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RESEARCH ARTICLE

Brucella abortus and *Brucella melitensis* in Iranian Bovine and Buffalo Semen Samples: The First Clinical Trial on Seasonal, Senile and Geographical Distribution Using Culture, Conventional and Real-time Polymerase Chain Reaction Assays [1]

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Abstract

Conventional and real-time PCR assays were developed for detection and identification of *Brucella* species in bovine and buffalo semen samples. Totally, 91 bovine and buffalo semen samples were collected from 4 major provinces of Iran in various seasons. The animals which their semen samples were collected for this study had less than 1 year old, 1-2 years old, 2-3 years old and more than 3 years old. Samples were cultured and DNA was extracted and novel primers have been designed using the IS711 target of *Brucella* species for conventional PCR. Positive results of PCR have been studied for presences of *Brucella abortus* and *Brucella melitensis*. Totally, 21.56% and 14.28% of bovine and buffalo semen samples, respectively, were positive for *Brucella* species. Khozestan had the highest while Sistan Va Balochestan had the lowest incidences of *Brucella* species in studied regions. Samples which were collected in spring season had the highest rate of infection. Also, samples which were collected from less than 1 year old bovine and buffalo semen samples had the highest incidence of *Brucella* species. Totally, 25 (24.50%) and 4 (3.92%) bovine semen samples and 14 (15.38%) and 1 (1.09%) buffalo semen samples were positive for *Brucella abortus* and *Brucella melitensis*.

Keywords: *Brucella abortus, Brucella melitensis, Seasonal distribution, Senile distribution, Geographical distribution, Semen, Iran*

İran'da Sığır ve Yaban Sığırları Semen Örneklerinde *Brucella abortus* ve *Brucella melitensis*: Kültür, Konvansiyonel ve Real-time Polimeraz Zincir Reaksiyonu Kullanılarak Mevsimsel, Yaşa Bağlı ve Bölgesel Dağılımı Üzerine İlk Klinik Çalışma

Özet

Sığır ve yaban sığırlarının semen örneklerinde Brusella türlerinin tespit ve tanımlanması amacıyla konvansiyonel yöntemler ve Gerçek Zamanlı Zincirleme Polimeraz Reaksiyonu kullanıldı. İran'da değişik mevsimlerde 4 ana bölgeden toplam 91 adet sığır ve yaban sığırı semen örnekleri toplandı. Semen örneklerinin toplandığı hayvanlar 1 yaş, 1-2 yaş, 2-3 yaş ve 3 yaş üzeri olarak gruplandırıldı. Örneklerden kültür hazırlandı, konvansiyonel PCR amacıyla DNA ekstraksiyonu yapılarak Brusella türleri için IS711 kullanılarak pimerler dizayn edildi. Pozitif sonuç veren örnekler *Brucella abortus* ve *Brucella melitensis* yönünden incelendi. Sığırların %21.56'sında yaban sığırların %14.28'inde Brusella tespit edildi. Çalışılan bölgeler arasında Khozestan en yüksek Sistan Va Balochestan ise en düşük Brusella oranına sahipti. İlkbaharda toplanan örneklerde oran en yüksek olarak bulundu. En yüksek Brusella oranı 1 yaşın altındaki hayvanlardan toplanan semen örneklerinde tespit edildi. *Brucella abortus* 25 sığırda (%24.50) ve 14 yaban sığırında (%15,38) *Brucella melitensis* ise 4 sığırda (%3.92) ve 1 yaban sığırında (%1.09) belirlendi.

Anahtar sözcükler: *Brucella abortus, Brucella melitensis, Mevsimsel dağılım, Yaşa bağlı dağılım, Bölgesel Yayılım, Semen, İran*

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INTRODUCTION

Brucellosis is a highly contagious zoonotic bacterial disease of human and many species of animals worldwide caused by gram-negative, aerobic and facultative intracellular bacterium of the genus *Brucella*. It is an important public health problem in many parts of the worlds, such as the Mediterranean littoral, the Middle East and parts of Latin America [1]. *Brucella* species are classically classified into 6 main species 2 of which are *Brucella abortus (B. abortus)* and *Brucella melitensis (B. melitensis)* cause abortions in ruminants [2].

In some countries, especially in Europe and Asia (including Iran), where animals like camelids and buffaloes are kept in close contact with infected sheep, goat and cattle, infections and abortions can also be caused by *B. melitensis* and *B. abortus* [3,4].

In Iran, *B. abortus* was first isolated from a bovine fetus in 1944 [5], and *B. melitensis* was first isolated from a sheep in Isfahan Province in 1952 [6]; brucellosis has been reported from various parts of Iran ever since. In the majority of cases of brucellosis in Iran, *B. abortus* and *B. melitensis* are the main pathogens. Artificial insemination is used to induce fertility in livestock in Iran and other sites of the world. Therefore, the quality and hygiene of semen samples should be considered. There are various assays for diagnosis of brucellosis such as culture, serological and molecular methods. Culture methods require a living host and are both time consuming and hazardous [7]. Previous study showed the low accuracy of serological methods for detection of brucellosis [8]. The usual method for detection and segregation of *Brucella* spp. is based on phenotypic traits, but it is associated with a high risk of laboratory-acquired infections and very time consuming [7].

Therefore, in order to avoid these problems and despite the high degrees of genetic similarity of *Brucella* spp., several conventional and real-time Polymerase Chain Reaction (PCR) assays that are easier, faster, safer and more accurate than traditional methods have been developed [9,10].

