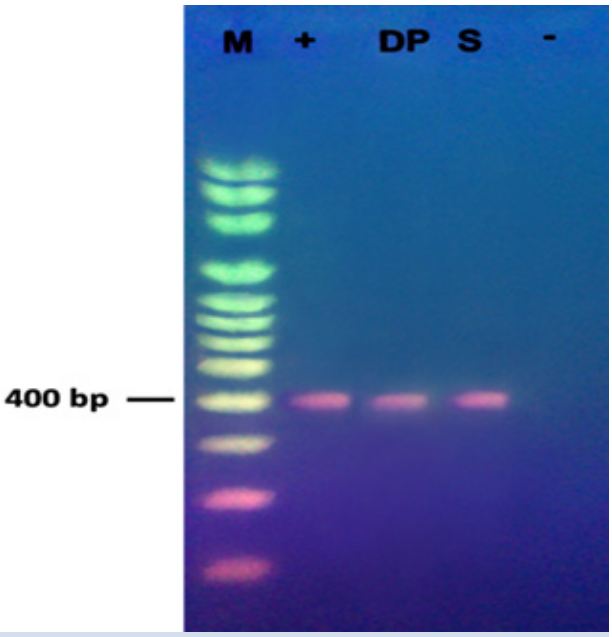

Figure 1. The gel electrophoresis images of genus - specific PCR of *Helicobacter* spp.: (M): Marker, (+): Positive control, (DP): Dental plaque, (S): Saliva, (-): Negative control

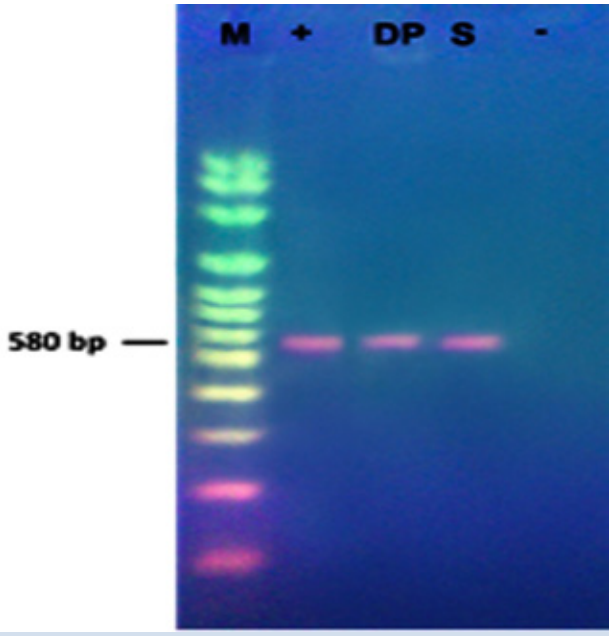

Figure 2. The gel electrophoresis images of species - specific PCR of *Helicobacter* spp.: (M): Marker, (+): Positive control, (DP): Dental plaque, (S): Saliva, (-): Negative control

DISCUSSION

The analysis methods such as rapid urease test (Berry and Sagar 2006) 13C-14C urea breath test (Gomes et al. 2002), histopathological examination (Lee and Kim 2015) and stool antigen test (Gulcan et al. 2005) are widely used in the diagnosis of Gastric Helicobacter as well as the cultural methods in which selective media are utilized. However, *Helicobacter* species are considered fastidious organisms which can be difficult to isolate and propagate (Handt et al. 1994; Yildirim 1997; Cantet et al. 1999; Sagnak and Ozgur, 2011) Optimization of methods, imparting selectivity of the culture media and sampling strategies (sampling region, transportation conditions) are important in increasing the isolation probability of *Helicobacter* species from heavy contaminated samples (Soltezs at al. 1992; Sturegard et al. 1998; Somily and Morshed 2015). In a study, *H. pylori* was able to be cultured from 3.44% of the gastric tissue of cats (Hantd et al. 1994). In a thesis study conducted on various animals (dogs, cats, chickens, rats and guinea pigs) in Turkey, *H. felis* was isolated in 2.6% of 75 dog and 25% of 16 cat stomach samples (Yildirim 1997). In this study, %5 defibrinated-horse blood and %5 defibrinated-sheep blood agar plates supplemented with 2.5µg/mL Amphotericin B, 20µg/mL Trimetoprim, 6µg/mL Vancomycin and 2.500 IU/L Polymixin were used. Helicobacter isolation was achieved in 10 (10%) of the 100 cats (10% of the saliva and 5% of the dental plaque) and these were further identified as *H. heilmannii*. The isolation rate is low when compared to the culture results yielded by the gastric mucosa of canine (Eaton et al. 1996; Jalava et al. 1998). But this can be explained as the antimicrobials participating in the medium cannot provide sufficient selectivity for Helicobacter isolation from oral cavity in which the microbial flora is much richer and more complex than the stomach flora has (Nigam 2015). However, the isolation rate has close similarity to those reported before (Handt et al. 1994; Yildirim 1997). The identified species, *H. heilmannii* is the sole species, is different as a reflection of some predictive factors. Even that *H. heilmannii* could be cultured at low rates suggests that Gastric Helicobacter protect the helical form in the oral cavity which makes it more recognisable unlike the stool samples have. In addition, the ability of Helicobacter to be cultured from the oral cavity of cats supports the views of that these agents, which are likely to have a zoonotic character, can contaminate food and many environmental samples (Neiger and Simpson 2000; van Duynhoven and De Jonge 2001; Guner and Telli 2012), transmit by oral - oral route (Jalava et al. 2001; Boyanova et al. 2007; Meining et al. 2009) and may be the most likely infection source of human beings (Ferguson et al. 1993; Megraud and Routet 2000).

