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Research Article

Genital Tract Pathologies of Cows Slaughtered at El-Harrach Abattoir in Algeria

Nora MIMOUNE [1,2] Rachid KAIDI [2] Mohamed Yassine AZZOUZ [3]
Rachid KEDDOUR [1] Ayed BELARBI [4] Salima Yamina DERDOUR [1]

[1] Higher National Veterinary School, Bab-Ezzouar, Algiers, ALGERIA
[2] Institute of Veterinary Sciences, LBRA, University Saad Dahleb, Soumaa, Blida, ALGERIA
[3] Veterinary Doctor, El-Harrach, Algiers, ALGERIA
[4] Laboratory of Pathological Anatomy of Douira Hospital, Algiers, ALGERIA

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Abstract

The aim of this study was to determine genital organ disorders in 2025 cows at El-Harrach abattoir in the capital of Algeria and to describe the microscopic changes associated with these cases. The results obtained showed a high incidence of slaughtered pregnant cows (16.49%). Majority of these cases were at the early gestation. The most frequently encountered defects were ovarian cyst, uterine infection, and inflammatory salpinx, which proved that contamination and infection are always present in the cowsheds from where animals were brought. Other observed abnormalities were cervicitis, mucometra, double cervix, unicornis uterus, horn malformation, uterine tumour, triple cervix (which is a new finding in the world), salpinx adhesion, pyosalpinx, hydrosalpinx, ovarobursal adhesion, ovarian inactivity, ovarian inflammatory changes, ovarian tumour and parovarian cysts.

Keywords: *Uterus, Oviduct, Ovary, Pathology, Histology, Abattoir*

Cezayir'de El-Harrach Kesimevinde Kesilen İneklerdeki Genital Kanal Patolojileri

Özet

Bu çalışmanın amacı Cezayir'in başşehrinde yer alan El-Harrach kesimevinde kesilen 2025 inekte gözlenen genital kanal bozukluklarını belirlemek ve bu vakalarla alakalı mikroskopik değişiklikleri tanımlamaktır. Sonuçlar çok sayıda gebe hayvanın (%16.49) kesime gönderildiğini ortaya koymuştur. Bu vakaların da çoğu gebeliğin erken dönemlerindeydi. En sık karşılaşılan bozukluklar ovaryum kistleri, uterus enfeksiyonu ve salfinks yangısıydı. Bu durum kesim için getirilen hayvanların getirildikleri ahırlarda enfeksiyonun ve kontaminasyonun sürekli olduğunu göstermekteydi. Diğer gözlenen bozukluklar servisitis, mukometra, çift serviks, unicornis uterus, rahim boynuz malformasyonları, uterus tümörü, triple serviks (yeni bir bulgu olarak), salfinks yapışması, pyosalfinks, hidrosalfinks, ovarobursal yapışma, ovaryum inaktivitesi, ovaryumun yangısal değişimleri, ovaryum tümörü ve parovarian kistler olarak kaydedildi.

Anahtar sözcükler: *Uterus, Ovidukt, Ovaryum, Patoloji, Histoloji, Kesimhane*

INTRODUCTION

The causes of low fertility can be multifactorial and complex. Malnutrition, infections, management errors, ovulatory or hormonal imbalances, and congenital [1] or acquired defects [2] can play significant roles in the failure of bovine breeding. To be able to minimize the economic losses, it is important to determine the incidence of the various genital pathologies especially those found at the slaughter-house since it constitutes a true source of study and provides a great deal of information [2,3]. Many studies about genital pathologies of cows were performed worldwide [4-6]. Some researchers reported that the most frequent lesion was uterine infection [4,7] while others noted that the incidence of ovariobursal adhesion was the highest, followed by endometritis and cystic ovary [3].

The present study was performed to investigate the incidence of reproductive tract abnormalities and pregnant cows at a slaughter house in Algiers (Capital of Algeria) and to describe the histological changes associated with these defects.

İletişim (Correspondence)
+213 79 0901606
nora.mimoune@gmail.com

MATERIAL and METHODS

Animals

Samples were obtained from an abattoir at which the genital tracts of 2025 cows and heifers of various breeds (Holstein, Montbeliard, Fleickveih, native and cross-breeds) were examined. The samples were collected between 01/01/2010 and 29/06/2014 (pregnant specimens were also collected), placed in separate plastic bags and transported to the laboratory as soon as possible.

Examination Procedures

Macroscopic Examination: In the external inspection, the cervix and the horns were measured (diameter and length). To note the existence of a volume modification and asymmetry between the two uterine horns, a difference >5 cm was regarded as asymmetry. After this examination, cervix and horns were incised in order to note the presence of possible abnormal intra-uterine secretions as well as congestion of the mucous membranes. -Cases of pregnancy were also noted after incision of the horns-. Attention to possible adhesion and modifications of volume, size, color and the content of the oviduct were also given. Inspection and palpation of the ovaries were performed in order to detect any various abnormal formations as well as the possible existence of ovariobursal adhesion. Macroscopic Aspect Of Cystic Ovary Was Also Studied.

Microscopic Examination: Microscopic examination of samples was carried out at the pathological anatomy laboratories of the NHSV (National High School of Veterinary) and of UHC (University Hospital Center) Mustapha Basha in Algiers. Samples of ovary, oviduct and uterus were fixed in 10% buffered formalin and embedded in paraffin wax. Sections cut at 5 µm thickness were stained with Hematoxylin and Eosin (H&E), and evaluated under a light microscope.

Statistical Analysis

Data were presented in percentages and the analysis was conducted using STATISTICA (Version 10, Stat Soft France, 2003). Differences in the position, nature of wall and number of cystic ovary were evaluated by Chi-square test. The level of significance was set at $P<0.05$.

RESULTS

It was observed that 334 (16.49%) out of 2025 cows inspected, were pregnant with one case of twin pregnancy, and 1691 (83.51%) cases were not pregnant. Of the non-pregnant cases 790 (46.72%) did not show any visible abnormality in the ovaries, the oviducts or the uteri. The remainder 53.28% had lesions that are described below.

As also shown in *Fig. 1*, corpus luteum was found in 85.14% of the cases with 57.03% on the right ovary and 42.97% on the left ovary. The incidence of cavitary corpus luteum was 7.76% *(Fig. 2)*. The frequency of the double ovulation was 7.09% with 53.12% of the cases were noted on the left ovary and 46.87% on the right ovary.

Macroscopic Abnormalities

Uterine Lesions: The macroscopic lesions were noted on those cases with infection (inflammatory signs and/or presence of abnormal secretions) and congenital defects. The results showed that uterine infection presented the highest frequency (12.47%) followed by cervicitis (3.84%), mucometra (1.18%), double cervix (0.71%) and uterine tumors (0.29%). The other abnormalities were presented in 0.06% of the cases (malformation and triple cervix) except the uterus unicornis (0.12%) *(Table 1) (Fig. 3, Fig. 4)*.

Oviduct Lesions: In *Table 2,* the list of various lesions observed on oviducts was shown.

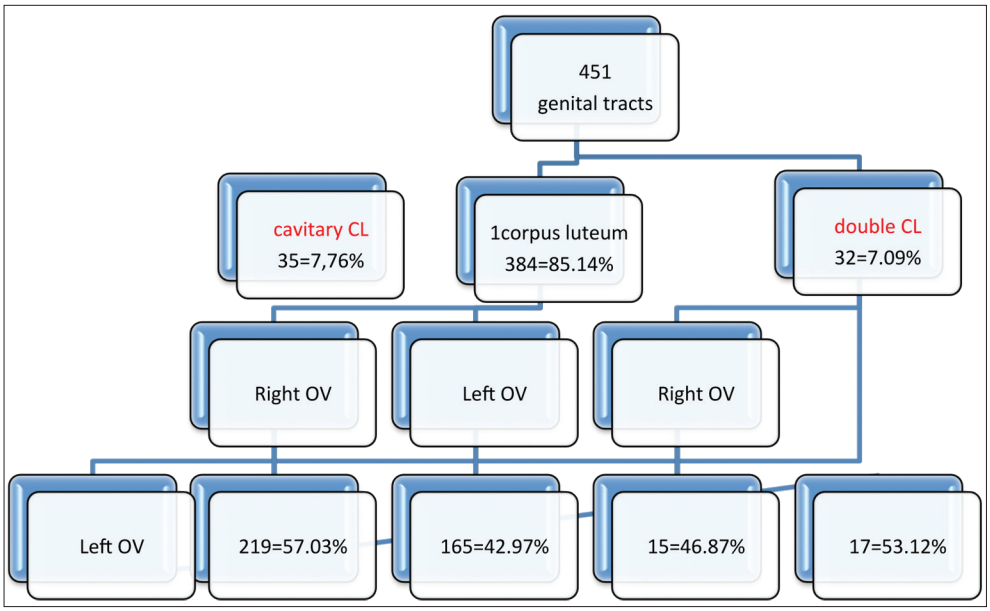


Fig 1. Distribution and incidence of genital tracts according to the number of the corpus luteum related to the ovaries

Şekil 1. Ovaryumlarda gözlenen corpus luteum sayılarının dağılım ve görünme sıklığı

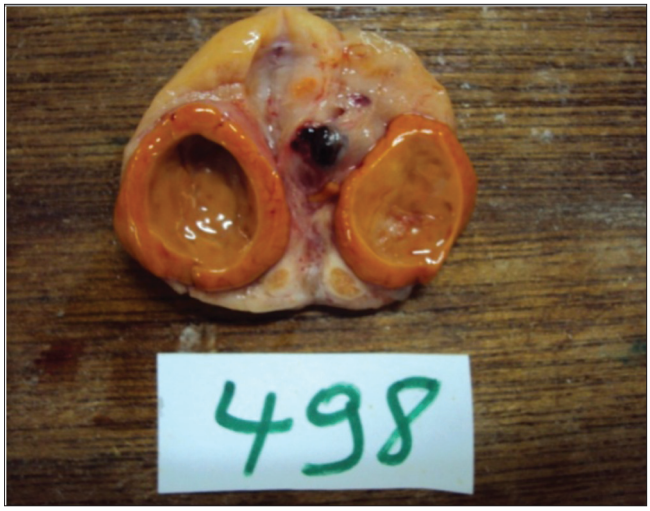


Fig 2. Cavitary corpus luteum
Şekil 2. Kaviter korpus luteum

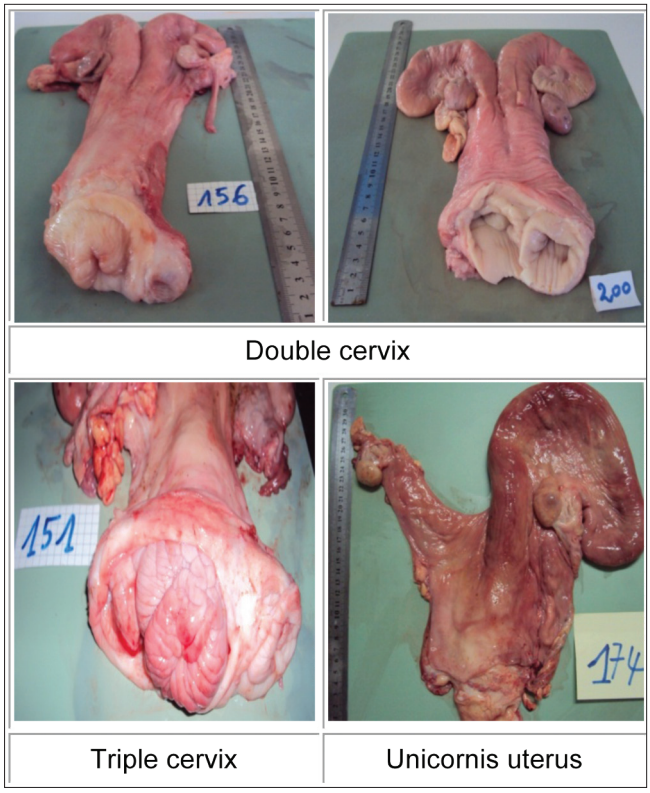


Fig 3. Uterine congenital pathologies
Şekil 3. Uterusda rastlanan konjenital patolojiler

Inflammatory salpinx and pyosalpinx were the most notewothy lesions (5.20% and 4.26%, respectively). Adhesion and hydrosalpinx showed the least frequencies with 1.54% and 1.30%, respectively.

Ovarian Lesions: The meticulous examination of the ovaries revealed the presence of several pathological changes as shown in *Table 3*.

Ovarian cyst was the most frequent lesion (14.25%)

Table 1. *Number & incidence of uteri presenting various macroscopic disorders*
Tablo 1. Çeşitli *makroskopik bozuklukları içeren uterus sayı ve sıklık oranları*

Lesion*	Number	Percentage (%)
Uterine infection	211	12.47
Cervicitis	65	3.84
Mucometer	20	1.18
Tumour	5	0.29
Double cervix	12	0.71
Triple cervix	1	0.06
Uterus unicornis	2	0.12
Horn malformation	1	0.06

** More than one lesion occurred in some cases*

Table 2. *Repartition of the genital tracts according to lesions revealed on the oviducts*
Tablo 2. *Oviduktta gözlenen lezyonlara göre vakaların ayırımı*

Lesion*	Number	Percentage (%)
Inflammation	88	5.20
Hydrosalpinx	22	1.30
Pyosalpinx	72	4.26
Adhesion	26	1.54

** More than one lesion occurred in some cases*

Table 3. *Repartition of the genital tracts according to the ovarian lesions*
Tablo 3. *Ovaryumda gözlenen lezyonlara göre vakaların ayırımı*

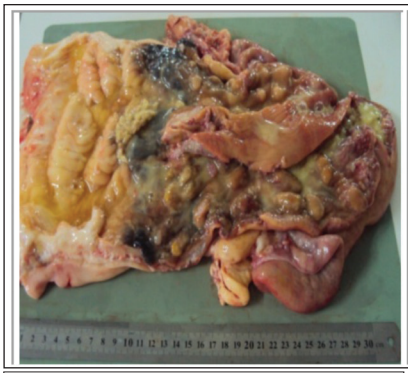
Lesion*	Number	Percentage (%)
Ovarian cysts	241	14.25
Smooth ovaries	33	1.95
Ovarobursal adhesion	91	5.38
Ovarian tumours	4	0.24
Inflammatory changes	4	0.24
Parovarian cysts	2	0.18

** More than one lesion occurred in some cases*

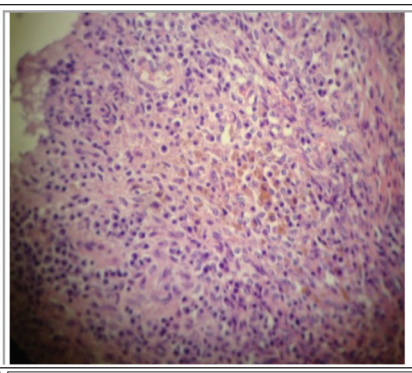
Table 4. *Distribution and incidence of cysts according to macroscopic aspect*
Tablo 4. *Makroskopik bakıda gözlenen kistik yapıların dağılım ve sıklığı*

Macroscopic Aspect of Cysts		n	%
Side	Right	156	64.73
	Left	70	29.05
	Right and left	15	6.22***
Wall	Thin	170	70.54
	Thick	70	29.05
	Hemorragic	1	0.41***
Number	Single	204	84.65
	Polycystic (3 or plus)	37	15.35***

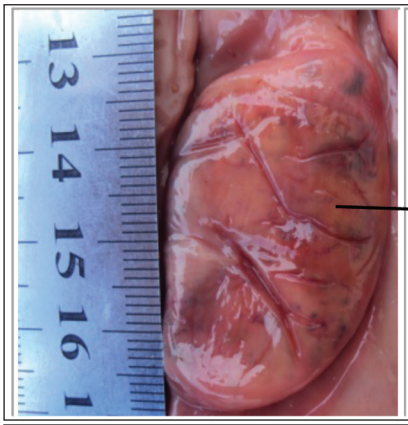
**** P<0.001*

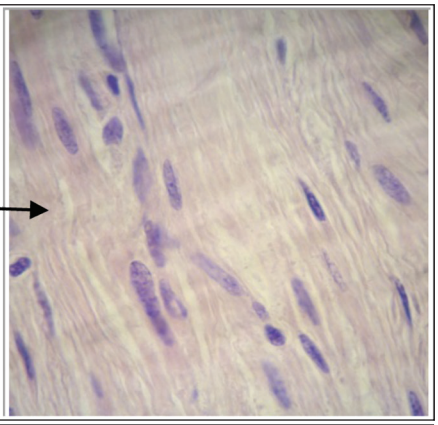


Uterine infection



Microscopic uterine infection: high infiltration of inflammatory cells with the destruction of epithelial surface (Gx400)

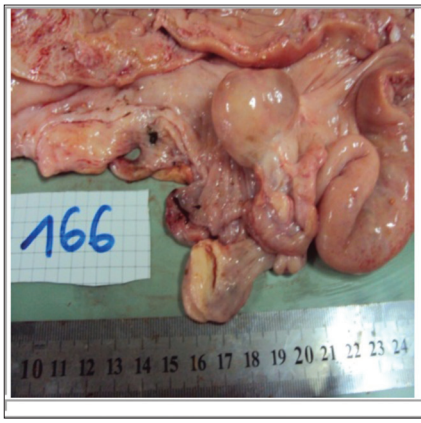




Leiomyoma: Aspect of cigar in the nuclei extremity (Gx1000

Fig 4. Uterine abnormalities

Şekil 4. Uterus anomalileri



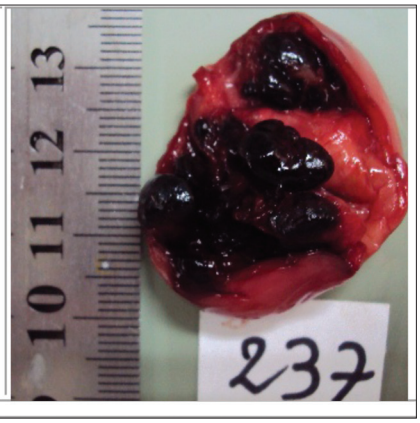


Fig 5. Ovarian cysts *(left)*, Microcystic hemorrhagic ovary *(right)*

Şekil 5. Yumurtalık kistleri *(sol)*, Mikrokistik hemorajik ovaryum *(sağ)*

followed by ovariobursal adhesion (5.38%) and smooth and small ovaries (01.95%) *(Fig. 5)*. The the least lesions noted in this study were inflammatory changes (0.24%), ovarian tumour (0.24%) *(Fig. 6)* and parovarian cysts (0.18%).

In this present study, cases of 241 cystic ovaries diagnosed are described in *Table 4*. The right ovary was the mostly affected (64.73%). Incidence in left ovary was 29.05%. The incidence of the 2 ovaries affected at the same time were 6.22% (P<0.001). The wall of cysts was thin in 70% (follicular cyst) whereas 29.05% of cysts had a thick wall (luteal cyst). In one case (0.41%), the wall was typically hemorrhagic microcystic (P<0.001). The cyst was single on the ovary in the majority of the cases (84.65%) and the polycystic ovary was found in 15.35% of the cases (P<0.001).

DISCUSSION

Usually, cows with reproductive problems and low

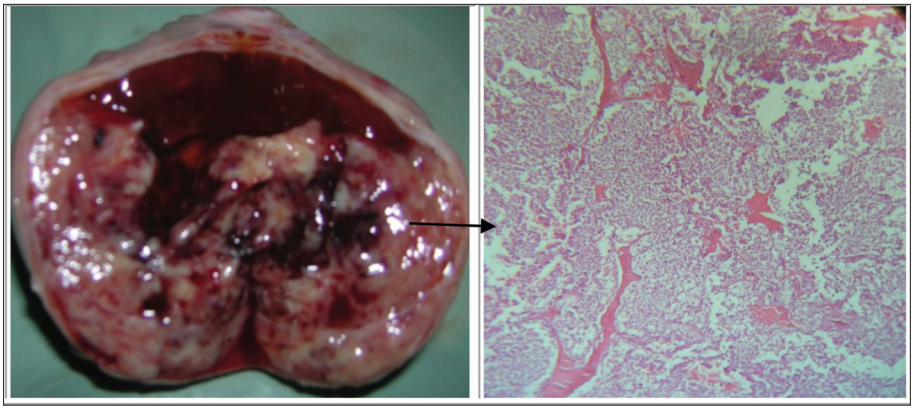


Fig 6. Ovarian tumor. Granulosa Cell Tumour (GCT): necrotic and hemorrhagic areas, uniform population of maldelimited cells, resembling granulosa cells, cytoplasm slightly mottled (GX 400)

Şekil 6. Yumurtalık tümörü. Granulosa hücre tümörü: Nekrotik ve hemorajik alanlar, granulosa hücrelerine benzeyen uniform yapılı hücre popülasyonu, sitoplazma az benekli görünümlü

milk production will be sold or sent to slaughterhouse. So, it is important that disorders of genital organs and their incidence must be defined to reduce the financial losses.

Among the 2025 reformed cows examined in this study, 16.49% were pregnant. The majority of the cases were at their beginning (less than 2 months), some cases were between 2 and 7 months. Concerning gestations of less than 2 months, it has been considered that these animals could not be diagnosed pregnant and that proves that the veterinary surgeons of the slaughterhouse find difficulties of establishing an early diagnosis of pregnancy per transrectal palpation. The finding of pregnancy cases in this study is between that reported by Al-Dahash and David [8] (23.36%) and that noted by Kaidi [9], which was 10.27%. The divergence of the results can be explained by the difference in the population of the examined cows, the areas of study and the material placed at the disposal of the veterinary surgeons at the slaughterhouse.

Double ovulation was noted on 7.07% of the non-pregnant genital tracts. Kidder et al.[10] and Lopez-Gatius et al.[11] reported high incidence (13.1% and 15.5%, respectively).

In the present study, any corpus luteum containing a cavity higher than 10mm of diameter and containing a liquid is regarded as cavitary. It was met in 7.76% of the cases. Few Studies reported the incidence of the cavitary corpus luteum, may be because it is not pathological and does not deteriorate the normal cycle of the cow [12,13]. So, "corpus luteum with a cavity" has been suggested to replace the classical term "cystic corpus luteum" [12].

Of the 1691 empty genital tracts examined in this macroscopic study, 53.28% presented abnormalities (at the level of the uterus, the oviducts or the ovaries). This incidence is lower than that reported by Elmarimi [14], which was 64.03%, but definitely higher than other findings in the literature, in which several authors reported varied frequencies. It was 11.9% by Perkins et al.[15], 8.4% by David et al.[16], and 9.78% by Nash et al.[17]. A similar frequency (9%) was noted by Drennan and Macpherson [18], in Canada. This difference could be due to the population of animals examined.

In the present study, the most common uterine disorder was the presence of infection (12.47%). The frequency of infection is lower than that obtained by Vallet et al.[19] who found 32.9% cases in 2024 cows. Result here was close to findings by Steffan [20] 17.2% and Elmarimi [14] who noted 6.30% of metritis and 7.50% of endometritis. Very low frequencies were reported by other authors; 1.23% [21], 2.5% [22] and 1.26% [7]. This divergence of results is surely due to the epidemiologic factors varied and different from an area to another, the number of examined animals and the time and criteria of diagnosis used by these authors. Endometritis was accompanied by inflammatory changes of other parts of the reproductive tract in some cases. These findings have supported some authors' opinions [7,23], that infectious agents in the vagina passing to cervix and uterine lumina may result in cervicitis and endometritis. In these cases, inflammatory lesions may have resulted from inadequate hygienic condition in the pospartum period and during parturition, retained placenta and traumatic laceration due to dystocia as reported by Jubb et al.[24]. Any congestion observed may also be due to the transrectal palpations carried out by the veterinary students during practical work.

The incidence of the congenital abnormalities was 0.95%. These included:

Twelve cases of double cervix (0.71%) in 4 cases, cervical canals were opened separately into the uterus. This result is higher than that noted by Hatipoglu et al.[7] (0.18%). According to Arthur et al.[25], these cases should conceive normally, but may generate dystocia due to a fetal limb entering each cervical canal.

One very rare case of triple cervix (0.06%), which has never been reported in the literature.

Unicornis uterus was detected in two cases (0.12%). Generally, this abnormality is known in the pathology as White Heifer Disease (WHD).

Malformation that resembles an obstruction at the level of the left horn was found in one case (0.06%).

The frequency of these abnormalities is in conformity

with those reported by Alam [4] and Kaidi [9].

Mucometra represents accumulation of strongly viscous aseptic liquid in uterine lumina [17]. It is associated with an anatomical lesion of the genital tract (congenital abnormality, abnormal long or tortuous cervix, adhesion, occlusion or obstruction) or with an ovarian pathology (especially the cystic ovary) [26]. In the present study, mucometra was found in 1.18% of cases and was associated with a tortuous cervix in six cases and in the rest with the ovarian cysts.

Leiomyoma is a uterine tumour with a nodular aspect, maroon, noninvasive, being able to reach 10-12 cm in diameter and is reported in cow, cat and especially bitch. Histologically, this tumour is formed by smooth muscle fibers with anarchistic disposition in the stroma [27]. This tumor was found in five cases (0.29%), with one case noted in the cervix.

The most common oviduct disorder was inflammatory changes (5.20%). The signs of inflammation (congestion and/or hypertrophy) were the basis for diagnosis. This disorder may be due to repeated transrectal palpations, realized by the veterinary students during their practical work on the cows whose genital apparatuses were the subject of this study. Presence of pus in oviduct was estimated by an incidence of 4.26% and it was associated in more than half of the cases with pyometra, which means ascending infection of the oviduct, in agreement with the report of McEntée [23]. The frequency of the hydrosalpinx was 01.30%. This lesion is associated with ovariobursal adhesion. It was bilateral in 12 cases, associated with cystic ovary. Oviduct adhesion was detected in 1.54% of the cases.

Ovariobursal adhesion is a structure constituted by fibrous bands between the surface of the ovary and the ovarian bursa and caused by an excessive follicular hemorrhage during ovulation, trauma by rectal examination and an infection from the uterus [28]. It can prevent the fertilization process when the fallopian tubes are blocked [29]. It was found in 5.38% of the genital apparatuses. Other results reported were 1.1% by David et al.[16], 2.7% by Roine [30], and 5.62% by Alam [4].

Smooth ovaries were regarded as the small ovaries on the 2 sides, right and left, with a surface which does not have any structure and having a congenital or acquired origin [31]. In the present study, these were found in 33 genital tracts with an incidence of 1.95%, not really close to that noted by David et al.[16] (0.33%), whereas it is definitely lower than that reported by Elmarimi [14] who found an incidence of 51.20%.

The microscopic examination performed on the fragments of four ovarian tumours found in this study revealed that in three cases, it was the Granulosa Cell Tumour (GCT), which is a type of sex cord-stromal tumour, composed primarily of neoplastic granulosa cells [32], and it is the most common bovine ovarian tumour in cattle [32-35]. In one case, it was the cystadenoma. This tumour is comparatively rare in domestic mammals and it has been mainly described in the bitch, sow and mare [36]. Pérez-Martınez et al.[34] reported a GCT frequency of 0.74% in 1489 reformed cows.

Typical purulent ovaries were observed (0.23%). The yellowish white aspect of the pus, recalling caseous necrosis pathognomonic of tuberculosis, presented in the different parts of genital tracts enabled us to pose the diagnosis of ovarian and endometritis tuberculosis. The low frequency noted was similar to the data in the literature [37].

Parovarian cysts are cystic structures that occur in the broad ligament close to the ovaries and the uterine tubes [15]. The parovarian cysts were noted by a weak frequency, in conformity with the results reported by some authors [3,38].

Follicular cysts in cows are hypertrophic follicles which do not ovulate in the estrus period [39]. They are involved in reduced fertility of dairy cows [40]. It is significant to note that the cystic follicles which exceeded 2.5 cm in diameter (except for the hemorrhagic microcystic ovary) were gathered under this pathology. Ovarian cyst was found in 241 cases (14.25%). This frequency is close to that reported by Silva et al.[41] which was 11% knowing that it always remains in the average reported in the literature which is 10 to 15% [42,43]. The factors which return in question of this difference in frequencies are mainly the methods and the criteria used to diagnose the cystic ovary, the epidemiological factors and the number of the examined animals.

According to the present study, the right ovary was more affected by ovarian cyst than the left one, which is in conformity with the result of Kaikimi et al.[44]. In the majority of the cases, the cyst was single (84.65%) whereas polycystic ovaries were found in 15.35%. The incidence of the latter recorded by Silvia et al.[45] exceeded our result (47%). Incidence of follicular cyst (FC) with thin wall was 70.57%. Luteal cysts (LC) were diagnosed in 29.05%. That is in conformity with the data of the literature [43] (70% FC and 30% LC).

The interpretation of the results obtained following the macroscopic and microscopic studies that were realized here on genital apparatuses recovered at the slaughterhouse in Algeria, enabled the following: A high frequency of the reformed pregnant cows; majority of these cases were at their beginning. Among the reproductive abnormalities, incidence of the ovarian cyst is the most significant followed by that of uterine infection, which proves that conditions of contamination leading to uterine infection are always present in the cowsheds and that anarchistic use of the treatments (synchronization and/or induction of estrus) and underfeeding would be probably the causes of ovarian cysts found in the present study. So, these results

will be important to the cattle breeders and clinicians. The majority of ovarian cysts diagnosed in this study had a thin wall macroscopically, and were classified as follicular cyst. In the authors' opinion, the discovery of the triple cervix opens the door for researchers to find more new abnormalities in the genital tracts.

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Research Article

Comparison of Three Fixation Methods for the Prevention of Wound Contractions in Diabetic and Non-Diabetic Mice with Full-Thickness Skin Excision [1]

Turgut ANUK [1] Savaş ÖZTÜRK [2] İsa ÖZAYDIN [2] Şahin KAHRAMANCA [3]
Sadık YAYLA [2] Özgür AKSOY [2] Zafer OKUMUŞ [4]
Uğur AYDIN [2] Bülent Çağlar BİLGİN [1] İbrahim DEMİRKAN [5]

[1] *Presented at 1st International Turkey Veterinary Surgery Congress (oral presentation), 11-14 May 2016, Erzurum, Turkey*
[1] Kafkas University, Faculty of Medicine, Department of General Surgery, TR-36100 Kars - TURKEY; [2] Kafkas University, Faculty of Veterinary Science, Department of Surgery, TR-36100 Kars - TURKEY; [3] Kars State Hospital, Depaetment of General Surgery, TR-36300 Kars - TURKEY; [4]Atatürk University, Faculty of Veterinary Science, Department of Surgery, TR-25240 Erzurum - TURKEY; [5] Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Surgery, TR-03100 Afyon - TURKEY

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Abstract

The most crucial problem in experimentally induced excisional wounds in animals with slim-skinned and poor subcutaneous attachment is wound contraction formed during recovery period. The contraction is mainly induced by myofibroblasts leading to early wound closure. This may be associated with false outcomes of the study. Recently, various trials have been made to reduce the contraction in full-thickness excisional models of wounds. This study aimed at comparing different fixation methods on the minimization of wound contraction. Study was carried out in two main groups as non-diabetic (Group I, n=24) and diabetic (Group II, n=24) and one control group. The animals in each group were divided into four equal subgroups (Group I-A, I-B, I-C, I-D; Group II-A, II-B, II-C, II-D) and 1.2x1.2 cm-sized full-thickness of skin excisions were generated on the dorsum of animals. No fixations were applied in subgroups I-A and II-A however, interrupted suture in subgroups I-B and II-B, suture passing from a couple of points through skin and subcutaneous tissues placed in each side of wound tied only in corners in subgroups I-C and II-C and using a polyethylene fixatives that fixed in wound edges with interrupted sutures in subgroups I-D and II-D were achieved to produce wound fixation methods. Digital photographs of the wound were taken on the 3rd, 7th, 14th and 21st days of the study, reduction ratios of the wounds were compared and the efficiency of methods for the prevention of contraction was examined. When wounds were evaluated comparatively after fixation, it was observed that three different applied fixation methods were efficient in prevention of wound contraction in all phases in comparison to control groups. Moreover, it was observed that contraction was highly impeded in interrupted suture groups (I-B, II-B) and polyethylene-reinforced interrupted suture groups (I-D, II-D) when compared to suture groups tied only in corners. The results revealed that wound surface area shrinkage should be taken into consideration in experimental studies to avoid misleading consequences.

Keywords: *Wound, contraction, Full-thickness skin, Excision, Fixation, Mice*

Tam Katmanlı Deri Eksizyonu Oluşturulmuş Diyabetik ve Non-Diyabetik Farelerde Yara Kontraksiyonunun Önlenmesi İçin Üç Farklı Fiksasyon Yönteminin Karşılaştırılması

Özet

İnce derili ve zayıf derialtı bağlantılı deney hayvanlarında oluşturulan eksizyonel yara modellerinde en önemli sorun iyileşme döneminde şekillenen yara kontraksiyonudur. Daha çok myofibroblastların neden olduğu bu durum yaranın erken kapanmasına ve yanıltıcı sonuçların ortaya çıkmasına neden olabilmektedir. Son zamanlarda, tam katmanlı eksizyonel yara modellerinde kontraksiyonu redükte etmek için çeşitli denemeler yapılmıştır. Bu çalışma ile yara kontraksiyonunun minimize edilmesine yönelik üç farklı fiksasyon yönteminin karşılaştırılması amaçlandı. Çalışma, non-diyabetik (Grup I, n=24) ve diyabetik (Grup II, n=24) olmak üzere 2 ana grupta yürütüldü. Her bir gruptaki hayvanlar 4 eşit alt gruba ayrılarak (Grup I-A, I-B, I-C, I-D; Grup II-A, II-B, II-C, II-D) hayvanların sırt bölgesinde 1.2x1.2 cm ölçülerinde tam katmanlı deri eksizyonu oluşturuldu. I-A ve II-A gruplarında herhangi bir fiksasyon uygulanmazken, I-B ve II-B gruplarında ayrı dikiş, I-C ve II-C gruplarında deri ve deri altı dokulardan birkaç noktadan geçecek şekilde her bir yara kenarına yerleştirilip sadece köşelerde düğümlenen dikiş, I-D ve II-D gruplarında ise polietilen bir fiksasyon materyalinin ayrı dikişlerle yara kenarına tespit edildiği fiksasyon modelleri oluşturuldu. Çalışmanın 3, 7, 14 ve 21. günlerinde yara bölgesinin dijital fotoğrafları alınarak yaraların küçülme oranları karşılaştırıldı ve kullanılan yöntemlerin kontraksiyonu engellemedeki etkinlikleri değerlendirildi. Fiksasyon sonrası yara alanları karşılaştırmalı olarak değerlendirildiğinde, uygulanan her üç fiksasyon yönteminin bütün aşamalarda kontrol gruplarına göre yara kontraksiyonunu engellemede etkili olduğu gözlendi. Ayrıca, tüm kontrol günlerinde ayrı dikiş (I-B, II-B) ve polietilen destekli ayrı dikiş (I-D, II-D) gruplarında, sadece köşelerde düğümlenen dikiş gruplarına göre kontraksiyonun daha fazla engellendiği de saptandı. Çalışmanın sonuçları, yara alanının küçülmesine dayalı deneysel çalışmalarda, biyolojik bir süreç olan yara kontraksiyonunun dikkate alınması ve yanıltıcı sonuçlardan kaçınmak için deney protokollerinin uygun bir fiksasyon eşliğinde yürütülmesi gerektiğini ortaya koymuştur.

Anahtar sözcükler: *Yara kontraksiyonu, Tam katmanlı deri eksizyonu, Yara fiksasyonu, Fare*

İletişim (Correspondence)
+90 532 6974498
turgutanuk@gmail.com

INTRODUCTION

Wound healing can be defined as tissue regeneration of mechanic, cellular and biochemical process initiated after trauma. The process continues at three successive stages: hemostasis and inflammation phase, proliferation (granulation) phase and maturation phase [1,2]. In human and veterinary medicine, experimental studies on animal models are widely used as an important biological approach to clarify the pathophysiology of wound healing and to identify new strategies [3,4]. Even if different animal species are used for this purpose, mice and other rodents are extensively preferred because their care and feeding are easy, they reproduce and grow quickly, suitable for application of surgical procedures and their biology is thoroughly investigated [3,5]. On the other hand, an excessive skin contraction is a reality and edges of the wound move rapidly towards the centre. While the wound closes, the central portion undergoes keratinization; thus premature and bad wound healing occurs. This may be associated with that these animals' skin has thin and loosed skin, weak subcutaneous connection and little adipose tissue. It is known that the wound contraction accomplished by fibroblasts, particularly myofibroblasts is more effective in the first 2-8 weeks of wound healing [6]. Since the studies on the contraction-based early wound closure especially those investigating various medical drugs on wound healing may produce misleading and erroneous results. It has been advocated that the experiment regarding full-thickness excisional wounds should be carried out with skin fixation [7]. A few fixation materials and methods were reported to fix the edges of the wound [2,6-8]. Nevertheless, it is difficult to emphasize an ideal fixation method that can be used for all the wound models.

In this study, the efficiency of skin fixation simplified from previous complex models in the prevention of wound contraction after full-thickness excisional skin wounds were evaluated in mouse model. Also, to determine whether diabetes affects wound contraction, the study was carried out in two groups as a diabetic and a non-diabetic. The wound area and the wound area reduction rate were digitally measured.

MATERIAL and METHODS

Ethical Approval

The study was performed after the approval by the local ethical committee (No: KAÜ-HADYEK 2014/060).

Animals

A total of 48 male, Swiss Albino mice, aged 8-12 week were included in the study. Mice were homed in separate cages under standard laboratory conditions (12 h dark/12 h daylight, 45%-55% of humidity rate and room temperature at 20-22°C). Animals were fed with a standard feed and water was supplied as *ad libitum*.

Study Groups

Animals were allocated into two main groups as non-diabetic (group I, *n*=24) and diabetic (group II, *n*=24). Each group were further divided into four equal subgroups (groups I-A, I-B, I-C, I-D; II-A, II-B, II-C, II-D). The following procedures were applied;

- *Control groups (I-A and II-A):* Skin was incised but sutures were not applied *(Fig. 1-A)*,

- *Simple interrupted suture groups (I-B and II-B):* Wound edges were fixed using 12 sutures in equal numbers for each wound side after skin excision *(Fig. 1-B)*,

- *Groups tied of sutures in the corners of wound (I-C and II-C):* Sutures were tied in the corners of wound by firmly stretching the knots *(Fig. 1-C)*.

- *Polyethylene-supported suture groups (I-D and II-D):* Wound was fixed with 12 sutures using a polyethylene material made from a fluid infusion bag according to the wound size *(Fig. 1-D)*.

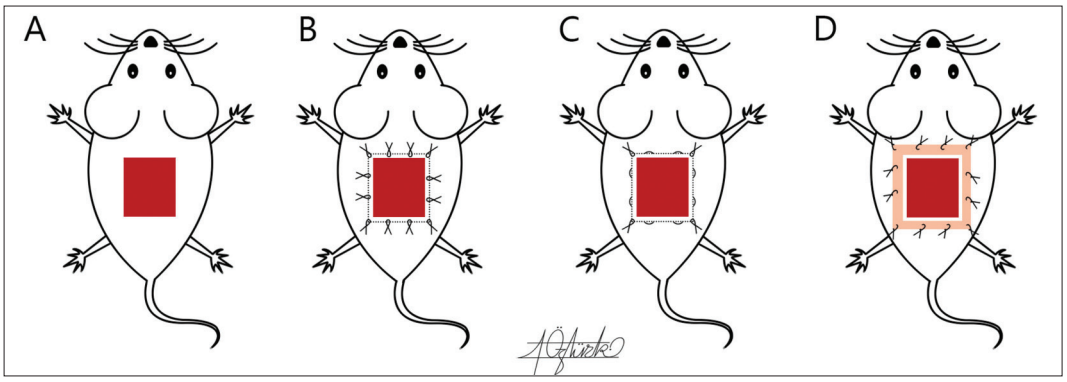


Fig 1. Excision and fixation models. **A-** Excised wound (control groups), **B-** Excision + simple interrupted suture, **C-** Excision + suture tied in the corners of the wound, **D-** Excision + polyethylene-supported simple interrupted suture

Şekil 1. Eksizyon ve fiksasyon modelleri. **A-** Yara eksizyonu (kontrol grupları), **B-** Eksizyon + basit ayrı dikiş, **C-** Eksizyon + yara köşelerinde düğümlenen dikiş, **D-** Eksizyon + polietilen materyal destekli basit ayrı dikiş

Induction of Diabetes

To produce diabetic animals, freshly prepared streptozotocin in 0.1M sodium citrate buffer solution (pH 4.5) [9] was intraperitoneally administered in a single dose (200 mg/kg). On the 3rd day of injection, preprandial blood glucose level was measured by application of a capillary tube to medial eye canthus. Mice showing over 250 mg/mL (13.9 mMol/dL) level were considered as diabetic [9,10].

Excisional Wound and Sutures

General anesthesia was induced by injecting the mixture of 10 mg/kg xylazine HCl (Rompun, 2%, Bayer) and 100 mg/kg ketamine HCl (Ketalar, 50 mg/ml, Pfizer) intraperitoneally. Dorsal regions of animals were clipped and disinfected with povidone iodine + 70% ethanol. Then, square shaped skin incision in size of 12x12 mm was made by a surgical knife. Skin and adipose tissues were dissected with the help of forceps and surgical scissors creating full-thickness skin excision *(Fig. 1)*. In control subgroups, no surgical intervention was applied (subgroups I-A and II-A) *(Fig. 1-A)*. In simple interrupted suture subgroups (I-B and II-B), 12 sutures were placed on wound edges in 3 mm apart from incision line. Skin was fixed to subcutaneous tissues and *panniculus carnosus (Fig. 1-B)*. In suture tied in the corners of wound subgroups (I-C and II-C; *Fig. 1-C)*, sutures located on each wound edge (in a way to pass through skin and subcutaneous tissues) were tied to their adjacent sutures stretched enough for secure fixation. In polyethylene-supported groups (I-D and II-D), a squarely-prepared polyethylene material was placed on skin after its central part was spread out more widely than the size of wound and then it was fixed with 12 simple interrupted sutures *(Fig. 1-D)*. Thus, the polyethylene material generated more force for fixation by exerting pressure on the skin. In all suture groups, 4/0 polyglactin 910 (Vicryl - Ethicon) was used as suture material. Wounds were left open in all groups and any local and/or systemic treatment was not administered during the entire study.

Wound Area Measurement and Wound Area Reduction Calculation

On the 0th, 3rd, 7th, 14th and 21st days of the study, wound area of all the subjects were logged on graph papers via a digital camera (Samsung WB2100). Digital image analyses were performed using a software program (Stereo Investigator 7.0; MBF Bioscience US). All image measurements were calibrated for each image with the help of graph paper therefore error probability originating from digital systems was eliminated. Wound areas were specified and they were measured with a 0.5 mm-sized point grid. Data were figured in mm^2. Wound area reduction rate (%) was calculated by means of following equation [2]:

$$\frac{\text{Created wound area} - \text{Measured wound area}}{\text{Created wound area}} \times 100$$

Statistical Analysis

Data generated in the study were evaluated by Anova, one-way test after being subjected to normality test (Minitab 17 Packed Programme). The significance level was set at $P<0.05$.

RESULTS

Measurements of wound sizes on the 0th day, and after excision and fixation days of 3, 7, 14 and 21 in non-diabetic and diabetic groups were given in *Table 1*. On the 3rd day after surgery showed that wound area in both non-diabetic and diabetic control groups decreased significantly ($P<0.05$) compared to fixation groups. In non-diabetic simple interrupted suture group the wound area was larger with respect to other fixation groups. While wound area in non-diabetic control group decreased significantly ($P<0.05$), on the 7th day, no significant difference was seen among fixation groups.

In diabetic group, wound areas in subgroups II-C and II-D were significantly larger then not only in II-A and II-B

Tablo 1. *Non-diyabetik ve diyabetik deney gruplarında kontrol günlerinde saptanan yara yüzey alanı değerleri (ortalama± standart sapma)*
Table 1. *The values of wound area determined in non-diabetic and diabetic experimental groups on control days (mean± standard deviation)*

Day	Wound Area (mm^2)							
	Non-diabetic (n=24)				Diabetic (n=24)			
	I-A (n=6)	I-B (n=6)	I-C (n=6)	I-D (n=6)	II-A (n=6)	II-B (n=6)	II-C (n=6)	II-D (n=6)
0	143.8±1.8 [a]	144.0.2±1.4 [a]	143.5±0.9 [a]	144.01±0.9 [a]	143.08±1.2 [a]	144.01±1.4 [a]	143.07±1.6 [a]	144.27±2.2 [a]
3	54.31±4.8[a]	103.66±11.3[b]	77.69±6.7[c]	83.63±7.4[bc]	60.94±6.2 [a]	86.38±7.6 [bc]	89.94±5,2 [bc]	83.88±1.6 [bc]
7	29.31±2.2[a]	43.06±3.8 [b]	41.94±3.8 [b]	40.42±4.6[b]	40.56±4.6[b]	45.38±5.3 [b]	61.94±4.9 [c]	68.25±5.6 [c]
14	3.25±0.75 [a]	11.88±7.0 [b]	15.22±7.1 [b]	11.93±0.5[b]	4.50±2.00 [a]	14.81±4.7 [b]	20.06±1.3 [c]	31.25±2.3[d]
21	1.43±1.5 [a]	3.68±0.5 [a]	2.81±1.4 [a]	3.43±1.5 [a]	3.00±1.4 [a]	7.50±1.0 [b]	3.44±1.4 [a]	16.06±1.4 [c]

[a,b,c,d] *Different letters on each line show statistical differences (P<0.05)*

subgroups but also those in non-diabetic subgroups. Wound area reduction in non-diabetic control group showed significant difference in comparison to fixation groups on the 14th day as for the 7th day ($P<0.05$). The difference among in subgroups I-B, I-C and I-D were not significant, while wound area in diabetic control group decreased significantly in comparison to other diabetic subgroups at the same period. Wound area values in subgroup II-C were significantly larger with respect to subgroup II-B. Moreover wound area value in subgroup II-D was significantly larger than subgroups II-B and II-C ($P<0.05$). According to wound area measurements in non-diabetic and diabetic groups, on the 21st day, values in diabetic subgroups II-B and II-D were significantly different between in-groups and inter-groups *(Table 1)*.

Reduction ratios in wound areas were presented respectively in *Fig. 2* and *Fig. 3* with respect to non-diabetic and diabetic groups. While the least reduction ratio in wound area was observed in subgroup I-B on the 3rd day. Reduction rate on the 7th and 14th day was higher only in control subgroup than other groups. However there was no significant difference between groups on the 21st day. Despite no significant difference in diabetic groups with regard to reduction rates of wound areas on the 3rd day, it was noted that subgroups II-C and II-D on the 7th and 14th day and group II-D on the 21st day showed less reduction in comparison to other groups.

DISCUSSION

Since *in vitro* models remain insufficient to generate conclusive wound healing process the *in vivo* settings mandatory to be performed. Different aspects of wound healing and pathophysiology have to be understood at least for designing new therapeutic strategies. It is necessary to create models that incision, tissue loss, flap, graft and diabetes for rational approach towards different clinical problems. The use of experimental animals is highly crucial biological tools in human and veterinary medicine and it seems to remain reliable option as it was. Rodents are mostly chosen as experimental animals for acute and chronic wound model because their care and feeding are easy, they reproduce and grow quickly and their biological characteristics are well understood [3-5,11,12]. On the other hand, they create a significant problem during experimental studies in wound healing due to a loose connection between skin and subcutaneous tissues in these highly thin-skinned animals. Panniculus carnosus muscle is responsible for the wound contraction [2,6]. This biological event where myofibroblasts generally take an active role causing the wound to close earlier by pulling wound edges rapidly towards its centre [2]. For this reason, in full-thickness models of open wound in rodents, experimental protocols have to be implemented after providing skin fixation to achieve correct measurements. The adequate

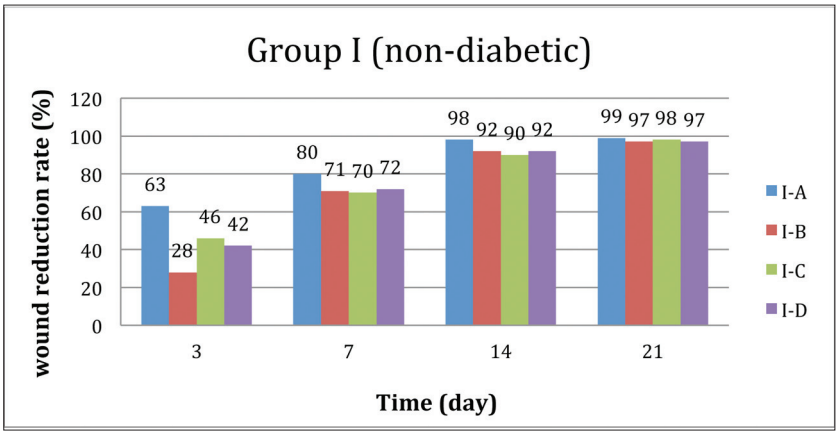
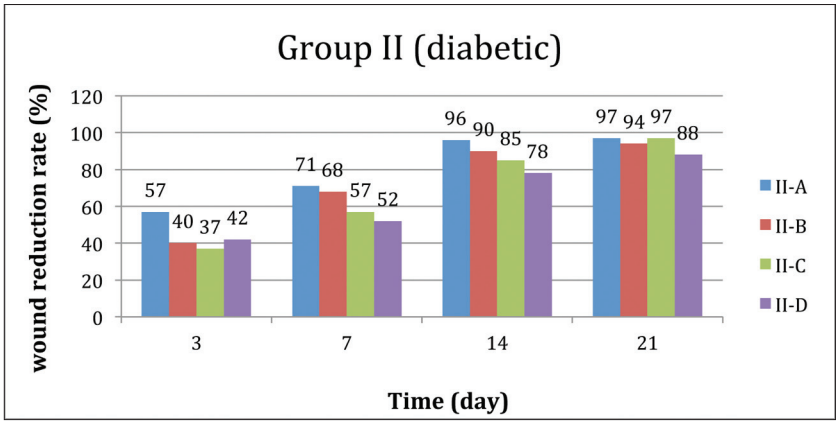


Fig 2. Reduction rates of wound area in Group-I (non-diabetic) (%)

Şekil 2. Group-I (non-diabetic) yara alanı küçülme oranları (%)

Fig 3. Reduction rates of wound area in Group-II (diabetic) (%)

Şekil 3. Group-II (diabetic) yara alanı küçülme oranları (%)



and correct models are one of the fundamental aspects of studies on the formulation of therapeutic strategies [3,13]. This study, without consideration of any therapeutic goal, aimed at measuring how full-thickness excisional acute wounds in mice can be affected by no treatment or fixation by various options in terms of wound contraction. Since wound contraction generally progress together with inflammation (1-4 days) and proliferation (4-21 days) process, this was taken into consideration while controlling animals. Different methods have been described with the purpose of wound fixation in rodents. The basic approach of these studies is based on developing fixation materials that are glued or stitched to wound edges producing resistance to wound contraction. Metals (titanium, steel), splints prepared in shape of silicon-like circle or variants [6-8,14], adhesive materials such as Tegaderm, Duoderm, and polyvinylchloride are some of the materials used for this purpose. It was shown that some methods were successful but others inadequate for the prevention of contraction [2]. Therefore, it is difficult to explain an ideal fixation material and method for all wound management, now. The aims of the experiment or wound characteristics are all important aspects for determining fixation methods. An ideal experimental model of wound should sufficiently reflects new tissue formation process and re-epithelization associated with biology of wound healing however it should have a minimum effect on wound contraction. The cost of fixation method should be affordable, easily applicable and effective in minimization of contraction [8].

In experimental animal models, some coworkers prefer to study on auricle (rabbit), scalp and tail skin where wound contraction is minimum instead of skin and subcutaneous tissues of other parts of the body that create strong contraction [3,13]. Three different fixation methods used in this study were significantly effective in non-diabetic and diabetic models for reduction of contraction in comparison with corresponding control groups. These effects were observed more specifically on 3rd, 7th and 14th days of wound area measurements in particular, whereas any superiority was not identified between groups where suture and suture-supported polyethylene material fixation were used. Wound areas in diabetic subgroups where simple uninterrupted suture (II-B) and polyethylene material-supported suture (II-D) used failed to display considerable wound closure compared to other groups on the 21st day. The wound reduction rate in diabetic control group was almost similar to non-diabetic groups. This may suggest that the diabetes may not play significant role in wound contraction. Hence, this condition may be largely explained that the wound healing is disruption due to diabetes but not wound contraction.

This study showed that three different employed fixation methods are considered as efficient in reduction of wound contraction in full-thickness excisional wound models in rodent. Thus, it was concluded that the wound fixation models presented here were simple and easy to apply for the determination of duration and degree of granulation formation, tissue proliferation and re-epithelialization for wound healing studies.

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Research Article

Effects of Subclinical Mastitis on Serum Estradiol and Tumour Necrosis Factor Alpha Levels During Estrus in Dairy Cows [1]

Bayazıt MUSAL [1] Süheyla TÜRKYILMAZ [2] Hakkı Bülent BECERİKLİSOY [1]
Cevdet PEKER [1] Eyyüp Hakan UÇAR [1]

[1] *This study was financially supported by the Scientific Reseach Fund of Adnan Menderes University by the project number VTF-14002, 2014*

[1] Adnan Menderes University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, TR-09010 Aydin - TURKEY
[2] Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, TR-09010 Aydin - TURKEY

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Abstract

The effect of subclinical mastitis on serum estradiol and tumor necrosis factor alpha (TNF-α) levels during oestrus and subsequent fertility was investigated in dairy cows. 40 cows were divided into two groups as healthy control (n=20) and subclinical mastitis (n=20), according to the results of California Mastitis Test (CMT) and bacteriological isolation and identification. Cows were synchronised with a standart 7 day Ovsynch protocol. Following prostaglandin F2alpha (PGF2α) administration, cows were examined with trans-rectal ultrasonography at 24, 36 and 48th h, dominant follicle diameters were recorded and blood samples were collected. Sixteen h after the second gonadotrophin-releasing hormone (GnRH) administration, cows were inseminated and a final examination of ovaries were performed and dominant follicle diameters were recorded. estradiol and TNF-α concentrations were analysed with ELISA in serum samples. No significant differences were found between the follicular diameters and growth patterns (P>0.05) of the two groups while estradiol concentations were significantly higher in the subclinical mastitis group than the control group at 24 and 48 h after PGF2α injection (P=0.017 and P=0.036 respectively). Also TNF-α levels were significantly higher in cows with subclinical mastitis than the control group (P=0.03). Positive correlations were observed between estradiol and TNF-α levels, in both groups (Control Group: R=0.512, P=0.021; Subclinical Mastitis Group: R=0.826, P<0.001). Overall pregnancy rate was higher in the control group (40%) than the subclinical mastitis (25%) group however the difference was not statistically significant (P>0.05). In conclusion estradiol and TNF-α concentrations were found higher in cows with subclinical mastitis during estrus and this data may be due to a luteal insufficiency during the initiation of synchronization, however further studies are required.

Keywords: *Cow, Subclinical mastitis, Estradiol, TNF-α*

Sütçü İneklerde Östrüs Sırasında Serum Östradiol ve Tümör Nekrozis Faktör Alfa Düzeyleri Üzerine Subklinik Mastitislerin Etkisi

Özet

Süt ineklerinde subklinik mastitislerin serum östradiol ve tümör nekrozis faktör alfa (TNF-α) düzeyleri ve fertilite üzerine etkileri araştırıldı. Kaliforniya Mastitis Test (CMT) ve bakteriyolojik izolasyon identifikasyon sonuçlarına göre 40 inek sağlıklı kontrol (n=20) ve subklinik mastitisli (n=20) olmak üzere iki gruba ayrıldı. İnekler standart bir 7 günlük Ovsynch protokolü ile senkronize edildi. Prostaglandin F2 alfa (PGF2α) uygulamasını takiben inekler 24, 36 ve 48. saatlerde transrektal ultrasonografi ile muayene edildi, dominant follikül çapları kaydedildi ve kan örnekleri toplandı. İkinci gonadotropin salgılatıcı hormon (GnRH) uygulamasını takiben 16. saatte inekler tohumlandı, son bir ovaryum muayenesi yapıldı ve dominant follikül çapları kaydedildi. Serum örneklerinde östradiol ve TNF-α düzeyleri ELISA ile belirlendi. Folikül çapları ve folliküler gelişim özelliği yönünden iki grup arasında önemli bir fark görülmezken (P>0.05), serum östradiol konsantrasyonları subklinik mastitis grubunda kontrol grubuna göre PGF2α enjeksiyonunu takiben 24 ve 48. saatlerde belirgin ölçüde yüksek bulundu (sırasıyla, P=0.017 ve P=0.036). Benzer şekilde TNF-α düzeyleri subklinik mastitisli ineklerde kontrol grubuna göre anlamlı düzeyde yüksekti (P=0.03). Her iki grupta da östradiol ve TNF-α düzeyleri arasında pozitif korelasyonlar bulundu (Kontrol Grubu: R=0.512, P=0.021; Subklinik Mastitis Grubu: R=0.826, P<0.001). Toplam gebelik oranı kontrol grubunda (%40) subklinik mastitisli gruba (%25) göre daha yüksek bulundu ancak bu fark istatistiksel açıdan anlamlı değildi (P>0.05). Sonuç olarak subklinik mastitisli ineklerde östrus sırasında östradiol ve TNF-α düzeylerinin yükseldiği, bunun senkronizasyonun başlangıcındaki bir luteal yetersizliğe bağlı olabileceği ve bu konuda daha fazla çalışmanın gerekli olduğu belirtildi.

Anahtar sözcükler: *İnek, Subklinik mastitis, Östradiol, TNF-α*

İletişim (Correspondence)
+90 532 4935443
bmusal@adu.edu.tr

INTRODUCTION

It has been well documented that both clinical and subclinical mastitis have negative impact on reproductive performance of dairy cows [1-4]. The interval for first post-partum insemination were delayed and the service for conception rate significantly increased in cows experiencing clinical mastitis compared to non-mastitic cows [2,4,5]. Long term nature of subclinical infections can damage the long process of follicular growth at various time points. It was observed that 32% of cows having subclinical mastitis exhibited low follicular estradiol concentrations compared to the uninfected and other subclinically infected cows. Low follicular estradiol levels in these cows were thought to be originated from the abnormally low expression of luteotrophic hormone (LH) receptor and steroidogenic genes in both theca and granulosa cell layers. However follicular estradiol concetrations in the remaining two-thirds of the subclinically infected cows were not affected and the reason of this difference remained unclear [6].

The release of proinflammatory cytokines such as interleukins, tumor necrosis factor-α (TNF-α) and eicosanoids, as a result of intramammary infections, have been suspected to be the possible causes of alterations in steroid production inside the follicular environment. The release of these substances in milk have been clearly observed during acute phase of challenge with experimental *Escherichia coli* Lipopolysaccharide (LPS) endotoxin [7]. Moreover in-vitro treatment by TNF-α reduced theca and granulosa cells androstenedione and estradiol production, respectively whilst the in-vivo effects were less pronounced [8]. Although these results have been observed clearly in clinical mastitis cases caused by Gram negative bacteria or via exposure to LPS endotoxin, mucopeptide layers of Gram positive pathogens also have been reported to possess the capability for inducing pyretic and cytokine responses like TNF-α [9].

Most of the previous studies have been focused on the effect of clinical mastitis on reproduction while the information about the subclinical cases remains relatively low. In two studies the increase in levels of milk TNF-α in naturally occured subclinical mastitis cases have been reported [10,11]. Only in one of these systemic concentrations of TNF-α were measured and found to be unaffected [10]. In the present study our aim was to observe follicular growth patterns, estradiol and TNF-α concentrations in serum of healthy cows and cows with subclinical mastitis in order to detect a possible detrimental effect of subclincal mastitis on these parameters thus pregnancy rates obtained at a single fixed time artificial insemination.

METERIAL and METHODS

The study was conducted in a dairy herd with a total size of 120 Holstein cows in the district of Izmir city. The cows were housed in tie-stall barns, connected with open air pens year-round without pasturing. Cows had free access to water, mainly fed with corn silage and concentrate feed (Crude Protein: 16.72%, 2800 kcal/kg) and bedded on mats inside the housing. The cows were milked twice daily and average milk production in the course of study was 32 kgs. The study was conducted between November 2014 - May 2015. A total of 40 multiparous cows with an average of 145 days in milk, were included in the study during monthly visits as groups consisting of 8 cows approximately. Cows (mean age: 4.8 years) free from any kind of infectious disease, especially checked out for clinical mastitis, metritis and foot problems were chosen for the study. All procedures used in the study were approved by the Local Ethical Comittee of Adnan Menderes University (ADÜ-HADYEK-Session number: 64583101/2014/059).

Primarily a standard Ovsynch protocol were administered starting by an injection of 2.5 mL of GnRH (Buserelin acetate, 0.004 mcg/mL, i.m. inj. sol., Receptal®, İntervet, Turkey) regardless of the stage of the cycle. On Day 7 a single dose of 5 ml $PGF_2\alpha$ (Dinoprost thromethamine, 5 mg/mL i.m. inj. sol., Dinolytic®, Zoetis, Turkey) was injected. On 24th, 36th and 48th h after $PGF_2\alpha$ injection, both ovaries were examined for dominant follicle by a portable real time B-mode ultrasonography (USG) equipped with a 5-7.5 MHz linear probe (Hasvet 838, Hasvet, Antalya) and the size of the dominant follicles were recorded. In addition to the examinations, each time a blood sample were collected into silicone coated tubes from *vena jugularis*. The blood samples were kept in room temperature for several h for clot formation and then sera were harvested following centrifugation with 3.000 rpm for 10 min. The serum samples were dispensed into microcentrifuge tubes, labeled and stored in -20°C until the time of hormone analysis. On 48th h upon transrectal ultrasonography and blood samplings the second GnRH injection were administered. Following this, at 16-18 h a final USG examination performed, the size of the follicle recorded and the cows were inseminated by the herd' s veterinarian. Finally pregnancy diagnoses were performed by transrectal USG around day 45 following artificial insemination to all cows.

On the first day of examinations, in other words at the 24th h following the $PGF_2\alpha$ administration in the milking parlor California Mastitis Test (CMT) was used to assign the cows into groups as healthy control (n=20) and subclinical mastitis (n=20). Cows having 2 or more mammary lobes CMT +1 or higher scores were included into the subclinical mastitis group. Following this milk samples from all cows were collected aseptically into tubes and these samples were taken immediately to the Laboratory of Department of Microbiology, Faculty of Veterinary Medicine, Adnan Menderes University (Aydin city).

Isolation and Identificiation of Pathogens

Milk samples were inoculated into blood agar and incubated at 37°C for 24 h. Samples obtained from the

colonies were than passed into brain heart infusion agar with 20% glycerine and stored in -20°C for further molecular analysis of bacteria type. At the end of the field study all of the stored samples were thawed and isolated bacterial DNA were extracted using Instagen DNA extraction kits (Instagene matrix, Bio-Rad, CN: 732-6030). Using PCR 16s rRNA, gene fragments were amplified and sequential analysis were done for bacterial identification. For this purpose S16S20 (5' AGA GTT TGA TCC TGG CTC AG 3') and 16S1390 (5' GAC GGG CGG TGT GTA CAA) universal primers were used. PCR products were visualised in 1% gel. These amplicons were sent to a commercial laboratory (Macrogen Inc./Korea) for sequential analysis. Later on the results of the sequential analysis were compared with the gene bank and species identification of the isolated bacteria were completed.

Analysis of Estradiol and TNF-α Levels

Stored serum samples were sent to a commercial laboratory (Farmasina, Istanbul/Turkey) for the analysis of estradiol and TNF-α using bovine species specific ELISA kits (Sunred Biotechnology Company®, China). The catalogue numbers, sensitivities and assay ranges and inter-assay and intra-assay coefficients of variations of these kits were as follows, respectively: Bovine estradiol ELISA kit (CN:21-04-210), 0.801 pg/mL, 1-300 pg/mL, <9%, <11%; Bovine TNFα ELISA kit (CN:201-04-0007), 14.155 ng/L, 15-4000 ng/L, <9%, <11%. While estradiol concentrations were measured in all of the samples, TNF-α were measured only in the first (24th h) samples.

Statistical Analysis

Statistical analyses were performed using the SPSS software version 22. For all variables tested, normality was checked by Shapiro-Wilk test. Intergroup relationships of the parameters (follicular size, estradiol, TNF-α) were analysed with Independent Samples T-test. The observed differences in parameters (follicular size, estradiol) by time were analysed with variance tests for repeated measures and Bonferroni pairwise comparisons. Correlations were analysed by Pearson correlation tests. Differences with P values less than 0.05 were considered to be significant. The results were presented as the mean ± SEM.

RESULTS

Intramammary Infections

The aseptically collected milk samples have revealed a variety of mastitis pathogens mostly consisting of Gram positive cocci from the 20 subclinically infected cows. All of the cows in the mastitis group had two or more mammary lobes infected and most of these cows had multiple pathogens residing in each mammary lobe. The types and distribution of these pathogens are demonstrated in *Table 1*.

Follicular Growth

Mean follicle diameters at 24, 36, 48 and 64th h were all slightly larger in the controls compared to the subclinically infected cows however these differences were not statistically significant (P>0.05). Also no statistically significant differences were observed in the follicular growth patterns throughout the repeated examinations inside each group (P>0.05). Follicle diameters throughout the study are presented in *Table 2*.

Table 1. *The isolated and identified mastitis pathogens*
Tablo 1. *İzolasyonu ve identifikasyonu yapılan mastitis patojenleri*

Isolated Patogens	No of Lobes Isolated	No of Cows Isolated
Gram Positive Pathogens		
Staphylococcus aureus	3	2
Streptococcus agalactiae	9	8
Coagulase negative Staphylococci (CNS)	13	9
Corynebacterium spp.	18	11
Aerococcus viridans	23	13
Bacillus spp.	7	4
Others	5	5
Gram Negative Pathogens		
Escherichia coli	4	3
Others	2	1

Table 2. *Follicle diameters and estradiol concentrations at h after PGF$_2$α* administation. Results are expressed as mans±standart errors
Tablo 2. *PGF2α uygulamasını izleyen saatlerde folikül çapları ve* östradiol konsantrasyonları. sonuç*lar ortalama±standart hata olarak ifade edilmiştir*

Parameter	Group	Hours			
		24th h	36th h	48th h	64th h
Follicle Diameters (cm)	Control	1.85±0.06	2.01±0.07	2.1±0.07	2.13±0.07
	SubclinicalMastitis	1.79±0.05	1.99±0.06	2.05±0.05	2.07±0.05
P values		NS	NS	NS	NS
Estradiol (pg/mL)	Control	44.2±1.94^{a}	45.89±3.59	41.4±2.39^{a}	-
	SubclinicalMastitis	63.85±7.65^{b}	60.95±7.1	59.94±8.19^{b}	-
P values		P=0.017	NS	P=0.036	

Different superscripts in the same column (a,b) indicate significant differences, ***NS:*** *Not significant (P>0.05)*

Hormone Concentrations

Serum estradiol concentrations (pg/mL) of the subclinically infected cows were higher than the control group at 24 (63.85±7.65 and 44.2±1.94, respectively); 36 (60.95±7.1 and 45.89±3.59, respectively) and 48 (59.94±8.19 and 41.4±2.39, respectively) h while these differences were significant at 24 and 48th h (P=0.017 and P=0.036 respectively). The decrease in the mean estradiol concentration from 45.89±3.59 to 41.4±2.39 in the control group throughout 36 to 48th h were also significant (P=0.043).

TNF-α (ng/L) concentrations measured from the samples at 24th h after the $PGF_2α$ injection in the subclinically infected and the control groups were 954.16±89.97 and 732.54±39.89 respectively and the difference was statistically significant (P=0.03).

Conception rate in the control group was higher than in the subclinical mastitis group and they were 40% (8/20) and 25% (5/20) respectively. However the difference was not found statistically significant (P>0.05).

Serum TNF-α was significantly correlated with estradiol concentrations at 24th h in both groups *(Table 2)*. However no significant correlations were found between follicular size and estradiol concentrations (P>0.05).

DISCUSSION

PCR based bacterial identification methods like used in this study makes it possible to detect bacteria more sensitively at the species level [12]. The isolated pathogens were mostly consisted of *Aerococcus viridans, Corynebacterium spp.*, coagulase-negative staphylococci (CNS) and *S. agalactiae.* The first three bacteria most frequently isolated in this study, are known to reside on the cows teat skin, around ostium papillare and sometimes cause subclinical mastitis with mild rise in somatic cell counts [13]. *Aerococcus viridans* has been isolated from clinical and subclinical mastitis cases recently and the source of the bacteria reported as composted manure bedding [14,15]. Similar to this study *Aerococcus viridans* were isolated frequently from milk samples and cows could freely access to open pens where manure content was high. *Corynebacterium spp.* were not considered as causal agents of mastitis previously, however it was identified from cases of subclinical and clinical mastitis cases [12]. In this study also *Corynebacterium spp.* were isolated from 18 mammary quarters of 11 subclinically infected cows. CNS causes milder milk loss and lower somatic cell counts (SCC) compared to the major mastitis pathogens however they are reported to be an important cause of mastitis especially in well managed dairy farms [16,17]. The high frequency of the above bacteria isolated from quarters of subclinical mastitic cows and rather limited number of major mastitis pathogens such as *S. aureus*, *S. agalactiae* and *E. coli* indicates a similar profile where the opportunistic bacteria leads to milder subclinical infections.

In this study follicular growth patterns were similar in both groups and no statistically significant differences were observed between groups at any time points (P>0.05). The mean diameters for control and mastitic groups at 24th h of $PGF_2α$ injection were 1.85±0.6 and 1.79±0.5 respectively and in the proceeding examinations follicular sizes in all cows increased gradually. Compared to the previous studies concerning GnRH - PG protocol synchronized cows, our data shows larger ovulatory follicles [6,18,19]. Recently in a study multiparous cows which are subjected to the 7 day Ovsynch protocol demonstrated larger follicles than primiparous cows and the conception rate was not affected by follicle size but pregnancy loss between 32 to 60 days increased due to larger follicle sizes [20]. Likewise, in the present study all of the cows were multiparous and 7 day Ovsynch protocol were used which may explain the larger follicle diameters. Aside from this the follicular growth pattern in both groups were similar to each other and this was in accordance with the studies of Lavon et al.[6] and Lavon et al.[21].

Decrased steroid production capacity in the preovulatory follicles of cows with either clinical or subclinical mastitis have been reported previously [6,21]. Follicular estradiol concentrations in one thirds of subclinically infected cows were significantly lower than healthy cows [6]. Also an extended estrus - ovulation interval accompanied with lower plasma estradiol concentrations were observed in 30% of cows with mastitis. However in this study serum estradiol concentrations were all higher in the subclinical mastitis group than the control cows and at 24th and 48th h and the differences were significant (P<0.05). Furman et al.[22] have reported that experimental subclinical intramammary infection did not cause an immediate decline in follicular estradiol but rather a marked, delayed decline in estradiol concentrations 16 days after mastitis induction was terminated. Moreover large portion of cows (especially consisting of Gram positive group) were not affected by the administration and follicular estradiol levels remained normal. These variations among cows were presumed to be related to individual, genetic differences. Authors have also suggested that the antral and medium sized follicles are more susceptible to stress and the effect of subclinical intrammammary infections (IMI) thus occurs belatedly [22]. In the present study as previously discussed above the mean follicular diameters of cows were higher than observed in the other studies and consequently larger follicles may be less affected from subclinical mastitis.

Timed AI programs such as used in this study depends on the effect of an initial GnRH injection to ovulate or luteinize the existing follicles. The presence of a functional luteal structure has been shown to be an important factor in the development of the new follicular wave thus the preovulatory follicle. Progesterone supplementation via a

CIDR device to cows that do not have a functional luteal structure at the initiation of ovsynch protocol have significantly increased circulating progesterone levels and subsequent fertility [23]. During early stages of diestrus progesterone concentrations are lower and LH pulses occur with greater frequency. Before the corpus luteum develops after ovulation, large estrogen-active follicles develop in the ovaries. As the luteal structure advances to mid phase, progesterone concentrations are increased and LH pulses are decreased. Administration of progesterone to ovariectomized cows also suppresses the release of LH, and both exogenous and endogenous sources has a similar supressive action in inhibiting release of LH pulses from the anterior pituitary [24]. TNF-α and IL-1β release may cause endogen $PGF_2α$ release from the endometrium which in turn causes luteolysis [25]. In the present study higher serum TNF-α levels in the subclinical mastitis group may have caused insufficient development of luteal structure causing lower progesterone levels at the time of first GnRH injection thus leading to greater release of LH pulses from the anterior pituitary causing higher estradiol levels. Higher LH pulses during the development phase of follicular wave may have caused estrogen active persistent follicles, which may not respond to GnRH treatment as indicated by Lopez-Gatius et al.[26]. Consequently overall fertility may be decreased in the subclinical mastitis group due to low ovulation and/or fertilization rates, however the difference was not significant (P>0.05).

No significant correlations between follicular diameters and estradiol levels at any time point in both groups were observed where this is a finding that could be expected in fixed time AI protocols especially in cows that did not exhibit estrus. Nearly 60% of cows did not display estrus behaviour during fix time AI protocols and follicular diameters were not correlated with serum estradiol concentrations in these cows [27].

A series of studies have reported high milk and serum concentrations of TNF-α in natural or experimentally induced *E. coli* mastitis cases [7,11,28,29]. Riollet et al.[10] also have observed that in chronical *S. aureus* infections interleukin together with TNF-α, other cytokines such as IL-1α, IL-1β, IL-6, IL-10 and IL-12 regulatory cytokine mRNA were synthesized in cells derived from infected mammary glands however cell subpopulations in blood from infected cows were not modified, indicating that immune responses to chronic intramammary infections were not manifested systemically. However as TNF-α is an important marker in the modulation of immune responses to infections and pathogen - host related variations may occur during intramammary infections, researchers of the present study aimed to re-test the possible role of TNF-α in mastitis related follicular/steroidogenic alterations during estrus in cows. Our results demonstrated that at 24th h following $PGF_2α$ injection, mean serum TNF-α levels of subclinically infected cows were found significantly higher than the control cows (P<0.05). This result is in contrast with the findings of Riollet et al.[7]. It can be said that in the present study multiplicity and variety of the pathogens may have caused this condition. However most of the pathogens were in Gram positive nature and a large portion of them were oppurtunistic mastitis bacteria causing mild to moderate rises in somatic cell counts. In addition serum TNF-α levels in both groups were at considerably low levels when compared to the researches demonstrating the TNF-α levels during endotoxin challange [7,30]. This seems to be in accordence with the mild nature of isolated pathogens that they may have caused much lower TNF-α release than endotoxemic bacteria but not totally absent.

Another interesting data in this study was that estradiol and TNF-α levels were significantly correlated in both subclinical mastitis group (P<0.001, R=0.826) and the control group (P=0.021, R=0.512). Kahl et al.[30] have reported that the production of TNF-α during endotoxin challange is enhanced during the follicular phase compared to the luteal phase in beef heifers. The above mentioned data in the present study may represent a similar effect of estradiol on the production of TNFα. Not presuming the effect of subclinical mastitis on TNF-α unsound, estradiol may have caused an amplifying effect on TNF-α levels. Sakumoto et al.[31], have reported that in cell cultures obtained from theca and granulosa cells of bovine small (2-5 mm) and large (12-18 mm) follicles, exposure to TNF-α resulted in inhibition of estradiol secretion in small follicles, but did not in large follicles. The reason for this was the low TNF-α receptor expression in the granulosa and theca cells of preovulatory follicles. Concerning this data and regarding the follicular growth patterns and serum estradiol concentrations observed in this study, it can be said that preovulatory follicules were not affected by the TNF-α content in serum.

As this study was focused on follicular growth, estradiol and TNFα levels in cows during estrus, data concerning pre and post-synchronisation progesterone levels, luteal structures and ovulation response to the initial GnRH injection were lacking. Together with these and levels of other cytokines examined during diestrus and estrus more comprehensive data would be obtained.

In conclusion follicular growth patterns were not affected by subclinical mastitis while estradiol and TNF-α was higher in the cows with subclinical mastitis than in the control group. Estradiol and TNF-α concentrations were positively correlated and this may indicate a possible increasing effect of estradiol on TNFα. Authors have hypothesised that high estradiol levels in the cows with subclinical mastitis could be a result of luteal deficiency due to cytokine release, however this requires further studies with intense data.

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Research Article

The Protective Effect of Thiopental Againts Renal Ischemia/ Reperfusion Damage in Rats

İsmail COŞKUNER [1] Meltem TÜRKAY [2] Hüseyin YILDIZ [3]
Emin SİLAY [3] Hamide SAYAR [4] Zeynep BAYAT [5]

[1] Department of Anesthesiology and Reanimation, Ozel Istanbul Hospital, TR-65030 Van - TURKEY
[2] Department of Anesthesiology and Reanimation, Bagcilar Training and Research Hospital, TR-34040 Istanbul - TURKEY
[3] Department of Anesthesiology and Reanimation, Kahramanmaras Sütcü Imam University, TR-46300 Kahramanmaras - TURKEY
[4] Department of Pathology, Kahramanmaras Sütcü Imam University, Faculty of Medicine, TR-46300 Kahramanmaras - TURKEY
[5] Department of Medical Biochemistry, Kahramanmaras Sutcu Imam University, Institute of Health Sciences, TR-46300 Kahramanmaras - TURKEY

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Abstract

The aim of this study was to determine optimal protective thiopental dose in renal ischemia/reperfusion (I/R) damage. This study was carried out on 42 rats. After abdominal midline incision, left renal artery was occluded. After 60 min of ischemia and reperfusion, a left nephrectomy was performed. Surgical intervention was made for the six rat groups (excluding sham group). In control group, 60 minutes of ischemia and reperfusion were applied to left kidneys. Thiopental (3, 10, 30, 60, 80 mg/kg) was applied intra-peritoneally to the rats except sham and control groups 15 minutes before reperfusion. After reperfusion, IL-1α, IL-6, TNF-α levels; SOD activity; MDA and NO levels were measured. There was no decrease in IL-6 or TNF-α levels in groups at all doses. IL-1α levels only decreased in the group receiving 3 mg/kg thiopental. Only the group receiving 80 mg/kg observed decrease in MDA levels. The rats receiving 30 mg/kg showed significant increase in SOD levels. The rats receiving 3 mg/kg detected significant increase in glutathione peroxidase (GPx) levels. The groups receiving 10, 30, and 60 mg/kg thiopental showed decrease in nitric oxide (NO) levels. According to study results, different doses of thiopental affected each biochemical indicator in renal I/R damage.

Keywords: *Renal Ischemia/Reperfusion, Thiopental, Rat*

Ratlarda Böbrek İşemi/Reperfüzyon Hasarına Karşı Tiyopentalin Koruyucu Etkisi

Özet

Çalışmada ratlarda farklı dozlarda uygulanan tiyopentalin renal I/R hasarına karşı koruyucu optimal dozunun saptanması amaçlandı. Bu çalışma 42 ratta yapıldı. Abdominal orta hat insizyonu sonrası sol renal arter okluzyonu yapıldı. Altmış dakika işemi ve reperfüzyondan sonra sol nefrektomi yapıldı. Altışar rattan oluşan gruplarda (sham grup dışında) cerrahi prosedür uygulandı. Kontrol grubunda sol böbreklere 60 dk işemi ve reperfüzyon uygulandı. Sham ve kontrol grubu dışındaki ratlara reperfüzyondan 15 dk önce tiyopental (3, 10, 30, 60, 80 mg/kg) intra-peritoneal olarak uygulandı. Reperfüzyon sonunda IL-1α, IL-6, TNF-α seviyeleri; SOD aktivitesi; MDA ve NO düzeyleri ölçüldü. Gruplarda IL-6 ve TNF-α seviyelerinde azalma olmadı. Yalnızca 3 mg/kg tiyopental uygulanan grupta IL-1α seviyesi azaldı. MDA düzeylerinde düşme yalnızca 80 mg/kg tiyopental uygulanan grupta görüldü. 30 mg/kg tiyopental uygulanan ratlarda SOD düzeylerinde anlamlı yükseklik saptandı. 3 mg/kg tiyopental uygulanan ratlarda glutatyon peroksidaz (GPx) düzeylerinde anlamlı yükseklik saptandı. Nitrik oksit seviyesindeki düşme 10, 30 ve 60 mg/kg tiyopental uygulanan gruplarda görüldü. Çalışma sonuçlarına göre, renal işemi reperfüzyon hasarında her bir biyokimyasal belirteci tiyopentalin farklı dozları etkiledi.