It seems that several risk factors like seasons, age and geographical area have an effective role in epidemiology and prevalence of *Breucella* spp. in animal hosts [11,12] but they are unknown in Iran. Therefore, this present investigation was carried out in order to study the seasonal, senile and geographical distribution of *Brucella* spp. in bovine and buffalo semen samples and identification of *B. abortus* and *B. melitensis* in positive cases.

MATERIAL and METHODS

Samples

From January 2011 to January 2012 (in various seasons of the year), a total of 102 bovine and 91 buffalo semen samples were collected randomly from 43 commercial herds in various parts of Iran *(Table 1)*. Those were commercial herds producing semen. The animals from which semen samples were collected for this study were clinically healthy and were classified into 4 age groups (less than 1 year old, 1-2 years old, 2-3 years old and over 2 years old) (animal age has been obtained from the history taking). All of these animals have been classified into two groups (with and without histories of orchitis). From each animal, 10 ml of semen were collected with using an artificial vagina. All semen samples showed normal physical characteristics including color and density. All samples were collected under sterile hygienic conditions and were immediately transported at 4°C to the laboratory in a cooler with ice packs. All of them were cultured and then the remaining semen samples were kept at -20°C until processing.

Brucella Culture Method

A trial of bacterial isolation from the samples was performed on blood agar base (Oxoid) supplemented with 5% defibrinated sheep erythrocytes and antibiotics (vancomycin, nalidixic acid, bacitracin, nystatin and cycloheximide at the dose recommended in the OIE manual, 2000). Cultures were incubated for 10 days with 5% CO_2 at 37°C. The isolated bacteria were identified according to the conventional procedures [13].

DNA Extraction

For *Brucella* DNA extraction, previous method that was introduced by Consuelo Vanegas et al.[14] was used. Purification of DNA was achieved using a genomic DNA purification kit (Fermentas GmbH, St. Leon-Rot, Germany), and the total DNA was measured at an optical density of 260 nm according to the method described by Sambrook and Russell [15].

Primer Designs and Conventional PCR

In the present study, the conventional PCR assay has been designed by the authors. This PCR to screen the *Brucella* spp. detected the DNA sequence of the gene coding the IS711 target reported for *Brucella* in the GenBank database of the National Center For Biotechnology Information (NCBI) (GenBank No: AF242533.1). In order to design primers, recorded sequences of the IS711 target have been gotten from the NCBI. The CLS sequence viewer software (Version 6/4) has been used for alignments of the IS711 target. Forward and reverse primers have been designed based on the protected area in these sequences. Thermodynamic properties of designed primers were studied using the Gene Runner software (Version 3.05). In order to ensure the specificity of designed primers, the Basic Logical Alignment Search Tool (BLAST) service, has been used. The forward primer sequence was 5'-GCGGTCAATGTTTTCTCGCA-3', and the reverse primer sequence was 5'-TGGGGCATGTCATTGCTGAT-3'.

All of the semen samples were analyzed for presence of *Brucella* using the novel conventional PCR assay. The PCR reaction was performed in a total volume of 25 µl containing 10 µl DNA concentrated in 2 µl of DNA sample, 0.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.8 µM each primers and 0.5 U/reaction of Taq DNA polymerase. Reactions were initiated at 94°C for 5 min, followed by 30 cycles of 94°C for 50 sec, 57°C for 1 min, 72°C for 1 min and a final elongation step at 72°C for 5 min, with a final hold at 4°C in a DNA thermal cycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). A negative control (sterile water), and a positive control DNA from *B. abortus* strain S19 (S19 vaccine strain) (Razi Institute, Karaj, Iran), were included in each amplification run.

Gel Electrophoresis

The PCR-amplified products (IS711: 223 bp) were examined by electrophoresis (120 V/208 mA) in a 1.5% agarose gel, stained with a solution of ethidium bromide (0.004 µg/ml) and examined under UV illumination.

Real-Time PCR

The real-time PCR for species segregation was based on unique genetic loci of *B. melitensis* and *B. abortus*. The primer set (which was designed by the author) consisted of BMEII0466 (5′-TCGCATCGGCAGTTTCAA/CCAGCTTTT GGCCTTTTCC-3′) (112 bp) with the Cy5-CCTCGGCATGG CCCGCAA-BHQ-2 (5′Fluorophore→3′Quencher) internal probe for *B. abortus* and BruAb2-0168 (5′-GCACACT CACCTTCCACAACAA/CCCCGTTCTGCACCAGACT-3′)(222bp) with the FAM-TGGAACGACCTTTGCAGGCGAGATC-BHQ-1 internal probe for *B. melitensis*. In this study, the starting quantity of DNA from each serial dilution was plotted as a function of threshold cycle (CT) values to obtain a standard curve. On the other hand, the CT is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. All of the positive samples on conventional PCR assays were studied for presences of *B. abortus*, *B. melitensis*, both bacteria and other species of *Brucella*.

A typical 25 µl reaction contained 12.5 µl TaqMan® Universal PCR Master Mix (Foodproof® Brucella Detection Kit), a 300 nM concentration of each forward and reverse primer (Bioneer Corporation, Daejeon, South Korea), a 200 nM concentration of the probe (Bioneer Corporation, Daejeon, South Korea), and 2.5 ng of sample DNA. TaqMan Master Mix real-time PCR reactions were carried out using a Rotor-Gene 6000 instrument (Corbett Research, Mortlake, NSW, Australia). The reaction mixture was initially incubated for 10 min at 95°C. Amplification was performed with 45 cycles of denaturation at 95°C for 20 sec, annealing and extension at 62°C for 1 min. In this reaction, the Foodproof® Brucella Internal Control (White cap) and Foodproof® Brucella Control Template (Purple cap) were used as an internal and positive control, respectively.