Due to some difficulties encountered in cultural processes of Helicobacter, it is emphasized that PCR

techniques are more advantageous for investigation of the bacteria from materials such as stool and saliva (Dunn et al. 1997). There are many researches on identification of Gastric Helicobacter by PCR, especially from gastric and stomach specimens of cats. Handt et al. (1994) investigated 29 gastric tissues of cats sampled from pet shops and identified *H. pylori* in only one sample by culture and PCR methods.Strauss-Ayalia et al. (2001), reported that *Helicobacter* was detected in 17 of the 45 gastric biopsy specimens taken from a group of sick and healthy cats and identified that 9 of them were *H. heilmannii* and 4 were *H. felis* (*H. heilmannii* and *H. felis* are common in 3 samples), while 7 were not typed. Ghil et al. (2009) investigated the saliva and stool samples of 165 cats in Korea by PCR and obtained Helicobacter positivity in 91.1% of stray cats and in 56.3% of owned cats. However, none of the samples were identified as *H. pylori* or *H. felis*. The most striking examples of the colonization of Gastric Helicobacter in cats have been reported by Dag et al. (2016) and Neiger et al. (1998) in which Gastric Helicobacter-like organisms were detected in 93.3% of the stomach biopsy samples of stray cats by immunohistochemically and PCR and *H. heilmannii* was detected in 78% of the stomach biopsy samples of cats with using PCR, respectively. In this study, *Helicobacter* DNA was detected in 65 (65%) of 100 stray cats. While this ratio shows that cats are *Helicobacter* carriers as that can be seen in many of the above mentioned studies, it supports the idea that the differences in the prevalence values may be related to the determinants such as regional, genetic and age as mentioned by Kuipers (1999). Moreover, the prevalence in our study is affected by the fact that we know nothing about the medical record of these cats.

Several *Helicobacter* species (*H. heilmanii, H. felis*,*H. pylori, H. pametensis* and *H. baculiformis* sp. nov.) are naturally found in cats, while the host preferences of Gastric Helicobacter were different (Handt et al. 1994; Nieger et al. 1998; Cantet et al. 1999; Baele et al. 2008). In contrast to the high prevalence in humans, *H. pylori* and *H. felis* are reported to be very low in cats (Norris et al. 1999; Goh et al. 2011). Similar to this *H. felis* and/or *H. pylori* was not identified from the samples either culture method or PCR method. In many studies, *H. heilmannii* was identified as the dominant species in cats (Neiger et al. 1998; Cantet et al. 1999; Neiger and Simpson 2000). In this study, 19 (38%) of 50 saliva and 10 (50%) of 20 dental plaque samples were detected as *H. heilmannii* with the PCR using the *urease B* gene primers specific for Gastric Helicobacter. This result is similar to other studies (Neiger et al. 1998; Cantet et al. 1999; Neiger and Simpson 2000) and indicating that *H. heilmannii* is the predominant species in cats studied in this region. Unidentified species are thought to be useful for analysis of presence of the other *Helicobacter* species (such as *H. bizzozernii, H. salomonis,* etc.) or unknown new species reported to be present in cats.