Anahtar sözcükler: *Renal İşemi/Reperfüzyon, Tiyopental, Rat*

İletişim (Correspondence)
+90 505 6849750
coskuner40@hotmail.com

INTRODUCTION

Ischemia is defined as the circulatory system's inability to provide the oxygen and metabolites needed by tissues to remove waste products [1]. It is caused by arterial and/or venous blockage. Shock, sepsis, renal transplantation, some vascular surgeries, myocardial infarction, cerebrovascular events, liver rejection, and trauma are among the important causes of ischemia [2,3].

Reperfusion is when circulation is provided again to ischemic tissue. During reperfusion, while the oxygen and metabolic needs of the tissue are met [4], introducing oxygen into the cell creates free oxygen radicals, and more severe damage occurs compared to the damage caused by the ischemia [5]. Free oxygen radicals deform the structure of the cell membrane and disrupt the function of the transport system in the cell membrane due to lipid peroxidation. DNA is damaged by the oxidative modification of proteins, and the energy production of the cell is hindered [6]. In addition to the characteristic emerging of free oxygen radicals [7] in ischemia/reperfusion (I/R) damage, polymorphonuclear leucocytes activation [8], increase in eicosanoid release [9], activation of the complement system [10], and increase in cytokine release [11] are observed.

With the developments in renal transplantation surgery, renal I/R damage has become an important clinical problem [12]. Tubular epithelial cell damage and ischemic acute renal failure can occur due to renal I/R damage, and this is accompanied by high rates of mortality [13]. The protective effects of various intravenous anesthetics, such as ketamine, propofol, etomidate, and thiopental [14-16], against renal I/R damage have been studied [12,13]. Thiopental is known to reduce the release of free oxygen radicals from neutrophils in renal I/R damage, inhibit lipid peroxidation, and demonstrate antioxidant effects by reducing hemolysis of red blood cells caused by free oxygen radicals [17]. However, there are no studies in the literature that posit at what doses thiopental is effective against renal I/R damage. In our study, we aimed to detect the optimal protective dose of thiopental against renal I/R damage.

MATERIAL and METHODS

Animals

This study was approved by the Animal Experimentation Ethics Committee of Kahramanmaraş Sütçü İmam University (05.04.2011, No: 7), and all of the surgical procedures were performed in accordance with the rules of the National Institutes of Health, Guide for the Care and Use of Laboratory Animals. All observations were conducted by a researcher blinded to the study groups.

The weights of the animals were ranging from 250 to 280 g; totally 42 male rats were used in this study. The rats were brought to the research center one week before the beginning of the study to become accustomed to the environment, which was heat-controlled cages with 12-h night and day cycles. They were fed standard water and food.

Experimentation Design

After the rats were anesthetized with intraperitoneal 1 g/kg urethane (99% Ethyl carbamate: Sigma-Aldrich; 94300-50 g), the abdominal area was shaved and sterilized with povidone iodine. A midline incision was made, and the abdominal viscera were moved to the right. The left renal hilus was dissected and renal artery occluded with a microvascular clamp, and the intestines were replaced in the abdominal cavity. After 60 min of ischemia, the clamp was removed and 60 minutes of reperfusion were provided; then, a left nephrectomy was performed. The rats were randomized into seven random groups, with six animals in each group:

- Group SG (sham group, n=6) rats received the described surgical procedure, except for renal I/R,
- Group CG (control group, n=6) rats received 60-min ischemia followed by 60-min reperfusion in the left kidney,
- Group TG 3 (n=6) rats received 3 mg/kg intraperitoneal thiopental (Pental, 0.5 g, İbrahim Etem Ulugay İlaç Sanayi Türk A.Ş.) 15 min before the reperfusion phase,
- Group TG 10 (n=6) rats received 10 mg/kg intraperitoneal thiopental 15 min before the reperfusion phase,
- Group TG 30 (n=6) animals received 30 mg/kg intraperitoneal thiopental 15 min before the reperfusion phase,
- Group TG 60 (n=6) rats received 60 mg/kg intraperitoneal thiopental 15 min before the reperfusion phase,
- Group TG 80 (n=6) rats received 80 mg/kg intraperitoneal thiopental 15 min before the reperfusion phase.

At the end of the reperfusion period, the tissue was incised for biochemical evaluation. IL-1α, IL-6, TNF-α levels; SOD activity; MDA and NO levels; were measured.

Antioxidant Study

In order to determine antioxidant levels, 1x1 cm^2 diameter tissue samples were taken. The samples were maintained in a deep freezer until the examination. The tissues were homogenized with 1.15% KCl at ice coldness in three volumes. Antioxidant enzyme activities and lipid peroxidation levels were measured in the supernatant obtained using a 14.000 rpm (18.400Xg) centrifuge. Superoxide dismutase (SOD) activity was measured using the method described by Fridovich [18]. Lipid peroxidation levels in the tissue samples were emphasized with malondialdehyde (MDA) and measured according to the procedure of Okhawa et al.[19]. Protein concentration was determined according to Lowry's [20] method.

Statistical Evaluation

In evaluating the data, the Kruskal-Wallis test was used for comparisons between groups and Dunn's multiple comparison test was used for sub-group comparisons, in addition to supplementary statistical methods (mean, standard deviation, median, interquartile range). The results were evaluated at a significance level of P<0.05 and confidence interval of 95%.

RESULTS

IL-1α, IL-6, and TNF-α means of the groups are shown in *Table 1*.

IL-1α levels in Group SG were found to be significantly lower than those of Group CG (P=0.002). There was no statistically significant difference in IL-1α levels between Group CG and Group TG 3 (P=0.655); however, the levels were found to be significantly higher in Group CG than in Group TG 10, Group TG 30, Group TG 60, and Group TG 80 (P=0.002, P=0.003).

While there was no statistically significant difference in IL-6 levels between Group CG and Group SG (p=0.055), the IL-6 level was statistically significantly higher in Group CG than in Group TG 3, Group TG 10, Group TG 30, Group TG 60, and Group TG 80 (P=0.003, P=0.002).

TNF-α levels in Group SG were lower compared to Group CG (P=0.002). TNF-α levels were significantly higher in Group CG than in Group TG 3, Group TG 10, Group TG 30, Group TG 60 and Group TG 80 (P=0.002) *Table 2*.

MDA, SOD, glutathione peroxidase (GPx), and nitric oxide (NO) means of the groups are shown in *Table 3*.

While there were no statistically significant differences in MDA nmol/mg prot values between Group CG and Group SG, Group TG 3, Group TG 10, Group TG 30, and Group TG 60 (P=0.949, P=0.406, P=0.565, P=0.848, P=0.180), there was a statistically significant difference between Group CG and Group TG 80 (P=0.018).

While there was no significant difference in SOD levels between Group CG and Group TG 3, Group TG 10, Group TG 60, and Group TG 80 (P=0.406, P=0.565, P=0.655, P=0.749), SOD level was significantly higher in Group CG than in Group SG and Group TG 30 (P=0.002).

While GPx levels were significantly higher in Group CG than in and Group SG and Group TG 3 (P=0.013, P=0.035), there were no statistically significant differences between Group CG and Group TG 10, Group TG 30, Group TG 60, and Group TG 80 (P=0.848, P=0.225, P=0.565, P=0.180).

While there were no statistically significant differences in NO levels between Group CG and Group SG, Group TG 3, and Group TG 80 (P=0.565, P=0.277, P=0.406), there was a statistically significant difference between Group CG and Group TG 10, Group TG 30, and Group TG 60 (P=0.003, P=0.009, P=0.002) *Table 4*.

Table 1. *IL-1α, IL-6 and TNF-α means of groups*
Tablo 1. *Grupların IL-1α, IL-6 ve TNF-α ortalamaları*

Group	IL-1α (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)
SG	90.37±19.23	92.63±9.29	48.31±5.57
CG	160.5±27.52	110.33±18.82	123.41±26.05
TG 3	182.34±71.82	218.57±77.39	292.9±54.87
TG 10	250.21±37.38	381.7±46.84	271.77±41.8
TG 30	321.93±60.85	203.81±37.85	337.73±39.35
TG 60	640.67±80.68	527.29±163.88	593.07±68.4
TG 80	387.57±49.99	452.94±40.77	495.53±108.24
P	0.0001*	0.0001*	0.0001*

** P<0.05, (means±SD)*

Table 2. *Comparison of IL-1α, IL-6 and TNF-α means of groups with the control group*
Tablo 2. *Grupların IL-1α, IL-6 ve TNF-α ortalamalarının kontrol grubu ile karşılaştırılması*

Dunn's Multiple Comparison Test	IL-1α (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)
Group SG / Group CG	0.002*	0.055	0.002*
Group CG / Group TG 3	0.655	0.003*	0.002*
Group CG / Group TG 10	0.003*	0.002*	0.002*
Group CG / Group TG 30	0.002*	0.002*	0.002*
Group CG / Group TG 60	0.002*	0.002*	0.002*
Group CG / Group TG 80	0.002*	0.002*	0.002*

** P<0.05*

DISCUSSION

In ischemia/reperfusion injury; oxygen deficiency in ischemic period and also cytotoxic events which occurs during reperfusion cause cell damage and apopitosis [21]. Prophylactic antiapoptotic treatment can be an effective therapeutic strategy for the prevention of I/R injury [22]. Thiopental is a highly lipid-soluble anesthetic, which has indicated antioxidant effect by inhibiting lipid peroxidation in ischemia/reperfusion injury [17]. Protective effects of thiopental for renal ischemia/reperfusion injury was searched in several and compared with the other intravenous agents [13,17,23]. Yüzer et al.[13] reported that propofol and thiopental anesthesia protects against biochemical, and morphological damage better than control in renal I/R injury. They also reported in the same study; thiopental decreases the kidney I/R damage via free oxygen radicals in rats, and this effect is significant compared to etomidate [13]. Basu et al.[23] studied oxidative stres after renal transplantation and inflamatory response and they compared propofol and thiopental. As a result they reported that propofol depressed the inflamatory response [23].

Table 3. *MDA, SOD, GPx and NO means of the groups*
Tablo 3. *Grupların MDA, SOD, GPx ve NO ortalamaları*

Group	MDA (nmol/mgprot)	SOD (U/mgprot)	GPx (U/mgprot)	NO (U/mgprot)
SG	2.05±0.83	48.55±20	0.33±0.07	0.06±0.04
CG	1.91±0.88	9.45±2.28	0.21±0.07	0.06±0.03
TG 3	1.24±0.63	10.99±3.62	0.29±0.06	0.06±0.07
TG 10	1.41±0.8	8.75±3.46	0.2±0.06	0.15±0.05
TG 30	1.85±0.65	103.78±33.07	0.26±0.1	0.33±0.26
TG 60	2.44±0.9	8.47±2.42	0.18±0.03	0.57±0.33
TG 80	0.85±0.58	9.2±2.52	0.24±0.03	0.12±0.09
P	0.029*	0.0001*	0.002*	0.0001*

** P<0.05, (means±SD)*

Table 4. *Comparison of MDA, SOD, GPx and NO means of the groups to the control group*
Tablo 4. *Grupların MDA, SOD, GPx ve NO ortalamalarının kontrol grubu ile karşılaştırılması*

Dunn's Multiple Comparison Test	MDA (nmol/mgprot)	SOD (U/mgprot)	GPx (U/mgprot)	NO (U/mgprot)
Group SG / Group CG	0.949	0.002*	0.013*	0.565
Group CG / Group TG 3	0.406	0.406	0.035*	0.277
Group CG / Group TG 10	0.565	0.565	0.848	0.003*
Group CG / Group TG 30	0.848	0.002*	0.225	0.009*
Group CG / Group TG 60	0.180	0.655	0.565	0.002*
Group CG / Group TG 80	0.018*	0.749	0.180	0.406

** P<0.05*

Contrary to this Doğan et al.[17] reported that thiopental has beter antioxidant effects than propofol according to their comparative study.

Li et al.[24] detected a significant increase in MDA levels in renal I/R damage. Plasma and tissue MDA levels are an indicator of increased free oxygen radical production and cell membrane damage as a result of the reperfusion process [19]. In our study, only the Group that received the highest dose of thiopental, 80 mg/kg, showed a decrease in MDA levels. Doğan et al.[17] reported that antioxidant effects of thiopental against renal ischemia/reperfusion injury is more prominent in high dose. Our study has the same result. It's reported that for other intravenous agents like ketamine and propofol, high doses increase the antioxidant effect [17].

It is known that I/R damage leads to an increase in levels of cytokines such as IL-1α, IL-6, and TNF-α [11]. In our study, IL-1α, IL-6, and TNF-α levels were lower in Group SG than in the control group. However, the difference in IL-6 levels was not statistically significant. We associated this finding with the fact that I/R was not applied in Group SG. However, in the thiopental doses we applied, there was no decrease in IL-6 or TNF-α levels in any of the groups. Only in the group receiving 3 mg/kg thiopental did IL-1 α level increase compared to the control group. This result leads us to think that 3 mg/kg thiopental is enough to prevent IL-1α release.

Superoxide dismutase, present in all cells that metabolize oxygen, is a defense mechanism that works with the catalase and glutathione system against the damage caused by oxygen radicals [26,27]. This enzyme protects aerobic organisms against the harmful effects of superoxide [27]. There was only a significant increase in SOD levels in the rats that received the 30 mg/kg dose of thiopental.

The GPx enzyme that carries selenium as the prosthetic group in erythrocytes and other tissues catalyzes the decomposition of hydrogen peroxide and lipid peroxides by reduced glutathione [28]. There was a significant increase in GPx levels in the group that received the 3 mg/kg dose of thiopental.

Nitric oxide is a free radical. Although it is a weak reducing agent, it causes peroxynitrite formation through interaction with the peroxide radical. The peroxynitrite radical is a highly reactive agent and leads to lipid peroxidation and protein damage [29,30]. In our study, a decrease in nitric oxide levels was observed in the groups that received 10, 30, and 60 mg/kg doses of thiopental. Although the NO level was suppressed in those dosage groups, we are of the opinion that the lowest thiopental dose, 10 mg/kg, is enough to prevent lipid peroxidation.

In conclusion, according to the results of our study, different doses of thiopental were found to be effective for each biochemical indicator in renal I/R damage. We

are of the opinion that further studies are required on this subject.

Conflicts of Interest

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

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Research Article

Induction and Recovery Characteristics and Cardiorespiratory Effects of Verapamil on Ketamine-Induced Anaesthesia in Dogs

Hadi IMANI [1] Bahman MOSALLANEJAD [1] Narges KAVOSI [1] Faezeh ZARMEHI [1]

[1] Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz - IRAN

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Abstract

The present study was designed to evaluate the induction and recovery characteristics as well as the cardiorespiratory effects of verapamil on ketamine-induced anaesthesia in dogs. Six healthy male mix-breed dogs weighing 19.5±3.0 kg and aged 1.5-2.5 years were used in a randomized crossover design. After premedication with acepromazine (0.025 mg/kg) and morphine (0.25 mg/kg) intramuscularly, dogs received either treatments of verapamil (0.1 mg/kg; IV) or normal saline intravenously. Anaesthesia was induced with ketamine (10 mg/kg; IV) and maintained with isoflurane. Induction and recovery quality were acceptable in both treatments. Recovery tended to be prolonged in verapamil compared with SAL. Heart rate in normal saline, after administration of ketamine, showed significant increased values ($P<0.05$); whereas, heart rate was relatively stable in verapamil with no significant changes ($P\geq0.05$). Mean arterial blood pressure, during inhalation anaesthesia, was below the reference ranges and showed significant decreases in both treatments ($P<0.05$). Respiratory rate decreased in both groups at several time points compared with the base ($P<0.05$). It was concluded that addition of verapamil to ketamine, had no effect on induction and recovery quality and could potentially prolong recovery. Verapamil resulted in relatively stable HR compared to that of control group.

Keywords: *Anaesthesia, Verapamil, Ketamine, Dog*

Köpeklerde Ketaminle İndüklenmiş Anestezi Üzerine Verapamilin İndüksiyon ve Rekover Özellikleri ve Kardiyorespiratuar Etkileri

Özet

Bu çalışma, köpeklerde ketamin-kaynaklı anestezi üzerine verapamil'in kardiyorespiratuar etkilerinin yanı sıra indüksiyon ve iyileşme özelliklerini değerlendirmek için tasarlanmıştır. Altı adet sağlıklı erkek melez-cins köpek (19.5±3.0 kg ağırlığında ve 1.5-2.5 yaşlı) rasgele seçilmiş bir çapraz tasarımda kullanıldı. Köpeklere, kas-içi asepromazin (0.025 mg/kg) ve morfin (0.25 mg/kg) ile premedikasyon sonrası, ya intravenöz yolla verapamil (0.1 mg/kg; IV) ya da normal tuzlu su tedavisi yapıldı. Anestezi ketamin ile anestezi (10 mg/kg; IV) indüklendi ve izofluran ile sürdürüldü. Her iki tedavide indüksiyon ve iyileşme kalitesi kabul edilebilir idi. İyileşme, normal tuzlu su ile karşılaştırıldığında verapamilde uzama eğiliminde idi. Normal tuzlu sudaki kalp atım hızı, ketamin verilmesinden sonra, önemli artan tarzda değişiklikler ($P<0.05$) gösterirken; oysa, verapamilde ki kalp atım hızına oranla istikrarlı olup, önemli değişiklikler göstermedi ($P\geq0.05$). Ortalama arteriyel kan basıncı, inhalasyon anestezisi sırasında, referans aralıkları altında idi ve her iki tedavide önemli düşüşler gösterdi ($P<0.05$). Solunum hızı, bazal seviye ile karşılaştırıldığında her iki grupta da çok sayıda zaman noktasında azaldı ($P<0.05$). Dolayısıyla; ketaminine verapamilin eklenmesinin, indüksiyon ve iyileşme kalitesi üzerine hiçbir etkisinin olmadığı ve potansiyel olarak rekoveri geciktirdiği sonucuna varıldı. Verapamil, kontrol grubuna kıyasla nispeten istikrarlı HR ile sonuçlandı.

Anahtar sözcükler: *Anestezi, Verapamil, Ketamin, Köpek*

INTRODUCTION

General anaesthesia is used frequently for various procedures in small animals. It can be induced and maintained with injectable and/or inhalant anaesthetics. Since it has not yet been introduced a specific anaesthetic agents without undesirable effects, research, to find a combination of pharmacological agents with more efficacy and lesser side effects, are continuing. Furthermore, in some situations such as in some diseases, it is needed to attenuate unfavourable effects of anaesthetic drugs to diminish the associated risks to the patients.

Ketamine hydrochloride is a common injectable anaesthetic agent. It can be used for induction and maintenance of anaesthesia. Ketamine is an N-methyl-D-

İletişim (Correspondence)
+98 913 3823324
h.imani@scu.ac.ir

aspartate receptor antagonist with anti-hyperalgesic and anaesthetic properties. The cardiovascular stimulation associated with ketamine is characterized by increases in heart rate (HR), cardiac output (CO), and arterial blood pressure as a result of increased sympathetic efferent activity [1]. Anaesthesia with ketamine may be associated with little muscle relaxation or even muscle rigidity as well as spontaneous movement and violent recovery. Administration of CNS depressant such as benzodiazepines and α2-agonists is recommended to prevent or to reduce undesirable effects of ketamine [2].

Verapamil, a phenylalkylamine, is a calcium channel blocker (CCB), calcium entry blocker, and calcium antagonist. CCBs belong to the class IV antiarrhythmic agents [3]. These drugs have been approved for the management of ischemic heart disease, hypertension and some forms of cardiac dysrhythmias in human medicine [4]. CCBs have also been shown that have some beneficial effects on preventing and treatments of different types of convulsions [5-8]. Verapamil decreases HR, cardiac contraction and has some vasodilatory effects [9]. It is indicated for the acute termination of supraventricular tachycardia in dogs [3].

The present study was designed to evaluate the effect of verapamil on the characteristics of induction and recovery as well as cardiorespiratory status in dogs anesthetized with ketamine. It was hypothesized that the addition of verapamil to ketamine, possibly because of the inhibitory effects of verapamil on stimulating reactions and the attenuating effects of verapamil on cardiovascular properties of ketamine, results in more convenient induction and recovery and more stability in cardiovascular variables in dogs.

MATERIAL and METHODS

Six healthy male mix-breed dogs weighing 19.5±3.0 kg and aged 1.5-2.5 years were used in a randomized crossover design. The dogs were considered healthy on the basis of physical examination findings, complete blood count (CBC) and total protein (TP). Each dog was used in two experiments at one-week interval; in each experiment, one of the two treatment protocols was evaluated. The animals had free access to feed and water but remained off feed for 12 h, and water was withheld for two h prior to the trials. The Institutional Animal Care and Research Committee approved all the procedure in this study.

On the day of experiments, dogs were transferred to the study place and allowed to acclimate to the environment for at least 30 min prior to any medication. Acepromazine (0.025 mg/kg, Neurotranq, 10 mg/mL, Alfasan, Holland) and morphine (0.25 mg/kg, Morphine sulphate, 10 mg/mL, Darou Pakhsh, Iran) were mixed into the same syringe, administrated intramuscularly (IM) for premedication. Twenty min later, dogs were transferred onto a table and a 20-gauge, 1.25-inch intravenous (IV) catheter was placed into the left cephalic vein.

For the dogs in treatment group (VER), 30 min after premedication, verapamil (0.1 mg/kg, Lekoptin, 5 mg/2 mL, Lek, Slovenia) was administered IV as a bolus. Five min later, anaesthesia was induced with ketamine (10 mg/kg, Ketamine hydrochloride, 50 mg/mL, Rotexmedica, Germany) administered IV in small increments over 30 seconds. Trachea, when the tongue movement was stopped, was intubated with an appropriate cuffed endotracheal tube. All intubations were performed by the same researcher. After intubation, all dogs were positioned in right lateral recumbency. Anaesthesia was maintained with isoflurane (1.5%) in oxygen (2 L/min) delivered through a rebreathing circuit system. All dogs were maintained under inhalation anaesthesia for 30 min. Normal saline was administered intra-operatively at a rate of 10 mL/kg/h from catheterization until the extubation was performed. Protocol in control group (SAL) was similar to that of treatment group but normal saline was administered IV instead of verapamil. At the end of anaesthesia, isoflurane was discontinued and animals were allowed to recover. Extubation was performed when the animal started to chew and no longer tolerated tracheal intubation.

Induction and recovery were evaluated subjectively and were scored based on a previous study [10]: induction: 1- no sign of excitement, intubation within 60 seconds after administration of ketamine; 2- mild signs of excitement, some struggling during intubation; and 3- hyperkinesis, restlessness, no intubation; recovery: 1- no struggling, standing and walking without difficulty; 2- some struggling, long-lasting sternal recumbency; 3- sever struggling, inability to be in sternal recumbency or walking. All scores were given by the same researcher who was not aware to the treatments. The times from induction of anaesthesia to extubation, head upraising, sternal recumbency, and standing position were recorded.

Heart rate (HR), respiratory rate (f_R), and rectal temperature (RT) were recorded before premedication (base), 30 min after premedication (sedation), and at 1, 5, 10, 15, 20, 30, 40, 50 and 60 min after induction. Except for inhalation anaesthesia period; HR was recorded using a stethoscope, f_R was counted by chest movement, and RT was measured using a digital thermometer. After induction of anaesthesia, animals were connected to a multiparameter monitoring system (Burtons, Guardian Industrial Estate, UK), and HR, *fr*, RT, systolic, diastolic and mean arterial blood pressure (SAP, DAP, and MAP, respectively), haemoglobin oxygen saturation (SpO_2), and end-tidal carbon dioxide tension ($ETCO_2$) were recorded in a predetermined intervals.

The data were evaluated for normal distribution using Kolmogorov-Smirnov test. All normally distributed data were presented as mean ± SD. Nonparametric data were shown as median (range). Induction and recovery quality

were compared using Friedman test. A paired sample t-test was employed for comparison the chronological sequences of recovery events. HR, f_R, RT, SPO_2, SAP, DAP, MAP, and $ETCO_2$ values were compared by analysis of variance for repeated measure followed by LSD test. Statistical analysis was undertaken by IBM SPSS Statistics for Windows Version 22 (IBM Corporation, NY, USA) with significant level of P<0.05.

RESULTS

The body weights of the animals were not significantly different at the time of each treatment: 19.2±0.2 kg for SAL and 19.8±3.1 for VER (P≥0.05). There were no significant differences in pre-anaesthetic behaviour of the dogs and all of them evaluated as normal. All dogs were adequately sedated before induction of anaesthesia. Induction of anaesthesia was satisfactory in both groups in which no distress or complications were seen. Intubation was relatively rapid (in less than 60 seconds) and achieved without difficulty. There were no differences between induction scores *(Table 1*; P≥0.05).

Recovery was relatively smooth in both treatments without significant differences between recovery scores *(Table 1*; P≥0.05). Recovery in a dog with SAL and a dog with VER were long lasting. A dog in SAL had a violent recovery with severe paddling and a dog in VER showed some trembling during recovery. The times to the recovery events were presented in *Table 2*. The time to the head upraising in SAL was significantly shorter in comparison with that in VER (P<0.05). The times to the extubation, sternal recumbency and standing did not show significant differences between treatments (P≥0.05).

Comparison of cardiovascular variables (HR, SAP, DAP and MAP; *Table 3)* between two groups showed a significant lower value of HR at T1 in VER than that of SAL (P<0.05). HR in SAL showed significant increases, after ketamine administration, in comparison with the base (P<0.05). HR in VER was relatively stable and no significant changes were detected during evaluation period (P≥0.05). SAP, DAP and MAP were significantly lower at T1 in SAL compared to VER (P<0.05). Statistically significant changes were observed in SAP, DAP and MAP in both groups (P<0.05).

Table 4 contains the results of the f_R, SPO_2, $ETCO_2$, and RT at selected time points. Apnoea was observed in all dogs at 1 min after induction of anaesthesia. In this time all animals received manual ventilation. Apnoea was resolved in all dogs within 2-3 min after induction except for a dog in SAL which apnoea lasted until seven min after induction.

Table 1. *Median (range) of scores of induction and recovery in dogs (n=6) received either intravenous (IV) normal saline (SAL) or verapamil (VER; 0.1 mg/kg) before induction of anaesthesia with ketamine (10 mg/kg; IV)*

Tablo 1. *Ketamin (10 mg/kg; IV) ile anestezi indüksiyonu öncesi, intravenöz (IV) serum fizyolojik (SALT) veya verapamil (VER; 0.1 mg/kg) verilen köpeklerde (n=6) ortanca (aralık) indüksiyon ve iyileşme puanları*

Variable	SAL	VER
Induction	1 (1-2)	1 (1-3)
Recovery	1 (1-2)	1 (1-3)

Table 2. *Mean ± SD of recovery times (min) in dogs (n=6) received either intravenous (IV) normal saline (SAL) or verapamil (VER; 0.1 mg/kg) before induction of anaesthesia with ketamine (10 mg/kg; IV)*

Tablo 2. *Ketamin (10 mg/kg; IV) ile anestezi indüksiyonu öncesi, intravenöz (IV) serum fizyolojik (SALT) veya verapamil (VER; 0.1 mg/kg) verilen köpeklerde (n=6) ortalama (± SD) iyileşme süreleri (dak)*

Variable	SAL	VER
Extubation	19±5.1	21±4.5
Head upraising	30±3.6	39±8.6 *
Sternal recumbency	42.5±6.9	47.5±11.7
Standing	56.3±4.7	63.7±20.0

** Significantly different between treatments (P<0.05)*

Table 3. *Cardiovascular variables (heart rate (HR); systolic, diastolic, and mean arterial blood pressure (SAP, DAP, MAP, respectively)) at selected time points in dogs (n=6) received either intravenous (IV) normal saline (SAL) or verapamil (VER; 0.1 mg/kg) before induction of anaesthesia with ketamine (10 mg/kg; IV)*

Tablo 3. *Ketamin (10 mg/kg; IV) ile anestezi indüksiyonu öncesi, seçilen zaman noktalarında intravenöz (IV) normal tuz (SALT) veya verapamil (VER; 0.1 mg/kg) verilen köpeklerde (n=6) kardiyovasküler değişkenler; (kalp hızı (KH); sistolik, diyastolik ve ortalama arteriyel kan basıncı (SAP, DAP, MAP, sırasıyla))*

Parameter	Base	Sedation	Time (min)								
			1	5	10	15	20	30	40	50	60
HR											
SAL	76±7	77±6	100±15 *	120±17 *	101±14 *	91±12 *	88±7 *	93±19 *	104±20 *	101±17 *	96±17 *
VER	85±8	76±12	82±23 †	94±28	87±30	84±34	94±13	93±15	93±20	93±21	87±22
SAP											
SAL	ND	ND	98±14	111±16	101±15	95±19	93±19	93±22	ND	ND	ND
VER	ND	ND	112±19 †	112±35	103±21	99±18	103±15	112±18	ND	ND	ND
DAP											
SAL	ND	ND	65±9	72±7 *	62±16	58±8 *	59±7 *	62±20	ND	ND	ND
VER	ND	ND	77±17 †	69±23	63±19	58±15 *	63±16 *	75±19	ND	ND	ND
MAP											
SAL	ND	ND	76±11	83±7 *	74±15	67±19 *	69±17 *	70±21	ND	ND	ND
VER	ND	ND	89±11 †	80±23	75±18	70±16 *	76±14 *	86±17	ND	ND	ND

** Significantly different from the base in each treatment (P<0.05). † Significantly different between treatments at that time point (P<0.05),* ***ND:*** *not determined*

Table 4. *Mean ± SD of respiratory rate (f_R), haemoglobin oxygen saturation (SpO_2), end-tidal carbon dioxide tension ($ETCO_2$), and rectal temperature (RT) at selected time points in dogs (n=6) received either intravenous (IV) normal saline (SAL) or verapamil (VER; 0.1 mg/kg) before induction of anaesthesia with ketamine (10 mg/kg; IV)*

Tablo 4. *Ketamin (10 mg/kg; IV) ile anestezi indüksiyonu öncesi, seçilen zaman noktalarında intravenöz (IV) normal tuz (SALT) veya verapamil (VER; 0,1 mg/kg) verilen köpeklerde (n=6) ortalama (± SD) solunum hızı (f_R), hemoglobin oksijen satürasyonu (SpO_2), end-tidal karbondioksit basıncı ($ETCO_2$), ve rektal sıcaklık (RT)*

Parameter	Base	Sedation	Time (min)								
			1	5	10	15	20	30	40	50	60
f_R											
SAL	29±6	21±6	Apnoea	9±1 *	11±3 *	12±6 *	17±9	16±6	30±7	28±6	29±5
VER	30±9	21±4	Apnoea	9±3 *	10±2 *	11±2 *	14±7 *	15±3	21±11	21±9 †	24±11
SPO_2											
SAL	ND	ND	97±3	96±1	97±1	97±2	97±1	96±1	ND	ND	ND
VER	ND	ND	96±2	97±3	96±3	96±3	97±2	97±1	ND	ND	ND
$ETCO_2$											
SAL	ND	ND	Apnoea	41±5	37±3	38±3	38±4	38±2	ND	ND	ND
VER	ND	ND	Apnoea	35±7	37±6	38±6	37±7	38±5	ND	ND	ND
RT											
SAL	39.3±0.5	38.6±0.7	38.2±0.7 *	37.3±1.0 *	36.6±2.0 *	37.6±0.7 *	37.4±0.8 *	37.2±0.8 *	37.3±0.7 *	37.6±0.9 *	37.3±0.7 *
VER	39.2±0.6	38.8±0.8	38.3±1.0 *	37.4±1.2 *	37.9±0.7 *	37.7±0.8 *	38.0±1.0 *	37.4±0.8 *	37.6±1.0 *	37.4±0.8 *	37.4±0.9 *

** Significantly different from the base in each treatment (P<0.05). † Significantly different between treatments at that time point (P<0.05).* ***ND:*** *not determined*

Statistically significant lower values of f_R were detected in comparison with the base in both groups (P<0.05). SPO_2 and $ETCO_2$ did not show significant difference between and within both groups in the evaluated time points (P<0.05). Significant decreases in RT were detected in SAL and VER (P<0.05). There were no significant differences in RT between SAL and VER (P≥0.05).

DISCUSSION

In the current study, using of verapamil, as a co-induction agent, with ketamine resulted in no differences in induction and recovery quality. Ketamine when used alone is considered, in general, as an anaesthetic agent with uneven induction and violent recovery. Thus, it is recommended to use ketamine with central nervous system (CNS) depressants such as benzodiazepines [1]. However, verapamil is generally considered to be devoid of CNS depressant effects, it has been shown that verapamil possesses some anticonvulsant properties [5,7,8]. One of the rational reasons for using verapamil, in the present study, was to achieve more convenient induction and recovery possibly, via the effect of verapamil on inhibition of some stimulating reactions of ketamine; nevertheless, induction and recovery in both groups were acceptable. The lack of differences between two treatments may be explained by expedient sedation produced by premedication agents subsequently resulted in more desirable induction and recovery.

However, just head upraising was significantly seen later in VER compared with SAL, but the times of extubation, sternal recumbency, and standing were also tended to be longer in VER. This finding is in accordance with studies which showed that pre-treatment with verapamil in ketamine-induced anaesthesia could lengthen sleeping time in mice [11,12]. In addition, verapamil prolonged sleeping time in broiler chickens in which anaesthesia induced with either propofol or xylazine-ketamine [13]. The effect of verapamil on prolongation of anaesthesia can be due to the direct effect of verapamil on inhibition of hepatic P450 metabolizing enzymes and neuronal calcium channel blockade produced by this agent [12]. It has also been shown that verapamil has a role on inhibition of P-glycoprotein (Pgp) which is a drug efflux pump. Inhibition of Pgp results in increased plasma level and subsequent longer duration of action of other drugs used with verapamil [14].

HR in SAL showed significant increases after induction of anaesthesia, which is remained high in the whole assessment period compared with the base. Increase in HR after induction of anaesthesia with ketamine has been reported on previous studies in dogs [1,15]. Administration of ketamine has been shown that indirectly, by catecholamine release, stimulates the cardiovascular system and subsequently leads to increased HR [16]. In the current study, HR in VER was more stable than SAL and no significant changes were observed during evaluation period. It appears depressant effect of verapamil on cardiac function has prohibited increase in HR produced by ketamine. Verapamil has been used to induce hemodynamic depression during various inhalant anaesthesia in dogs [17]. Verapamil has also decreased HR in pentobarbital-anesthetized dogs; however, it has increased HR in conscious dogs [18]. This increase in HR presumably is due to increase in sympathetic tone caused by vasodilation following verapamil administration [3].

However, SAP did not show significant differences in both groups; DAP and MAP in SAL and VER showed significant decreases at T15 and T20 compared with T1. MAP in both treatments was below reference range [19]. Ketamine can increase MAP via the same mechanism

explained for increase in HR [16]. Indeed, the increase in HR after administration of ketamine has a key role in any increase in MAP [1]. It was expected, in the present study, that increase in HR in SAL consequently resulted in increase in MAP, but MAP just showed a slight increase at T5 and then decreased. The condition in VER was similar except for increasing in MAP, which was not observed in this group. The overall decrease in MAP in both treatments can be explained by the cardiovascular depressant effect of isoflurane [20]. Lack of improved MAP in VER can also be attributed to the vasodilatory effect of verapamil [3]. In SAL, HR showed increased values; whereas, MAP did not increase. Lack of changes in MAP, despite of increase in HR, has been reported in humans and dogs anesthetized with ketamine and ketamine-diazepam, respectively [21-23]. Henao-Guerrero and Ricco [22] accounted the increase in cardiac output with no changes in stroke volume for the lack of changes in MAP in dogs anesthetized with ketamine-diazepam and ketamine-propofol.

f_R decreased after premedication with acepromazine and morphine in SAL as well as VER; nevertheless, the changes were not statistically significant. Pre-anaesthetic administration of the aforementioned agents is employed to produce mild to moderate sedation in dogs [24,25]. Acepromazine may decrease f_R in dogs [26]. On the other hand the ability of opioids to induce respiratory depression in a dose-dependent manner has been previously documented [25]. It seems both acepromazine and morphine contributed in inducing respiratory depression after premedication. Apnoea was observed in both treatments at 1 min after induction of anaesthesia. In addition, f_R during isoflurane anaesthesia showed lower values compared with the base in both groups. f_R returned to the level of the base after disruption of isoflurane administration. The incidence of apnoea after ketamine administration and the decrease in f_R during isoflurane anaesthesia can be explained by the effect of premedication agents and isoflurane on exacerbating respiratory depression [25]. Furthermore, it is plausible that ketamine when used in combination with other CNS depressants, has a role on aggravating respiratory depression [2].

In the current study, RT in both groups was significantly at lower values after induction of anaesthesia compared with the base, while RT did not show any significant differences between two treatments at any time points. Reduction in body temperature can be attributed to the impairment of thermoregulatory mechanisms and vasodilation of cutaneous vessels resulting from premedication and anaesthetic agents, respectively [25].

Results of the present study indicated that verapamil when used as co-induction of anaesthesia with ketamine had no effect on induction and recovery quality. Verapamil had a potential trend to prolong recovery induced by ketamine. Addition of verapamil to ketamine resulted in relatively stable HR compared to that of control group. Further studies are needed to determine the effectiveness of verapamil on ketamine-induced anaesthesia in different clinical situations.

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Research Article

Nitric Oxide and Glial Fibrillary Acidic Protein (GFAP) Expression in the Liver Parenchyma in Carbon Tetrachloride-Induced Hepatotoxicity

Güngör Çağdaş DİNÇEL [1] Ayhan ATASEVER [2] Duygu YAMAN [2]

[1] Laboratory and Veterinary Health Program, Eskil Vocational School, University of Aksaray, TR-68800 Aksaray - TURKEY
[2] Department of Pathology, Faculty of Veterinary Medicine, University of Erciyes, TR-38000 Kayseri - TURKEY

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Abstract

Carbon tetrachloride (CCl_4)-induced liver injury causes necrosis and fibrosis, which may lead to cirrhosis and liver failure. Although a lot of study to understand the mechanism of liver fibrosis, our knowledge of the molecular level of this disease is still incomplete. The aim of this study was to investigate the effect and regulation of nitric oxide (NO) and glial fibrillary acidic protein (GFAP) on the severity of hepatic injury in male Wistar albino rats with CCl_4-induced hepatotoxicity and elucidate the underlying mechanism. A total of 20 male rats were randomly divided into healthy control group (A) and CCl_4-induced hepatic fibrosis model group (B), with 10 rats in each group. The results of this study suggest CCl_4-induced hepatic injury is mediated by excessive NO production and up-regulated by inducible and endothelial NO synthase ($P<0.05$), which may plays a role in increasing hepatic injury. Additionally, liver injury induced by CCl_4 demonstrated significant increases in GFAP ($P<0.05$). Histological and immunohistochemical analyses of the CCl_4-treated group exhibited increased inflammatory process and liver necrosis/fibrosis. Inflammatory response especially NO and GFAP expression is identified to play an important role in the development of liver injury and fibrosis induced by CCl_4.

Keywords: *Carbon tetrachloride, Nitric oxide, Glial fibrillary acidic protein, Fibrosis*

Karbon Tetraklorür İle Oluşturulan Hepatotoksisite Karaciğer Paranşimindeki Nitrik Oksit ve Gliyal Fibriller Asidik Protein (GFAP) Sunumları

Özet

Karbon tetraklorür (CCl_4) karaciğerde nekroz ve fibrozis meydana getirerek siroza ve karaciğer yetmezliğine neden olabilmektedir. Günümüzde karaciğer fibrozis mekanizmasının anlaşılmasına yönelik çok fazla çalışma olmasına rağmen, oluşum mekanizması ile ilgili moleküler düzeydeki bilgiler hala eksiktir. Bu çalışmada CCl_4 ile hepatotoksisite oluşturulan erkek Wistar albino sıçanlarda fibrozisin oluşum aşamasında nitric oksit (NO) ve glial fibriller asidik protein (GFAP) etkilerinin araştırılması ve altında yatan mekanizmanın aydınlatılması amaçlanmıştır. Çalışmada 20 adet erkek sıçan, her grupta toplam 10 sıçan olmak üzere sağlıklı kontrol grubu (A) ve CCl_4 ile oluşturulan karaciğer fibrozu grubu (B) olarak rastgele ikiye ayrıldı. Histolojik ve immunohistokimyasal analizlerle CCl_4 grubunda yangısal değişiklikler ile karaciğer nekroz/fibrozis tespit edildi. On iki hafta süreyle CCl_4 verilerek oluşturulan karaciğer hasarının oluşumunda, indüklenebilen ve endotelyal NO sentazın ($P<0.05$) yüksek sunumlarına bağlı olarak artan NO üretiminin önemli bir rol oynadığı belirlendi. Ayrıca, CCl_4 grubunda karaciğerde GFAP ($P<0.05$) sunumlarında önemli artış da gösterildi. Sonuç olarak, CCl_4 kaynaklı karaciğer hasarı ve fibrozisin gelişiminde NO ve GFAP'ın önemli bir rol oynadığı tespit edildi.

Anahtar sözcükler: *Karbon tetraklorür, Nitrik oksit, Gliyal fibriller asidik protein, Fibrozis*

INTRODUCTION

Carbon tetrachloride (CCl_4) is a strong hepatotoxin that causes centrilobular necrosis in experimental animals and is currently used to induce a model of chemical liver injury in experimental studies [1,2]. The hepatotoxicity and carcinogenic properties of CCl_4 have been well defined in humans [3]. Animal models of CCl_4 demonstrate a significant similarity to the symptoms of toxicity in humans including the morphological, biochemical, and pathological

İletişim (Correspondence)
+90 532 2897255
gcdincel@yahoo.com.tr

manifestations. Because of the superficial similarity of cirrhotic responses induced experimentally by CCl_4 in animals, as compared to humans, CCl_4 has been commonly used as a hepatotoxin in experimental studies [2,4-6].

Nitric oxide (NO) is a free radical synthesized from L-arginine by NO synthases activation in parenchymal and non-parenchymal cells [7-9]. In mammals, NO can be generated by three different isoforms of the enzyme NO synthase (NOS). Neuronal NOS (nNOS), which belongs to the constitutive class and is usually localized in neurons and endothelial NOS (eNOS), which is localized in the endothelial cells and is responsible for the regulation of vascular tone, shear stress, and receptor-dependent substances such as bradykinin and acetylcholine. Inducible NOS (iNOS) is expressed in various cells, especially macrophages and plays a part in the inflammatory response [9,10].

Nitric oxide, potentially has both cytotoxic and cyto-protective roles in the liver, which are critical. The amount, location, and duration of NO generated determines the predominance of its protective or harmful effects [11]. Previous studies demonstrated degenerations resulting from severe NO accumulation in the tissues, which triggered apoptosis [11]. In addition, NO creates severe oxidative stress by inducing high level of reactive oxidants from the parenchymal and non-parenchymal cells in the liver [8,12,13]. Furthermore, there are studies suggesting that NO protects the liver against oxidative damage and peroxidation in experimental CCl_4 intoxication [14]. It also contributes to the removal of CCl_4 from the liver by developing the microcirculation in the liver [15,16].

There are studies examining CCl_4 intoxication under conditions where the generation and accumulation of NO were simultaneously investigated [14,15,17,18]. However, the pathophysiology of severe liver injury has not been clarified yet. This is because of the complex role of NO in liver diseases and the conflicting effects that it demonstrates. It is believed that elucidation of the potential role of NO in liver injury, as well as determination of the isoforms of NOS responsible for its generation, would contribute to the development of alternative treatments.

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein that is expressed by cells of the central nervous system and generally considered to be specific for astrocytes [19,20]. GFAP has been described in non neural tissues including liver [21-23]. Hepatic stellate cell (HSC), which can express GFAP, proliferation plays an important role in fibrosis as a result of chronic inflammatory liver diseases [24-26].

In this study, the interactions between NO and GFAP in CCl_4-induced hepatotoxicity as well as the possible roles of NO in the pathogenesis of the associated liver injury have been investigated. In addition, the relationship between the severity of the resulting liver injury and the source of NO generated have been determined.

MATERIAL and METHODS

Ethics Statement

This study was performed in strict accordance with the recommendations in the Guide of The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Erciyes University (Permit Number: 05-11/59).

Animal Model

Wistar albino male rats weighing 200-250 g were used for all. Rats were randomly divided into two groups of ten animals each (n = 10). To induce chronic liver injury, a 1:1 (v/v) mixture of the CCl_4 (Merck, 1.02222) and corn oil was injected intraperitoneally (i.p., 0.25 mL/kg) twice a week for twelve weeks. Control animals received corn oil at a dose of 0.25 mL/kg in the same manner. Rats were fasted and sacrificed while under sodium pentobarbital anesthesia (65 mg/mL) 48 h after administration of the last dose of CCl_4 and corn oil.

Necropsy and Histopathologic Examination

The livers were collected for histopathology and immunohistochemistry. After harvesting, the livers were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at Ph 7.4 for 48 h and then were thoroughly rinsed overnight, under tap water. After performing the routine tissue preparation procedures of dehydration using graded alcohol and xylene, the tissue samples were embedded in paraffin blocks. Paraffin serial sections were cut at a thickness of 4-5 µm and placed onto poly-L-lysine-coated glass slides. Hematoyxlin-Eosin (H&E) and immunohistochemical staining were performed, and the sections were histo-pathologically and immunohistochemically analyzed using a binocular light microscope.

Antibodies

Commercial anti-rat antibodies against eNOS (Thermo-scientific, USA, PA1-037), iNOS (Thermo Scientific, USA, PA1-036), nNOS (Santa Cruz Biotechnology, USA sc-5302), and GFAP (Thermo Scientific, USA, MS-1376) were used in the present study. GFAP antibody was diluted to 1:100, ready to use eNOS antibody was used, nNOS antibody was diluted to 1:100 and ready to use iNOS antibody was used.

Immunoperoxidase Examination

Immunohistochemistry was performed to investigate eNOS, iNOS, nNOS, and GFAP expressions. Commercial antibodies were visualized on 4- to 5-µm-thick paraffin sections using an indirect streptavidin/biotin immuno-peroxidase kit (HRP; Thermo Scientific, USA, PHL100413). All steps were carried out following the procedure described by Dincel and Yıldırım [27]. Tissue sections were incubated with the primary antibody (eNOS, iNOS, nNOS, and GFAP)

for 60 min in a humidified chamber at room temperature. Finally, sections were incubated in aminoethylcarbazole chromogen (Thermo Scientific, USA, HA24938) for 5-10 min to induce the color reaction. Mayer's hematoxylin was applied as a counterstain for 30 sec. As a control for non-specific endogenous peroxidase and biotin activities in each test, the primary antibody step was omitted.

Masson Trichrome with Anilin Blue Staining

The extent of liver fibrosis was evaluated by Masson trichrome staining from the CCl_4-treated and control groups rats. After deparaffinization and rehydration, liver sections were stained with Masson's trichrome. Masson trichrome staining was performed following the manufacturer's instructions (Bio Optica, CND Code: W01030799, Milano).

Histomorphometric Analysis and Statistics

The density of immunpositive staining was measured using a computerized image system composed of a Leica CCD camera DFC420 (Leica Microsystems Imaging Solutions, Ltd., Cambridge, UK), connected to a Lecia DM4000 B microscope (Leica Microsystems Imaging Solutions, Ltd.) was used according to the procedure described by Dincel and Atmaca [28]. The pictures of five random fields selected and consecutive 20x objective microscopic fields were captured by the Leica QWin Plus v3 software (Leica Microsystems Imaging Solutions) at a setting identical to the image system. For examining the staining for each antibody, we used the same setting for all slides. Integrated optical density of all the positive staining of eNOS, iNOS, nNOS and GFAP in each photograph was measured. For the quantification of mean was quantified as the eNOS-, iNOS-, nNOS- and GFAP-positive area/total area were measured and calculated by Leica Qwin Plus on the pictures. Data were statistically described in terms of mean and standard deviation (mean±SD) for area %. For evaluating the non-parametric data, Mann-Whitney U-test was performed to compare eNOS, iNOS, nNOS and GFAP immunoreactive cells and immunopositively stained areas in the CCl_4-treated animals versus the healthy controls. A P value of < 0.05 was considered significant. The data were presented as means ± SD. All statistical analyses and graphs were prepared using GraphPad Prism version 6.0 (GraphPad Software, La Jolla California, USA).

RESULTS

Histopathologic Findings

Histopathological examination of rat livers from healthy control group showed normal hepatic lobular architecture *(Fig. 1a)*. The most prominent histopathological findings in the livers of CCl_4-treated rats were characterized diffuse vacuolar fatty changes, large areas of extensive pericentral/centrilobular necrosis/fibrosis, loss of hepatic architecture and hemorrhage *(Fig. 1b)*. Fatty degeneration and coagulative necrosis, especiallyamong hepatocytes settled in the central zone and midzone, were clearly identified in the CCl_4-treated group *(Fig. 1b)*. In addition, necrotic cells were evident in centrilobular areas, and these were surrounded by inflammatory cells.

Masson Trichrome with Anilin Blue Staining Findings

The degree of liver fibrosis in CCl_4-treated group was more severe than that in the control group. The collagen fibers were heavily deposited around portal tracts and central veins in CCl_4-treated group *(Fig. 1c)*. In addition, pseudolobules formations were showed in CCl_4-treated rats.

Nitric Oxide Synthase (NOS) and Glial Fibrillary Acidic Protein (GFAP) Expression

In this study, immunohistochemical staining score that iNOS, eNOS and GFAP immunoreactivity was dramatically enhanced in activated rat parenchymal and nonparenchymal liver cells. iNOS, eNOS and GFAP expressions in the livers of CCl_4-treated rats were higher than healthy control group rats (P<0.05) *(Table 1)*. Increased expression of GFAP expression was observed in the more extensively injured liver samples *(Fig. 2 a,b,c)*. High GFAP expression was observed in limited to the fibrosis area in HSC *(Fig. 2 a,b)*. In addition to GFAP expression increased significantly in the vascular endothelial cells *(Fig. 2b)*, which was also significantly higher than the levels in the healthy control group. The liver sections from CCl_4-treated rats showed strong GFAP immunopositive bands lining the hepatic sinusoids and pericentral hepatocyte cytoplasm *(Fig. 2c)*. GFAP-positive cells exhibited diffuse distribution in the livers of CCl_4-treated rats.

Kupffer and endothelial cells iNOS and eNOS immunoreactivity were evaluated as a remarkable finding in the

Table 1. *Immunoperoxidase test results and statistical data*
Tablo 1. *İmmünoperoksidaz test sonuçları ve istatistiksel verileri*

Animals	N	eNOS		P <	iNOS		P <	nNOS		P <	GFAP		P <
		Mean	Sd		Mean	Sd		Mean	Sd		Mean	Sd	
Control animals	10	5.379	0.732	0.014	5.117	0.453	0.001	1.800	0.509	0.821	2.937	0.423	0.009
CCl_4-treated animals	10	6.379	0.735		6.935	0.670		1.880	0.382		3.548	0.525	

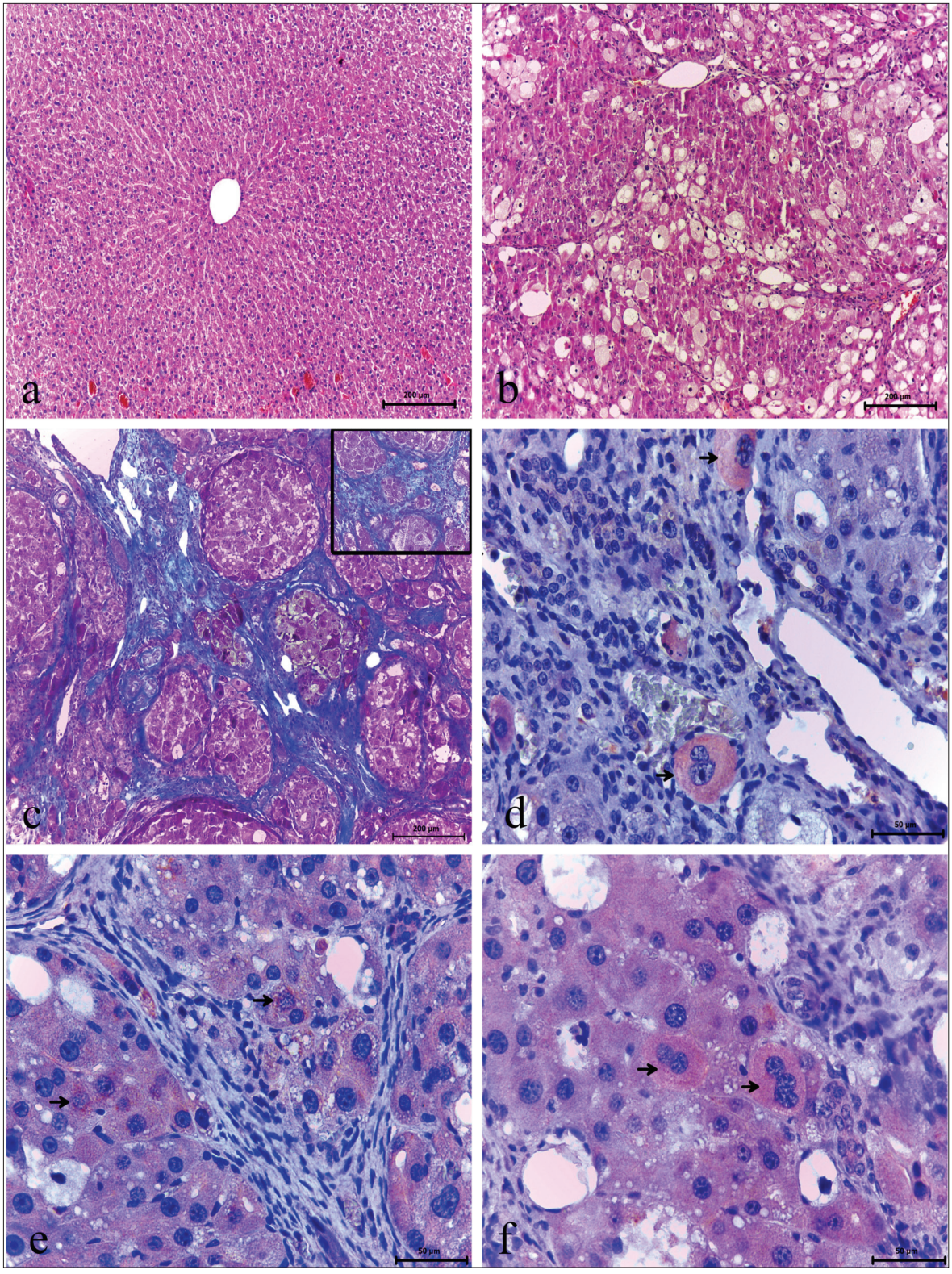

Fig 1. (a) Healthy liver tissue, H&E; **(b)** In the CCl_4 group, observed more severe degeneration and balooned/necrotic hepatocytes, H&E; **(c)** Excessive collagen fibers deposition. Collagen fibers of the connective tissues are identified by their blue color. Masson's trichrome stain and upper small photo large magnification, 40 µm; **(d,e,f)** Strong expression of iNOS in degenerated hepatocytes *(arrows)*. ABC technique (anti-iNOS), Mayer's hematoxylin counterstain

Şekil 1. (a) Sağlıklı karaciğer dokusu, H&E; **(b)** CCl_4 group, şiddetli dejeneratif ve balonumsu/nekrotik hepatositler, H&E; **(c)** Yoğun kolajen fiber depolanması. Konnektif dokudaki kolajen fiberler mavi görülüyor. Masson's trichrome boyaması ve küçük fotoğraf büyük büyütme, 40 µm; **(d,e,f)** Dejeneratif hepatositlerde *(oklar)* şiddetli iNOS sunumları. ABC teknik (anti-iNOS), Mayer›s hematoksilin arka plan boyaması

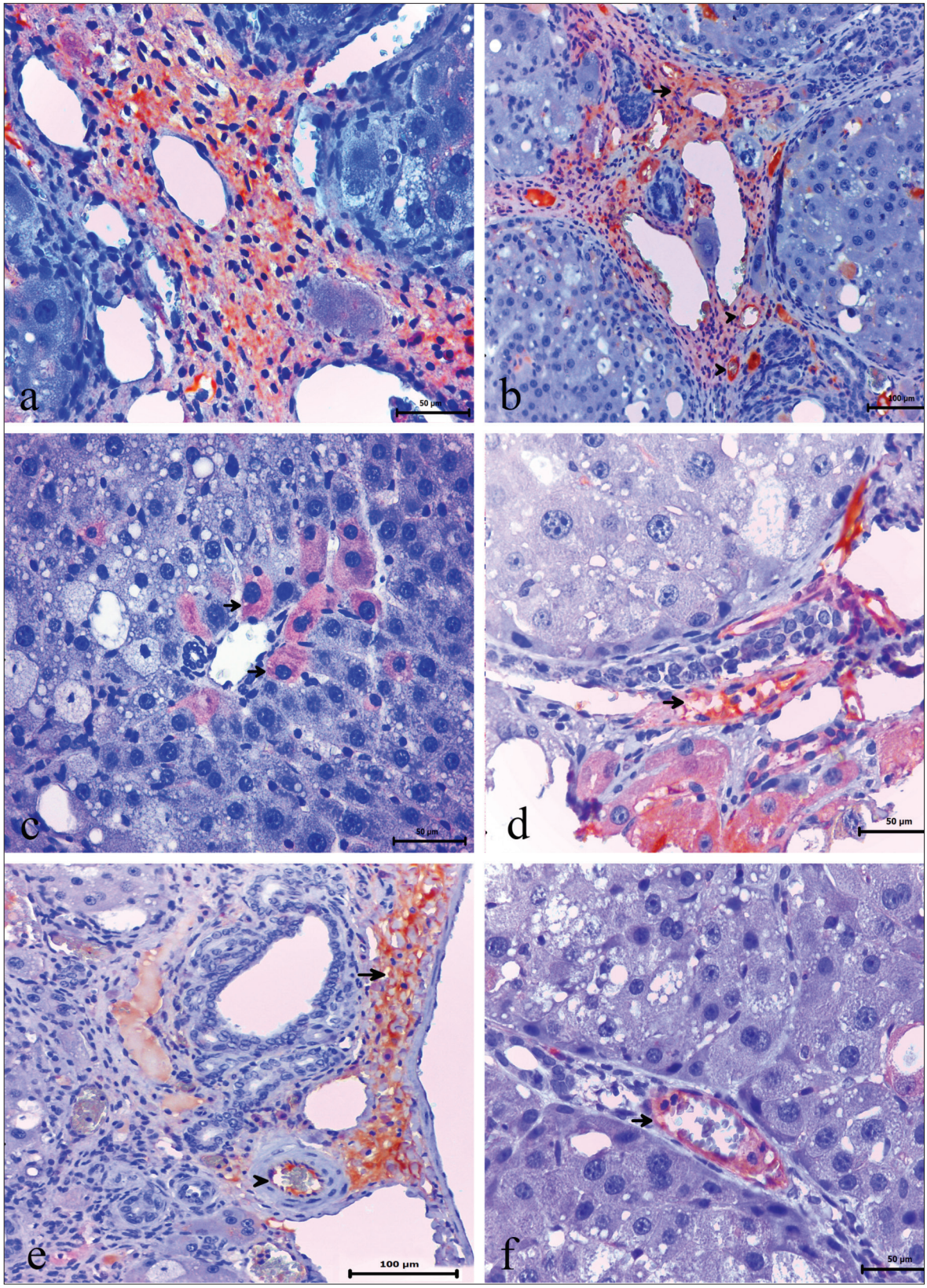

Fig 2. (a,b,c) In the CCl_4 group, strong expression of GFAP in scar and degenerated area *(arrow)*, endothelial cells (arrowhead), pericentral hepatocytes. ABC technique (anti-GFAP), Mayer's hematoxylin counterstain; **(d,e,f)** Modarete expression of eNOS in the endothelial cells *(arrow)*, strong expression of eNOS in some scar area *(arrow)* and endothelial cells *(arrowhead)*. ABC technique (anti-eNOS), Mayer's hematoxylin counterstain.

Şekil 2. (a,b,c) CCl_4 group, skar ve dejenere olmuş alanlarda *(ok)*, endotelyal hücrelerde (okbaşları) ve perisentral hepatositlerde şiddetli GFAP sunumları. ABC teknik (anti-GFAP), Mayer's hematoksilin arka plan boyaması; **(d,e,f)** Endotel hücrelerde orta şiddette eNOS sunumları *(ok)*, skar bölgesinde *(ok)* ve endotel hücrelerde *(okbaşı)* şiddetli eNOS sunumları. ABC teknik (anti-eNOS), Mayer›s hematoksilin arka plan boyaması

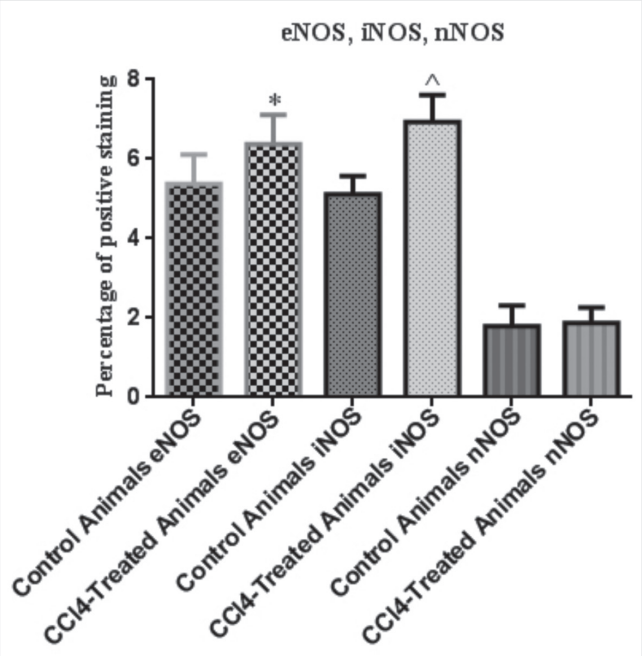

Fig 3. Comparison of eNOS, iNOS and nNOS immunopositivity. '*' indicates values that are significantly greater than those of the healthy control group for eNOS. '^' indicates values that are significantly greater than those of the healthy control group for iNOS.

Şekil 3. eNOS, iNOS ve nNOS immünopozitifliklerin karşılaştırılması. eNOS için '*' ve iNOS için '^' istatistiksel olarak sağlıklı kontrol grubundan yüksek olduğunu vurgulamaktadır

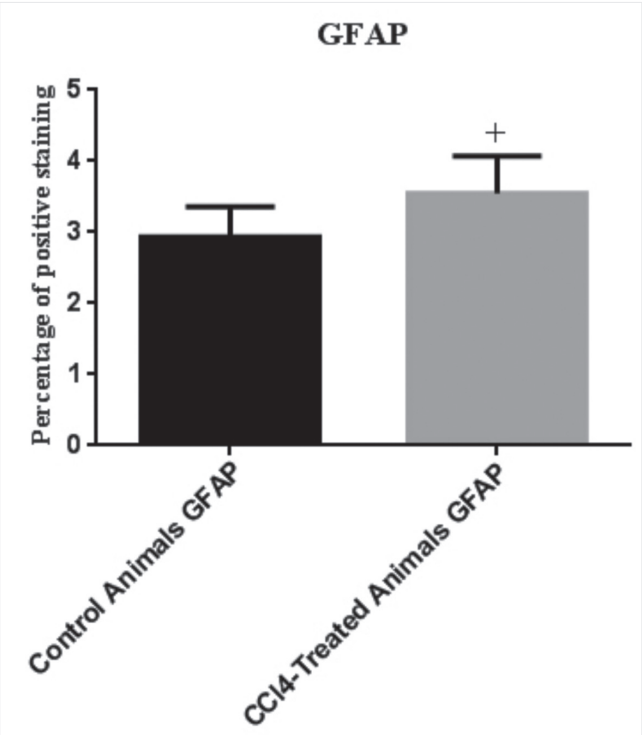

Fig 4. Comparison of GFAP immunopositivity. '+' indicates values that are significantly greater than those of the healthy control group for GFAP

Şekil 4. GFAP immünopozitifliklerin karşılaştırılması. '+' istatistiksel olarak sağlıklı kontrol grubundan yüksek olduğunu vurgulamaktadır

fibrosis area. The more severe iNOS immunoreactivity was determined in parenchymal cells and infiltrating inflammatory cell (lymphocytes) area *(Fig. 1 d,e,f)*. Exclusively in the expression of eNOS a certain amount of increase was observed in endothelial cells *(Fig. 2 d,e,f)*.

nNOS immunopositive stainings was observed in vascular endothelia, hepatocytes and Kupffer cells in livers of CCl_4-treated rats and healthy control group animals. However, the differences was not statistically important between in CCl_4-treated and healthy control group ($P>0.05$) *(Fig. 3)*.

iNOS and eNOS expressions in CCl_4-treated group were higher when healthy control group difference was statistically important *(Fig. 3)*. In addition to GFAP expressions in CCl_4-treated group were higher when healthy control group Difference was statistically important *(Fig. 4)*.

DISCUSSION

In this study, HSC numbers activated with GFAP staining and NO generation in the liver, have been studied immunohistochemically. It has been determined that the NO generated was from the non-parenchymal cells (endothelial, Kupffer, and natural killer cells related to the liver hepatic stellate cells) and from parenchymal cells. It was noteworthy that compared to the healthy control, stellate cell numbers in rats with CCl_4 toxicity, demonstrated a significant increase in iNOS, eNOS and GFAP ($P<0.05$). There was no change in nNOS generation ($P>0.05$).

Liver fibrosis is the pathologic result of chronic liver diseases, and it is characterized by HSC proliferation [24]. HSCs, which express GFAP in normal livers, play important roles in hepatic fibrogenesis [25,26]. This study demonstrated that a high level of GFAP immunopositivity in the HCS was associated with CCl_4 toxicity and activated the HCS considerably. Additionally, the presence of intense GFAP immunopositivity, particularly in the perisinusoidal regions suggests that the fibrogenesis commences from these regions. Furthermore, increasing GFAP immunoreactivity suggests that active fibrogenesis may still be on going. Immunohistochemical demonstration of GFAP expressing HCS count was strongly predictive of the degree of liver fibrosis in CCl_4-induced hepatotoxicity.

Kupffer cell infiltration and activation lead to significantly increases the level of activation of stellate cells. In addition, Kupffer cells create reactive oxygen species (ROS) in the liver. In this case, there is an apparent increase in the stellate cell activation and collagen synthesis [29]. However, the increase in the stellate cell activation is thought to be linked to CCl_4 hepatotoxicity because there are studies that suggest that NO could modulate the activation of stellate cells and thereby, decrease fibrosis [11]. In this study, we speculate that Kupffer and stellate cells serve as critical effector cells in the liver damage linked fibrosis induced CCl_4 toxicity. Furthermore, it is obvious that these

cells have complicated the underlying functions in the molecular mechanism of CCl_4 toxicity.

It has been observed that inhibition of iNOS activity enhances the sensitivity of the liver of rats to hepatotoxic agents. Furthermore, in sensitized rats, the severity of CCl_4-induced liver necrosis increased [18]. In previous studies, it was demonstrated that the increase in hepatic iNOS in rats with liver injury [17]. This finding is consistent with other studies demonstrating that NO increases in CCl_4-induced hepatotoxicity [15,17,30]. In this study, NO is thought to be one of the most significant mediators in activated macrophages and contribute greatly to the pathogenesis of CCl_4 hepatotoxicity. Collectively, these findings support the idea that the NO generated in macrophages and hepatocytes has both protective and pathological roles in the development of CCl_4-induced liver damage.

Cytokine production in healthy liver is very low [31]. Additionally, the balance between the types and in the amounts of cytokines produced is an important factor in the recovery of the liver [32]. However, the roles of cytokines in the regenerative process are still not clear. In pathological conditions, a significant increase has been observed in the release of effector molecules. In this situation, cytokines have a serious role in the pathogenesis of liver diseases. It has been shown that Kupffer cells are involved in the chemical liver damage induced by CCl_4 and alcohols [33-35]. It has also been shown that Kupffer cells enhance tissue damage in the liver through mediators such as biologically active ROS and cytokines [36]. In this study, it is thought that Kupffer cells contribute to the pathogenesis of chemical-mediated liver damages, by releasing biologically active mediators.

In this study, we determined that hepatic NO generation increases significantly, and the major enzyme involved in its production is iNOS. Furthermore, it has been shown that there is a significant increase in eNOS, which also contributes to the existing NO. However, it is not clear which of the NOS isoforms contribute to the change in the hepatic arterial blood flow in acute liver damage. In addition, we speculated that the high level of iNOS expression suggests that this isoform maybe responsible for generating the NO that regulates the hepatic circulation during inflammation and reduces the effect of degeneration. Additionally, endothelial cells express both constitutive NOS and iNOS [37]. Another effect that was observed with chronic CCl_4-induced hepatotoxicity was the iNOS-mediated vasodilation, which appeared to reduce the liver damage. However, more studies need to be conducted to clarify the mechanism involved.

Our findings suggest that NO plays an important role in the advanced phases of the pathogenesis of liver degeneration and fibrosis. In summary, the enhanced NO expression persists over a long period, and contributes to the liver fibrosis. We speculate that the persistent generation of NO observed in chronic CCl_4 toxicity plays an important role in the continuous degeneration and fibrosis, rather than in hepatoprotection.

In liver diseases, different chemical toxins show variations in the rate and time of toxicity. We believe that the increased activation and numbers of HSCs in the livers of CCl_4-treated rats depending on degeneration of liver tissue occurring as a result of the pathological production of NO. The obvious relationship between NO and GFAP in the liver should be further evaluated using different immunohistochemical markers and molecular studies. The results of such in-depth studies would be expected to contribute to providing further explanations for the possible liver damage and repair mechanisms. In addition, these studies may even be beneficial to the process of developing new therapeutic interventions.

In conclusion, the differences in the NO levels observed in CCl_4 hepatotoxicity may be critical to the patho-physiology of NO-induced liver damage. This suggests that the existing major source of NO is also mainly iNOS-generated. The increase in the NO level may be responsible for the hemo-dynamic changes occurring in patients experiencing CCl_4 toxicity. Maintaining the stability of NO levels or an increase under certain conditions may imply that a protective mechanism connected to NO levels can be activated. Furthermore, we saw that there is a definite relationship between stellate cell counts and CCl_4 toxicity. GFAP expressing stellate cells may play an active role in the chemical-induced liver pathology and hepatic fibrogenesis and it count had predictive values for perisinusoidal fibrosis in CCl_4-induced hepatotoxicity.

Conflict of Interests

The authors declare no conflict of interest.

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Research Article

Serum Iron Status and Its Relation with Haematological Indexes Before and After Parturition in Sheep

Huseyin CIHAN [1] Ethem Mutlu TEMIZEL [1] Zeki YILMAZ [1] Yesim OZARDA [2]

[1] Uludag University, Faculty of Veterinary Medicine, Animal Teaching Hospital, Department of Internal Medicine, Gorukle Campus, TR-16059 Nilufer, Bursa - TURKEY
[2] Uludag University, Faculty of Medical Science, Department of Biochemistry, Gorukle Campus, TR-16059 Nilufer, Bursa - TURKEY

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Abstract

Erythrocyte indices, total iron binding capacity (TIBC) and unsaturated iron binding capacity (UIBC) values provide important information for the diagnosis of anaemia in iron deficiency conditions and inflammatory diseases. The aim of this study was to evaluate the serum iron status and its relation with haematological indexes before and after parturition in sheep. Blood samples were collected from pregnant (n=30) and non-pregnant aged-matching Ivesi sheep (n=30), housed under the same conditions, at 1 week (wk) (±2 days) before expected parturition, in parturition (baseline), at day 1 and at 1, 2, 3, 4, 6, 8 and 12th wk subsequent to the parturition, to determine the serum levels of iron (SI), TIBC and UIBC as well as the haematological parameters. Erythrocyte (RBC) count, haematocrit (Hct) and haemoglobin (Hb) values dropped at 12 wk ($P<0.05$) according to 1 wk (±2 days) before expected parturition and during parturition. Mean corpuscular volume concentrations were also found lower ($P<0.05$) at 4-12 wk after parturition, in comparison with their baselines. After the parturition, SI concentration decreased at day 1 ($P<0.05$) and then remained low ($P<0.05$) during the study. TIBC was higher for the first 2 wk, but was lower at 6-12 wk compared with its baseline ($P<0.05$). UIBC values were in parallel to TIBC values after the parturition. In conclusion; negative effects on leukocytes of metabolic stress after delivery should be taken into consideration, as it can trigger the potential problems during this period. In postpartum period, SI levels along with erythrocyte indices can be useful parameters to follow the iron loss by fetus and colostrum. Incompatible results of TIBC and UIBC levels with SI and erythrocyte indices in this study showed that TIBC and UIBC may not be useful biomarkers to evaluate serum iron values, particularly in postpartum period.

Keywords: *Iron, Binding Capacity, Unsaturated, Erythrocyte Indices, Sheep*

Koyunlarda Doğum Öncesi ve Sonrası Serum Demir Durumu ve Hematolojik Endekslerle İlişkisi

Özet

Eritrosit belirteçleri, total demir bağlama kapasitesi (TIBC) ve demir bağlama kapasitesi (UIBC) değerleri demir eksikliği durumları ve inflamatuar hastalıklarda, aneminin tanısı için önemli bilgiler sağlar. Bu çalışmanın amacı, doğum öncesi ve sonrası koyunlarda serum demir durumu ve hematolojik endekslerle ilişkisini değerlendirmektir. Serum demir seviyesi (SI), TIBC, UIBC ve hematolojik parametreleri belirlemek üzere kan örnekleri aynı yaş ve barındırılma koşullarındaki gebe (n=30) ve gebe olmayan (n=30) İvesi koyunlarından doğumdan tahmini 1 hafta (±2 gün) önce, doğum zamanı ve doğumdan 1 gün sonra ve 1, 2, 3, 4, 6, 8 ve 12. haftalarda toplandı. Eritrosit (RBC), hematokrit (Hct) değer ve hemoglobin (Hb) seviyeleri postpartum dönemin 12. haftasına doğru ($P<0.05$) doğumdan 1 hafta (±2 gün) önceki ve doğum sırasındaki değerlerine oranla daha düşüktü. Ayrıca doğum sonrası 4-12. haftalardaki ortalama eritrosit hacmi konsantrasyonları da doğum sırasındaki değerlerine oranla düşük bulundu ($P<0.05$). Doğum sonrası SI seviyesi 1. günde düşmüş ($P<0.05$) ve çalışma boyunca da düşmeye devam etmiştir ($P<0.05$). TIBC doğum sonrası ilk 2 hafta boyunca yüksek iken, doğum anındaki değere kıyasla 6-12. haftalarda daha düşüktü ($P<0.05$). UIBC değerleri doğumdan sonraki TIBC değerlerine paralel seyretmiştir. Sonuç olarak, potansiyel problemleri tetikleyebileceği için, doğum sonrası metabolik stresin lökositler üzerindeki negatif etkisi bu periyodda dikkate alınmalıdır. Postpartum dönemde eritrosit indeksleri ile birlikte serum demir düzeyinin belirlenmesi fetüs ve kolosturum aracılığı ile meydana gelen demir kaybının takip edilebilmesi açısından faydalı parametrelerdir. Bu çalışmada saptanan TIBC ve UIBC düzeyleri ile SI ve eritrosit indeksleri arasındaki uyumsuzluk, TIBC ve UIBC'nin özellikle postpartum dönemde SI düzeylerinin değerlendirilmesinde yararlı biyobelirteçler olmayabileceğini göstermiştir.

Anahtar sözcükler: *Demir, Bağlama Kapasitesi, Doymamış, Eritrosit Belirteçleri, Koyun*

İletişim (Correspondence)
+90 224 2940813
hcihan@uludag.edu.tr

INTRODUCTION

Iron is an essential constituent of the haem portion of haemoglobin (Hb), and as the Hb in aged RBCs is broken down and fresh Hb synthesized, so the iron in the body is continually re-cycled. Its transport within the body is in the plasma, attached to a beta-1 globulin known as transferrin [1].

Iron derived from the degradation of Hb by the mononuclear phagocyte system (MPS) may be stored in the MPS (liver, spleen and bone marrow) in the form of ferritin or hemosiderin [2]. However, most of the iron passes into the plasma, becomes bound to transferrin and is placed within newly formed RBCs and released into the circulation [1].

The total iron binding capacity (TIBC) is a measure of the total serum transferrin concentration. TIBC is the amount of iron that serum transferrin can bind when all iron - binding sites are saturated. The concentration of the iron standard minus the unbound iron is called the unbound iron-binding capacity (UIBC), which is used to calculate the TIBC (UIBC plus total serum iron concentration). TIBC is increased in iron deficiency in humans, horses, cattle, and pigs, but does not appear to be increased in clinical iron deficiency in dogs [2,3]. In a study by [4], TIBC levels in sheep exposed to dietary limitation were higher than that of control group. Also, TIBC decreased or in the low - normal range in inflammatory diseases [2].

Iron is required for Hb synthesis. Both true and functional iron deficiency result in deficient Hb synthesis. Because Hb concentration signals cessation of cell division, iron deficient cells undergo one or two more cell divisions resulting in formation of microcytes [2]. Both mean corpuscular volume (MCV) and mean corpuscular Hb concentration (MCHC) tend to be decreased in iron deficiency [5], but in some cases MCHC may be normal when the MCV is mildly decreased [2]. Although it can be artefactual, microcytosis in routine practice is considered as a hallmark of iron deficiency, usually associated with hyposideremia, while it can also occur in other pathophysiological conditions [6]. To authors' best knowledge, there is no data yet regarding iron status and its related parameters, TIBC and UIBC, in a controlled randomized long-term study with healthy pregnant and non-pregnant sheep. Thus, the aim of this study was to evaluate serum levels of iron, TIBC and UIBC, as well as to compare between erythrocyte indices and iron status before and after parturition in sheep.

MATERIAL and METHODS

Admission of Animals

This study was conducted in the town of Nilufer in Bursa, Turkey. The herd consisted of totally 150 Ivesi sheep. In different compartments of the same pen 85 sheep, 65 goats, all animals were fed with alfalfa, as forage wheat bran, sunflower meal and soybean meal, as well as a commercial mixture for lambs as concentrate which consists of ground corn, barley, calcium carbonate, and vitamin premix. All sheep had free access to water. Blood sampling and treatments were performed under the control of the farm health coordinator in accordance with the Animal Welfare Guidelines [7]. The study was approved by Committee of the Animal Experimentation of Uludag University (1.06.2004/2).