Sequencing

In order to confirm the PCR results, a sequencing method was used. For this reason, PCR products of some positive samples were purified with a High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's recommendations. Single DNA strands were sequenced with an ABI 3730 XL device and Sanger sequencing method (Macrogen, Seoul, South Korea). The sequence of each gene was aligned with the gene sequences recorded in the GenBank database on the NCBI.

Statistical Analysis

Data were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using the Statistical Package for the Social Sciences (SPSS) 18.0 statistical software (SPSS Inc., Chicago, IL, USA), Analysis of Variance (ANOVA) test analyses were performed for study the differences between incidence of bacteria in various seasons and various ages, and differences were considered significant at values of $P<0.05$. In this study, the distribution of CT values was compared between bovine and buffalo semen samples using an ANOVA test.

RESULTS

The results of this present study showed that 22 out of 102 bovine semen samples (21.56%) and 13 out of 91 buffalo semen samples (14.28%) were positive for *Brucella* spp. *Table 1* presents the seasonal distribution of *Brucella* spp. in bovine and buffalo semen samples collected from various geographical regions.

Table 2 presents the senile distribution of *Brucella* spp. in bovine and buffalo semen samples collected from various geographical regions.

Totally, 25 bovine (24.50%) and 17 buffalo (18.68%) of samples had the typical 223 bp fragment in gel electrophoresis *(Fig. 1)* which were positive for *Brucella* spp. positive conventional PCR results were studied using Taqman real-time PCR. Totally, 25 out of 102 (24.50%) and 4 out of 102 (3.92%) bovine semen samples were positive for *B. abortus* and *B. melitensis*, respectively. Also, 14 out of 91 (15.38%) and 1 out of 91 (1.09%) buffalo semen samples were positive for *B. abortus* and *B. melitensis*, respectively. Results showed that only a bovine semen sample were positive for presence of both *B. abortus* and *B. melitensis*. Also, 2 bovine semen samples (1.96%) and 2 buffalo semen samples (2.19%) were positive for other species of *Brucella (Table 3)*.

The sensitivity and specificity of our novel primers for detection of *Brucella* spp. in bovine and buffalo semen samples were 100% and 96% and 100% and 94%, respectively *(Table 4)*.

Table 1. *Seasonal distribution of Brucella spp. in bovine and buffalo semen samples collected from various geographical regions*
Tablo 1. *Değişik bölgelerdeki sığır ve yaban sığırlarından toplanan semen örneklerinde Brucella spp. mevsimsel dağılımı*

Provinces	No. of Samples		Culture Positive (%)		Seasonal Distribution (%)							
	Bovine	Buffalo	Bovine	Buffalo	Summer		Autumn		Winter		Spring	
					Bovine	Buffalo	Bovine	Buffalo	Bovine	Buffalo	Bovine	Buffalo
Khozestan	30	29	9 (30)	6 (20.68)	-	1 (16.66)*	2 (22.22)	2 (33.33)	1 (11.11)	-	6 (66.66)	3 (50)
Boshehr	27	21	6 (22.22)	3 (14.28)	1 16.66)	-	1 (16.66)	1 (33.33)	-	-	4 (66.66)	2 (66.66)
Hormozgan	24	21	4 (16.66)	2 (9.52)	-	-	1 (25)	-	1 (25)	-	3 (75)	1 (50)
Sistan Va Balochestan	21	20	3 (14.28)	2 (10)	-	-	1 (33.33)	-	-	-	2 (66.66)	2 (100)
Total	102	91	22 21.56)	13 14.28)	1 (4.54)	1 (7.69)	5 (22.72)	3 (23.07)	2 (9.09)	-	15 68.18)	8 (61.53)

** Positive samples from a total of culture positive samples*

Table 2. *Senile distribution of Brucella spp. in bovine and buffalo semen samples collected from various geographical regions*
Tablo 2. *Değişik bölgelerdeki sığır ve yaban sığırlarından toplanan semen örneklerinde Brucella spp.yaşa bağlı dağılımı*

Provinces	No. of Samples		Culture Positive (%)		Senile Distribution (%)							
	Bovine	Buffalo	Bovine	Buffalo	Bovine				Buffalo			
					1>	1-2	2-3	3<	1>	1-2	2-3	3<
Khozestan	30	29	9 (30)	6 (20.68)	5 (55.55) *	2 (22.22)	1 (11.11)	1 (11.11)	3 (50)	1 (16.66)	1 (16.66)	1 (16.66)
Boshehr	27	21	6 (22.22)	3 (14.28)	3 (50)	2 (33.33)	1 (16.66)	-	2 (66.66)	1 (33.33)	-	-
Hormozgan	24	21	4 (16.66)	2 (9.52)	3 (75)	1 (25)	-	-	1 (50)	1 (50)	-	-
Sistan Va Balochestan	21	20	3 (14.28)	2 (10)	2 (75)	1 (25)	-	-	2 (100)	-	-	-
Total	102	91	22 (21.56)	13 (14.28)	13 (59.09)	6 (27.27)	2 (9.09)	1 (4.54)	8 (36.36)	3 (23.07)	1 (4.54)	1 (7.69)