CONCLUSIONS

The identification of *H. heilmannii* at certain ratios from saliva and dental plaque of cats suggests that cats may play a role in the transmission of *H. heilmannii* in humans. Furthermore, to investigate the Gastric Helicobacter such as *H. felis, H. pylori* and other possible species will be important to identify the bacterial habitat of oral cavity of cats in terms of Helicobacters.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Case report
Κλινικό περιστατικό

Giardia sp. and *Cryptosporidium* sp. in Iberian Wolf

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Abstract: A subsample consisting of fifty fecal samples from wild Iberian Wolf (*Canis lupus signatus*), from the northwest of Spain were collected in the field. The samples were analyzed for cysts of *Giardia* sp. and oocysts of *Cryptosporidium* sp. using a direct immunofluorescence antibody test (IFA). *Giardia* sp. and *Cryptosporidium* sp. were found in 20.0 % of the samples examined. Simple infections were more frequent (90.0 %) with seven (14.0 %) positive for *Giardia* sp. and two (4.0 %) positive for *Cryptosporidium* sp. To the authors' knowledge, this is the first report of occurrence of *Cryptosporidium* sp. in Iberian Wolf.

Keywords: *Cryptosporidium*; *Giardia*; Spain; Parasites; Wolf

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INTRODUCTION

The Iberian Wolf (*Canis lupus signatus*), one of the major wildlife predator that circulates between Portugal and Spain in the Iberian Peninsula, carries a cultural and historical stigma that contributed to its historical decline. However the populations are currently increasing across Europe (Chapron et al., 2014). In human-dominated landscapes, the occurrence of wolves is the result of a complex interaction among several environmental and human factors (Llaneza et al., 2012). It is recognized that wolves can be a reservoir of many zoonotic agents (Torres et al., 2000) but on the other hand, humans and/ or livestock can be a risk for wolves like other wildlife species (Castro-Hermida et al., 2011).

Giardia sp. and *Cryptosporidium* sp. are considered ubiquitous protozoan parasites and can be a cause of gastrointestinal diseases in many mammals (Reboredo-Fernández et al., 2014). Both protozoan are included in the WHO Neglected Diseases Initiative in 2004 (Savioli et al., 2006) with special concern for the species of *Giardia duodenalis* and *Cryptosporidium parvum*, that can be a cause of human mortality (Plutzer and Karamis, 2009). Cysts of *Giardia* sp. and oocysts of *Cryptosporidium* sp. (infective stages) can feature a high fecal excretion in the environment and can remain on the ground surface or soil even after the feces have decomposed (Kloch et al., 2005; Ryan and Cacció, 2013).

MATERIALS AND METHODS

The fecal samples were collected in the Northwest of Spain. Given the nature of the sampling procedure no information was available regarding the sex or age of the animals. Differentiation of the wolf´s feces from, namely dog feces, was achieved by indirect signs such as the composition/ morphology of feces (shape, size, and contents like hair and pieces of bones with a characteristic odor) and typical localization (Llaneza et al., 2012). Samples were collected during the summer (May, June, July and August) and autumn (September and October) seasons of two consecutive years (2013 and 2014). The collected fecal samples were frozen at -20 ºC, and maintained in this condition until analysis.

From total fecal samples collected, fifty samples were randomly selected to determine the presence of *Cryptosporidium* oocysts and *Giardia* cysts. For that purpose, a commercial direct immunofluorescence assay (Cellabs® Pty Ltd, Brookvale, Australia) was used. Feces (50 µL or 5 mm diameter) were diluted (1:10) with phosphate-buffered saline (PBS). PBS was prepared by diluting 0.2 g potassium chloride, 0.2 g potassium dihydrogenphosphate, 1.2 g anhydrous disodium hydrogen phosphate and 8 g sodium chloride in 1 L of distilled water, with pH adjusted to 7.4 through the use of 1 M HCl and 0.1 M NaOH. In a microscope slide, 20 µL of the fecal specimen was placed and allowed to completely air dry. The slides were fixed for five minutes in acetone and allowed to air dry. The probed-antibody (25 µL) was added to the fixed specimen and positive control, covering all area. The slides were incubated at 37 ºC in a humid chamber for 30 minutes, and then rinsed gently in a bath of PBS for one minute. The slide was drained and the excess moisture around the well was removed with tissue. A drop of mounting fluid (RMG) was added to the slide well, and a coverslip was placed on top of the drop and the air bubbles were removed. The entire specimen was immediately scanned using a fluorescence microscope.