Collection of Samples

Animals were divided into two groups: pregnant (n=30) (during second breeding season) and non-pregnant (n=30) (four months after second parturition), 2-3 years old Ivesi sheep. All animals were housed under the same conditions. Synchronization was not applied and sheep were mated by ram. Pregnancy examinations were performed by conducting an ultrasound of the abdominal area, and rectally during the 3rd month post-mating. According to the examination results, clinically healthy pregnant sheep were included to the study. All ewes were let to graze on pasture during periods when seasonal conditions were appropriate. When the ewes returned to the sheep corral, appropriate forage and concentrated feed were provided after the nutritive value of the pasture was taken into consideration. Routine health controls and deworming were applied to all animals after mating.

Blood samples were collected at 1 week (wk) (±2 days) before expected parturition, at parturition (baseline), day 1, and 1, 2, 3, 4, 6, 8 and 12th wk subsequent to the parturition. Blood was sampled one time from non-pregnant group. Samples were collected from all sheep in the study between Fabruary and May. The heparinized blood (Vacusera 4 mL, lithium heparin tube, Turkey) obtained through jugular vena puncture was centrifuged to separate the plasma and stored at -20°C until biochemical analysis. Additionally, blood for haematological evaluation was collected into 2 mL vacutainer tube with EDTA (Hema&Tube®, Turkey) from the same vein and analysed within 2 h. The haematological parameters (complete cell counts with 5-parts leucocyte differentials) were analysed using an automatic analyser (Cell-Dyne 3500®, Abott Inc., USA), and white blood cell (WBC) count, red blood cell (RBC) count, Hct, Hb and MCV were recorded for this study.

Serum iron (SI), UIBC and TIBC concentrations were measured on Architect ci8200 System (Abbott Laboratories, IL, USA) according to the manufacturer's instructions.

Statistical Analysis

Data between non-pregnant and pregnant sheep (before parturition) was analysed by student t test. Data obtained 1 wk before parturition, during parturition (baseline), 1 day after parturition and weekly until 12 wk was analysed by one-way analysis of variance (SigmaStat 3.1, GmbH,

Germany). Tukey test was used as post-hoc test. Results were expressed as mean ± standard deviation (SD), and a P value less than 0.05 was considered as statistically significant.

RESULTS

A statistical significance was detected for WBC between week 2 and baseline, day 1 and week 8 (P<0.05). Erythrocyte indices and SI, TIBC and UIBC in non-pregnant and in sheep 1 week before (± 2 days) parturition, and during parturition (baseline) and post-partum periods are shown in *Table 1* and *Table 2* respectively. In non-pregnant and pre-partum sheep, there were statistically significant differences in RBC, Hct, Hb and MCV (P<0.05). In post-partum period, RBC, Hct, Hb levels in baseline levels were higher than values period after week 3 (P<0.05). MCV, TIBC and UIBC values in day 1 were different from values in period after week 6. There were also statistically differences between baseline for UIBC value (P<0.05). For serum iron, there were statistically differences (P<0.05) in all the time (except week 1 and week 3) compared with baseline.

Table 1. *Erythrocyte indices, serum iron (SI), TIBC and UIBC results in non-pregnant and in sheep 1 week before parturition*

Tablo 1. *Gebe olmayan ve doğumdan bir hafta önceki koyunlarda eritrosit belirteçleri, serum demir, TIBC ve UIBC sonuçları*

Parameters	Non-pregnant (n=30)	1 Week (± 2 days) Before Parturition (n=30)
WBC (x10^9/L)	8.4±3.3^a	8.6±3.7^a
RBC (10^{12}/L)	7.1±8.1^a	7.6±1.1^b
Hct (%)	35.2±0.4^a	38.5±0.5^b
Hb (g/L)	12±0.1^a	13±0.^{b}
MCV (fl)	31±0.3^a	36±0.3^b
Iron (µg/dL)	187.2±15.4^a	183.1±10.1^a
TIBC (µg/dL)	477.8±13.6^a	466.3±11.7^a
UIBC (µg/dL)	265.7±26.3^a	317.3±17.7^a

^{a,b} Differences between the values involving different letters in the same row were found to be statistically significant at P<0.05

DISCUSSION

WBC counts may increase in post parturient period due to various causes, such as ketosis, retentio secundinarum, infectious disorders, mastitis, metritis and the combination of disorders [8,9]. Increasing numbers of leucocytes toward the time of delivering [10,11] were also found in the sheep studied herein, which were with normal clinical findings. In the present study, baseline WBC counts markedly increased in 2 weeks after parturition. None of pregnant sheep examined showed any clinical manifestations during the observation periods. Reproductive examinations of sheep were normal during after parturition. New born lambs were also apparently healthy, grew and were weaned normally. Thus, observed increases in WBC count in the study might be the result of the metabolic stress [10-12] associated with parturition and lactation.

RBC indices are the most important parameters in the evaluation of iron deficiency anaemia and total iron status. Our results were consistent with those found in a study which was conducted on Tsigain sheep with varying physiological states, such as healthy non-pregnant, pregnant, and lactating, and determined that Hb values were within physiological ranges in all 3 groups, while showing a decrease in the postpartum period as compared to the prepartum period [13]. On the contrary, no any statistically significant difference between prepartum and postpartum RBC indices were observed in another study [14]. However, in the same study [14], the finding of higher RBC and Hct values in the postpartum period as compared to the prepartum period. On the other hand, in another study, the authors reported elevated postpartum Hb and Hct values as compared to the prepartum period [15]. In this study, postpartum RBC indices (RBC, Hct, Hb, and MCV) were found to be lower than levels in 1 wk (±2 days) before expected parturition. Hemodilution during prepartum period can cause decrease in Hct and RBC volume [16-18]. However, this condition may continue within a week after parturition [18]. After this period, during a decrease in hemodilution, an increase in Hct and RBC count can

Table 2. *Erythrocyte indices, serum iron (SI), TIBC and UIBC results in sheep during parturition and postpartum periods*

Tablo 2. *Doğum sırasında ve postpartum dönemdeki koyunlarda eritrosit belirteçleri, serum demir, TIBC ve UIBC sonuçları*

Parameters	Baseline (n=30)	Day 1 (n=30)	Week 1 (n=30)	Week2 (n=30)	Week 3 (n=30)	Week 4 (n=30)	Week 6 (n=30)	Week 8 (n=30)	Week 12 (n=30)
WBC (x10^9/L)	8.1±3.6^b	8.1±7.6^b	8.6±6.8^{a,b}	12.5±7.4^{a,b}	9.2±3.4^a	9±4.0^{a,b}	8.6±3.3^{a,b}	8.3±3.5^b	9.3±4.2^{a,b}
RBC (10^{12}/L)	7.6±1.1^a	7.3±1.06^{a,b}	7.2±1.5^{a,b}	7.4±1.5^{a,b}	7.1±1.0^b	7.0±1.1^b	6.9±1.1^b	6.7±1.2^b	6.6±1.3^b
Hct (%)	38.5±0.5^a	36.4±0.4^{a,b}	34.4±0.5^b	36.1±0.6^{a,b}	35.2±0.5^b	34.5±0.6^b	34.3±0.5^b	31.6±0.8^b	30.4±0.7^b
Hb (g/L)	12.8±0.2^a	12.2±0.1^{a,b}	12±0.2^{a,b}	12.2±0.2^{a,b}	11.8±0.2^b	11.6±0.2^b	11.5±0.2^b	10.7±0.2^b	10.2±0.2^b
MCV (fl)	36±0.3^{a,b}	36±0.4^a	35±0.5^{a,b}	36±0.4^{a,b}	35±0.3^{a,b}	34±0.3^{a,b}	34±0.2^b	34±0.3^b	33±0.3^b
Iron (µg/dL)	183.1±10.1^a	122.7±17.1^b	137.9±6.1^{a,b}	120.4±15.2^b	138.4±7.4	125.2±7.7^b	133.8±8.0^b	126.4±13.2^b	118.4±8,5^b
TIBC (µg/dL)	466.3±11.7^{a,b}	532.8±15.9^a	532.7±20.3^{a,b}	520.0±10.6^{a,b}	478.4±15.8^{a,b}	476.8±18.1^{a,b}	430.2±10.1^b	410.3±11.0^b	389.7±13.6^b
UIBC (µg/dL)	317.3±17.7^b	412.5±30.2^a	398.4±23.2^{a,b}	403.6±14.9^{a,b}	339.1±18.7^{a,b}	351.5 ±15.3^{a,b}	308.7±13.1^b	264.9±13.4^b	280.7±25.1^b

^{a,b} Differences between the values involving different letters in the same row were found to be statistically significant at P<0.05

be detected. In the present study, while the presence of a decrease in RBC and Hct levels due to hemodilution within one week was observed, a slight increase in these values were detected in second week. However, this rise is expected to continue in the other coming weeks [18,19], a decrease was observed in the present study. In case of the decreases in these parameters were within reference intervals, it was thought that decreases in erythrocytes and Hct in lactation period can be caused by lactation stress which can cause increases in osmotic fragility [20].

In studies conducted in cows and buffaloes, were reported to have lower serum iron levels in postpartum according to prepartum period [21,22]. Miltenburg et al.[21] conducted a study on the level of plasma iron in dams and calves and found that during parturition and early in postpartum period dams had lower plasma iron level than prepartum period in contrast with the calves' plasma iron levels. One of the recent study indicated that the low level of iron in postpartum period of cattle can be hardly increased by mineral injection including iron chloride [23]. In the present study, postpartum serum iron levels between 1-12 weeks were lower than those in non-pregnant sheep and those in 1 wk before parturition. This study revealed decreased iron levels in sheep after parturition as seen in cattle and buffaloes [21-23]. Miltenburg et al.[21] suggested that the use of dam's iron stores by fetus at delivery was associated with these low iron levels in dams. Also, in the same study, it was noted that calving and expansion of colostrum intake can lower the plasma iron level in dams. In our study, the tendency of SI to decrease was parallel to the tendency of RBC indices to fall. Moreover, several complications such as; hemorrhage during parturition in sheep with high body score and acute/chronic infections, which were not observed in our study, might also lead to reduced SI status. However, the cause of fluctuations in serum iron levels was not fully understood, their levels are known to be affected with the amount of iron taken together with last food intake before sampling [24].

In the present study, TIBC value did not differ statistically between non-pregnant and pregnant sheep. Rising TIBC levels within the first 2 weeks after delivery may be associated with elevated transferrin level. Although transferrin levels were not evaluated in the study, transferrin, secreted as an acute phase reactant from the liver, is known to rise in the presence of acute inflammation and 1 week after parturition [25,26]. In a study, performed on Anatolian Black Goats in Siirt Province, Turkey, it was found that serum transferrin levels in blood samples taken 1 week after delivery were statistically significantly higher than those measured before delivery [26], which was a result consistent with our explanations. As compared to the baseline values, TIBC started to drop 2 weeks after the delivery and continued to decrease until the end of the study, indicating the physiological variation in SI and TIBC in the postpartum period. The factors behind this response may be reduced iron level in the ration along with decreased levels of serum transferrin and its tissue receptors. Serum transferrin value is an important parameter that affects the TIBC level [2]. Similarly, in a study, conducted on healthy pregnant sheep, was observed physiological variation between prepartum and 2-week postpartum TIBC levels [2].

In the present study, UIBC was 265.7±26.3 µg/dL in non-pregnant animals and 317.3±17.7 µg/dL in sheep 1 wk before parturition. In a study on healthy Awassi sheep and those with iron deficiency anaemia, UIBC value was found 149±5 µg/dL, 175±7 µg/dL and 154±9 µg/dL in the non-pregnant sheep, and in sheep in prepartum and postpartum periods, respectively [27]. In another study, the mean prepartum UIBC value was 157.2±5.3 µg/dL in Akkaraman sheep [28]. In the present study, the UIBC values were higher than those found in both of the above mentioned studies [27,28], which may be associated with variation between the sheep, or factors influencing the transferrin and iron levels.

In conclusion; although data detected from the study were within the reference ranges, negative effects on leukocytes of metabolic stress after delivery should be taken into consideration, as it can trigger the potential problems during this period. In postpartum period, serum iron levels along with erythrocyte indices can be useful parameters to follow the iron loss by fetus and colostrum. Incompatible results of TIBC and UIBC levels with serum iron and erythrocyte indices in this study showed that TIBC and UIBC may not be useful biomarkers to evaluate serum iron values, because particularly in postpartum period, they can be effected by factors such as involution, inflammation and malnutrition.

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Research Article

Investigation of Intermediary Margins in the Marketing of Beef and Lamb Meat in Ankara Province, Turkey

Yılmaz ARAL [1] Mustafa Bahadır ÇEVRİMLİ [1] Nil AKDOĞAN [1] Erol AYDIN [2]
Mehmet Saltuk ARIKAN [1] Ahmet Cumhur AKIN [1] Doğukan ÖZEN [3]

[1] Ankara University, Faculty of Veterinary Medicine, Department of Animal Health Economics and Management, TR-06110 Ankara - TURKEY
[2] Kafkas University, Faculty of Veterinary Medicine, Department of Livestock Economics, TR-36100 Kars - TURKEY
[3] Ankara University, Faculty of Veterinary Medicine, Department of Biostatistics, TR-06110 Ankara - TURKEY

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Abstract

This research was conducted to determine intermediary margins in the marketing system of slaughtering animals and meat in Ankara Province of Turkey in the form of livestock-wholesale, wholesale-retail and livestock-retail. The material of the study consists of the records of sale transactions in the livestock and meat exchanges subject to Ankara Commodity Exchange. Intermediary margins (2008-2011) in marketing were calculated with current and fixed prices in percentages, making use of the producer price index (PPI) and consumer price index (CPI) of the Turkish Statistical Institute with 2003 set as the base year to rule out the effect of inflation. The study revealed that average livestock-wholesale marketing margins of beef were 18.96%, 19.94%, 16.69%, 11.25%, average wholesale-retail marketing margins of beef were 38.94%, 34.85%, 32.41%, 35.75%, and average livestock-retail marketing margins of beef were 47.71%, 45.34%, 40.99%, 39.48% respectively with fixed prices. The calculations based on monthly averages of livestock-retail intermediary margins showed that the share of producer in the retail beef price varied in a wide range, between 47.05% and 66.32%. On the other hand, a high level of correlation was found between retail beef prices and live cattle, wholesale beef prices, livestock-wholesale, wholesale-retail and livestock-retail marketing margins ($p<0.01$). In this study, average current livestock-wholesale margins were 18.41%, 26.99%, 18.55%, 20.08%, wholesale-retail margins were 27.72%, 27.89%, 29.70%, 28.50% and livestock-retail margins in lamb meat marketing were 38.23%, 45.12%, 39.83%, 40.13% respectively. In the same period, the share transferred to the producer through current retail sales prices of lamb meat was 59.17% as average. The correlation was found significantly as $r=+0.936$ and $r=+0.960$ ($P<0.01$) between fixed and current intermediary margins for livestock-retail and fixed and current retail lamb prices in marketing. As a result, due to the high level intermediary margins of beef and lamb meat marketing, red meat prices is increasing in Turkey. The possible reduction of the intermediary margins at each stage in marketing structure will provide to buy meat from the appropriate prices by the consumers and to contribute market regulation.

Keywords: *Marketing, Slaughtering animal, Beef and lamb meat, Price, Intermediary margin*

Türkiye'de Ankara İli Örneğinde Dana ve Kuzu Eti Pazarlamasında Oluşan Aracı Marjlarının Araştırılması

Özet

Bu araştırma, Türkiye'de Ankara ili örneğinde kasaplık dana ve kuzu ile dana ve kuzu eti pazarlama sistemi içerisinde oluşan aracı marjlarının canlı-toptan, toptan-perakende ve canlı-perakende olmak üzere belirlenmesi amacıyla yürütülmüştür. Araştırmanın materyalini Ankara Ticaret Borsasına bağlı canlı hayvan ve et borsalarında gerçekleştirilen satış işlemlerine ilişkin kayıtlar oluşturmuştur. Pazarlamadaki aracı marjları (2008-2011), enflasyon etkisini ortadan kaldırmak için Türkiye İstatistik Kurumu'nun üretici fiyat endeksi (ÜFE) ve tüketici fiyat endeksinden (TÜFE) faydalanılarak 2003 yılı baz alınarak cari ve sabit fiyatlarla ve yüzde olarakta hesaplanmıştır. Araştırmada sabit fiyatlarla ortalama dana eti canlı-toptan pazarlama marjı sırasıyla %18.96, %19.94, %16.69, %11.25, dana eti ortalama toptan-perakende pazarlama marjı %38.94, %34.85, %32.41, %35.75, dana eti ortalama canlı-perakende pazarlama marjı ise %47.71, %45.34, %40.99, %39.48 olarak hesaplanmıştır. Canlı-perakende aracı marjlarında aylık ortalamalar üzerinden yapılan değerlendirmede, tüketicilerin perakende dana eti için ödedikleri fiyattan üreticilerin eline geçen bölümün %47.05 ile %66.32 arasındaki geniş bir aralıkta değiştiği belirlenmiştir. Diğer taraftan yapılan analizde dana eti perakende fiyatları ile canlı hayvan ve karkas dana eti fiyatları, canlı-toptan, toptan-perakende ve canlı perakende aracı marjları arasında yüksek düzeyde korelasyon tespit edilmiştir ($P<0.01$). Çalışma kapsamında kasaplık kuzu ve kuzu eti pazarlamasında canlı-toptan aracı marjı sırasıyla %18.41; %26.99; %18.55, %20.08, toptan-perakende aracı marjı %27.72; %27.89; %29.70, %28.50 ve canlı-perakende cari marj ise sırasıyla %38.23; %45.12; %39.83, %40.13 olarak saptanmıştır. Aynı dönemde kuzu eti perakende cari satış fiyatları üzerinden üreticiye aktarılan pay ortalama olarak %59.17 olarak gerçekleşmiştir. Kuzu eti pazarlamasında canlı-perakende sabit ve cari aracı marjları ile perakende kuzu eti sabit ve cari fiyatları arasında önemli düzeyde korelasyon $r = +0.936$ ve $r = +0.960$ ($P<0.01$) bulunmuştur. Sonuç olarak, Türkiye'de dana ve kuzu eti pazarlamasında yüksek düzeyde bulunan aracı marjlarına bağlı olarak kırmızı et fiyatları artmaktadır. Pazarlama yapısı içindeki aracı marjlarının azalması tüketicinin daha uygun fiyat seviyesinden et satın alabilmesini sağlayacak ve piyasanın düzenlenmesine katkı sunacaktır.

Anahtar sözcükler: *Pazarlama, Kasaplık hayvan, Dana ve kuzu eti, Fiyat, Aracı marjı*

İletişim (Correspondence)
+90 474 2426807/5099
dr-erolaydin@hotmail.com

INTRODUCTION

The main objectives of marketing is to maintain the existing market volume, to increase the effective demand, to enhance productivity and quality of products in conformity with today's changing and developing conditions and to increase the volume of marketing by acquiring new markets. In marketing, price is one of the most powerful factors, which is always effective, and reflects the consumer demand to producers. In the marketing system, price usually increases in the direction from producers to consumers.

In the process from production to consumption in marketing of meat and animals for slaughter, the reactions of supply, demand, price and cost factors on the basis of various times, places and forms need to be assessed [1,2].

Producers offering services to consumers, the meat processing industry, and retailers face additional costs to meet various demands of consumers. These additional costs make up the marketing service fee. Demand and supply factors, marketing costs, and competitive relationship between intermediaries in the marketing channel affect the marketing margins [3].

The passing of slaughtering animals and meat through the hands of numerous intermediaries to reach consumers via marketing channels and the increasing intermediary margins result in a decrease in the earnings of producers and an increase in the price paid by consumers for these products [4].

Balling [5], dealt with the concept of branding in the beef industry in Germany, assessed the factors affecting the amount of beef consumption and purchase of beef, and underlined the importance of improving the intermediary services in the marketing of beef as well as of formulating effective policies for production, distribution and pricing of beef.

In a study conducted by Kanlı [6], the producers' share in the price paid by consumers for 1 kg of beef in Izmir between 1980 and 1984 years was found to be 58.2% in 1980, 59.4% in 1981, 44.5% in 1982, 43.3% in 1983 and 28.9% in 1984. Another study conducted in Ankara between 2001 and 2004 years, the period averages of the livestock-retail, livestock-wholesale, and wholesale-retail marketing margins for slaughtering cattle and beef were found to be 55.36%, 39.81% and 25.25%, respectively [7].

Kuosmanen and Niemi [8], reported that with the development of supermarket chains particularly in food products and the retail sector intensified rapidly in Finland. The study notes that the butchers' and retailer companies' price margin for retail mincemeat, which was 45% in early 1980s, rose above 60% in early 2000s.

Tiffin and Dawson [9], emphasize that lamb prices has seasonality both at producer level and retail level, there is a long-term relationship between the two prices, and producers face different marketing margins depending on the price level forming at the retail phase. The study further notes that the lamb price is set in the retail market, and that producer margins have been increasingly declining, often due to imperfect price transitions and structural breaks.

The present study aims to determine and analyse in detail the intermediary margins for livestock-wholesale, wholesale-retail and livestock-retail in the beef and lamb meat marketing chain with respect to current and fixed prices in Ankara province of Turkey and to offer solutions to the structural problems in the marketing system.

MATERIAL and METHODS

This study, conducted on the beef and lamb meat marketing structure in Ankara and the recent intermediary margins in the marketing system, first deals with the issues that are important in livestock and meat marketing in Turkey, and then analyses the intermediary margins for livestock-wholesale, livestock-retail and wholesale-retail in the marketing of beef and lamb meat in light of the data and information obtained within the scope of the study, and finally addresses the problems faced in red meat marketing.

The material of the study consists of the records relating to the sale transactions carried out in livestock and meat exchanges affiliated with Ankara Commodity Exchange between 2008 and 2011. The study also uses the average retail sale prices for beef and lamb meat in Ankara given in official statistics for each month [10]. On the basis of the data entered into Microsoft Excel and SPSS 17.0 software package, the intermediary margins were calculated and analysed in tables.

In determining the livestock-wholesale and livestock-retail intermediary margins, the retail and wholesale prices were calculated assuming that carcass yield is 60% for beef and 55% for lamb and adding an offal premium of 20% of the live animal price for both. The values obtained were converted into meat price of 1 kg of LW (live weight). On the basis of the wholesale and retail meat prices determined at current and fixed prices, the margin between the meat prices and livestock prices was calculated as a percentage (%).

On the basis of the daily average price data obtained (livestock, wholesale and retail beef and lamb meat prices), the monthly average prices and current intermediary margins were determined. The intermediary margins in marketing were calculated as percentage on the basis of fixed prices, with 2003 being the base year, by using the producer price index (PPI) and consumer price index (CPI) issued by the Turkish Statistics Institute (TUIK) in order to eliminate the effect of inflation.

Besides, in the analysis conducted within the scope of the study, the correlation between slaughtering animals

and meat prices and livestock-wholesale, wholesale-retail and livestock-retail intermediary margins were examined.

RESULTS

The change in intermediary margins determined on the basis of current and fixed prices in the marketing of beef and lamb meat in Ankara for the period between 2008 and 2011 years is given below *(Table 1, 2, 3* and *4)*.

The correlations between current and fixed prices of slaughtering animals, wholesale and retail beef and lamb meat and intermediary margins for such products in Ankara for the period between 2008 and 2011 years are given in *Table 5* and *Table 6*.

In the study, the average livestock-wholesale marketing margins for beef at fixed prices for 2008, 2009, 2010 and 2011 were calculated to be 18.96%, 19.94%, 16.69% and 11.25%, respectively. The average wholesale-retail marketing margins for beef were calculated to be 38.94%, 34.85%, 32.41% and 35.75%, respectively. The average livestock-retail marketing margins for beef were calculated to be 47.71%, 45.34%, 40.99% and 39.48%, respectively. In an assessment based on the monthly averages of livestock-retail intermediary margins, the portion earned by producers from the price paid by consumers for retail beef was found to vary in a wide range from 47.05% to 66.32%.

A high level of correlation was found between the retail prices of beef and cattle and wholesale carcass prices, and livestock-wholesale, wholesale-retail and livestock-retail intermediary margins ($P<0.01$).

It was found that there was a significant relationship between wholesale and retail prices of beef carcass in the same direction on the basis of current and fixed prices. At fixed prices, carcass price and retail price of beef were found to be correlated at a level of $r = +0.957$, and at current prices, carcass price and retail price of beef were found to be correlated at a level of $r = +0.983$.

Table 1. *Current and fixed average prices of livestock, wholesale carcass and retail beef*
Tablo 1. *Canlı hayvan, toptan karkas ve perakende dana eti ortalama cari ve sabit fiyatları*

Years	Current Prices			Fixed Prices		
	Cattle-Livestock (TRY/Kg)	Wholesale Carcass Beef (TRY/Kg)	Retail Beef (TRY/Kg)	Cattle-Livestock (TRY/Kg)	Wholesale Carcass Beef (TRY/Kg)	Retail Beef (TL/Kg)
2008	5.62	9.79	15.60	3.54	6.16	10.08
2009	6.28	11.11	17.42	3.90	6.90	10.58
2010	9.28	15.62	23.61	5.31	8.94	13.23
2011	9.39	14.61	22.26	4.84	7.53	11.72
Average of the Period	7.64	12.78	19.72	4.40	7.38	11.40

** 2008 (1 USD=1.29 TRY), 2009 (1 USD=1.54 TRY), 2010 (1 USD=1.50 TRY, 2011 (1 USD=1.67 TRY)*

Table 2. *Changes in current and fixed livestock-wholesale, livestock-retail and wholesale-retail ıntermediary margins of beef marketing*
Tablo 2. *Dana eti pazarlamasında cari ve sabit olarak canlı-toptan, canlı-perakende ve toptan-perakende aracı marjlarındaki değişim*

Years	Cattle Livestock-Retail Beef Intermediary Margin with Current Prices (TRY/Kg)	Cattle Livestock-Wholesale Beef Intermediary Margin with Current Prices (TRY/Kg)	Wholesale-Retail Beef Intermediary Margin with Current Prices (TRY/Kg)	Cattle Livestock-Retail Beef Intermediary Margin with Fixed Prices (TRY/Kg)	Cattle Livestock-Wholesale Beef Intermediary Margin with Fixed Prices (TRY/Kg)	Wholesale-Retail Beef Intermediary Margin with Fixed Prices (TRY/Kg)
2008	4.87	1.38	5.81	3.23	0.87	3.93
2009	5.43	1.64	6.31	3.24	1.02	3.69
2010	6.75	1.95	7.99	3.69	1.12	4.29
2011	5.84	1.25	7.66	3.16	0.64	4.19
Average of the Period	5.72	1.56	6.94	3.33	0.91	4.03
Years	**Current Average Cattle Livestock-Retail Beef Intermediary Margin (%)**	**Current Average Cattle Livestock-Wholesale Beef Intermediary Margin (%)**	**Current Average Wholesale-Retail Beef Intermediary Margin (%)**	**Fixed Average Cattle Livestock-Retail Beef Intermediary Margin (%)**	**Fixed Average Cattle Livestock-Wholesale Beef Intermediary Margin (%)**	**Fixed Average Wholesale-Retail Beef Intermediary Margin (%)**
2008	46.42	19.72	37.24	47.71	18.96	38.94
2009	46.35	20.73	36.21	45.34	19.94	34.85
2010	42.10	17.38	33.84	40.99	16.69	32.41
2011	38.36	11.75	34.39	39.48	11.25	35.75
Average of the Period	43.31	17.40	35.42	43.38	16.71	35.49

Table 3. *Current and fixed average prices of livestock, wholesale carcass and retail lamb meat*
Tablo 3. *Canlı hayvan, toptan karkas ve perakende kuzu eti ortalama cari ve sabit fiyatları*

Years	Current Prices			Fixed Prices		
	Lamb-Livestock (TRY/Kg)	Wholesale Carcass Lamb (TRY/Kg)	Retail Lamb Meat (TRY/Kg)	Lamb-Livestock (TRY/Kg)	Wholesale Carcass Lamb (TRY/Kg)	Retail Lamb Meat (TRY/Kg)
2008	5.31	9.89	13.69	3.34	6.22	8.85
2009	6.45	13.71	19.02	4.01	8.52	11.57
2010	9.72	18.16	25.83	5.56	10.40	14.48
2011	10.48	20.02	28.01	5.40	10.32	14.74
Average of the Period	7.99	15.45	21.64	4.58	8.87	12.41

** 2008 (1 USD=1.29 TRY), 2009 (1 USD=1.54 TRY), 2010 (1 USD=1.50 TRY, 2011 (1 USD=1.67 TRY)*

Table 4. *Changes in current and fixed livestock-wholesale, livestock-retail and wholesale-retail ıntermediary margins of lamb meat marketing*
Tablo 4. *Kuzu eti pazarlamasında cari ve sabit olarak canlı-toptan, canlı-perakende ve toptan-perakende aracı marjlarındaki değişim*

Years	Lamb Livestock-Retail Intermediary Margin with Current Prices (TRY/Kg)	Lamb Livestock-Wholesale Intermediary Margin with Current Prices (TRY/Kg)	Lamb Wholesale-Retail Intermediary Margin with Current Prices (TRY/Kg)	Lamb Livestock-Retail Intermediary Margin with Fixed Prices (TRY/Kg)	Lamb Livestock-Wholesale Intermediary Margin with Fixed Prices (TRY/Kg)	Lamb Wholesale-Retail Intermediary Margin with Fixed Prices (TRY/Kg)
2008	3.28	1.20	3.80	2.20	0.92	2.63
2009	5.30	2.38	5.30	3.16	1.68	3.05
2010	6.43	2.21	7.67	3.51	1.55	4.08
2011	7.02	2.63	7.98	3.79	1.63	4.42
Average of the Period	5.51	2.11	6.19	3.16	1.44	3.54
Years	**Current Average Lamb Livestock-Retail Intermediary Margin (%)**	**Current Average Lamb Livestock-Wholesale Intermediary Margin (%)**	**Current Average Lamb Wholesale-Retail Intermediary Margin (%)**	**Fixed Average Lamb Livestock-Retail Intermediary Margin (%)**	**Fixed Average Lamb Livestock-Wholesale Intermediary Margin (%)**	**Fixed Average Lamb Wholesale-Retail Intermediary Margin (%)**
2008	38.23	18.41	27.72	39.70	17.69	29.67
2009	45.12	26.99	27.89	44.08	26.04	26.36
2010	39.83	18.55	29.70	38.68	17.82	28.17
2011	40.13	20.08	28.50	41.23	19.31	29.98
Average of the Period	40.83	21.01	28.45	40.92	20.21	28.55

Moreover, the current carcass prices and livestock-retail and wholesale-to-retail intermediary margins for beef were found to be highly and significantly correlated at a level of r = +0.809 and r = +0.806, respectively (P<0.01). Likewise, the retail prices of beef and livestock-retail and wholesale-retail intermediary margins for beef were found to be highly and significantly correlated at a level of r = +0.865 and r = +0.901, respectively (P<0.01).

The current livestock-retail intermediary margins in the marketing of slaughter lamb and lamb meat in Ankara from 2008 were found to be 38.23%, 45.12%, 39.83% and 40.13%, respectively. The livestock-wholesale intermediary margins for lamb were found to be 18.41%, 26.99%, 18.55% and 20.08%, respectively. Finally, the wholesale-retail intermediary margins for lamb were found to be 27.72%, 27.89%, 29.70% and 28.50%, respectively. The portion earned by producers from the current retail price of lamb meat paid by consumers in Ankara between 2008 and 2011 years was 59.17% in average.

Furthermore, the correlations between livestock-retail intermediary margins for lamb and current and fixed prices of slaughtering lamb in Ankara between 2008 and 2011 years were found to be r = +0.769 and r = +0.858, respectively (P<0.01).

On the basis of these results, it is clear that livestock-retail intermediary margins for lamb and fixed and current prices of slaughtering lamb are significantly related in the same direction. In other words, as the price of lamb increases, the livestock-retail margin increases as well, or as the price of lamb decreases, the livestock-retail margin decreases as well.

Similarly, the fixed and current livestock-retail intermediary margins in the marketing of lamb meat in Ankara between 2008 and 2011 years were found to be highly correlated with the fixed and current retail prices of lamb meat, at a level of r = +0.936 and r = +0.960, respectively (P<0.01). Accordingly, there seems to be a strong and

Table 5. *The correlations between prices of livestock, wholesale and retail beef and intermediary margins*
Tablo 5. *Canlı hayvan, toptan ve perakende dana eti fiyatları ve aracı marjları arasındaki korelâsyonlar*

Beef Cross-Correlation Table

Parameters		FLP	FCP	FRP	FLRM	FLWM	FWRM	CLP	CCP	CRP	CLRM	CLWM	CWRM
FLP	r	**1**	.919**	.901**	.320*	.073	.394**	.957**	.949**	.933**	.693**	.251	.767**
FCP			**1**	.957**	.575**	.459**	.356*	.829**	.931**	.907**	.838**	.606**	.723**
FRP				**1**	.700**	.392**	.612**	.839**	.926**	.947**	.917**	.553**	.865**
FLRM					**1**	.734**	.687**	.257	.460**	.532**	.860**	.793**	.627**
FLWM						**1**	.011	-.061	.219	.191	.558**	.970**	.100
FWRM							**1**	.444**	.444**	.578**	.671**	.130	.817**
CLP								**1**	.960**	.950**	.666**	.153	.795**
CCP									**1**	.983**	.809**	.423**	.806**
CRP										**1**	.865**	.395**	.901**
CLRM											**1**	.700**	.877**
CLWM												**1**	.272
CWRM													**1**

*** P<0.01, * p<0.05; **FLP:** Fixed Livestock Price, **FCP:** Fixed Carcass Price, **FRP:** Fixed Retail Price, **FLRM:** Fixed Livestock-Retail Intermediary Margin, **FLWM:** Fixed Livestock-Wholesale Intermediary Margin, **FWRM:** Fixed Wholesale-Retail Intermediary Margin, **CLP:** Current Livestock Price, **CCP:** Current Carcass Price, **CRP**: Current Retail Price, **CLRM:** Current Livestock-Retail Intermediary Margin, **CLWM:** Current Livestock-Wholesale Intermediary Margin, **CWRM:** Current Wholesale-Retail Intermediary Margin*

Table 6. *The correlations between prices of livestock, wholesale and retail lamb meat and intermediary margins*
Tablo 6. *Canlı hayvan, toptan ve perakende kuzu eti fiyatları ve aracı marjları arasındaki korelâsyonlar*

Lamb Cross-Correlation Table

Parameters		FLP	FCP	FRP	FLRM	FLWM	FWRM	CLP	CCP	CRP	CLRM	CLWM	CWRM
FLP	r	**1**	.922**	.945**	.769**	.481**	.839**	.975**	.932**	.947**	.844**	.530**	.890**
FCP			**1**	.978**	.917**	.782**	.768**	.899**	.970**	.955**	.945**	.811**	.830**
FRP				**1**	.936**	.696**	.884**	.938**	.976**	.988**	.970**	.740**	.923**
FLRM					**1**	.842**	.823**	.782**	.902**	.911**	.986**	.874**	.843**
FLWM						**1**	.388**	.466**	.698**	.640**	.784**	.984**	.449**
FWRM							**1**	.872**	.828**	.901**	.865**	.460**	.977**
CLP								**1**	.958**	.968**	.858**	.549**	.898**
CCP									**1**	.987**	.945**	.767**	.863**
CRP										**1**	.960**	.706**	.933**
CLRM											**1**	.828**	.902**
CLWM												**1**	.503**
CWRM													**1**

*** P<0.01; **FLP:** Fixed Livestock Price, **FCP:** Fixed Carcass Price, **FRP:** Fixed Retail Price, **FLRM:** Fixed Livestock-Retail Intermediary Margin, **FLWM:** Fixed Livestock-Wholesale Intermediary Margin, **FWRM:** Fixed Wholesale-Retail Intermediary Margin, **CLP:** Current Livestock Price, **CCP:** Current Carcass Price, **CRP:** Current Retail Price, **CLRM:** Current Livestock-Retail Intermediary Margin, **CLWM:** Current Livestock-Wholesale Intermediary Margin, **CWRM:** Current Wholesale-Retail Intermediary Margin*

significant relationship between retail prices of lamb meat and fixed and current livestock-retail intermediary margins in the same direction. In other words, as the retail price of lamb meat increases, the livestock-retail margin increases as well, or as the retail price of lamb meat decreases, the livestock-retail margin decreases as well.

Additionally, the fixed and current livestock-wholesale intermediary margins for lamb meat and fixed and current wholesale prices of lamb carcass were found to be highly and significantly correlated (r = +0.782 and r = +0.767, P<0.01).

DISCUSSION

Livestock breeding is performed in different ways depending on various factors such as the enterprise structure, genetic level of the breed, pasture conditions, methods of raising and feeding, market conditions, and livestock policies of the country. Profitability of breeding depends on the production of abundant amounts of high-quality meat within economically-optimal fattening time at low costs. As is the case in all economic enterprises, the purpose of cattle and lamb fattening enterprises

is to maximize profits.

In fattening enterprises, the live weight increases, feed consumption and feed conversion ratio of animals taken into fattening process as well as the interrelations between such factors are the factors that directly affect profitability [11].

In the analysis conducted on the basis of the sale prices in livestock and meat exchanges affiliated with Ankara Commodity Exchange between 2008 and 2011 years, the intermediary margins for beef and lamb meat were examined, and the average livestock-wholesale intermediary margins for beef at current prices were found to be 19.72% in 2008, 20.73% in 2009, 17.38% in 2010 and 11.75% in 2011. The average livestock-wholesale intermediary margins for lamb meat at current prices were found to be 18.41% in 2008, 26.99% in 2009, 18.55% in 2010 and 20.08% in 2011.

The average wholesale-retail intermediary margins for beef at current prices in the marketing were found to be 37.24% in 2008, 36.21% in 2009, 33.84% in 2010 and 34.39% in 2011. The current livestock-retail intermediary margins for beef in Ankara as from 2008 were found to be 46.42%, 46.35%, 42.10% and 38.36%, respectively.

The wholesale-retail intermediary margins for lamb meat at current prices for the period between 2008 and 2011 years were calculated to be 27.72%, 27.89%, 29.70% and 28.50%, respectively, and the livestock-retail intermediary margins for lamb meat were calculated to be 38.23%, 45.12%, 39.83% and 40.13%, respectively.

In a study conducted by Keskin et al.[12], it was shown that the lamb meat marketing margins between 1993 and 2007 years had varied between 15.93% and 20.82% in the wholesale-retail phase, that is approximately 1/5 of the price paid by consumers had been earned by intermediaries following the wholesale phase.

In the assessment on the basis of the research data, it was found that annual average live lamb prices in the lamb meat market between 2008 and 2011 years increased by 97.36% from TRY5.31/kg to TRY10.48/kg, that the price of lamb carcass increased by 102.83% from TRY9.89/kg to TRY20.02/kg, and that the retail price of lamb meat increased by 104.60% from TRY13.69/kg from TRY28.01/kg.

In a study conducted in Ankara between 1990 and 1994 years, the wholesale-retail intermediary margin for sheep was found to be 15% of sale price in 1990, 17% of sale price in 1991, 14% of sale price in 1992, 16% of sale price in 1993 and 17% of sale price in 1994. The study notes that the share of wholesaler margins in the sale price in the marketing of sheep decreased and the share of retailer margins in the sale price increased within the research period [1]. Another study conducted in Istanbul between 1996 and 1999 years, It was found that within this period the shares of wholesaler margins in retail price of mutton were 6.28%, 5.53%, 6.45% and 5.34% respectively, and the shares of retailer margins were 10.28%, 11.89%, 14.29% and 15.96% respectively [13].

According to Marsh and Brester [3], demand and supply, marketing costs, and competitiveness of intermediaries in the marketing channel constitute the major elements that affect marketing margins. Brester and Musick [14], found that the density of increasing lamb packagers in the slaughter and processing industry had relatively small but positive effects on the lamb marketing margins.

A study conducted in Spain by Kaabia and Gil [15], underlines that the main determinant factors playing a role in the change of marketing margins should be investigated, that market structures and degree of market-oriented integration as well as other meat sectors and products with different characteristics (branded products, processed products, long-life products, etc.) should be dealt with.

Ancelmo et al.[16], calculated the revenue generated from the sale of 1 kg of cold mutton carcass on the basis of cost-benefit ratios of the elements in the marketing chain. According to the study, producer earned $2.7 (47%) and intermediaries earned $3.1 (53%) from the sale of 1 kg of mutton in Mexico. In a study carried out in Pakistan, the shares received by intermediaries in return for marketing services in the marketing of mutton and goat meat were found to be 30% and 32%, respectively, with the remaining marketing margin earned by the producers [17].

In a study examining the relationship between supply and demand during fluctuations of the market price of lamb, Vere and Griffith [18], emphasised that the mutton market in New South Wales, Australia faced a significant decline in market competition due to beef not experiencing price break in the major export market. Research further noted that when increase in production reduces the prices, the immediate impacts should be determined expeditiously.

Bojnec [19], explained that even if the meat market is externally isolated and the market is internally regulated as the case in Slovenia in 1990s, the red meat market can act like a competitive market. It is also stated that advanced reforms, reconstructions and effective measurements relating to quality and price are needed to remedy the deficiencies in the beef and pork marketing chain in relation to raising, processing and marketing.

Dagdemir et al.[20], found that 70.99% of the final price paid by consumers in the marketing system for red meat obtained from ovine and caprine animals as of 1998 had been earned by farmers, and 29.01% had been earned by intermediaries. The study further noted that 74.59% of the price paid by consumers in the marketing system for red meat obtained from bovine animals as of 1998 had been earned by farmers, and 25.41% had been earned by intermediaries.

The degree of self-sufficiency of the red meat market

in Turkey in terms of production level, population and consumption needs to be revealed. Appropriate policies and practices should be formulated as soon as possible in order to handle the problems of the red meat sector in Turkey, including animal protein consumption deficit associated with insufficiency of production and the increasing prices in the red meat market.

It would be beneficial to examine in detail the existing system in Turkey for production of beef and lamb meat on the basis of socioeconomic status, structures and sizes of enterprises, production level, carcass yields, specialisation in production, recording and inspection in all stages from production to consumption, degree of organisation of producers, number of intermediaries and marketing efficiency, and to regulate the system in such a manner as to improve it.

Therefore, in a modern, effective and producer-oriented marketing system, it would be for the benefit of the sector to establish a market structure where the share of intermediary margins in the consumer price gradually declines and the portion of the price earned by producers rises.

In Turkey, the bargaining power of producers is low for reasons such as the insufficiency of organisation among producers and the high number of intermediaries involved in marketing. It is essential for increasing the revenues of producers by reducing the intermediary margins in the marketing system.

Measures taken for the beef and lamb meat production and the red meat sector in Turkey are incapable of increasing production at the desired level and providing producers with a certain level of welfare. In order to enhance the productivity of slaughter animals and ensure regular flow of raw materials into the industry, permanent and stable policies on livestock sector should be implemented by the government, aiming to solve structural problems in various areas such as animal breeding, care and feeding, sizes of enterprises, marketing, training and organisation.

In conclusion, optimal and rational use of resources in such a manner as to reduce the meat production costs and enhance productivity and quality in Turkey will be accompanied by favourable outcomes such as improvement in the sizes and level of specialisation of enterprises, establishment of an effective marketing system fulfilling the expectations of producers and consumers, regulation of the red meat market, ensuring of price stability, reduction in intermediary margins in marketing, increase in production, achievement of self-sufficiency, and gaining of a competitive position in foreign trade. As a result, due to the high level intermediary margins of beef and lamb meat marketing, red meat prices is increasing in Turkey. The possible reduction of the intermediary margins at each stage in marketing structure will provide to buy meat from the appropriate prices by the consumers and to contribute market regulation.

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Research Article

Protective and Antigenotoxic Effects of Silymarin and Curcumin in Experimental Cyclophosphamide Intoxication in Rats [1]

Hamdi AVCI [1] Selim SEKKİN [2] Murat BOYACIOĞLU [2] Hasan AKŞİT [3]
Recai TUNCA [1] E. Tuğrul EPİKMEN [1] S. Serap BİRİNCİOĞLU [1]

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[1] Department of Pathology, Faculty of Veterinary Medicine, University of Adnan Menderes, TR-09016 Aydin - TURKEY
[2] Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Adnan Menderes, TR-09016 Aydin - TURKEY
[3] Department of Biochemistry, Faculty of Veterinary Medicine, University of Balikesir, TR-10100 Balikesir - TURKEY

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Abstract

The aim of this study was to investigate the protective effects of silymarin (SLY) and curcumin (CUR), which have strong antioxidant properties, against the toxic effects of high dose of cyclophosphamide (CP) on the liver and kidneys of rats. For this purpose, a total of 36 adult Wistar albino female rats at the weight of, 250±10 g were used. Biochemical parameters were found to be significantly higher in the CP-only group than in the other groups. In the groups in which SLY and CUR were administered concurrently with CP, biochemical parameters were found to be significantly lower than in the CP-only group. In the SLY+CP and CUR+CP groups, biochemical parameters were correlated with the severity of histopathological findings. In genotoxic examination, the comet parameter levels in the SLY+CP and CUR+CP-treated groups were lower than those in the CP-treated group. The present study established that both SLY and CUR are effective against the toxic effects of high doses of CP.

Keywords: *Cyclophosphamide, Silymarin, Curcumin, Biochemistry, Pathology, Comet assay*

Ratlarda Deneysel Siklofosfamid Toksikasyonunda Silimarin ve Curcuminin Koruyucu ve Antigenotoksik Etkileri

Özet

Bu çalışmada, yüksek dozda siklofosfamid (CP) verilen ratların karaciğer ve böbrekler üzerindeki toksik etkilerine karşı kuvvetli antioksidan özellikleri bulunan silimarin (SLY) ve curcuminin (CUR) koruyucu etkileri araştırıldı. Bu amaçla çalışmada toplam 36 adet, ağırlıkları 250±10 gram olan erişkin Wistar albino ırkı dişi ratlar kullanıldı. Yalnızca CP verilen grupta biyokimyasal parametreler kontrol grubuna göre istatistiksel olarak anlamlı bir şekilde yüksek bulundu. CP ile birlikte eş zamanlı olarak SLY ve CUR verilen gruplarda biyokimyasal parametreler, yalnızca CP verilen gruba göre istatiksel olarak anlamlı bir şekilde düşük bulundu. Genotoksik incelemede comet parametreleri CP ile birlikte eş zamanlı SLY ve CUR verilen gruplarda ise yalnızca CP verilen gruba göre istatistiksel olarak anlamlı bir şekilde düşük bulundu. Çalışmada CP'nin toksik etkilerine karşı, kuvvetli antioksidan özelliklere sahip SLY ve CUR'un koruyucu alternatif birer kimyasal madde olarak kullanılabileceği görüldü.

Anahtar sözcükler: *Siklofosfamid, Silimarin, Curcumin, Biyokimya, Patoloji, Comet assay*

INTRODUCTION

Cyclophosphamide (CP) (*N,N*-bis(2-chloroethyl tetrahydro-2H-1,3,2-oxphosphorin-2-amine, 2-oxide monohydrate), also known Cytoxan or Endoxan, is a pharmaceutical product used as an antineoplastic agent in the treatment of a wide range of cancers, including Hodgkin's disease, many types of leukaemia, multiple myeloma and neuroblastomas [1,2]. Despite its therapeutic importance, a wide range of adverse effects, including nephrotoxicity [2], hepatotoxicity [3], cardiotoxicity [4] and male reproductive toxicity [5], have been reported for the drug.

Since the 1980s, medicinal plants and their active ingredients have received increasing attention [6]. Medicinal

İletişim (Correspondence)
+90 256 2470700/234
havci@adu.edu.tr

plants serve as therapeutic alternatives, safer options or, in some cases, the only effective treatment, and an increasing number of these plants and their extracts have been shown to produce beneficial therapeutic effects, including antioxidant, anti-inflammatory, anticancer, antimicrobial and immunomodulatory effects [7-9]. Among the promising medicinal plants, silymarin (SLY) [10-12] and curcumin (CUR) [7,8,13] have been reported to have multiple pharmacological activities, including antioxidant, hepatoprotectant and anti-inflammatory, antibacterial, and antineoplastic effects. SLY is the polyphenolic fraction from the seeds of milk thistle (*Slybum marianum*) and is composed of various flavonoids, including silybin (major component), silydianin, and silychristine [14]. It has been reported that silymarin acts as an antioxidant, reducing free-radical-mediated injury in tissues and inhibiting lipid peroxidation [14,15]. CUR is a hydrophobic polyphenol derived from the rhizome of herb *Curcuma longa* belonging to family zingiberaceae and has been used for centuries in indigenous medicine [16]. A large body of evidence suggests that CUR has a diverse range of molecular targets, including transcription factors, growth factors, and their receptors, cytokines, enzyms, and genes regulating cell proliferation and apoptosis [13,16].

The aim of the present study was to evaluate and compare the protective roles of SLY and CUR in CP-induced hepatotoxicity, nefrotoxicity and genotoxicity in rats.

MATERIAL and METHODS

Chemicals, Animals and Experimental Design

CP (Endoxan) was purchased from Eczacıbaşı-Baxter (Istanbul, Turkey). SLY (code; S0292) and CUR (code; C1386) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Thirty-six healthy adult female Wistar albino rats (3 months old, 250±10 g body weight) were used. The rats were obtained from Adnan Menderes University Experimental Research Centre (Aydin, Turkey) and were housed under standard laboratory conditions (24±3°C, 40-60% humidity, 12-h light-dark cycle). A commercial pellet diet and fresh drinking water were available *ad libitum*. All animals received humane care according to criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health, and the experimental protocol was approved by the University of Adnan Menderes Institutional Animal Ethics Committee (No: B.30.2.ADU.0.00.00.00/050.04/2011/043).

Rats were randomly divided into six experimental groups containing six rats each, as follows: Group I (control group; nothing was administered), Group II (CP group; 30 mg/kg/day CP administered intraperitoneally to each animal for seven days), Group III (SLY group; 100 mg/kg/day SLY by gavage for 14 days), Group IV (CUR group; 100 mg/kg/day CUR by gavage for 14 days), Group V (SLY + CP group; 100 mg/kg/day SLY by gavage for 14 days plus 30 mg/kg/day CP intraperitoneally starting from the eighth day) and Group VI (CUR + CP group; 100 mg/kg/day CUR by gavage for 14 days plus 30 mg/kg/day CP intraperitoneally starting from the eighth day). All administrations were applied by gavage as an emulsion in 0.5 mL corn oil.

Biochemistry

The rats were killed under slight ether anaesthesia at the end of 14 days. Blood samples were collected and then transferred directly into plain vacutainer tubes, centrifugated at 1.200 g for 10 min at 4°C and stored at -20°C until analysis. Serum ALT (alanine aminotransferase, Archem, A2221, Turkey), AST (aspartate aminotransferase, Archem, A2212, Turkey) and creatinine (Archem, A2162, Turkey) were assayed using a Biochemistry Auto Analyzer (Sinnowa D280, China).

For the determination of malondialdehit (MDA) levels and superoxide dismutase (SOD) activity in the liver and kidneys, the dissected tissues were immediately rinsed in ice-cold phosphate-buffered saline. Tissues were homogenised (2000 rpm/min for 1 min, 1/10 w/v) using a Teflon-glass stirrer (IKA Overhead Stirrer; IKA-Werke GmbH & Co. KG, Staufen, Germany) in a 10% 150 mM phosphate buffer (pH 7.4) in an ice bath. The homogenate was centrifuged (Hettich Zentrifugen, Mikro 200 R, Tuttlingen, Germany) at 6.000 g for 10 min at 4°C. The supernatants were frozen at -80°C (Glacier Ultralow Temperature Freezer, Japan) until analysed and then used for determination of MDA levels and SOD activity. The lipid peroxidation levels were determined according to the concentration of thiobarbituric acid reactive substances, and the amount of MDA produced was used as an index of lipid peroxidation. Absorbance was measured with a spectrophotometer at 532 nm. The MDA concentration was calculated by the absorbance complex (absorbance coefficient $\varepsilon = 1.56 \times 10^5$/M/cm) and expressed as nmol/mg of tissue protein [17]. SOD activity was determined according to the method of Sun *et al.*[18], and the absorbance was measured with a spectrophotometer at 560 nm. This method is based on the inhibition of nitro blue tetrazolium reduction using the xanthine-xanthine oxidase system as a superoxide generator. SOD activity was then measured by the degree of inhibition of this reaction. The protein levels in the tissues were determined by the method described by Lowry *et al.*[19].

For the measurement of DNA fragmentation levels, the liver and kidneys samples were removed and protected against light, and then stored at -20°C until analyses. The extent of apoptosis was evaluated by the measurement of DNA fragmentation. This was assessed by quantification of cytosolic oligonucleosome-bound DNA by using the Cell Death Detection ELISA Plus kit (Roche, Mannheim, Germany). The liver and kidneys of rats were treated with a homogeniser (Stuart SHM1, UK). The 0.2 g homogenate

was made with the lysis buffer and then centrifuged at 20.000 g for 10 min at 4°C. The supernatant fraction was used as the antigen source for the immunoassay. This assay is based on the quantitative sandwich ELISA principle using mouse monoclonal antibodies directed against histones (coating antibody) and DNA (peroxidase-labelled antibody), respectively. The amount of peroxidase retained in the immunocomplex is determined photometrically with ABTS (2,29-azino-di-(3-thylbenzthiazoline sulfonate) as a substrate (Thermo Multiskan FC Microplate Photometer, USA). This allows the specific determination of mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysates.

Histopathology

For histopathological examinations, samples of the liver and kidneys were taken. The liver was cut into thinner sections and each kidney was cut into two pieces along its transverse axis. Parts of the liver and kidneys were fixed in 10% neutral buffered formalin. After fixation, sections were embedded in paraffin wax and sectioned at 4-6 µm and stained with haematoxylin-eosin. The histopathological results were semiquantitatively assessed under a light microscopy with ocular grids and x 10, x 20 and 40 objectives. In total, 10 high-power fields were randomly chosen in each section. Changes in the experimental histopathological findings were graded as follows: 0, no changes, 1, mild changes, 2, moderate and 3, severe changes [20].

TUNEL Assay

Apoptotic cells were detected using the DeadEnd Colorimetric TUNEL system following the manufacturer's protocol (GenScript, USA). In brief, tissue sections after deparaffinisation and rehydration were permeabilised with proteinase K for 30 min at ambient temperature. Thereafter, the sections were quenched of endogenous peroxidase activity using 0.3% hydrogen peroxide for 10 min. After thorough washing with 3 x phosphate buffered saline (PBS), sections were incubated with equilibration buffer for 10 min, and terminal deoxynucleotidyl transferase reaction mixture was then added to all the sections, except for the negative control, and incubated at 37°C for 1 h. The reaction was stopped by immersing the sections in 2x saline sodium citrate buffer (SSC) for 15 min. Sections were then treated with conjugated horseradish peroxidase streptavidin (1:500) for 30 min at room temperature, and after repeated washing, sections were incubated with 3,30-diaminobenzidine for 90 s for colour development. Sections were mounted after dehydration and observed under 400 x magnification for TUNEL-positive cells. The results of the TUNEL assay were assessed using a grading system based on the total number of positive cells. For quantification of the TUNEL assay, the tissue areas with the highest density of positive staining were chosen. All positive cells from a total of 10 high-power fields measured 0.025 mm^2 were counted. To calculate the total number of positively stained cells, an ocular grid of 100 (10x10) squares was used at 20X microscope objective.

Lymphocyte Isolation and DNA Analysis by Comet assay

The comet assay used here was adapted from the method described previously [21-23]. For this purpose, fresh blood samples were mixed with the PBS solution for the determination of DNA fragmentation of blood lymphocytes. Lymphocytes were isolated with histopaque and suspended in a freezing medium. Isolated lymphocytes were slowly frozen in aliquots of 1 ml at - 80°C. Conventional end-frosted slides were pre-coated with 1% normal melting agarose. This suspension was mixed with pre-warmed low - melting - point agarose. The positive control slide cells were dipped in an H_2O_2 solution for 5 min at 4°C. Following lysis, slides were aligned in a horizontal gel electrophoresis tank (CSL - COM20, Cleaver Scientific, UK) that was connected to a recirculating cooler (FL300, Julabo, Germany) set at 4°C and filled with freshly made alkaline electrophoresis solution. Electrophoresis (CS - 300V, Cleaver Scientific, UK) was carried out at approximately 1 V/cm for 20 min. Measurements of the tail intensity and tail moment of comets were made using a computer-based image analysis system (Comet Assay IV, Perceptive Instruments, UK). The mean value of the % Tail DNA and Mean Tail Moment parameters was calculated and used to assess the DNA damage.

Statistical Analysis

All data were checked for normal distribution with the Shapiro-Wilk test and for homogeneity of variance with Levene's test. The data were compared among groups using the Kruskal-Wallis analysis of variance (ANOVA) or one-way ANOVA, according to whether data were normally distributed or not. Post hoc multiple comparisons were performed using the Mann-Whitney *U* test with Bonferroni corrected or Duncan's test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. Differences were considered statistically significant if $P<0.05$, $P<0.01$ and $P<0.001$. All data were expressed as mean and standard error [24].

RESULTS

Biochemistry

The levels of biochemical parameters including liver (ALT and AST) and kidney (creatinine) enzymes, the lipid peroxidation (MDA) and antioxidant (SOD) levels, and DNA fragmentation (ELISA) in the liver and kidneys of all groups are presented in *Table 1*. A significant increase in serum ALT, AST and creatinine levels were observed in the CP-treated group compared to the control and other treated groups. However, it was observed that AST and creatinine activity in the SLY+CP- and CUR+CP-treated groups were significantly lower than those in the CP group.

Table 1. *Biochemical Parameters, TUNEL and Comet assay results in all groups*
Tablo 1. *Tüm gruplara ait biyokimyasal parametreler, TUNEL ve Comet assay sonuçları*

Biochemical Parameters, TUNEL and Comet Assay Results	Experimental Groups						
	Control	CP	SLY	CUR	SLY+ CP	CUR+ CP	*P*
Liver							
ALT (U/L)	20.33±5.16 ^{b}	30.16±2.60 ^{a}	16.00±2.08 ^{b}	15.50±1.33 ^{b}	22.00±1.39 ^{b}	21.66±0.95 ^{b}	**
AST (U/L)	61.16±6.75 ^{c}	147.33±5.67 ^{a}	62.66±3.41 ^{c}	57.66±6.37 ^{c}	125.66±0.95 ^{b}	117.00±3.95 ^{b}	***
MDA(nmol/mg protein)	47.54±2.32 ^{b,c,d}	110.13±2.49 ^{a}	45.16±2.18 ^{c,d}	41.66±3.22 ^{d}	53.91±1.54 ^{b}	52.09±3.14 ^{b,c}	***
SOD (U/mg protein)	4.02±0.22 ^{a,b}	2.69±0.14 ^{c}	4.21±0.18 ^{a,b}	4.40±0.12 ^{a}	3.72±0.15 ^{b}	3.97±0.20 ^{a,b}	***
DNA fragmentation (U/mg protein)	0.81±0.04 ^{b}	1.49±0.06 ^{a}	0.78±0.07 ^{b}	0.74±0.13 ^{b}	1.06±0.09 ^{b}	1.03±0.20 ^{b}	**
TUNEL	8.00±0.00 ^{c}	20.00±0.00 ^{a}	5.33±0.33 ^{d}	5.00±0.00 ^{d}	15.00±0.00 ^{b}	10.00±0.00 ^{b}	***
Kidney							
Creatinine (mg/dl)	0.65±0.14 ^{c}	1.27±0.05 ^{a}	0.41±0.13 ^{c}	0.40±0.13 ^{c}	0.96±0.05 ^{b}	0.95±0.02 ^{b}	***
MDA (nmol/mg protein)	87.41±3.68 ^{c,d}	188.08±3.87 ^{a}	86.98±3.14 ^{c,d}	81.66±2.22 ^{d}	107.57±3.43 ^{b}	98.46±5.71 ^{b,c}	***
SOD (U/mg protein)	5.00±0.15 ^{b}	2.05±0.25 ^{c}	5.18±0.22 ^{a,b}	5.97±0.40 ^{a}	4.51±0.35 ^{b}	4.71±0.19 ^{b}	***
DNA fragmentation (U/mg protein)	1.09±0.03 ^{c}	2.82±0.17 ^{a}	1.22±0.02 ^{b}	1.09±0.03 ^{c}	1.50±0.10 ^{b}	1.41±0.15 ^{b}	***
TUNEL	9.33±0.42 ^{c}	62.50±2.14 ^{a}	7.50±0.22 ^{d}	7.33±0.21 ^{d}	33.33±1.05 ^{b}	22.50±1.11 ^{b}	***
Comet Assay							
Olive tail moment	22.83±3.06 ^{b}	46.03±1.18 ^{a}	15.35±1.46 ^{c}	11.76±1.18 ^{c}	28.20±4.22 ^{b}	17.17±2.75 ^{b,c}	***
Tail DNA (%)	54.94±4.11 ^{b}	82.71±1.73 ^{a}	50.63±2.85 ^{b}	42.06±3.24 ^{b,c}	62.78±4.92 ^{b}	52.94±7.35 ^{b}	***

$^{a, b, c, d}$ *Different letters indicate statistically significant differences in the same row, * P<0.05; ** P<0.01, *** P<0.001;* ***CP:*** *Cylophosphamide,* ***SLY:*** *Silymarin,* ***CUR:*** *Curcumin*

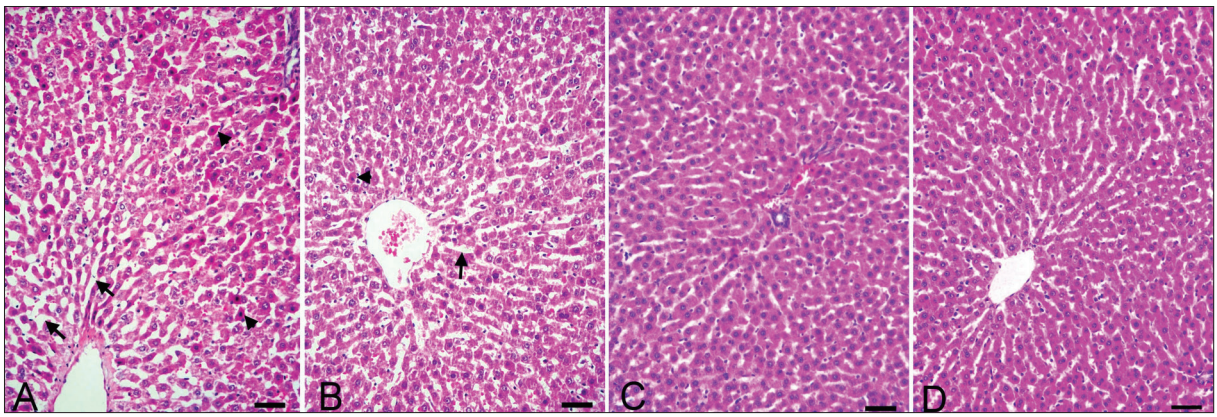

Fig 1. Liver. **A.** Severe dilatations in sinusoids *(arrows)* and eosinophilic staining in hepatocytes *(arrowheads),* CP group, HE, Bar: 50 µm, **B.** Mild dilatations in sinusoids *(arrow)* and eosinophilic staining in hepatocytes *(arrowhead)*, CUR + CP group, HE, Bar: 50 µm, **C.** Normal histological appearance of liver, CUR group, HE, Bar: 50 µm, **D.** Histological appearance of liver in control group, HE, Bar: 50 µm

Şekil 1. Karaciğer. **A.** Sinuzoidlerde şiddetli dilatasyon *(oklar)* ve hepatositlerde eozinofilik boyanma *(okbaşları),* CP grup, HE, Bar: 50 µm, **B.** Sinuzoidlerde hafif dilatasyon *(ok)* ve hepatositlerde hafif eozinofilik boyanma *(okbaşı)*, CUR + CP grup, HE, Bar: 50 µm, **C.** Karaciğerde normal histolojik görünüm, CUR grup, HE, Bar: 50 µm, **D.** Kontrol grubunda karaciğerin normal histolojik görünümü, HE, Bar: 50 µm

CP administration caused significant increases in MDA levels and decreases in SOD levels in the liver and kidneys when compared with the control and other treated groups. However, the CUR+CP-treated group in all tissues had lower MDA levels and higher SOD levels compared to the SLY+CP group, but the differences were not significant.

Levels of the tissues' DNA fragmentation in the CP-treated group were higher than those found in the control group and other treated groups, and the difference was statistically significant (P<0.001). The values of DNA fragmentation levels of the kidney tissues in the SLY+CP- and CUR+CP-treated groups were significantly higher than those in the control group. However, no statistically significant differences were observed in the values of the DNA fragmentation of the liver in the SLY+CP- and CUR+CP-treated groups compared to the control group.

Table 2. *The degree of histopathological findings in liver and kidney tissues*
Tablo 2. *Karaciğer ve böbrekte histopatolojik bulguların dağılımı*

Histopathological Findings	Experimental Groups						
	Control	CP	SLY	CUR	SLY+ CP	CUR+ CP	*P*
Liver							
Disorganization of hepatic cord	00.00±0.00^{c}	3.00±0.00^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.83±0.30^{b}	1.66±0.21^{b}	***
Sinusoidal dilatation	00.00±0.00^{c}	2.83±0.16^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.66±0.21^{b}	1.50±0.22^{b}	***
Hyperemia	00.00±0.00^{c}	2.83±0.16^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.66±0.21^{b}	1.50±0.22^{b}	***
Haemorrhage	00.00±0.00^{c}	1.00±0.25^{a}	00.00±0.00^{c}	00.00±0.00^{c}	0.16±0.16^{b}	0.16±0.16^{b}	***
Degeneration	00.00±0.00^{c}	3.00±0.00^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.83±0.30^{b}	1.66±0.21^{b}	***
Single cell necrosis	00.00±0.00^{c}	2.83±0.16^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.66±0.21^{b}	1.33±0.21^{b}	***
Nuclear changes	00.00±0.00^{c}	2.83±0.16^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.66±0.21^{b}	1.50±0.22^{b}	***
Lipid degeneration	00.00±0.00^{c}	3.00±0.00^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.50±0.22^{b}	1.16±0.16^{b}	***
Bile duct hyperplasia	00.00±0.00^{c}	2.33±0.33^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.33±0.21^{b}	1.16±0.16^{b}	***
Mononuclear cell infiltration	00.00±0.00^{b}	1.00±0.25^{a}	00.00±0.00^{b}	00.00±0.00^{b}	0.16±0.16^{b}	0.16±0.16^{b}	***
Kidney							
Oedema	00.00±0.00^{c}	2.66±0.21^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.66±0.21^{b}	1.16±0.16^{b}	***
Glomerular congestion	00.00±0.00^{c}	2.66±0.21^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.66±0.21^{b}	1.16±0.16^{b}	***
Interstitial haemorrhage	00.00±0.00^{c}	2.66±0.21^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.00±0.00^{b}	0.50±0.22^{b}	***
Tubular dilatation	00.00±0.00^{c}	2.83±0.16^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.66±0.21^{b}	1.33±0.21^{b}	***
Tubular degeneration and desquamation	00.00±0.00^{c}	2.83±0.16^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.50±0.22^{b}	1.16±0.16^{b}	***
Tubular necrosis	00.00±0.00^{c}	2.83±0.16^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.50±0.22^{b}	1.33±0.21^{b}	***
Intraluminal hyaline cast	00.00±0.00^{c}	2.50±0.22^{a}	00.00±0.00^{c}	00.00±0.00^{c}	1.50±0.22^{b}	1.33±0.21^{b}	***
Mononuclear cell infiltration	00.00±0.00^{b}	1.33±0.21^{a}	00.00±0.00^{c}	00.00±0.00^{c}	0.33±0.21^{b}	0.16±0.16^{b}	*

$^{a, b, c, d}$ *Different letters indicate statistically significant differences in the same row, * P<0.05; ** P<0.01, *** P<0.001;* ***CP:*** *Cylophosphamide,* ***SLY:*** *Silymarin,* ***CUR:*** *Curcumin*

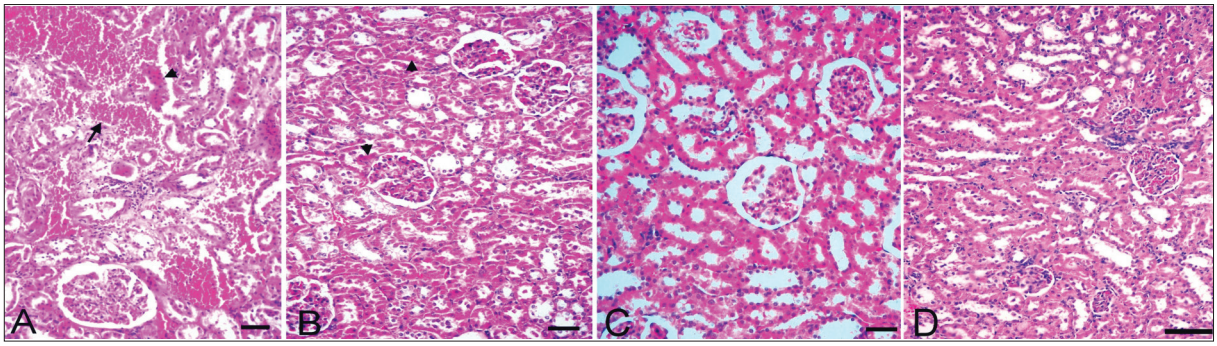

Fig 2. Kidney. **A.** Severe interstitial haemorrhage (arrow) and severe necrosis in the tubular epithelial cells *(arrowhead),* CP group, HE, Bar: 50 µm, **B.** Mild necrosis in the epithelia of the tubules *(arrowheads)* SLY + CP group, HE, Bar: 50 µm, **C.** Normal histological appearance of kidney, SLY group, HE, Bar: 50 µm, **D.** Histological appearance of kidney in control group. HE, Bar: 80 µm

Şekil 2. Böbrek. **A.** Şiddetli kanama *(ok)* ve tubulus epitellerinde şiddetli nekroz *(okbaşı),* CP grup, HE, Bar: 50 µm, **B.** Tubulus epitellerinde hafif nekrozlar *(okbaşları)*, SLY + CP grup, HE, Bar: 50 µm, **C.** Böbrekte normal histolojik görünüm, SLY grup, HE, Bar: 50 µm, **D.** Kontrol grubunda böbreğin normal histolojik görünümü, HE, Bar: 80 µm

Histopathological Findings

Microscopic findings of the liver and kidney were summarized in *Table 2*. In all the tissues, normal morphological findings were seen in the control, SLY and CUR groups. Severe histopathological changes in the liver and kidneys were consistently observed in the CP-treated group. Histopathological findings in the SLY+CP- and CUR+CP-treated groups were similar to those in the CP-treated group, but there was a prominent decrease in the frequency of pathological results.

In the liver, central veins and sinusoids were dilated. Focal haemorrhages were also noted. Disorganisation

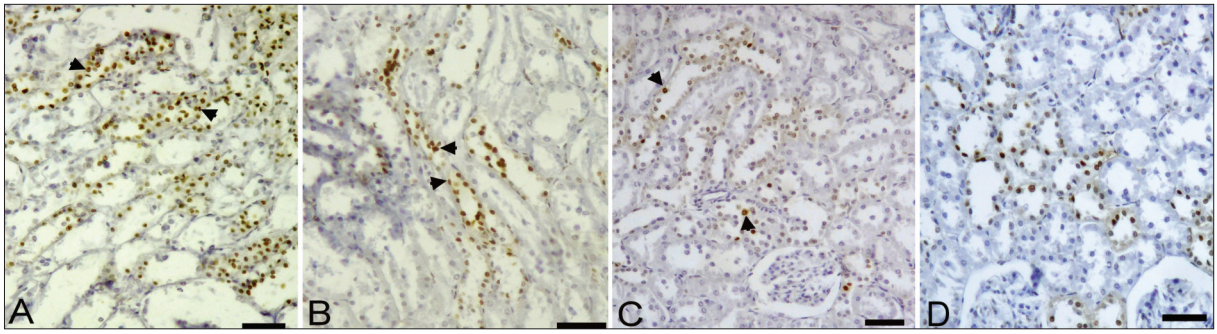

Fig 3. Kidney. **A.** A significant increases in the percentage of the TUNEL positive cells *(arrowheads),* CP Group, TUNEL, Bar: 50 µm, **B.** A decreases in the percentage of the TUNEL positive cells *(arrowheads)*, CUR + CP Group, TUNEL, Bar: 50 µm, **C.** The percentage of the TUNEL positive cells (arrowheads) in the CUR group. CUR group, TUNEL, Bar: 50 µm, **D.** The percentage of the TUNEL positive cells in the Control group, TUNEL, Bar: 50 µm

Şekil 3. Böbrek. **A.** TUNEL pozitif hücrelerin oranında artışlar *(okbaşları)*, CP Grup, TUNEL, Bar: 50 µm, **B.** TUNEL pozitif hücrelerin oranında azalma *(okbaşları),* CUR + CP Grup, TUNEL, Bar: 50 µm, **C.** CUR grubunda TUNEL pozitif hücreler *(okbaşları),* CUR grubu, TUNEL, Bar: 50 µm, **D.** Kontrol grubunda TUNEL pozitif hücreler, Kontrol grubu, TUNEL, Bar: 50 µm

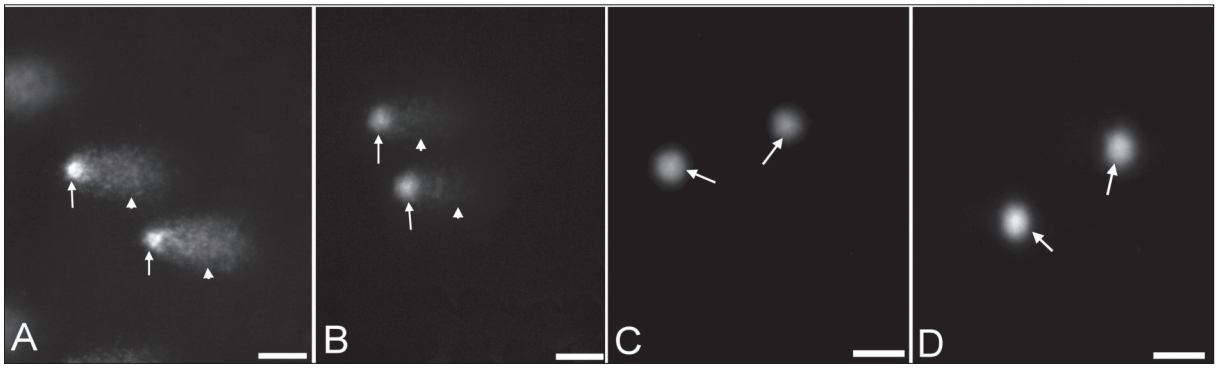

Fig 4. The fluorescence microscopy images of lymphocytes. **A.** CP group, Bar: 30 µm, **B.** CUR + CP group, Bar: 30 µm, **C.** CUR group, Bar: 30 µm, **D.** Control group, Bar: 30 µm; *Arrows:* head of lymphocytes, *arrowheads:* tail migration of lymphocytes

Şekil 4. Lenfositlerin floresan mikroskopik incelenmesi. **A.** CP grup, Bar: 30 µm, **B.** CUR + CP grup, Bar: 30 µm, **C.** CUR grup, Bar: 30 µm, **D.** Kontrol grubu, Bar: 30 µm; *oklar:* lenfosit, *okbaşları:* lenfosit kuyruk yoğunluğu

of the hepatic cord, hydropic degeneration and lipid degeneration were common histopathological findings. These findings were generally more pronounced in the sentroaciner areas. Most hepatocytes in the degenerative areas were so swollen, and several hepatocytes had ruptured. Hepatocytes with eosinophilic cytoplasm with picnotic nuclei were also detected in the same areas *(Fig. 1)*. In some hepatocytes, the nuclei were swollen, and enlarged, and in some cells they were a few times larger than normal. However, many hepatocytes had moderate to severe cytoplasmic vacuolation, indicating that fatty changes. These fatty droplets were confirmed with Oil Red O. In addition, mononuclear cell infiltrations was seen in portal areas. In kidney tissues, haemorrhages appeared mostly in the cortex *(Fig. 2)*. In areas with severe degeneration, cellular integrity was disrupted in many tubular epithelia, and the cytoplasms of intact epithelia had a granular appearance. Nuclei had a picnotic appearance, and the cytoplasm was eosinophilic in epithelia with necrotic changes. In the lumens of many tubules, eosinophilic hyalin casts of varying sizes and irregular structures were present In some regions, these casts filled in the lumens of tubules completely.

TUNEL assay

The number of cells undergoing apoptosis in the liver and kidneys was determined by a TUNEL assay. The percentage of TUNEL-positive cells per total cells in the liver and kidney *(Fig. 3)* tissues was evaluated and is presented in *Table 1*. A significant increase in the percentage of TUNEL-positive cells was detected in the CP-treated group compared to the control and other treated groups. However, there were significant differences in the percentage of TUNEL-positive cells in the SLY+CP- and CUR+CP-treated groups compared to the control group. The percentage of TUNEL-positive cells in all the tissues of the CUR+CP-treated groups was lower than that of the SLY+CP-treated groups, but the differences did not reach statistical significance.

Comet Assay

Comet-assay examination revealed that the CP administrated group showed a statistically significant

increase in the olive tail moment and the percentage of DNA in the tail levels compared to the control and other treated groups *(Fig. 4)*. The statistical differences of comet assay parameters in all groups are summarised in *Table 1*. However, the comet parameter levels in the SLY+CP- and CUR+CP-treated groups were lower than those in the CP-treated group, and these values reached the levels of the control and SLY- and CUR-treated groups.

DISCUSSION

CP is an alkylating agent widely used in cancer chemotherapy, and its cytotoxic effects are the result of chemically reactive metabolites that alkylate DNA and protein, producing cross-links [1,2]. The major limitation of using CP is injury to normal tissues, which gives rise to numerous side effects [25]. It has been reported that oxidative-stress-mediated disruption of redox balance after CP exposure generates biochemical and physiological disturbances [5,7]. To date, a number studies have reported that CP has a prooxidant character and that generation of oxidative stress after CP administration leads to a decrease in the activities of antioxidant systems and increases in lipid peroxidation in some tissues of mice and rats [5]. In both human and veterinary medicine, when tumours are resistant to antineoplastic drugs or when the doses of such drugs are inadequate, many drugs used in chemotherapy, including CP, should be administered at high doses [26]. The most important concern regarding high CP doses is the drug's toxic effects on the tissues [27,28]. It has been reported that CP's toxic effect is mediated by the degradation of antioxidant defence systems by acrolein, which is formed by the metabolisation of CP, resulting in excessive production of free radicals [2,3]. In the present study, SLY and CUR, which have strong antioxidant characteristics, were chosen in order to make it possible to use CP in high doses.

Although oxidative stress plays a part in many pathological cycles in the body, excessive amounts of free radicals arising in these processes are counterbalanced by the antioxidant system; if this balance is not maintained, tissue injury occurs [29,30]. It was reported that these radicals lead to peroxidation and modification by oxidising carbohydrates, lipids, proteins and DNA in the cell, exerting a quite toxic effect [29]. One of the reactive metabolites formed during lipid peroxidation, associated with the reactions of free radicals, is MDA [29]. In this study, tissue MDA levels were significantly higher in the CP-only group, indicating that CP increased lipid peroxidation and induced oxidative stress significantly, as reported in the literature [7,30]. However, It has been suggested that lipid peroxidation might be a contributing factor to the development of hepatic and renal toxicity. It is likely that the actions of SLY and CUR in reducing the membrane damege is partially related to theirs ability to scavenge lipid peroxidation initiating agents [7,8,30,31].