** Positive samples from a total of culture positive samples*

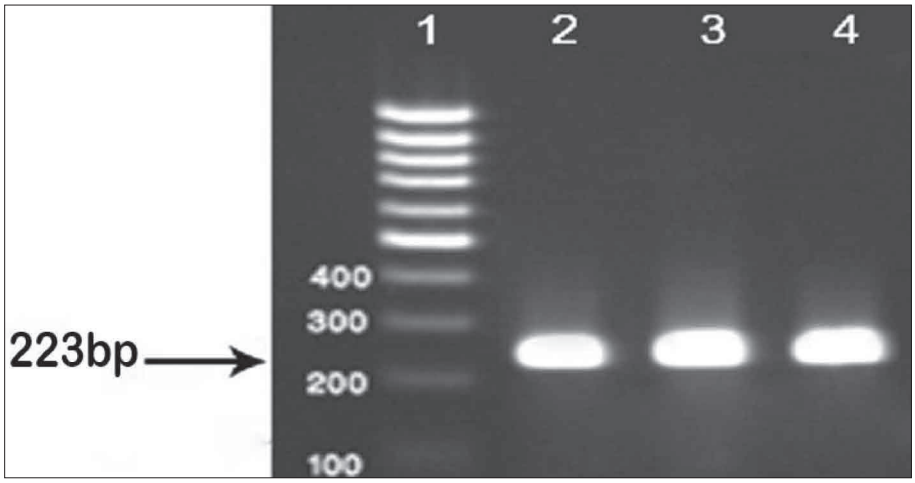

Fig 1. Novel conventional PCR for detection of *Brucella* spp. Lane 1 is a 100 bp ladder, lanes 2 and 3 are positive samples for bovine and buffalo semen samples (223 bp) and lane 4 is the positive control

Şekil 1. *Brucella* spp.'nin PCR ile tespiti. 1. sıra 100 bp merdiveni, 2. ve 3. sıralar sırasıyla sığır ve yaban sığırı için pozitif örnekler (223 bp) ve 4. sıra pozitif control

DISCUSSION

Our study showed that the semen samples of infected bovine and a buffalo possibly plays an important role in distribution of brucellosis in Iran. Unfortunately, despite the high incidences of these bacteria in bovine and buffalo semen samples, very little research concerning detection of *Brucella* in semen samples has been performed. In addition, in the majority of cases, the semen samples are not well screened for the presence of *Brucella* and other pathogens. When laboratories do screen for them, they commonly use traditional diagnostic methods like the enzyme-linked immunosorbent assay (ELISA). Diagnosis of brucellosis by the ELISA method is not recommended because it can be unspecific and subsensitive due to cross-reaction with other pathogens including *Yersinia*

Table 3. *Incidences of B. melitensis, B. abortus and other species of Brucella in bovine and buffalo semen samples collected from various geographical regions*
Tablo 3. *Değişik bölgelerdeki sığır ve yaban sığırlarından toplanan semen örneklerinde B. abortus, B. melitensis ve diğer Brucella türlerinin yoğunluğu*

Provinces	No. of Samples		Conventional PCR (%)		Novel Real-time PCR (%)							
					Bovine				Buffalo			
	Bovine	Buffalo	Bovine	Buffalo	*B. abortus*	*B. melitensis*	Unknown	Both bacteria	*B. abortus*	*B. melitensis*	Unknown	Both bacteria
Khuzestan	30	29	11 (36.66)	8 (27.58)	8 (26.66)*	2 (6.66)	1 (3.33)	1 (3.33)	6 (20.68)	-	2 (6.89)	-
Bushehr	27	21	7 (25.92)	4 (19.04)	6 (22.22)	-	1 (3.70)	-	3 (14.28)	1 (4.76)	-	-
Hormozgan	24	21	4 (16)	3 (14.28)	3 (12.5)	1 (4.16)	-	-	3 (14.28)	-	-	-
Sistan Va Baluchestan	21	20	3 (14.28)	2 (10)	2 (9.52)	1 (4.76)	-	-	2 (10)	-	-	-
Total	102	91	25 (24.50)	17 (18.68)	25 (24.50)	4 (3.92)	2 (1.96)	1 (0.98)	14 (15.38)	1 (1.09)	2 (2.19)	-

** Positive results of total samples*

Table 4. *Evaluation of sensitivity and specificity of conventional PCR for detection of Brucella spp. in the bovine (A) and buffalo (B) semen samples*
Tablo 4. *Konvansiyonel PCR'ın sığır (A) ve yaban sığırlarında (B) Brucella spp. tespit etmedeki duyarlılığı ve özgüllüğü*

A

Sensitivity	Culture Positive	Culture Negative	Total
Conventional PCR positive	22^{*a}	3^{c}	25
Conventional PCR negative	$-^{b}$	74^{**d}	74
Total	22^{a+b}	77^{c+d}	99

$$*\text{Sensitivity: } \frac{a}{a+b} = 100\%$$

$$**\text{Sensitivity: } \frac{d}{d+c} = 96\%$$

B

Sensitivity	Culture Positive	Culture Negative	Total
Conventional PCR positive	13^{*a}	4^{c}	17
Conventional PCR negative	$-^{b}$	74^{**d}	74
Total	13^{a+b}	78^{c+d}	91

$$*\text{Sensitivity: } \frac{a}{a+b} = 100\%$$

$$**\text{Sensitivity: } \frac{d}{d+c} = 94\%$$

enterocolitica, *Salmonella*, *Escherichia coli* O:157 and other *Brucella* spp.[16-18]. Therefore, this makes PCR as an accurate, safe, sensitive, fast and specific assay for detection and differentiation of *Brucella* spp., so essential in these cases. Furthermore, the real-time PCR assay has some advantages compared with the conventional PCR. It is an important diagnostic tool yielding reliable and reproducible results, does not require post-PCR analysis (gel electrophoresis, hybridization), and has a limited risk of cross contamination compared with the conventional method; however, real-time PCR is more expensive than conventional PCR. Many studies have shown that the conventional method for detecting *Brucella* spp. is technically more time-consuming and labor-intensive than real-time PCR assay [8,19].