RESULTS

Cryptosporidium oocysts (2-6 µm in size) appeared with a round or oval shape with bright green fluorescence. A fold or suture could be seen on the surface. *Giardia* cysts appeared elliptical in shape, with bright green fluorescence. The test was considered positive if one or more oocysts and cysts were present. Of the fifty samples analyzed, ten (20.0 %) were positive for both *Giardia* sp. and *Cryptosporidium* sp. *Giardia* cysts and *Cryptosporidium* oocysts were detected in simple infections with more frequency (90.0 %), with seven (14.0 %) samples positive for *Giardia* sp., and two (4.0 %) samples positive for *Cryptosporidium* sp. and one sample (2.0 %) positive for both agents.

DISCUSSION

Our results show that *Giardia* sp. were more frequently found than *Cryptosporidium* sp. but on the other hand most of the studies on wolves do not have a large number of samples, especially in Europe where this specie (*Canis lupus*) is endangered and the samples are difficult to collect. We found a lower prevalence compared to other studies (Kloch et al., 2005; Paziewska et al., 2007; Stronen et al., 2011) which may be due to the fact that our samples were frozen and the sensitivity may be lower. However, this study reveals that Iberian wolf is susceptible to the presence of these agents and at the same time can play a role on dissemination, as it was reported for other species from different environments in Spain.

In northwest of Spain, these protozoa were previously reported in some wild species. In otters (*Lutra lutra*), a prevalence of 6.8 % (30/437) for *Giardia* sp. and 3.9 % (17/437) for *Cryptosporidium* sp. was reported by Méndez-Hermida et al. (2007). Reboredo-Fernández et al. (2015) analyzed 70 fecal samples of aquatic species, found that 2.8 % were positive for *Giardia* sp. and 5.7 % positive for *Cryptosporidium* sp. In addition, *Giardia* sp. (6.0 %) and *Cryptosporidium* sp. (9.0 %) were detected in common dolphins (*Delphinus delphis*). In eight of the analyzed samples (n=133) *G. duodenalis* was identified whereas *C. parvum* was identified in three samples (Reboredo-Hermida et al., 2014). Castro-Hermida et al. (2011) compared different environments (635 samples collected from a coastal area and 851 samples from an inland area) with samples of wastewater (untreated and treated). The results showed a difference between coastal (positive for *Giardia* sp. 15.9 % and positive for *Cryptosporidium* sp. 9.2 %) and inland area (positive for *Giardia* sp. 26.7 % and positive for *Cryptosporidium* sp. 13.7 %). In wild birds 2.1 % (9/433) of the fecal samples were found positive for *Giardia* sp. and 8.3 % (36/433) positive for *Cryptosporidium* sp. *Giardia* sp. was identified in two species of raptors and *Cryptosporidium* sp. was identified in 7.1 % of the samples (Reboredo-Fernández et al., 2015). In roe deer (*Capreolus capreolus*), Castro-Hermida et al. (2011) also reported *Giardia duodenalis* (5.3 %) and *Cryptosporidium parvum* (1.3 %). The same study detected *Giardia* sp. (1.3 %) and *Cryptosporidium* sp. (7.6 %) in 381 fecal samples of wild boars. Recently, a study in Spain, in wild carnivores reported one positive sample for *Giardia duodenalis*, out of six analysed fecal samples of wolves (Mateo et al., 2017).

Outside Spain, both protozoan parasites have already been reported in wolves. In Poland, Kloch et al. (2005) found a prevalence of 45.5 % for *Giardia* sp. and 54.9% for *C. parvum* (n=57). Also in Poland, Paziewska et al. (2007) reported *Cryptosporidium* sp. with a prevalence of 35.5 % (n=14). In Canada, the protozoan parasites were also detected by Stronen et al. (2011) with a prevalence of 29.5 % for *Giardia* sp. and 1.2 % for *Cryptosporidium* sp., but with a larger number of samples (n=601).

To the authors´ knowledge, no studies reported the presence of *Cryptosporidium* oocysts in the wolf packs of Iberian Peninsula. *Giardia* sp. in canids can cause chronic diarrhea, weight loss, lethargy and growth retardation. *Cryptosporidium* sp. is normally asymptomatic in canids, but in immunosuppressed animals can be a cause of chronic diarrhea (Taylor et al., 2007) From the obtained results, it is reasonable to suggest that accidental infection of humans, livestock or companion animals can ensue, especially *Giardia duodenalis*, which has been reported in many mammals, including humans (Ryan et al., 2013). On the other hand, *Cryptosporidium* sp. has a negative impact in immunocompromised population, humans being infected by many species of *Cryptosporidium* (Plutzer and Karanis, 2009). The social behavior of wolf packs can further contribute to the dissemination due to their ability to migrate for long distances and the increasing proximity with humans and livestock. Wolf marking behavior by means of visual and scent marks such as feces and urine (Mech and Boitani, 2003; Zub et al., 2003; Llaneza et al., 2014) in unpaved roads and trails, and that could further contribute to the dissemination, as cysts and oocysts can remain in the soil for long periods of time, even under adverse conditions, such as rain or snow.