It has been reported by many investigators that SLY [6,12,31] and CUR [7,8,16] have protective activity against oxidative stress caused by various chemical agents. In the present study, MDA levels were found to be significantly lower in the tissues of rats administered SLY and CUR concurrently with CP than in the tissues of rats administered CP by itself, which is consistent with the literature. Although it was established that CUR, by preventing lipid peroxidation, decreased tissue MDA levels more than SLY did, the difference was not statistically significant. SOD enzyme are among the most important defence mechanisms against the damage caused by free oxygen radicals, and it has been reported that toxic superoxide radicals play an important role in the defence against harmful effects by accelerating dismutation to hydrogen peroxide and molecular oxygen [18]. Many studies have investigated the effects of SLY and CUR on antioxidant enzymes in various tissues [30,32]. In the present study, SOD enzyme activity levels were found to be significantly lower than those in the control group in the group CP-only group, which suggests that CP may markedly induce superoxide anion radical formation [5]. In the present study, SOD enzyme activity levels in the groups in which SLY and CUR were administered concurrently with CP, were found to be significantly different from those in the CP-only group, suggesting that both SLY and CUR influence the antioxidant system via SOD in the liver and kidneys, as in other chemical agents reported in the literature [4,8].

Serum ALT and AST values have been reported to be influenced by chemical agents having a toxic effect on hepatocytes in the liver, and ALT is considered a more important parameter than AST [33]. Creatinine is known to be an important biochemical parameter indicating kidney function disorders [34]. In the present study, in the CP-only group, liver ALT and AST levels and kidney creatinine levels were found to be significantly higher than those in the other groups, as reported in other studies [3,34]. However, in the histopathological examination of liver and kidney tissues in the present study, pathological findings were more severe in the CP-only group, indicating that histomorphological changes should also be used in the evaluation of these parameters when they are applied to liver and kidney tissues, which is consistent with previous reports [32,34]. In the groups in which SLY and CUR were administered concurrently with CP, ALT levels were found to be significantly lower than those in the CP-only group, suggesting that both SLY and CUR may affect serum ALT levels. Serum AST levels were found to be significantly lower in the groups in which SLY and CUR were administered concomitantly with CP than in the CP-only group. Nevertheless, because there was a significant difference between these groups and the control group in terms of AST level, we speculated that the impact of SLY and CUR on these groups may be weak. In the present study, serum kidney creatinine levels were also significantly lower in the groups in which SLY and CUR were administered

concurrently with CP than in the CP-only group, indicating that SLY and CUR affected kidney creatinine levels, but the difference between them and the control group was not statistically significant.

In the present study, the liver and kidneys of rats in all groups were examined microscopically and macroscopically, and the most common and severe findings were in the CP-only group. These findings are compatible with those of many studies aiming to decrease the toxic effect of CP [3,7,35]. In the present study, there was seen a decreasing in the severity of histopathological changes in the liver and kidney tissue of rats in the groups in which SLY and CUR were administered concurrently with CP than in the CP-only group, demonstrating that these two antioxidants have protective characteristics at the histomorphological level. Our results suggest that the protective mechanism of SLY and CUR might be due to the strong antioxidant property i.e. they helped for healing of liver and kidney parenchyma and regeneration of hepatocytes and tubular epithelial cells.

In order to determine apoptosis in tissues, various techniques, including morphological, immunohistochemical, biochemical, immunological and molecular biology methods, are employed [36,37]. In the present study, in order to measure apoptotic DNA breakages in living tissue, ELISA was chosen from among immunological methods [37], and for use in paraffin sections, TUNEL was chosen from among immunohistochemical methods [37]. According to the ELISA and TUNEL results, the rate of apoptotic cells was significantly higher in the tissues of rats to which CP only was administered, as reported in the literature [38], indicating that high doses of CP are quite effective on apoptosis in tissues. In addition, significantly lower rates of apoptosis were found in the groups in which SLY and CUR were administered together with CP than in the CP-only group, which suggests that both antioxidants decrease the rate of apoptosis caused by the toxic effects of CP.

Single-cell gel electrophoresis or the comet method is a noninvasive, rapid and sensitive fluorescent microscopic method used to determine the rate of DNA damage at the cellular level [21,22]. It has been reported that in the quantitative determination of DNA damage using the comet method, olive tail moment and the percent tail DNA are the most commonly used parameters [21]. In the present study, as in previous research, olive tail moment and the percent tail DNA were used as indicators of DNA damage [22,23]. The significant increase in the aforementioned parameters in the CP-only group compared to the groups in which CP was administered together with SLY and CUR suggests that CP has a marked genotoxic effect on DNA, and it was concluded that both SLY and CUR may be used reliably against the genotoxic effect of CP in cases where it has to be used in high doses. In addition, the lack of a statistically significant difference between the values in these groups and those in the control group supports this conclusion.

In conclusion, in the present study, the most severe pathological findings were seen in the CP-only group, and all serum and tissue biochemical parameters were influenced adversely in this group, indicating that CP exerted a markedly toxic effect on the tissues examined. In addition, it was observed that the findings obtained with the comet method were higher in the CP-only group than in the other groups. Better biochemical, pathological and genotoxic results were obtained in the groups in which SLY and CUR were administered concurrently with CP; this suggests that these antioxidants can be used in conditions when CP has to be administered at high doses. The present study established that both SLY and CUR are effective against the toxic effects of high doses of CP.

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Research Article

Prevalence and Molecular Characterization of *Cryptosporidium* in Dairy Cattle from Farms in Algeria

Nassim OUCHENE [1] Nadjet Amina OUCHENE-KHELIFI [1] Manal KHELIFI [5]
Fayçal ZEROUAL [3] Idir BITAM [2] Ahmed BENAKHLA [3]
Rachid KAIDI [1,4] Abu-Bakr ABU-MEDIAN [4]

[1] Laboratory of Biotechnology Related to Animal Reproduction, Institute of Veterinary Science, University Saad Dahlab, Blida, ALGERIA
[2] Laboratory of Biodiversity and Environment: Interactions, Genomes. Faculty of Biological Sciences, University of Science and Technology Houari Boumediene, Algiers, ALGERIA
[3] Instituteof Veterinary Sciences, University of El Tarf, BP73, 36000 El Tarf, ALGERIA
[4] School of Veterinary Medicine and Science, University of Nottingham, UNITED KINGDOM
[5] Hospital Public Establishment of Bordj Menaiel, Street Madaoui Ali BP 70, Boumerdes, 35000, ALGERIA

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Abstract

Faecal samples were collected from 338 less than 03 month-old dairy calves from 34 cattle herds located in three regions of north-eastern Algeria in order to characterize *Cryptosporidium* oocyst output using Ziehl-Neelsen, Heine and immunofluorescence methods and to identify different species of *Cryptosporidium* using a PCR 18S. Faecal samples were processed, firstly, through concentration and modified Ziehl-Neelsen staining techniques. Then, 93 faecal samples (not concentrated) were randomly selected and analyzed with the Heine staining technique. Positives samples revealed by both methods, Heine and Ziehl-Neelsen, were analyzed by direct immunofluorescent antibody test and by PCR 18S. Twenty-three herds out of thirty four (68%) were found positive and 108 (32%) calves were shedding oocysts. The prevalence of excretion peaked when calves were 15-30 days of age. However, it was found that the majority of infected calves were coupled to diarrhoea in 1-14 day old calves. Moreover, calves aged 1-14 days showed a higher level of oocyst shedding with OPG equal to 5.2×104. Three species were identified by sequencing of the 18S PCR products; *Cryptosporidium parvum, Cryptosporidium bovis* and *Cryptosporidium andersoni*. These results represent the first report on the genetic identification of *Cryptosporidium* species in dairy cattle in Algeria and confirm the frequent occurrence of *Cryptosporidium* in diarrhoeic calves.

Keywords: *Cryptosporidium, PCR 18S, calves, Algeria*

Cezayir Çiftliklerinde Sığırlardaki *Cryptosporidium* Prevalansı ve Moleküler Karakterizasyonu

Özet

Kuzey-doğu Cezayir'de 3 farklı bölgedeki 34 sığır çiftliğinden toplam 338 adet 3 aylıktan daha küçük buzağıya ait dışkı örnekleri Ziehl-Neelsen, Heine ve immunfloresan yöntemleri kullanılarak *Cryptosporidium* oositlerini karakterize etmek ve 18S PCR ile farklı türlerini belirlemek amacıyla toplandı. Dışkı örnekleri konsantrasyon ve sonrasında modifiye Ziehl-Neelsen yöntemi ile işlendi. Takibinde, 93 dışkı örneği (konsantre edilmemiş) kör olarak seçildi ve Heine boyama tekniği ile analiz edildi. Heine ve Ziehl-Neelsen tekniklerinin her ikisiyle birlikte pozitif bulunan örnekler direkt immunfloresan ve 18S PCR ile analiz edildi. Otuz dört çiftlikten 23'ü (%68) pozitif olarak belirlenirken 108 (%32) buzağının oosit yaydığı tespit edildi. Oosit yayma oranı buzağılar 15-30 günlükken en yüksek seviyedeydi. Ancak enfekte buzağıların çoğunun 1-14 günlükken ishalli oldukları belirlendi. 1-14 günlük buzağılar her bir gram dışkı örneğinde 5.2×104 oosit ile daha yüksek seviyede oosit yayma oranı gösterdiler. 18S PCR ile *Cryptosporidium parvum, Cryptosporidium bovis* ve *Cryptosporidium andersoni* olmak üzere üç tür belirlendi. Bu çalışma ile Cezayir'de sütçü sığırlarda *Cryptosporidium* türlerinin ilk defa genetik identifikasyonu yapılarak ishalli buzağılardaki bulunma sıklığı tespit edildi.

Anahtar sözcükler: *Cryptosporidium, PCR 18S, buzağı, Cezayir*

İletişim (Correspondence)
+213 66 1224250
ouchenassim@gmail.com

INTRODUCTION

Cryptosporidium is a major agent of neonatal diarrhoea of calves and infection has been associated with economic losses due to the occurrence of the diarrhoea and more rarely to the death of animals [1].

Cryptosporidium was first isolated in cattle in 1971 [2]. Since then, there have been numerous reports of cryptosporidiosis in different countries [3-6]. More than 20 *Cryptosporidium* species have been described. Cattle have been reported to be infected with four species of *Cryptosporidium*: *Cryptosporidium parvum, Cryptosporidium bovis, Cryptosporidium ryanae* and *Cryptosporidium andersoni* [3,7-9].

To date, little information regarding the epidemiology of *Cryptosporidium* infection in cattle from Algeria has been reported and no studies have been conducted to identify the different *Cryptosporidium* species. The studies realized by Akam et al.[10], Khelef et al.[11] and Ouchene et al.[12] in Algeria, indicate only a prevalence of *Cryptosporidium* infection in cattle (from 16.66% to 24.7%) using Ziehl-Neelsen acid-fast method but no species have been identified.

Consequently, the present study was designed to assess the prevalence of *Cryptosporidium* in dairy calves and, for the first time in Algeria, as a preliminary approach, to try to identify different species of *Cryptosporidium* using PCR assay.

MATERIAL and METHODS

Samples

This study was performed in north Algeria during the period between January and December 2010. Samples were taken from thirty-four dairy cattle herds: 12 from Setif region, 15 from El Tarf region and 7 from Constantine region. Faecal samples were collected once from a total of 338 calves aged less than 3 month-old of which 105, 165 and 68 were sampled in Setif, El Tarf and Constantine regions, successively.

Faecal samples were taken directly from rectum and transported directly to the laboratory in a cool box to be stored and processed in the following 3 days.

Laboratory Analyses

- Oocyst concentration: Faecal samples (n=388) were concentrated as previously described by Castro-Hermida et al.[13]. The sediment was removed and washed in distilled water by centrifuging at 1000× g for 5 min at 4°C. The volume of the oocyst suspension was adjusted to 1 ml using distilled water.

- Ziehl-Neelsen acid-fast method: The oocyst suspension was used to prepare thin faecal smear on slide for staining with Ziehl-Neelsen technique modified by Henriksen and Pohlenz [14]. After staining, faecal smears were observed under an optical microscope at 1000×.

The intensity of excretion was evaluated semi-quantitatively according to the average number of oocysts in 30 randomly selected fields: 0: absence of oocyst; +1: <1oocysts per field; +2: 1-10 oocysts per field; +3: >10 oocysts per field.

- Heine staining method: From the 338 faecal samples,93 (not concentrated) were randomly selected to be analyzed with the Heine staining technique [15]. The intensity of excretion was also evaluated semi-quantitatively according to the average number of oocysts in 10 randomly selected fields: 0: absence of oocyst, 1: <1 oocyst per field, 2: 1-10 oocysts per field, 3: 11-20 oocysts per field, 4: 21-30 oocysts per field, 5: >30 oocysts per field.

- Immunofluorescence examination and molecular characterization: Positives samples revealed by both methods Heine and Ziehl-Neelsen (n=16), were analyzed by direct immunofluorescent antibody test (IFAT) (MeriFluor® *Cryptosporidium/Giardia*, Meridian Bioscience Europe, Nice, France) to calculate the number of oocysts per gram of faeces (OPG) and by PCR 18S to identify different species of *Cryptosporidium*.

For the IFAT, 10 µl of oocyst suspension was fixed on the slides using acetone at 4°C for 10 min. The slides were then processed according to the manufacturer's instructions. The oocysts were observed 400 × magnifications under fluorescence microscopy. The OPG was calculated by using this formula: number of oocysts seen in the well × 100/2.

For the PCR 18S, 250 µl of the oocyst suspension was processed using the UltraClean Faecal DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, USA) according to the manufacturer's instructions. The eluted DNA was dissolved in 50 µl.

A two-step nested PCR protocol was used to amplify an 830 bp segment of the 18S rRNA gene using primers 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAA ACAGGA-3' for primary PCR and 5'-GGAAGGGTTGTATTTAT TAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3' for secondary PCR [16,17].

The amplification runs were performed in a Bio-Rad iCycler thermal cycler as: 10 min at 94°C, 40 cycles of 45 s at 94°C, 55 s at 55°C and 1 min at 72°C, with a final 5min elongation step at 72°C. Amplification products (10 µl) were analyzed on 2% agarose gel and visualized after ethidium bromide staining under UV light.

PCR products were sequenced using an ABI 3730XL sequencer (Applied Biosystems, Warrington, UK). Sequence accuracy was ensured by two-directional sequencing, and all electrophoregrams were manually checked and edited as deemed necessary. Sequences were compared with

known sequences by BLAST-analysis against the NCBI database.

Statistical Analysis

The Chi-square test was used to compare between the different prevalences and Pearson correlation test was used to compare between the results of both methods Ziehl-Neelsen and Heine. The statistical program used was R i386 3.0.2 for Windows GUI front-end. Differences were considered as significant when p value was less than 0.05.

RESULTS

Ziehl-Neelsen Acid-fast Method

Twenty-three herds out of thirty-four (68%) were found positive and 108 (32%) calves were shedding *Cryptosporidium* oocysts. Within-herd prevalence of infection ranged from 6% to 100% and in different regions, varies from 28% (29/105), 30% (49/165) and 44% (30/68) in Setif, El Tarf and Constantine, respectively.

Prevalence of infection showed the highest values of 46% in the 15-30 day-old calf group when compared to other age groups (P<0.001) *(Table 1)*.

The overall percentage of diarrhoeic calves at the time of sampling was 20% (67/338). This general figure varied according to the age and showed a higher value (28%) for the 1-14 day-old calves compared to the other age groups (P<0.01). The majority of infected calves were coupled to diarrhoea in 1-14 day old calves (59%) compared with other age groups (P<0.001) *(Table 1)*.

A significantly higher level of oocysts shedding (3+) was observed in 1-14 day-old animals compared to the other levels (+1 and +2) (P<0.01). In contrast, in the other age groups (15-30 days and 31-90 days), the oocysts excretion level was low (+1) (P<0.01). The same observations have been reported in diarrhoeic calves *(Table 1)*.

Heine Staining Method

Heine staining method showed that 19% (18/93) of calves were infected; this prevalence is lower than that reported by Ziehl-Neelsen method above (P<0.001).

However, a correlation was observed between the prevalence of *Cryptosporidium* infection reported by Ziehl-Neelsen method and that reported by Heine method according to age of calves (P<0.01).

Moreover, *Cryptosporidium* was commonly found among 15-30-day-old calves (35%) (P<0.05) and1-14day-old calves were found to be most affected by diarrhoea (55%) with a prevalence of *Cryptosporidium* infection of 100% in diarrhoeic calves (P<0.01). The intensity of oocyst shedding (2-5) was the highest in 1-14 day-old calves compared to other age groups *(Table 2)*.

Immunofluorescence Examination

The mean oocyst excretion calculated using the IFAT indicated that calves 1-14 days-old were the most excreting of *Cryptosporidium* oocysts with OPG equal to 5.2×10^4. This result was in accordance with the finding of Ziehl-Neelsen and Heine methods. The OPG in 15-30 days and 31-90 day-old calves was 2.4×10^4 and1.6×10^2, respectively.

Molecular Characterization

Three species were identified by sequencing of the 18S PCR products; *Cryptosporidium parvum* in only one 20-day old calf, *Cryptosporidium bovis* in two calves 21 and 60 days

Table 1. *Prevalence and intensity of excretion of Cryptosporidium oocysts according to age of calves and to the score of diarrhoea (Ziehl-Neelsen methods)*
Tablo 1. *Yaşa ve ishal derecesine göre Cryptosporidium oositlerinin atılma prevalansı ve sıklığı (Ziehl-Neelsen metodu)*

Age Groups	Overall Diarrhoea Frequency	Cryptosporidiosis Individual Prevalence	% of Diarrhoeic Positive Calves	Oocyst excretion level				Oocyst Excretion Level from Diarrhoeic Calves			
				0	+1	+2	+3	0	+1	+2	+3
1-14 d (n=72)	28% (20/72)	37% (27/72)	59% (16/27)	62% (45/72)	19% (5/27)	30% (8/27)	52% (14/27)	9% (4/45)	6% (1/16)	31% (5/16)	62% (10/16)
15-30 d (n=83)	23% (19/83)	46% (38/83)	39% (15/38)	54% (45/83)	47% (18/38)	29% (11/38)	24% (9/38)	9% (4/45)	40% (6/15)	33% (5/15)	27% (4/15)
31-90 d (n=183)	15% (28/183)	23% (43/183)	26% (11/43)	76% (140/183)	70% (30/43)	16% (7/43)	14% (6/43)	12% (17/140)	55% (6/11)	36% (4/11)	9% (1/11)

Table 2. *Prevalence, intensity of excretion of Cryptosporidium oocysts according to the age of calves (Heine method)*
Tablo 2. *Yaşa göre Cryptosporidium oositlerinin atılma prevalansı ve sıklığı (Heine metodu)*

Age Categories	Overall Diarrhoea Frequency	Prevalence of Excretion	Proportions of Diarrhoeic Positive Calves	Intensity of Excretion
1-14 days (n=22)	55% (12/22)	18% (4/22)	100% (4/4)	2-5
15-30 days (n=31)	39% (12/31)	35% (11/31)	73% (8/11)	1-3
31-90 days (n=40)	45% (18/40)	7% (3/40)	0% (0/3)	1-2

old and *Cryptosporidium andersoni* in only one 35 day old calf.

DISCUSSION

Cryptosporidium parvum is one of the major causes of neonatal calf diarrhea [1,12,18]. However, so far, studies on the prevalence of *Cryptosporidium* in Algeria [10,12] have been conducted without molecular characterization.

The present study indicates that *Cryptosporidium* remains widespread in Algeria. The results of Ziehl-Neelsen method showed that 68% (23/34) of dairy cattle were infected and the prevalence of *Cryptosporidium* infection in calves was 32% (28%-44%) which is similar to the results reported by other authors in Algeria [10-12] and Spain [18] and higher to the results showed in Turkey [19-21].

However, our results showed that the parasite prevalence on farms ranged from 6.25% to 100% of the sampled animals. This observation was in accordance with results in France [22] and Michigan (USA) [23].

In order to increase the sensitivity of the assays, the concentration method was utilized [12,24] which explains, in the present study, the great prevalence revealed by Ziehl-Neelsen technique (32%) compared to that reported by Heine technique (19%). However, according to the age calves, a correlation of the prevalence of *Cryptosporidium* infection was observed between the two techniques ($P<0.01$).

Higher frequency of oocyst shedding was observed in 15-30 day-old calf groups (both Ziehl-Neelsen and Heine methods) what is a steady and characteristic trait of *Cryptosporidium* infection in young cattle [25]. The same results were reported in Algeria [10,12]. Decreasing prevalence of *Cryptosporidium* infection with age in cattle has been constantly observed [19,26,27].

Our results showed that the prevalence of *Cryptosporidium* infection in 1-14 day-old calves was significantly associated with diarrhoea and high level of oocysts shedding. This suggested a highly significant association between the intensity of shedding and the presence of diarrhea in young calves. Such positive relationship between diarrhoea and *Cryptosporidium* oocyst output has been described in calves with simultaneous occurrence of diarrhoea and high oocyst counts [26].

The peak of cryptosporidiosis prevalence in young calves could reflect the immaturity of the immune status and the low excretion of *C. parvum* oocysts in older calves might be related to the development of immunity that also protected the animal against a secondary infection [28].

Cattle have been reported to be infected with four species of *Cryptosporidium*: *Cryptosporidium parvum*, *Cryptosporidium bovis, Cryptosporidium ryanae* and *Cryptosporidium andersoni* [9]. Our study represents the first report on the genetic identification of *Cryptosporidium* species in dairy cattle in Algeria. Three species were identified; *Cryptosporidium parvum*, *Cryptosporidium bovis* and *Cryptosporidium andersoni*.

Several authors reported that the species *C. parvum* constituted the majority of infections in pre-weaned calves whereas *C. bovis* in post-weaned calves and *C. andersoni* in adult cows [7,26,29-32]. In our study, *C. parvum* was identified in only one 20 day-old calf, *C. bovis* in two calves 21 and 60 day-old and *C. andersoni* in only one 35 day old calf.

In Belgium, *C. parvum* is predominant in young dairy calves (<1month) [33]. *C. bovis* was found in dairy calves at 2 or 3 weeks of life in India and China [27].

Our study confirms the frequent occurrence of *Cryptosporidium* in diarrhoeic calves in Algeria. As several species and/or genotypes may be involved according to the different age classes of cattle, further studies are needed to have a better understanding of *Cryptosporidium* infection in cattle farms and its zoonotic potential. Hence, considerable attention should be paid to preventing the spread of the infection.

These results represent the first report on the prevalence and genetic identification of *Cryptosporidium* species in dairy cattle in Algeria and may contribute to a better understanding of *Cryptosporidium* epidemiology in cattle.

However, calf neonatal diarrhoea in the field is a more complex and multifactorial disease that involves *Cryptosporidium* and other pathogens exposure as well as environmental and management factors and this should be considered.

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Research Article

Evaluation of Serum C-Reactive Protein and Natural Antibodies in Cows with Endometritis [1]

Semra KAYA [1] Cihan KAÇAR [1] Metin ÖĞÜN [2] Mushap KURU [1]
Hasan ÖZEN [3] Murat Can DEMİR [1] Levent ŞAHİN [4] Abuzer Kafar ZONTURLU [5]

[1] *This study was supported by the Scientific Research Project Coordinator's Office at Kafkas University (2015-TS-31)*

[1] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, University of Kafkas, TR-36300 Kars - TURKEY
[2] Department of Biochemistry, Faculty of Medicine, University of Kafkas, TR-36300 Kars - TURKEY
[3] Department of Pathology, Faculty of Veterinary Medicine, University of Kafkas, TR-36300 Kars - TURKEY
[4] Department of Obstetrics and Gynecology, Faculty of Medicine, University of Kafkas, TR-36300 Kars - TURKEY
[5] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, University of Harran, TR-63200 Sanliurfa - TURKEY

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Abstract

The aim of this study is to assess serum natural antibody (NAb) bindings keyhole limpet hemocyanin (KLH) titer and C-reactive protein (CRP) levels, two of the most important elements of the natural defense system in the uterus. The study was conducted on 77 Brown Swiss cows (3-7 years of age) with body condition scores (BCS) of 2.6±0.1. The diagnosis of endometritis in cows on day 30-32 postpartum was made by rectal examination, vaginoscopy, ultrasonography and cytobrush technique. Blood samples were collected immediately after the examinations to measure serum NAb titer and CRP concentrations. NAb binding KLH titers were determined using the indirect ELISA method and the CRP level by using the bovine CRP ELISA kit. Mean NAb titers were 5.36±0.16, 7.43±0.15, 8.55±0.19 and 9.64±0.27 respectively in healthy cows (Control, n = 29), cows with mild endometritis (ME, n = 21), cows with moderate endometritis (E, n = 17) and cows with severe endometritis (SE, n = 10). Mean serum CRP concentrations were 48.88±3.92 µg/mL, 82.86±4.28 µg/mL, 98.86±8.2 µg/mL and 122.34±12.72 µg/mL respectively in groups control, ME, E and SE. NAb binding KLH titer and CRP levels increased significantly in cows with endometritis (P<0.001). BCS did not change serum NAb titer and CRP level (P>0.05). Parity did not cause a significant difference in serum NAb titer and CRP levels (P>0.05), but serum NAb level were higher in multiparous cows with ME (P<0.05). In conclusion, it is thought that serum NAb titer and CRP level can be used effectively as an indicator to determine uterus infections.

Keywords: *Cow, Endometritis, NAb, CRP*

Endometritisli İneklerde Serum C-Reaktif Protein ve Doğal Antikor Düzeylerinin Değerlendirilmesi

Özet

Sunulan çalışmada uterusun doğal savunma sisteminin en önemli bileşenlerinden olan keyhole limpet hemosiyanine (KLH) serum doğal antikor (NAb) titresi ve C-reaktif protein (CRP) düzeyinin belirlenmesi amaçlanmıştır. Çalışma 3-7 yaşlı, ortalama vücut kondisyon skoru (BCS) 2.6±0.1 olan 77 İsviçre Esmeri inekte yapıldı. Postpartum 30-32. günlerde bulunan ineklerde endometritis teşhisi; rektal, vaginoskopik, ultrasonografik muayene yöntemleri ve cytobrush tekniğiyle yapıldı. Muayenelerden hemen sonra KLH bağlı NAb titresi ve CRP konsantrasyonlarının belirlenmesi için kan örnekleri alındı. Serum NAb titresi indirekt ELISA yöntemiyle, CRP düzeyi ise bovine CRP ELISA kiti kullanılarak belirlendi. Serum NAb titresi sağlıklı (Kontrol, n = 29), hafif endometritis (ME, n = 21), orta derecede endometritis (E, n = 17) ve şiddetli endometritisli (SE, n=10) ineklerde sırasıyla ortalama 5.36±0.16, 7.43±0.15, 8.55±0.19 ve 9.64±0.27 olduğu belirlendi. Serum CRP konsantrasyonlarının ise kontrol, ME, E ve SE'de sırasıyla ortalama 48.88±3.92 µg/mL, 82.86±4.28 µg/mL, 98.86±8.2 µg/mL ve 122.34±12.72 µg/mL düzeyinde olduğu tespit edildi. Endometritisli ineklerde serum NAb titresi ve CRP düzeyinin önemli oranda arttığı belirlendi (P<0.001). BCS'nin serum NAb ve CRP düzeylerini değiştirmediği saptandı (P>0.05). Doğum sayısının serum NAb titresi ve CRP düzeyinde önemli bir değişikliğe neden olmadığı (P>0.05), sadece ME grubundaki multipar ineklerde serum NAb titresini artırdığı belirlendi (P<0.05). Sonuç olarak ineklerde uterus enfeksiyonlarının belirlenmesinde serum NAb titresi ve CRP düzeyinin indikatör olarak etkin bir şekilde kullanılabileceği düşünülmektedir.

Anahtar sözcükler: *İnek, Endometritis, NAb, CRP*

İletişim (Correspondence)
+90 474 2426807/5221
semra-kafkas@hotmail.com

INTRODUCTION

Uterus infections result economic losses by causing decreases in reproductive performance and milk production, extending the time intervals between parturition-first insemination and between parturition-conception [1-5]. The incidence of clinical endometritis reportedly ranges between 9-31% (mean 17%) depending on the diagnostic method used, the way the herd being studied is managed and the season in which the study is being conducted [2,6-8]. *Trueperella pyogenes, Fusobacterium necrophorum, Escherichia coli* [9,10] and *Campylobacter fetus* the most important micro-organisms causing endometritis [11]. The regional and systemic defense system of the uterus attempt to destroy [12] the microorganisms that enter the uterus due to the fact physical protective barriers are removed at parturition [13,14]. Neutrophils and lymphocytes play a fundamental role in the uterine immune system [13,15]. Pathogens opsonized with the humoral defense system are increasingly phago-cytosed by neutrophils [15]. Natural antibodies (NAb) are the most significant elements of the humoral defense system from birth [16]. They are present in body serum even in non-vaccinated organisms [17]. They are produced by B1 cells without needing any antigenic stimulation [18]. They constitute the first line of defense against pathogens and activate the complement system [19]. Acute phase proteins also play an important role in the system that fights infection. One of the most important acute phase proteins is C-reactive protein (CRP) [20,21]. CRP is a pentameric protein capable of binding to various pathogenic bacteria [20]. It regulates the immune system in the early periods of infection. It also plays a significant role in the destruction of infectious agents, protecting the tissue against greater damage, and tissue repair and regeneration [22]. Furthermore, it mediates phagocytosis by binding to specific receptors on phagocytic cells, stimulates the production of anti-inflammatory cytokines and contributes to the acquired defense system by activating the complements [23]. In reviewing the literature, we encountered a limited number of studies measuring serum CRP level and NAb titer in cows diagnosed with different severities of endometritis and reporting the differences observed in CRP level and NAb titer according to the severity of endometritis. The aim of this study were to determine the serum NAb titer as a humoral marker and serum CRP level as an acute phase protein in cows with endometritis.

MATERIAL and METHODS

Animals

The present study was conducted on 77 Brown Swiss cows ages 3-7 (between their 1st and 5th parity) at the Kafkas University Veterinary School Research and Application Farm fed on meadow grass, silage (live weight x 1.5/100) and dairy cattle feed (20% crude protein, 2700 energy) with mean body condition score (BCS) of 2.6±0.1.

The Classifications and Determination Endometritis

Cows with a history of caesarean operation, retentio secundinarum, vaginal tear and severe systemic-metabolic disease were excluded. Postpartum examinations were carried out between postpartum days 30-32. Initially the tail, vulva and perineum was examined for the presence of discharge. Then a vaginoscopy was performed, and the character of the discharge on the vaginal wall or coming from the cervix uteri was recorded. Rectal palpation findings (location of the uterus, symmetry of the cornua, etc.) and ultrasonographic (USG, 7.5 MHz, Titan®, Sonosite, USA) images of the uterus (diameter of the cornua, the presence of fluid in the uterine lumen, uterine wall thickness) were recorded. The diameter of the cervix uteri was measured using USG. Endometrial samples were obtained using the cytobrush technique from cows that showed no evidence of discharge, had a normal cervix uteri diameter and presented no symptoms of endometritis in the USG. The cytobrush technique was performed using a brush approximately 3 cm long on a stainless steel rod, 65 cm in length and 4 mm in diameter. Plastic sheaths were used to prevent contamination of the cytobrush in the vagina. Endometrial cytology samples were obtained by rotating cytobrushes a few times in the uterine endometrium. The samples obtained were smeared on slides and dried. They were fixed in methanol (Sigma Aldrich®, Turkey) for fifteen minutes and stained with Giemsa solution (Merck®, Turkey) for 30 min. Then the neutrophil to leukocyte ratio was calculated using microscopy (Olympus CX23®, Olympus corp, Japan) (by counting a minimum of 100 cells at 400x magnification). Cows having a neutrophil to leukocyte ratio >18% [24] were classified as subclinical endometritis. Cows that were determined to be healthy were put in the control group. Endometritis was classified and evaluated as reported by LeBlanc et al.[2] Cows' body condition scores were assessed immediately after the examinations as reported by Edmonson et al.[25] (1=thin; 5=fat; in increments of 0.25). Cows classified according to the degree of endo-metritis were later separated into subgroups based on BCS and number of parities; their serum NAb titer and CRP level were compared. Blood samples were collected from the vena coccygea of all animals into vacuum gel serum tubes (8.5 mL). The blood samples were centrifuged at 1200 g for 10 min. The serums were then transferred into eppendorf tubes and stored at -20°C until the measurements were made.

Analysis of NAb Binding KLH Titer and CRP Level

C-reactive protein was estimated using the bovine CRP ELISA kit purchased from Mybiosource® (USA). Since KLH is a metalloprotein found in the hemolymph, cows are sensitive to it [26]. Therefore, it is reported that unlike the determination of specific serum immunoglobulins of cows, KLH is a good antigen with which to measure NAb [27]. NAb binding KLH titer were determined using an indirect ELISA procedure described by van Knegsel et al.[28]. Plates were coated with 2 µg of KLH/mL (Sigma Aldrich®, USA)

(100 µL/well) dissolved in carbonate buffer (10.6 g/L Na_2CO_3, pH 9.6). After incubating overnight at 4°C and then washed once with tap water and 0.05% Tween-20 (Sigma Aldrich®, USA). After washing, serial dilutions of plasma (1:4) in PBS, 0.05% Tween-20, and than 2.5% rabbit serum (Sigma Aldrich®, USA) were added. Plates were incubated with in PBS with 0.05% Tween-20 for at 1 h at room temperature. Serial dilutions of plasma (1:4), and 2.5% rabbit serum were added, dilutions started at 1:40 for plasma. After being washed with tap water and 0.05% Tween-20, binding of bovine antibodies was detected using 1:20.000 diluted rabbit-anti-bovine IgG conjugated to peroxidase (Sigma Aldrich®, USA) 100 µL/well. After washing, tetramethyl-benzidine (Sigma Aldrich®, USA) 100 µL/well were added and incubated for 10 min at room temperature. The reaction was stopped by adding 1.25 M H_2SO_4 50 µL/well. Extinctions were measured with ELISA Reader (Epoch®, Biotek, USA) at a wavelength of 450 nm.

This study was conducted after obtaining approval from the Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK - Submission: 18.12.2014/040).

Statistical Analysis

Statistical analyses were performed using the SPSS® (SPSS 20, IL, USA) software program. NAb titer and CRP level observed in each group were tested for normality using the Shapiro-Wilk test. Differences in NAb titer and CRP level between the groups were compared using ANOVA - Tukey HSD tests. The effects of parity and BCS on NAb titer and CRP level were compared using the Independent T-test. The results were analyzed and found to be X±SE. P values <0.05 were considered statistically significant.

RESULTS

Postpartum Examinations Results

In postpartum examinations, mild endometritis (ME) was diagnosed in 21 cows, moderate endometritis (E) in 17 cows, severe clinical endometritis (SE) in 10 cows, healthy (control) in 29 cows. No differences were found between control group (2.61±0.11) and cows with endometritis (ME = 2.63±0.12, E = 2.74±0.12, SE = 2.28±0.13) in terms BCS (P>0.05).

NAb Binding KLH Titers

Serum NAb titer in each group are illustrated in *Fig. 1*. Serum NAb titer were higher in groups with endometritis (ME = 7.43±0.15, E = 8.55±0.19, SE = 9.64±0.27) than in control group (5.36±0.16) and this difference was statistically significant (P<0.001). The more severe the endometritis, the higher the serum NAb titer raise and this difference was considered statistically significant (ME-E = P<0.001; ME-SE = P<0.001; E-SE = P<0.005).

CRP Levels

Serum CRP level in control group and groups with endometritis are presented in *Fig. 2*. Serum CRP level were significantly higher in groups with endometritis (ME = 82.86±4.28 µg/mL, E = 98.86±8.2 µg/mL, SE = 122.34±12.72 µg/mL) than in control group (48.88±3.92 µg/mL, P<0.001). Serum CRP level in the ME group were lower than the SE group (P=0.002). However, no statistically significant difference was observed between the groups ME and E and the groups E and SE in terms of serum CRP level (P>0.05).

Serum NAb Binding KLH Titer and CRP Levels were Compared According to BCS

The number of cows with a body condition score ≤2.5 was control = 14, ME = 10, E = 8, SE = 8, and the number of cows with BCS >2.5 was control = 15, ME = 11, E = 9, SE = 2. When serum NAb titer and CRP level were compared according to BCS, it was found that NAb titer and CRP level were higher in the group with a BCS ≤2.5 (7.23±0.29, 81.94±6.86 µg/mL respectively) than the group with a BCS >2.5 (7.14±0.28, 75.24±4.81 µg/mL); however, this difference was not considered statistically significant (P>0.05). When groups were compared (cows with a BCS ≤2.5 and those with a

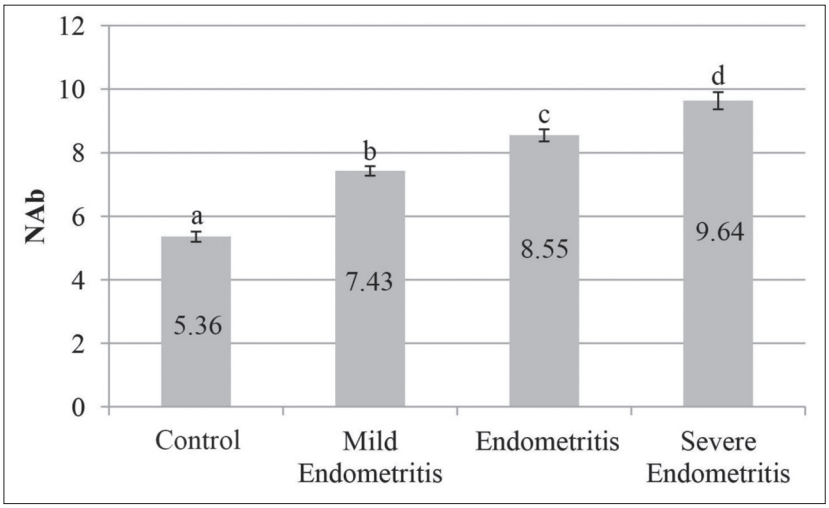

Fig 1. Comparison of NAb titer binding KLH for the groups (a:b:c:<0.001; a:b:d<0.001; c:d=0.005)

Şekil 1. Gruplarda KLH'ye bağlanan NAb titrelerinin karşılaştırılması (a:b:c:<0.001; a:b:d<0.001; c:d=0.005)

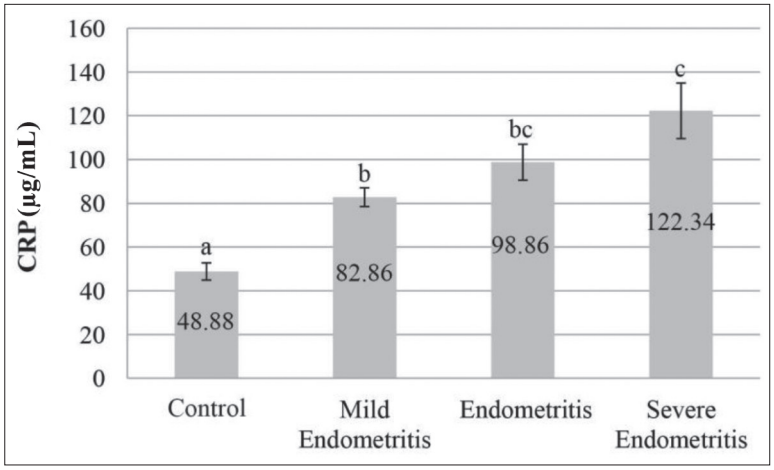

Fig 2. Comparison of serum CRP levels in each group (a:b<0.001; a:bc<0.001; a:c<0.001; b:c=0.002; b:bc>0.05; bc:c>0.05)

Şekil 2. Gruplarda serum CRP seviyelerinin karşılaştırılması (a:b<0.001; a:bc<0.001; a:c<0.001; b:c=0.002; b:bc>0.05; bc:c>0.05)

Table 1. *Determination of NAb titer and CRP level based on body condition scores*
Tablo 1. *Vücut kondisyon skoruna göre NAb titresi ve CRP düzeyinin belirlenmesi*

Groups	BCS		Variables	BCS		P
	≤2.5 (n)	>2.5 (n)		≤2.5	>2.5	
Control	14	15	NAb	5.26±0.20	5.46±0.24	>0.05
			CRP (µg/mL)	41.51±4.09	55.76±6.15	>0.05
Mild Endometritis	10	11	NAb	7.20±0.20	7.64±0.22	>0.05
			CRP (µg/mL)	83.00±6.44	82.74±5.99	>0.05
Endometritis	8	9	NAb	8.33±0.28	8.76±0.24	>0.05
			CRP (µg/mL)	101.33±13.56	96.67±10.49	>0.05
Severe Endometritis	8	2	NAb	9.61±0.27	9.75±1.05	>0.05
			CRP (µg/mL)	131.99±13.66	83.75±13.75	>0.05

n *= cows number,* ***BCS*** *= Body condition score*

Table 2. *Determination of NAb titer and CRP level based on parity*
Tablo 2. *Doğum sayısına göre NAb tiresi ve CRP düzeyinin belirlenmesi*

Groups	Primiparous (n)	Multiparous (n)	Variables	Parity		P
				Primiparous	Multiparous	
Control	16	13	NAb	5.48±0.22	5.22±0.21	>0.05
			CRP (µg/mL)	45.53±4.80	53.00±6.46	>0.05
Mild Endometritis	12	9	NAb	7.17±0.2*	7.78±0.20*	0.043*
			CRP (µg/mL)	77.46±5.09	90.06±6.92	>0.05
Endometritis	9	8	NAb	8.63±0.31	8.46±0.20	>0.05
			CRP (µg/mL)	96.26±9.47	101.79±14.47	>0.05
Severe Endometritis	4	6	NAb	9.60±0.50	8.74±0.52	>0.05
			CRP (µg/mL)	133.3±18	115.03±18.13	>0.05

n *= cows number, * istatistically significant*

BCS >2.5), no statistically significant difference was observed in serum NAb titer and CRP level *(Table 1*; P>0.05)

Serum NAb Binding KLH Titer and CRP Level were Compared According to Parity

The number of primiparous cows in the study was control = 16, ME = 12, E = 9, SE = 4 while the number of multiparous cows was control = 13, ME = 9, E = 8, SE = 6. Serum NAb titer were significantly higher in multiparous cows with ME than in primiparous cows (P<0.05). Although a lower mean serum NAb titer was found in multiparous cows compared to primiparous cows in other groups, no statistically

significant difference was observed (P>0.05). When groups were compared in terms of serum CRP levels, no statistically significant difference was found (P>0.05; *Table 2*).

DISCUSSION

The purpose of this study was to assess serum CRP level and NAb titer according to the degree of endometritis in cows. As is known, the innate defense system serves as a barrier against infections and fights organisms causing the infection. The most important element of this defense system is NAb [29]. Furthermore, NAb is also related to the acquired defense system. It helps clean out pieces of circulating pathogens in order to prevent them from damaging vital organs [18]. Nutrition, environmental conditions and factors related to the cow may individually change the NAb titer. In order to eliminate such differences in the titer of NAb, cows at similar postpartum days, raised under the same conditions of care and feeding were included in the present study. Natural antibodies (IgM, IgG and IgA) are present in the blood serum and do not require any antigenic stimulation [30]. They prevent bacterial load from binding to the uterine endometrium [31] and opsonizes bacteria for phagocytosis [31,32]. They increase the phagocytic capacity of neutrophils by opsonizing microorganisms [15,33]. The titer of NAb binding to KLH in plasma shows the efficiency of the innate humoral defense system [29]. Resistance to disease is reportedly higher in cows with a high titer of NAb [27,34,35]. Machado et al.[27] reported an association between higher circulating titer of serum NAb around parturition and decreased incidence of clinical endometritis. van Knegsel et al.[28] report in their study that an increase in the titer of NAb in milk may indicate a mammary infection. The NAb titer in plasma has been shown to decrease in the peripartum period [36]. The mean titer of NAb binding to KLH in healthy controls on days 27-195 postpartum was 8.5±0.35 [29]. In the present study, the mean titer of NAb binding to KLH in control group was 5.36±0.16. NAb binding KLH titer were higher in groups with endometritis than in control group, and the more severe the endometritis was the higher the NAb titer rose. These results imply that postpartum uterine infections may increase NAb levels in blood. This increase in NAb titer is thought to arise from NAb production by the natural defense system of the body to facilitate phagocytosis by neutrophils.

C-reactive protein is one of the most important elements of the innate defense system playing a role in pathogen response [23]. CRP is an important acute phase protein in humans [37], dogs [38,39], horses [40] and pigs [41]. Since peripheral CRP level changes minimally in cows during inflammation [42] it is not generally considered an acute phase protein for cows [42,43]. However, some studies have shown that infections increased serum CRP levels in cows [44,45]. A lower level of CRP is reported in cows raised under the best conditions and management methods compared to other cows. It is known that the potential for diseases is higher under poor conditions. In this respect, it is thought that there is a relation between health conditions in dairy farming and CRP levels [45]. The increase in CRP levels are reported to be useful in the diagnosis of mastitis [44]. The mean CRP level was found to be 39.8±47.5 µg/mL in healthy cows [46]. In an another study was found 22.4 ng/mL in CRP level of healthy cows [44]. Li et al.[47] reported in their study that serum CRP levels were significantly higher in cows with clinical endometritis on day 28 postpartum (262.47±8.69 µg/mL) than in healthy cows (1.39±0.04 µg/mL). Consistent with the findings of Li et al.[47], our study found that serum CRP levels were higher in groups with endometritis than in control group (48.88±3.92 µg/mL). CRP levels in the SE group were approximately 3 times higher than in the control group. This difference in CRP levels suggests that serum CRP levels are sensitive to uterine infections and can be a significant indicator in the diagnosis of endometritis.

It is known that the energy balance in animals cannot be measured directly and that BCS is the most important factor showing the amount of food consumed. The negative energy balance (NEBAL) resulting from the reduction in food intake and the start of lactation causes BCS to decline [48,49]. The increase in NEBAL and NEBAL-associated variables in the postpartum period affects innate cellular and humoral activities. The titer of NAb binding to KLH is negatively related to the plasma non-esterified fatty acid (NEFA) concentration increased in NEBAL. This indicates a significant relationship between metabolic health and plasma NAb titer [28]. Similarly, a study conducted by Lee et al.[45] showed a significant relation between serum CRP levels and BCS (serum CRP level is higher in cows with a low BCS than in other cows). However, since cows were classified according to their BCS in the present study (BCS≤2.5 and BCS>2.5), no significant difference could be detected in the serum NAb titer and CRP level. Absence of a difference is thought to be due to the fact that the cows inlcuded in the study had similar BCS (generally between 2.50 and 3.25).

There are studies which report that parity raises NAb binding to KLH [36] but that it has no effect on serum CRP levels [45]. The increase of NAb titer depend on parity in cows corresponds with the that exogenous stimuli enhance the NAb formation [50]. In this study, NAb titer were found to be significantly higher only in multiparous cows in the ME group compared to primiparous cows (P<0.05). Parity had no effect on serum NAb titer in the other groups. Smilarly, parity had no effect on serum CRP levels (P>0.05).

In conclusion, serum NAb titer increased significantly in uterine infections, and the severity of endometritis was significant in this increase. Furthermore, it was determined that CRP, which has been shown to change only minimally in inflammatory disease in cows, increased significantly in uterus infections. It was determined that BCS did not

change serum NAb titer and CRP levels. It is thought that serum NAb titer and CRP level can be effectively used as an indicator for detecting uterus infections in cows. Future studies should confirm these results and shed light on the value of serum NAb titer and CRP levels in evaluating of uterus health.

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Research Article

Immune Cell Counts, Plasma Immunoglobulin Contents and INF-γ Gene Expression in Rats Exposed to Bisphenol A

Amir ELHAMI [1] Ali Asghar SADEGHI [1]

[1] Department of Animal Science, Science and Research Branch, Islamic Azad University, Tehran, IRAN

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Abstract

The purpose of this study was to evaluate the effect of low-dose graded of bisphenol A (BPA) on immune cell counts, immunoglobulin contents and gamma-interferon (INF-γ) gene expression in rats. Bisphenol A was injected intraperitoneally to male Wistar rats at doses of 15, 30 and 45 µg/kg body weight for 5 weeks. Rats were anesthetized with diethyl ether and blood samples collected, plasma separated and spleen inserted in liquid nitrogen. Total and differential white blood cells, antibody titers, and gene expression of INF-γ were measured. Plasma malondialdehyde levels increased linearly as BPA doses increased. Bisphenol A at dose of 45 µg/kg body weight resulted in significant increase of plasma cortisol level as compared with other treatments. Red blood cell counts decreased linearly as doses of BPA increased. There were no significant differences among treatment for eosinophil, monocytes and basophil counts. The gene expression of INF-γ increased as doses of BPA increased. Rats received 45 µg BPA/kg body weight had 3.2 folds higher gene expression of INF-γ than the control group. The plasma IgG and IgA levels increased linearly as bisphenol doses increased. There was no significant difference among treatments for plasma IgM level. Based on the results of this study, BPA at low doses may result in increase of immune responses in a dose dependent manner.

Keywords: *bisphenol A, Blood cells counts, INF-γ, Immune response, Rat*

Bisfenol A'ya Maruz Bırakılmış Sıçanlarda İmmun Hücre Sayısı, Plazma İmmunglobulin İçeriği ve INF-γ Gen Ekspresyonu

Özet

Bu çalışmanın amacı, sıçanlarda immun hücre sayısı, immunoglobulin içeriği ve gama-interferon (INF-γ) gen ekspresyonu üzerine bisfenol A'nın (BPA) kademeli düşük doz etkisini değerlendirmekti. Bisfenol A Erkek Wistar ratlara 5 hafta boyunca 15, 30 ve 45 ug/kg canlı ağırlık (c.a.) dozlarında intraperitonal olarak enjekte edildi. Sıçanlar dietil eter ile anestetize sonra kan örnekleri toplandı, plazma ayrıldı ve dalak sıvı azot içine konuldu. Toplam ve diferansiyel beyaz kan hücreleri, antikor titreleri, ve INF-γ gen ekspresyonu ölçüldü. Plazma malondialdehit düzeyleri BPA dozları arttıkça doğrusal olarak arttı. Bisfenol A'nın 45 ug/kg c.a. dozu, diğer tedaviler ile karşılaştırıldığında plazma kortizol seviyesinde önemli bir artışla sonuçlandı. BPA dozları arttıkça kırmızı kan hücresi sayımları da doğrusal azaldı. Tedavi grupları arasında eozinofil, monosit ve bazofil sayıları yönünden anlamlı bir fark saptanmadı. BPA dozları arttıkça INF-γ gen ekpresyonu da arttı. 45ug/kg c.a. BPA verilen sıçanlar kontrol grubuna göre 3.2 kat daha yüksek INF-γ gen ekpresyonuna sahipti. Plazma IgG ve IgA düzeyleri bisfenol dozları arttıkça doğrusal olarak arttı. Plazma IgM düzeyi için tedavi grupları arasında anlamlı bir fark yoktu. Bu çalışmanın sonuçlarına göre; düşük dozlardaki BPA, doz-bağımlı bir şekilde immun tepkilerin artmasına neden olabilir.

Anahtar sözcükler: *bisfenol A, Kan hücre sayımı, INF-γ, İmmun yanıt, Sıçan*

INTRODUCTION

Bisphenol A (BPA) is a monomer of plastics and epoxy resins that is pervasive in the soil, water and food. Recently, exposure of human and animals to BPA has been increased. The presence of BPA in urine of 95% of human urine samples was reported [1], which indicates that environmental exposure is widespread. In the last two decades an increasing interest can be observed on biological effects of this compound. Most studies on endocrine disrupters including bisphenol A have been focused on reproductive toxicology and carcinogenesis [2-5]. It acts as endocrine disrupter with estrogenic activities in the body. Estrogen has stimulatory effects on humoral immune responses [6,7]. More recent studies demonstrate that estrogen increases the secretion of interferon-γ (IFN-γ) from splenic lymphocytes, which play a major role in regulating the function of all key immune cells [8,9]. Yoshino et al.[10] suggested that

İletişim (Correspondence)
+98 21 44868536
a.sadeghi@srbiau.ac.ir

prenatal exposure to BPA result in the increase of IFN-γ secretion and up-regulation of immune responses. Youn et al.[11] reported that IFN-γ production induced by BPA treatment after it suppressed IL-4 production. It was not clear that these effects occur in mRNA formation of IFN-γ.

In addition to estrogenic activity, BPA could initiate nitrosative and oxidative stress. Rats prenatally exposed to a human-relevant exposure dose of bisphenol showed increased reactive nitrogen species [2,12] and it also increased reactive oxygen species [3,13,14]. Reactive nitrogen species act together with reactive oxygen species to damage cells, causing nitrosative and oxidative stress. This type of stress is one of the factors that cause immune dysfunctions [14].

Therefore, it is of interest to determine whether BPA influences the immune system in a manner similar to estrogen or reduce immune response because of inducing the stress. There are discordances in the literature and some controversy over the resulting about the effect of BPA on immune parameters. Moreover, information about its effect on blood cells counts and expression of immune genes is also scarce. Therefore, the purpose of this study was to evaluate the effect of low range doses of BPA on immune cell counts, immunoglobulin contents and gene expression of INF-γ in rats.

MATERIAL and METHODS

The study was approved by the Ethics Committee of Islamic Azad University, Science and Research Branch (approval date: 30.06.2014; no: 93530).

Chemicals

Bisphenol A was purchased from Sigma Chemical Company (CAS Registry No. 80-05-7). BPA was dissolved in 5% ethanol solution. Malondialdehyde kit was also provided by Pars Azmoon Company (Tehran, Iran). Immunoglobolins kits were provided from Life Diagnostics Inc. (West Chester, PA, USA). Cortisol ELISA Kit was provided from Mono Bind Company (Lake Forest, CA, USA). All other materials were of analytical grade, obtained from standard sources.

Animals and Experimental Design

Twenty male Wistar albino rats (50-55 g body weight) were purchased from the Razi Institute (Karaj, Iran). The animals were housed in polycarbonate cages, fed a standard laboratory diet and water *ad libitum*. Rats were exposed to a 12 h light/dark cycle, and maintained at 20±2°C. After one week of acclimatization to the animal house, rats were weighted and randomly divided into four experimental groups (5 rats in each). The first group served as control group and was injected 5% ethanol solution intraperitoneally. Rats of the second, third and fourth groups received BPA at doses of 15, 30 and 45 µg/kg body weight four times per week for 5 weeks. The doses of bisphenol were calculated according to the animal's body weight before each injection.

Blood Sampling and Measurements

At the end of experiment, rats (weight, 170 g; age, 8 weeks) were anesthetized with diethyl ether and blood samples were collected into vacuum tubes from heart. Immediately after collection, 500 µL of blood were transferred to micro-tube containing 100 µL sodium citrate solutions (3.85 mg/100 µL) and immediately mixed. These tubes transferred to laboratory (Kharazmi Lab., Tehran, Iran) for counting red blood cells and total and differential white blood cells. Remainder of blood sample was transferred to glass tubes containing heparin and centrifuged at 1500 × *g* for 15 min. Plasma was obtained and stored at -20°C until analyses of malondialdehyde (MDA), cortisol and antibody titers.

Plasma malondialdehyde level was determined using commercial kit (Pars Azmoon, Tehran, Iran) based on method described by Dropper et al.[15]. Red blood cells, total and differential white blood cells counts were done in hemocytometer (T-890, Culter, USA). Giemsa-stained blood films were used for differential white blood cell (WBC) counts.

The rat IgG, IgA and IgM ELISA kit (Life Diagnostics Inc., PA, USA) was used for measurement of plasma IgG, IgA and IgM. The assay uses goat anti-rat IgG, IgA or IgM for solid phase immobilization and horseradish peroxidase (HRP) conjugated goat anti-rat IgG, IgA or IgM antibodies for detection (Life Diagnostics Inc., PA, USA). Plasma cortisol level was measured by rat cortisol EIA Kit (Mono Bind Co., CA, USA).

Quantification of INF-γ Gene Expression

At the end of experiment, spleen were removed and immediately stored in liquid nitrogen for messenger RNA (mRNA) extraction using extraction kit (Vivantis Company, Malaysia). cDNA synthesis was done by reverse transcriptase according to the kit (Vivantis Company, Malaysia). Real time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA).

Statistical Analysis

Collected data were analyzed using completely randomized design using ANOVA procedure of SAS (SAS Institute, Cary, NC). To evaluate the differences between the control and treatments, significant means were analyzed using Duncan's multiple range tests. In all cases, $P \leq 0.05$ were considered significant.

RESULTS

In the present study, BPA was administered intraperitoneally in 5% ethanol solution every forty-eight hours for 5 weeks on male Wistar rats. Body weight of rats

received different doses of BPA did not show significant differences and when compared to the control group.

The effect of different treatments on plasma MDA concentration is shown in *Fig. 1*. Duncan's test showed significant differences among treatments for plasma MDA concentration. The lowest MDA concentration was found in control group and the highest was for rats received 45 µg BPA/kg body weight. Based on orthogonal contrast, MDA concentration increased linearly as doses of bisphenol A increased.

There was no significant difference for plasma cortisol level among rats in control group and those in groups received 15 and 30 µg BPA/kg body weight *(Fig. 2)*. Administration of 45 µg BPA/kg body weight to rats resulted in significant increase of plasma cortisol level as compared with other treatments.

Red blood cell counts decreased linearly as doses of BPA increased *(Table 1)*. The highest counts of white blood cells were observed in rats received 30 µg BPA/kg body weight and the lowest one found in control group. There was no significant difference for white blood cells count among rats received different doses of bisphenol A, but difference among these groups and control group was significant. The highest lymphocyte count was found in rats received 15 µg BPA/kg body weight and the lowest count found in rats received 45 µg BPA/kg body weight. There was significant difference for neutrophil count

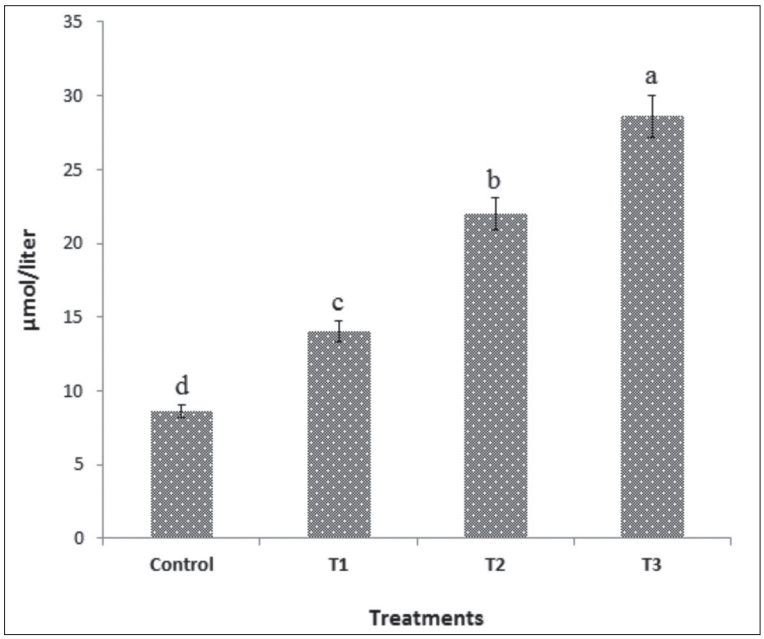

Fig 1. The effect of graded doses of bisphenol A on plasma malondialdehyde level of rats. Control: control group injected i.p. 5% ethanol solution; T1, T2 and T3: injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively

Şekil 1. Sıçanlarda bisfenol A'nın kademeli dozlarının plazma malondialdehit düzeyi üzerine etkisi. Kontrol: %5 i.p. etanol çözeltisi enjekte edilen kontrol grubu; T1, T2, T3: sırası ile 15, 30 ve 45 ug/kg c.a. dozunda bisfenol A enjekte edilmiş

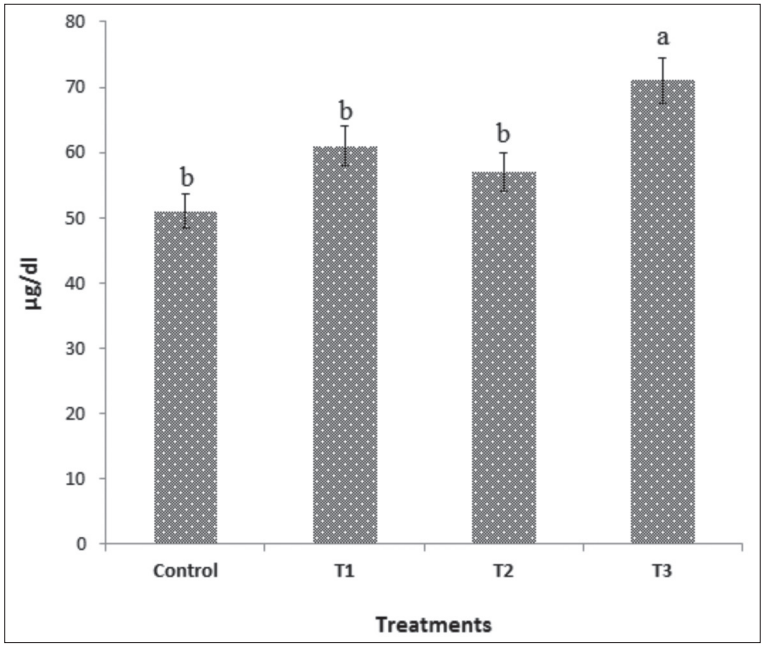

Fig 2. The effect of graded doses of bisphenol A on plasma cortisol level of rats. Control: control group injected ip 5% ethanol solution; T1, T2 and T3: injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively

Şekil 2. Sıçanlarda kademeli bisfenol A dozlarının plazma kortizol seviyesi üzerine etkisi. Kontrol: % 5 i.p. etanol çözeltisi enjekte edilen kontrol grubu; T1, T2, T3: sırası ile 15, 30 ve 45 ug/kg c.a. dozunda bisfenol A enjekte edilmiş

Table 1. *Plasma immunoglobulin contents of rats exposed to graded doses of bisphenol*

Tablo 1. *Bisfenol'ün farklı dozlarına maruz bırakılan sıçanlarda plazma immünoglobulin içeriği*

Treatments*	IgG (mg/L)	IgM (mg/L)	IgA (mg/L)
Control	1113^{c}	788	90^{b}
T1	1565^{b}	831	97^{b}
T2	1752^{b}	846	101^{b}
T3	1980^{a}	855	121^{a}
SEM	117.2	36.8	6.79
Linearity	0.001	0.20	0.002

^{abc} *Means without a common superscript letter differ within each part of a column (P<0.05); * Control: control group injected ip 5% ethanol solution, T1, T2 and T3 injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively*

The gene expression of INF-γ increased as doses of PBA increased *(Fig. 3)*. Rats received 45 µg BPA/kg body weight had 3.2 folds higher gene expression than the control group.

The plasma IgG level increased linearly with increases in BPA doses *(Table 2)*. There was no significant difference among treatments for plasma IgM level. Increase in doses of bisphenol resulted in significant linear increase in plasma IgA level.

DISCUSSION

The reference dose for BPA accepted by the United States Environmental Protection Agency's is 50 µg/kg/day, which is the recommended safe level of exposure. Doses of

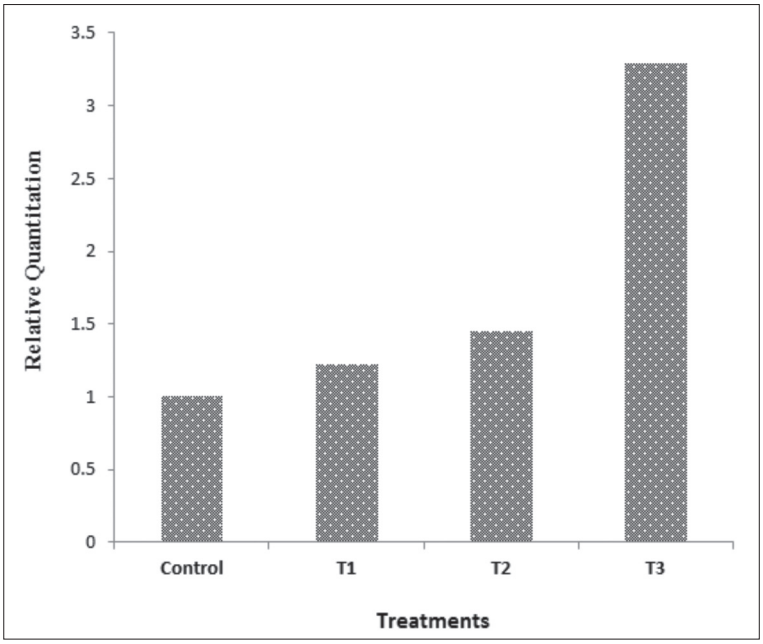

Fig 3. The effect of graded doses of bisphenol A on gene expression of INF-γ. Control: control group injected ip 5% ethanol solution; T1, T2 and T3: injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively

Şekil 3. Bisfenol A'nın kademeli dozlarının NF-y gen ekspresyonu üzerine etkisi. Kontrol: % 5 i.p. etanol çözeltisi enjekte edilen kontrol grubu; T1, T2, T3: sırası ile 15, 30 ve 45 ug/kg c.a. dozunda bisfenol A enjekte edilmiş

Table 2. *Blood cells counts of rats exposed to graded doses of bisphenol A*

Tablo 2. *Kademeli dozlarda bisfenol A'ya maruz bırakılan sıçanların kan hücre sayıları*

Treatments*	RBC ×10^{6}/mm^{3}	WBC ×10^{3}/mm^{3}	Lymph ×10^{3}/mm^{3}	Neutr ×10^{3}/mm^{3}	Eosino ×10^{3}/mm^{3}	Monocyt ×10^{3}/mm^{3}	Basophyl ×10^{3}/mm^{3}
Control	8.0^{a}	3.8^{b}	1.6^{ab}	8.2^{bc}	0.036	0.016	0.007
T1	8.7^{a}	9.8^{ab}	8.6^{a}	5.2^{c}	0.004	0.013	0.007
T2	3.7^{ab}	3.10^{a}	6^{ab}	2.3^{b}	0.032	0.014	0.006
T3	5.6^{b}	7.9^{ab}	6.5^{b}	6.3^{a}	0.029	0.01	0.006
SEM	0.68	0.84	0.42	0.24	0.007	0.007	0.004
Linearity	0.08	0.07	0.053	0.002	0.4	0.8	0.97

^{abc} *Means without a common superscript letter differ within each part of a column (P<0.05); * Control: control group injected ip 5% ethanol solution, T1, T2 and T3 injected 15, 30 and 45 µg bisphenol A/kg body weight, respectively*

.among treatments. The lowest count was for rats received 15 µg BPA/kg body weight and the highest count found in rats received 45 µg BPA/kg body weight. There were no significant differences among treatment for eosinophil, monocytes and basophil counts.

BPA in this study were selected below the reference dose for BPA accepted by this agency and previously tested in a rat study [14]. In the literature, information about the effect of BPA on immune cells and response was scarce, especially effects of important factors such as dose,

duration of exposure and exposure age. Rats used in this study received BPA only for 5 weeks, while humans receive BPA from different sources (metal food cans, water, foods, dental sealants, printed papers, etc.) for a longer period. The main objective of this study was to evaluate the dose effects of BPA on immune cells and response in mature rats (average final weight 170 g, age of 8 weeks) that received BPA from weaning (55 g body weight, age of 5 weeks).

The results of this study show that the levels of MDA increased in dose dependent manner after exposure to BPA. In agreement to our results, a significant increase in the serum MDA level were found in rats exposed to BPA compared to the control group [16]. Also, study of Kourouma et al.[17] showed exposure of rats to BPA resulted in significant increase of liver MDA level. Several studies [2,3,12,13,18,19] demonstrated that exposure to BPA generate reactive oxygen species, but reduce antioxidant content and activity. This condition named oxidative stress. Oxidative stress has proven to be related to BPA toxicity in animal models for years. A study [14] revealed that injection of BPA induces overproduction of hydrogen peroxide in the mouse organs. Hydrogen peroxide is easily converted to hydroxyl radicals. Their results have also revealed decrease in the levels of GSH and increase in the levels of oxidized glutathione by hydroxyl radicals [14]. Therefore, BPA not only increases the free radical formation but also decreases body ability to detoxify reactive oxygen species. So, BPA induces formation of superoxide radicals may cause tissue damage leading to increase in the plasma MDA level.

The plasma level of cortisol increased in group received 45 µg BPA/kg body weight. There are several mechanisms by which BPA disrupts normal endocrine function. BPA can act as a weak estrogen, binding to the estrogen receptor [5] and induce some estrogenic activities. Increases in cortisol level in rats received BPA may be related to estrogenic activity. The study of Edwards and Mills [20] showed that estrogen administration lead to elevated plasma cortisol level. An interesting study [21] demonstrated that bisphenol A, similar to estrogen [22], could increase cortisol production by enhancing phosphorylation of CREB (cAMP response element-binding protein) in normal human adrenocortical cells. Another study [23] showed that BPA could induce corticotropin-releasing hormone expression in the placental cells.

Red blood cells count decreased with increases in doses of BPA. In agreement to our result, Ulutas et al.[24] and Yamasaki and Okuda [25] found that BPA induced a significant decrease in red blood cell count, hemoglobin concentration and packed cell volume. The decrease in the red blood cells may indicate a disruption of erythropoiesis. The administration of estrogens has been known to reduce erythropoiesis in male rats [24]. The present data revealed that BPA induce change in total white blood cells counts or differential count when compared with the control. Inconsistence with our results, Ulutas et al.[24] reported that BPA in rats induce no effect on white blood cells. Increases in reactive oxygen species (as shown by increase in MDA) and also plasma cortisol level of rats received BPA at doses higher than 15 µg BPA/kg body weight may be resulted in decrease of total white blood cells and lymphocyte counts and increase in neutrophil count. BPA changed immune response as it increased gene expression of INF-γ and increased IgG and IgA. In some studies [4,26,27] reported that BPA has multiple actions on patterns of cytokine and antibody production, response to infection, and autoimmune disease progression, T cell subsets, B cell functions, and dendritic cell and macrophage biology. The immunological activities of BPA may be mediated through estrogen receptor signaling, arylhydrocarbon receptor, and the peroxisome proliferator-activated receptor family of nuclear receptors [5]. Estrogen was shown to have immunomodulatory effects, particularly with respect to humoral immunity, and immunosuppressive effects [6,7], particularly with respect to cellular immunity [28]. More recent studies demonstrate that estrogen increases the secretion of IFN-γ from splenic lymphocytes, which play a major role in regulating the function of all key immune cells [8,9]. In a study [28], administration of concanavalin-A, an estrogenic substance, resulted in increase of IFN-γ secretion from thymocytes and splenic lymphocytes. Prenatal exposure to BPA resulted in the increase of IFN-γ secretion and up-regulation of immune responses [10]. Based on report of Youn et al.[11], the production of a strong Th-1 type cytokine (INF-γ) was induced while Th-2 type (IL-4) was suppressed by BPA treatment. Authors [11] suggested that stimulation of prolactin production by estrogenic effects of BPA would affect cytokine profiles, and lead to imbalanced cellular immune response.

In conclusion, bisphenol A could change immune parameters through estrogenic activity and inducing of reactive oxygen species, but its effect on various immune parameters is different. Treatment with BPA decreased count of lymphocytes, but increased IgG, IgA and gene expression of cytokine interferon-γ. Further study is needed to clear the mode of action of bisphenol A on immune parameters.

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Research Article

Evaluation of Survival of *Salmonella* Typhimurium in Homemade Kefir

Filiz YILMAZ AKSU [1] Karlo MURATOĞLU [2] Sema SANDIKÇI ALTUNATMAZ [1]
Deniz AKTARAN BALA [1] Özge ÖZGEN ARUN [2‡]

‡ *Senior Author*

[1] Istanbul University, Faculty of Veterinary Medicine, Vocational High School, Food Processing Department, Food Technology Programme, TR-34320 Avcılar, Istanbul - TURKEY

[2] Istanbul University, Veterinary Faculty, Food Hygiene and Technology Department, TR-34320 Avcılar, Istanbul - TURKEY

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Abstract

Kefir is a fermented probiotic dairy product which is fondly consumed with its peculiar, pleasant taste. However, like all dairy products, kefir may also constitute a risk for pathogenic microorganisms, which is particularly associated with its relatively long fermentation period that requires numerous processing steps. The objective of this study was to investigate the putative growth and risk status of *Salmonella* during the production and shelf life of kefir. For this purpose, kefir samples prepared with *Salmonella* reference culture-spiked milk, were divided into two groups; one was fermented at cold temperatures while the other was left to fermentation at ambient temperature. *Salmonella* enumeration and pH measurements of each group was performed at every 24 hours for six days. According to data obtained, *Salmonella* count has increased to 5.60 log cfu/ml on the first day of incubation at ambient temperature, which then started to decrease with ongoing fermentation. However, the bacteria was still present at the 6th day. Bacterial count of refrigerated kefir did not exhibit a sudden logarithmic rise, reaching its highest value at 2.87 log cfu/ml level and did not reveal a marked drop in comparison to the initial count, which instead sustained at 1.98 log cfu/ml level and thus bacteria survived until the end of the determined shelf life.

Keywords: *Kefir, Salmonella Typhimurium, Foodborne pathogens, Probiotic, Growth potential*

Ev Yapımı Kefirde *Salmonella* Typhimurium'un Canlılığının İncelenmesi

Özet

Kefir, probiyotik özelliği, hoş ve karakteristik lezzeti ile sevilerek tüketilen fermente süt ürünüdür. Buna karşın, tüm süt ürünlerinde olduğu gibi kefir de patojen mikroorganizma gelişimi açısından risk teşkil etmektedir. Özellikle, kefirin çok sayıda işlem gerektiren ve nispeten uzun olan fermantasyon aşaması bu riski akla getirmektedir. Bu çalışmada, önemli patojenlerden olan *Salmonella*'nın kefir üretimi ve raf ömrü boyunca gelişme durumunun ve risk seviyesinin incelenmesi amaçlanmıştır. Bunun için, referans *Salmonella* kültürü ile aşılanan sütlerden hazırlanan kefir örnekleri 2 gruba ayrılarak bir grubu soğuk muhafaza koşullarında, diğeri ortam sıcaklığında fermantasyona bırakılmıştır. Her iki grubun da 6 gün süresince 24 saatte bir *Salmonella* sayılarının tespiti ve pH ölçümleri yapılmıştır. Elde edilen sonuçlara göre ortam sıcaklığında inkübe edilen kefirde *Salmonella* sayısı inkübasyonun 1. gününde 5.60 log kob/ml seviyelerine kadar yükselmiş, daha sonra ilerleyen fermantasyonun da etkisi ile düşmeye başlamış, ancak 6. günün sonuna kadar canlılığını sürdürmüştür. Soğuk muhafaza koşullarında fermente edilen kefir ise ani bir logaritmik artış göstermemiş, en yüksek 2.87 log kob/ml seviyelerine kadar yükselmiş ve başlangıç seviyesine oranla çok belirgin bir düşüş göstermeyip, 1.98 log kob/ml seviyelerinde kalarak, 6. gün sonuna kadar canlılığını sürdürmüştür.

Anahtar sözcükler: *Kefir, Salmonella Typhimurium, Gıda patojenleri, Probiyotik, Gelişim potansiyeli*

INTRODUCTION

Kefir, which is a slightly acidic fermented dairy product, is produced from cow, ewe, goat and mare milk with the addition of kefir grains through in tandem fermentation of ethyl alcohol and lactic acid [1]. According to the Turkish Food Codex and Turkish Fermented Dairy Product Regulation, kefir is defined as fermented dairy product in which kefir

İletişim (Correspondence)
+90 532 5573291
filiz.aksu@istanbul.edu.tr

grains or starter cultures including different strains of specific fermenting bacteria like *Lactobacillus kefiri*, *Leuconostoc*, *Lactococcus* and *Acetobacter* along with lactose-fermenting (*Kluyveromyces marxianus*) or non-fermenting yeasts (*Saccharomyces unisporus*, *Saccharomyces cerevisiae* and *Saccharomyces exiguous)* are used [2]. Kefir grains contain both yeasts and bacteria in their proteinaceous and carbohydrate matrix. Commonly obtained microorganisms from kefir grains are yeasts (*Kluyveromyces, Candida, Torulopsis* and *Saccharomyces* spp), different species of *Lactobacillus* genus *(L. brevis, L. casei, L. kefiri, L. acidophilus, L. plantarum, L. kefiranofaciens subsp. kefiranofaciens, L. kefiranofaciens subsp. kefirgranum, L. parakefir, L. helveticus, L. delbruecki) as well as Streptecocci (Streptecoccus salivari), Lactococcus (Lc. lactis subsp. lactis, Lc. lactis ssp. thermophilus), Leuconostoc (L. mesenteroides*), and *Acetobacter* [1,3]. These lactic acid bacteria and yeasts present in kefir grains function in fermentation of milk by producing numerous metabolites like acetic acid, lactic acid, CO_2, bacteriocin, low concentration of alcohol and aromatic substances (acetaldehyde, acetone, diacetil), which constitute the probiotic properties of kefir as a beverage [4]. These materials produced during the fermentation of kefir and other probiotic foods show inhibitory activity against growth of pathogenic bacteria like *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp. by preventing the invasion of these pathogens into the intestinal epithelium [5,6]. Kefir provides health-promoting effects and support immune system since it contains metabolites of beneficial microorganisms as well as all of the nutrients of milk [6]. Along with its health benefits, kefir is known to exhibit prophylactic and therapeutic effects in miscellaneous diseases. Kefir and kefir grains were demonstrated to have antibacterial, antifungal, antitumoral, hypocholesterolemic and immunomodulatory effects by both in vivo and in vitro studies [7].

Salmonella is a commonly pronounced microorganism included among most important pathogens responsible for food-borne poisoning. Prevalence of salmonellosis has increased in different regions of the world since mid-1980. Salmonellosis was diagnosed in 91034 people in Europe in 2002. In USA, incidence of salmonellosis was reported to be 1 million people per year, 19000 cases of which required hospitalization while 380 people died [8]. Prevalence of this disease may be associated with the acid tolerance response of *Salmonella* Typhimurium as well as its increased adaptation capability to stress factors such as heat, salt and organic acids [9-11].

Pasteurized cow milk or grayish white kefir grains are commonly used in the production of kefir. Recent public belief in homemade probiotics as being healthier products has popularized their homemade production. As a matter of course, no standardization or quality criteria are available with respect to the properties of raw material, production conditions, type of fermentation process (at room temperature or refrigerating) and shelf-life period in homemade kefir. Insufficiency in the pasteurization of milk as the raw material, overall inefficiency in performing hygienic tasks including the disinfection of equipment and utensils, filtration process of kefir yeasts and frequency of bare hand contact increase the risks for contamination. Nevertheless, even though commercially available kefir products are produced under relatively optimized industrial conditions, studies regarding the microbiological quality of the product revealed Enterococcus, Enterobacter, coliform bacteria, fecal coliforms and *E. coli* contamination [12,13].

The objective of this study was to investigate the growth potential of *Salmonella* in fermented kefir at different temperatures.

MATERIAL and METHODS

Single use natural kefir culture was used in kefir production (Danem Milk and Dairy Products Ltd.Co/Isparta). Ultra high temperature (UHT) processed, 3% fat milk (manufactured by Pınar Company) was used in the kefir production. The kefir was spiked with certificated reference culture (ATCC 14028) of *Salmonella* Typhimurium.

Preparation of Stock Culture

Lyophilized culture was initially activated according to the manufacturer's instructions and inoculated on Plate Count Agar (PCA, Merck 1.05463) to give single colonies. This stock cultures on PCA were stored at 4°C until used.

Enumeration of Salmonella in the Inoculum

To prepare a working culture solution was a single colony of *Salmonella* stock culture was transferred in to 5 ml BHIB (Brain Heart Infusion Broth, Oxoid CM1135) tube and incubated at 35°C for 18 h. The colony count in the culture solution was estimated by MacFarland densitometry (Biosan, 050102-11080341). For the enumeration of absolute *Salmonella* count, serial dilutions and plating on PCA were performed from this working culture and then incubated at 35°C for 48 h.