To our best knowledge, this study has been introduced a pair of primer which had the high sensitivity and specificity for detection the IS711 target of *Brucella* spp. Also, this study is the first prevalence report of seasonal, senile and geographical distribution of *Brucella* spp. in bovine and buffalo semen samples in Iran. Our results showed that 24.50% of bovine and 18.68% of buffalo

semen samples were positive for *Brucella* spp. Also, 24.50% and 3.92% of bovine semen samples were positive for *B. abortus* and *B. melitensis*, respectively and 15.38% and 1.09% of buffalo semen samples were positive for *B. abortus* and *B. melitensis*, respectively. Statistical analysis showed significant differences ($P<0.01$) between *B. abortus* and *B. melitensis* in bovine and buffalo semen samples and between the presence of *Brucella* spp. in bovine with buffalo semen samples ($P<0.05$).

A previous study showed that Iran, Saudi Arabia, Jordan, Syria and Oman had the highest incidences of brucellosis among the countries of the Near East region [20]. Studies about brucellosis from various parts of Iran [12,21] and various species such as sheep [22], goats [23], cattle [24], buffaloes [21] and humans [6] indicated that brucellosis is one of the most important endemic zoonotic diseases in Iran. Brucellosis causes great economic losses in Iran. A previous report from Iran indicated that the prevalence of brucellosis was 0.037% in humans, 3.4% in sheep and goats and 0.56% in cattle in Eastern Iran during 2002-2006 [25]. The prevalence of *B. abortus* observed in bovine in this study (24.50%) is higher than those in Egypt (5.44%) [26], Ethiopia (4.9%) [27], and India (18.81%) [28], Punjab region (20.67%) [29] but lower than those in the Sokoto State (25.25%) [30] and Kenya (77.5%) [31]. The incidence of *B. abortus* observed in buffaloes in the present study (15.38%) is higher than those in Egypt (0.3%) [20] and the Punjab region (India) (13.4%) [10] but is lower than that in Africa (30%) [32]. It appears that there is no prevalence report of brucellosis caused by *B. melitensis* in bovine and buffaloes, making the present study is the first prevalence report of *B. melitensis* observed in bovine and buffalo semen samples in the world.

The high incidence of brucellosis in semen samples of an unspecific host (buffalo) in the present study possibly indicates that these animals were maintained in close association with infected sheep and cattle. In addition, the high prevalence of brucellosis in animals in Khozestan Province probably represents the low number of veterinary facilities in this province, geographical and climate conditions and importation of infected livestock from neighboring countries like Iraq.

The semen samples collected in spring seasons had the highest prevalence of *Brucella* spp. in bovine (68.18%) and buffalo (61.53%) semen samples. Statistical analyses were significant ($P<0.05$) for the prevalence of *Brucella* spp. between spring season and winter. The main reason for this finding is the fact that temperature and climate maybe have effect on the prevalence of *Brucella* spp. in bovine and buffalo semen samples. An explanation for the highest prevalence of *Brucella* spp. in spring season might be that during this time some climatic events such as heat, rain, and thunderstorms, as well as variation in barometric pressure might have occurred and may have influence on the autonomic nervous system. These events is known to cause reduction in the levels of animal immunity. Therefore, several infections might also have been occurred.

Seasonal distribution of the brucellosis were expressed previously with the highest occurrence in May (15.9%), June (16.3%) and July (15.1%) (spring) [11], which was in agree with our results. In Germany, the largest number of cases was recorded in August and September [33]. In Central Greece, the largest number of outbreaks was reported from spring [34]. In countries with temperate or cold climates there is a marked seasonal variation in the incidence of acute brucellosis, with most cases occurring in spring and summer.

Also, the results from this study indicated that Khozestan had the highest while Sistan Va Balochestan had the lowest prevalence of *Brucella* spp. in bovine and buffalo semen samples. Statistical analysis were significant for the prevalence of *Brucella* spp. in both bovine and buffalo semen samples between Khozestan and while Sistan Va Balochestan provinces ($P<0.05$). After analyzing the average temperatures of these 4 seasons and provinces, it has been found that spring season and Khozestan province had the most constant temperature. The high prevalence of *Brucella* spp. seems reasonable in spring season and Khozestan province of Iran since this bacterium needs moderate temperature with high degree of moisture for growth and survival. Similarly, regional differences have also been reported previously from Azerbaijan [12].

Our results showed that there were strong age distributions for the incidence of *Brucella* spp. in bovine and buffalo semen samples in Iran. Results showed that 59.09% less than 1 year old bovine and 36.36% of less than 1 year old buffalo semen samples were positive for *Brucella* spp. Also, statistical analyses were significant for the prevalence of *Brucella* spp. between young bovines and buffaloes and old bovines and buffaloes ($P<0.05$). This may be explained by the fact that younger bovines and buffaloes have weaker immune system. Thus, several infection and illnesses maybe expected. Seasonal distributions of *Brucella* have been reported previously [33].

This study showed that both *B. abortus* and *B. melitensis*, can infect bovine and buffaloes, but the incidence of *B. abortus* was higher than *B. melitensis*. It appears that buffaloes are unlikely the primary hosts for *Brucella*, but they can be infected with both *B. abortus* and *B. melitensis*. Consequently, the prevalence of brucellosis in buffaloes is dependent on the infection rate of primary hosts being in contact with. On the other hand, spread of brucellosis in buffaloes depends on the *Brucella* spp. prevalence in other animals sharing their habitat and on the husbandry methods of the different species.