CONCLUSIONS

More studies using molecular assays are needed to characterize the parasite fauna of remote and endangered wildlife especially to assess to infectious status in living animals. Furthermore, molecular assays can clarify the trophic relationships of wild carnivores and their prey species especially in human-dominated landscapes and with these studies we can establish the source of the infection of the wolves.

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CONFLICT OF INTEREST

The authors report no conflicts of interest

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Case report
Κλινικό περιστατικό

Uterine stump adenocarcinoma in a bitch with an ovarian remnant: A case report

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Αδενοκαρκίνωμα κολοβώματος μήτρας σε σκύλα με υπόλειμμα ωοθήκης: Αναφορά περιστατικού

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ABSTRACT. A 3-year-old female spayed dog was presented with a history of sanguineous vaginal discharge of 2 month duration. The dog was spayed 1.5 years before presentation. Clinical examination revealed a large, solid, ovoid mass in the caudal abdomen, recognized by diagnostic imaging as an enlargement at the top of the uterine stump. Additionally, high serum progesterone concentration was measured, confirming the presence of functional ovarian tissue in dioestrus. With a suspicion for a related uterine stump pyometra or less likely, for other enlargements, a coeliotomy was performed. A mass at the apex of the uterine body and a right side ovarian remnant were found. Both structures and the remaining uterine stump were excised. The uterine remnant mass was histologically diagnosed as uterine adenocarcinoma, herein detected for the first time at the uterine stump in the bitch. Unsuccessful, incomplete ovariohysterectomy may permit even neoplastic transformation of uterine stump tissues.

Keywords: adenocarcinoma, uterine stump, ovarian remnant

ΠΕΡΙΛΗΨΗ. Θηλυκός σκύλος, ηλικίας 3 ετών, προσκομίστηκε 1,5 χρόνο μετά από υποτιθέμενη στείρωση, διότι παρουσίαζε επί 2 μήνες αιμορραγικό έκκριμα από το αιδοίο. Στην κλινική εξέταση ψηλαφήθηκε ευμεγέθες, συμπαγές, ωοειδές μόρφωμα στην οπίσθια κοιλία, το οποίο στην απεικονιστική διερεύνηση προσδιορίστηκε ως διογκωμένο κολόβωμα μήτρας. Επιπλέον διαπιστώθηκε υψηλή συγκέντρωση προγεστερόνης στον ορό του αίματος, αποδεικτική παρουσίας λειτουργικού ωοθηκικού ιστού σε δίοιστρο. Με υποψία πυομήτρας του κολοβώματος της μήτρας και λιγότερο άλλου είδους μορφώματος πραγματοποιήθηκε μέση λαπαροτομή. Βρέθηκε μόρφωμα – μάζα στο πρόσθιο άκρο του κολοβώματος το οποίο εξαιρέθηκε μαζί με το υπόλοιπο σώμα της μήτρας, καθώς και υπόλειμμα δεξιάς ωοθήκης. Το μόρφωμα προσδιορίστηκε ιστοπαθολογικά ως αδενοκαρκίνωμα και διαπιστώθηκε για πρώτη φορά σε κολόβωμα μήτρας σκύλας. Η αποτυχημένη, μερική ωοθηκυστερεκτομή μπορεί να επιτρέψει ακόμη και την εξαλλαγή των ιστών του κολοβώματος της μήτρας.

Λέξεις κλειδιά: αδενοκαρκίνωμα, κολόβωμα μήτρας, παραμονή ωοθηκικού ιστού

CASE HISTORY

A 3-year-old female spayed Labrador retriever weighing 29 kg was presented with a 2-month history of persisting sanguineous vaginal discharge following a recent copulation. The dog underwent a complete ovariohysterectomy (OHE) 1.5 years prior presentation. The dog was bright and alert at presentation. Clinical examination revealed a palpable mass in the caudal abdomen and no further abnormalities were noted. Vaginoscopy confirmed the presence of sanguineous secretions and a moderate wrinkling of the vaginal wall; a smear obtained from the mucosa had cytological characteristics suggestive of dioestrus. Routine blood tests results were within normal limits. Serum hormonal analysis showed oestradiol-17β concentration of 5.00 pg/ml and progesterone concentration of 17.37ng/ml (reference values: basal levels: <0.6 ng/ml, ovulation levels: 4.0-6.0 ng/ml) revealing functional ovarian tissue in dioestrus.