Preparation of Kefir Culture

For the ambient temperature fermented samples, single use kefir culture was directly added to the milk samples without prior processing. Preliminary trials showed that kefir culture was not satisfactorily activated at cold temperature; therefore it was subjected to pre-activation process in 100 ml milk (6 h at 25°C) and then added to 1 liter of milk for cold temperature fermentation.

Inoculation of the Samples with Salmonella and Incubation

The experimental design included three groups: ambient temperature fermentation group (AT group),

cold temperature fermentation group (CT group) and the control group (ATC and CTC). Milk samples with kefir grains were not inoculated with *Salmonella* consisted the control group. All of the experimental samples apart from control groups were contaminated with 100 cfu/ml *Salmonella* bacteria. All groups were subdived in to 2 parallel subgroups. After inoculation, AT group and the control samples were left at 25°C for 48 h for fermentation in an incubator (Nüve-EN 400) and then taken in to cold storage (5±1°C, Siemens). CT group and its control samples were directly left to fermentation at 5±1°C. Microbiological analyses and pH measurements were performed every 24 h during 6 days. The whole experimental procedure was repeated 3 times at different time intervals.

Microbiological Analyses

Two parallel microbiological analyses were performed for each sample and mean of these parallel results were used for data analysis. In addition, natural microbial load of the raw material (milk) was determined by enumeration of total mesophilic aerobic bacteria and detection of *Salmonella* [14,15]. Control samples were analyzed for coliform bacteria, *E. coli*, and *Salmonella* counts [15,16].

For the enumeration of *Salmonella* in spiked samples, 25 ml sample was homogenized in 225 ml 0.1% peptone water to make a 1:10 dilution. Further, serial dilutions were carried out also with 0.1% peptone water. The relevant diluted solutions were spread on selective media (XLD, Merck 1.05287 and SS, Merck 1.07667) and incubated at 35°C for 24±3 h and typical colonies were counted [15,17]. Furthermore, all samples were subjected to enrichment process to detect the presence of viable *Salmonella* below countable limit [15].

pH Measurement

pH values of the samples were measured by a pH meter (Hanna HI 2211-02) every 24 h during 6 days in two experimental (AT and CT) and in the control groups (ATC and CTC) [18].

RESULTS

In this study kefir samples were spiked with *Salmonella* and left for fermentation at two different conditions (cold and ambient temperature) and the *Salmonella* load of these samples were enumerated every 24 h for 6 days. According to the results of this study the *Salmonella* count of AT group was 2.40 log cfu/ml in the first day and decreased to 1.00 log cfu/ml at 6 day after making a peak at first 24 h and reached to 5.60 log cfu/ml. In CT group *Salmonella* count was almost steady and only increased to 2.87 log cfu/ml from 2.49 log cfu/ml at the first 24 h and it has decreased to 1.98 log cfu/ml on the last day *(Table 1, Fig. 1)*.

Microbiological analyses of raw milk did not reveal the presence of *Salmonella* and a total count of mesophilic aerobic bacteria was not determined. Likewise, *Salmonella* was not detected in the control samples (ATC and CTC) of each experimental group.

pH measurements were also carried out in *Salmonella*-

Table 1. *Growth of Salmonella Typhimurium in kefir samples of AT and CT groups (log cfu/ml)*
Tablo 1. *AT ve CT gruplarındaki kefirde Salmonella Typhimurium'un gelişimi (log kob/ml)*

Group	***Salmonella* Typhimurium Count**						
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
AT	2.40	5.60	4.00	2.50	1.00	1.60	1.00
CT	2.49	2.87	2.74	2.37	2.16	2.29	1.98

AT: *Kefir group fermented at ambient temperature (Trials: mean values of AT1. AT2. AT3),* ***CT:*** *Kefir group fermented at cold temperature (Trials: mean values of CT1. CT2. CT3)*

Fig 1. Changes in *Salmonella* Typhimurium quantity in AT and CT groups (log cfu/ml)

Şekil 1. AT ve CT gruplarında *Salmonella* Typhimurium'un değişimi (log kob/ml)

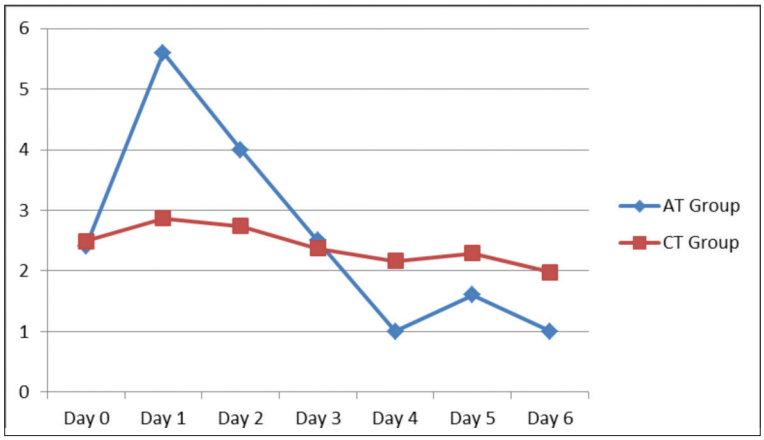

Table 2. *Changes in pH values of kefir samples in AT and CT groups*
Tablo 2. *AT ve CT gruplarındaki kefirde pH değerinin değişimi*

Group	pH						
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
AT	6.78	4.48	4.24	4.28	4.30	4.28	4.31
CT	6.78	5.73	5.54	5.46	5.35	5.23	5.13

AT: *Kefir group fermented at ambient temperature (Trials: mean values of AT1. AT2. AT3),* ***CT:*** *Kefir group fermented at cold temperature (Trials: mean values of CT1. CT2. CT3)*

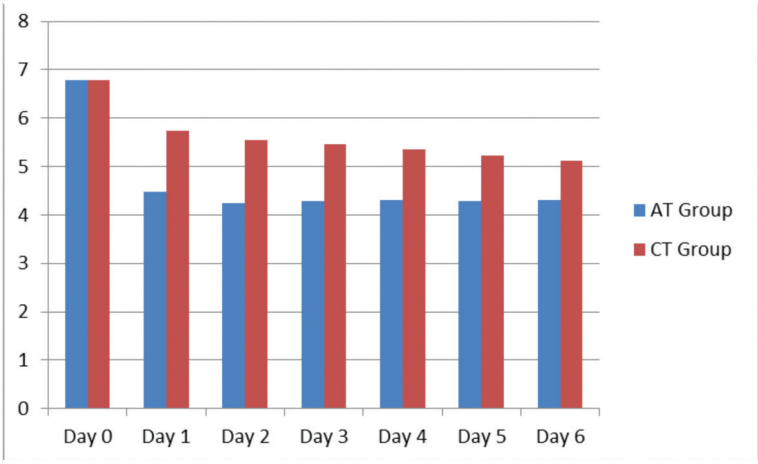

Fig 2. View of changes in pH levels in AT and CT groups

Şekil 2. AT ve CT gruplarında pH'ın değişimi

inoculated kefir at both fermentation temperatures during the whole procedure. According to the results, initial pH value of 6.78 in AT group decreased to a 4.31 level at the end of day 6 whereas that of CT group 5.13 at the end of the procedure *(Table 2, Fig. 2)*.

To monitor the unintentional contamination of samples during experimental kefir production, control samples (ATC and CTC) detected for *Salmonella* in parallel to the spiked samples. No positive detection was observed with these samples. pH measurement results of the control groups did not indicate, in comparison to the *Salmonella* contaminated groups.

DISCUSSION

According to the results of this study the growth rate of *Salmonella* was different in AT and CT groups during the first 24 h. While *Salmonella* count of AT group significantly raised from 2.40 log cfu/ml to 5.60 log cfu/ml level, CT group showed a slight change from 2.49 log cfu/ml to 2.87 log cfu/ml during the same period. Growth potential of *Salmonella* during fermentation kefir samples was previously investigated in some other studies aswell [3,6,19]. Karagozlu et al.[6] investigated the growth potential of some pathogens like *E. coli* O157:H7, *Salmonella* Typhimurium and *S. aureus* during the fermentation process of kefir at 23±1°C for 24 h. Their results showed that *Salmonella* count has increased to 4.64±0.67 log cfu/ml from 2.37±0.20 log cfu/ml at 10^2 cfu/ml *Salmonella* Typhimurium spiked samples while it has increased to 5.60±0.10 log cfu/ml from 3.52±0.07 log cfu/ml in 10^3 *Salmonella*-spiked samples at first 24 h. Our findings are mostly compatible with those of this study. Dias et al.[3] in a similar study evaluated the growth potential of *E. coli* O157:H7, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Staphylococcus aureus* and *Listeria monocytogenes* in contaminated kefir samples fermented at 20°C for 72 h, assuring a final concentration of 10^3 cfu/ml levels. According to their findings both *Salmonella* Typhimurium and *Salmonella* Enteritidis survived after 24 h. In another study by Gulmez and Guven [19] after a 24-h fermenting period (28±1°C, 20-24 h), pathogenic load of kefir regarding *E. coli* O157:H7, *L. monocytogenes* 4b and *Yersinia enterocolitica* O3 increased from 4.68±0.9 log cfu/ml to 5.32±1.1 log cfu/ml; from 4.32±0.80 log cfu/ml to 7.7±0.6 log cfu/ml and from 6.24±1.0 log cfu/ml to 7.03±1.1 log cfu/ml, respectively. In accordance with our findings and those of similar studies, when kefir is fermented at ambient temperature pathogenic microorganisms in the flora exhibits a logarithmic rise of statistical significance during the first 24-28 h [3,6,19]. In our study further fermentation after 48 h resulted with a slight decrease in *Salmonella* count in CT group, whereas it significantly dropped in AT group and at the end of the fermentation process of 6 days, AT group samples contained approximately 1.00 log cfu/ml lower *Salmonella* count than CT. Nevertheless, *Salmonella* survived at each incubation temperature till the end of determined shelf life. Likewise, Gulmez and Guven [19] demonstrated in a study that pathogenic agents in modified kefir and in pasteurized modified kefir samples survived until the 21st day of the trial with low temperature preservation. These

findings in accordance with our results proved that most pathogenic microorganisms had the capability to adapt to a changing environment like lactic acid fermentation and cold storage conditions and thus survived during the shelf-life of the product [6,8,19-21]. In contrast with our study, Dias et al.[3] did not isolate *Salmonella* in kefir samples even after 48 h of fermentation which might readily be associated with the one week enrichment process of the kefir grains they used, which eventually resulted a stronger competitive flora.

In our study, changes in pH levels were also monitored in AT and CT groups every 24 h for a 6-day period *(Table 2, Fig. 2)*. In AT group, initial pH value of 6.78 decreased to 4.48 after 24 h. This sudden change in pH level only 24 h of fermentation was consistent with the findings of several other researches [3,6,22]. Survival of *Salmonella* at such an acidic condition was considered to be associated with the acid resistance nature of the bacteria [23,24]. pH level in AT group did not show a marked change from day 2 to 6 and reached to a value of 4.3 which could be explained by the fact that growth of lactic acid bacteria started to decline in kefir matrix depending on time [22].

Initial pH value of 6.78 in CT group decreased to the levels of 5.73 in the first 24 h and 5.13 on the last day of the experiment, respectively *(Table 2, Fig. 2)*. The insignificant change in pH levels in this group could be explained by the limited activation capacity of lactic acid bacteria in kefir culture. Kefir culture contains lactic acid bacteria as well as yeasts [25]. If fermentation has occurred at low temperature, yeasts exhibit predominance in kefir flora hence alcohol fermentation develops [22].

Despite being a favorable probiotic beverage of recent years, kefir may constitute a risk for public health in case of contamination either in homemade or by industrial manufacturing. On the basis of our findings, *Salmonella* Typhimurium survived for at least six days in kefir samples fermented both at ambient and cold temperatures. Moreover, if we take into consideration that homemade kefir is usually consumed after 48-72th h of processing it is very like to expose to higher count of bacteria when fermented at ambient temperature. Therefore milk to be used as raw material should be pasteurized and utmost care should be taken in performing hygienic tasks regarding equipments and utensils, staff and the kefir culture to be used.

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Research Article

Evaluation of Age Releated Anti-Müllerian Hormone Variations in Domestic Cat

İlknur PİR YAĞCI [1] İbrahim Mert POLAT [1] Mert PEKCAN [2]

[1] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Kırıkkale University, TR-71450 Kırıkkale - TURKEY
[2] Department of Biochemistry, Faculty of Veterinary Medicine, Ankara University, TR-06110 Ankara - TURKEY

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Abstract

Anti-Müllerian hormone (AMH) belongs to the Transforming Growth Factor-ß (TGF-ß) family and produces only in ovaries by the granulosa cells of growing follicles in females. Therefore the measurement of AMH levels plays an important role for the detection of the presence or absence of the ovaries. The aim of this study was to evaluate the individual AMH levels before and after ovariohysterectomy (OHE), to demonstrate mean AMH alterations in the absence of ovaries with blood sampling in different days after OHE, to demonstrate the age-related changes in serum AMH levels and investigate the usability of serum AMH levels as a diagnostic tool for detecting the presence of functional ovarian tissue in domestic cats. Totally 30 domestic cats were used for this study. The animals were allocated into 2 groups according to their ages; <1 year old (Group 1, n=13) and >1 year old (Group 2, n=17). Mean serum AMH concentrations of all cats at day 0 and day 3rd were found 3.15±2.25 ng/mL and 0.38±0.21 ng/mL respectively. Ten days after surgery, AMH levels of all spayed cats were below the minimum detectable concentration of the ELISA kit (<0.08 ng/mL). According to the age classification, the age of cats were correlated with AMH levels at day 0 (r_s=0.293, P<0.05) and at day 3 (r_s=0.410, P<0.01). It can be concluded that measurement of serum AMH concentrations is adequate for assessing the functional ovarian tissue in cats.

Keywords: *Anti-Müllerian hormone, Cat, Ovariohysterectomy, Intact*

Evcil Kedilerde Yaş İlişkili Anti-Müllerian Hormon Değişimlerinin Değerlendirilmesi

Özet

Anti-Müllerian hormon (AMH) Transforming Growth Factor Beta ailesinin bir üyesidir ve dişilerde sadece ovaryumlardaki büyümekte olan granüloza hücrelerinden üretilir. Bu nedenle, ovaryumların varlığını ya da yokluğunu belirlemede AMH ölçümleri önemli rol oynamaktadır. Bu çalışmanın amacı evcil kedilerde ovariohisterektomi (OHE) öncesi ve sonrası bireysel AMH düzeylerinin değerlendirilmesi, OHE sonrası farklı günlerde alınan kan örneklerinde ortalama AMH değişimlerinin gösterilmesi, yaşa bağlı AMH düzeylerindeki değişimlerin belirlenmesi ve AMH düzeylerinin fonksiyonel ovaryum dokusunun belirlenmesinde bir tanı aracı olarak kullanılabilirliğinin araştırılmasıdır. Bu çalışmada toplam 30 evcil kedi kullanıldı. Hayvanlar yaşlarına göre iki gruba ayrıldı; <1 yaş (Grup 1, n=13), >1 yaş (Grup 2, n=17). Tüm kedilerin serum AMH konsantrasyonları 0 ve 3. günlerde sırasıyla 3.15±2.25 ng/mL ve 0.38±0.21 ng/mL olarak belirlendi. Operasyondan 10 gün sonra tüm kısırlaştırılmış kedilerin AMH düzeyleri ELISA kitinin ölçebildiği minimum değerden daha düşüktü (<0.08 ng/mL). Yaş sınıflandırmasına göre ise kedilerin yaşları ile AMH düzeyleri arasında 0. gün (r_s=0.293, P<0.05) ve 3. günde(r_s=0.410, P<0.01) korelasyon belirlendi. Sonuç olarak kedilerde fonksiyonel ovaryum dokusunun değerlendirilmesinde serum AMH konsantrasyonlarının ölçümü yeterli olacağı düşünüldü.

Anahtar sözcükler: *Anti-Müllerian hormon, Kedi, Ovariohisterektomi, Kısırlaştırılmamış*

INTRODUCTION

The female cat is classified as a seasonally polyestrous carnivore having an induced ovulation process after with vaginal stimulation or mating. Puberty depends on body weight (2.3-2.5 kg) and the photoperiod (14 hours bright light by day). Therefore in most cats, pubertal estrous observed at 4-12 months of age but young cats (<1 age) may also have irregular estrous cycles. Overt behavioral sings of estrus may not be observed in all cats even the cat is in the mating season. Consequently, the owners may not identify the estrus signs of young cats. Cats have a long reproductive life and optimal breeding age is between 1.5-7 years [1,2].

İletişim (Correspondence)
+90 318 3574242/6068
ilknurpiryagci@gmail.com

Anti-Müllerian hormone (AMH) belongs to the Transforming Growth Factor-ß (TGF-ß) family and plays an important role in male sex differentiation with testosterone and insulin like growth factor-3 [3] and in females, it is known as a marker of ovarian follicular reserve and ovarian aging, tumor marker or tumor inhibitor and have roles in follicle development and oocyte maturation [4,5]. AMH have an inhibitory function in the antral follicle growth [4]. In females, AMH is produced only in ovaries by the granulosa cells of growing follicles. Therefore the measurement of AMH levels plays an important role for the diagnosis the presence or absence of the ovaries which have increasingly gain popularity [6-8].

The diagnosis of the spayed or intact status of female cats with unknown gynecologic history or Ovarian Remnant Syndrome (ORS) is based on history, clinical symptoms of estrus, vaginal cytology (recommended in follicular phase of the estrus cycle), hormonal analysis (estrogen and progesterone analysis with or without hormonal stimulation such as GnRH stimulation test), and exploratory laparotomy [9-11]. In ORS cases, the time period of presenting estrus following OHE may be observed within 12 months or may extend up to 9 years (mean 3 years) [9]. Because of this wide time gap, and the possibility of owning a cat elderly, it may be hard to determine whether the cat is intact or neutered.

Moreover, abdominal ultrasonography can enlighten the determination of the presence of ovaries in cats however its efficacy is depends on the practitioner's skills, the size of ovarian remnant tissue [10] and the stage of the cycle [12]. In recent years, the practicability of serum AMH levels to diagnose the presence of the ovaries in female cats is discussed in some studies [6-8] but additional studies are required to establish the references ranges of serum AMH levels in domestic cat.

The present study was aimed (1) to evaluate the individual AMH levels before and after ovariohysterectomy (OHE), (2) to demonstrate mean AMH alterations in the absence of ovaries with blood sampling in different days after OHE, (3) to demonstrate the age-related changes in serum AMH levels and (4) to investigate the usability of serum AMH levels as a diagnostic tool for detecting the presence of functional ovarian tissue in female domestic cats.

MATERIAL and METHODS

Study Material

This study was conducted in 30 healthy cats (ages ranged from 6 months to 5 years) which were referred for ovariohysterectomy in our clinics. The study was approved by the Kırıkkale University Local Animal Ethical Committee (16/01-16/12). All owners signed informed consequent. OHE sections were performed during mating season at interestrous stages.

In the present study, the animals were divided into 2 groups according to their age; <1 year old (Group 1, 13 cats) and >1 year old (Group 2, 17 cats). According to history, no estrus signs was observed in group 1 (except 4 cats) until OHE, in addition, all animals in group 2 showed at least one distinctive estrus cycle before the surgery.

Collection of blood samples

The blood samples were collected 3 consecutive times from all the animals *cephalic vein* into vacuum tubes. The collection times points were as follows (1) one h before the surgery, day 0, (2) three day after the surgery, day 3 and (3) ten day after the surgery, day 10. All the samples were stored at -20°C until analysis.

AMH analyses

The commercially enzyme-linked immunosorbent assay kit (Beckman Coulter®, AMH Gen II, USA) was used for determination of serum AMH levels according to the manufacturer instructions. The ELISA kit used in this study is based on two site immunoassay, utilizing two monoclonal antibodies of which the secondary antibodies labeled with biotin. Primary antibodies were coated in 96 well plates. All individual reagents (anti AMH biotin conjugate and streptavidin-HRP) and samples or standards are incubated one at a time. Extensive washing cycles were performed after each incubation period. Following final wash plates were incubated with chromogen solution before addition of the stop solution. The dual-wavelength absorbance measurements were taken at 450 and 620 nm.

Statistical Analysis

Data presented as the arithmetic mean and standard error of the mean. The Shapiro-Wilk test was used to control for the distribution of normality of the data. Serum AMH levels of cats before and after ovariohysterectomy was checked with *Wilcoxon* test. Pearson correlation test was used for determination of age and serum AMH interactions. $P<0.05$ was considered to be significant. All analyses were carried out using commercial software (SPSS 14.0).

RESULTS

Mean serum AMH concentrations of Group 1 and 2 were given in *Table 1*.

Mean serum AMH concentrations of all cats without grouping at day 0, day 3rd and day 10th were found 3.15±2.25 ng/mL, 0.38±0.21 ng/mL and non-detectable concentrations, respectively *(Fig. 1)*. There was a sharp decrease of serum AMH levels three days after surgery in all cats. Ten days after surgery, AMH levels of all spayed cats were below the detection limit of the ELISA kit (<0.08 ng/mL).

According to ages of cats, in Group 1 and Group 2, serum AMH levels were found 1.86±1.73 ng/mL and 3.57±2.28

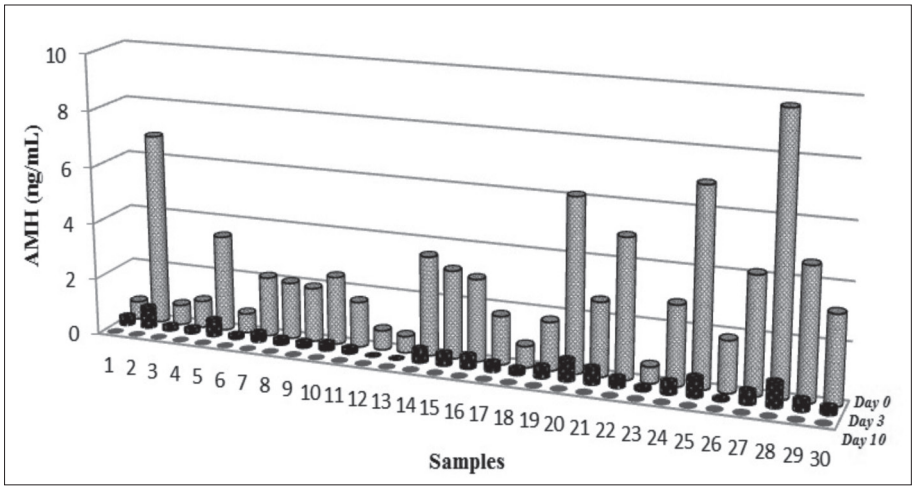
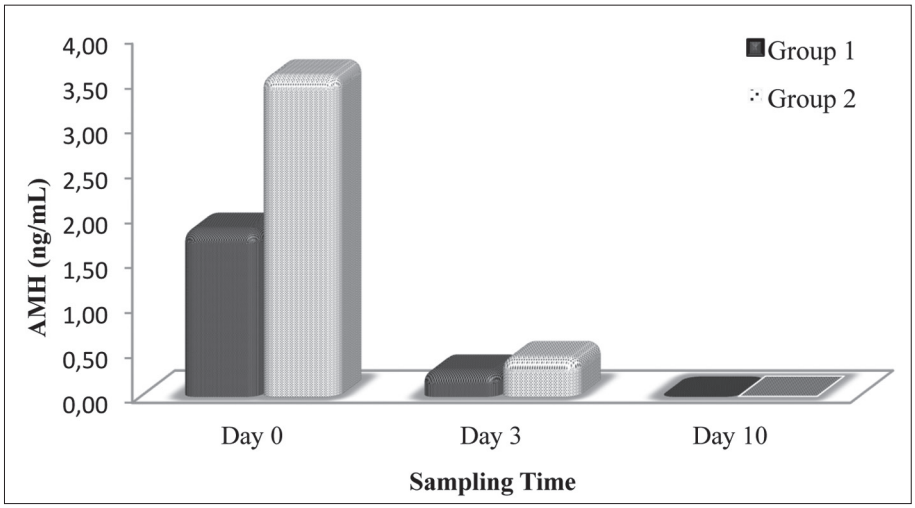


Fig 1. Mean serum AMH concentrations of all cats

Şekil 1. Tüm kedilerin ortalama AMH konsantrasyonları

Fig 2. Mean serum AMH concentrations of study groups at different days

Şekil 2. Çalışma gruplarında farklı günlerde ortalama serum AMH konsantrasyonları



Tablo 1. *Mean serum AMH concentrations of study groups*
Table 1. *Çalışma gruplarında ortalama serum AMH konsantrasyonları*

Groups	Day 0	Day 3	Day 10
Group I (n=13)	1.86±1.73[a] ng/mL	0.26±0.20[b] ng/mL	ND
Group II (n=17)	3.57±2.28[a] ng/mL	0.42±0.20[b] ng/mL	ND

Values are means±S.E.M, ***ND:*** *not detectable in all samples (<0.08 ng/mL).* [a,b] *Within rows, values with different superscripts are significantly different (P<0.01)*

ng/mL in intact cats *(Fig. 2)*, respectively (P<0.01). Serum AMH levels were dramatically decreased in both groups three days after OHE (P>0.05). The age of cats were highly correlated with AMH levels at day 0 (r_s=0.293, P<0.05) and at day 3 (r_s=0.410, P<0.01).

DISCUSSION

Anti Müllerian hormone is secreted from ovaries, especially preantral and antral follicles in woman and most of the domestic mammals. Ovaries are the unique source of this hormone which is an indicator for follicular population and reserve [13]. In cats, it is difficult to determine the ovarian activity via vaginal cytology and endocrinology during the off season. Consequently, some methods are needed to determine the ovarian activity such as ovarian stimulation test which requires multiple sampling and repetitious visiting of the patient [1]. The best way to confirm the functional ovarian tissue is vaginal cytology however in the cat, cytological changes are not distinct and it is better to perform examinations during behavioral estrus [14]. These procedures are laborious, time-consuming and stressful for both owners and patients. Recently, AMH was suggested as an important indicator for the detection of presence of the ovaries in animals with unknown history and diagnosis of ORS cases as well in veterinary medicine. In a previous study [7], serum AMH levels were decreased after OHE in cat and also in bitch [6,8]. In the present study, similar to Axner *et al.*[7] and others [6,8], mean serum AMH levels (3.15 ng/mL) before OHE was dramatically decreased three days after OHE and dropped down to non-detectable concentrations at ten days after OHE in all cats (below 0.08 ng/mL). These findings support the hypothesis that the unique source of AMH is ovaries. Single sampling of AMH measurements is adequate to determine the presence of functional ovarian tissue and diagnose the ORS cases included in this study.

Bristol-Gould and Woodruff [15] reported that; TGF-β signals are present in primordial, primary and secondary follicles in cat ovary. Thus, all of the activation signals are ready for triggering the follicular selection process in domestic cats when they reach the pubertal age. AMH might have an important role in the regulation of follicles mainly in growing process and follicular selection for ovulation [13]. In this study, high serum AMH levels in sexually active cats, (Group 2) could be caused by currently presence of ovarian activity. Similar to Place *et al.*[6] our results showed that cats had higher AMH concentrations when compared to bitches. In addition, the numbers of primordial follicles in ovarian cortex are greater in cats than other domestic mammals [14] might be reflect the high AMH production and secretion in this study. The production and secretion of AMH is independent and it is not affected from the stage of estrus cycle [15] even if sexual cycle variations are present in domestic mammals [16,17]. In a previous report [7], administration of buserelin injections did not affect the serum AMH levels in intact cats whereas the serum estradiol concentration which is a marker of terminal follicular development is altered after injections. To study cyclic variations of serum AMH levels in cats, sampling from higher number of animals are required for demonstration of AMH dynamics. Although, serum AMH concentrations are decreased after OHE in bitches [8] when compared to our results, these levels were non-detectable ten days after OHE (<0.08 ng/mL). This indicates that AMH might be a better indicator for detecting the presence of ovaries in cats than dogs. Moreover, measuring the serum AMH levels is useful for assessing the ovaries in cats with unknown reproductive history as well.

In conclusion, serum AMH levels showed a descent trend three days after OHE and undetectable concentrations were observed ten days after OHE. However there were individual variations of serum AMH levels between age comparison, a single sampling for evaluation of serum AMH concentrations ten days after OHE, appears to be adequate for assessing the functional ovarian tissue in cats with unknown reproductive history or ORS suspected cases.

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Research Article

Differences in the Follicular Morphology of Young and Aged Bitches and Their Correlation with the Anti-Müllerian Hormone

Ömer KORKMAZ [1] Deniz KORKMAZ [2] İbrahim Mert POLAT [3] İlknur PİR YAĞCI [3]
Mert PEKCAN [4] Birten EMRE [1] Abuzer Kafar ZONTURLU [1]

[1] Harran Üniversitesi, Veteriner Fakültesi, Doğum ve Jinekoloji Anabilim Dalı, TR-63200 Eyyubiye, Şanlıurfa - TÜRKİYE
[2] Harran Üniversitesi, Veteriner Fakültesi, Histoloji-Embroyoloji Anabilim Dalı, TR-63200 Eyyubiye, Şanlıurfa - TÜRKİYE
[3] Kırıkkale Üniversitesi, Veteriner Fakültesi, Doğum ve Jinekoloji Anabilim Dalı, TR-71450 Yahşihan, Kırıkkale - TÜRKİYE
[4] Ankara Üniversitesi, Veteriner Fakültesi, Biyokimya Anabilim Dalı, TR-06110 Dışkapı, Ankara - TÜRKİYE

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Abstract

This study aimed to investigate the structural, morphological and cellular differences of follicles at different stages of follicular development as well as to determine the correlation of the ovarian follicle population with serum Anti-Müllerian Hormone (AMH) levels in young and aged bithches. Sixteen bitches were divided into two groups according to their ages. Group A included young bitches aged 2 years (n=8) while Group B constituted of those aged 8 to 10 years (n=8). Diameters of the primordial, primary and preantral follicles were found to be significantly larger in Group B, in comparison to Group A. In Group A, the mean number of granulosa cells was 91.65±2.23 in the secondary follicles and 301.31±4.16 in the preantral follicles. In Group B, the same values were found to be 89.46±2.68 and 270.25±3.54, respectively. The mean serum AMH levels in Group A and Group B were 0.233±0.046 ng/mL and 0.099±0.008 ng/mL, respectively (P<0.05). In conclusion, the results indicated that the number of primordial and primary follicles as well as the numbers of granulosa cells in secondary and preantral follicles decreased with advanced age which resulted in lower serum AMH levels in aged bitches. The results suggested that the AMH, which is used as a fertility parameter in humans, could also be used for the same purpose in dogs.

Keywords: *Bitch, Follicular morphology, Granulosa cell, Anti-Müllerian hormone*

Genç ve Yaşlı Dişi Köpeklerde Foliküler Morfolojinin Farklılıkları ve Anti Müllerien Hormon ile İlişkisi

Özet

Sunulan çalışmada genç ve yaşlı dişi köpeklerde, foliküler gelişimin farklı aşamalarında, foliküllerin yapısal morfolojik ve hücresel farklılıklarının ortaya konması, ovaryum follikül popülasyonunun Anti Müllerien Hormon (AMH) ile ilişkilerinin araştırılması ve bu hormonun genç ve yaşlı köpeklerdeki düzeylerinin belirlenmesi amaçlanmıştır. Çalışma, materyalini 8 adet, 2 yaşında, genç (grup A) ve 8 adet, 8-10 yaş aralığında yaşlı olmak üzere (grup B) toplam 16 dişi köpek oluşturmuştur. Primordial, primer ve preantral folikül çapları grup B de grup A'ya göre daha yüksek ölçülürken sekunder folikül çapları yönünden gruplar arası fark istatistiki olarak önemsiz bulunmuştur. Grup A'da Sekunder foliküllerdeki granuloza hücreleri ortalama 91.65±2.23 adet preantral foliküllerde ise 301.31±4.16 adet sayılmıştır. Grup B'de ise bu değerler sırasıyla 89.46±2.68 ve 270.25±3.54 adet olarak kaydedilmiştir. Çalışmada serum AMH sonuçlarına bakıldığında Grup A' da bu değer ortalama 0.233±0.046 ng/mL tespit edilmiştir. Grup B'de ise bu ortalamanın 0.099±0.008 ng/mL'ye düştüğü gözlemlendi (P<0.05). Sonuç olarak köpeklerde artan yaş ile primordial ve primer folikül sayılarının ve özellikle sekunder ve preantral foliküllerdeki granuloza hücre sayılarının azalması ve AMH hormonunun da buna paralellik göstermesi, insanlarda fertilite parametresi olarak kullanılan AMH hormonunun köpeklerde de kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Köpek, Foliküler morfoloji, Granuloza hücre, Anti Müllerien hormon*

INTRODUCTION

Although bitches reach puberty at the age of 6-14 months, the optimal age range for mating is 2-6 years. The number of ovulatory follicles decrease with age, and the ovarian activity, which declines from 6 years onwards, nearly diminishes by the age of 10. In bitches older than 8 years, the oestrous cycle becomes irregular, fecundation

İletişim (Correspondence)
+90 414 3183859/3914
ahuramazda@harran.edu.tr

and fertility decrease, and the frequency of abortion increases. Therefore, it is suggested that dogs older than 7-8 years should not be used for breeding purposes [1].

Canine ovarian follicles are categorised into 5 classes including primordial, primary, secondary, early antral (pre-antral) and advanced antral (graafian) follicles on the basis of follicle morphology, follicle size, type and number of follicle cell layers, and the presence of follicular fluid [2]. Primordial follicles contain a small oocyte with a mean diameter of 25 µm, which lacks a zona pellucida (ZP) and is lined by a single layer of squamous granulosa cells [3]. By day 120, all primary follicles contain a small, pale oocyte with a distinct ZP, and have a single layer of cuboidal granulosa cells [4]. Secondary follicles are lined by two or more layers of granulosa cells [5]. Early antral follicles are observed with fluid-filled cavities among the granulosa cells [2]. In pre-pubertal bitches, the transition from a primordial follicle to an early antral follicle requires a time period of 70-150 days. Antral follicles are found in bitches at the age of 6 months, and shortly before proestrus [6,7]. Nonetheless, only a limited number of studies are available on folliculogenesis and the ovarian follicle population in dogs.

Granulosa cells, which provide the physical support and microenvironment required for oocyte development, are capable of active differentiation. Differentiation of the granulosa cells is regulated by several hormones and growth factors [8]. Granulosa cells bear specific receptors for several hormones and growth factors such as follicle stimulating hormone (FSH), luteinizing hormone (LH) [9] epidermal growth factor (EGF) [10], insulin-like growth factor (IGF) [11], and Anti-Müllerian hormone (AMH) [12].

The AMH known as the Müllerian inhibitory substance, is a homodimeric glycoprotein hormone belonging to the transforming growth factor-β superfamily [13] and is secreted by the sertoli cells in males [14,15] and by the granulosa cells of the secondary, preantral and small antral follicles in females. By inducing the regression of the Müllerian ducts, the AMH enables foetal sex determination and regulates the development of primordial follicles in females [8,16-18]. In male dogs, AMH is expressed from the time of sexual differentiation to puberty and is responsible for regression of the paramesonephric duct during sexual differentiation [19].

The AMH reduces the sensitivity of growing follicles to the FSH, and thereby, limits the number of actively growing follicles, and also acts in follicular development. In this way, the AMH contributes to the maintenance of oocyte reserves [17,20]. Detection of the AMH level has found common use in women, for determination of the ovarian reserve, which refers to the number of follicles that can be successfully recruited for a possible pregnancy, as well as for the monitoring of transition into menopause, the diagnosis of the polycystic ovary syndrome and granulosa cell tumours, the detection of low ovarian response, and prognosis in *in vitro* fertilization studies [16,20].

This study aimed to investigate the structural, morphological and cellular differences of follicles at different stages of follicular development as well as to determine the correlation of the ovarian follicle population with serum Anti-Müllerian Hormone (AMH) levels in young and aged bitches.

MATERIAL and METHODS

Sixteen dogs, including eight 2-year-old young bitches, which were known to have undergone at least one proestrus bleeding and were referred to the clinic for routine ovario-hysterectomy (Group A), and eight 8 to 10-year-old aged bitches (Group B), constituted the study material. Before operation clinical examinations, a complete blood count, a regular vaginal cytological examination and gynaecological ultrasonography were performed, to ensure that healthy animals with no medical problem and gynaecological pathology were included in the study. This study was approved by the Dollvet Animal Experiments Local Ethics Committee (Aproval number: Dollvet A.Ş. HADYEK 2016/04).

Prior to surgery, blood samples were taken from each animal into dry tubes. After coagulation the blood samples were centrifuged at 3.000 rpm for 15 min, and the collected sera were stored at -20°C until being analysed. The serum AMH levels were measured with the enzyme-linked immunosorbent assay (ELISA) (DRG Instruments Elisa Mat 2000), using a commercial kit (Beckman Coulter, AMH Gen II, USA). All serum assays were performed in duplicate. The minimum detectable concentration of the assay was 0.375 ng/mL. The lower and upper limits of detection were 0.375 ng/mL and 150 ng/mL, respectively. The respective intra and inter assay coefficients of variations were <8% and <10%, respectively. Standard curve ranges were 0.07-22.5 ng/mL. The limit of detection value of the ELISA kit was <0.1 ng/mL.

The ovarian tissues obtained by routine ovariohysterectomy were fixed in 10% formaldehyde for 24 h and then washed. After being passed through a series of graded alcohol and xylol solutions, the tissue samples were embedded in paraffin. For each animal, four serial sections of 5 µm thickness cut from the paraffin blocks were stained with Crossman's modified triple staining technique [21]. The serial sections were examined under an Olympos Cover 018 model research microscope and measured using a Bs200Pro image analysis programme (BAB software).

In order to determine the number of follicles in different stages of the follicular development four sections pertaining to each animal were counted.

The follicles were categorised into five classes according to Songsasen et al.[2] on the basis of the characteristics described below.

1- Primordial follicle: No ZP surrounding the oocyte and the oocyte lined by a single layer of squamous granulosa cells.

2- Primary follicle: The oocyte surrounded by a ZP and lined by a single layer of cuboidal granulosa cells.

3- Secondary follicle: Oocyte lined by two or more layers of granulosa cells.

4- Preantral follicle: Oocytes surrounded by granulosa cells with small cavities (follicular antrum) among the granulosa cells or segmented cavities with two or more compartments.

5- Antral follicle: The formation of a single, large, continuous cavity (antrum).

Follicle diameters were determined as described by Griffin et al.[22], by averaging two measurements, at right angles, at the widest cross-section of the follicles.

The results obtained in the present study were analysed by the Statistical Package for Social Sciences (SPSS) Version 14.01 (Serial number: 9869264). The statistical significance of the differences observed between the groups for the values, which met the parametric assumptions of the test and were determined by measurement, including diameter (primordial, primary, secondary, preantral and antral follicle diameters) and theca folliculi wall length (in the secondary, preantral and antral follicles), was determined with Student's t-test, whereas the significance of the differences for follicle (primordial and primary follicles) and granulosa cell (of secondary and antral follicles) numbers, and quantitative features that did not meet the parametric assumptions of the test, was analysed by the Mann-Whitney U test. Furthermore, the significance of the correlation of the AMH with follicle number, follicle diameter, and granulosa cell number was determined with the Pearson correlation test.

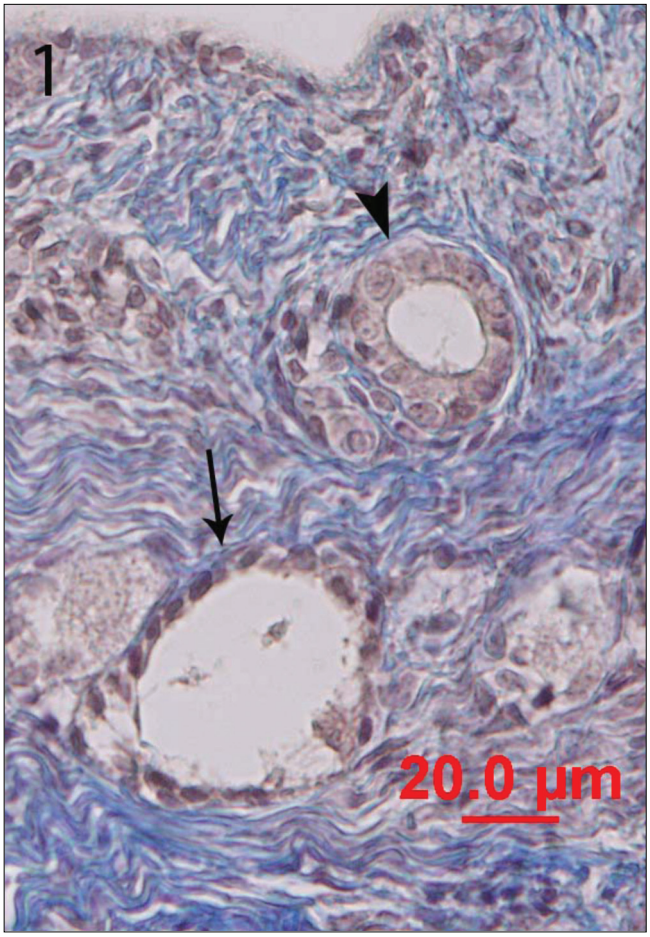


Fig 1. Primordial follicle *(arrow head)* and primary follicle *(arrow)*
Şekil 1. primordial folikül *(ok başı)* ve primer folikül *(ok)*

RESULTS

The diameters of the primordial *(Fig. 1)*, primary *(Fig. 1)*, secondary *(Fig. 2)*, preantral *(Fig. 3)* and antral *(Fig. 4)* follicles and the theca folliculi wall lengths of the secondary, preantral and antral follicles observed in the ovarian sections of the group of young bitches (Group A) and the group of aged bitches (Group B) are presented in *Table 1*.

While the diameters of the primordial, primary and preantral follicles were found to be larger in Group B in comparison to Group A (P<0.001). The difference between the two groups for secondary follicle diameter was found to be statistically insignificant.

The numbers of the primordial and primary follicles and the granulosa cell numbers of the secondary and preantral follicles of both groups are presented in *Table 2*.

In the present study, the numbers of primordial and primary follicles were counted in an area of 1 mm^2 of the histological sections. The mean number of primordial follicles counted in this area was significantly higher in Group A than in Group B (P<0.05). Similarly, the mean number of primary follicles was higher in Group A, when compared to Group B, however, this was non-significant. In Group A, the mean number of granulosa cells averaged 91.65 in the secondary follicles and 301.31 in the preantral follicles. In Group B, both parameters were lower (89.46 vs 270.25). While the number of granulosa cells in the preantral follicles differed significantly between groups (P<0.001), this was not the case in the secondary follicles. The serum AMH concentrations measured in Groups A and B are presented in *Table 3*.

The assessment of the serum AMH concentrations demonstrated that the values averaged 0.166 ng/mL and ranged from 0.092 ng/mL to 0.478 ng/mL for both groups. In Group A, the average serum AMH level was significantly higher than in Group B (P<0.05).

The correlation of the serum AMH levels with the numbers and diameters of the primordial and primary follicles, and the diameters and the number of granulosa cells of the secondary and preantral follicles is presented in *Table 4*.

A significant correlation between serum AMH and the numbers of primordial (r=0.611) as well as primary follicles

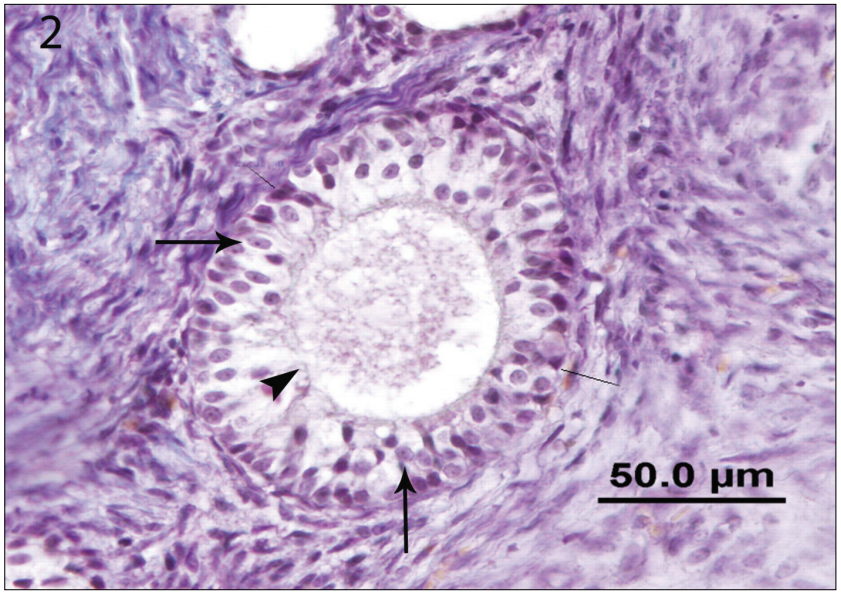
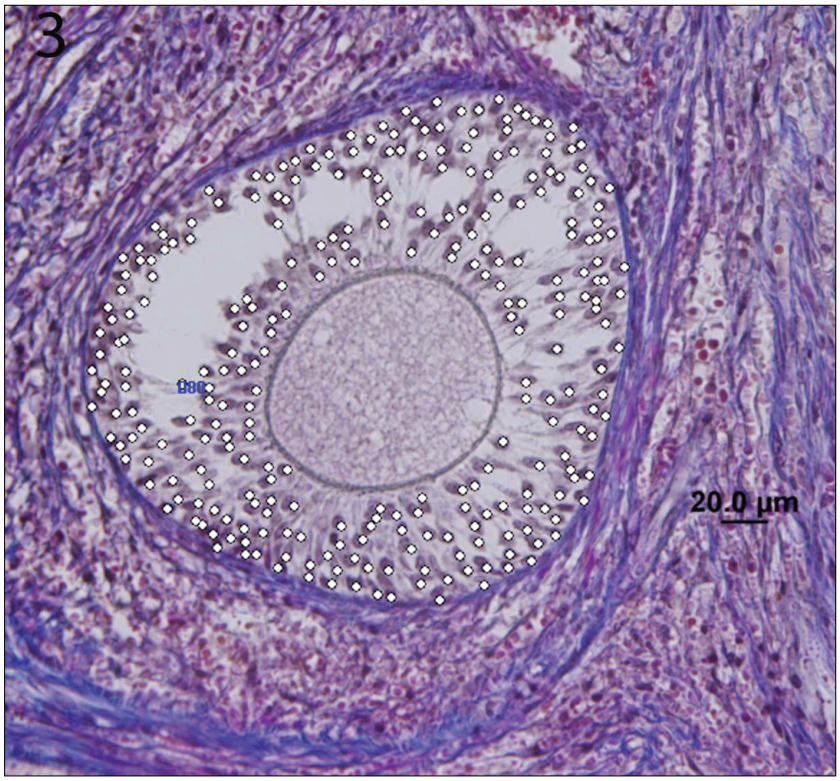


Fig 2. Secondary follicle. *Straight line:* teca follicle wall, *arrow head:* oocyte, *arrows:* granulosa cells

Şekil 2. Sekunder folikül. *İnce çizgi:* teka folikül duvarı, *ok başı:* oosit, *ok:* granuloza hücreler

Fig 3. Preantral follicle. *White circles:* granulosa cells

Şekil 3. Preantral folikül. *Beyaz noktalar:* granuloza hücreler



(r=0.572) was assessed (P<0.05); furthermore, between the serum AMH levels and the diameter of the primordial follicles (r=-0.593) (P<0.05). As the diameter of the primary, secondary and preantral follicles increased, the serum AMH levels decreased, however this correlation was non-significant (P>0.05).

DISCUSSION

In mammals, female individuals are born with the pool of primordial follicles that develop throughout the reproductive life span of the female [8]. Clusters of primordial and primary follicles are referred to as 'egg nest', and are generally observed in mammalian species characterized by the ovulation of more than one oocyte at a time [4]. In the present study, it was observed that the numbers of the primordial and primary follicles were higher in the young bitches. This finding was in agreement with the report of Dolezel et al.[23] who, showed that the total number of follicles was lower in aged bitches, when compared to young bitches.

Studies of Griffin et al.[22] on ovarian follicle diameter in

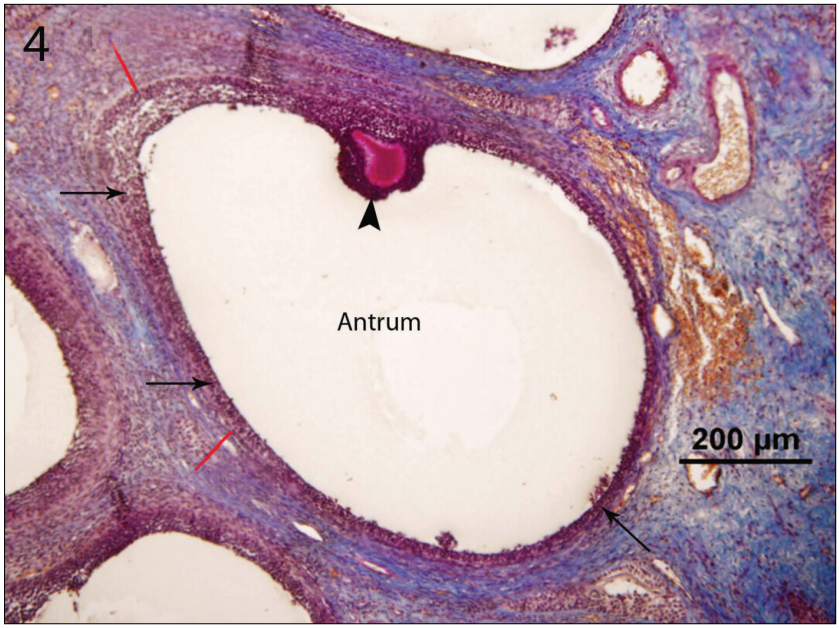


Fig 4. Antral follicle. *Straight line:* teca follicle wall, *arrow head:* corona radiata, *arrows:* granulosa cells

Şekil 4. Antral folikül. *İnce çizgi:* teka folikül duvarı, *ok başı:* korona radiyata, *ok:* granuloza hücreler

Table 1. *Follicle diameters and theca folliculi wall lengths in Group A and Group B*
Tablo 1. *Grup A ve Grup B deki folikül çapları ve teka duvarı uzunlukları*

Diameter or Length	Group A (young)		Group B (aged)		t	P
	n	$\bar{x}\pm_x$ (µm)	N	$\bar{x}\pm_x$ (µm)		
Primordial follicle diameter	80	24.53±0.56	80	30.38±0.44	8.068	***
Primary follicle diameter	80	59.73±0.62	80	67.29±0.49	9.570	***
Secondary follicle diameter	45	137.87±2.64	48	143.71±2.36	1.654	-
Wall length of secondary theca	40	13.15±0.37	48	21.12±0.63	10.427	***
Preantral follicle diameter	19	201.15±5.78	20	234.94±5.89	4.089	***
Wall length of preantral theca	19	34.58±1.85	20	42.58±1.57	3.312	**
Antral follicle diameter	13	367.14±22.51	6	508.88±40.87	3.295	**
Wall length of antral theca	13	92.54±2.99	6	96.01±7.31	0.530	-

*-: P>0.05: non-significant, * P<0.05, ** P<0.01, *** P<0.001*

Table 2. *Number of primordial and primary follicles and granulosa cell numbers of the secondary and preantral follicles in both groups*
Tablo 2. *Her iki gruba ait köpeklerde primordial ve primer follikül sayıları ile sekunder ve preantral folliküllerdeki granuloza hücre sayıları*

Number	Group A (young)			Group B (aged)			P
	n	$\bar{x}\pm_x$	Median	N	$\bar{x}\pm_x$	Median	
Primordial follicle number	8	43.50±3.86	46.50	8	16.57±2.10	14.00	*
Primary follicle number	8	29.88±6.07	22.00	8	15.57±3.54	11.00	-
Granulosa cell number of secondary follicles	48	91.65±2.23	89.50	48	89.46±2.68	90.00	-
Granulosa cell number of preantral follicles	32	301.31±4.16	298.00	32	270.25±3.54	264.00	***

*-: P>0.05: non-significant, * P<0.05, ** P<0.01, *** P<0.001*

mice, hamsters, pigs and humans have demonstrated that differences exist between these four mammalian species. The follicle diameters measured in the present study were found to be close to the follicle diameters measured by Griffin et al.[22] in hamsters. Furthermore, it was determined that the antral follicle diameter of the dog was smaller than that of human and pig, and larger than that of mouse. The differences observed between dogs and other animal species were attributed to the proportional relationship of the body mass index of a given species with its ovarian and follicle diameters. In the present study, it was observed that during the transition from the primordial follicle to the antral follicle, the follicle size displayed a nearly 15-fold increase in young animals, and a 17-fold increase in

Table 3. *Blood serum AMH levels in both groups*
Tablo 3. *Her iki gruba ait köpeklerde kan serum AMH değerleri*

Group	n	$\bar{X}\pm_{\bar{X}}$ (ng/ml)	Z	P
Group A	8	0.233±0.046	-2.941	0.03
Group B	8	0.099±0.008		

Table 4. *Correlation of serum AMH levels with follicle number, follicle diameter, and granulosa cell number*
Tablo 4. *Serum AMH düzeyi ile follikül sayısı, çapları ve granuloza hücre sayıları arasındaki korelasyon*

Number or Diameter	Anti-Müllerian Hormone	
	R	P
Primordial follicle number	0.611	0.016
Primary follicle number	0.572	0.026
Primordial follicle diameter	-0.593	0.015
Primary follicle diameter	-0.464	0.07
Secondary follicle diameter	-0.228	0.397
Granulosa cell number of secondary follicles	0.193	0.473
Preantral follicle diameter	-0.359	0.208
Granulosa cell number of preantral follicles	0.093	0.733

aged animals. The greatest increase in follicle diameter was observed during the transition to the antral follicle, similar to findings previously reported by Songsasen et al.[2].

Diameters of the primary and secondary follicles measured in the young dogs in the present study were found to be similar to those measured by Dolezel et al.[23]. Furthermore diameters of primary and secondary follicles measured in the young dogs in the present study were also similar to those reported by Songsasen et al.[2] while the antral follicle diameters determined in the present study were found to be larger. The follicle diameters measured in the aged dogs were larger than those in the young dogs and those reported in previous studies. The differences between the values observed in the present study and those reported by the researches above can be attributed to the differences in the age of the experimental animals used. The age of the animals included in the study conducted by Songsasen et al.[2] ranged from 5 months to 7 years which support this presumption. Hence, while the follicle diameters measured in the aged dogs were found to be larger than the diameters reported by Songsasen et al.[2], the follicle diameters measured in the young dogs, which were at an age close to that of the animals used by Songsasen et al.[2], were found to be similar to the measurements of these researchers.

In females although AMH is also secreted by the granulosa cells lining the secondary, preantral and small antral follicles [8] the major source of this hormone is the granulosa cells of early antral follicles [16,17]. In the present study, both the granulosa cells number and the diameter of the secondary follicles were similar to those reported by two study groups. An interesting finding was the granulosa cell number of the preantral follicles being smaller in the aged dogs (P<0.001).

It was considered that, the differences observed in the aged dogs for follicle diameter and granulosa cell number could be related to the ageing-related changes in the hormones regulating follicular development [24], the factors regulating follicular development (kit ligand, leukaemia inhibitory factor, bone morphogenic proteins 4 and 7, thrombocytic growth factor, basic fibroblast growth factor) [8,25] and the factors inhibiting follicular development (AMH and stromal cell factor-1) [26].

When compared to the values reported by Place et al.[27], the serum AMH levels measured in the young dogs in the present study were found to be lower (0.36 ng/mL) but were observed to be higher than the values measured in dogs with the ovarian remnant syndrome (0.195 ng/mL). This reflects that more clinical studies are required to develop the reference concentration ranges of serum AMH in bitches.

The lower serum AMH levels of the aged dogs in comparison to young dogs were probably attributed to the higher number of the granulosa cells of the preantral follicles in young animals, since these cells are directly involved in AMH synthesis. The negative correlation observed between serum AMH levels and age in dogs is in accordance with the decrease determined in the serum AMH levels of women in parallel with the decrease observed in gonad functions and ovarian reserves with advanced age [28-30]. In women, this is supposed to be caused by the aging process and changings towards menopause; even though in carnivores no menopause is described, the ovarian activity and fertility decrease in geriatric dogs.

Interestingly, even though the serum AMH levels increased with increased granulosa cell numbers of the secondary and preantral follicles, there was no correlation between serum AMH and follicle number, follicle diameter and granulosa cell number, which cannot be explained at present.

In conclusion, the decrease observed with advanced age in the number of primordial and primary follicles, and in particular, in the granulosa cell numbers of the secondary and preantral follicles, as well as the parallel changes determined in the serum AMH levels in bitches, makes us suggest that the AMH, which is used as a fertility parameter in humans, could be used for the same purpose in dogs. In this context, serum AMH measurement seems to be an interesting tool for the diagnosis and treatment of fertility problems in dogs, as well as for the selection of breeding animals.

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Research Article

Antibacterial Activity of *Citrus limon* Peel Essential Oil and *Argania spinosa* Oil Against Fish Pathogenic Bacteria

Canan ÖNTAŞ [1] Esin BABA [1] Erhan KAPLANER [2]
Selçuk KÜÇÜKAYDIN [2] Mehmet ÖZTÜRK [2] Menekşe Didem ERCAN [1]

[1] Muğla Sıtkı Kocman University, Faculty of Fisheries, Department of Aquaculture, Diseases, Division TR-48000 Muğla - TURKEY
[2] Muğla Sıtkı Koçman University, Department of Chemistry, Faculty of Sciences, TR-48000 Muğla - TURKEY

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Abstract

The main objective of the study was the identification of antibacterial activity of lemon (*Citrus limon* L.) peel essential oil and argan (*Argania spinosa* L.) oil against fish pathogenic bacteria. Antibacterial activity was determined against six different fish pathogens (*Yersinia ruckeri, Aeromonas hydrophila, Listonella anguillarum, Edwarsiella tarda, Citrobacter freundii and Lactococcus garvieae*). Essential oil derived from lemon peel and argan oil were applied against the bacteria using the disc diffusion and micro dilution method under in vitro conditions. The disc diffusion results indicated that essential oil of naturally *C. limon* peel and argan oil significantly inhibited the growth of *Y. ruckeri, A. hydrophila, L. anguillarum* and *C. freundii*. Our results suggested that the use of lemon peel essential oil and argan oil induced a stronger antibacterial effect.

Keywords: *Essential oil, Citrus limon, Argania spinosa, Fish pathogen, Antibacterial*

Balık Patojenlerine Karşı Limon *(Citrus limon)* ve Argan *(Argania spinosa)* Yağının Antibakteriyel Aktivitesi

Özet

Bu çalışmanın amacı limon kabuğu yağı (*Citrus limon* L.) ve argan (*Argania spinosa* L.) yağının bakteriyel balık patojenlerine karşı etkisinin belirlenmesidir. Antibakteriyel aktivite altı farklı balık patojenine (*Yersinia ruckeri, Aeromonas hydrophila, Listonella anguillarum, Edwarsiella tarda, Citrobacter freundii and Lactococcus garvieae*) karşı belirlenmiştir. Limon kabuğundan elde edilen yağ ve argan yağının antibakteriyel etkileri in vitro koşullar altında disk difüzyon ve mikrodilüsyon metodu kullanılarak belirlenmiştir. Disk difüzyon sonuçlarına göre limon ve argan yağının önemli ölçüde özellikle *Y. ruckeri, A. hydrophila, L. anguillarum* ve *C. freundii* patojenlerinin gelişimini engellediği saptanmıştır. Çalışmada kullanılan limon ve argan yağının güçlü antibakteriyel etkilerinden dolayı kullanılabileceği önerilmiştir.

Anahtar sözcükler: *Temel yağ, Citrus limon, Argania spinosa, Balık patojeni, Antibakteriyel*

INTRODUCTION

Fish pathogens such as *Yersinia ruckeri*, *Aeromonas hydrophila*, *Listonella anguillarum*, *Edwarsiella tarda*, *Citrobacter freundii* and *Lactococcus garvieae* are known to be causes of serious disease in aquaculture with high economic losses. In aquaculture, antibiotics are widely used for treatment and control of these pathogens. Due to conscious or unconscious excessive use of antibiotics, bacteria can improve resistance of these antibiotics [1]. Also antibiotics can accumulate in soil or sediment and become harmless for environment. Medical plants are very popular products of pre-treatment, treatment and immunostimulation in fish health. The ability of natural plants to inhibit activity of bacteria having potential interest as fish pathogens has been documented [2]. Many essential oils and plant extracts have been shown to be effective against fish pathogens [3-8].

Lemon is an important medicinal plant of *Citrus* genus (Rutaceae). Citrus essential oils mainly exist in fruit peels which are usually discarded as waste. Thus, citrus essential oil could be manufactured at a more affordable price than plant essential oils [9]. Citrus fruit peels exhibiting antimicrobial activity are rich with flavonoid glycosides,

İletişim (Correspondence)
+90 252 2111904; Fax: +90 252 2111887
esinbaba48@hotmail.com

coumarins, β and ɤ- sitosterols, and volatile oils [10]. Of course the chemical ingredients are responsible for their antimicrobial activity. Argan oil is obtained from *Argania spinosa* seeds belonging to the Sapotaceae family [11]. It contains mainly oleic (47.7%) and linoleic acid (29.3%) which are essential unsaturated fatty acids [12]. Several biological activities of *A. spinosa* such as antiproliferative [13], antiatherogenic, antiradical and anti inflammatory activities [14] and immunomodulatory activities [15] have been investigated in animals.

Lemon peel essential oil and argan oil were selected for the study, because both have different biological activities. To the best of our knowledge, though, both of them were investigated for food borne pathogens, none of them have been investigated for fish pathogens. Therefore, the aim of the present study was to investigate the antibacterial activity of essential oil of lemon peels and argan oil against six fish pathogenic bacteria; namely, *Yersinia ruckeri, Aeromonas hydrophila, Listonella anguillarum, Edwarsiella tarda, Citrobacter freundii* and *Lactococcus garvieae*. In addition, the chemical constituents of lemon pell essential oil and argan oil were analyzed by GC and GC-MS.

MATERIAL and METHODS

Chemicals

α-thujene, α-pinene, camphene, β-pinene, α-terpinene, terpinolene, borneol, terpinene-4-ol, α-terpineol, cis-geraniol, geranyl acetate, β-caryophyllene, valencene and caryophyllene oxide were obtained from Sigma-Aldrich GmbH, Sternheim, Germany. *p*-cymene, γ-terpinene, limonene and linalool were obtained from Fluka, GmbH, Sternheim, Germany. Myristic acid (C14:0), pentadecanoic acid (C15:0), palmitoleic acid (C16:1), palmitic acid (C16:0), margaric acid (C17:0), linoleic acid (C18:2), oleic acid (C18:1), stearic acid (C18:0), nonadecanoic acid (C19:0), eicosanoic acid (C20:0), heneicosanoic acid (C21:0), eruric acid (C22:1), docosanoic acid (C22:0), tricosanoic acid (C23:0), tetracosanoic acid ($C_{24:0}$) were obtained from sigma-aldrich GmbH, Sternheim, Germany. All other chemicals are in analytical grade.

Plant Material and Extraction of the Essential Oil

The lemon peels were gathered from fruit juice industry in Muğla - Ortaca, Turkey, March 1st, 2015. The citrus essential oil was obtained from fresh peels using hydro-distillation method by a Clevenger type apparatus. For this purpose, 100 g of lemon peels were used and 1 mL of essential oil was obtained after 3 h hydrodistillation. The oil was stored at 4°C in a dark bottle until usage.

Derivatization of Argan Oil

Commercially provided argan *(A. spinosa*; Mecitefendi 20 mL, Yeşilvadi) oil was used in this study, as well. In order to analyze its constituents, argan oil was derivatized to its fatty acid methyl esters. Argan oil (10 mg) was dissolved in 2 mL methanol in a 25 mL flask on which 2 mL 0.5 M NaOH was added. After the flask was heated at 50°C using a water bath, 2 mL BF_3:MeOH was added. The mixture was boiled for 2 min. After cooled down the volume was completed to 25 mL with saturated NaCl solution. The esters were extracted with *n*-hexane. The hexane layer was washed with a potassium bicarbonate solution (4 mL, 2%) and dried with anhydrous Na_2SO_4 and filtered. The organic solvent was removed under reduced pressure by a rotary evaporator to give methyl esters [16].

Analysis of Essential Oil and Argan Oil

The essential oil and argan oil constituents were analyzed with a Shimadzu GC-17 AAF, V3, 230 V series gas chromatograph (Japan); GC–MS analyses were carried on a Varian Saturn 2100T (USA) system equipped with an ion trap analyzer (IT). The essential oil was diluted with hexane 1:50 v/v, ratio, and the methyl derivative of argan oil diluted with chloroform 1:20 v/v, ratio before injection to the GC, and GC-MS. The standards were prepared in 40 ppm. For these purpose 1 mg of standard sample was diluted in 25 mL of chloroform.

Gas Chromatography (GC) Analysis Conditions

A DB-1 fused silica capillary non-polar column containing dimethylpolysiloxane (30 m × 0.25 id., film thickness 0.25 µm; J&W Scientific) and a flame ionisation detector (FID) were used for GC analyses. The injector temperature and detector temperature were adjusted to 250 and 270°C, respectively. Carrier gas was He at a flow rate of 1.4 mL/min. Sample volume was 1.0 µL with a split ratio of 50:1. For the essential oil analysis, initial oven temperature was held at 60°C for 5 min, then increased up to 280°C with 4°C/min increments and held at this temperature for 15 min. For the methylated argan oil, however, column temperature program started at 100°C for 5 min, then increased to 238°C with the rate of 3°C/min and held at this temperature for 15 min. The percentage compositions of the essential oil were determined with the Class GC10 GC computer programme [16]. The Retention indices were calculated according to the following equation:

n, n+i = Carbon number of reference hydrocarbon, i = 1 or 2

t_{Rx} = Retention time of Analyte

t_{Rn} = Retention time of Reference hydrocarbon before analyte

t_{Rn+i} = Retention time of Reference hydrocarbon after analyte

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Same analytical column and oven temperature program were used for the GC-MS analysis. Sample size was 0.2 µL

with a split ratio of 50:1.70 eV was used for electron ionization. Injector, transferline and manifold temperatures were adjusted to 250, 290 and 240°C respectively. For the determination of the constituents, NIST 2005 library, retention time index comparison as well as co-injection of standards were used [16].

Bacterial Fish Pathogens

Six bacterial fish strain were tested for the antibacterial activity of essential oils from lemon and argan oil. Different species of bacteria were isolated from sick fish. The antibacterial activity of essential oil of lemon and argan were tested against *Yersinia ruckeri, Aeromonas hydrophila, Lactococcus garvieae, Listonella anguillarum, Edwarsiella tarda,* and *Citrobacter freundii (Table 1)*. These isolates were stored in Triptic soya agar at 4°C for further use. Also bacterial strains were examined by phenotypic tests. Identification was carried out by conventional biochemical tests and API 20E as described by Austin and Austin [17].

Antibacterial Assay

The antibacterial effects were tested by the disc diffusion method [18]. The final concentrations of lemon and argan oil (dissolved in methanol) were at 0.5, 1%, 2.5%, 5%, 7.5%, or 10% [6]. The microorganisms used were: *Y. ruckeri, A. hydrophila, L. anguillarum, E. tarda, C. freundii*, which are Gram-negative bacteria, and *L. garvieae*, which is a Gram-positive bacteria. The previously prepared bacteria strains inoculums were adjusted to 0.5 Mc Farland standards, which are equal to $1x10^8$ CFU/mL and then the MHA plates were seeded with 100 µL of the standardized inoculum of each tested organism. The inoculum was spread evenly over plate with loop or sterile glass spreader. Afterward 25 µL of each lemon essential oil and argan oil were inoculated onto wells, plate culture of each microbial isolates were made in the spread. After incubation, each essential oil was noted for zone of inhibition for all isolates. The diameters of the zone of tested bacteria were measured by measuring scale in millimeter (mm). Thirteen different antibiotics (OTC:Oxytetracycline 20 µg, N: Neomycin 30 µg, AX: Amoxicilin 25 µg, NV: Novobiocin 30 µg, CIP: Ciprofloxacin 5 µg, SXT: Sulphamethoxazole 25 µg, CN: Gentamicin 10 µg, S: Streptomycin 10 µg, UB: Flumequine 30 µg, C: Chloramphenicol 30 µg, P: Penicillin 10 µg, TE: Tetracycline 30 µg, ENR: Enrofloxacin) were used as a positive, the methanol as a negative control. The tests were carried out in triplicate.

Table 1. *The bacterial strains and origin*
Tablo 1. *Bakteri suşları ve kökenleri*

Bacteria	Origin
Yersinia ruckeri	Rainbow trout *(Oncorhynchus mykiss)*, Fethiye
Aeromonas hydrophila	Common carp *(Cyprinus carpio)*, Çanakkale
Lactococcus garvieae	Rainbow trout *(Oncorhynchus mykiss)*, Fethiye
Listonella anguillarum	Mullet *(Mugil cephalus)*, Muğla
Edwarsiella tarda	Nil tilapia (*Oreochromis niloticus*), Çanakkale
Citrobacter freundii	Rainbow trout *(Oncorhynchus mykiss)*, Çanakkale

Minimum Inhibitory Concentration (MIC) Assay

The mininum inhibition concentration (MIC) of lemon peel essential oil and argan oil was determined according to the method of Eloff [19] with slight modification. The bacterial suspensions were prepared as described in antibacterial assay section. A twofold serial dilution of each oil (100 µL) in methanol was prepared in 96-well micro plates. 100 µL bacterial suspensions were added to each well. The methanol was included as negative control in each assay. The plates were incubated overnight at 22-25°C. After, incubation was measured OD=630 nm. MIC values were recorded as the lowest concentration of the oils that completely inhibited bacterial growth.

Statistical Analysis

The data were expressed as arithmetic means with standard error (SEM). Statistical analysis of data involved one-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison tests. Different letters in tables represent the significant difference at $P<0.05$.

RESULTS

Chemical Composition of Essential Oil and Argan Oil

The yellowish essential oil of *C. limon* with a yield of 2% was obtained by hydro-distillation. The essential oil constituents analyzed by GC-MS were given in *Table 2* along with LOD, LOQ, coefficient of determination and *m/z* values of the compounds. The major constituents of essential oil were elucidated as limonene (54.4%), γ-terpinene (%12.0), β-pinene (8.81%), α-terpineol (3.45%), myrecene (2.96%) and terpinolene (2.08%). *Table 3* shows the percentage concentration of the fatty acid composition of *A. spinosa* oil, analyzed by GC and GC-MS along with LOD, LOQ, coefficient of determination and m/z values of the compounds. Oleic acid (40.9%), linoleic acid (28.4%), palmitic acid (15.0%) and stearic acid (10.2%) were detected as major fatty acid constitutes.

Biochemical Test Results

Bacterial groups were determined on the criteria of shape, motility, catalase and oxidase reactions, oxidation-fermentation test. API® 20E system (BioMerieux, France) was furthermore used in order to identify oxidase positive and negative bacteria, respectively, at species level.