We also claim by this study that both bovine and buffalo can be important reservoirs for transmission of this zoonotic disease to humans in Iran. Our results revealed that bovines and buffaloes less than 1 year old in spring season and Khozestan province had the highest prevalence of *Brucella* spp. in Iran. Several control programs should be performed on Khozestan province in spring seasons especially on younger bovines and buffaloes. The present study shows that molecular methods such as conventional and real-time PCR are accurate, reliable and rapid assays for detection and identification of *B. abortus* and *B. melitensis* in bovine and buffalo semen samples but that the real-time PCR assay is better. It seems that this study is the first report of direct detection and segregation of *B. melitensis* and *B. abortus* by application of conventional and real-time PCR assays in bovine and buffalo semen samples in Iran. We hope that the real-time PCR method introduced in this study as an accurate, safe, fast, sensitive and specific assay for detection and segregation of *B. melitensis* and *B. abortus* in clinical samples.

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RESEARCH ARTICLE

Karacabey Merinosu, Karya ve Kıvırcık Kuzularda Sütten Kesim Döneminde Kabuk Yağı Kalınlığı ve *Musculus longissmus dorsi thoracis et lumborum* (MLD) Derinliğinin Ultrason Ölçümleri

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Özet

Bu çalışma, Karya, Kıvırcık ve Karacabey Merinosu kuzularda sütten kesim dönemi canlı ağırlığı yanında *Musculus longissmus dorsi thoracis et lumborum* (MLD) kası özelliklerine ait ultrason ölçüm parametrelerinin ortaya konması amacıyla yapılmıştır. Anılan ölçümler, Karacabey Merinosu (258), Karya (168) ve Kıvırcık (241) ırkında 2012-2013 üretim sezonunda doğan toplam 667 baş kuzuda gerçekleştirilmiştir. Yaklaşık 3 aylık (115 gün) yaşta kuzularda kabuk yağı kalınlığı, deri+kabuk yağı kalınlığı, kas derinliği, ortalama günlük canlı ağırlık artışı ve sütten kesim canlı ağırlığı için en küçük kareler ortalamaları sırasıyla 0.31 cm, 0.55 cm, 2.09 cm, 229.57 g ve 29.20 kg olarak bulunmuştur. Yağ kalınlığı, deri+yağ kalınlığı, kası derinliği ve ortalama günlük canlı ağırlık ortalamaları ile ilgili bulgular ırklar arasında canlı ağırlığın değişimi ile uyumludur. Kuzularda etlenme ve yağlanma düzeyinin tahmininde kullanılan MLD parametrelerinin her biri ile canlı ağırlık arasında pozitif ve önemli fenotipik korelasyon katsayıları elde edilmiştir.

Anahtar sözcükler: *Karacabey Merinosu, Karya, Kıvırcık, MLD, Kabuk yağı, Ultrasonik ölçümler*

Ultrasound Measurements of Backfat Thickness and *Musculus longissmus dorsi thoracis et lumborum* (MLD) Depth at the Weaning on Karacabey Merino, Karya and Kıvırcık Lambs

Abstract

The experiment was conducted to determine the ultrasonic measurements of backfat thickness and *Musculus longissmus dorsi thoracis et lumborum* (MLD) characteristics at weaning. Ultrasonic measurements of MLD in Karacabey (258), Karya (168) and Kıvırcık (241) were performed in 667 lambs born in 2012-2013 lambing season. Least square means for backfat thickness, skin+fat thickness, muscle depth, average daily gain and weaning weight, at the mean age of 115 days, were 0.31 cm, 0.55 cm, 2.09 cm, 229.57 g and 29.20 kg respectively. Results for backfat thickness, skin+backfat thickness, muscle depth of MLD and average daily gain and weaning weight were compatible with differences for live weight among the breeds. All the correlation coefficients between ultrasound measurements of MLD properties, used to determine the effect of conformation and fatness status, and live weight were positive and statistically significant.

Keywords: *Karacabey Merino, Karya, Kıvırcık, MLD, Backfat, Ultrasonic measurements*

GİRİŞ

Türkiye'nin özellikle Batı Anadolu bölgesinde koyunculuk kuzu eti üretimine yönelik olarak gerçekleştirilmektedir. Son 30-40 yıllık süreçte, başlıca kuzu ihraç eden ülkeler (Yeni Zelanda ve Avustralya) ve kimi Orta Doğu ülkeleri hariç kuzu eti tüketiminde bir azalmadan bahsedilebilir. Etin, lezzetini etkilemeyecek düzeyde olabildiğince yağsız olması tüketicilerin en önemli istekleri arasındadır [1-4]. Kuzu eti kompozisyonunun tüketici istekleri doğrultusunda geliştirilmesi için bir çok ülkede, ırklar içi ve ırklar arası varyasyona dayalı ıslah çalışmaları sürdürülmektedir [5-7].