Plain abdominal radiography revealed a well-defined, oval-shaped soft tissue opacity at the caudal aspect of the abdominal cavity (**Figure 1**), compatible with a stump pyometra. Subsequent ultrasonographic assessment showed a mass, isoechoic to adjacent muscles, spotted by some hyperechoic foci, which casted clear shadows (**Figure 2**). No ovaries were identified during ultrasonographic examination. No lymph nodes enlargement was evident. Given the diagnostic imaging results, the dog was directly prepared for surgery.

Thirty minutes after premedication with acepromazine (Acepromazine, Alfasan) 0.02 mg/kg b.w., intramuscularly, morphine (Morphine 1 mg/ml, EFET-HFA) 0.1 mg/kg b.w., intramuscularly and meloxicam (Loxicom, Norbrook) 0.1 mg/kg b.w., intravenously, anaesthesia was induced with propofol (Propofol MCT/LCT Fresenius 1%, Fresenius Kabi) 3 mg/kg b.w., intravenously and maintained with isoflurane ~ 2% (IsofFlo®, Zoetis) in oxygen and constant rate infusion of fentanyl (Fentanyl®, Janssen) 0.05 μg/kg/min, intravenously. A midline coeliotomy was performed. Findings included the absence of both uterine horns, the presence of a sizeable (2.7 x 2 x 1.8 cm) remnant tissue on the right mesovarium with an active vascular bed, a tiny remnant tissue on the left mesovarium with poor vascular supply and a large, almost ovoid, encapsulated mass (size: 6.3 x 4.5 x 4.3 cm) associated with the uterine stump. Extensive adhesions of the mass with the adjacent organs were present (**Figure 3**). The caudal vagina and the uterine cervix were not affected. The tissues suspected as ovarian remnants and the mass including the uterine body and cervix were excised. Following mass resection, several slide imprints were taken and the mass was submitted for histopathological examination. Cytological examination revealed numerous round cells

with neoplastic characteristics including anisokaryosis, nucleoli presence, euchromatin, and scanty plasma cells and neutrophils with phagocytosed bacteria (**Figure 4**). Histopathological examination confirmed the presence of adenocarcinoma arising from the uterine stump, where a neoplastic population was organized in arbitrary lobules separated by thin bundles of fibrovascular stroma. The mass was moderately cellular, consisted by epithelial cells arranged in branched cords supported by a delicate fibrous net. Neoplastic cells had variable distinct cell borders, small amount of eosinophilic cytoplasm, oval or round nuclei with coarsely clumped chromatin and one variably distinct nucleolus. Anisokaryosis was moderate and 4 mitoses per High Power Field (HPF) were detected. The neoplastic mass was multifocally infiltrated by foamy macrophages, lymphocytes and plasma cells (**Figure 5**). Histopathological examination also showed that the tissue excised from the right mesovarium was an almost anatomically complete ovary with four corpora lutea, a small follicular cyst and at least two developing follicles. The tissue excised from the left mesovarium proved to be granulation tissue with fibroblasts and dense collagen stroma, infiltrated by few lymphocytes and plasma cells. No ovarian structures were found.

The dog recovered uneventfully from anaesthesia and was discharged from the clinic 2 days after surgery. On re-examination 1, 4 and 13 months post-surgery the dog was clinically healthy. The results of radiographic and ultrasonographic re-examination at 13 months post-surgery were unremarkable, since neither recurrence nor metastatic disease was noted.

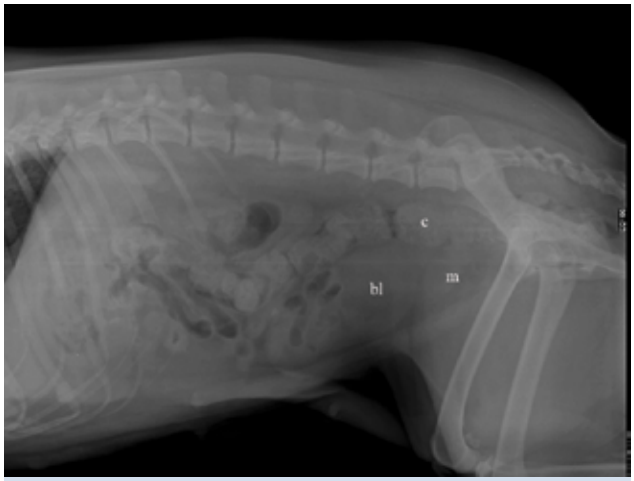

Figure 1. Plain abdominal radiograph of a 3-year-old bitch. Well-defined, oval-shaped soft tissue opacity (m) measured 72x57 mm (LxH) was detected at the caudal aspect of the abdominal cavity, dorsal to the urinary bladder (bl) and ventral to the descending colon (c).