Antibacterial Activity Disc Diffusion Test Results

Results of antibacterial activity of lemon peel essential oil and argan oil against Gram positive and negative isolates by the disc diffusion method were shown on

Table 2. *Chemical composition of C. limon essential oil*
Tablo 2. *C. limon uçucu yağının kimyasal içeriği*

No	Analyte	Molecular ion(m/z)[a]	Fragments MS[b]	Ionization Mode	RI[c]	$R^{2,d}$	LOD/LOQ (µg/L)[e]	Concentration (%)	Identification method
1	α-thujene	136	136 (8.2), 94 (8.6), 93 (99.9), 92 (30.8), 91 (34.9), 79 (9.9), 77 (34.2), 41 (11.2), 39 (9.9), 27 (8.4)	Poz	901	0.9925	13.3/ 44.6	0.82	Co-GC[f], MS[g], RI[h]
2	α-pinene	136	121 (13.6), 105 (10.2), 93 (99.9), 92 (34.9), 91 (32.3), 79 (22.7), 77 (28.5), 41 (20.0), 39 (18.9)	Poz	914	0.9977	18.2 / 57.1	0.20	Co-GC[f], MS[g], RI[h]
3	Camphene	136	121 (58.2), 93 (99.9), 91 (37.3), 79 (39.9), 77 (28.0), 67 (29.4), 41 (31.7), 39 (33.5), 27 (19.7)	Poz	925	0.9939	16.3 / 52.7	0.26	Co-GC[f], MS[g], RI[h]
4	β-pinene	136	93 (99.9), 92 (15.2), 91 (30.8), 79 (27.4), 77 (27.5), 69 (35.0), 53 (13.4), 41 (60.9), 39 (31.8), 27 (21.4)	Poz	960	0.9912	18.3 / 54.8	8.81	Co-GC[f], MS[g], RI[h]
5	Myrecene	136	93 (85.5), 91 (95.0), 79 (13.8), 77 (11.0), 69 (79.6), 67 (11.0), 53 (14.1), 41 (99.9), 39 (29.9), 27 (28.0)	Poz	977			2.96	MS[g], RI[h]
6	α-phellandrene	136	136 (15.6), 94 (85.0), 93 (99.9), 92 (25.3), 91 (33.2), 79 (64.0), 77 (30.6), 41 (14.1), 39 (11.2), 27 (10.1)	Poz	989			0.19	MS[g], RI[h]
7	α-terpinene	136	136 (35.8), 121 (30.3), 105 (9.90), 93 (99.9), 92 (21.4), 91 (37.2), 79 (18.5), 77 (28.1), 43 (17.2), 41 (9.90)	Poz	1003	0.9951	17.2 / 57.3	0.73	Co-GC[f], MS[g], RI[h]
8	*p*-cymene	134	134 (25.4), 120 (10.0), 119 (99.9), 117 (9.10), 115 (4.40), 91 (15.8), 77 (5.20), 65 (4.70), 41 (5.90), 39 (4.80)	Poz	1007	0.9947	14.7 / 51.6	0.74	Co-GC[f], MS[g], RI[h]
9	Limonene	136	136 (16.4), 107 (18.0), 94 (22.4), 93 (50.0), 92 (18.1), 79 (16.4), 68 (99.9), 67 (44.4), 53 (17.5), 39 (22.0)	Poz	1018	0.9987	12.1 / 31.4	54.4	Co-GC[f], MS[g], RI[h]
10	β-cis-ocimene	136	93 (99.9), 92 (22.5), 91 (27.2), 80 (19.1), 79 (31.0), 77 (25.8), 53 (16.5), 41 (35.6), 39 (27.5), 27 (20.5)	Poz	1026			0.20	MS[g], RI[h]
11	β-trans-ocimene	136	93 (99.9), 92 (24.5), 91 (28.2), 80 (15.6), 79 (29.7), 77 (26.9), 53 (16.5), 41 (35.6), 39 (27.5), 27 (20.5)	Poz	1037			0.37	MS[g], RI[h]
12	γ-terpinene	136	136 (31.1), 121 (28.5), 93 (99.9), 92 (24.7), 91 (56.5), 79 (24.5), 77 (41.5), 43 (13.9), 41 (15.0), 39 (15.0)	Poz	1047	0.9963	16.3 / 55.2	12.0	Co-GC[f], MS[g], RI[h]
13	Terpinolene	136	136 (61.4), 121 (78.3), 107 (16.5), 105 (26.0), 93 (99.9), 91 (61.7), 79 (45.7), 77 (42.8), 41 (24.0), 39 (25.3)	Poz	1074	0.9969	23.9 / 73.2	2.08	Co-GC[f], MS[g], RI[h]
14	*cis-p*-mentha-2,8 dienol	152	137 (41.5), 134 (91.9), 119 (82.0), 93 (42.1), 91 (99.9), 79 (76.1), 77 (45.3), 43 (84.4), 41 (63.2), 39 (59.2)	Poz	1080			0.29	MS[g], RI[h]
15	Linalool	154	121 (20.6), 93 (59.1), 80 (24.1), 71 (99.9), 69 (38.1), 55 (46.9), 43 (64.0), 41 (64.2), 39 (21.7), 27 (19.8)	Poz	1082	0.9923	8.0 / 22.0	0.76	Co-GC[f], MS[g], RI[h]
16	Fenchol	154	84 (20.7), 82 (18.8), 81 (99.9), 80 (52.8), 72 (21.0), 71 (23.1), 69 (24.2), 67 (16.9), 43 (26.3), 41 (24.6)	Poz	1110		4.1 / 13.3	0.19	MS[g], RI[h]
17	Borneol	154	139 (8.5), 110 (19.1), 96 (8.6), 95 (99.9), 93 (8.9), 71 (7.2), 69 (7.4), 67 (7.9), 55 (10.3), 41 (15.9)	Poz	1132	0.9941	9.7 / 33.1	0.15	Co-GC[f], MS[g], RI[h]
18	Terpinene-4-ol	154	154 (14.8), 111 (49.8), 93 (43.4), 86 (27.2), 69 (21.2), 68 (15.2), 55 (17.2), 43 (29.0), 41 (23.1)	Poz	1142	0.9977	10.7 / 35.6	2.11	Co-GC[f], MS[g], RI[h]
19	α-terpineol	154	136 (47.3), 121 (58.3), 95 (22.3), 93 (67.7), 81 (31.7), 68 (27.3), 67 (21.7), 59 (99.9), 43 (32.0), 41 (19.0)	Poz	1150	0.9938	9.1 / 30.4	3.45	Co-GC[f], MS[g], RI[h]
20	*cis*-carveol	152	134 (34.8), 119 (78.3), 117 (30.0), 109 (24.1), 93 (20.8), 92 (29.4), 91 (99.9), 77 (35.4), 65 (21.1), 39 (26.3)	Poz	1189			tr[i]	MS[g], RI[h]
21	*cis*-Geraniol	154	93 (12.8), 84 (11.8), 69 (99.9), 68 (24.5),67 (15.8), 53 (12.8), 41 (93.4), 39 (20.1), 29 (12.9), 27 (13.8)	Poz	1206	0.9951	2.6 / 8.8	0.32	Co-GC[f], MS[g], RI[h]
22	β-citral	152	109 (9.4), 94 (13.0), 84 (19.7), 69 (85.3), 67 (8.8), 53 (10.7), 41 (99.9), 39 (24.8), 29 (12.0), 27 (25.4)	Poz	1208			0.37	MS[g], RI[h]
23	*trans*-Geraniol	154	123 (8.5), 93 (9.4), 84 (6.8), 70 (7.5), 69 (99.9), 68 (19.8), 67 (8.0), 55 (6.5), 41 (65.3), 29 (10.0)	Poz	1224			0.49	MS[g], RI[h]
24	Geranial	152	109 (9.4), 94 (16.2), 91 (8.9), 84 (26.7), 83 (12.5), 69 (99.9), 67 (10.8), 53 (13.1), 41 (74.6), 39 (25.5)	Poz	1242			0.15	MS[g], RI[h]
25	Citronellol acetate	198	123 (62.8), 95 (76.8), 82 (67.2), 81 (70.4), 69 (99.9), 68 (43.8), 67 (49.9), 53 (49.3), 43 (81.2), 41 (69.5)	Poz	1332			0.46	MS[g], RI[h]
26	Neryl acetate	196	136 (13.1), 121 (15.5), 93 (39.1), 80 (17.4), 69 (99.9), 68 (40.2), 67 (12.9), 43 (43.3), 41 (60.1), 39 (10.5)	Poz	1339			1.81	MS[g], RI[h]
27	Geranyl acetate	196	136 (31.7), 121 (23.0), 93 (41.1), 80 (23.0), 69 (99.9), 68 (69.3), 67 (28.1), 43 (84.0), 41 (76.6), 39 (21.3)	Poz	1358	0.9965	5.1/17.9	1.60	Co-GC[f], MS[g], RI[h]
28	β-Caryophyllene	204	133 (92.1), 120 (44.7), 107 (48.3), 105 (62.3), 93 (99.9), 91 (85.8), 79 (76.3), 77 (43.9), 69 (75.4), 41 (76.9),	Poz	1415		6.3/19.7	0.66	Co-GC[f], MS[g], RI[h]
29	α-*trans*-bergamotene	204	119 (99.9), 107 (22.9), 105 (17.1), 93 (68.6), 91 (25.7), 77 (14.3), 69 (34.3), 55 (17.1), 41 (57.1), 39 (17.2)	Poz	1434			0.79	MS[g], RI[h]
30	(*Z*)-β-Farnesene	204	133 (22.8), 120 (17.7), 93 (46.4), 91 (17.5), 81 (21.1), 79 (20.1), 69 (80.1), 67 (23.6), 53 (18.7), 41 (99.9)	Poz	1476			0.11	MS[g], RI[h]
31	Valencene	204	204 (57.6), 161 (99.9), 135 (36.6), 133 (40.9), 119 (48.0), 107 (50.6), 105 (56.9), 93 (53.5), 91 (46.1), 79 (46.1)	Poz	1486	0.994	5.2/17.5	0.46	Co-GC[f], MS[g], RI[h]
32	α-selinene	204	107 (54.0), 105 (58.1), 93 (59.6), 91 (57.0), 81 (42.3), 79 (68.5), 67 (53.5), 53 (42.6), 41 (99.9), 39 (57.9)	Poz	1489			0.16	MS[g], RI[h]
33	β-bisabolene	204	204 (24.5), 161 (17.3), 109 (23.8), 107 (17.6), 94 (26.5), 93 (69.0), 79 (23.8), 69 (99.9), 67 (25.5), 41 (68.6)	Poz	1498			1.74	MS[g], RI[h]

Table 2. *Chemical composition of C. limon essential oil* ***(Continue...)***
Tablo 2. *C. limon uçucu yağının kimyasal içeriği* ***(Devam...)***

No	Analyte	Molecular ion(m/z)[a]	Fragments MS[b]	Ionization Mode	RI[c]	$R^{2,d}$	LOD/LOQ (µg/L)[e]	Concentration (%)	Identification method
34	Caryophyllene oxide	220	95 (42.0), 93 (66.1), 91 (57.1), 81 (37.3), 79 (88.5), 69 (40.7), 67 (37.7), 55 (39.3), 43 (99.9), 41 (92.7)	Poz	1561	0.9981		tr[i]	Co-GC[f], MS[g], RI[h]
35	α-bisabolol	222	119 (39.7), 109 (50.0), 95 (27.6), 93 (38.5), 69 (80.3), 67 (28.1), 55 (35.3), 43 (99.9), 41 (89.3), 39 (26.1)	Poz	1653			tr[i]	MS[g], RI[h]
				Monoterpene hydrocarbons: 83.7 Monoterpenoids: 12.2 Sesquiterpene hydrocarbons: 3.9 Total identified: 99.8					

[a]***Parent ion (m/z):*** *Molecular ions of the standard compounds (mass to charge ratio),* [b]***MS:*** *Fragments for the related molecular ions,* [c]***RI:*** *Retention index on DB-1 fused silica column,* [d]***R^2:*** *coefficient of determination,* [e]***LOD/LOQ (µg/L):*** *Limit of detection/Limit of quantification,* [f]***Co-GC:*** *co-injection of standards,* [g]***MS:*** *Mass spectra comparison with NIST 2005 library,* [h]***RI:*** *Retention Indices on literature,* [i]***tr:*** *Trace*

Table 3. *Fatty acid composition of A. spinosa oil*
Tablo 3. *A. spinosa yağının yağ asidi içeriği*

Peak no	Compounds	Molecular ion peak (m/z)[a]	Fragments MS[b]	$R^{2,c}$	LOD/LOQ (µg/L)[d]	Concentration (%)	Identification Methods
1	Myristic acid ($C_{14:0}$)	228	228 (44.1), 129 (43.7),73 (99.9), 71 (37.6), 60 (95.2), 57 (72.7), 55 (56.9), 43 (86.3), 41 (72.9), 29 (41.6)	0.9922	13.3/35.7	0.49	Co-GC[e], MS[f]
2	Pentadecanoic acid ($C_{15:0}$)	242	242 (30.2), 73 (92.2),71 (32.7), 69 (33.9), 60 (91.9), 57 (64.4), 55 (68.1), 43 (99.9), 41 (87.1), 29 (54.1)	0.9944	13.2/38.1	0.19	Co-GC[e], MS[f]
3	Palmitoleic acid ($C_{16:1}$)	254	97 (29.8), 83 (43.8), 69 (69.1), 67 (32.4), 56 (32.4), 55 (99.9), 43 (54.2), 41 (84.2), 29 (34.2), 28 (33.3)	0.9970	3.4/9.7	0.33	Co-GC[e], MS[f]
4	Palmitic acid ($C_{16:0}$)	256	73 (90.5), 71 (28.5), 69 (31.0), 60 (83.0), 61 (21.8), 57 (63.4), 55 (61.6), 43 (99.9), 41 (74.9), 29 (41.4)	0.9939	33.4/95.9	15.0	Co-GC[e], MS[f]
5	Margaric acid ($C_{17:0}$)	270	270 (67.5), 129 (50.7), 73 (99.9), 71 (46.3), 69 (31.6), 60 (85.8), 57 (78.4), 55 (43.0), 43 (76.3), 41 (44.9)	0.9946	31.8/96.1	0.30	Co-GC[e], MS[f]
6	Linoleic acid ($C_{18:2}$)	280	96 (54.7), 95 (62.7), 82 (73.0), 81 (87.8), 69 (35.9), 68 (60.2), 67 (99.9), 55 (59.5), 54 (44.9), 41 (54.1),	0.9928	33.2/99.0	28.4	Co-GC[e], MS[f]
7	Oleic acid ($C_{18:1}$)	282	97 (44.3), 83 (59.3), 70 (32.0), 69 (75.7), 67 (35.3), 57 (43.9),56 (34.5), 55 (99.9), 43 (54.9), 41 (75.2),	0.9919	30.5/89.2	40.9	Co-GC[e], MS[f]
8	Stearic acid ($C_{18:0}$)	284	129 (31.8), 73 (84.0), 71 (37.1), 69 (35.2), 60 (80.6), 57 (75.8), 55 (63.7), 43 (99.9), 41 (69.5), 29 (38.1)	0.9948	10.3/27.5	10.2	Co-GC[e], MS[f]
9	10-Nonadecenoic acid ($C_{19:1}$)	296	97 (40.5), 87 (38.6), 84 (37.6), 83 (50.7), 74 (58.4), 69 (65.2), 57 (33.8), 55 (99.9), 43 (59.4), 41 (75.8)	0.9952	14.8/43.1	0.10	MS[f]
10	Nonadecanoic acid ($C_{19:0}$)	298	298 (40.6), 73 (74.9), 71 (33.7), 69 (33.6), 60 (68.6), 57 (71.9), 55 (61.3), 43 (99.9), 41 (69.8), 29 (38.8)	0.9973	13.8/38.6	0.15	Co-GC[e], MS[f]
11	10,13-Eicosadienoic acid ($C_{20:2}$)	308	109 (44.3), 96 (71.3), 95 (81.7), 82 (74.7), 81 (98.2), 69 (52.1), 68 (53.0), 67 (99.9), 55 (74.7), 41 (49.5)	0.9971	4.1/13.6	0.10	MS[f]
12	11-Eicosenoic acid ($C_{20:1}$)	310	292 (52.0), 97 (46.0), 84 (39.0), 83 (56.0), 74 (57.0), 69 (73.0), 57 (39.0), 55 (99.9), 43 (63.0), 41 (72.0)	0.9937	4.5/13.9	1.52	MS[f]
13	Eicosanoic acid ($C_{20:0}$)	312	85 (26.1), 73 (70.9), 71 (42.9), 69 (35.2), 60 (60.5), 57 (78.7), 55 (65.9), 43 (99.9), 41 (68.8), 29 (30.8)	0.9921	5.4/15.9	1.42	Co-GC[e], MS[f]
14	Heneicosanoic acid ($C_{21:0}$)	326	326 (99.9), 129 (27.3), 73 (53.6),71 (32.1), 69 (26.0), 60 (45.3), 57 (58.7), 55 (39.7), 43 (65.5), 41 (36.1)	0.9929	*4.4/12.3*	*tr*[g]	Co-GC[e], MS[f]
15	Eruric acid ($C_{22:1}$)	338	97 (36.7), 83 (48.4), 70 (25.8), 69 (63.5), 57 (41.7), 56 (29.1), 55 (99.9),43 (60.1), 41 (78.1)	0.9936	*4.2/13.5*	*tr*[g]	Co-GC[e], MS[f]
16	Docosanoic acid ($C_{22:0}$)	340	340 (99.9), 129 (38.3), 97 (28.9), 83 (26.3), 73 (41.3), 71 (35.4), 60 (28.1), 57 (51.3), 55 (29.2), 43 (39.3)	0.9940	4.2/12.9	0.48	Co-GC[e], MS[f]
17	Tricosanoic acid ($C_{23:0}$)	354	354 (75.2), 129 (35.4), 73 (62.2), 71 (36.9), 69 (37.8), 60 (63.2), 57 (67.5), 55 (70.8), 43 (99.9), 41 (68.8)	0.9939	*3.5/8.8*	*tr*[g]	Co-GC[e], MS[f]
18	15-Tetracosenoic acid ($C_{24:1}$)	366	348 (28.0), 97 (26.0), 83 (41.0), 74 (54.0), 69 (57.0), 67 (25.0), 57 (35.0), 55 (99.9), 43 (60.0), 41 (65.0)	0.9933	*4.4/11.5*	*tr*[g]	MS[f]
19	Tetracosanoic acid ($C_{24:0}$)	368	368 (39.8), 129 (50.7), 73 (79.7), 71 (55.6), 69 (41.6), 60 (61.3), 57 (99.8), 55 (58.2), 43 (99.9), 41 (50.6)	0.9927	4.3/11.7	0.17	Co-GC[e], MS[f]
	Saturated Unsaturated Total identified					28.8 71.4 99.8	

[a]***Parent ion (m/z):*** *Molecular ions of the standard compounds (mass to charge ratio),* [b]***MS:*** *Fragments for the related molecular ions,* [c]***R^2:*** *coefficient of determination,* [d]***LOD/LOQ (µg/L):*** *Limit of detection/Limit of quantification,* [e]***Co-GC:*** *co-injection of standards,* [f]***MS:*** *Mass spectra comparison with NIST 2005 library,* [g]***tr:*** *Trace*

Tables 4 and *5* respectively. The lemon peel essential oil produces a maximum zone of inhibition 19.00±0.58 mm against *Y. ruckeri* followed by *L. anguillarum* (18.00±1.15), *A. hydrophila* (17.00±1.20), *C. freundii* (17.00±0.57), *E. tarda* (11.00±0.57). The minimum were 10.33±0.28 mm zone inhibition produced against *L. garvieae*. The strongest

Table 4. *Antibacterial activity of C. limon peel essential oil against different bacterial fish pathogens (The diameter of the zone of inhibition, mm)*
Tablo 4. *C. limon kabuğu yağının farklı bakteriyel balık patojenlerine karşı antibakteriyel aktivite (inhibisyon zon çapı, mm)*

Bacteria	Lemon (C. limon) peel essential oil different concentration (%)				
	1%	2.5%	5%	7.5%	10%
Y. ruckeri	8.33±0.33	10.33±0.88	12.00±0.57	13.00±0.57	19.00±0.58
A. hydrophila	9.00±0.57	10.66±0.66	12.33±0.33	14.00±0.57	17.00±1.20
L. anguillarum	8.00±0.57	9.00±0.56	11.66±1.45	13.66±1.33	18.00±1.15
C. freundii	8.33±0.33	10.00±0.57	12.66±0.66	14.00±0.46	17.00±0.57
E. tarda	7.33±0.33	8.00±0.57	8.33±0.76	9.33±0.33	11.00±0.57
L.garvieae	7.33±0.33	8.34±0.42	10.00±0.57	11.00±0.33	10.33±0.28

Inhibition zones>15 mm were declared as strong (bold), from 8 to 15 mm as moderate and from 1 to 8 mm as weak activities (M±SE; indicates Mean ± Standard error)

Table 5. *Antibacterial activity of A. spinosa oil against different bacterial fish pathogens (The diameter of the zone of inhibition, mm)*
Tablo 5. *A. spinosa kabuğu yağının farklı bakteriyel balık patojenlerine karşı antibakteriyel aktivite (inhibisyon zon çapı, mm)*

Bacteria	Argan (Argania spinosa) oil different concentration (%)				
	1%	2.5%	5%	7.5%	10%
Y. ruckeri	9.00±0.57	10.33±0.88	12.00±0.57	13.00±0.66	18.33±0.88
A. hydrophila	9.00±0.57	10.33±0.88	12.33±0.33	14.00±0.57	17.00±0.57
L. anguillarum	7.00±0.57	9.33±0.33	12.00±1.52	12.33±1.45	17.00±0.57
C. freundii	8.33±0.33	10.00±0.57	12.66±0.66	14.00±0.57	17.00±0.56
E. tarda	7.33±0.33	8.00±0.57	8.33±0.33	10.00±0.57	9.66±0.88
L. garvieae	7.33±0.33	8.33±0.33	10.00±0.57	11.00±0.57	11.33±0.88

Inhibition zones>15 mm were declared as strong (bold), from 8 to 15 mm as moderate and from 1 to 8 mm as weak activities (M±SE; indicates Mean ± Standard error)

Table 6. *Antibiotic suspectibility test results against different bacterial fish pathogens (The diameter of the zone of inhibition, mm)*
Tablo 6. *Farklı bakteriyel balık patojenlerine karşı antibiyotik duyarlılık testi*

A	Bacteria					
	Y. ruckeri	***A. hydrophila***	***L. anguillarum***	***C. freundii***	***E. tarda***	***L. garvieae***
OTC	25.00±0.57	21.66±1.20	26.00±0.57	22.33±1.45	20.66±0.88	10.33±0.88
N	19.66±0.88	16.33±0.88	18.66±0.88	12.00±1.15	10.33±0.88	12.00±0.57
AX	20.33±0.88	10.00±1.15	18.00±0.57	10.00±0.57	24.00±0.57	23.66±0.88
NV	12.66±1.45	19.00±0.57	24.33±1.20	0	26.33±0.88	25.00±0.57
CIP	28.66±1.20	30.00±0.57	20.00±0.57	30.33±0.88	15.66±1.20	16.33±0.88
SXT	29.66±0.88	35.00±0.57	25.00±0.57	30.00±0.57	10.00±0.57	10.33±0.88
CN	15.33±0.88	13.66±0.88	16.33±0.88	15.00±0.57	9.66±0.88	13.00±0.57
S	16.33±0.88	13.00±0.57	20.00±0.57	15.33±0.88	8.00±0.57	9.33±0.88
UB	30.66±1.76	27.00±0.57	38.00±1.15	31.00±0.57	12.66±1.20	8.33±0.88
C	25.33±0.88	35.00±0.57	30.00±1.15	20.00±0.57	19.66±0.88	20.33±1.45
P	0	0	0	0	15.66±0.88	15.66±0.88
TE	21.00±1.54	11.00±0.57	30.66±0.88	29.66±0.88	21.33±0.88	10.00±0.57
ENR	30.00±0.57	25.00±0.57	27.00±3.60	17.92±1.68	19.66±0.88	22.00±1.15

*(A: Antibiotics, **OTC:** Oxytetracycline 20 µg, **N:** Neomycin 30 µg, **AX:** Amoxicilin 25 µg, **NV:** Novobiocin 30 µg, **CIP:** Ciprofloxacin 5 µg, **SXT:** Sulphamethoxazole 25 µg, **CN:** Gentamicin 10 µg, **S:** Streptomycin 10 µg, **UB:** Flumequine 30 µg, **C:** Chloramphenicol 30 µg, **P:** Penicillin 10 µg, **TE:** Tetracycline 30 µg, **ENR:** Enrofloxacin 5 µg)*

antibacterial activities were obtained by lemon essential oil with inhibition zones of 19 mm against *Y. ruckeri (Table 4)*. The argan oil produces a maximum zone of inhibition 18.33±0.88mm against *Y. ruckeri* followed by *L. anguillarum* (17.00±0.57), *A. hydrophila* (17.00±0.57), *C. freundii* (17.00±0.56), *L. garvieae* (11.33±0.88) whereas the minimum were 9.66±0.88 mm zone inhibition produced against *E. tarda*. The strongest antibacterial activities were obtained by lemon essential oil with inhibition zones of 18.33 mm against *Y. ruckeri (Table 5)*. Summarizing the results, it can be concluded that the most antibacterial effective lemon and argan oil were against *Y. ruckeri*, *L. anguillarum*, *A. hydrophila* and *C. freundii*.

Inhibition zone profiles against different antibiotics of bacterial isolated from fish was shown on *Table 6*. Antibiotic susceptibility test showed that *Y. ruckeri, L. anguillarum* and *C. freundii* isolates were susceptible to enrofloxacin but the isolates were resistant to penicillin. *A. hydrophila* isolates were found to be susceptible to sulphamethoxazole and chloramphenicol and to be resistant to penicillin. Also *E. tarda* and *L. garvieae* isolates were susceptible to Novobiocin.

Minimum Inhibitory Result (MIC)

The result of minimum inhibitory concentration (MIC) of oil of lemon and argan is shown in *Tables 7* and *8*. The lemon peel essential oil against *Y. ruckeri* and *L. anguillarum* showed a higher MIC values (62.5 µL/mL), followed by *A. hydrophila* and *C. freundii* (125 µL/mL), *E. tarda* and *L. garvieae* (250 µL/mL) *(Table 7)*. Argan oil against *Y. ruckeri* showed a higher MIC values of 62.5 µL/mL followed by *L. anguillarum, A. hydrophila* and *C. freundii* (125 µL/mL), *E. tarda* and *L. garvieae* (250 µL/mL) *(Table 8)*.

DISCUSSION

Essential oils can inhibit pathogenic bacteria because of its chemical compounds which are thymol, carvacrol, phenolic acids, ascorbic acid, polyphenols and dietary fiber [20]. Numerous studies have confirmed that the citrus show antimicrobial, antioxidant and anticancer activities [21,22]. In this study major essential oil components identified from *C. limon* essential oil were limonene, γ-terpinene, β-pinene, α-terpineol, myrecene and terpinolene. Various trials have documented the inhibitory effects of citrus against different pathogens [23,24]. Argan oil contains vanillic acid, syringic acid, ferulic acid, tyrosol, catechol, resorcinol, epicatechin, catechin [25]. In the current study, important fatty acid ingredients identified from *A. spinosa* were oleic acid, linoleic acid, palmitic acid and stearic acid. Fatty acids, particularly oleic, linoleic and linolenic acids which are the long chain fatty acids attribute to inhibit growth of bacteria as antimicrobial agents [26,27]. The effect of dietary argan oil on the immune system was also evaluated on rats. Those studies showed that argan oil effects on immune cells, which is similar to that of olive oil [28].

This study demonstrates the antibacterial activity of lemon *(C. limon)* peel essential oil and argan oil against fish pathogenic bacteria. The isolated strains from diseased fish were used in this study. Because, reference bacteria which are clinical strains were possible to lost their pathogenicity caused by subculturing. For this purpose, essential oils derived from lemon peel and argan oil were applied against isolated bacteria using the disc diffusion and micro dilution method *in vitro* conditions. Lemon essential oil and argan oil inhibited the growth of all

Table 7. *Minimum inhibitory concentrations of lemon peel essential oils against different fish pathogens*
Tablo 7. *Limon yağının farklı balık patojenlerine karşı MİK değerleri (µL/mL)*

Amount (µL/mL)	Bacteria					
	Y. ruckeri	A. hydrophila	L. anguillarum	E. tarda	C. freundii	L. garvieae
500	-	-	-	-	-	-
250	-	-	-	+	-	+
125	-	+	-	+	+	+
62.5	+	+	+	+	+	+
31.25	+	+	+	+	+	+
15.62	+	+	+	+	+	+
7.8	+	+	+	+	+	+
3.9	+	+	+	+	+	+
1.95	+	+	+	+	+	+
0.975	+	+	+	+	+	+
0.48	+	+	+	+	+	+
0.24	+	+	+	+	+	+
0.12	+	+	+	+	+	+
0.06	+	+	+	+	+	+
Control	+	+	+	+	+	+

(+): Reproduction, (-): No reproduction

Table 8. *Minimum inhibitory concentrations of A. spinosa essential oil against different fish pathogens*
Tablo 8. *Argan yağının farklı balık patojenlerine karşı MİK değerleri (µL/mL)*

Amount (µL/mL)	Bacteria					
	Y. ruckeri	*A. hydrophila*	*L. anguillarum*	*E. tarda*	*C. freundii*	*L. garvieae*
500	-	-	-	-	-	-
250	-	-	-	-	-	-
125	-	-	-	+	-	+
62.5	-	+	+	+	+	+
31.25	+	+	+	+	+	+
15.62	+	+	+	+	+	+
7.8	+	+	+	+	+	+
3.9	+	+	+	+	+	+
1.95	+	+	+	+	+	+
0.975	+	+	+	+	+	+
0.48	+	+	+	+	+	+
0.24	+	+	+	+	+	+
0.12	+	+	+	+	+	+
0.06	+	+	+	+	+	+
Control	+	+	+	+	+	+

(+): Reproduction, (-): No reproduction

bacteria. Among the strains tested, both oils were possessed remarkable activity against *A. hydrophila*, *L. anguillarum* and *C. freundii*. However, they exhibited lesser activities against *Y. ruckeri*. Both also demonstrated more or less trivial activity against *E. tarda* and *L. garvieae*. Both essential and fatty acid extracts indicated inhibitory effects on same pathogents, mentioned in this study, which is parallel to other several reports. Hindi and Chabuck [8] reported antimicrobial effect of different aqueous lemon extracts against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus agalactiae* (6 Gram-positive) and *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Escherichia coli*,

Salmonella typhi, *Proteus spp.*, *Moraxella catarrhalis*, *Acinetobacter spp.* (8 Gram-negative) and *Candida albicans* isolates. Hayes and Markovic [29] reported the antimicrobial activity of lemon against *Escherichia coli, S. aureus, P. aeruginosa* and *C. albicans*. The argan oil inhibited the growth of Gram positive and Gram negative bacteria along with yeasts and molds [30]. Antibacterial effect of *C. limon* against *S. aureus, P. aeruginosa* and *P. vulgaris* were revealed [5]. Furthermore, some essential oil, except citrus family members, was investigated antibacterial activity against fish pathogens. Ekici et al.[6] investigated the antibacterial properties essential oils of thyme *(Origanum vulgaris)*, melissa *(Melissa oleum)*, lavandula oil *(Lavandulae romanae oleum)*, rosemary oil *(Rosmarinus officinalis)* and ginger *(Zingiber officinale)*. Essential oils possessed significant antibacterial activity against *Yersinia ruckeri, Aeromonas hydrophila, Vibrio anguillarum, Flavobacterium psychrophilum* and *Lactococcus garvieae*. Starliper et al.[31] were reported that cinnamon *(Cinnamomum cassia)*, oregano *(Origanum vulgare)*, lemongrass *(Cymbopogon citratus)* and thyme *(Thymus vulgaris)* oils were reduced growth of *Aeromonas salmonicida subsp. salmonicida*.

In the present study, limonene was the main ingredient of essential oil. In a recent paper, the essential oil containing limonene as the major compound obtained from sweet orange peel were mixed to fish fed [32]. This prepared fish fed indicated resistance against *Streptococcus iniae* in Mozambique tilapia *in vivo*. Hematologic and immunologic parameters of the recent study also exhibited that orange peel oil showed no negative effect to fish health and growth performance [32].

As a result, this study showed that lemon essential oil and argan oil had antibacterial potentials against some fish pathogens. Since the lemon essential oil mainly obtained from fruit peels which are discarded as waste, the essential oil to be used for fish fed will be produced economically cheaper than those of other plants. Therefore, both can be used to prevent fish diseases by adding to fish fed or to prepare solutions for immersion treatment. However, further studies, particularly *in vivo* studies are necessary.

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Research Article

The Serologic and Molecular Prevalence of Heartworm Disease in Shelter Dogs in the Thrace Region of Turkey [1]

Handan ÇETİNKAYA [1] İbrahim AKYAZI [2] Mete ÖZKURT [3] Erdal MATUR [2]

[1] *This study was supported by the Research Fund of Istanbul University (Project Number: 625/28062007)*

[1] Department of Parasitology, Faculty of Veterinary Medicine, Istanbul University, TR- 34320 Avcılar, Istanbul - TURKEY
[2] Department of Physiology, Faculty of Veterinary Medicine, Istanbul University, TR- 34320 Avcılar, Istanbul - TURKEY
[3] Department of Physiology, Medical Faculty, Eskisehir Osmangazi University, TR- 26480 Meşelik Yerleşkesi, Eskişehir - TURKEY

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Abstract

Dirofilaria immitis is an important nematode of dogs and cats which causes fatal heartworm disease in their hosts. The aim of this study was to investigate the prevalence of *D. immitis* by serologic and molecular methods in shelter dogs, in the Thrace Region of Turkey. Blood samples were collected from 402 dogs in shelters which were located in four cities (Istanbul, Edirne, Tekirdag and Kirklareli) in this region. The blood smears were examined for the presence of *D. immitis* microfilariae under the light microscope. The commercial Snap3Dx test kit and PCR assay for amplifying the ITS-2 gene region were used for the serological and molecular analyses, respectively. The serologic and molecular prevalence of *D. immitis* was determined as 6.7% and 2.7%, respectively. *D. immitis* microfilariae were also determined in the blood smears of three dogs (0.8%). The distribution of the infection according to the provinces was 14.7% in Edirne, 11% in Kirklareli, 1% in Tekirdag and 0% in Istanbul. The occult infection rate was determined as 59.3%. This study revealed the presence of *D. immitis* for the first time in the Thrace Region of Turkey. This region is the transition point to Europe and obtained data in this study could contribute to the control of heartworm disease in the area.

Keywords: *Heartworm Disease, Dog, Serology, PCR, Thrace Region, Turkey*

Türkiye'nin Trakya Bölgesindeki Barınak Köpeklerinde Kalp Kurdu Hastalığının Serolojik ve Moleküler Prevalansı

Özet

Dirofilaria immitis, konaklarında ölümcül Kalp Kurdu Hastalığı'na neden olan köpek ve kedilerin önemli bir nematodudur. Bu çalışmanın amacı, Türkiye'nin Trakya Bölgesi'ndeki barınak köpeklerinde *D. immitis*'in prevalansının serolojik ve moleküler yöntemlerle araştırılmasıdır. Kan örnekleri, bu bölgedeki dört şehirde (İstanbul, Edirne, Tekirdağ ve Kırklareli) lokalize olmuş barınaklardaki 402 köpekten alındı. Kan yaymaları *D. immitis*' in mikrofilerlerinin varlığı için ışık mikroskobu altında incelendi. Snap 3Dx test kiti ve ITS-2 gen bölgesini çoğaltmak için PCR yöntemi sırasıyla serolojik ve moleküler analizlerde kullanıldı. *D. immitis*'in serolojik ve moleküler prevalansı sırasıyla %6.7 ve %2.7 olarak belirlendi. Ayrıca 3 köpeğin kan yaymalarında *D. immitis*' in mikrofilerleri saptandı (%0.8). İllere göre enfeksiyonun dağılımı Edirne'de %14.7, Kırklareli'nde %11, Tekirdağ'da %1 ve İstanbul'da %0 olarak bulundu. Gizli enfeksiyon oranı %59.3 olarak saptandı. Bu çalışma *D. immitis*'in varlığını, Trakya Bölgesinde ilk kez ortaya koydu. Bu bölge Avrupa'ya geçiş noktası olup, bu çalışmada elde edilen veriler Kalp Kurdu Hastalığı'nın bölgedeki kontrolüne katkı sağlayabilir.

Anahtar sözcükler: *Kalp Kurdu Hastalığı, Köpek, Seroloji, PCR, Trakya Bölgesi, Türkiye*

INTRODUCTION

Dirofilaria immitis is the causative agent of canine heartworm disease which leads to serious health problems even death in dogs. It threatens not only the animals' health but also the humans as it is a zoonotic disease. While adult worms are found in pulmonary arteries, heart and vena cava, microfilariae are found in the blood of dogs. Different species of mosquitoes belonging to the family of Culicidae (e.g. *Anopheles* spp., *Aedes* spp., *Culex* spp.,) transmit the infection between the dogs [1]. *Aedes vexans* and *Culex pipiens* were reported as the potential vectors of *D. immitis* in Central Turkey [2]. Temperature has influence not only on the survival of mosquitos but also on

İletişim (Correspondence)
+90 532 4659754, Fax: +90 212 4737241
handancet@hotmail.com, handan@istanbul.edu.tr

the development of infective microfilariae (L3) in them. The amount of microfilariae in the peripheral blood of the dogs varies daily and seasonally [3]. Occult (amicrofilaremic) infections can occur in consequence of the presence of only the same gender, prepatent infection or sterile adult worms depending on drug-induced [4].

There are kind of methods for detection of adult *D. immitis* and their microfilariae in peripheral blood of dogs. Many commercial serologic kits detecting the antigen of adult female *D. immitis* can be used for diagnosis. Microfilariae of the nematode can be determined in the blood samples by the concentration methods, filter tests, histochemical staining and PCR assay [5]. Among these techniques, PCR assay is a sensitive and rapid assay for differentiation of *D. immitis* microfilariae from the microfilariae belong to other filarial worms living in dogs [6,7].

Heartworm disease has worldwide distribution and the rate of this infection has been increasing through the central and northern Europe [1]. Many researchers [8-10] reported the presence of the infection in different countries around the world. In Europe, the prevalence range of the infection reported between 0.2% [11] and 28.7% [12]. *Dirofilaria immitis* also reported from different cities of Turkey by serologic and molecular assays and the prevalences were between 1.5% [13] and 46.2% [14].

Best of the authors' knowledge, *D. immitis* has not been reported in the Thrace Region of Turkey. This region is border to Europe and has an intensive movement of pet animals. The aim of this study was to determine the prevalence of *D. immitis* in shelter dogs in four provinces of the Thrace Region of Turkey.

MATERIAL and METHODS

This study has been approved by the ethical committee of Istanbul University (ref: 2006/168).

A total of 402 blood samples were collected from the shelter dogs in Istanbul (Avcilar, Altinsehir, Bakirkoy, Beyoglu, Gurpinar, Kemerburgaz, Silivri, Zeytinburnu districts), Edirne (center, Kesan district), Tekirdag (center, Corlu, Cerkezkoy, Saray districts), and Kirklareli (center, Luleburgaz, Igneada districts) provinces located in the Thrace Region of Turkey. The blood samples were taken from the cephalic vein of the dogs into the tubes containing EDTA. Blood smears were prepared and stained with May-Grünwald Giemsa. The blood smears were examined for the presence of the microfilariae of *D. immitis* under the light microscope. Identification of the microfilaria species was based on morphological and morphometric characteristics according to Genchi et al.[5].

Serologic Assay

A commercial kit SNAP3Dx (IDEXX Laboratories, Westbrook, ME, USA) was used to detect *D. immitis* antigens. This kit includes two antibodies (one for capture and the other for detection) which are specific to antigens of adult female *D. immitis*. Whole blood was used according to the kit manufacturer's instructions.

PCR Assay

DNA extraction was carried out with a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. PCR assay was performed using the primers D.imm-F1 CAT CAG GTG ATG ATG TGA TGA T and D.imm-R1 TTG ATT GGA TTT TAA CGT ATC ATT T which targeted to the ITS2 region of *D. immitis* [6]. The PCR reaction mix consisted of 2.5 µl 10X Taq polymerase buffer, 1.5 µl $MgCl_2$ (25 mM), 0.5 µl of each primer (200 mM), 0.12 µl (5 U) Taq polymerase (MBI, Fermentas, Lithuania), 0.5 µl dNTPs (200 mM each) and 5 µl template DNA with a final volume of 25 µl. All reagents except DNA were added to negative control. PCR was carried out in a thermal cycler (MaxyGene Gradient Thermal Cycler, Axygen Scientific, USA) with the following conditions: initial denaturation 3 min at 94°C, followed by 35 cycles of denaturation 45 sec at 94°C, annealing 45 sec at 60°C, extension 45 sec at 72°C and a final extension 7 min at 72°C. PCR products were electrophoresed through 1.5% agarose gels containing ethidium bromide (10 mg/ml) and the expected DNA fragments of 302 bp were visualised under UV.

Sequencing

All positive PCR products were sent to a commercial company (BGI, Shenzhen, China) for sequencing in order to confirm the validity of PCR amplifications. Sequences were compared with sequences of *D. immitis* available in the GenBank using Blast analysis.

Statistical Analysis

Haematological parameters of the infected dogs were compared with non-infected dogs using independent samples t-test. The differences of prevalence with serologic and molecular assays according to the cities were analysed with Chi- square test. $P<0.05$ was considered as significance level in both statistical analyses.

RESULTS

The results of serologic, molecular and microscopic analyses according to the provinces are given in *Table 1*. Out of 402 blood samples, 27 (6.7%) were positive serologically and 11 (2.7%) were positive by molecular analysis. While there was no significant difference in prevalence between Edirne and Kirklareli, these two cities had significant differences between Istanbul and Tekirdag with both serologic and molecular assays (P=0.001 and P=0.009 respectively) *(Table 1)*. The seropositivity was higher in Edirne (14.7%) and Kirklareli (11%) than Tekirdag (1%). None of the dogs from Istanbul was found to be

Table 1. *Results of serologic, PCR and microscopic examination assays for the detection of D. immitis in examined dogs according to the provinces in the Thrace Region*

Tablo 1. *İncelenen köpeklerde D. immitis'in belirlenmesinde kullanılan serolojik, PZR, mikroskobik inceleme yöntemlerinin Trakya bölgesindeki illere göre sonuçları*

Sampling Provinces	Number of Seropositive Dogs by Serologic Test	Number of Positive Dogs by PCR	Number of Positive Dogs by Microscopic Examination
Edirne (n = 102)	15 (14.7%) [a]	5 (4.9%) [a]	0
Kırklareli (n = 100)	11 (11%) [a]	6 (6%) [a]	3 (3%)
Tekirdag (n = 100)	1 (1%) [b]	0 [b]	0
İstanbul (n = 100)	0 [b]	0 [b]	0
Total (n = 402)	27 (6.7%)	11 (2.7%)	3 (0.8%)
X^2	26.174	11.498	
P	0.001	0.009	

[a, b] *Different letters within same column indicate significant differences between cities*

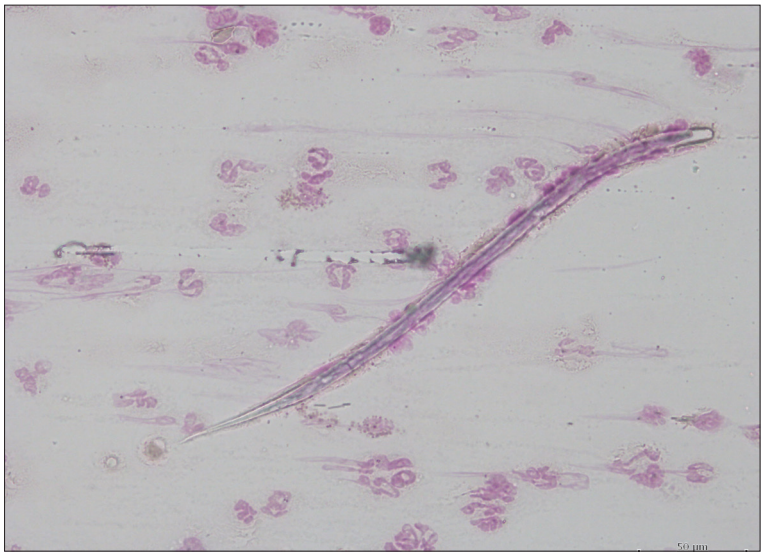

Fig 1. Microfilaria of *Dirofilaria immitis*

Şekil 1. *Dirofilaria immitis'*in mikrofileri

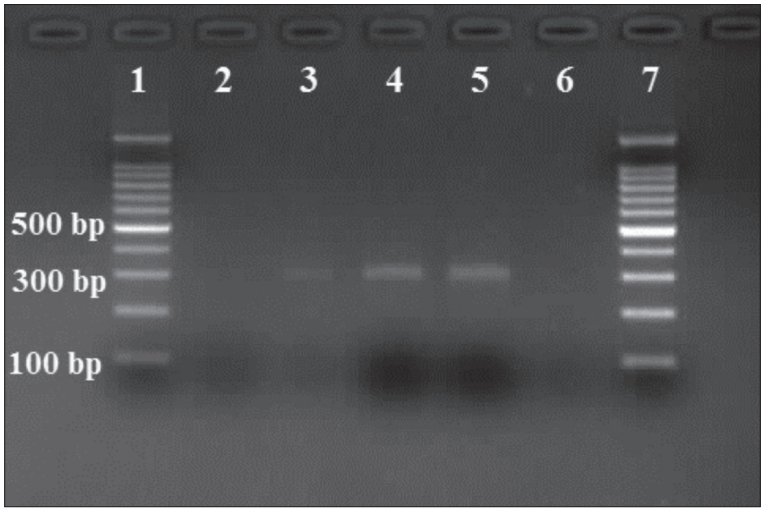

Fig 2. Agarose gel images of positive PCR products belong to *D. immitis*. Lane 1: 100 bp DNA marker, Lane 2: negative control, Lane 3, 4, 5: *D. immitis* positive samples (302 bp), Lane 6: negative control, Lane 7: 100 bp DNA marker

Şekil 2. *D. immitis'*e ait pozitif PZR ürünlerinin agaroz jel görüntüleri. şerit 1: 100 bp DNA marker, şerit 2: negatif kontrol, şerit 3, 4, 5: *D. immitis* pozitif örnekler (302 bp), şerit 6: negatif kontrol, şerit 7: 100 bp DNA marker

positive either by serologic or molecular assays. 16 of 27 seropositive blood samples were negative by both PCR and microscopic analyses result in 59.3% occult infection. Microfilaria *(Fig. 1)* was only found in blood smears from three dogs (0.8%) which were also positive with serologic and PCR assays by microscopic examination. The microfilariae were 290-295 µm in length and 6-7 µm in width with straight tail. The positive samples gave the expected amplification products of 302 bp for *D. immitis* by PCR and the gel images of DNA are shown in *Fig. 2*.

Sequences of positive samples were analysed by BLAST and were found closely related to DNA sequences of *D. immitis* available in GenBank (accession numbers: JX481279.1, EU182329.1, AF217800.2). There were no significant differences in haematological parameters between infected and non-infected dogs (data not shown).

DISCUSSION

Dirofilariasis is a fatal disease in dogs with worldwide distribution. In the current study, this infection was investigated for the first time in shelter dogs located in the Thrace Region of Turkey by serologic, molecular and microscopical assays. The seroprevalence of the infection was reported as 1% in Iran [15], 18% in India [16], 13.5% in China [10] and 55.3% in Russia [8]. In Europe, the seroprevalence was recorded as 9.6% in Italy [17], 2.4% in Spain [18] and 2.1% in Portugal [19]. The first case report of *D. immitis* was notified in an imported dog in Turkey, in 1951 [20]. Tasan [21] reported the prevalence of the infection as 5% for the first time in native dog breed. Until the year 2000, the studies on the prevalence of *D. immitis* had been performed using necropsy and/or Knott technique [21-27]. After development of commercial serologic tests for the diagnosis of *D. immitis,* many researchers used them in Turkey. The seroprevalence was 2% in Şanlıurfa [28] and 2.4% in Diyarbakır [29] which were lower than this study (6.7%). Considering the other provinces, the seroprevalence was found to be 9.1 % in Elazıg [30], 9.3% in Ankara [31], 9.6% in Kayseri [32], 17.8% in Van [33], 26% in Hatay [34], 34.5% in Kırıkkale [35], 40% in Igdır [36], 46.2% in Van [14] which were higher than the seroprevalence determined in the current study. There are limited studies on the diagnosis of this nematode in dogs by molecular techniques in Turkey. The frequency of the infection was reported as 5.4% in Kars [37], 19.6% in Igdır [37] and 8.1% in Erzurum [38] by PCR which were higher than the current study. PCR assay was also used for determination of *D. immitis* microfilariae in the mosquitos as the vectors in Turkey and the ratio of infected pools in *Aedes vexans* and *Culex pipiens* were reported as 0.32% and 1.24%, respectively [2].

First case report of *D. immitis* in three dogs in Istanbul was reported in 2005 [39]. In another study [13], in Istanbul the seroprevalence of the infection was reported as 1.5%. However, no infection was found in the examined dogs from Istanbul in the current study. To our knowledge, there was no information about the heartworm disease in the provinces of Edirne, Kirklareli and Tekirdag until this study. The seroprevalence of the dirofilariasis was 16.2% in Bulgaria [40], 17.9% in Greece [41] and 28.7% in Romania [12]. These countries have borders to the provinces of Edirne and Kirklareli and the seroprevalence in these countries were higher than the rate obtained with this study.

Occult infection rates were reported as 61.4% [34], 36.1% [37], 35.5% [42], 29.6% [32] and 27.5% [35] in other studies conducted in Turkey. The high rate of occult infections (59.3%) was also observed in this study. The reason of this high rate could be attributed to the low parasite burden, the presence of only the same genders of nematodes in the host, the prepatent infection or the treatment with a microfilaricide in the area which were also mentioned by several researchers [4,32,34]. The time of the blood collection in this study might also affect the results of the microfilariae detection assays. The amount of the microfilariae in the circulating blood varies daily [3] and the blood of dogs were not taken at a certain time interval in this study.

It was reported that the prevalence of *D. immitis* was higher in outdoor, stray and suburban dog population than the indoor, owned and urban dogs [9,33,34]. Parallel to these reports, the dog population was consisting of stray dogs which were living in shelters in the present study.

In conclusion, the prevalence of *D. immitis* was determined in dog populations for the first time in the Thrace Region of Turkey with this study. This region has border to Europe and is the transition point. The results of this study might contribute to prevention and control measures against the spreading of the disease.

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Research Article

Bovine Hypodermosis in the Maghreb: Sero-epidemiological Study in Algeria by Indirect ELISA

Khelaf SAIDANI [1,3] Ceferino LÓPEZ-SÁNDEZ [2] Pablo DÍAZ-FERNÁNDEZ [2]
Eva CABANELAS-DOPAZO [2] Ana PEREZ-CREO [2] Abdenour KADDOUR [3]
Patrocinio MORRONDO-PELAYO [2] Pablo DÍEZ-BAÑOS [2]
Ahmed BENAKHLA [4] Rosario PANADERO-FONTÁN [2]

[1] Superior National Veterinary School of Algiers, Issad Abbes Street, Oued Smar, ALGERIA
[2] Departament of Patología Animal (INVESAGA GROUP), Lugo Veterinary Faculty, University of Santiago de Compostela, 27071 Lugo, SPAIN
[3] Institute of Veterinary Sciences, 270 road of Soumaa, Saad Dahleb University, Blida - ALGERIA
[4] Department of Veterinary Sciences, Faculty of Natural and Life Sciences, University of El-Tarf, ALGERIA

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Abstract

During May-July 2014, a total of 837 cattle serum samples were collected from 4 departments in northern Algeria and tested for the presence of *anti-Hypoderma* antibodies by indirect ELISA. Warble counts and some intrinsic (age, breed, sex) and extrinsic factors (location, husbandry system) were also considered. The overall seroprevalence by indirect ELISA was 49.8%, whereas by clinical inspection only 25.7% of the animals were infested. Except for the sex, all the factors included in the study were significantly associated with seroprevalence. The husbandry system was identified as the factor with more influence in seroprevalence, so cattle kept under extensive (79%) or semi-extensive (50.6%) husbandry systems have 74.10 and 18.13 more risk than those under an intensive system (5.8%), respectively. Both the seroprevalence and the intensity of infection are inversely related with the age of the animals. So, the youngest animals (<3 years) are less frequently infested but display the highest intensities of infection. The seroprevalence detected in northern Algeria reflects a high exposition of cattle to *Hypoderma* spp. To minimize the economic losses due to this myiasis it is essential to establish control programs based on serological surveillance prior to the administration of macrocyclic lactones.

Keywords: *Bovine hypodermosis, Maghreb, Algeria, Indirect ELISA, Seroprevalence, Risk factors*

Cezayir - Mağrip'te Bovine Hipodermozisi: İndirek ELİSA İle Sero-epidemiyolojik Bir Çalışma

Özet

Mayıs - Temmuz 2014 tarihleri arasında Kuzey Cezayir'de 4 bölgeden toplam 837 sığır serum örneği indirek ELİZA ile anti-Hipoderma antikorlarını belirlemek amacıyla toplandı. Çıban sayımları ve bazı iç (yaş, tür, cinsiyet) ve dış (lokasyon, barınma koşulları) faktörler dikkate alındı. İndirek ELİZA ile seroprevalans %49.8 olarak tespit edilirken klinik incelemede hayvanların %25.7'i enfeste olarak belirlendi. Cinsiyet hariç diğer tüm faktörlerin anlamlı derecede seroprevalans ile ilişkili olduğu tespit edildi. Barınma koşullarının seroprevalansı en fazla etkileyen faktör olduğu belirlendi. Eksentif (%79) veya yarı-eksentif (%50.6) şartlarda barındırılan hayvanlarda intesif şartlara oranla (%5.8) risk sırasıyla 74.10 ve 18.13 olarak tespit edildi. Hem seroprevalans hem de enfeksiyon yoğunluğu hayvanların yaşı ile ters orantılıydı. Genç hayvanlar (<3 yaş) daha az sıklıkla infeste olurken en şiddetli enfeksiyonu gösterdiler. Kuzey Cezayir'de belirlenen seroprevalans sığırlarda yüksek düzeyde *Hipoderma* spp. maruziyetini göstermektedir. Bu miyazise bağlı ekonomik kayıpları minimalize etmek için makrosiklik lakton uygulamalarından önce serolojik taramalar ışığında kontrol programlarının geliştirilmesi önemlidir.

Anahtar sözcükler: *Bovine hipodermozis, Mağrip, Cezayir, İndirekt ELİSA, Seroprevalans, Risk faktörleri*

INTRODUCTION

Cattle warble fly infestation (WFI), caused by *Hypoderma bovis* and *H. lineatum*, is a common myiasis found in all continents of the northern hemisphere. Despite the absence of ostensive clinical signs, *Hypoderma* spp. can cause economic losses by reducing milk production, increasing the meat trim and damaging the hides [1].

İletişim (Correspondence)
+213 554209276
kamel_khelaf@yahoo.fr

Currently, control measures against bovine hypodermosis are mainly focused on the administration of broad-spectrum macrocyclic lactone compounds, involving some potential environmental risks [2]. Despite effective chemotherapy, warble flies continue infecting cattle in North America [3,4], some countries in Europe [5,6], northern Africa [7] and Asia [8,9].

There are several diagnostic methods designed to detect the disease including larval detection by grub monitoring or the examination of carcasses at slaughter. However, by using the traditional method of grub detection by back palpation, the infestation is diagnosed at very late stages when most of the damage has already been done; thus an earlier diagnosis is needed to minimize the economic losses associated to this myiasis. The detection of antibodies against first instars (L1) at the beginning of the migratory phase allows systemic treatment, avoiding the damage caused by the parasite on the host tissues [10]. The Enzyme Linked Immunosorbent Assay (ELISA) has been widely used to monitor the occurrence of hypodermosis in Britain [11] and other European countries for a number of years [12] and has been recommended for surveillance when clinical diagnosis becomes impractical because of low infestation levels [3,13]. Anti-*Hypoderma* antibodies appear within 4-8 weeks post infection during the migration of L1 and persist at positive levels for 3 or 4 months after the emergence of the third instar larvae [13,14].

Bovine hypodermosis is a major economic problem in the Maghreb, particularly affecting the leather industry [15]. In Morocco, Sahibi *et al*.[16] reported a global seroprevalence of 49.5%. Jemli *et al*.[17], in northern Tunisia found by clinical examination a herd and individual prevalence of 90% and 30%, respectively. Northern Algeria is in the southern limit of distribution of hypodermosis. Up to date, little is known about the distribution of WFI in this country and all the published data make reference to clinical studies based on warble counts, with prevalences ranging from 18.1% [7] to 84% [18]. Unfortunately, in Algeria, like in other Maghrebian countries cattle breeders are confronting with severe economic losses in the absence of warble management programs.

Bearing in mind the importance of ELISA for the early detection of hypodermosis in cattle, the present study was designed to determine the seroprevalence of this myiasis in Algerian cattle by using indirect ELISA. In addition, relationships between seroprevalence and intensity of infection with some intrinsic and extrinsic risk factors such us age, breed, sex, husbandry system and location were also analyzed.

MATERIAL and METHODS

Study Area

Northern Algeria is in the temperate zone and has a mild Mediterranean climate. However, its broken topography provides sharp local contrasts in both temperatures and precipitations. This area, comprising from the coast to the Tell Atlas, is inhabited by more than the 90% of Algeria´s population, because it is the most fertile region in the country. The geographic distribution of cattle, very scarce in southern Algeria, follows almost the same pattern as human population.

The present study has been conducted in 4 departments from 2 bioclimatic areas of northern Algeria: The humid area (Departments of Tizi Ouzou and Boumerdes), with mild temperatures and annual precipitations around 1.000 mm, and the Semi-arid area (Bouira and Bourdj Bou Arreridj) with scarce precipitations and large differences between high and low temperatures [19].

Animals and Samples

During May-July 2014, a total of 837 bovine blood samples were randomly collected from northern Algeria. Simultaneously, clinical examination by back palpation of the animals was carried out to assess the intensity of infection.

Different factors (age, sex, breed, location, grazing pattern) were recorded during the sampling in order to analyze their influence on warble fly prevalence. Variables were grouped and categorized for statistical analysis as follows:

Age groups: 1 (<3 years), 2 (3-5 year), 3 (>5 year).

Climatic area: 1 (Humid area), 2 (Semi arid area).

Department: 1 (Tizi Ouzou), 2 (Boumerdes), 3 (Bouira), 4 (Bourdj Bou Arreridj).

Breed: 1 (Montbéliard), 2 (Crossbreed), 3 (Atlas Brown).

Husbandry system: 1 (Intensive), 2 (Semi-extensive), 3 (Extensive).

Sex: 1 (Male), 2 (Female).

Indirect ELISA Protocol

Vinyl flat bottom microtiter plates (Thermo Scientific) were coated with a total extract, obtained from L1 of *H. lineatum* collected at the slaughterhouse. The antigen was added at a concentration of 5.5 µg/mL in phosphate-buffered saline (PBS, pH 7.5) and incubated at 37°C for 30 min in agitation. Unspecific unions were blocked for 30 min at 37°C with PBS containing 0.2% Tween-20 (PBS-T) and 1% skimmed milk powder as dilution buffer (PTL). Serum samples were tested in duplicate at 1:10. A positive and negative pooled serum was used as standards in each plate. Following the addition of sera, plates were incubated for 1 h at 37°C. After washing, Immuno-Conjugate (Horseradish peroxidase rabbit antibovine IgG (H + L); Bio Rad Laboratories) was used at 1:800 dilution in PT and the incubated for 30 minutes at 37°C. Finally, the reaction was revealed with *O*-phenylenediamine (SigmaFast OPD tablets; Sigma-Aldrich) and stopped by the addition of

100 µL of 3N H2SO4. After 2-5 min the absorbance was measured at 492 nm using a spectrophotometer (680XR; Bio-Rad Laboratories).

Cut-off Estimation

The cut-off value was determined by means of the receiver operating characteristic (ROC) analysis on sera from positive and negative control populations. Positive sera (n=27) came from naturally infected cattle which have palpable grubs and negative sera (n=51) from cattle with no previous exposure to *Hypoderma*. Levels of sensitivity were plotted against 1-specificity at each cut-off point on an ROC curve [20]. The area under the ROC curve is a simple and well suited overall measure of diagnostic test accuracy. In this study, the optimal combination of sensitivity and specificity was determined as 92.3% and 94.1% respectively, for a cut-off value of absorbance 0.740 *(Fig. 1)*.

Statistical Analysis

In order to assess the influence of some intrinsic (age, sex and breed) and extrinsic factors (husbandry system and location) in both seroprevalence and clinical prevalence of WFI, a logistic regression algorithm was applied. The dependent variable was the presence of warbles in each animal. Factors indicated previously were introduced in a backward conditional method and removed from the model one by one (on the basis of the highest p-value) until the best model was built [21]. Next, all pairwise interactions that were biologically plausible were evaluated. Odds ratio were computed by raising e to the power of the logistic coefficient over the first category of each factor, not over the last.

A multifactorial ANOVA over positive animals was used for the examination of the intensity of infestation; the dependent variable –number of nodules counted in animals– has been analyzed after having verified the variance homogeneity. Tukey HSD post hoc test was used to detect the differences between pairs.

Chi-squared automatic interaction detector has been performed to stratify risk factors in order of importance. CHAID algorithm identified factors that divide cattle in subgroups with different positive/negative ratio. CHAID is a tool to identify mayor factors using as criteria the significance of a Chi-squared test and successively splitting data in increasingly homogeneous nodes in relation to dependent variable (warble presence) until the classification tree is fully grown.

Spearman rank correlation was applied to measure the strength of a potential association between the number of warbles and the optical density corresponding to each warbled animal.

Statistical analyses were done using R statistical package v. 3.2.0 (22). CHAID algorithm was performed with CHAID function [22].

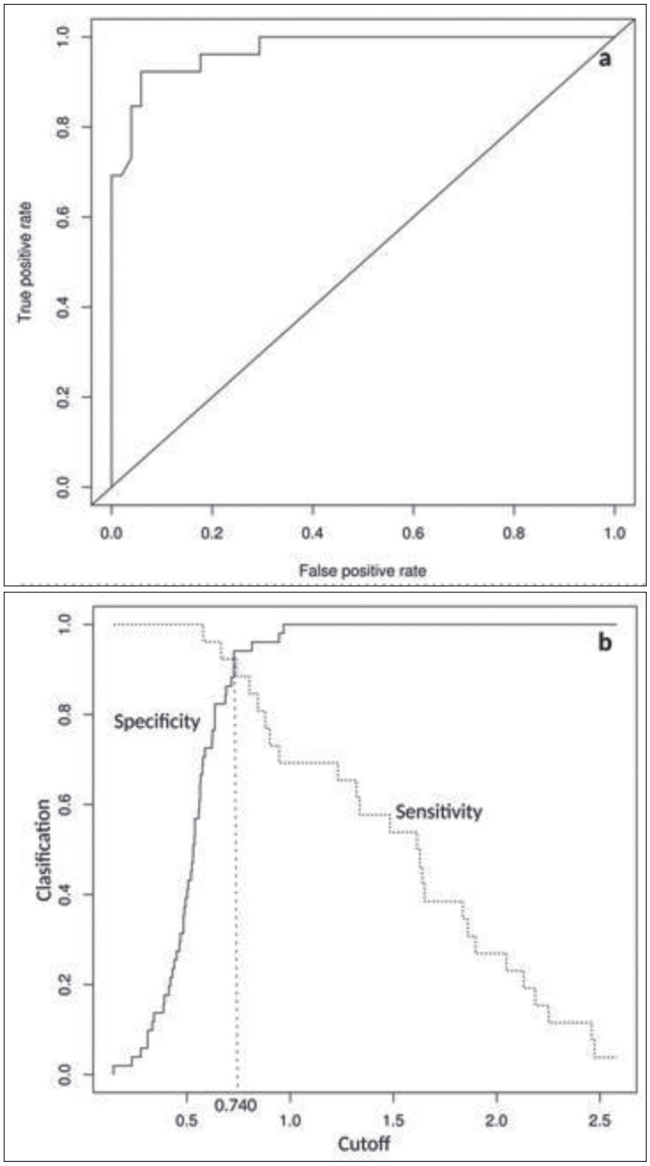

Fig 1. (a) Relative operating characteristic curve analysis of the results achieved with the indirect ELISA (b) Representation of the sensibility and specificity of the ELISA by using *Hypoderma* positive and negative control populations to obtain the cutoff value

Şekil 1. (a) İndirek ELİZA ile elde edilen sonuçların karakteristik analiz eğrisi (b) Hipoderma pozitif ve negatif kontrol popülasyonları kullanılarak elde edilen eşik değeri ile belirlenen ELİZA'nın duyarlılığı ve özgüllüğü

RESULTS

The overall seropositivity by *Hypoderma* spp. in cattle from northern Algeria was 49.8% (417 out of 837); when considering warble counts only the 25.7% of the animals were infested.

The results of the serological survey are summarized in *Table 1*. WFI was detected in all the departments included in the study; the highest prevalence was recorded in Tizi Ouzou (63.9%) located in the humid area and the lowest in Bourdj Bou Arreridj (42.4%) placed in the semiarid area.

Table 1. *Seroprevalence and intensity of infection by Hypoderma spp. in cattle from northern Algeria*
Tablo 1. *Kuzey Cezayir'de sığırlarda Hipoderma spp. seroprevalansı ve enfeksiyon yoğunluğu*

Factor	Levels	Number of Animals	Seroprevalence (%)	95% Confidence Interval	Intensity of Infection * Mean (SD)
Location	Tizi Ouzou	183	63.9	56.5–70.8	9.0(3.30)
	Boumerdes	217	44.6	37.9–51.6	9.58(4.38)
	Bouira	194	52.0	44.8–59.2	8.93(3.77)
	B. B. Arreridj	243	42.4	36.1–48.9	9.22(4.79)
Age	<3 years	205	42.4	35.6–49.5	11.6(3.07)
	3-5 years	371	47.4	47.4–52.7	7.8(4.05)
	>5 years	261	59.0	52.8–65.0	7.5(3.20)
Sex	Male	161	50.9	43.0–58.8	8.8(3.71)
	Female	676	49.6	45.7–53.4	9.3(4.07)
Husbandry System	Intensive	52	5.8	1.5–16.9	1.0(0)
	Semi extensive	708	50.6	46.8–54.3	8.6(4.09)
	Extensive	77	79.2	68.2–87.3	11.3(2.44)
Breed	Monbéliard	56	53.6	39.9–66.8	10.2(3.90)
	Crossbreed	396	52.2	47.5–57.5	9.2(3.60)
	Atlas Brown	385	47.7	42.2–52.4	9.0(4.33)

** Over warbled cattle*

Table 2. *Last step of the Logistic regression analysis when considering ELISA data*
Table 2. *ELİSA verisi dikkate alındığında Lojistik regresyon analizinin son basamağı*

Factors	Estimate	S.E.	Z Value	P	OR*	Lower Upper 95% CI for OR	
B.B. Arreridj							
Bouira	0.2814	0.2845	1.377	0.1684	1.3249	0.8878	1.9773
Boumerdes	0.1071	0.2008	0.528	0.5976	1.1118	0.7500	1.6479
Tizi Ouzou	1.1071	0.2408	4.599	<0.001*	3.0257	1.8875	4.8501
Monbeliarde							
Crossbreed	0.6045	0.4333	1.761	0.0783	1.8303	0.9339	3.5870
Local breed	0.9609	0.3582	2.683	0.0073*	2.6139	1.2955	5.2743
< 3 years							
3-5 years	0.4594	0.1915	2.399	0.0164*	1.5833	1.0877	2.3842
> 5 years	0.8623	0.2038	4.230	<0.001*	2.3685	1.5884	3.5316
Intensive							
Semi extensive	2.8978	0.6096	4.754	<0.001*	18.1338	5.4904	59.8929
Extensive	4 .3055	0.6714	6.412	<0.001*	74.1085	19.8763	276.3126

** Odds ratio were computed by raising e to the logistic estimation over the first category of each factor*

Analysis of ELISA results by logistic regression *(Table 2)* showed that cattle from Tizi Ouzou are 3.02 times more likely to acquire this myiasis than cattle from Bourdj Bou Arreridj. This binary logistic test indicated that in addition to location, other factors like age, breed and husbandry system were also significantly associated with seroprevalence. So, respect to the age cattle older than 5 years are 2.36 times more likely to be seropositive than cattle younger than 3 years. Likewise, the likelihood of being infected is 2.61 times higher for the local breed, Atlas Brown, than for the Montbéliard. Finally, the risk to get infested in cattle kept under extensive or semi-extensive husbandry systems is 74.10 and 18.13 times higher than in those under an intensive system, respectively.

Chi-squared automatic interaction detector stratified the husbandry system as the most influencing factor for warble fly seroprevalence, followed by the age *(Fig. 2)*.

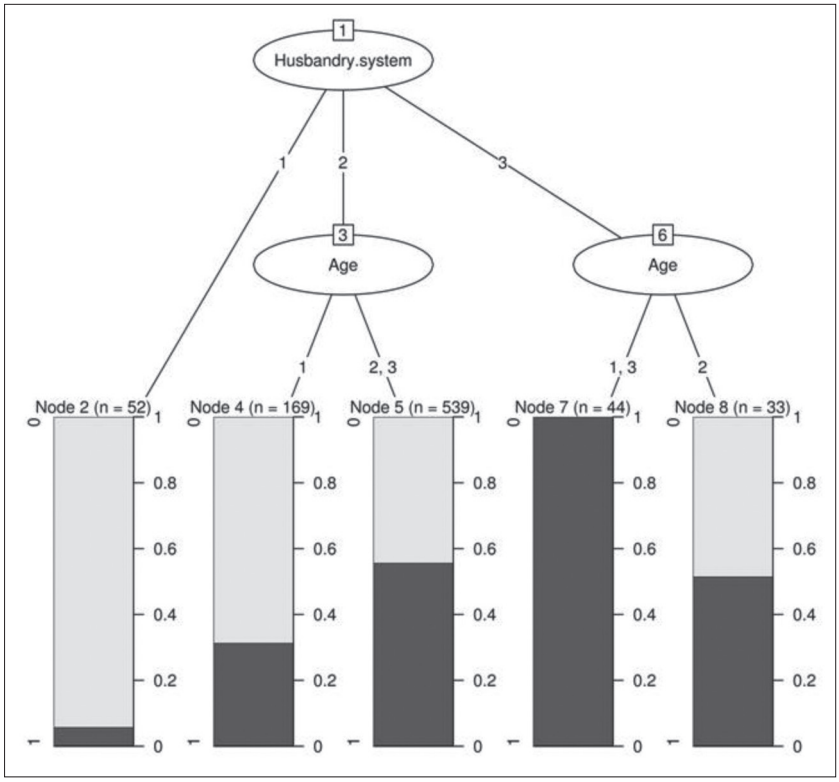

Fig 2. Classification tree produced by the CHAID algorithm when considering the different factors included in the study

Şekil 2. Çalışmada kullanılan farklı faktörler dikkate alınarak CHAID algoritması ile oluşturulan sınıflandırma ağacı

Table 3. *Last step of the Logistic regression analysis when considering clinical data*
Table 3*. Klinik veri dikkate alındığında Lojistik regresyon analizinin son basamağı*

Factors	Estimate	S.E.	Z Value	P	OR*	Lower Upper 95% CI for OR	
B. B. Arreridj							
Bouira	0.5416	0.2501	2.165	0.0383*	1.7188	1.052	2.8064
Boumerdes	0.5324	0.2504	2.126	0.0335*	1.7030	1.0424	2.7820
Tizi Ouzou	1.2641	0.2716	4.654	<0.001*	3.5397	2.0786	6.0276
< 3 years							
3-5 years	-0.7391	0.2096	-3.527	<0.001*	0.4775	0.3166	0.7201
> 5 years	-0.9060	0.2291	-3955	<0.001*	0.4041	0.2579	0.6331
Monbeliarde							
Crossbreed	0.3973	0.3852	1.031	0.3823	1.4877	0.6993	3.1652
Local breed	0.8755	0.4034	2.178	0.0299*	2.4000	1.0005	5.2913
Intensive system							
Semi extensive	1.9234	0.7351	2.616	0.0088*	6.844	1.6201	28.9120
Extensive	3.6715	0.7712	4.761	<0.001*	39.3109	8.9713	178.2122

** Odds ratio were computed by raising e to the logistic estimation over the first category of each factor*

Analysis of the presence or absence of warbles by logistic regression *(Table 3)* showed the location, age, breed and management system as risk factors for the presence of warbles. The results were in accordance to those obtained with the ELISA, with exception of the age of the animals. In this case, oldest cattle have less risk to present warbles on the back than animals under 3 years of age.

As regard to the intensity of infection *(Table 1)*, the number of warbles ranged from 1 to 24 nodules. Multifactorial ANOVA showed significant differences in the intensity of infection when considering the sex (P=0.002) and the age of the animals (P<0.001); with females and animals younger than 3 years showing the highest burdens. Husbandry system also had a significant influence in the number of warbles/animal (P<0.001). Post hoc Tukey's test revealed significant differences between all the categories both of age and husbandry system.

The Spearman rank correlation test (rho= 0.4579, <0.001) applied over warbled animals, revealed a very high association between OD values obtained by ELISA and warble counts.

DISCUSSION

The overall seroprevalence of hypodermosis in cattle from northern Algeria was 49.8%, which means that almost a half of those animals exposed to the parasite. This percentage was very similar to the seroprevalence (49.5%) detected by Sahibi *et al.*[16] in Morocco, and slightly higher than those recorded in other Mediterranean countries such as Turkey (38.6%) [23] and Albania (41.28%) [24].

The prevalence obtained by ELISA is higher than that resulting in the clinical examination. The diagnosis based on grub detection is laborious and relatively insensitive, resulting in an underestimation of infestation levels unless animals were inspected regularly throughout the emergence period [25]. In addition, it detects the infestations when larvae have finished their migrations, i.e. when the damage is done. On the contrary, the ELISA method is more valuable than grub monitoring for epidemiological surveys, and it was used in many countries in the diagnosis of WFIs, because, it has the advantage that it can be applied to many animals rapidly and easily, is also relatively cheap and it can used either milk or serum samples [26]. Moreover, ELISA provides a sensitive indication of how many individuals actually are exposed to infestation and reveals the number of animals that will harbor larvae that would die in the host prior to reaching the back where they are clinically detectable [3].

In this study the results showed that WFI prevails in all the studied departments of Northern Algeria, although in humid areas have more risk to become infected than in semi-arid areas, indicating more favorable conditions for the development and survival of pupal and adult stages [27]. Similar results were recorded by Sahibi *et al.*[16] in Morocco who found higher seropositivity in sub-humid areas (60%) in relation to semi-arid areas (50%).

The outcome of the statistical analysis is consistent with the biological cycle of *Hypoderma* spp. and confirms the free grazing as a major risk factor for WFI. Indeed, the husbandry system is known to exert a major effect on both prevalence and intensity of infection in bovine hypodermosis since extensive husbandry systems are the most favorable for the occurrence of this myiasis, because flies have more chances to contact the animal. This fact agrees with several previous studies [7,9,27-30]. Moreover, the autochthonous local breed Atlas Brown which is raised under semi-extensive and extensive husbandry systems presented higher seroprevalence than dairy breeds like the Montbéliard that is mostly kept intensively.

Both the seroprevalence and the intensity of infection are inversely related to the age of cattle. So, the youngest animals (<3 years) were less frequently infested but displayed the highest intensities of infection. This finding can be easily explained, because this category consisted of a great proportion of calves younger than one year, with no chances to be in contact with the parasite. In contrast, animals younger than 3 years were most heavily infested because the intensity of infection decline with age after repeated infestations [14,31]. According to these authors, cattle develop acquired resistance after repeated exposures to *Hypoderma* larval antigens. This resistance is recognized as an important factor in controlling grub populations and depends on the host's age and the number of larvae invading the host [32].

In addition to husbandry system and age, multifactorial ANOVA also showed significant differences in the intensity of infection when considering the sex of the animals; with females, young and free grazing animals showing the highest grub burdens. This result could be explained by the fact that cows in Northern Algeria are generally raised in semi-extensive and extensive management systems, whereas bulls are mainly kept in stables, decreasing the risk of infection.

Both Pearson's and Spearman's Rank correlations applied over clinically positive animals identified a high positive relationship between OD values and warble burden. Although Pruett and Barrett [33] reported an absence of correlation between circulating antibody levels and the number of grubs achieving their biological cycle, Panadero *et al.*[6] found a positive correlation between IgG levels and warble burdens at different time points throughout the course of the infection.

In conclusion, the seroprevalence detected in northern Algeria reflects a high exposition of cattle, especially those maintained under extensive conditions, to *Hypoderma* spp. To minimize the economic losses due to this myiasis, it is crucial to establish control programs based on serological surveillance to be conducted prior to the administration of macrocyclic lactones.

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Conflict of Interest

The authors declare that they have no competing interests.

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Research Article

Antimicrobial Activity Potential of *Enterococcus* spp. Isolated from some Traditional Turkish Cheeses

Sine ÖZMEN TOĞAY [1] Mustafa AY [2] Sema SANDIKÇI ALTUNATMAZ [3]
Filiz YILMAZ AKSU [3] Özlem EROL TINAZTEPE [2] Ghassan İSSA [4] Serkan Kemal BÜYÜKÜNAL [5]

[1] Department of Food Engineering, Faculty of Agriculture, Uludag University, TR-16053 Bursa - TURKEY
[2] Institute of Natural and Applied Sciences, Çanakkale Onsekiz Mart University, Terzioglu Campus, TR-17100 Canakkale - TURKEY
[3] Food Technology Programme, Vocational High School, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Istanbul - TURKEY
[4] Culinary Programme, Avrupa Vocational High School, TR-34010 Istanbul - TURKEY
[5] Department of Nutrition and Dietetics, School of Health Sciences, Istanbul Arel University, TR-34537 Istanbul - TURKEY

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Abstract

Enterococci can produce enterocins which have antimicrobial activity against Gram-positive and also Gram-negative pathogenic, toxigenic and food-spoilage bacteria. The aim of this study was to determine the antimicrobial activity of *Enterococcus* spp. isolated from traditional Turkish cheeses such as Kashar, *Manyas, Sepet, Kelle, Mihalic, Tulum*. The isolates were tested against *Listeria monocytogenes, Listeria innocua, Listeria ivanovii, Staphylococcus aureus* and *Enterococcus faecalis* and also detected the presence of *ent*A and *ent*B genes of these isolates. Total 66 of enterococcal isolates were obtained from 34 of cheese samples and 25 of these isolates showed antimicrobial activity against tested reference bacteria by using agar spotting method. Also it was determined most of *Enterococcus* spp. carried enterocin encoding *ent*A and *ent*B genes. We concluded that these isolates or their enterocins may have a potential for food preservation, however they should be evaluated in terms of food safety.

Keywords: *Enterococcus, Antimicrobial activity, Enterocin, entA, entB, Traditional cheese*

Bazı Geleneksel Türk Peynirlerinden İzole Edilen *Enterococcus* spp.'nin Antimikrobiyal Aktivite Potansiyeli

Özet

Enterokoklar, patojenik, toksijenik ve gıdalarda bozulma yapan Gram-pozitif ve hatta Gram-negatif bakterilere karşı antimikrobiyal etkiye sahip enterosinler üretebilmektedir. Bu çalışmanın amacı, Kaşar, *Manyas*, *Sepet, Kelle, Mihaliç, Tulum* gibi geleneksel Türk peynirlerinden elde edilen *Enterococcus* spp. izolatlarının antimikrobiyal aktivitelerinin belirlenmesidir. İzolatların, *Listeria monocytogenes, Listeria innocua, Listeria ivanovii, Staphylococcus aureus* ve *Enterococcus faecalis*'e karşı aktiviteleri test edilmiş ve ayrıca izolatlarda *ent*A ve *ent*B gen varlığı araştırılmıştır. Çalışmada 34 peynir örneğinden toplam 66 adet enterokok izole edilmiş ve yapılan testte bunların 25 tanesinin test edilen bakterilere karşı antimikrobiyal aktivite gösterdiği belirlenmiştir. Ayrıca bu izolatların çoğunun enterosin kodlayan *ent*A ve *ent*B genlerini taşıdıkları görülmüştür. Bu izolatların ya da bu izolatlardan elde edilecek enterosinlerin gıda koruyucusu olarak kullanım potansiyeli olduğu ancak izolatların gıda güvenliği yönüyle değerlendirilmesinin gerektiği düşünülmektedir.

Anahtar sözcükler: *Enterococcus, Antimikrobiyal aktivite, Enterosin, entA, entB, Geleneksel peynir*

INTRODUCTION

In Turkey, there are more than 50 cheese varieties and the main produced cheeses are Turkish white cheese, Kashar, *Tulum*, *Lor* and *Cokelek*, etc. The cow's, ewe's and goat's milk are used in the production of these cheeses. Beside of these, there are some local cheeses such as *Abaza*, *Mihalic*, *Sepet* cheese, *Ezine* Goat's cheese, etc.[1-3].

Enterococci are important members of cheese microbial flora during ripening period [1,4] and have an important effect in developing taste and flavour of fermented cheeses, by their proteolytic and lipolytic activities [5-8]. Certain strains of *Enterococcus* spp. may be used as starter cultures, co-cultures or probiotics in the food industry [6-8]. Furthermore, it is known that some enterococci have pathogenic potential [9-13].

İletişim (Correspondence)
+90 224 2941490, Fax: +90 224 2941402
sineozmen@gmail.com, sinetogay@uludag.edu.tr

Food safety, especially the control of food-borne pathogen bacteria such as *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus* have become an increasingly important concern in worldwide. The use of chemical preservatives in food industry has also increased and it is needed to create more natural food preservatives. So, naturally produced antimicrobial agents have a great interest in terms of food processing and also consumer concern [14,15]. Lactic acid bacteria and also enterococci may produce natural biopreservatives called bacteriocin. Bacteriocins show antagonistic effect especially against Gram-positive bacteria [6,16,17] and also Gram-negative pathogenic, toxigenic, and food-spoilage bacteria [17]. It is thought that bacteriocins have bactericidal mechanisms through pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA and inhibition of peptidoglycan synthesis [16]. It is suggested that bacteriocins can be more effective when used in combination with other antimicrobial hurdles such as organic acids, chelating agents or essential oils. This combine effect may provide to reduce the required bacteriocin levels for inhibition. It is expected to find novel bacteriocins with enhanced specificity and potency in the future perspective [15].

Enterococci may produce multiple bacteriocins. Enterocins A and B are most common bacteriocins that can produce by enterococcal isolates and may be found in the same isolates [18,19].

In this study, it was investigated the antimicrobial activity potential and enterocin encoding *ent*A and *ent*B genes of *Enterococcus* spp. isolated from some traditional Turkish cheeses against certain Gram positive bacterial strains such as *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivonovii*, *Staphylococcus aureus* and *Enterococcus faecalis*.

MATERIAL and METHODS

Isolation of Enterococcus spp. from the Cheese Samples

Thirty four samples including Kashar, *Manyas, Sepet, Kelle, Mihalic, Tulum, Orgu* cheeses and Turkish white cheeses were supplied from different cities in Turkey such as Manisa, Izmir, Balikesir, Trabzon and Tekirdag. Ten grams of each cheese sample were homogenized with 90 mL of a Maximum Recovery Diluent (MRD, Oxoid, United Kingdom). Decimal dilution series of samples were prepared in sterile MRD. Following inoculation on Kanamycin Aesculin Azide agar (Oxoid, United Kingdom) including kanamycin sulphate supplement, the samples were incubated at 37°C for 24-48 h, under aerobic conditions. The typical 1-3 black colonies surrounded by black zones were selected from each sample. Purification of colonies was done streaking onto Tryptic Soy agar (Merck, Germany). These isolates were detected at the genus level using Gram staining, catalase test and growth at 6.5% NaCl, 10°C, 45°C, and pH 9.6. Then, they were stored at -20°C in glycerol [20-22].

Determination of Antimicrobial Activity Potential of the Isolates

The antimicrobial activity potentials of 66 of *Enterococcus* spp. isolated from traditional Turkish cheeses were investigated by using agar spotting and well diffusion methods against some pathogens and spoilage bacteria such as *Listeria monocytogenes* ATCC 7644, *Listeria ivanovii* ATCC 19119, *Listeria innocua* ATCC 33090, *Staphylococcus aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 29212.

Agar spotting test were performed by spotting 3 µL of an overnight *Enterococcus* spp. isolates onto the surface of Brain Heart Infusion agar (BHI, Oxoid, United Kingdom) plate and incubating at 37°C for 18-24 h. The plates were overlaid with 10 mL of BHI soft agar (0.7% agar) inoculated with 10 µL of the test culture (*Listeria monocytogenes* ATCC 7644, *Listeria ivanovii* ATCC 19119, *Listeria inocua* ATCC 33090, *Staphylococcus aureus* ATCC 6538 and *Enterococcus faecalis* ATCC 29212). After overnight incubation at 37°C for 18-24 h the plates were examined for clear inhibition zones at around spotted enterococcal isolates. The clear zone diameters were evaluated including the spotted culture [23].

Cell-free supernatants (CFS) were obtained from the enterococcal isolates which showed antimicrobial activity with agar spotting test. CFS which obtained by centrifugation at 12,000g at 4°C for 10 min were used in well diffusion methods for determination of antimicrobial activity potential of the isolates. CFS was then adjusted to pH 7.0 with 1 N NaOH. The wells were made using sterile hollow punches in freshly prepared lawns of the test culture (70 µL of an overnight culture grown in 10 mL BHI broth containing 0.7% agar). Thirty microliter of each neutralized CFS was placed into each well and plates were incubated aerobically for 18-22 h at 37°C. Antimicrobial activity was measured after incubation as a clear inhibition zone around the wells [24,25].

To test the proteinaceous nature of the inhibitors, the active cell-free supernatants, were subjected to various enzymes treatment trypsin and proteinase K, at 1 mg/mL at 37°C during 2 h. Then, the residual activity was detected against indicator strains as above. The absence of inhibition zones indicated protease sensitivity. The heat sensitivity was evaluated by exposing the supernatants to heat at 60°C, 100°C and 121°C for 15 min, then, the inhibitory activity was checked by well diffusion method [26].

Investigation of entA and entB Genes in the Isolates

Enterococcus spp. isolates (n = 25) which showed antimicrobial activity against tested pathogen bacteria were also evaluated in point of presence of *ent*A and *ent*B genes. The isolates were grown overnight at 37°C in BHI broth and the genomic DNAs of all isolates were extracted by phenol-chloroform procedure for the detection of enterocin encoding genes [22]. The bacteriocin (enterocin) encoding genes (*ent*A, *ent*B) were investigated by using

Polymerase Chain Reaction (PCR) with specific primers listed in *Table 1* [26]. The PCR protocole and also PCR components and concentrations were shown in *Table 2* and *Table 3*, respectively.

RESULTS

Total 66 of *Enterococcus* spp. were isolated from 34 of traditional Turkish cheeses samples including Kashar, *Manyas, Sepet, Kelle, Mihalic, Tulum, Orgu* cheeses and Turkish white cheeses.

The *Enterococcus* spp. were examined for their antimicrobial activity against some pathogen and food-spoilage bacteria and 25 of these isolates showed mono- or multi-antimicrobial activity against tested reference bacteria with a different zone diameter *(Table 4)*.