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Hayvanların et verimi bakımından damızlık değerlerinin tahmini önceleri karkas derecelendirme, ağırlık artışı ve konformasyon bilgilerine dayandırılmakta ve karkas değerlendirme büyük oranda hayvan kesildikten sonra yapılmaktadır [3]. Canlı hayvanda karkas değerlendirme, vücut kondisyon skoru gibi geleneksel ve kullanımı pratik olan ancak hata varyasyonunun kimi zaman çok yüksek olduğu subjektif yöntemler ile yapılabilmektedir. Karkas kalitesinin geliştirilmesine yönelik olarak birçok yöntem geliştirilmiştir. Bu yöntemlerde genel olarak, düşük maliyete, kolay uygulanabilirliğe, kesinlik ve yüksek güvenilirliğe sahip olması gerektiği üzerinde durulmuştur [1,3,8]. İlk defa Wild [9] tarafından biyolojik olarak dokuların ölçülmesi ve dokulardaki değişikliklerin tespit edilmesi amacıyla kullanılan ultrason teknolojisi daha sonra Temple et al.[10] tarafından canlı hayvanlarda karkas özelliklerinin tahmin edilmesinde kullanılmıştır. Bu teknoloji zaman ve emek açısından büyük tasarruf sağlaması ve yüksek güvenilirliğe sahip olması nedeniyle günümüzde et kalitesini hedef alan ıslah programlarında yaygın olarak kullanılmakta, et kalitesinin tanımlanmasında ve tahmin parametrelerinin elde edilmesinde gittikçe önem kazanmaktadır. Ultrason teknolojisi temel olarak iki amaca yönelik kullanılabilmektedir. Bunlardan birincisi, genetik ilerleme programlarında ultrasona dayalı ölçüm sonuçlarının birer seleksiyon kriteri olarak kullanılması, ikincisi ise, kesime gönderilecek kuzularda optimum seviyedeki yağ oranının belirlenebilmesidir [11,12]. Canlı hayvanda ultrason ölçümü yapılacak bölge hayvanın dış anatomik yapısından kolayca saptanabilmeli ve bu bölgede yapılan doku derinliği ve alan ölçümleri tüm vücut veya karkas kompozisyonunu doğru bir şekilde yansıtmalıdır.

Ultrason ölçümleri için daha çok 12-13. kaburgalar arasına denk gelen sırt bölgesinden yararlanılmaktadır. Canlı hayvanlarda *Musculus longissmus dorsi thoracis et lumborum* (MLD) ve yağ kalınlığı ölçümleri ile kesim sonrası yapılan ölçümler arasında yüksek oranda korelasyon belirlenmiştir [1,2,4,11,13,14].

Batı Anadolu bölgesinde kasaplık kuzuların pazarlanması direk sütten kesimde ya da sütten kesimden hemen sonra süt kuzu olarak yapılmaktadır. Bu çalışmada, Batı Anadolu bölgesinde oldukça yaygın bir şekilde yetiştirilen Karya (KR), Kıvırcık (KIV) ve Karacabey Merinosu (KM) kuzularının pazarlama dönemi canlı ağırlığı ve MLD özelliklerinin ultrasonla tespiti amaçlanmıştır.

MATERYAL ve METOT

Çalışmanın hayvan materyalini Batı Anadolu'da yaygın olarak yetiştirilen Karya (KR), Kıvırcık (KIV) ve Karacabey Merinosu (KM) ırklarından *(Şekil 1)* sütten kesim döneminde ortalama 115 günlük yaşta 667 baş kuzu oluşturmuştur.

Hayvan materyali için örnekleme yapılan lokasyonlar ve örnek büyüklükleri *Tablo 1*'de verilmiştir.

Ultrason ölçümü yapılan Kıvırcık, Karya ve Karacabey Merinosu sürülerinde kuzulara 1 aylık yaşa kadar sadece ana sütü 1 aylık yaştan sütten kesime kadar ana sütü ve kuzu büyütme yemi verilmiştir. Kuzuların yeme alışması için Karacabey Merinosu ve Karya ırkında süzek yemleme yöntemi uygulanmıştır. Kıvırcık ırkında ise analar meraya çıktıktan sonra yemleme serbest olarak yapılmıştır. Kuzu canlı ağırlıkları 50 g hassasiyete sahip elektronik baskül ile belirlenmiştir. MLD ölçümleri 12 ve 13. kaburgalar arası bölgede 6 cm tarama alanına sahip linear prob (8 MHz) kullanılarak ultrason cihazı (Pie Medical Falco 100) ile gerçekleştirilmiştir. Ölçümlerde kas derinliği (KD) ile kabuk yağı kalınlığı (KYK) ve deri + kabuk yağı kalınlığı (D+KYK) belirlenmiştir *(Şekil 2)*.

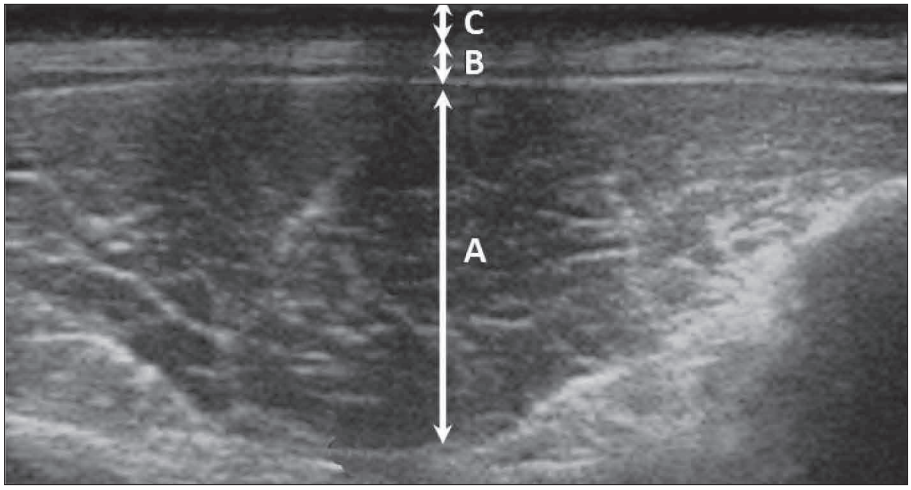
Ele alınan özellikler, sistematik çevre etmenleri de modele dahil edilerek varyans analizine tabi tutulmuş ve en küçük kareler ortalamaları elde edilmiştir. Ayrıca, korelasyon analizi uygulanarak ele alınan özellikler arası fenotipik korelasyon katsayıları belirlenmiştir. Analizler