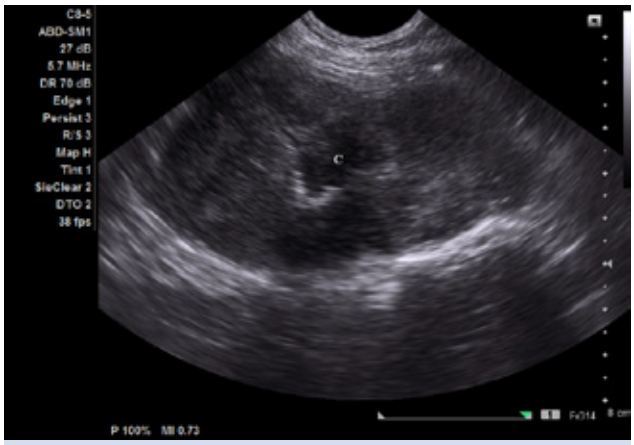

Figure 2. Abdominal ultrasound of the dog of fig 1 shows an isoechoic mass with hypoechoic center (c), compatible with fluid accumulation. Some hyperechoic foci throughout the mass casting a clear shadow were also evident.

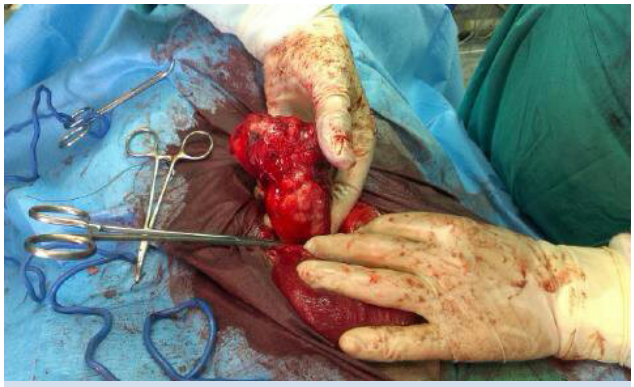

Figure 3. Intraoperative view of the dog of fig 1 following coeliotomy, showing that the mass (s) was associated with the uterine stump. Urinary bladder (bl), cervix (c).

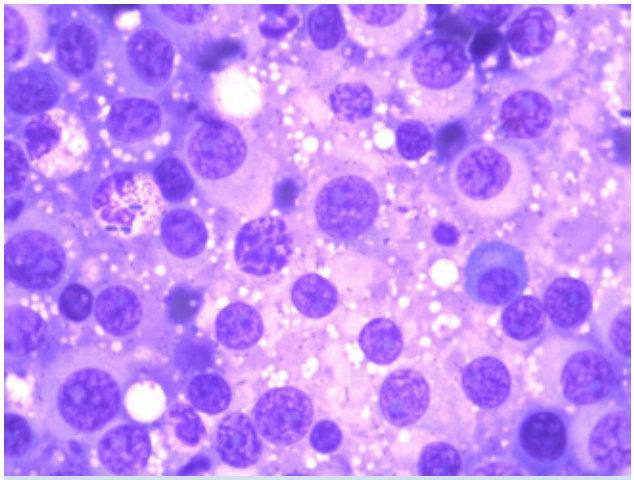

Figure 4. Microphotograph of a stained impression smear from the mass of fig 3 was indicative of a round cell tumour (quick staining technique). Neoplastic cell (nc), plasmacyte (p), neutrophil (n).

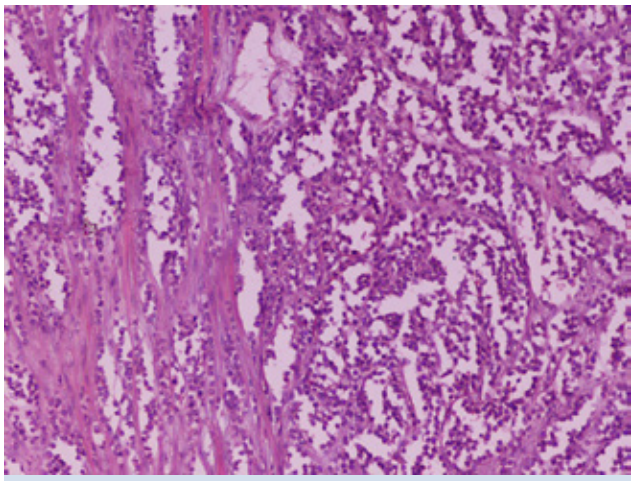

Figure 5. Histopathological section of the mass of fig 3 was compatible with uterine stump adenocarcinoma (magnification x100). Stain H-E.