After evaluation of antimicrobial activity potential by using CFS in well diffusion method, pH adjustment, protease and heat sensitivity testing, it was observed the antimicrobial activity all 25 of *Enterococcus* spp. isolates *(Table 4)*. Twelve isolates had antimicrobial activity against *L. monocytogenes* ATCC 7644, seven, nineteen, sixteen and one isolates showed antimicrobial activity with different zone diameter against *Listeria innocua* ATCC 33090, *Listeria ivanovii* ATCC 19119, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 6538, respectively. Most of the isolates (n = 16) showed also multi-antimicrobial activity against tested pathogen and food-spoilage bacteria. It was observed that most of antimicrobial activity zone formed against *Listeria ivanovii* ATCC 19119 and *Enterococcus faecalis* ATCC 29212 test bacteria.

Table 1. *Specific primers of entA and entB genes*
Tablo 1. *entA ve entB genlerinin spesifik primerleri*

Enterocin Genes	Oligonucleotide Sequence	Product Size (bp)
*ent*A	f: AAATATTATGGAAATGGAGTGTAT	126
	r: GCACTTCCCTGGAATTGCTC	
*ent*B	f: GAAAATGATCACAGAATGCCTA	159
	r: GTTGCATTTAGAGTATACATTTG	

Table 2. *The PCR protocol for detecting enterocin structural genes*
Tablo 2. *Enterosin yapısal genlerinin belirlenmesinde kullanılan PZR protokolü*

Program	Temperature (°C)	Time	Cycle
First Denaturation	95	2 min	-
Denaturation	95	30 sec	
Anneling	56	30 sec	35
Extension	72	30 sec	
Final Extension	72	5 min	-

Table 3. *PCR components and concentrations for detecting enterocin structural genes*
Tablo 3. *Enterosin yapısal genlerinin belirlenmesinde kullanılan PZR bileşenleri ve derişimleri*

PCR Components	*ent*A (µL/tube)	*ent*B (µL/tube)	Final Concentration
Sterile bidistilled H_2O	15.7	16.2	-
10X buffer	2.5	2.5	1X
$MgCl_2$ (25 mM)	3.0	2.5	2.5 mM-3.0 mM
dNTP (2.5 mM)	0.3	0.3	0.3 mM/25 µL
Primer forward (10 pmol/µL)	1.0	1.0	10 mM/25 µL
Primer reverse (10 pmol/µL)	1.0	1.0	10 mM/25 µL
Taq polymerase (5U/µL)	0.5	0.5	2.5 U/25 µL
DNA (150 ng/µL)	1.0	1.0	150 ng/25 µL
Final volume	25.0	25.0	-

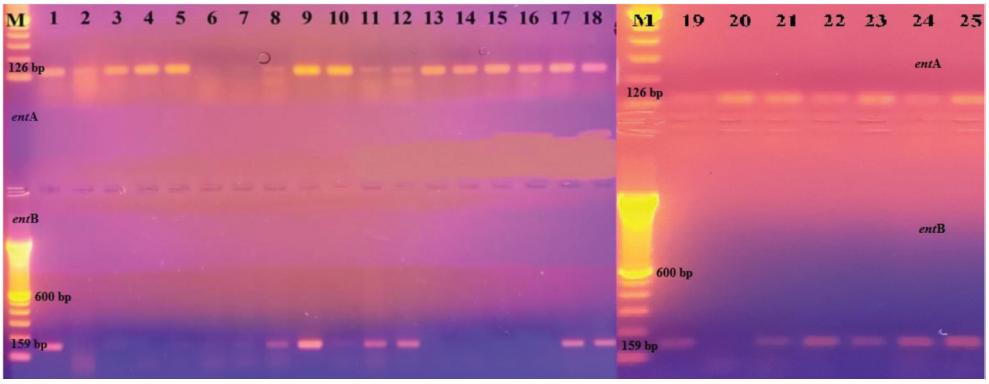

Fig 1. Enterocin structural genes in *Enterococcus* spp. isolated from traditional Turkish cheeses (1. LE 3-1, 2. LE 3-3, 3. LE 4-1, 4. LE 8-4, 5. LE 13-1, 6. LE 13-2, 7. LE 13-3, 8. LE 13-4, 9. LE 14-3, 10. LE 15-1, 11. LE 15-2, 12. LE 19-2, 13. LE 20-1, 14. LE 20-2, 15. LE 20-4, 16. LE 27-1, 17. LE 27-2, 18. LE 29-4, 19. LE 33-3, 20. LE 38-3, 21. LE 47-1, 22. LE 47-2, 23. LE 49-2, 24. LE 49-5, 25. LE 50-3)

Şekil 1. Geleneksel Türk peynirlerinden izole edilen *Enterococcus* spp. suşlarında enterosin yapısal genleri (1. LE 3-1, 2. LE 3-3, 3. LE 4-1, 4. LE 8-4, 5. LE 13-1, 6. LE 13-2, 7. LE 13-3, 8. LE 13-4, 9. LE 14-3, 10. LE 15-1, 11. LE 15-2, 12. LE 19-2, 13. LE 20-1, 14. LE 20-2, 15. LE 20-4, 16. LE 27-1, 17. LE 27-2, 18. LE 29-4, 19. LE 33-3, 20. LE 38-3, 21. LE 47-1, 22. LE 47-2, 23. LE 49-2, 24. LE 49-5, 25. LE 50-3)

Table 4. *Zone diameters of entA and/or entB positive Enterococcus spp. isolates against some test bacteria (mm)*
Tablo 4. *Test edilen bazı bakterilere karşı entA ve/veya entB pozitif Enterococcus spp. izolatlarının zon çapları (mm)*

Isolate No	Source	*L. ivanovii*	*L. innocua*	*L. monocytogenes*	*E. faecalis*	*S. aureus*	*entA*	*entB*
LE 3-1	Kashar cheese	15	-	7	15	12	+	+
LE 3-3	Kashar cheese	-	-	7	-	-	+	-
LE 4-1	Kashar cheese	9	-	-	7	-	+	+
LE 8-4	*Kashar cheese*	11	7	7	12	-	+	-
LE 13-1	*Manyas cheese*	11	-	-	13	-	+	+
LE 13-2	*Manyas cheese*	12	-	-	13	-	+	+
LE 13-3	*Manyas cheese*	11	-	10	10	-	+	+
LE 13-4	*Manyas cheese*	14	-	-	15	-	+	+
LE 14-3	*Kelle cheese*	17	15	20	15	-	+	+
LE 15-1	*Kelle cheese*	11	15	15	15	-	+	+
LE 15-2	*Kelle cheese*	-	-	15	-	-	+	+
LE 19-2	*Kelle cheese*	-	-	11	-	-	+	+
LE 20-1	*Sepet cheese*	10	-	10	10	-	+	-
LE 20-2	*Sepet cheese*	-	-	-	10	-	+	-
LE 20-4	*Sepet cheese*	7	-	-	10	-	+	-
LE 27-1	*Cerkes cheese*	10	-	-	-	-	+	-
LE 27-2	*Cerkes cheese*	12	17	-	-	-	+	+
LE 29-4	*Kelle cheese*	10	10	-	-	-	+	+
LE 33-3	*Mihalic cheese*	-	-	-	7	-	+	+
LE 38-3	*Orgu cheese*	12	12	15	15	-	+	-
LE 47-1	*Tulum cheese*	7	-	-	-	-	+	+
LE 47-2	*Tulum cheese*	15	-	-	-	-	+	+
LE 49-2	Kashar cheese	10	-	-	10	-	+	+
LE 49-5	Kashar cheese	-	-	20	-	-	+	+
LE 50-3	Turkish white cheese	8	10	13	12	-	+	+

It was showed that enterocin structural *ent*A and *ent*B genes in *Enterococcus* spp. isolated from traditional Turkish cheeses in *Fig. 1*. Eighteen of *Enterococcus* spp. isolates had both of *ent*A and *ent*B genes, while all of *Enterococcus* spp. isolates carried only *ent*A gene and eighteen of the isolates had only *ent*B gene *(Table 4)*. The LE 3-1 isolate from Kashar cheese, showed wide antimicrobial activity against *Listeria ivanovii* ATCC 19119, *L. monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 6538, respectively. The LE 14-3 isolate from *Kelle* cheese and LE 49-5 isolate from Kashar cheese showed most effective antibacterial activity against *L. monocytogenes* ATCC 7644 with 20 mm zone diameter *(Table 4)*.

DISCUSSION

Enterococcus species are found in human gastrointestinal tract, farm animals, and different foods such as meats, milk and cheeses [27]. In recent years, there has been enormous increase in the reports about enterococci used as starter cultures, co-cultures and probiotics [6-9]. The ability of enterococci to produce bacteriocins and to adapt to different environmental conditions are important characteristics for the food industry [6,8].

Several researches on determination of antimicrobial activity potential and enterocin encoding genes in *Enterococcus* species isolated from cheese samples were published [16,17,28-31]. Many of the findings are consistent with the results of our study. In a study performed by Renye et al.[29] 33 enterococcal isolates from Hispanic-style cheeses were screened for the production of bacteriocins and 5 *Enterococcus faecium* and 1 *Enterococcus durans* isolates which were inhibited the growth of *Listeria* spp. A PCR screen revealed that four *E. faecium* isolates contained nucleic acid sequences for multiple enterocins (*ent*A, *ent*P, *ent*L50AB and *ent*B). Similar to these results, enterococcal isolates contained multiple enterocin structural genes (*ent*A and entB) and showed antimicrobial activity against *Listeria* spp. (*L. monocytogenes*, *Listeria innocua* and *Listeria ivanovii) (Table 4)*. A study by Tuncer [30] also reported that three of *E. faecium* and one of *E. durans* isolates were found as bacteriocin producer strain obtained from Turkish Tulum cheese samples and most of these isolates showed antimicrobial activity against *Listeria innocua*. Favaro et

al.[16] determined bacteriocinogenic *E. faecium* isolates from Bulgarian homemade white brine cheese and it was reported that the isolates showed antilisterial activity and carried bacteriocin-encoding genes (*ent*A, *ent*B, *ent*P, *ent*L50B).

The *Enterococcus* spp. isolates which carried *ent*A and/or *ent*B genes did not show antimicrobial activity against all tested Gram positive bacteria such as *L. monocytogenes*, *S. aureus* and *E. faecalis* in this study. It was concluded that these enterococcal isolates may carry incomplete or nonfunctional bacteriocin genes or produce additional bacteriocins except *ent*A and *ent*B as mentioned by Abriouel et al.[32].

In conclusion, the single and/or multiple enterocin encoding genes (*ent*A and *ent*B) and also antimicrobial activity were detected in *Enterococcus* spp. isolates from traditional Turkish cheeses in this study. Although enterococcal isolates are widely used as starter and/or probiotic culture in food industry, this genus should be evaluated in terms of presence of virulence genes and acquired antibiotic resistance at the strain level. It is well known that enterococci, especially some of *E. faecium* and *E. faecalis* strains may have pathogenic potential, and cause infectious diseases in humans. However, the antimicrobial activity of *Enterococcus* isolates against *L. innocua*, *L. ivanovii, E. faecalis*, *L. monocytogenes* and *S. aureus* may create an opportunity for use them in food preservation.

Further studies are needed to purify and optimize the isolated enterocins. Safety evaluation of virulence genes and antibiotic resistance in these isolates is also necessary.

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Research Article

The Effects of Exon 2 of Inhibin βB Gene and Exon 3 of FSHB Gene on Litter Size in Akkaraman and Bafra Sheep Breeds [1,2]

Ilke UNLUSOY [1] Okan ERTUGRUL [2]

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[2] *A part of this study was presented at 9. Ulusal Zootekni Bilim Kongresi 2015 in Konya-Turkey as an abstract*

[1] International Livestock Centre for Research and Training, Department of Breeding and Genetics, TR-06852 Lalahan/ Ankara - TURKEY

[2] Ankara University Faculty of Veterinary Medicine, Department of Genetics, TR-06110 Diskapi/Ankara - TURKEY

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Abstract

Inhibin and follicle-stimulating hormone (FSH) are hormones which directly affect ovulation rate in mammals. The aim of this study was to evaluate beta B subunit of inhibin (INHBB: inhibin βB) and beta subunit of follicle-stimulating hormone (FSHB) in terms of prolificacy. For this reason, some polymorphisms of INHBB and FSHB genes were determined with DNA sequencing method in two Turkish native sheep breeds, one of which was Bafra ewes with high prolificacy and the other of which was Akkaraman ewes with low prolificacy. According to GeneBank references, four SNPs, three of which were in INHBB gene and one of which was in FSHB gene, were determined. In Akkaraman ewes, three SNPs, two of which were in INHBB gene and one of which was in FSHB gene, were polymorphic while one SNP in INHBB gene was monomorphic. In Bafra ewes, only one SNP in INHBB gene was polymorphic while the others were monomorphic. All SNPs were investigated in terms of the relationship of litter size within breed and between breeds. In the evaluation of relationship of litter size between breeds, Akkaraman ewes were taken as a low prolific breed to compare Bafra and so it was found that there was no SNP unique in Bafra ewes. In the evaluation of relationship of litter size within breed, variance analyses and logistic regression analyses were used, and each polymorphic SNP was handled individually and together with others (SNPs combination). The effect of SNP and SNPs combination on litter size was insignificant in terms of statistics (P>0.05). Consequently the polymorphisms determined in INHBB and FSHB genes were not related with litter size in Bafra and Akkaraman ewes.

Keywords: *FSHB, Gene, INHBB, Litter size, Sheep*

Akkaraman ve Bafra Koyun Irklarında İnhibin βB Geni Ekzon 2 Bölgesi ve FSHB geni Ekzon 3 Bölgesinin Yavru Verimine Etkisi

Özet

İnhibin ve folikül stimülan hormon (FSH) memelilerde ovulasyon oranını doğrudan etkileyen hormonlardandır. Bu çalışmanın amacı inhibin beta B (INHBB: inhibin βB) ve folikül stimülan hormon B (FSHB) genlerini yavru verimi yönünden değerlendirmektir. Bunun için, Türkiye yerli koyun ırklarından yavru verimi yüksek Bafra koyunları ve yavru verimi düşük Akkaraman koyunlarında INHBB ve FSHB genlerindeki bazı polimorfizmler dizi analiz yöntemi ile tespit edilmiştir. Gen bankası referans dizisine göre üç tanesi INHBB geni içinde bir tanesi FSHB geni içinde olmak üzere toplam dört adet SNP belirlenmiştir. Akkaramanlarda ikisi INHBB geninde biri FSHB geninde olmak üzere üç SNP'de polimorfizm gözlenirken biri monomorfik olarak gözlenmiştir. Bafralarda ise sadece INHBB geninde bir SNP polimorfik olarak gözlenirken diğerleri monomorfik olarak gözlenmiştir. Bu SNP'ler ile yavru verimi arasındaki ilişkiler ırklar arası ve ırk içi araştırılmıştır. Irklar arası değerlendirmede, Akkaraman ırkı yavru verimi düşük ırk olarak alınmış buna göre Bafra ırkına özgü herhangi bir SNP'e rastlanmamıştır. Irk içi değerlendirmede ise polimorfik SNP'lerin tek başlarına ve beraber bulunma durumlarının (SNP kombinasyonları) yavru verimi ile ilişkisine varyans analizi ve lojistik regresyon analizi ile bakılmıştır. Buna göre SNP ve SNP kombinasyonlarının yavru verimi üzerine etkisi istatistiki açıdan önemsiz bulunmuştur (P>0.05). Sonuç olarak INHBB ve FSHB genlerinde tespit edilen polimorfizmlerin Bafra ve Akkaraman koyunlarında yavru verimi ile ilişkili olmadığı belirlenmiştir.

Anahtar sözcükler: *FSHB, Gen, INHBB, Koyun, Yavru verimi*

İletişim (Correspondence)
+90 312 8651196
ilke.unlusoy@gmail.com

INTRODUCTION

Follicle-stimulating hormone (FSH) secreted by hypophysis is the primary hormone regulating mammalian gonad functions [1,2]. FSH included in the follicle growth from early antral stage to ovulation is necessary for the follicle proliferation and survival [3].

FSH has a common α subunit and a possible β subunit. β subunit brings a functional specificity to the hormone [2,4-6]. β subunit of FSH called FSHB has a major effect on FSH level. Therefore, it directly relates with oocyte quantity ovulated [7]. In a study, some polymorphisms on FSHB gene were related with litter size of goats and it was indicated that FSHB could be a candidate gene affecting prolificacy [3].

Inhibin belonging to TGF- β upper family is a growth factor, and a heterodimeric protein which contains a common α subunit and a possible β subunit. The heterodimer of inhibin A consists of α and βA subunits (INHBA), while the heterodimer of inhibin B consists of α and βB subunits (INHBB) [2,8,9].

Oocyte growth depends on FSH level during the oestrus cycle [10]. Inhibin has an important role on the negative feedback mechanism of FSH. When FSH level increases in blood, inhibin is secreted by granulosa cells of antral follicles as a response to decreasing FSH level [11]. Furthermore, FSHB is affected negatively by the presence of inhibin [12]. This mechanism affects the number of oocyte which would ovulate [11]. It was informed that some polymorphisms of INHBB in some sheep breeds were related with multiple births and it was declared that INHBB gene might be a candidate gene effecting on litter size [13].

Bafra sheep breed derived from two Turkish native sheep breeds, Sakiz and Karayaka, has high prolificacy coming from Sakiz sheep breed [14,15]. It was indicated that the litter size of Bafra ewes was between 1.78 and 2.20 [14,15] while the litter size of Akkaraman ewes was between 1.20 and 1.30 [16].

The aims of this study were to detect the polymorphisms in exon 2 and some part of intron closed to exon 2 of INHBB gene, and exon 3 and some part of intron closed to exon 3 of FSHB gene in Akkaraman ewes with low prolificacy and Bafra ewes with high prolificacy and to investigate its relationship with litter size. This study is important in terms of gaining some information about if INHBB and FSHB could be major genes effecting litter size in sheep.

MATERIAL and METHODS

This study was approved by Animal Experimentation Ethics Committee of International Center for Livestock Research and Training (Approval Numbers: 66 and 106).

Animals

Fifty Akkaraman ewes and 49 Bafra ewes were selected for this study. The ages of ewes were 3 to 5 for both breeds. Approximately 200-300 g additional feeding was given to the animals before mating season. After lambing season (February to May), their litter sizes were recorded. While twins in Akkaraman ewes were rarely appeared (litter size: 1.16), multiple births from 2 to 5 in Bafra ewes were frequently appeared (litter size: 2.10). Blood samples were collected from the animals and were stored at -20°C.

DNA Isolation and PCR Amplification

DNA extractions from blood sample were achieved by phenol-chloroform method described by Sambrook and Russell [17]. UV spectrophotometer was used to determine the quality and quantity of DNA extracted. GeneBank references of INHBB gene (Accession number: FJ167874.1) and FSHB gene (Accession number: NC019472.1) were used to design the gene specific primers *(Table 1)*.

For PCR mix, 0.5 µL primer (10 pmol), 2.5 µL 10XTaq Buffer (with KCl), 0.5 µL dNTP (10 µM), 0.3 µL Taq (5U/µL), 5% DMSO, 2 µL genomic DNA (50 ng/µL) were added, and total volume was completed to 25 µL with ddH_2O. Initial denaturation was at 95°C for 3 min, it was followed by denaturation at 95°C for 30 sec, extension was at 72°C for 1 min, final extension was at 72°C for 5 min. The PCR products were controlled in 2% agarose gel with 100bp ladder *(Fig. 1)*.

Sequence PCR

GF-1 PCR Clean-Up Kit (Vivantis, Malaysia) was used for purification of PCR products. Sequence PCR was achieved with two different mixes that were prepared with either F or R primer for each sample. The reaction mixtures consisted of 2 µL primer (3.6 pmol), 2 µL sequencing buffer,

Table 1. *Primers and PCR conditions*
Tablo 1. *Kullanılan primerler ve PCR koşulları*

Primer	Primer Sequence 5´-3´	GC Content	Amplicon Size	Annealing Temperature (°C)	Mg (mM)
Primer-INHBB-Exon-2_F	AGTGGTATTTCTGGTCAGGACGG	52%	897bp	60	1
Primer-INHBB-Exon-2_R	ACTCCTCCACGATCATATTGGGC	52%			
Primer-FSHB-Exon-3_F	TTCAATCCCTGTCTCATTTTG	38%	584bp	53	3
Primer-FSHB-Exon-3_R	AAGCACCCTCGTGTCTGTAAG	52%			

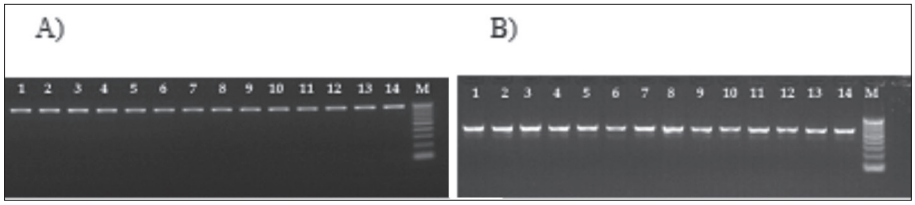
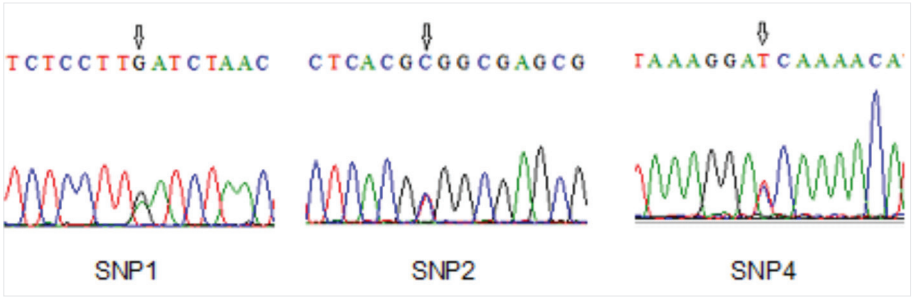


Fig 1. A: INHBB gene, B: FSHB gene (1-14: PCR products, M: 100bp marker)

Şekil 1. A: INHBB geni, B: FSHB geni (1-14: PCR ürünleri, M: 100bp'lik belirteç)

Fig 2. Heterozigosities of SNPs

Şekil 2. Heterozigotluk gözlenen SNP'ler



2 µL sequencing standard, 2 µL PCR product and 2 µL ddH_2O. Initial denaturation was at 96°C for 1 min, the rest of denaturation was at 96°C for 10 sec, annealing was at 50°C for 5 sec, extension was at 60°C for 4 min. The PCR products were precipitated to get rid of salt and heavy metals. Sequence was achieved by the DNA sequencing system.

Bioinformatics and Statistical Analyses

All sequence data were aligned with Bioedit software [18] and the polymorphisms were determined by using GeneBank reference sequences of INHBB gene (Accession number: FJ167874.1) and FSHB gene (Accession number: NC019472.1). Hence a number was given to each polymorphism determined. Then each SNP was controlled if there was the same SNP reported to GeneBank. PLINK 1.9 software [19] was used to compute minor allele frequencies (MAF), expected heterozygosity, observed heterozygosity and Hardy-Weinberg equilibrium.

The relationship of these polymorphisms with litter size was evaluated within breed and between breeds. In the evaluation of relationship of litter size between breeds, Akkaraman ewes were used as low prolific group and all the polymorphisms were controlled if any polymorphisms were specific to Bafra ewes. In the evaluation of relationship of litter size within breed, variance analyses and logistic regression analyses were used to relate the polymorphisms with litter size. Each polymorphism was evaluated individually and together with others (SNPs combination). In the evaluation of polymorphisms individually, genotypes of a polymorphic SNP observed in a breed was analyzed to relate with litter size statistically. In the evaluation of polymorphisms together, a combination was created with a genotype of a polymorphic SNP with the other genotype of the other polymorphic SNPs. SNPs combinations were evaluated with statistical analyses to relate with litter size. Binary model was used for logistic regression analyses.

RESULTS

Four SNPs were determined, three of which were in INHBB gene and one of which was in FSHB gene. SNPs determined were coded as 1, 2, 3, 4. Thus SNP1 was at nucleotide position 1186 of the reference sequence of INHBB and AA, GA, GG genotypes were determined in both breeds. SNP2 was at nucleotide position 1495 in the reference sequence of INHBB and CC, CT, TT genotypes was determined in Akkaraman ewes, while only CC genotype was determined in Bafra ewes. SNP3 was determined at nucleotide position 1611 in the reference sequence of INHBB and only CC genotype which caused the amino acid change from Glutamate (Glu: E) to Alanine (Ala: A) was found in both breeds. SNP4 was determined at nucleotide position 2691 in the reference sequence of FSHB, and CC, CT, TT genotypes were found in Akkaraman ewes while only CC genotype was determined in Bafra ewes. Accordingly, SNP1 was observed in intron of INHBB while SNP2 and SNP3 were observed in exon 2 of INHBB. SNP4 was observed in intron of FSHB. The heterozygosities of SNP1, SNP2 and SNP4 are presented in *Fig. 2*. Minor allele frequency (MAF), expected heterozygosity, observed heterozygosity and Hardy-Weinberg equilibrium of SNPs are presented in *Table 2*.

When SNPs were evaluated between breeds, there was no SNPs particular to Bafra ewes. Thus, the high prolificacy which is the genetic feature in Bafra ewes was not able to be related with the SNPs in question.

Variance analyses and logistic regression analyses were used to evaluate SNPs within breed. In Bafra breed, only SNP1 could be evaluated as polymorphic because the other SNPs were found monomorphic *(Table 3)*. And there was no significant relationship between genotypes of SNP1 and litter size in Bafra ewes (P>0.05). In Akkaraman breed, SNP3 has been found monomorphic, for this reason it was not evaluated. Hence, SNP1, SNP2 and SNP4 were evaluated individually and in SNPs combination *(Table 4)*.

Table 2. *A: Allele evaluation of Akkaraman ewes, B: Allele evaluation of Bafra ewes (CHR: chromosome, A1: minor allele, A2: major allele, NMA: non-missing allel, GENO: genotype ratio, Ho: observed heterozygosity, He: expected heterozygosity, p: p values for Hardy-Weinberg equilibrium)*

Tablo 2. *A: Akkaraman koyunlarının allel değerlendirmesi, B: Bafra koyunlarının allel değerlendirmesi (CHR: kromozom, A1: minör allel, A2: majör allel, NMA: okunan allel sayısı, GENO: genotip oranı, Ho: gözlenen heterozigotluk, He: beklenen heterozigotluk, p: Hardy-Weinberg p değeri)*

A								
CHR	**SNP**	**A1**	**A2**	**MAF**	**NMA**	**GENO**	**Ho**	**He**	**p**
2	SNP1	G	A	0.4082	98	8/24/17	0.4898	0.4831	1
2	SNP2	T	C	0.2500	100	5/15/30	0.3000	0.3750	0.1495
2	SNP3	0	C	0	100	0/0/50	0	0	1
15	SNP4	T	C	0.1700	100	1/15/34	0.3000	0.2822	1
B								
CHR	**SNP**	**A1**	**A2**	**MAF**	**NMA**	**GENO**	**Ho**	**He**	**p**
2	SNP1	G	A	0.3878	98	7/24/18	0.4898	0.4748	1
2	SNP2	0	C	0	98	0/0/49	0	0	1
2	SNP3	0	C	0	98	0/0/49	0	0	1
15	SNP4	0	C	0	98	0/0/49	0	0	1

Table 3. *Evaluation of Bafra ewes within breed*

Tablo 3. *Bafra koyunlarının ırk içi değerlendirilmesi*

SNPs	Genotypes	n	Litter Size Average	Variance Analyses P Value	Logistic Regression P Value
SNP 1	SNP1=GA	24	2.13	0.888	0.720
	SNP1=AA	18	2.17		0.417
	SNP1=GG	7	2.00		0.999

DISCUSSION

In this study, SNPs determined in INHBB gene and in FSHB gene were evaluated. Only few investigators had previously appealed to DNA sequencing method to study INHBB and FSHB genes as candidate genes of prolificacy in small ruminant [13,20].

SNP1 was matched up with the SNP whose accession number was rs409298247 while SNP4 was matched up with the SNP at the position 261 of the nucleotide sequence whose accession number was AY853254 in GeneBank. It was remarkable that SNP2 and SNP3 were the SNPs had not been notified to GeneBank.

All SNPs were in Hardy-Weinberg equilibrium within breeds. SNP1 was a common SNP (MAF>0.05) in both sheep breeds. Therefore SNP1 can be used for other association studies in both breeds. SNP2 and SNP4 were common SNPs (MAF>0.05) in Akkaraman sheep breed. Therefore SNP2 and SNP4 can be used for other association studies in Akkaraman sheep breed.

SNP2 and SNP3 were determined in the exon. It did not change the amino acid structure because SNP2 which was in exon 2 of INHBB gene was silent in codon. SNP3 which was in exon 2 of INHBB was monomorphic in both breeds and caused an amino acid change from Glutamate (Glu: E) to Alanine (Ala: A). However, it was found that this change did not have any effects on litter size. We could not discover any polymorphisms on exon 3 of FSHB gene.

All SNPs even polymorphic or not were evaluated between breeds and it was not found any SNPs which was specific to only Bafra breed. In the evaluation of within breed, polymorphic SNPs and SNPs combinations did not have any effects on the differences of litter size statistically.

Zhang et al.[20] detected a change from Glutamine (Gln) to Arginine (Arg) at exon 3 of FSHB in goat. They called the genotypes of the polymorphism as AA, AB, BB in four goat breeds and declared that this polymorphism had significant effect on litter size. But we could not define any polymorphism at the same nucleotide position in the present study.

Chu et al.[13] defined a SNP at intron of INHBB gene in prolific Hu sheep. The researchers called the genotypes of this SNP as AA, AB, BB and notified that BB genotype had significant effect on litter size compared with AA genotype. But we could not evaluate this polymorphism because this nucleotide position was not between the sequence borders in the present study. SNP1, SNP2 and SNP3 that we determined were not revealed by these researchers.

As a result, it was concluded that the SNPs, which we have evaluated, could not be candidate genes affecting litter size for Akkaraman and Bafra ewes. It could be recommended

Table 4. *Evaluation of Akkaraman ewes within breed. a: One way ANOVA, b: Kruskal Wallis*
Tablo 4. *Akkaraman koyunlarının ırk içi değerlendirilmesi. a: Tek yönlü ANOVA, b: Kruskal Wallis*

SNPs	Genotypes	n	Litter Size Average	Variance Analyses P Value	Logistic Regression P Value
SNP1	GA	25	1.16	0.995[a]	0.564
	AA	17	1.16		0.302
	GG	8	1.17		1.000
SNP2	CT	14	1.17	0.512[a]	0.475
	CC	31	1.14		0.478
	TT	5	1.27		0.230
SNP4	CT	15	1.13	0.388[b]	0.827
	CC	33	1.18		0.999
	TT	2	1.00		0.999
SNP1, SNP2 combination	GA, CT	13	1.18	0.671[b]	0.853
	GA, CC	12	1.14		0.362
	GG, CT	1	1.00		0.362
	GG, CC	2	1.00		1.000
	GG, TT	5	1.27		0.999
	AA, CC	17	1.16		0.174
SNP2, SNP4 combination	CC, CT	12	1.14	0.472[b]	0.700
	CC, CC	18	1.15		0.910
	CC, TT	1	1.00		-
	TT, CT	3	1.11		0.380
	TT, CC	2	1.50		-
	CT, CC	13	1.18		0.713
	CT, CT	1	1.00		-
SNP1, SNP4 combination	GA, CT	4	1.33	0.256[b]	0.722
	GA, CC	19	1.14		0.130
	GA, TT	2	1.00		-
	GG, CT	4	1.08		0.464
	GG, CC	3	1.33		0.482
	GG, TT	1	1.00		-
	AA, CT	7	1.05		0.352
	AA, CC	10	1.23		0.788
SNP1, SNP2, SNP4 combination	GA, CT, CC	13	1.18	0.182[b]	0.954
	GA, CC, CT	4	1.33		0.383
	GA, CC, CC	7	1.05		0.392
	GA, CC, TT	1	1.00		-
	GG, CT, TT	1	1.00		-
	GG, CC, CT	1	1.00		-
	GG, CC, CC	1	1.00		-
	GG, TT, CT	3	1.11		0.999
	GG, TT, CC	2	1.50		-
	AA, CC, CT	7	1.05		0.392
	AA, CC, CC	10	1.23		0.999

that subsequent studies on prolificacy in Bafra and Akkaraman ewes should focus on the other exons and introns of these genes or other possible candidate genes.

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Research Article

Gemifloksasinin *Galleria mellonella* L. (Lepidoptera: Pyralidae) Erginlerinin Bazı Biyolojik Özelliklerine Etkisi [1]

Pınar HIZ [1] Meltem ERDEM [2] Ender BÜYÜKGÜZEL [3] Kemal BÜYÜKGÜZEL [4]

[1] *Bu çalışma "Bülent Ecevit Üniversitesi Bilimsel Araştırma Fonu Tarafından Desteklenmiştir (Proje No: 2012-10-06-07)*

[1] Uludağ Üniversitesi, Fen Edebiyat Fakültesi, Tıp Fakültesi, İmmünoloji Anabilim Dalı, TR-16120 Bursa - TÜRKİYE
[2] Bülent Ecevit Üniversitesi, Ahmet Erdoğan Sağlık Hizmetleri Meslek Yüksekokulu, TR-67100 Zonguldak - TÜRKİYE
[3] Bülent Ecevit Üniversitesi, Fen Edebiyat Fakültesi, Moleküler Biyoloji ve Genetik Bölümü, TR-67100 Zonguldak - TÜRKİYE
[4] Bülent Ecevit Üniversitesi, Fen Edebiyat Fakültesi, Biyoloji Bölümü, TR-67100 Zonguldak - TÜRKİYE

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Özet

Büyük bal mumu güvesi *Galleria mellonella* L. mikrobiyal enfeksiyonların ve klinik ilaçların çalışılmasında memeli modellerine alternatif bir model olarak bilindiği gibi insektisit etkinliği çalışmalarında da model böcek olarak yaygın kullanıma sahiptir. Bu böceğin larvaları yapay besin ortamında beslenerek fluorokinolon sınıfından bir antibiyotik olan gemifloksasinin böceğin eşey oranı, dişi ve erkek ömür uzunluğu, yumurta verimi, açılma oranı gibi ergin biyolojik özellikleri üzerine etkisi laboratuvar şartlarında incelendi. Böceğin birinci evre larvaları %0.001, 0.01, 0.1 ve 1.0 oranında gemifloksasin içeren yapay besinler ile ergin evreye kadar beslendi. Gemifloksasinin denenen konsantrasyonlarını içeren besinler erkek ve dişi eşey oranı ile erginlerin ömür uzunluğu üzerinde etkili olmadığı halde yumurta verimini önemli derecede düşürdüğü belirlendi. Artan besinsel gemifloksasin konsantrasyonları ile ters orantılı olarak yumurta verimi ve açılma oranı azaldı. Kontrol besini ile yetiştirilen dişiler günde 134.46 adet yumurta üretirken gemifloksasinin yüksek miktarlarında dişiler 26.75 ve 53.5 yumurta bıraktılar. Yumurtaların açılma oranı da gemifloksasinin %0.01'lik konsantrasyonundan itibaren önemli derecede azalmış olup, en yüksek konsantrasyon bu oranı %53.71'e düşürdü. Bu çalışma erginlerin biyolojik özelliklerinin gemifloksasin tarafından önemli derecede etkilendiğini ve bu etkilerin antibiyotiğin konsantrasyonlarına bağımlı değişimler olduğunu gösterdi.

Anahtar sözcükler: *Galleria mellonella, Gemifloksasin, Yumurta verimi, Ömür uzunluğu*

The Effect of Gemifloxacin on Some Biological Traits of *Galleria mellonella* (Lepidoptera: Pyralidae) Adults

Abstract

Greater wax moth, *Galleria mellonella* L. is most commonly used as model insects for studying insecticidal efficiency of chemicals as well as it has been known as an alternative model to mamalian model for studying microbial infections and clinical drugs. The effects of a fluoroquinolone antibacterial agent, gemifloxacin, on sex ratio, male and female adult longevity, fecundity and hatchability of this insect were investigated by rearing the first instar larvae on artificial diets in the laboratory condition. The insect was reared from first-instar larvae to adult stage on an artificial diets containing gemifloxacin at 0.001, 0.01, 0.1 or 1.0%. Gemifloxacin did not affect male and female sex ratio and adult longevity while it significantly decreased egg number. Fecundity and hatchability were significantly and inversely decreased by increasing gemifloxacin concentrations. The females reared from control diet produced 134.46 per day, whereas high concentrations of gemifloxacin decreased the egg number to 26.75 and 53.5. Hatchability was also significantly decreased by 0.01% and above concentrations of gemifloxacin whereas, the highest concentration of antibiotic lowered the hatchability to 53.71%. This study indicated sublethal effects of gemifloxacin are likely to have a significant impact on adult biological traits and these effects show concentration-dependent variation in biological traits of the insect.

Keywords: *Galleria mellonella, Gemifloxacin, Fecundity, Longevity*

GİRİŞ

Memeli konak modellerin dışındaki omurgasız hayvanların, özellikle önemli bir grubunu oluşturan böceklerin, laboratuvar ortamında yapay besinler ile yetiştirilmesi ve biyolojik model olarak kullanılması klinik amaçlı ilaç denemesi ve hastalık etkeni mikroorganizmaların etkinliğinin araştırılmasında son zamanlarda yaygınlaşmıştır. Bu uygulama tarımsal ürün zararlısı böcekler ile etkin ve çevreye duyarlı yeni kimyasal mücadele yöntemlerinin geliştirilmesi

İletişim (Correspondence)
+90 372 2911757
buyukguzelk@hotmail.com

için, laboratuvar şartlarında yetiştirilmesinin zor olduğu bu böceklerin yerine, diğer model böceklerin kullanılması kolaylığını da getirmiştir.

Böceklerin laboratuvar şartlarında yapay besinler ile yetiştirilmesi çalışmaları sırasında meydana gelebilecek mikrobiyal kontaminasyonların önlenmesi amacıyla geleneksel bazı inhibitörler kullanılmıştır [1]. Son zamanlarda bu amaçla antimikrobiyal kimyasallar olan yeni kuşak antibakteriyel, antifungal, antiviral ve hatta antiprotozoal antibiyotikler denenmiştir [2-4]. Bu antibiyotiklerin yüksek konsantrasyonlarda böceğin ergin evreye kadar yaşama, gelişmesi ile ömür uzunluğu, yumurta verimi ve açılma oranına olumsuz etkileri belirlenmiştir [5-7]. Bu çalışmalardan da anlaşılacağı gibi omurgasız ökaryotik organizma olan böceklerin ergin biyolojik özelliklerine yeni kuşak sentetik bakteriyel DNA giraz inhibitörlerinin etkilerini inceleyen herhangi bir çalışma bulunmamaktadır. Bu antibiyotiklerin düşük konsantrasyonlarında ise muhtemel kontaminasyonlara karşı önlem alınarak hem besin maddelerinin böcek tarafından etkin kullanılması hem de larvaların gelişiminde kontaminasyonların görülemeyen etkilerinin ortadan kaldırılması sağlanmıştır. Bu yaklaşım eski yıllarda besi hayvancılığında olduğu gibi tüm yararlı hayvanların beslenmesi açısından antibiyotiklerin çok düşük miktarlarda verimi artırmak amacıyla besinlere ilave edilmesi ile benzerdir. Et ve süt verimini artırmak amacıyla besi hayvancılığında kullanılan antibiyotiklerin etkilerine [8] benzer bir şekilde böcek beslenmesi amacıyla kullanılan antibiyotiklerin çok düşük konsantrasyonlarda böceklerin yaşama oranı ve gelişme süresi üzerinde olumlu etkiler yaptığı da belirlenmiştir [2,9]. Diğer taraftan bu antibiyotikler ipek böcekciliği yetiştiriciliğinde kullanıldığında ipek proteini miktarı üzerinde olumlu etki yaptığı gibi [10] böceklerin vücut bileşimleri ve bazı fizyolojik özellikleri üzerinde de olumlu etkiler yaptığı gözlenmiştir [11].

Galleria mellonella L. larvaları bal arısı kovanlarında bal peteği, mum ve bal gibi kovan ürünleri ile beslenerek önemli ekonomik zarara neden olmaktadır [12]. Bu böceğin larvaları, kültürünün daha ucuz olması, laboratuvar şartlarında daha kolay sürdürülebilmesi, özel laboratuvar ekipmanlarına gerek duyulmaması ve diğer omurgasız modeller *Caenorhabditis elegans* ve *Drosophila melanogaster*' in aksine 37°C de yaşamını sürdürebilmesi sebebiyle deney hayvanı modeli olarak kullanılmaktadır. Bu böceği laboratuvar ortamında yetiştirirken gerek besinlerdeki mikrobiyal kontaminasyonu önlemek gerekse biyolojik, fizyolojik, biyokimyasal ve ekolojik araştırmalar için mikroorganizmadan arındırılmış böcekler yetiştirmek amacıyla bazı antibakteriyel antibiyotikler kullanılmıştır [13-15]. Diğer taraftan, *G. mellonella* larvaları yeni antimikrobiyal ilaçların etkinliği ile hastalık etkeni mikroorganizmaların (bakteriyel ve fungal) patojenitelerini belirlemede memeli model konaklara alternatif model olarak kullanılmaktadır [16,17].

Gemifloksasin fluorokinolon sınıfına dahil yeni kuşak bir antibiyotiktir. Bakterisit etkili olup insanların ve hayvanların enfeksiyon hastalıklarının tedavisinde yaygın olarak kullanılır. Gemifloksasin, bakterilerin DNA giraz (DNA gyrase; topoizomeraz II)) ve topoizomeraz IV enzimlerini inhibe ederek DNA replikasyonunu engeller [18]. Klinik amaçlı kullanılan antibiyotiklerin zararlı böceklere yönelik insektisit olarak veya laboratuvar şartlarında böceklerin biyolojik özelliklerinin iyileştirilmesinde kullanılıp kullanılamayacağı henüz tartışma halindedir. Bu çalışma insan ve hayvanların enfeksiyon hastalıklarının tedavisinde yaygın olarak kullanılan yeni kuşak antibakteriyel antibiyotiklerin böceklerin üzerindeki etkisinin incelemesi bakımından önemlidir.

Bu çalışmada *G. mellonella* larvalarını beslemek için kullanılan yapay besinlere ilave edilen, bakteriyel DNA giraz inhibitörü fluorokinolon grubu bir antibakteriyel antibiyotik olan gemifloksasinin böceğin eşey oranı, ömür uzunluğu, yumurta verimi ve açılma oranı gibi ergin biyolojik özelliklerine etkileri araştırıldı.

MATERYAL ve METOT

Böceklerin Yetiştirilmesi

Yumurtadan yeni çıkmış *G. mellonella* larvaları laboratuvar ortamında yapay besinde aseptik olmayan şartlarda beslenerek böcek kültürü oluşturuldu. Böceklerin yetiştirilmesi inkübatörde (Nüve, ES 500) 28±2°C ve %65±5 bağıl nemde yapıldı. Deneylerde yumurtadan yeni çıkmış birinci evre larvaları kullanıldı.

G. mellonella larvalarını laboratuvar şartlarında yetiştirmek için Bronskill [19] tarafından geliştirilen yapay besin kullanıldı. Besin, 420 g buğday kepeği, 150 ml süzme bal, 150 ml gliserin (Merck, Darmstadt, Germany), 20 g öğütülmüş koyu renkli eski petek ve 30 ml saf su içermektedir. Hazırlanan besin bir litrelik cam kavanozların (80x180 mm) yaklaşık 1/3'ine kadar doldurulduu. Kavanozun içine konulacak dişilerin yumurta bırakması ve yeni açılan larvaların beslenmesi için besinin üzerine küçük bir parça bal peteği bırakıldı. Bu kavanozların içine 10-15 adet dişi bırakılarak ağızları tel kafes yerleştirilmiş kapak ile kapatıldı. Gelişimlerini tamamlayan olgunlaşan larvalar (7. evre) pup olmaları için diğer bir kavanoza aktarıldı. Bu kavanozun içine, larvaların pup olmaları için kuru ortam sağlamak üzere, katlanmış pelur kağıt parçaları bırakıldı [20]. Oluşan puplardan erginleşen bireylerin büyük bir çoğunluğu böcek kültürünün devamı, bazı erginler ise gemifloksasinin farklı konsantrasyonları ile ilgili beslenme çalışmaları için gerekli yumurtaların elde edilmesinde kullanıldı.

Beslenme Deneyleri

Deneylerde kullanılacak larvaların elde edilmesi için, 30 ml'lik geniş ağızlı, vida kapaklı plastik kapların (ORLAB, L190030, 35x55 mm) iç yüzeyine dişiler tarafından yumurta bırakılması sağlandı. Bırakılan yumurtalar 28±2°C ve %65±5 bağıl nemde bekletilerek açılması sağlandı. Bu yumurtalardan açılan *G. mellonella* larvaları beslenme deneylerinde

kullanıldı. Açılan larvalar, yumuşak uçlu bir fırça (No: 0, Goya Toray) ile tel kafes kapaklı cam kavanozların (60 x120mm) içindeki besine (yaklaşık 200 g) bırakıldı.

Gemifloksasin (gemifloksasin mesilat, %100, beyaz kristal toz, çalışmanın ilgili bölümlerinde gemifloksasin olarak verilecektir) antibiyotiği Abdi İbrahim İlaç Sanayi ve Ticaret A.Ş. (Maslak, İstanbul)'den temin edildi. Gemifloksasin mesilatın besine ilave edilmesi ile yürütülen beslenme deneylerinde denenen miktarların konsantrasyonu 100 gram besin başına gram antibiyotik (% a/a) olarak ifade edildi. Gemifloksasin, besinin hazırlanması sırasında doğrudan besine ilave edilerek homojenizasyonun sağlanması için besinler homojenizatörde 5 dk süre ile karıştırıldı. Gemifloksasinin %0.001, 0.01, 0.1 ve 1.0 olmak üzere 4 farklı konsantrasyonu denendi. Kontrol deneylerinde ise gemifloksasin içermeyen besin kullanıldı. Bu çalışmada denenecek gemifloksasin konsantrasyonları *G. mellonella* [14] ve bazı parazitoit böcek türleri [2] üzerinde antibiyotiklerin etkisinin araştırıldığı önceki çalışmalar temel alınarak belirlendi. Bu çalışmaların ışığında, denenecek konsantrasyonların aralığını belirlemek amacıyla ön beslenme deneyleri yapıldı. Böceklerin ergin evreye kadar gelişimini tamamlayabileceği konsantrasyon aralıkları belirlendi. Gemifloksasinin *G. mellonella* üzerindeki konsantrasyonları belirlenerek böceğin eşey oranı, erkek ve dişi ömür uzunluğu, dişilerin yumurta verimi, yumurtaların açılma oranı üzerine etkisi incelendi.

Eşey Oranı

Kontrol besini ve antibiyotiğin her konsantrasyonunu içeren besinlerin bulunduğu beslenme kaplarına (Cam kavanozlar, 60 x120 mm) 20 larva bırakıldı ve deneyler dörder defa tekrarlandı. Gelişimlerini tamamlayan olgun 7. evre larvaları alınarak pup olmak üzere 30 ml'lik plastik örnek kaplarına (ORLAB, L190030, 35x55 mm) her kapta bir larva olacak şekilde aktarıldı. Puplardan erginleşen bireylerin eşeyleri belirlenerek erkek ve dişi oranı hesaplandı. Denemede kullanılan erginlerin eşey ayrımı, erginlerinin vücut büyüklüğüne ve abdomenlerinin son segmentindeki genital yapıya göre yapıldı.

Ergin Ömür Uzunluğu

Gemifloksasinin erginlerin ömür uzunluğuna etkisini belirlemek için birinci evre larvaları gemifloksasinin miktarlarını içeren yapay besinler ile ergin evreye kadar beslendi. Her konsantrasyon için 10 adet ergin kullanıldı ve deneyler 4'er defa tekrarlandı. Erginleşen bireyler 30 ml'lik, geniş ağızlı, şeffaf, delikli kapaklı plastik kaplara (ORLAB, L190030, 35x55 mm) 1'er adet bırakıldı. *G. mellonella* erginleri besin almadığı (Charrière and Imdorf 1997) için deney süresince herhangi bir besin verilmedi. Bu erginler böcek kültürünün devam ettirildiği ortam şartlarında bekletildi. Erginler, her gün belirli saatte kontrol edilerek en son erginin ölümüne kadar her erginin yaşadığı süre belirlendi.

Yumurta Verimi ve Açılma Oranı

Yeni erginleşmiş ve döllenmemiş bir günlük *G. mellonella* dişileri geniş ağızlı, delikli kapaklı, plastik kaplara (15 ml, ORLAB) her kapta 1 adet dişi olacak şekilde bırakıldı. Bırakılan yumurtalar siyah bir zemin üzerine konulan petri kutusu içinde sayıldı. Yapılan ön denemeler erginleşen dişilerin ilk 48-72 saat içinde yumurtalarını bıraktığını göstermiştir. Bu yüzden ilk 2-3 gün içinde bırakılan yumurtalar sayılarak açılması için stok kültürün devam ettirildiği ortam şartlarında bekletildi. Dişinin yumurta verimi, 1 günde dişi başına bırakılan yumurta sayısı ele alınarak değerlendirildi. Yumurtaların açılma oranı (fertilite) her gün açılan larvalar siyah bir zemin üzerinde sayılarak kaydedildi. Her bir deney için 10 adet dişi kullanıldı ve deneyler 4'er defa tekrarlandı.

Deneylerin tümü kısa bir günlük inceleme periyodu hariç sürekli olarak karanlıkta tutuldu. Besinin hazırlanması ve larvaların aşılanması hariç beslenme deneylerinin tümü böceklerin yetiştirildiği şartlarda yürütüldü. Besinin hazırlanması, yumurtaların elde edilmesi, bu yumurtalardan çıkan larvaların besine konulması işlemleri tamamen aseptik olmayan şartlarda yapıldı [14].

İstatistiksel Analizler

Erginlerin ömür süresi, dişilerin yumurta verimi ve yumurtaların açılma oranı, ile ilgili verilerin değerlendirilmesinde tek yönlü "Varyans Analizi" (ANOVA) [21], ortalamalar arasındaki farkın önemini saptamak için "LSD Testi" [21], eşey oranı ile ilgili verilerin değerlendirilmesinde ise "χ^2 (Chi square) Testi" [22] kullanıldı. Ortalamalar (± S.H.) arasındaki farkın önemi 0.05 olasılık seviyesinde analiz edildi.

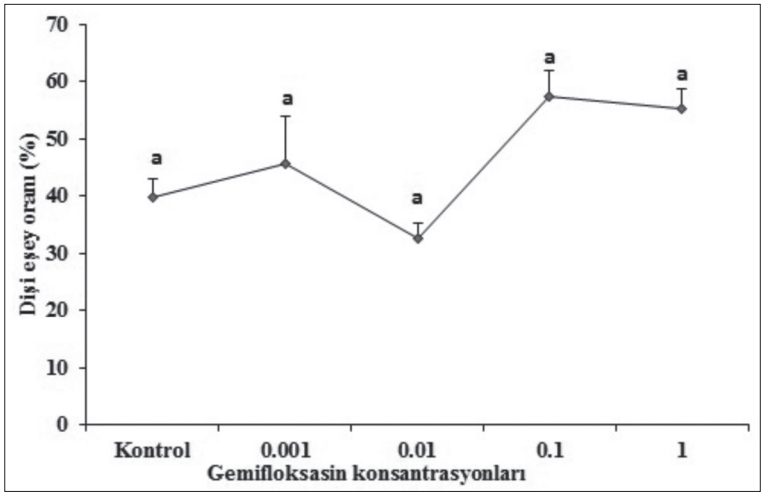
BULGULAR

Eşey Oranı

Kontrol besini ile beslenen başlangıçtaki larvaların %78.75'i erginleşti. Bu besin üzerinde beslenen larvalardan %60.11 erkek, %39.89 dişi ergin meydana geldi. Kontrol besini ile karşılaştırıldığında, gemifloksasinin denenen konsantrasyonlarını içeren besinler erkek ve dişi birey oranını etkilemedi. Ancak en yüksek gemifloksasin konsantrasyonunu içeren besin ile beslenen larvalardan kontrole göre düşük oranda erkek birey meydana gelirken dişi birey sayısında artış oldu *(Şekil 1, Şekil 2)*. Gemifloksasinin %1.0'ini (en yüksek konsantrasyon) içeren besin erkek oranını %60.11'den %44.80'e düşürürken, dişi olma oranını ise %39.89'dan %55.20'ye artırdı.

Ömür Uzunluğu, Yumurta Verimi ve Açılma Oranı

Antibiyotiğin denenen en düşük miktarı, bu antibiyotiği içermeyen kontrol besinine göre erkek erginlerin ömür uzunluğunu ortalama 2.0 gün uzattığı halde istatistiksel bakımdan önemli bir etki oluşturmadı. Bu antibiyotiğin

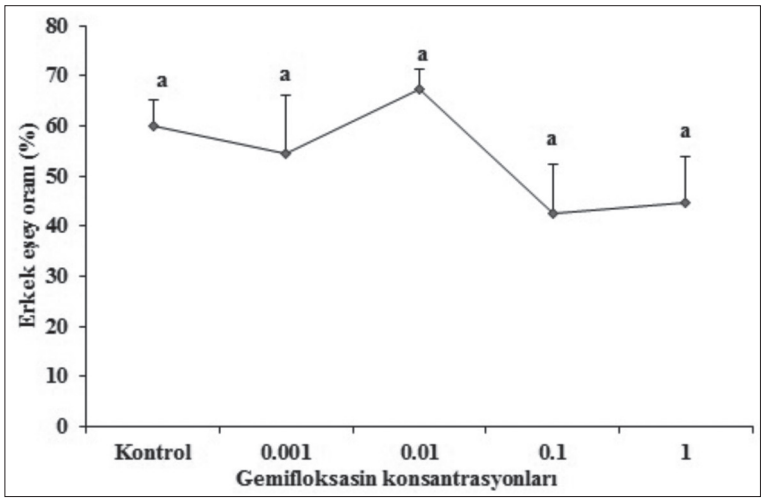


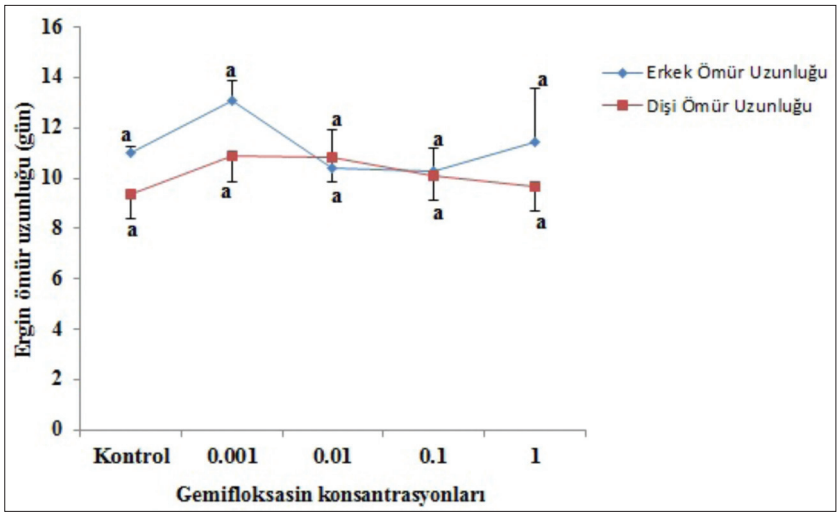
Şekil 1. Gemifloksasinin *G. mellonella*'nın dişi eşey oranına etkisi. Veriler 4 tekrarın ortalaması (± S.H) olarak verilmiştir, her tekrarda 20 larva kullanıldı. Aynı harfi içeren değerler birbirinden farklı değildir, $P>0.05$ (X^2 testi)

Fig 1. The effects of gemifloxacin on female sex ratio of *G. mellonella*. Data are the average of 4 replicates (± S.E), with 20 larvae per replicate. Values followed by the same letter are significantly not different from each other ($P>0.05$) (X^2 test)

Şekil 2. Gemifloksasinin *G. mellonella*'nın erkek eşey oranına etkisi, 4 tekrarın ortalaması (± S.H), her bir tekrar için 20 larva kullanıldı. Aynı harfi içeren değerler birbirinden farklı değildir, $P>0.05$ (x^2 testi)

Fig 2. The effects of gemifloxacin on male sex ratio of *G. mellonella*. Data are the average of 4 replicates (± S.E), with 20 larvae per replicate. Values followed by the same letter are significantly not different from each other ($P>0.05$) (x^2 test)



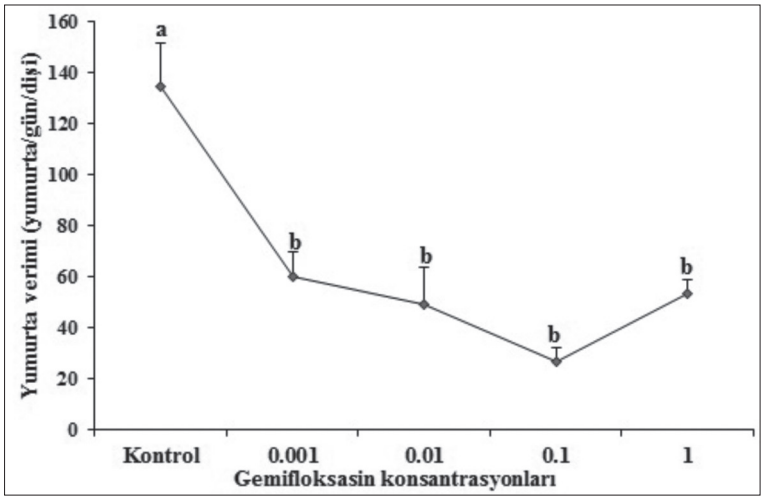


Şekil 3. Gemifloksasinin *G. mellonella* ergin ömür uzunluğuna etkisi, 4 tekrarın ortalaması (± S.H), her bir tekrar için her eşeyden 10 ergin kullanıldı. Aynı harfi içeren değerler birbirinden farklı değildir, $P>0.05$ (LSD testi)

Fig 3. The effects of gemifloxacin on adult longevity of *G. mellonella*. Data are the average of 4 replicates (± S.E), with 10 adults per replicate. Values followed by the same letter are significantly not different from each other ($P>0.05$) (LSD test)

denenen en düşük miktarı benzer bir etkiyi dişi ömür uzunluğu üzerinde de gösterdi. Sonuçta gemifloksasinin denenen konsantrasyonlarını içeren besinler erkek ve dişi erginlerin ömür uzunluğu üzerinde önemli derecede etkili olmadığı görüldü *(Şekil 3)*. Gemifloksasinin %0.001-0.1 oranında düşük konsantrasyonlarını içeren besinler dişilerin ömür uzunluğunda kontrol grubuna göre yaklaşık 1'er gün uzamaya neden oldu, ancak bu etki istatistiksel olarak önemli olmadı.

Gemifloksasinin denenen konsantrasyonlarını içeren besinler ile beslenen larvalardan erginleşen dişilerin kontrol

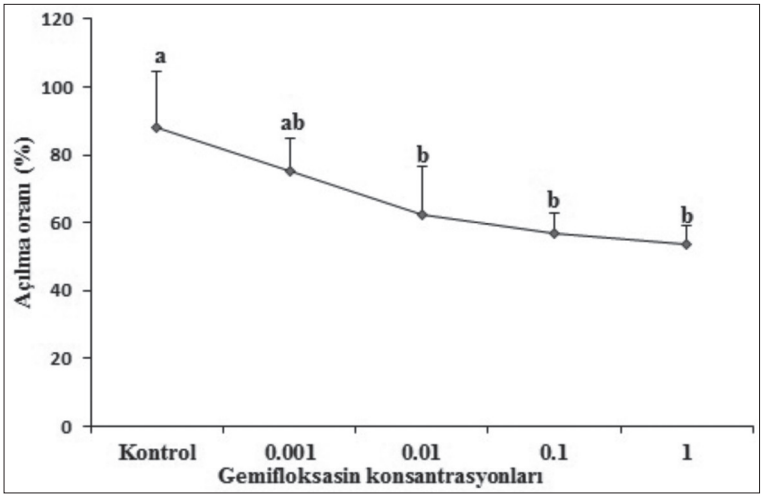


Şekil 4. Gemifloksasinin *G. mellonella* dişilerinin yumurta verimine etkisi, 4 tekrarın ortalaması (± S.H), her bir tekrar için 10 dişi kullanıldı. Aynı harfi içeren değerler birbirinden farklı değildir, $P>0.05$ (LSD testi), (a-b) %0.001, 0.01, 0.1 ve 1.0'lik gemifloksasin konsantrasyonları kontrolden farklıdır ($P<0.05$)

Fig 4. The effects of gemifloxacin on fecundity of *G. mellonella*. Data are the average of 4 replicates (± S.E), with 10 female per replicate. Values followed by the different letter are significantly different from each other ($P>0.05$) (LSD Test). (a-b) 0.001, 0.01, 0.1 or 1.0% of gemifloxacin are different from control ($P<0.05$)

Şekil 5. Gemifloksasinin *G. mellonella* dişilerinin bıraktığı yumurtaların açılma oranına etkisi, 4 tekrarın ortalaması (± S.H), her bir tekrar için 10 dişi kullanıldı. Aynı harfi içeren değerler birbirinden farklı değildir, $P>0.05$ (LSD testi), (a-b) %0.01, 0.1 ve 1.0'lik gemifloksasin konsantrasyonları kontrolden farklıdır ($P<0.05$), (ab) %0.001'lik gemifloksasin konsantrasyonu kontrolden ve %0.01, 0.1, 1.0'lik gemifloksasin konsantrasyonlarından farklı değildir ($P>0.05$)

Fig 5. The effects of gemifloxacin on hatchability of *G. mellonella*. Data are the average of 4 replicates (± S.E), with 10 female per replicate. Values followed by the different letter are significantly different from each other ($P<0.05$) (LSD Test). (a-b) 0.01, 0.1 or 1.0% of gemifloxacin are different from control ($P<0.05$), (ab) 0.001% of gemifloxacin are not different from control or 0.01, 0.1, 1.0% of gemifloxacin ($P>0.05$)



besini ile beslenen larvalardan erginleşen dişilere göre, yumurta verimi önemli derecede azaldı. Kontrol besini ile yetiştirilen dişiler günde 134.46 adet yumurta bırakırken %0.001'lik gemifloksasin ile yetiştirilen dişilerin yumurta verimi 60.26'ya düştü. Buna karşılık besine %0.1 oranında gemifloksasinin ilave edilmesi dişilerin bıraktığı yumurta sayısını önemli derecede azaltarak *(Şekil 4)* bir günde ortalama 26.75 adet yumurta bırakılmasını sağladı. Gemifloksasinin besindeki en yüksek miktarı (%1) gemifloksasinin %0.1'lik konsantrasyonu ile karşılaştırıldığında, yumurta sayısını iki katı artırdı. Antibiyotiğin bu iki konsantrasyonu etkileri bakımından kendi aralarında karşılaştırıldığında önemli bir istatistiksel fark oluşturmadı.

Yumurtaların açılma oranı bu antibiyotiğin denenen en düşük konsantrasyonunu içeren besin tarafından istatistiksel olarak önemli olmayan derecede düşürüldü. Gemifloksasin bırakılan yumurtaların açılma oranını besindeki artan antibiyotik konsantrasyonu ile ters orantılı olarak önemli derecede azalttı *(Şekil 5)*. Gemifloksasin içermeyen kontrol besini ile yetiştirilen dişilerin bıraktığı yumurtaların %87.86'sı açılırken bu oran gemifloksasinin en yüksek konsantrasyonu tarafından %53.71'e düşürüldü.

TARTIŞMA ve SONUÇ

Bu çalışmanın sonuçları, klinik amaçlı olarak kullanılan yeni kuşak sentetik antibiyotik gemifloksasinin *G. mellonella* dişilerinin yumurta verimi ve açılma oranını olumsuz yönde etkilediğini açıkca gösterdi. Bu etkiler besindeki gemifloksasin miktarına bağlı olarak değişti. Klinik öneme sahip geleneksel antibiyotikler, penisilin ve streptomisinin, *G. mellonella*'nın yaşama, gelişme, vücut ağırlığı ve total protein miktarına etkisi böceğin gelişme evreleri (larva, pup ve ergin) ile antibiyotiklerin türü ve dozuna bağlı olduğu gösterilmiştir [14,15]. Bu çalışmada denenen gemifloksasinin *G. mellonella* erginlerinin yumurta verimi ve açılma oranını artan antibiyotik konsantrasyonu ile ters orantılı olarak azalttığı belirlendi. Yapay besinlere küf ve mantar kontaminasyonunu önlemek için ilave edilen nistatin, sodyum benzoat ve metil p-hidroksibenzoat gibi bazı geleneksel antifungaller endoparazitoit *Trichogramma* türlerinin yumurtalarının açılma oranını azaltmış, farklı gelişme evrelerindeki ölüm oranını artırmıştır [6]. Benzer şekilde, bazı immün ve antioksidan savunmaya aracılık eden sinyal moleküllerinin biyosentezini engelleyen inhibitörlerin çeşitli böceklerin yaşama ve gelişmesi üzerinde önemli

derecede etkili olmadığı ancak yumurta veriminde önemli bir azalmaya sebep olduğu ortaya çıkarılmıştır [23].

DNA giraz inhibitörü novobiyosinin, en düşük miktarını içeren besin endoparazitoit bir hymenopter türü olan *P. turionellae* larvalarının beşinci evreye ulaşmak için gereken süreyi kısaltmış, pup ve ergin yüzdesini önemli derecede artırmıştır. Oksolinik asitin en düşük miktarı yaşama üzerinde etkili olmazken böceğin gelişmesini geciktirmiştir. Antibiyotiklerin yüksek miktarları genellikle gelişme süresini uzatmış, yaşamayı düşürmüştür. Nalidiksik asitin denenen bütün miktarları ise yaşamayı dikkate değer bir şekilde artırmıştır [2]. Bu geleneksel DNA giraz inhibitörleri antibiyotiklerin bazı üçlü besinsel konsantrasyonları ergin parazitoitlerin erginlerinde böceklerde toplam protein miktarını ve yaş ağırlığı artırmıştır [11]. Gerek bizim çalışmamızdan elde edilen sonuçlar gerekse diğer böcek türleri ile yapılan benzer çalışmaların sonuçları yapılan etkinin yeni kuşak bir DNA giraz inhibitörü olan gemifloksasinin besindeki miktarı yanında böcek türüne göre de değişebildiğini göstermektedir. Bu çalışmadan elde edilen sonuçlar *A. affinis* ile yapılan çalışmadan elde edilen sonuçlar ile karşılaştırıldığında *G. mellonella*'nın *A. affinis*'e göre [5] genelde antibiyotiklerin daha yüksek miktarlarına tolerans gösterdiğini ortaya çıkardı. Bu böcek türlerinde böyle bir ayrıcalığın bulunması larval evredeki sindirim kanalının fiziksel ve kimyasal olarak farklı olmasından ileri gelebilir [7]. Lepidoptera takımına ait böceklerin sindirim kanalı alkali özellikte yüksek bir oksidasyon ve redüksiyon potansiyeline sahiptir [24].

Larval evrede alınan doğal ve yapay besin maddelerinin kalitesi ve besinsel dengesi böceklerin erginlerinin üreme ve diğer bazı özellikleri ile ömür uzunluğu (hayatta kalma süresi) üzerinde etkilidir [25]. Zararlı bir dipter tür olan Akdeniz meyve sineği *Ceratitis capitata*'nın larval evrede aldığı besinin ergin oluşumunu, vücut büyüklüğünü, eşeysel olgunluğu, yumurta bırakma davranışını ve yaşama süresini etkilediği bilinmektedir [26]. Gemifloksasinin denenen besinsel karışımlarının hiç biri *G. mellonella* erkek ve dişi erginlerinin ömür uzunluğu üzerinde istatistiksel olarak etkili olmamıştır. Bu sonuçlar böceğin ergin öncesi gelişme evresinde ve erginleşen bireylerin hayatta kalma süresi içerisinde bu antibiyotiği tolere edebildiği ancak erginlerin fizyolojik işlevlerinde bu antibiyotiğin subletal etkilerine karşı bazı adaptasyon tepkileri verebildiğini göstermiştir. Gemifloksasinin yalnızca yumurta verimi ve açılma oranını önemli derecede düşürmesi bu görüşü desteklemekte olup aynı zamanda farklı bir mekanizma ile etkili olabileceği düşünülmektedir. *G. mellonella*'nın ergin ömür uzunluğu mevsime (kış ve yaz ayları) göre değişmekte olup eşey oranları ise her mevsimde 1:1,1 (populasyon içi paylar %50: %55) oranındadır [27]. Bu çalışmada gemifloksasin böceğin eşey oranını ve ömür uzunluğunu önemli derecede etkilememiş olup özellikle yüksek antibiyotik konsantrasyonlarında erkeklere göre daha yüksek oranda dişi meydana geldiği görüldü. Bu sonucun antibiyotiğin etkisine bağlı olup olmadığını araştırmak için birkaç nesil boyunca eşey oranı ve ömür uzunluğu izlenmelidir.

Gemifloksasinin tüm miktarlarını içeren besinlerde yetiştirilen dişilerin yumurta veriminde önemli bir azalma oldu, denenen yüksek gemifloksasin konsantrasyonlarında bırakılan yumurtaların büyük bir çoğunluğunun embriyonik gelişimi tamamlayamadığı ve normal olarak açılamadığı görüldü. Ekonomik olarak zarara sebep olan böceklerin yumurta sayısının azalması ve bu yumurtaların canlılık oranının düşük olması böceklerin kontrolünde önemlidir. Çeşitli antibiyotiklerin bazı takımlara ait böceklerin yumurta üretimini çoğunlukla olumsuz yönde etkilediği bilinmektedir [28]. Örneğin toprakta bulunan *Folsomia fimetaria* L. ve *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae)'nın ergin yaşama oranı ve verdikleri yeni nesil birey sayısının üzerine oksitetrasiklin ve tilozin antibiyotikleri düşük bir toksik etki yapmıştır. Oksitetrasiklin yaşama oranını etkilemezken tilozin önemli olmayan bir azalmaya sebep olmuştur. Buna karşılık oksitetrasiklin bu iki böceğin üreme oranını önemli derecede düşürmüştür [29]. Bazı antimikrobiyal maddelerden metronidazol, olankindoks, tiyamulin ve antihelmintik ivermektin *Folsomia fimetaria*'nın üreme oranını düşürmüştür [30]. Gemifloksasinin etkisinin araştırıldığı *G. mellonella* ile yaptığımız bu çalışma antibiyotiklerin böceklerdeki etkisinin belirlenmesinde ergin ömür uzunluğuna göre üreme ile ilgili özelliklerin daha hassas kriterler olduğunu gösterdi. Diğer çalışmaların sonuçları bizim çalışmamızın sonuçları ile uyumludur. Örneğin, rifampisin *F. candida* Willem'in yumurta sayısını etkilemezken yumurtaların açılma oranını düşürmüştür [31]. Buna karşılık Pike ve Kingcombe [32] rifampisinin yumurta sayısında ve açılma oranında bir azalmaya sebep olduğu ancak tetrasiklinin yumurta sayısını düşürmediğini belirtmiştir. Kimyasal mücadelede kullanılan bazı nükleozit türevi antiviral maddelerin heteropter böceklerden *Pyrrhocoris apterus* (L.)'da ve *Dysdercus cingulatus* (Fabr.)'da ovaryum ve folikül hücrelerinin yapısının bozulmasına, embriyo gelişimi için önemli olan vitellin proteininin sentezinin önlenmesine bağlı olarak embriyonik gelişimin gerilemesine sebep olmuştur [33]. Bu nükleozit türevi antiviral maddelerin böceklerde ovaryumlardaki yumurta verimini jüvenil hormon (JH) ve ovaryum ekdisteroidlerinin seviyesini değiştirmek suretiyle etkilediği ileri sürülmüştür [34-36]. Bu çalışmada denenen gemifloksasinin besinsel karışımları *G. mellonella* dişilerinin yumurta gelişimi ile ilgili hormonların işlevinde önemli değişikliklere sebep olabilir, ancak bu konuda kesin bir sonuca varabilmek için detaylı çalışmalara gerek bulunmaktadır.

Gemifloksasinin gerek besinin kimyasal bileşimleri arasındaki dengeyi gerekse böceğin bağırsak osmotik basıncını ve pH'ını değiştirmesi sonucu larvaların beslenme davranışı değişmiş ve besin tüketim oranı azalmış olabilir. Örneğin beta-laktam antibiyotiklerin, sefalosporinlerin ve gemifloksasin ile aynı grupta olan fluorokinolon yapısındaki ofloksasinin çeşitli canlıların bağırsaklarından emilim-

lerinde ise pH'ın önemli rol oynadığı belirtilmektedir [37]. Diğer taraftan, böcekleri beslemek için kullanılan besinlerin içeriğinin bozulması ve kalitesinin düşük olması erginlerin fizyolojik ve biyolojik özelliklerini olumsuz etkilediği bilinmektedir [25]. Omurgalı hayvanlar ile yapılan bir çalışmada beslenme şartlarına bağlı olarak genç evrelerde oluşan oxidatif stresin ergin özellikleri olumsuz etkilediği tespit edilmiştir [38]. Bu çalışmada besin tüketim oranının azalmasına bağlı olarak larvalar, erginleşecek bireylerin ömür uzunluğunu etkileyecek düzeyde gemifloksasin alamamış olabilir. Ancak tüm canlılarda olduğu gibi böceklerde de yumurta üretimi, embriyo gelişimi ve olgunlaşan embriyonun serbest kalması gibi üreme ile ilgili fizyolojik işlemler antibiyotiğin bu düşük miktarlarından etkilenmiş olabilir. Ancak bu konunun aydınlatılabilmesi için detaylı çalışmalara ihtiyaç duyulmaktadır.

Bu çalışmada gemifloksasin *G. mellonella*'nın yumurta verimi ve açılma oranını olumsuz yönde etkiledi. Buna karşılık eşey oranı ve ergin ömür uzunluğu etkilenmedi. Erginlerin üremesi üzerindeki subletal etkiler, bu antibiyotiğin konsantrasyonlarının hassas bir şekilde ayarlanmasıyla diğer zararlı böcek türlerinin mücadelesinde kullanılabileceğini göstermektedir. Diğer taraftan böyle bir çalışma bu böceklerin sentetik besin ortamlarında yetiştirilmelerinde önemli bir sorun olan mikrobiyal kontaminasyonları önlemeye yönelik çalışmalara da ışık tutacaktır.

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Research Article

Comparison of Alfalfa *(Medicago sativa)* Energy Values Estimated by Using the NRC-2001, Hohenheim and UC Davis Equations [1]

Mustafa Selçuk ALATAŞ [1] Kanber KARA [2] Fatma İNAL [1]
Oğuzhan KAHRAMAN [1] Abdullah ÖZBİLGİN [1] Behiç COŞKUN [1]

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[1] Selçuk Üniversitesi Veteriner Fakültesi, Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı, Konya - TÜRKİYE
[2] Erciyes Üniversitesi Veteriner Fakültesi, Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı, Kayseri - TÜRKİYE

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Abstract

In this study, our aim was to compare estimated metabolizable energy (ME) and net energy lactation (NEL) contents in dried alfalfa samples in their different growth stages, using models such as NRC-2001, Hohenheim$_{Menke}$ and University of California at Davis (UC Davis). A total of 73 alfalfa hay samples obtained during three different growth stages (Vegetative, Bud and Bloom) were used. Chemical analyses were performed for each sample. Energy values of the alfalfa hay samples were calculated with NRC-2001 equations from chemical analysis results, and with Hohenheim$_{Menke}$ and UC Davis equations from *in vitro* gas production volumes. Gas production in alfalfa samples in vegetative period (S1) was higher than other periods (P<0.001). Again, ME values calculated with all three methods in the alfalfa hay samples of this period were significantly higher than the samples in bud and bloom periods (P<0.001). In addition, energy values obtained with Hohenheim$_{Menke}$ equation in all periods were found to be higher than the averages obtained with NRC-2001 and UC Davis equations. It is concluded that, for the alfalfas in vegetative period, a correlation of 85.6% between energy values obtained with NRC-2001 and Hohenheim$_{Menke}$ equations, a correlation of 81.8% between energy values obtained with UC Davis and NRC-2001 equations, and over 99% correlation between energy values obtained with Hohenheim$_{Menke}$ and UC Davis equations were determined (P<0.000).

Keywords: *Alfalfa, NRC-2001, UC Davis, In vitro gas production*

Yoncanın *(Medicago sativa)* NRC-2001, Hohenheim ve UC Davis Eşitlikleri Kullanılarak Tahmin Edilen Enerji Değerlerinin Karşılaştırılması

Özet

Çalışmada farklı büyüme dönemlerinde biçilerek kurutulmuş yonca örneklerinde, NRC-2001, Hohenheim$_{Menke}$ ve Davis Kaliforniya Üniversitesi (UC Davis) gibi modeller kullanılarak tahmin edilen metabolize olabilir enerji (ME) ve net enerji laktasyon (NEL) içeriklerinin kıyaslanması amaçlandı. Üç farklı büyüme (vejetatif, tomurcuklanma ve çiçeklenme) döneminde elde edilmiş toplam 73 yonca kuru otu örneği kullanıldı. Her bir numunenin kimyasal analizleri yapıldı. Yoncaların enerji değerleri kimyasal analiz sonuçlarından NRC-2001 denklemleri ile ve *in vitro* gaz üretim miktarlarından Hohenheim$_{Menke}$ ve UC Davis eşitlikleri kullanılarak hesaplandı. Yonca örneklerinde vegetative dönemde (S1) gaz üretimi diğer dönemlerdekinden daha yüksek idi (P<0.001). Yine bu dönemdeki yoncalarda her üç yöntemle hesaplanan ME değerleri tomurcuklanma ve çiçeklenme dönemlerindeki yoncalarınkinden belirgin bir şekilde yüksekti (P<0.001). Ayrıca bütün dönemlerde Hohenheim$_{Menke}$ eşitliğinden elde edilen enerji değerleri NRC-2001 ve UC Davis eşitliklerinden elde edilen ortalamalardan yüksek bulundu. Sonuç olarak vejetatif dönemdeki yoncaların NRC-2001 ve Hohenheim$_{Menke}$ eşitliğinden elde edilen enerji değerleri arasında %85.6; UC Davis ve NRC-2001 eşitliklerinden elde edilen enerji değerleri arasında %81.8; Hohenheim$_{Menke}$ ve UC Davis eşitliklerinden elde edilen enerji değerleri arasında ise %99'un üzerinde bir korelasyon belirlendi (P<0.000).

Anahtar sözcükler: *Yonca, NRC-2001, UC Davis, In vitro gaz üretimi*

İletişim (Correspondence)
+90 332 2232698
selcukalatas@gmail.com

INTRODUCTION

It is very important to know energy and nutrient contents of feeds to be used during the ration formulation for ruminants. Nutrients can be determined by chemical analyses. However, it is difficult to determine the energy amount. While metabolizable energy (ME) contents of ruminant feeds can be estimated often by looking at chemical compositions of the feeds [1], alternatively, they can be evaluated with *in situ* or *in vitro* methods. For example, energy contents of feed materials can be learned by looking at amount of *in vitro* gas production [2-4]. Currently, the results obtained by providing real rumen environment with ruminal *in situ* methods are more reliable than the results obtained with *in vitro* methods. However, the limitations of *in situ* methods are that the laboratory and analysis costs are quite expensive, rumen cannulated animals are required and that these methods cannot measure the actual fermented amount of the feeds and can only measure the amount of the lost feed [5,6].

In calculation of energy values of ruminant feeds, *in vitro* methods based on gas production are more commonly used due to high costs of *in situ* methods. With Hohenheim method, metabolizable energy of the feeds can be evaluated with various chemical components and by calculating total gas amount formed by incubating them for 24 h in syringes using an equation [2,7]. This method has more advantages compared to *in vivo* tests, not only due to being more economical, but also due to providing more sustainable experimental conditions. In addition, *in vitro* gas production technique is often not considered ethical because of the fistulated animal requirement [8]. The difficulty in provision of sample rumen liquor required for gas production technique is also among the disadvantages of the method [9].

Apart from these methods, there are NRC-2001 equations that calculate TDN from nutrients with chemical formulas. By means of this model, accurate results can be obtained in a very practical way and free of charge, without any need for animal experiments and rumen liquor [10]. With analysis results of feeds and formulas including some coefficients, tdNFC, tdCP, tdFA, tdNDF and TDN are calculated, and digestible energy (DE), metabolizable energy (ME) and net energy lactation (NEL) calculations can be performed from TDN [11,12]. Although it was argued that TDN values calculated with NRC-2001 equations are not accurate for feeds in the tropic regions [13], Kishore and Parthasarathy [10] obtained very similar results when they compared their study where they estimated TDN values of various tropical herbs and leaves, using NRC-2001 equations, to *in vivo* tests. Similarly, in their study Şayan et al.[14] suggest that ME values can be calculated by means of regression equations developed for roughage and determination of 24 h gas production volumes of feeds with CP, EE, NDF and ADF should be used primarily as reliable parameters.

The UC Davis equation is a modification of the method of Menke and Steingass [2] that predicts ME from gas produced *in vitro* at 24 h of incubation, as well as estimates of the feed's levels of CP and fat.

In this study, from nutritional values of alfalfa hays in different vegetation periods, determined with chemical analyses, metabolizable energy (ME_{NRC}) and net energy lactation (NEL_{NRC}) values were calculated by using models in NRC-2001. It was also determined *in vitro* gas production of the same alfalfa hays with calculated energy values (ME_{NRC} and NEL_{NRC}), it was aimed to compare and interpret energy values (ME_{Menke}, NEL_{Menke} and ME_{UCD}) obtained from Hohenheim [2] and UC Davis [3].

MATERIAL and METHODS

Sample Collection

Alfalfa samples (Elçi, Bilensoy 80, Kayseri, Prosementi and Local variety) were obtained from fields of private producers in different districts of Konya, from 2-3. Cuttings using a quadrate of 50x50 cm from at least 4 different parts. A total of 73 alfalfa samples of different maturity stages were collected. The alfalfa samples were divided into 3 groups according to their maturity stages;

Stage 1: Vegetative (S1);

Stage 2: Bud (S2);

Stage 3: Bloom (Midbloom) (S3).

Numbers of alfalfa hays for each maturity stage were 27, 23, 23 respectively. The harvested alfalfa samples were chopped up in big pieces and dried in fan drying-ovens (VWR, Dry-Line) at 65°C to a constant weight. All dried samples for chemical analysis were ground to pass a 1 mm screen in a Retsch mill (Retsch GmbH, Haan, Germany).

Chemical Analysis

Milled samples were analyzed for DM, crude ash, crude protein (CP) and ether extract (EE) by AOAC [15]. Neutral detergent fiber (aNDF) and acid detergent fiber (ADF) were assayed according to the methods prescribed by Van Soest et al.[16] using Ancom^{200} Fiber Analyzer (Ancom Technology, Fairport, NY, USA). Neutral detergent fibre was determined with the inclusion of heat stable α-amylase and express inclusive of residual ash [16]. Sodium sulfite was used prior to neutral detergent extraction. The NDFn was also adjusted by: NDF-NDICP. Acid detergent lignin (ADL) was determined in ADF samples by soaking in 72% sulfuric acid for 3 h in beakers. The Neutral (NDICP) and acid (ADICP) detergent-insoluble CP was determined on the samples obtained from NDF and ADF residues [17]. Acid detergent insoluble protein (ADICP) and neutral

detergent insoluble protein (NDICP) were calculated as ADICP = 6.25×ADIN and NIDCP = 6.25 ×NDIN, respectively. All samples were analyzed in duplicate and repeated if chemical analysis error was in excess of 5%.

In Vitro Gas Production Technique

In vitro gas production of 73 samples was analyzed. Rumen fluid was obtained from a steer beef fed with a diet containing alfalfa hay (40%) and concentrates (60%). Rumen fluid was collected into a glass bottle (Isolab, Germany). The bottle was transported to laboratory in a sealed thermos container at 39±1°C and filtered through four layers of cheesecloth under CO_2 gas. The samples were incubated in rumen fluid and buffer mixture (in 100 mL glass syringes (Model Fortuna, Germany) following the procedures of Menke and Steingass [2]. About 200±10 mg dry samples were weighed in triplicate into glass syringes. The syringes were prewarmed at 39°C in a thermostatically cabinet (Lovibond, Switzerland), before 10 mL of rumen fluid and 20 mL of prewarmed buffer mixture were dispensed anaerobically in each syringe using an automatic bottle top dispenser (Isolab, Germany). Syringes were closed using one position polypropylene clamps and incubated at 39±0.5°C for 24 h. In addition, three blank syringes (no template; rumen fluid + buffer mixture) were used to calculate the total gas production. After 24 h of incubation, the total gas volume (mL) was recorded from the calibrated scale on the syringe.

Energy Values

Estimated energy contents for metabolizable energy (ME) and net energy lactation (NEL) were calculated separately using the three different equations as follows:

- *Using NRC predictive equations:* The NRC-2001 chemical formula is one method to estimate energy values for feeds for dairy cattle. This method is a chemical approach that uses analytical results to estimate the values of truly digestible nutrients (tdNFC, tdCP, tdFA, tdNDF, TDN). Metabolizable energy at production level of intake (ME_{NRC}) and net energy for lactation at production level of intake (NEL_{NRC}) were determined using a summative approach [18] from NRC [11]. The values of proximate analysis and fiber analysis were used in the following equations of NRC-2001 to predict the truly digestible (td) nutrients of the test feeds and then the digestibility values were summed up to arrive at the TDN content of the feeds.

NFC = 100 - [CP + EE + ash + (NDF - NDICP)]

tdNFC = 0.98 × {100 - [CP + EE + ash + (NDF - NDICP)]}× PAF

where;

PAF = Processing adjustment factor, 1 for alfalfa

$tdCP_{forage}$= CP × exp [-1.2 ×(ADICP CP)]

$tdCP_{concentrate}$= [1 - (0.4 ×(ADICP CP))] × CP

tdFA (fatty acid) = FA (FA = EE -1)

$tdNDF = 0.75 \times (NDFn - L) \times [1 - (L/NDFn)^{0.667}]$

where;

L = Acid detergent lignin and NDFn= NDF - NDICP

TDN = tdNFC + tdCP + (tdFA × 2.25) + tdNDF - 7

The energy values of digestible energy (DE) and metabolizable energy (ME) and net energy lactation (NEL) were estimated using the following equations of NRC [11].

DE (MJ/kg) = 0.04409 × TDN(%)/4.184

ME_{NRC} (MJ/kg) =1.01 × DE (Mcal/kg) - 0.45/4.184

NEL_{NRC} (MJ/kg) = (0.0245× TDN(%) - 0.12)/4.184

- *Using UC Davis predictive equations:* ME_{UCD} content was also estimated using the UC Davis equation proposed by Robinson et al.[3] and Tagliapietra et al.[4] resulting from a modification of that proposed by Menke and Steingass [2] as:

ME_{UCD} (MJ/kg DM) = 1.25 + 0.0292GP + (0.000143(CP – ADICP)) + 0.0246EE

(GP is 24 h *in vitro* gas production in ml/g of DM, and EE, CP and ADICP are as g/kg of DM).

- *Using Hohenheim predictive equations:* The metabolizable energy (ME_{Menke}) and net energy lactation (NEL_{Menke}) contents of alfalfa were calculated using equations of Menke and Steingas [2] as follows:

ME_{Menke} (MJ/kg DM)= 2.20 + 0.1357GP + 0.0057CP + $0.0002859EE^2$

NEL_{Menke} (MJ/kg DM)= 0.54 + 0.0959GP + 0.0038CP + $0.0001733EE^2$

(GP is 24 h net gas production in ml/200 mg of DM, and CP, EE are as g/kg of DM)

Statistical Analysis

Relationships between the ME values estimated by each of the predictive approaches were tested using standard ANOVA procedures within SPSS [19]. The paired t test procedure and Pearson correlation analysis were performed to establish the relationship between values of alfalfa by using NRC 2001, $Hohenheim_{Menke}$ and UC Davis predictive equations. The methods were compared by linear regression of the mean values of ME_{NRC}, ME_{Menke} and ME_{UCD} data obtained for each alfalfa hays. Treatment means were compared using the Duncan multiple range test.

RESULTS

Chemical analysis results of alfalfa hays in different maturity stages are shown in *Table 1*. According to the results, harvesting of alfalfa in different maturity stages had a great impact on chemical composition and *in vitro* gas production of alfalfa. A great difference was observed

especially in CP, Crude ash, ADF, NDF, ADL (P<0.000) in terms of maturity stages.

Minimum, maximum and average values of ME and NEL values of alfalfa hays in different maturation periods determined with NRC-2001, Hohenheim$_{Menke}$ and UC Davis equations are shown in *Table 2*. In all maturity stages, the energy value averages obtained from Hohenheim$_{Menke}$ technique were found to be higher than the averages obtained from NRC-2001 and UC Davis equations.

Although there is a correlation of 85.6% between ME and NEL values obtained with Hohenheim$_{Menke}$ and NRC-2001 methods in S1 alfalfa samples (P<0.000), a statistically significant difference between the average values was observed (P<0.000). The same difference was determined also in other maturity stages. However, as vegetation progressed, it was observed that this relationship reduced *(Table 3)*.

Equations and relations obtained after regression analysis between ME and NEL values of all alfalfa samples in the study, calculated with NRC-2001, Hohenheim$_{Menke}$ and UC Davis equations are shown in *Table 4*. The fact that

Table 1. *The chemical composition (g/kg DM) and gas production (mL/200 mg DM) of alfalfa hays*
Tablo 1. *Yonca kuru otlarının kimyasal kompozisyonları (g/kg DM) ve gaz üretimleri (mL/200 mg KM)*

Items	Maturity Stages			SEM	P
	S1	S2	S3		
DM	937.32^{b}	943.57^{a}	937.39^{b}	0.9	0.005
CP*	253.47^{a}	200.84^{b}	198.87^{b}	4.66	0.000
EE*	40.23^{a}	38.04^{ab}	34.98^{b}	1.03	0.108
NFC*	272.75	274.08	285.24	4.16	0.417
Ash*	93.44^{a}	79.74^{b}	80.32^{b}	1.7	0.000
NDF*	340.10^{b}	407.30^{a}	400.58^{a}	7.25	0.000
ADF*	259.60^{b}	313.09^{a}	309.66^{a}	6.14	0.000
Lignin*	67.56^{b}	97.61^{a}	99.08^{a}	3.72	0.000
NDICP*	20.83	19.72	22.42	0.68	0.287
ADICP*	12.83^{ab}	11.55^{b}	13.60^{a}	0.33	0.040
Gas Production**	54.20^{a}	44.72^{b}	45.32^{b}	1.24	0.001

*^{a,b} Means between different maturity stages of alfalfa having different letters are significantly different; * (in g/kg on DM basis; ** Net gas production (ml/200 mg DM)*

Table 2. *Comparison of ME and NEL obtained from NRC-2001, Hohenheim$_{Menke}$ and UCD equations in alfalfa different maturity stages*
Tablo 2. *Yoncanın farklı olgunlaşma dönemlerinde NRC-2001, Hohenheim$_{Menke}$ ve UCD eşitliklerinden elde edilen ME ve NEL'in kıyaslanması*

Method		ME,MJ/kg DM			Between Groups	NEL,MJ/kg DM			Between Groups
Maturity Stage		S1	S2	S3	P	S1	S2	S3	P
Menke	Mean	11.50^{aA}	9.84^{bA}	9.84^{bA}	0.000	7.00^{aA}	5.85^{b}	5.86^{bA}	0.000
	Min	8.61	7.87	8.05		5.01	4.46	4.6	
	Max	14.71	12.67	12.87		9.24	7.86	7.99	
	SEM	0.2				0.14			
NRC-2001	Mean	9.95^{aB}	8.99^{bB}	8.88^{bB}	0.000	6.22^{aB}	5.54^{b}	5.45^{bB}	0.000
	Min	8.09	7.82	7.66		4.89	4.71	4.59	
	Max	12.24	10.94	9.72		7.86	6.95	6.05	
	SEM	0.11				0.08			
UCD	Mean	10.19^{aB}	8.74^{bB}	8.75^{bB}	0.001	-	-	-	
	Min	7.17	6.54	6.95		-	-	-	
	Max	13.1	11.9	11.77		-	-	-	
	SEM	0.2				-			
Between Methods	P	0.001	0.003	0.005		0.000	0.086	0.034	

^{a,b} Means from different maturity stages of alfalfa having different letters are significantly different
^{A,B} Means obtained different methods having different letters are significantly different

determination coefficient is close to 1, and slope value being also close to 1 means ME_{Menke} and ME_{UCD} methods show similar change.

DISCUSSION

In Stage 1, which is the early growth period of alfalfas, CP, EE, Ash and gas production volume were higher than other periods *(Table 1)*. As plant matures, it was reported a decrease, similar to the literature [20-25], especially in CP, ash and EE volumes. While it was reported that cell wall elements (ADF and NDF) increased with maturation [20,21,26,27], in this study it increased during transition from vegetative period to budding [28], fiber content was affected very little after transition to flowering stage and no statistically significant difference was observed [25] *(Table 1)*.

As a reason for the decline in gas production, with the growth of alfalfa and extension of the stem, decreased digestibility with increased amount of fiber can be considered [29], *(Table 1)*. In the study they conducted by Getachew et al.[30] reported that gas production of many feeds evaluated in 7 different laboratories were 43.6 to 53.6 mL/200 mg, DM and ME values were 9.92 to 11.37 MJ/kg DM, and there were substantial differences between laboratories. Because animal which rumen liquor is received from, diet, time of receiving the liquor

Table 3. *Comparison and correlation analysis between NRC-2001, $Hohenheim_{Menke}$ and UCD equations in the determination energy values of alfalfa hays with different stages*

Tablo 3. *Farklı dönemlerdeki yonca kuru otlarının enerji değerlerini belirlemede NRC-2001, $Hohenheim_{Menke}$ ve UCD eşitlikleri arasındaki ilişki ve kıyaslanma*

Comparison NRC-2001, $Hohenheim_{Menke}$ and UCD Equations				Correlation Analysis NRC-2001, $Hohenheim_{Menke}$ and UCD Equations	
Types of Energy	x - y	Paired Differences		r_{xy}	P
		SEM	P (2-tailed)		
ME	$S1_{Menke}$ - $S1_{NRC}$	0.209	0.000	0.856	0.000
	$S2_{Menke}$ - $S2_{NRC}$	0.246	0.002	0.347	0.105
	$S3_{Menke}$ - $S3_{NRC}$	0.253	0.001	0.512	0.012
	$S1_{UCD}$ - $S1_{NRC}$	0.216	0.287	0.818	0.000
	$S2_{UCD}$ - $S2_{NRC}$	0.269	0.368	0.286	0.186
	$S3_{UCD}$ - $S3_{NRC}$	0.257	0.622	0.490	0.018
	$S1_{Menke}$ - $S1_{UCD}$	0.035	0.000	0.995	0.000
	$S2_{Menke}$ - $S2_{UCD}$	0.029	0.000	0.996	0.000
	$S3_{Menke}$ - $S3_{UCD}$	0.018	0.000	0.998	0.000
NEL	$S1_{Menke}$ - $S1_{NRC}$	0.147	0.000	0.856	0.000
	$S2_{Menke}$ - $S2_{NRC}$	0.175	0.087	0.332	0.121
	$S3_{Menke}$ - $S3_{NRC}$	0.177	0.034	0.516	0.012

Table 4. *Relationships between ME values (MJ/kg DM) of alfalfa hays (n=73) estimated according to different equations*

Tablo 4. *Yonca kuru otlarının farklı eşitlikler aracılığıyla belirlenen ME değerleri arasındaki ilişkiler*

Methods		Equations	SE (P)		R^2
(y)	(x)		Intercept	Slope	
ME_{Menke}	ME_{NRC}	y=-1.981+1.335x	0.141 (0.000)	1.320 (0.138)	0.558
ME_{Menke}	ME_{UCD}	y=0.982+1.021x	0.118 (0.000)	0.013 (0.000)	0.989
NEL_{Menke}	NEL_{NRC}	y=-1.146+1.289x	0.800 (0.156)	0.138 (0.000)	0.551
NEL_{NRC}	NEL_{Menke}	y=3.075+0.428x	0.293 (0.000)	0.046 (0.000)	0.551
ME_{NRC}	ME_{Menke}	y=4.943+0.418x	0.467 (0.000)	0.044 (0.000)	0.558
ME_{NRC}	ME_{UCD}	y=5.591+0.401x	0.460 (0.000)	0.049 (0.000)	0.488
ME_{UCD}	ME_{NRC}	y=-2.049+1.217x	1.385 (0.143)	0.148 (0.000)	0.488
ME_{UCD}	ME_{Menke}	y=-0.853+0.969x	0.126 (0.000)	0.012 (0.000)	0.989

ME_{NRC}: *ME estimated from NRC-2001 equations;* ***ME_{Menke}:*** *ME estimated from the equation in Menke and Steingass [2];* ***ME_{UCD}:*** *ME estimated from UCD equation*

and even the place where the liquor is received affect *in vitro* gas production volume obtained from Hohenheim technique, hence the obtained energy level [9]. The reason for the differences between the energy values obtained in different laboratories may be due to these factors. Low repeatability can also be considered as one of the problems of gas production technique.

Chemical differences between feeds affect *in vitro* gas production significantly [31]. In addition, gas production decreases as plant matures [32], *(Table 1)*. Although it was reported that gas production occurs as a result of fermentation of primary carbohydrates, and protein fermentation has a minor affect [33,34], in this study, gas production in the 1st stage of alfalfa is higher (P<0.001), yet NFC levels are similar in this stage, and NDF and ADF levels are higher in other stages. This result is in line with findings of Zinash et al.[35], Lee et al.[36], Kamalak et al.[37], Canbolat et al.[38], Karabulut et al.[39]. High protein levels, especially NPN and soluble protein (SP) rates can be considered as the reason for higher gas production during the first stage. On the contrary, however, there are also views suggesting that ammonia in the media binds to carbon dioxide and reduces the gas production [40]. Karabulut et al.[39], on the other hand, demonstrated a significant positive correlation between gas production and crude protein in alfalfa hay. Coblentz et al.[41], reported that in oat hays with high NDF, *in vitro* gas production is negatively correlated with NDF, lignin, HK; and positively correlated with water soluble carbohydrates (WSC) and non-fiber carbohydrates (NFC), however there is no such correlation in hay with low NDF.

While Canbolat and Karaman [42] found gas production in alfalfa hay in 24-h incubation as 52.9 mL/200 mg DM, similar to our study, Iantcheva et al.[43] found in 20 samples, Özkul et al.[44] found in 10 samples of alfalfa hay as 25.8-39.0 mL, which is lower.

As a result of analyses and calculations, energy values of alfalfa samples harvested in the S1 obtained with each of three equations were found to be higher than other periods (P<0.001) *(Table 2)*. With maturation of alfalfa hays, a decrease was observed in ME and NEL values calculated from all equations with reduction of digestible nutritional values, which is compatible with the results reported by some researchers [4,25,28,45].

In the study, ME and NEL values of the samples calculated with NRC-2001 equation were similar to the values determined by different researchers [4,25,28]. When some studies, where vegetation period of alfalfa hays are uncertain, were examined; ME_{NRC} and NEL_{NRC} values calculated in this study were found to be lower than ME values [46] calculated using CP and Ash data as well as ME and NEL levels [47] determined with *in vitro* gas production technique. However, in this study, average ME_{UCD} values determined with UC Davis equation of the alfalfas were found to be lower than or similar to [4] the values of different researchers [3].

While ME_{NRC} value averages were lower than ME_{Menke} with a rate of 5.69%, NEL_{NRC} values were found to be lower than NEL_{Menke} with a rate of 4.18%. In the study conducted by Tagliapietra et al.[4], on the other hand, the energy values obtained from NRC-2001 equation were found to be higher than energy values obtained from $Hohenheim_{Menke}$ equation. The reason for this may be that Tagliapietra et al.[4] have used tdNDF48 instead of tdNDF calculated from lignin in NRC-2001 equation. Because Robinson et al.[3] detected that the energy value calculated with tdNDF48 was higher than the energy value calculated with lignin.

Because of lignin level increases with maturation of alfalfa and NDF digestion based on lignin is used in calculation in NRC-2001, it depends on taking the 24-h gas production as basis in gas production method, an incubation 24-h might not be sufficient. A correlation of 81.8% was found between ME values obtained with UC Davis and NRC 2001 method (P<0.000) and results was not found to be statistically significant (P<0.287). Again relationship between the two methods in the subsequent stages decreased *(Table 3)*. Similarly over 99% correlation was observed between ME values of alfalfa samples in all maturity stages, calculated with $Hohenheim_{Menke}$ and UC Davis equations (P<0.000) and the average values were found to be statistically different from each other (P<0.000). The high correlation originates from the fact that 24-h *in vitro* gas production amounts were present in both methods *(Table 4)*.

In this study, when ME values of all alfalfa samples calculated with each of the three equations, regardless of development stages, were compared, the following sequence was obtained; $ME_{UCD}<ME_{NRC}<ME_{Menke}$ (9.23<9.27 <10.39 MJ/kg KM). When it is considered that the result obtained with NRC-2001 model was between the results obtained with $Hohenheim_{Menke}$ and UC Davis models, and also the difficulties in the implementation of *in vitro* gas production, it may give the impression that it is more advantageous for determination of energy content of alfalfa. However Tagliapietra et al.[4] obtained alfalfa hay ME content as 8.5<9.5<10.6 MJ/kg KM from $ME_{Menke}<ME_{UCD}<ME_{NRC}$ equations. While data obtained after 48-h incubation was used in ME_{NRC} calculation, in this study, lignin method in NDF digestibility was used. In studies where different equations were used, Robinson et al.[3] also obtained the lowest ME level from UC Davis equation, similarly to our study. Nuez-Ortín and Yu [12] reported no difference in energy levels in corn and wheat DDGSs obtained with NRC-2001 equations and *in situ* method, there was a strong relation between TDN and energy values obtained with both methods (P<0.05), and there were significant differences in tdNDF, tdCP, tdFA, tdNFC values. Yu et al.[48] reported energy values determined

in alfalfa and timothy according to the methods as $ME_{NRC}<ME_{Invitro}<ME_{Insitu}$.

In NRC-2001 equation, used to estimate energy value of ruminant feeds, it is suggested that the obtained from 48-h *in vitro* incubation should be used instead of calculating NDF digestibility according to lignin content [4]. However, there are also opinions suggesting that more accurate results can be obtained by decreasing incubation period [3,4,49,50]. In this study, only alfalfa hay was used as feed material and Robinson et al.[3] also found energy contents of alfalfa hay determined with *in vivo* and NRC-2001 model very similar. Therefore, it seems that NRC-2001 equations can be safely used to determine energy contents of alfalfa. On the other hand, Das et al.[8] found in their study that the energy contents of 14 different concentrated feeds, calculated with NRC-2001 equations were similar to those *in vitro* results, and reported that NRC-2001 equations were suitable to estimate energy values of tropical ruminant feeds. Magalhães et al.[51] also reported that use of 48-h *in vitro* NDF digestion, instead of 24-h gas production, in UC Davis for determination of energy values of feeds in tropical regions, and also NCR-2001 and Detmann equations allow more accurate estimates.

In ruminant feed, it is very important to know the energy values of roughages such as alfalfa hay, which will be used throughout the year. Due to difficulties in implementation of *in vivo* and *in vitro* methods in determining energy values, estimations obtained from equations based on nutrients is preferred. In this study, energy values of alfalfa hay were determined using NRC-2001, $Hohenheim_{Menke}$ and UC Davis equations and values obtained with NRC-2001 equations were found to be between the other two methods' values. It suggested more comprehensive studies with different feeds and different equations are needed.

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Research Article

Homeopathic Treatments in 17 Horses with Stereotypic Behaviours

Çağla PARKAN YARAMIŞ [1] Marie-Noelle ISSAUTIER [2] Sinem ULGEN SAKA [3]
Berjan DEMİRTAŞ [1] Dilek OLGUN ERDİKMEN [4] Mehmet Erman OR [3]

[1] İstanbul University, Vocational School of Veterinary Medicine, Equine and Equine Training Program, TR-34320 Avcilar, İstanbul - TURKEY
[2] Veterinary Surgeon, Expert and Consultant in Veterinary Homeopathy, Lyon - FRANCE
[3] İstanbul University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-34320 Avcilar, İstanbul - TURKEY
[4] İstanbul University, Faculty of Veterinary Medicine, Department of Surgery, TR-34320 Avcilar, İstanbul - TURKEY

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Abstract

Many stabled horses perform a variety of stereotypic behaviours such as cribbing, circling, wall kicking, self-mutilation, weaving and headshaking. It is difficult to know why exactly each horse performs these behaviours, there may be specific causal factors for these activities in the horse. Homeopathy can contribute to the treatment of these disorders, because it approaches the organism as the sum of the specific characteristics of an animal when it is in a state of health or illness, at the physical, mental and psychological level. The application of homeopathic laws during anamnesis allows us to achieve a deeper understanding of the horse, considering all its special characteristics. This study has been conducted with 17 horses showing one of the stereotypic behaviours of cribbing, circling, wall kicking and self mutilation. Homeopathic remedies are determined specifically according to the constitution and stereotypic behaviour problem of each horse. One month after the begining of the treatment, the symptoms of stereotypical behaviours were found to be decreased and after two months considerable regression were detected. Cribbing and self-mutilation were completely treated. Circling and wall kicking were decreased. To our knowledge, this study is the first report to cure the stereotypic behaviours of horses with using homeopathic remedies. This study suggests that homeopathy is as an effective therapy method which is used uncommonly in stereotypic behaviours of horses.

Keywords: *Horse, Stereotypic behaviours, Homeopathy*

Sterotipik Davranış Bozukluğu Gösteren 17 Atın Homeopati İle Tedavisi

Özet

Ahırda bakılan atlarda yel yutma, ahırda dönme, duvar tekmeleme, kendine zarar verme gibi sterotipik davranış bozukluklarına sıklıkla rastlanabilmektedir. Atların bu tip davranış bozukluklarını yapmalarının sebebi tam olarak bilinmemekle birlikte, her atın kendine ait nedensel faktörleri olabileceği düşünülmektedir. Homeopati organizmaya, sağlık veya hastalık durumunda, fiziksel, zihinsel ve psikolojik düzeyde, hayvanın tüm karakteristik özelliklerini bir bütün olarak görüp yaklaşır. Vakanın geçmişe ait hikayesini alırken uygulanan homeopati prensipleri, bize davranış bozukluğu gösteren atı daha derin bir anlayış ile tanıma olanağı sağlar. Çalışmamız yel yutma, ahırda dönme, duvar tekmeleme ve kendine zarar verme sterotipik davranışlarından birini gösteren 17 at ile yapılmıştır. Homeopatik ilaçlar her at için özel olarak, atın yapısal özelliklerine ve sterotipik davranışına göre belirlenmiştir. Tedavinin birinci ayının sonunda, sterotipik davranışlarda azalma; ikinci ayın sonunda ise önemli bir gerileme ve iyileşme saptanmıştır. Yel yutma ve kendine zarar verme tamamen iyileşmiş; ahırda dönme ve duvar tekmeleme belirgin şekilde azalmıştır. Çalışma, tarafımızca yapılan araştırmalara göre, atlarda sterotipik davranışların homeopati ile tedavisinde bildirilen ilk rapordur. Bu çalışmada, atların sterotipik davranışlarının tedavisinde ender kullanılan homeopatinin etkili bir sağaltım yöntemi olarak kullanılabileceği sonucuna varılmıştır.

Anahtar sözcükler: *At, Sterotipik davranışlar, Homeopati*

INTRODUCTION

There are a wide range of seemingly bizzare behaviour patterns considerƒed undesirable by horse owners for which they may seek veterinary advice and treatment. Stereotypies are invariant and repetitive behaviour patterns that seemingly have no function [1,2]. Development and continued performance of stereotypic behaviour have been linked to suboptimal environments [3]. Specifically, stereotypic behaviour can develop within the following contexts:

İletişim (Correspondence)
+90 530 6457484
cparkan@istanbul.edu.tr

When an animal is unable to execute a behaviour pattern that it is highly motivated to perform, such as feeding or foraging behaviour, when it can't escape or avoid a stressful or fearful situation, or when it is kept in confinement or social isolation [4,5].

The performance of stereotypic behaviour has been used as an indicator of poor welfare although it is often difficult to determine whether the behaviour is the result of poor welfare in the past or due to current adverse conditions [6]. Studies conducted in Canada [7] and the United Kingdom [8] have reported that greater than 13% of domesticated horses exhibit stereotypies. Stereotypic behaviour patterns observed in domestic horses can be listed as cribbing, weaving, circling, wall kicking, pawing, head shaking and self-mutilation which can be observed as flank-biting, chest-biting, shoulder-biting [9,10].

Behavioural disorders have minor effects on the horse's health or performance like cribbing, however, some of them like self-mutilation, head shaking in show horses and race horses probably can result in euthanasia [2,5]. There are many studies on the neuropharmacological, surgical or mechanical therapy of stereotypies, but little is known about their causation. Behavioral disorders can be treated with tranquilisers or anxiolytics. The most well-known tranquilisers or anxiolytics are those of the benzodiazepine family [11,12]. The common side effects of the conventionel theurapeutics drugs made the homeopathy increasingly popular in treatment of horse behavioural disorders and also homeopathic remedies can help enormously without fear of breaking the rules of International Federation of Equestrian Sports on prohibited substances or causing sedation [13].

Homeopathy is a safe, gentle, and natural system of healing that works with the body to relieve symptoms, restore itself and improve the overall health. Homeopathy is a 200 years old therapeutic method involving 'preparations of substances whose effects when administered to healthy subjects correspond to the manifestations of the disorder (symptoms, clinical signs, pathological states) in the individual patient'. The aspects of the mechanism of action still remain unclear however, the principle of the homeopathy is based on "like cures like". The term homeopathy is derived from two Greek words, 'homoios', meaning 'like', and 'pathos', which is translated as suffering [13-15].

Homeopathic remedies can contain an unlimited number of plant, animal and mineral compounds, can be supplied in a number of forms, including tablets, creams, powders, liquids, and pellets [16]. Remedies generally come in the form of tiny tablets or liquid and are designed to be absorbed through the tongue and not put down the throat; since neither tablets nor liquid has much taste, administration is usually quite easy. Homeopathic remedies are very safe to use. Generally, no side effects are observed, if the remedy is used correctly. However, in the hands of persons with little or no knowledge of homeopathy, remedies may be overused or, much more commonly, the wrong remedy is used, and no effect at all will be seen. This obviously does not harm the patient, but it does it no good either and is one of the reasons why, in the recent past, homeopathy was seen as a treatment with little or no effectiveness [17].

Homeopathy can be used to treat a surprising number of conditions in both large and small animals. It can be used as therapy in trauma, acute injuries and in many types of inflammatory conditions such as acute and chronic diarrhea, chronic gingivitis, acute and chronic respiratory conditions, renal insufficiency, behaviour disorders etc.[17,18]. Homeopathy can contribute to the treatment of these disorders, because it approaches the organism taking its idiosyncrasy into consideration. Idiosyncrasy is defined as the sum of the specific characteristics of an animal when it is in a state of heath or illness, at the physical, mental and psychological levels [19].

Veterinary studies in homeopathy to date has typically studied the effect of a pre-selected homeopathic medicine in groups rather then using individualised prescribing [15,17]. One of the most powerful ways of dealing with behavioural problems by homeopathy is using homeoapthic remedies according the complaint of the animal and using the constitutional remedies [13].

There are few studies about equine homeopathic treatments, however to our knowledge there is not a research article about the effects of homeopathic treatments on equine behavioural problems.

According to the books written by experienced veterinarians specialized in homeopathy, the basic homeopathic remedies used for equine behavioural problems are: Ignatia, Gelsemium, Pulsatilla, Phosphorus, Lycopodium, Arnica Montana, Hepar Sulfur, Arsenicum Album, Nux Vomica, Thuya Occidentalis, Argentum Nitricum, Lachesis, Stramonium. It has been reported that the most communly used ones are Ignatia and Gelsemium [13,18,20].

Ignatia is one of the homeopathic remedies most commonly used in horses. This is the principal remedy to consider in treating grief, both silent grief and for the deeper effects of these emotional problems [13,21]. Gelsemium is described as a remedy for a vareity of anxiety-like psychological and behavioral symptoms. In recent years, the effects of Ignatia and Gelsemium on emotional response are studied mostly in mice. It has been reported that Gelsemium reduced anxiety and fear in manner quantitavity comparable to the effects of normal anxiolytic drugs [22]. Marzotto et al.[23] have been reported that Ignatia has been shown to be able to modulate the emotional responses of mice.

Stereotypic behaviour problems are often observed in horses [7,8]. Horse owners try to solve this problem using chemical drugs or by traditional treatments. All these

so-called solutions can cause some harmful effects to the horses.

The aim of our study is to cure stereotypic behaviour problems which comprimise the welfare and health of the horses with homeopathic remedies which can be used as an alternative way to the conventional therapy have low costs, respond fast, easy in usage and acceptable for racing rules.

MATERIAL and METHODS

Subjects

This study has been conducted with 17 show jumping horses (12 geldings, 5 mares), consulted by the owners of two riding centers with the stereotypic behaviours, between 2014 and 2015. Horse breeds were Hannover (6 horses), Holsteiner (5 horses), Irish Sport Horse (3 horses), Oldenburger (2 horses), Trakehner (1 horses). The age of the horses was varied between 7 and 14 years old.

Before treatment, a researcher took detailed anamneses from horse caretakers asking about the constitution of each horse (e.g. dominance in character, fearfulness, aggresiveness, fragility in nails, fragility in digestive system, feeling cold easily, sweating easily, neural sensitivity to smell and voices, fear from thunder, excitement before competition, head shy, excessive attention-seeking), the previous history of any illness and the current complaints of the horses.

A researcher also asked the feeding and exercise regimes of the horses and thereafter visited the horse riding centers to observe the horses at their own places.

The daily routine schedule of feeding and exercise regimes were same in both of the riding centers. Horses were fed five times a day; as a roughage, hay was given three times a day at 07:00 am, 13:00 pm and 19:00 pm, as a concentrate, a mix of crushed barley, oat and bran was given twice a day at 08:00 am in the morning and 20:00 pm in the evening. Horses allowed to drink water *ad libitium*. Two hours before or after the feeding, horses exercised by the trainers, once a day for 45-60 min. The same feeding and exercise regimes have been continued in the same manner during the treatment as well.

According to the detailed anamneses and observations, it is diagnosed that these 17 horses showed the stereotypic behaviour patterns of cribbing, circling, wall kicking and self mutilation. The distribution of these stereotypic behaviours in horses is shown in *Table 1*. According to the anamneses the horses have had one of these stereotypic behaviours at least for 3 months and horses with cribbing and circling have not shown these behavioural patterns during feeding and exercising time and when they are in contact with people while horses with wall kicking and self mutilation have continued to show these patterns in minimal level during feeding but not during exercising time.

Four horse caretakers (owner, trainer, groom and veterinarian) who belonged to the same riding center were appointed as an observer team. They were considered to understand the horse sufficiently through ordinary care and daily exercising. A scoring survey scale was given to each person in observation team *(Table 2)*. For each horse, each person in observation team was asked to provide their impression about the pattern of stereotypic behaviour at the end of each month according to the everyday observations throughout the study. The observations were carried out especially during the times when the horses are not feeding or exercising since it has been reported that these steorotypic behaviour patterns had not been observed during feeding and exercising time. Each response was given based on a scale of 1-5, with one being never match with a diagnosed stereotypic behaviour pattern, 5 being the highest match in that pattern.

Treatment

Horses were treated with homeopathic remedies with the requests of horse owners. These remedies are bought as granulated form (granules) in plastic tubes from by Boiron Laboratoires, Lyon, France. The remedies were granules already diluted and dynamized in 15CH (centesimal dilutions).

Homeopathic remedies are determined spesifically according to the constitution and stereotypic behaviour problems of each horse *(Table 3)*. Homeopathic Ignatia (Ign) and/or Gelsenium (Gels) have been used for every horse according to the effects of each remedy on behavioural problem. Apart from these two remedy, additonally

Table 1. *The distribution of stereotypic behaviours observed in 17 horses*
Tablo 1. *Onyedi atta gözlemlenen sterotipik davranışların dağılımı*

Stereotypic Behaviour	Horses	
	n	%
Cribbing	7	41.17
Circling	3	17.64
Wall kicking	4	23.52
Self-mutilation	3	17.64
Total	17	100

Table 2. *Example of scoring survey for observers*
Tablo 2. *Gözlemci anket örneği*

Observer Name-Surname			
Horse 1	**A0**	**A1**	**A2**
1 (never), 2, 3, 4, 5 (highest)			

A0: *Stereotypic behaviour score before treatment;* ***A1:*** *Stereotypic behaviour score at the end of 1st month;* ***A2:*** *Stereotypic behaviour score at the end of 2nd month*

Table 3. *The homeopathic remedies used according to the constitution and the stereotypic behaviour of each horse*

Tablo 3. *Her atın yapısal özelliklerine ve sterotipik davranışına göre kullanılmış olan homeopatik remediler*

Horse Number, Sex, Age	Symptoms	Treatment
Horse 1 (female, 8)	Cribbing	Ign, Gels, Thuj
Horse 2 (gelding, 11)	Cribbing	Stram, Gels, Phos
Horse 3 (female, 9)	Cribbing	Nux-v, Ign, Gels
Horse 4 (gelding, 10)	Cribbing	Gels, Puls, Thuj
Horse 5 (gelding, 11)	Cribbing	Ign, Hyper
Horse 6 (gelding, 14)	Cribbing	Gels, Lyc, Phos
Horse 7 (female, 12)	Cribbing	Ign, Lyc, Phos
Horse 8 (gelding, 7)	Circling	Gels, Ign
Horse 9 (gelding, 10)	Circling	Puls, Gels
Horse 10 (female, 8)	Circling	Puls, Ign
Horse 11 (female, 12)	Wall kicking	İgn, Gels, Nux-v
Horse 12 (gelding, 9)	Wall kicking	Lach, Lyc, Gels
Horse 13 (gelding, 11)	Wall kicking	Gels, Arg-n
Horse 14 (gelding, 8)	Wall kicking	Ign, Nux-v
Horse 15 (gelding, 13)	Self-mutilation	Lach, Staph, Ign
Horse 16 (gelding, 8)	Self-mutilation	Lach, Staph, Gels
Horse 17 (stallion, 9)	Self-mutilation	Ars-a, Arg-n, Nux-v, İgn, Lach

Stramonium (Stram), Phosphorus (Phos), Nux vomica (Nux-v), Pulsatilla (Puls), Hypericum (Hyper), Lycopodium (Lyc), Argentum nitricum (Arg-n), Staphysagria (Staph), Arsenicum Album (Ars-a), Lachesis (Lach) and Thuya occidentalis (Thuj) were used as treatment remedies specific for each horse.

Five granules from each remedy in the recipe of each horse were put in 50 mL of brown bottle adding 7.5 mL pure alchohol and 42.5 mL water. The bottle was shaked until the granules dissolved and the name of horse, the beginning date of the treatment were written on the label of bottle. 15-20 seconds shaking before usage was also noted on the label of bottle.

The mix of remedies specific for each horse was shaked for 15-20 seconds before administration and 1 mL of mixed remedies was administered by adding on sugar [spraying 5 times] to the horse by groom. This treatment was applied every morning at 10:00 am for 2 months. Thuja occidentalis (Thuj) (horse 1, 4) and Lachesis (Lach) (horse 17) have been used only twice a week additionally to the prepared mixed remedies.

RESULTS

By treatment survey analysis, after one month evaluation the symptoms of stereotypical behaviours were found to be decreased and after two months considerable regression were detected. Cribbing and self-mutilation were completely treated. Circling and wall kicking were decreased *(Fig. 1)*.

Additionally, in 3 horses (horse 1, 3, 6) cribbing was very severe however, reduced considerably after 2 weeks and completely disappeared after one month. Self mutilation in all horses was reduced but still continued in the first month, thereafter disappeared completely at the beginning of second month *(Fig. 2a-b)*. One of the horses (horse 9) with circling was cured completely at the fifth week of the treatment while this behavioural pattern was reduced in other two horses (horse 8, 10) but not completly disappeared. In horses with wall kicking (horse 11, 12, 13, 14) recovery rate was very high at first month but then reduced towards the end of treatment but not complete recovery occurred in these horses.

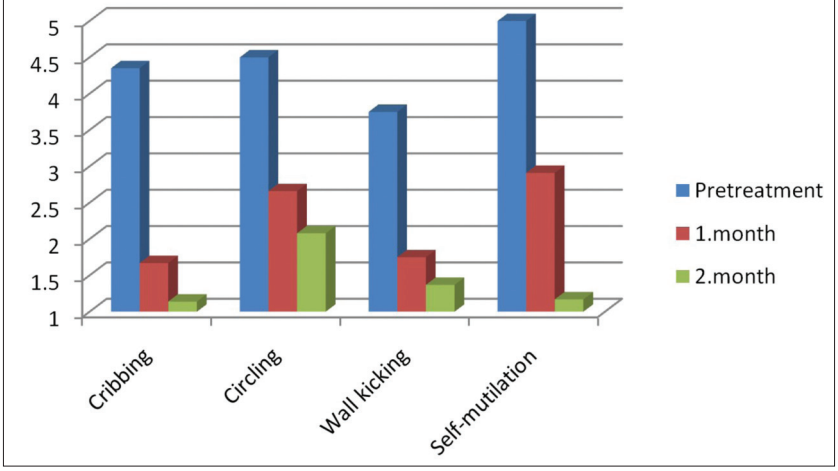

Fig 1. The alteration of stereotypic behaviours according to survey analysis at pretreatment, at the end of the first and the second month

Şekil 1. Gözlem anketlerine göre tedavi öncesi, tedavinin 1. ve 2. ayının sonundaki sterotipik davranışlarda gözlenen değişimler

DISCUSSION

Horses are naturally free-ranging, social, grazing herbivores. In stables, horses are provided with food, water and shelter, but their choice of feed, social interactions and movement are limited. Under these conditions a number of stereotypic behaviours can develop such as cribbiting, windsucking, wall kicking, headshaking, self-mutilation

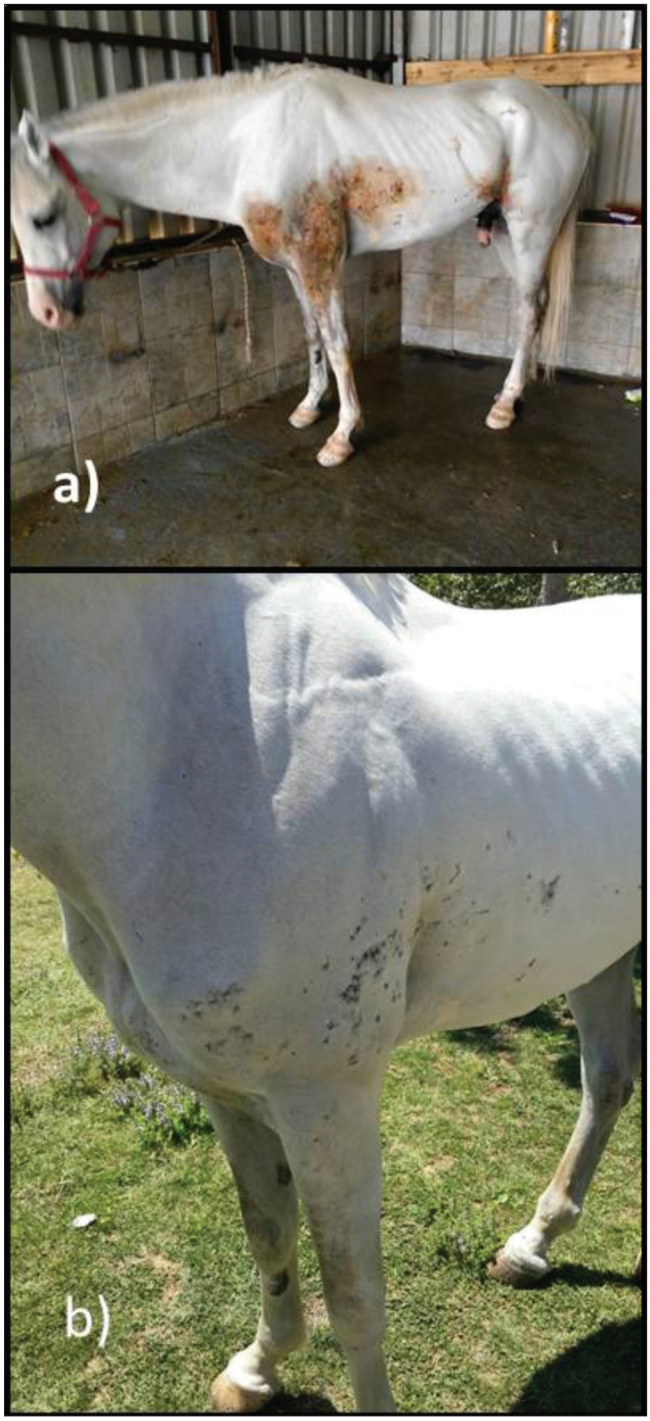

Fig 2. The appearance of the horse (horse 17) with self mutilation disorder before and after the treatment (flank chest, shoulder and hind leg bitting) **a-** Before treatment **b-** After treatment (in 2nd month)

Şekil 2. Onyedi numaralı atın tedavi öncesi ve sonrası görüntüsü. Göğüs cidarı, göğüs, omuz ve arka bacaktaki kendine zarar verme **a-** Tedavi öncesi görüntüsü **b-** Tedavi sonrası 2. aydaki görüntüsü

etc. Alternatively, these horses should be treated because their behaviour appears to reduce quality of life, welfare concern or reduce the value of the horse [24]. In many studies treatments for stereotypic behaviour have been applied due to the concern of economic values [24,25]. However, in our study horse owners main concerns were the welfare and the health issues of the horses.

Cribbing, circling, self-mutilation, wall kicking and other stereotypic behaviours are recognized as both a management and a welfare concern, and many owners attempt to physically prevent horses from engaging in these behaviours [26,27]. Number of responses to the stable environment are believed to be harmful. For example, wind-sucking and cribbiting are irregularly associated with teeth wear and potentially life threatening colic. Weaving and circling in stabled horses are also said to have harmful consequences such as muscle damage and fatigue [24]. In our study, 3 horses (horse 2, 5, 6) with cribbing showed colic symptoms together with this stereotypic behaviour pattern. However, colic symptoms have also been dissappered in these horses after treatment for cribbing. Additionally, all horses with circling had some musculoskeletal lesions due to this stereotypic behaviour pattern before treatment. However, these lesions had not been occured in any horse after treatment for circling. We suggest that treatments for stereotypic behaviours may reduce the occurrence of secondary diseseases developed after behavioural disorders.

It has been reported that horses with stereotypic behaviours are more sensitive to stress factors and repetitive movements of these stereotypic behaviours have been increased in these horses due to the stress factors [28]. Homeopathic treatment is a noninvasive and an easily applied treatment without causing any stressful effects on horses. Furthermore, easily appliable by horse caretakers can make the homeopathic remedies sustain the treatment. However, the treatment of the behavioural disorders require more time and more patience of the practicians. In our study, we applied the remedies everyday without any cessation. This might have helped to get a very fast response in our study.

Prevention of stereotypic behaviours in horses can be achieved with a variety of techniques including: physical barriers, such as weaving bars above stable doors or harnesses that restrict movement; punishment, such as nails hammered into cribbed wooden surfaces; and surgical intervention, such as cutting the muscles involved in cribbing or wind-sucking [9,24]. Temporary inhibition of the undesirable behavior can sometimes be achieved by physical restraint with special equipment (e.g., a cribbing collar, a muzzle or head cradle). The primary problem with physical prevention, for example, attempting to stop cribbing using a cribbing collar or by removing cribbing surfaces, is that this approach fails to address the underlying causes of the behaviour and may further reduce equine welfare and cosmetic outcome [10]. Cribbing colar as a physical restraint before homeopathic treatment was applied in one horse (horse 1) in our study. It was observed that the repetitive movements were reduced after this

application however, contact dermatitis occured on the neck of the horse as a side effect. Interestingly, after cribbing colar was taken off the repetitive movements reoccured. These kind of preventative treatments might solve the problem for a short period but can not provide permanent solutions without curing underlying causes of the stereotypic disorder.

Tranquilisers, anxiolytics and opiate receptor blockers can also treat the stereotypical horses for a temporary period but these drugs have some side effects [11]. It has been reported that opiate receptor blockers stop horses from cribbing for 20 min following a 20 min latency period. Intravenous naltrexone, another opiate blocker, suppressed cribbing for two to seven hours. Cribbing is supressed only while the opiate antagonists are present; the short half-life and expense of these drugs have made long-term opiate receptor blockage impractical [9]. Side effects, the short half-life, the expense of these drugs and the fear of breaking the rules of International Federation of Equestrian Sports made the homeopathy increasingly popular in the treatment of equine stereotypic behaviours.

The various surgical treatments for some stereotypes like cribbing and self-mutilation include buccostomy, spinal accessory neurectomy, myectomy or a combination of partial myectomy and spinal accessory neurectomy and castration. The success rates of these treatments vary from 0% to 70%. Cribbing often reoccurs. Despite meticulous intra-operative dissection and hemostasis, seroma, hematoma, wound drainage, and surgery site infection are the post-surgical complications [29]. None of our horses have been treated by surgically. Only horse 17 (self-mutilation) was castrated before homeopathic treatment however, no improvement was detected after this surgery. Surgical treatments are not preferred due to the factors such as wide range of success rates (between 0% and 70%), high recurrence rate, risks of anesthesia and high costs of operations [26,29].

According to our knowledge, there are not any study related to the treatment of the equine stereotypic behaviours with the homeopathic remedies.

Loukaki et al.[19] have reported that homeopathic remedies such as Phosphorus, Lachesis, Arsenicum album, Pulsatila, Argentum nitricum, Ignatia, Gelsemium were often indicated in the cases of separation anxiety in dogs. Bellavite [22] has reported that Gelsemium has reduced anxiety and fear in murine, in a manner quantitatively comporable to the effects of anxiolytic drugs without provoking any sedation side-effects. It has been also reported that Ignatia reduced anxiety and fear in mouse [23]. In our study, we also applied Ignatia and/or Gelsemium to the horses and our results imply that the same effects occur in the horses as in other animals.

Classical homeopathy, as well as most of the other alternative approaches, emphasises a holistic view of disease in which individual judgement and treatment are important. This implies that a homeopathic remedy must be chosen by taking into consideration the organism as a whole, including personality and behaviour, and not merely the symptoms in the affected organ system. Solid statistical evidence demonstrates that the effects of the different potency levels investigated (5CH, 7CH, 30CH) do not follow a classical dose-response relationship regarding their mere dilution level, confirming a phenomenon frequently observed with high homeopathic dilutions in other laboratory models [30]. In our study the symptoms of two horses (horse 8, 10) with circling were reduced considerably but not completely disappeared. The reason might be the dilution and dynamization of homeopathic remedies used in this study (15 CH) was not enough to cure the stereotypic behaviour completely. We suggest that the potency level of 30 CH may improve the course of the treatment and get better results. We recommend that further studies should be carried on using different dilutions and dynamizations to see the effect of different potency levels of these homeopathic remedies on stereotypic behaviours.

As a result, homeopathic treatments prepared specifically for each horse's constitution and complaints provided an effective therapy in a short time to cure equine stereotypic behaviours.

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Short Communication

The Effect of Different Doses of Intramuscular Xylazine HCl Administration on Intraocular Pressure in Rabbits

Elif DOGAN [1] Latif Emrah YANMAZ [1] Ugur ERSOZ [1]
Mumin Gokhan SENOCAK [1] Zafer OKUMUS [1]

[1] Department of Surgery, Veterinary Faculty, Ataturk University, TR-25240 Erzurum - TURKEY

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Abstract

This study was aimed to determine the effects of intramuscular Xylazine HCl administration on intraocular pressure (IOP) in rabbits at 5 mg/kg (XYL-5) and 10 mg/kg (XYL-10) doses. IOP measurements were recorded at baseline (T_{-1}), and at 5 (T_5), 10 (T_{10}), 15 (T_{15}), 20 (T_{20}), 30 (T_{30}), 45 (T_{45}) and 60 (T_{60}) minutes following Xylazine HCl administration. IOP values were significantly decreased at T_{15} in group XYL-5 (8.37±1.45 mm Hg) and at T_5 in group XYL-10 (8.00±1.13 mm Hg). In conclusion, intramuscular Xylazine HCl significantly reduced IOP in rabbits at the latest after 15 minutes of administration, regardless of the doses used in this study.

Keywords: *Intraocular pressure, New Zealand White rabbit, Xylazine HCl*

Farklı Dozlarda Uygulanan Ksilazin HCl'ün Tavşanlarda İntraoküler Basınç Üzerine Etkisi

Özet

Bu çalışmada tavşanlarda 5 mg/kg (XYL-5) ve 10 mg/kg (XYL-10) dozlarda uygulanan Ksilazin HCl'ün intraoküler basınç üzerine etkilerinin belirlenmesi amaçlandı. İntraoküler basınç ölçümleri Ksilazin HCl uygulamasını takiben bazal (T_{-1}), 5 (T_5), 10 (T_{10}), 15 (T_{15}), 20 (T_{20}), 30 (T_{30}), 45 (T_{45}) ve 60 (T_{60}) dakikalarda yapıldı. Grup XYL-5'te T_{15} (8.37±1.45 mm Hg) ve grup XYL-10'da T_5'te (8.00±1.13 mm Hg) önemli derecede IOP ölçümlerinin azaldığı belirlendi. Sonuç olarak, doza bakılmaksızın Ksilazin HCl, uygulamadan en geç 15 dakika sonra Beyaz Yeni Zelanda tavşanlarında intraoküler basıncı düşürdü.

Anahtar sözcükler: *Beyaz Yeni Zelanda tavşanı, İntraoküler basınç, Ksilazin HCl*

INTRODUCTION

Xylazine HCl is an alpha-2 adrenoceptor drug that has muscle relexant, sedative and analgesic properties and commonly combined with ketamine for routine procedures [1]. It is used as a premedicant to prevent side effects (e.g, respiratory depression) associated with ketamine administration [2,3]. Alpha-2 adrenoceptor drugs decrease intracellular cyclic adenosine monophosphate levels and results with less production of aqueous humor and increased uveoscleral outflow [4].

Maintaning intraocular pressure (IOP) at certain level is an important aspect for experimental studies especially in ocular surgery [5]. Because sudden increase in IOP may provoke complications such as vitreal hemorrhage or retinal decollement, IOP alterations should be minimized during the anesthesia [6]. Previous study has reported that topical and intraarterial administration of Xylazine HCl decreases IOP in rabbits, cats and monkeys [7]. Moreover, intravenous Xylazine HCl administration causes a decrease in IOP in horses [8]. It has shown that intramuscular (im) administration of 10mg/kg Xylazine HCl and 50 mg/kg Ketamine HCl combination results with lower IOP values in rabbits [6]. However, no significant alterations were observed in IOP after anesthesia achieved with the im combination of 5 mg/kg Xylazine HCl and 35 mg/kg Ketamine in rabbits [9].

Even though Xylazine HCl may be used alone for minor procedures in animals, to date no study has evaluated the im administration of Xylazine HCl alone on IOP in rabbits. For this reason, this study was aimed to detect the effects of 5 mg/kg and 10 mg/kg doses of Xylazine HCl administration on IOP in rabbits.

İletişim (Correspondence)
+90 442 2317161
elif.dogan@atauni.edu.tr

MATERIAL and METHOD

Atatürk University Local Board of Ethics Committee for Animal Experiments has approved the study protocol of this research (HADYEK decision no: 2015/188).

Eight, adult male, New Zealand White rabbits weighing 2.2-3.5 kg were used in this study. They were housed in individual cages with food and water *ad libitum*. The humidity ranged between 40 and 60%. A uniform temperature of 22±2°C was maintained thoroughout with a 12:12 h light:dark cycle. The rabbits were screened for pre-existing ocular disorders and clinical assessment was performed to ensure adequate health status. All animals were determined to be free of corneal and conjunctival diseases and they underwent a 14 days acclimization period. Food and water were not withdrawn before administration, and animals were weighed prior to experiment.

Animals received each drug treatment in random order in a crossover study, with a minimum interval of one week between treatments. All rabbits in group of XYL-5 received im 5 mg/kg Xylazine HCl (2% Rompun, Bayer, Istanbul, Turkey), and those in group of XYL-10 received im 10 mg/kg Xylazine HCl. The doses used in this study were selected based on previous studies [6,9]. All injections were performed to *quadriceps femoris* muscle by the same person who was unaware of the experimental design.

Handling of rabbit and calibration of tonometer were accomplished as previous report [10]. IOP was measured with a rebound tonometer (Tonovet, Icare, Vantaa, Finland). Anesthetic eye drops were not used during the measurements. IOP was recorded at the same time of the day (at 9:00 to 11:00) at baseline (T_{-1}), and at 5 (T_5), 10 (T_{10}), 15 (T_{15}), 20 (T_{20}), 30 (T_{30}), 45 (T_{45}) and 60 (T_{60}) min following Xylazine HCl administration. Measurements were discontinued until the use of im 0.70 mg/kg Atipamezole (Antisedan, Pfizer, Istanbul, Turkey) administration.

Each measurement of IOPs were taken by the same examiner who was unaware of performed medications. The left eye measurement was always performed prior to the right eye measurement. The mean of the left and right IOP was assumed as the animal's IOP.

Statistical Analysis

All data were analyzed using the SPSS 19 (IBM Company, Version 19.0, SPSS Inc, USA, 2010) statistical package. Data are reported as means±SD. Prior to statistical analysis data were subjected to Kolmogorow-Smirnow test to asses normality. An independent samples t-test was used to determine pre-treatment differences between groups. Post-treatment differences within each treatment group were evaluated using a paired samples t-test. A P-value of <0.05 was considered statistically significant.

RESULTS

The mean values of IOP at all time intervals are shown in *Table 1*. All data were expressed as mm Hg. The mean T_{-1} values of IOP in group XYL-5 and XYL-10 were 10.25±2.54 and 10.62±2.04 mm Hg, respectively. There were significant differences ($P<0.05$) observed between groups at T_{45} and T_{60} values. However, the differences in IOPs at the other predefined time (T_5, T_{10}, T_{15}, T_{20}, T_{30}) points were not statistically significant.

IOP values were significantly decreased at T_{15} in group XYL-5 (8.37±1.45 mm Hg) and at T_5 in group XYL-10 (8.00±1.13 mm Hg). The lowest IOP values were obtained at T_{60} in both groups. These were 8.12±1.40 mm Hg in group XYL-5 and 5.93±0.77 mm Hg in group XYL-10.

DISCUSSION

This study is the first report on the IOP alterations following different doses of im Xylazine HCl administration. The im Xylazine HCl significantly reduced IOP in rabbits at the latest after 15 min of administration, regardless of the doses used in this study.

Rabbits are commonly prefered in ocular experiments because of their large size of the eye, easy handling and meekness [11]. Normal IOP measurements using rebound tonometer in rabbits are ranged between 9.51±2.62 mm Hg [10]. In the current study, the mean IOP of baseline values for all rabbits were within the reference values. The IOP can vary throughout the day and environmental factors may

Table 1. *Mean and SD of intraocular pressure values during the predefined time points in two groups. Group XYL-5 received im 5 mg/kg Xylazine HCl, Group XYL-10 received im 10 mg/kg Xylazine HCl*

Tablo 1. *İki grubun tanımlanan zamanlarda intraoküler basınç ortalamaları ve standart sapma değerleri. Grup XYL-5'deki deneklere im 5 mg/kg Xylazine HCl, Grup XYL-10'daki deneklere im 10 mg/kg Xylazine HCl uygulanmıştır*

Groups	Predefined Time Points							
	T_{-1}	T_5	T_{10}	T_{15}	T_{20}	T_{30}	T_{45}	T_{60}
XYL-5	10.25±2.54	9.25±1.44	8.81±1.69	8.37±1.45*	8.12±1.12*	8.15±1.70*	8.62±1.15*a	8.12 ±1.40*a
XYL-10	10.62±2.04	8.00±1.13*	8.31±1.18*	8.25±1.95*	7.65±1.23*	8.31±1.66*	6.40±1.18*b	5.93 ±0.77*b

** Indicates significant differences within the groups ($P<0.05$); a,b indicates significant differences between the groups; T_{-1} Baseline value*

alter the measurements [12,13]. Based on these premises, IOP measurements of rabbits were recorded at the same time of the day and after two weeks of acclimatization period.

Previous study has reported that Xylazine HCl at the dose of 1 or 2 mg/kg do not cause any significant changes in IOP, whereas 4 or 8 mg/kg doses of Xylazine HCl administration results in lower IOP values in dogs [14]. In the present study significant decrease in IOP was observed 15 min after im administration of 5 mg/kg Xylazine HCl (T_{15}). Moreover, 10 mg/kg doses of xylazine used in this study reduced IOP 5 min after administration (T_5) in comparison with the baseline value (T_{-1}). In the current study, no significant decrease on IOP was observed between groups at all time intervals with the exception of T_{45} and T_{60}. Xylazine HCl at 10 mg/kg dose used in the present study more reduced IOP at 45 and 60 min, compared with at the dose of 5 mg/kg. A limitation to the present study is the rabbits that enrolled having healthy eyes. Effects of Xylazine HCl administration on IOP involving rabbits with ocular diseases is a worthy topic for future study.

It has been stated that reduced blood pressure results with lower IOP values [15]. Similarly in this study, Xylazine HCl administration causes reduced IOP in rabbits possibly due to xylazine-induced bradycardia [1].

In conclusion, im Xylazine HCl at 5 mg/kg or 10 mg/kg doses reduced IOP in rabbits. Comparison of two pre-anesthetic regimen suggests Xylazine HCl at 10 mg/kg is preferred over 5 mg/kg in rabbits that require lower intraocular pressure for procedures that longs more than 45 min. Because present study was carried out in only eight rabbits, additional studies involving larger numbers of rabbits are needed to confirm our results.

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Short Note

Aegypoecus guralpi, A Junior Synonym of *Neophilopterus incompletus* (Insecta: Phthiraptera: Philopteridae)

Bilal DİK [1]

[1] University of Selcuk, Faculty of Veterinary Medicine, Department of Parasitology, TR-42250 Konya - TURKEY

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Abstract

Aegypoecus guralpi Dik et al., 2015, a louse described from *Buteo rufinus* collected in southern Turkey, has been found to be conspecific with *Neophilopterus incompletus* (Denny, 1842) from *Ciconia ciconia.* Therefore, *A. guralpi* is now regarded as a junior synonym of *N. incompletus*.

Keywords: *Lice, Neophilopterus incompletus, Aegypoecus guralpi, new synonymy, Hatay, Turkey*

Aegypoecus guralpi, Neophilopterus incompletus (Insecta: Phthiraptera: Philopteridae)'un Yeni Bir Sinonimi

Özet

Güney Türkiye'de, Kızıl Şahin'den *(Buteo rufinus)* tanımlanmış bir bit olan *Aegypoecus guralpi* Dik et al., 2015'nin Ak Leylek'deki *(Ciconia ciconia) Neophilopterus incompletus* (Denny, 1842) ile aynı olduğu anlaşılmıştır. Böylece *A. guralpi N. incompletus*'un yeni bir sinonimi olmaktadır.

Anahtar sözcükler: *Bit, Neophilopterus incompletus, Aegypoecus guralpi, yeni sinonim, Hatay, Türkiye*

The genus *Aegypoecus* was erected by Clay & Meinertzhagen (1939) [1] for some philopterid lice parasitic on birds belonging to the subfamily Aegypinae, the Old World vultures. Dik et al.[2] described *Aegypoecus guralpi* from a long-legged buzzard, *Buteo rufinus* (Cretzschmar, 1829) (subfamily Accipitrinae) from southern Turkey. *Neophilopterus incompletus* was described from *Ciconia alba* (now = *Ciconia ciconia* (Linnaeus, 1758)) by Denny in 1842 [3]. This species has been reported on white storks *(Ciconia ciconia)* in Turkey by Dik & Uslu [4].

Nine lice collected from an unknown host in the Hatay Province, southern Turkey, and mounted on one slide were identified as *Neophilopterus incompletus* (Denny, 1842). Also, the type material of *Aegypoecus guralpi* Dik et al.[2], collected from *Buteo rufinus* in the same locality, was re-examined and compared with the *N. incompletus* sample.

A detailed study of several specimens of the chewing louse *Neophilopterus incompletus* collected on wild birds in the Hatay Province, southern Turkey, showed that they are conspecific with *Aegypoecus guralpi* Dik et al.[2]. Therefore, I propose that *A. guralpi* be regarded as a junior synonym of *N. incompletus*.

Two years ago, M.N. Muz - the second author of the paper by Dik et al.[2] - sent me a series of chewing lice collected from different wild birds in the Hatay province, southern Turkey. The host names on the labels were given as: 'Eagle A', 'Eagle B', 'Eagle C', 'Long-legged buzzard', 'Stork', etc. written in Turkish. I examined them under a stereo microscope and identified most of them to species, but some samples remained unidentified because the host was unknown or the specimens were not sufficiently clear. On one slide, there were 10 lice labelled as from a 'long-legged buzzard', i.e. *Buteo rufinus*. Using specimens from my collection, I compared the two species recorded from *B. rufinus* listed by Price et al.[5] against the new sample from the same host, but the latter did not belong to any of those two species. Then, I used the key to chewing lice genera found on Falconiformes in Price et al.[5], and I identified the specimens as belonging to the genus *Aegypoecus*. However, the hosts of all the species placed in the genus *Aegypoecus* are vultures of the subfamily

İletişim (Correspondence)
+90 332 2232736
bdik2005@yahoo.com

Aegypinae, while *B. rufinus* belongs to the Buteoninae. I checked papers dealing with species of *Aegypoecus* (e.g. Clay & Meinertzhagen [6]; Dhanda [7,8]; Pérez-Jiménez *et al*.[9], and my specimens showed some morphological differences from other described species. Subsequent clearing and slide-mounting of the lice allowed a detailed examination of their features, showing that they were a different species, which I believed was new and undescribed. Although I realised that the host association of the sample was unusual, we proceeded to describe it as the new species *Aegypoecus guralpi*.

Now I believe that either the sample was actually collected from a stork but was mistakenly labelled as from a 'long-legged buzzard' (i.e. *Buteo rufinus*), or the lice were mixed in the laboratory during the slide-mounting process. An alternative explanation for this incorrect host-louse association would be that the buzzard may have been feeding on a dead white stork and acquired the lice as stragglers.

In conclusion, I place *Aegypoecus guralpi* Dik et al.[2] as a junior subjective synonym of *Neophilopterus incompletus* (Denny, 1842). Also, I conclude that my incorrect generic identification of the lice due to using a key for louse genera from Falconiformes misguided us to describe a new species, when in fact it belonged to another genus and it was not a new species. This is a good example of the mistakes that can be made by placing too much emphasis on the host identity when identifying lice.

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Letter to the Editor

Diagnostic Importance of Transesophageal Echocardiography for Detecting Patent Ductus Arteriosus (PDA) in a Puppy (Yavru Bir Köpekte Patent Duktus Arteriosus (PDA) Tespiti İçin Transözefagal Ekokardiyografinin Tanısal Önemi)

Zeki YILMAZ [1] Meriç KOCATÜRK [1] Hakan SALCI [2] Pınar LEVENT [1]
Uygur CANATAN [2] Saim SAĞ [3] Sümeyye GÜLLÜLÜ [3]

[1] Department of Internal Medicine, Faculty of Veterinary Medicine, Uludag University, TR-165059 Bursa - TURKEY
[2] Department of Surgery, Faculty of Veterinary Medicine, Uludag University, TR-165059 Bursa - TURKEY
[3] Department of Cardiology, Medical School, Uludag University, TR-165059 Bursa - TURKEY

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Dear Editor;

Herein, we are presenting a case of patent ductus arteriosus (PDA) diagnosed by transesophageal echocardiography (TEE) in a puppy. Transthoracic echocardiography (TTE) is used as a traditional method during diagnostic work-up in patients with heart disease in human and veterinary medicine, but in some cases, it has a limitation to show the current problems due to low image resolution and anatomical disposition [1]. As compared with TTE, TEE offers superior visualization of dorsal cardiac structures due to close proximity of the esophagus to the dorsomedial heart with lack of intervening lung and bone. However, TEE procedure needs an anesthesia protocol as well as expensive equipment and experienced stuff [1]. There are limited case presentations on the utility of TEE in the diagnosis of congenital heart defects.

A dog (Labrador, Male, 2 months of age, 5.2 Kg) was presented to Animal Hospital (Dep. of Internal Medicine, Faculty of Veterinary Medicine, Uludag University, Bursa) with a history of respiratory distress increasing with exercise for two days. The dog was alert and tachypneic (36 breath/min). Cardiac auscultation revealed a grade V/VI continuous machinery murmur over the pulmonic area. ECG revealed sinus tachycardia and left ventricular (LV) enlargement (R: 7.6 mV/lead II). Thoracic radiographs showed marked left-sided cardiomegaly with enlarged pulmonary arteries (PA) and minimal pulmonary edema. These findings suggested the presence of a congenital heart defect such as pulmonic stenosis, aortic stenosis, PDA, tetralogy of Fallot or dilated cardiomyopathy [1-4]. Routine hematological and biochemical analyses were non-specific.

With a TTE (Caris plus, Esoate, Italy), the 2-dimensional right parasternal, short-axis view revealed enlarged left atrium (LA: 3.5 cm [1.0-1.6 cm]), whereas aortic diameter (Ao: 1.3 cm [1.0-1.5 cm]) was in reference range [1]. The M-mode echocardiogram showed the severe LV dilatation at diastole (4.48 cm [1.9-2.8 cm]) and systole (3.49 cm [1.1-1.9 cm]) as well as increased E-point to septal separation (1.22 cm [<0.6 cm]). A significant turbulence was observed on PA by color flow imaging, probably due to PA insufficiency, PA hypertension, aorticopulmonary window or PDA [1], but one of them could not be detected by TTE. Thus, to make a clear diagnosis, the use of TEE was decided (Vivid S5, General Electric). For this purpose, the dog was anesthetized with intravenous combination of ketamine HCl (10 mg/kg) (Alfamine®, Egevet, Turkey) and diazepam (0.5 mg/kg) (Diazem®, Deva, Turkey). The dog was intubated and vital parameters were controlled during TEE.

TEE revealed a severe mitral insufficiency at apical two-chamber view. Color flow imaging showed a marked turbulence over PA *(Fig. 1A)* and there was a connection (0.7 cm, *Fig. 1B)* between ascending Ao (Aa) and PA with a high flow velocity (Vmax: 2.97 m/s, gradient: 35.3 mmHg), indicating a large PDA [1,2]. Observed retrograde flow pattern on descending Ao supported the presence of blood access into Ao from PA, as well *(Fig. 1C)*.

After TEE, the anaesthesia of the dog was maintained by 2% isoflurane for surgery. Left 4th intercostal thoracotomy incision was performed for vascular exploration. Ao and PA were dissected and then PDA was exposed and ligated with 0 no silk *(Fig. 2)* as reported earlier [3]. Routine thoracic closure and chest drainage were carried out. Postoperative analgesia (carprofen, 5 mg/kg, 1x1, 5 days) and antibiotherapy (cefazolin Na, 20 mg/kg, im, 2x1, 5 days) were completed.

PDA is classified as small (type 1), medium (type 2) and large (type 3 and 4). Type 3 PDA is related with left to

İletişim (Correspondence)
+90 224 2940809
zyilmaz@uludag.edu.tr

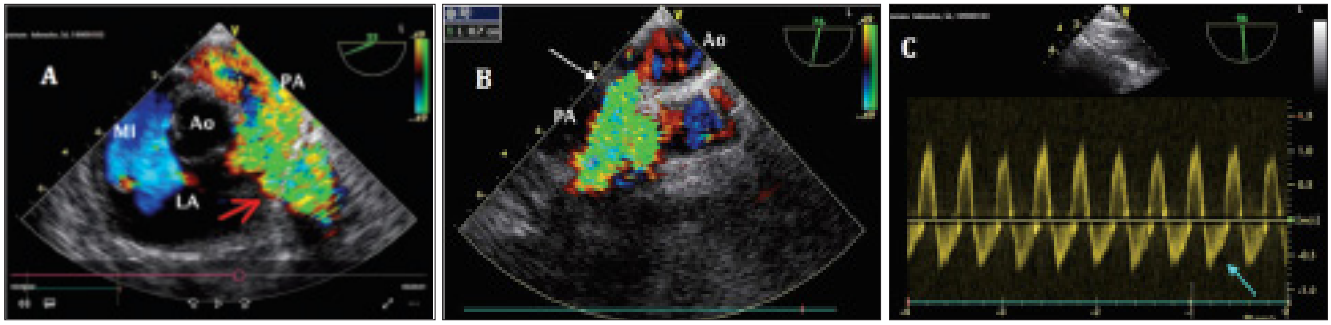

Fig 1. A: TEE short axis view demonstrating a significant turbulent flow *(mosaic color, red arrow)* in the pulmonary artery (PA), **B:** PDA jet and large defect *(white arrow)* between ascending aorta (Aa) and PA is seen at the 7 o'clock position, **C:** Retrograde flow in the descending aorta during diastole *(blue arrow)* revealing the presence of PDA

Şekil 1. A: TEE kısa eksende pulmoner arterde (PA) önemli türbülans akımı *(mozaik renk, kırmızı ok)* gösteriyor, **B:** Saat 7 pozisyonunda asending aorta (Aa) ve PA arasında PDA jeti ve büyük bir defekt *(beyaz ok)* görünüyor, **C:** Diyastol sırasında desending aortadaki ters yöndeki akım *(mavi ok)* PDA varlığını gösteriyor

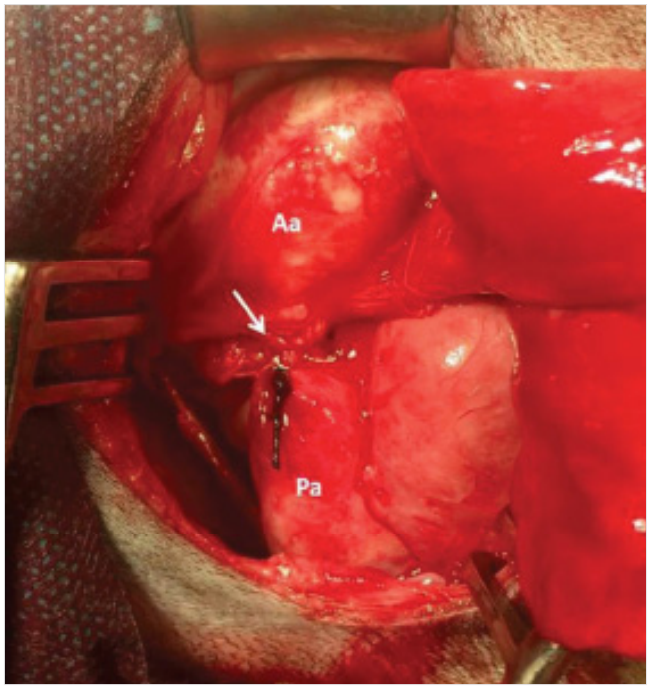

Fig 2. This perioperative view shows the localization of type 3 PDA. *Aa:* ascending aorta, *Pa:* pulmonary artery, *arrow:* ligated site

Şekil 2. Bu operasyon sırasındaki tip 3 PDA ile ilgili görünüm. *Aa:* asending aorta, *Pa:* Pulmonar arter, *ok:* ligature edilen yer

right shunt, but type 4 is related with right to left shunt, thus exhibiting cyanotic mucous membranes. Large PDA is sub-divided into those with (3a) and without (3b) congestive heart failure [3]. Since our dog had a continuous murmur and thrill over the left thorax, marked left heart enlargement, a significant increase in pulmonary vascular markings and R waves >5 mV/lead II, PDA was classified as type 3b. Surgery is recommended immediately in these cases [2,3]. Thus, this dog was operated; defect between Aa and PA was corrected *(Fig. 2)* as reported earlier [2]. Right after the operation, TTE revealed that high velocity flow pattern over the PA disappeared. Post-operative ECG showed lower R amplitude (4.6 mV/lead II) than the pre-operative value. These observations were compatible with the early cardiac responses (cardiac re-modelling) to new dynamics of the blood flow. Dog is still alive without cardiac medication for two months.

In conclusion, clinician should be kept in mind that respiratory problems might be due to congenital heart defects such as PDA in puppies, and in these cases comprehensive cardiological examinations including TEE utility should be performed. For symptomatic PDA, surgery should be definitive solution.

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YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup ve derleme türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.

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3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.

4- ***Makale Türleri***

Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır.

Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.

Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.

Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.

Gözlem (Olgu Sunumu), uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri Giriş, Olgunun Tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.

Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 sayfayı geçmemelidir.

Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri Giriş, Metin, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 sayfayı geçmemelidir.

5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.

6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.

Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.

Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.

Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.

Örnek: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI numarası bulunan kaynaklarda bu bilgi ilgili kaynak künyesinin sonuna eklenmelidir.

Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi, kaynak bilgilerinin sonuna eklenmelidir.

Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.

Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.

7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.

8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir alan editörü ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.

9- Yayınlanan yazılardan dolayı doğabilecek her türlü sorumluluk yazarlara aittir.

10- Yazarlara telif ücreti ödenmez.

11- Resim ve baskı masrafları için yazarlardan ücret alınır. Ücret bilgileri *http://vetdergi.kafkas.edu.tr/* adresinden öğrenilebilir.

12- Yazarlara 50 adet ayrı baskı ücretsiz olarak yollanır.

INSTRUCTIONS FOR AUTHORS

1- The Journal of the Faculty of Veterinary Medicine, University of Kafkas (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, and review and on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table must clearly be written in both Turkish and foreign language and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

During the submission, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the copyright transfer agreement form signed by all the authors should be send to the editorial office.

3- Authors should indicate the name of institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, editorial board may also request the official document of the ethical commission report.

4- Original (full-length) Manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, Introduction, Material and Methods, Results, Discussion, and References and it should not exceed 12 pages including text, tables and illustrations. Abstract should contain 200±20 words.

Short Communication Manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but each of the abstracts should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages. Additionally, they should not contain more than 4 figures or tables.

Preliminary Scientific Reports are short description (maximum 4 pages) of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles.

Case Reports describe rare significant findings encountered in the application, clinic and laboratory of related fields. The title and summary of these articles should be written in the format of full-length original articles and the remaining sections should follow Introduction, Case History, Discussion and References without exceeding the total of 4 pages.

Letters to the Editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases without exceeding 2 pages.

Reviews are original manuscripts gather the literature on current and significant subject along with the commentary and findings of the author on the particular subject. The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should follow Introduction, Text, Conclusion, and References without exceeding 12 page.

5- The necessary descriptive information (thesis, projects, financial supports etc) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of title.

6- References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4^{t}h ed. ,339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must be complied with the other references.

Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

7- The Latin expression such as species names of bacterium, virus, parasite and fungus and anatomical terms must be written in italic character keeping their original forms.

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