DISCUSSION

Surgical neutering of companion animals is considered to be the most common, reliable, effective and the least controversial contraception method (Concannon and Meyers-Wallen, 1991). However, complications may occur and affect the reproductive system itself, including ovarian remnant syndrome (ORS) and stump pyometra (Pearson, 1973). The ORS is an iatrogenic long term complication of OHE, occurring when the ovarian cortex is not fully removed. Signs of ORS typically mimic those of proestrus or oestrus and occasionally pseudopregnancy or pyometra (Ball et al., 2010). Ovarian cortex presence can be confirmed by assaying serum hormones concentration (Wallace, 1991), as in our case.

In some studies, the residual ovary or ovarian tissue was more often seen at the right side (Pearson, 1973; Wallace, 1991; Ball et al., 2010), as in our case. The sizable ovarian remnant found in our case, raises some suspicion about a voluntary deposit, which might be considered helpful for the bitch to avoid weight gain and express a normal inter-dog social behaviour, although the unintentional, accidental deposit seems more probable, since some difficulty in fully retracting the right ovary is often encountered, especially when the ovarian ligament is not torn (Ball, 2010).

The interval between previous OHE and the onset of signs of oestrus in bitches has been reported to range from three months to five years (Miller, 1995). In our case, sanguineous vaginal discharge was noticed by the owner 13 months after OHE. However, in a retrospective study of Ball et al. (2010), dogs with ORS and reproductive system tumours had a significantly longer interval between OHE and diagnosis of ORS (47 to120 months), compared with the respective interval of animals without neoplasms (1 to 60 months). In contrast, in our case the dog manifested clinical signs earlier, at 13 months after OHE.

Uterine tumours in the bitch occur rarely, representing 0.4% of all canine tumours, reflecting widespread neutering practices in bitches. Of those reported, leiomyoma accounts for 85-90% and the most common malignant neoplasm is leiomyosarcoma (Murphy et al., 1994). Other mesenchymal and epithelial tumours are less frequently seen (Taylor, 2010). Very few reports of canine uterine carcinoma exist in the literature mainly affecting middle-aged or geriatric bitches (Vos 1988; Pena et al., 2006; Patsikas et al., 2014); however, it has been seen in a 2-year-old bitch treated with combined sex hormones, but the cause-and-effect association was not confirmed (Payne-Johnson et al., 1986) and in a 10-month-old entire golden retriever (Cave et al., 2002). Recently, the immunohistochemical characteristics of a canine endometrial adenocarcinoma were studied (Pires et al., 2010). No predilections have been reported for uterine tumours in other dog breeds apart from a hereditary neoplastic syndrome in the German shepherd dog where renal cystadenocarcinomas, nodular dermatofibrosis and multiple leiomyomas are seen (Moe and Lium, 1997); a mutation of the canine Birt-Hogg-Dubé gene has been associated with the condition (Lingaas et al., 2003).

Several cases of ORS have been reported in combination with various neoplasms of the reproductive tract (Ball et al., 2010; Rota et al.,2013; Perez-Marin et al., 2014), but only a haemangiosarcoma has been associated with uterus (Wenzlow et al., 2009). Therefore, our case is the first report of uterine adenocarcinoma in a bitch with ORS. We did not detect any evident carcinogenic factor in the history of our case. Similar to our case, one cat with uterine stump adenocarcinoma and ORS, but with abdominal metastases as well, has been reported in the literature (Anderson and Pratschke, 2011). Additionally, a completely spayed cat with a uterine stump adenocarcinoma has also been reported (Miller et al., 2003).

According to the relevant literature, OHE is the treatment of choice for uterine tumours in dogs (Saba and Lawrence, 2013). The use of adjunctive chemotherapy is sporadically reported, though its efficacy is questioned (Cave et al., 2002; Pires et al., 2010).

In our case surgical excision of the neoplastic mass and ovarian remnant resulted in resolution of clinical signs, since the dog has been clinically healthy 13 months after surgery.

Conclusively, uterine carcinoma is an uncommon canine neoplasm and to the author's knowledge, this is the first case of uterine stump adenocarcinoma in an incompletely ovariohysterectomised bitch, reported in the literature. Prompt surgical intervention was curative and preventive of any further neoplastic growth and spreading.

CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest for the presented case report.

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