Tablo 1. *Hayvan materyalinin örnekleme sayıları ve lokasyonları*
Table 1. *Location and sample sizes of animal materials*

Irklar	N	Lokasyon	Sürü Tipi	İşletme Sayısı
KR	168	ADÜ-GKYP	Elit sürü	1
KIV	241	Aydın	Yetiştirici sürüleri	5
KM	258	KAİ	Elit sürü	1

KAİ: *Koyunculuk Araştırma İstasyonu,* ***Bandırma, Türkiye, ADÜ-GKYP:*** *Adnan Menderes Universitesi Grup Koyun Yetiştirme Programı, Aydın, Turkiye*

Şekil 1. Çalışmada kullanılan ırklar (**KR:** Karya, **KIV:** Kıvırcık, **KM:** Karacabey Merinosu)
Fig 1. Breeds in the study (**KR:** Karya, **KIV:** Kıvırcık, **KM:** Karacabey Merino)



Şekil 2. Ölçülen özellikler **A**- kas derinliği, **B**- kabuk yağı kalınlığı, **B+C**- deri + kabuk yağı kalınlığı

Fig 2. Measurement characteristics; **A**- muscle depth, **B**- backfat thickness, **B+C**- skin + backfat thickness

için SAS [15] paket istatistik programında bulunan GLM ve CORR prosedürleri kullanılmıştır.

BULGULAR

Çalışmada kullanılan kuzuların MLD ultrason ölçümlerinin yapıldığı döneme ait ortalama kuzu yaşı 115 gün olmuştur. Sütten kesim canlı ağırlığı, ortalama günlük canlı ağırlık artışı ve MLD ultrasonik ölçümlerine ait tanımlayıcı istatistikler *Tablo 2*'de verilmiştir.

Bu yaşta kuzuların canlı ağırlık ve MLD özelliklerinin çok değişken olduğunu *Tablo 2*'de verilen tanımlayıcı istatistikler ortaya koymaktadır. *Tablo 2* incelendiğinde kabuk yağı kalınlığı ve deri+kabuk yağı kalınlığının en yüksek varyasyona sahip özellikler olduğu görülmektedir.

KR, KIV ve KM ırklarına ait kuzuların sütten kesim canlı ağırlıkları ile MLD özelliklerinin ultrasonik ölçümleri için elde edilen en küçük kareler ortalama ve standart hataları *Tablo 3*'te verilmiştir. Kabuk yağı kalınlığı, deri+kabuk yağı kalınlığı ve kas derinliğine ait genel ortalamalar sırasıyla 0.31 cm, 0.55 cm ve 2.09 cm olmuştur. Ultrasonla gerçekleştirilen ölçümler üzerine ırk ve cinsiyetin etkisinin önemli (P<0.001), doğum tipi etkisinin ise önemsiz (P>0.05) olduğu görülmektedir. Bunun yanında, ölçüm dönemi kuzu canlı ağırlığının artışı kabuk yağı ve deri+kabuk yağı kalınlığında artışa yol açmıştır (P<0.001).

Kuzuların sütten kesim canlı ağırlığına ait genel ortalama 29.20 kg olarak tespit edilmiştir. Değerlendirmede dikkate alınan ırk, doğum tipi ve cinsiyet gibi kesikli etmenlerin ortaya koydukları varyasyon ile sürekli etmen olarak ele alınan kuzu yaşının sütten kesim ağrılığına etkilerinin (regresyon) tümü istatistiki olarak çok önemli (P<0.001) bulunmuştur. En düşük sütten kesim canlı ağırlık ortalaması KR ırkında gözlemlenmiştir. KM ırkında sütten kesim ağırlığı için elde edilen ortalama değerin diğer iki ırkın oldukça üzerinde olduğu dikkati çekmektedir. Beklenildiği gibi ikizler tek doğanlardan ve yine cinsiyet bakımından dişiler erkeklerden daha düşük canlı ağırlığa sahip olmuşlardır. Sürekli etmenlerden kuzu yaşının artışı canlı ağırlığın artışında önderlik etmiştir. Günlük ortalama canlı ağırlık artışı için elde edilen bulgular sütten kesim ağırlığı için elde edilen bulgulara benzerdir. Beklenildiği şekilde yüksek canlı ağırlığa sahip ırkların daha yüksek ortalama günlük canlı ağırlık artışına sahip oldukları görülmektedir.

Kuzularda etlenme ve yağlanma derecesinin tahminde kullanılan MLD ultrason ölçüm parametreleri ve sütten kesim ağırlığı arasındaki fenotipik korelasyon katsa-

Tablo 2. *MLD ölçümleri, sütten kesim ve ortalama günlük canlı ağırlık artışına ilişkin basit istatistikler*
Table 2. *Basic statistics for ultrasound measurements of MLD, weaning weight and avarage daily gain*

Değişkenler	N	X ± Sx	Min.	Mak.	VK (%)
KYK (cm)	667	0.32±0.004	0.12	0.81	34.02
D+KYK (cm)	667	0.57±0.007	0.21	1.40	32.40
KD (cm)	667	2.13±0.019	1.02	3.25	23.16
SKCA (kg)	667	30.96±0.267	15.20	51.20	22.31
OGCAA (g)	667	238.6±2.800	94.77	432.22	30.29
Kuzu Yaşı (gün)	667	115.24±0.876	47.00	165.00	19.63

KYK: *kabuk yağı kalınlığı,* ***D+KYK:*** *deri+kabuk yağı kalınlığı,* ***KD:*** *kas derinliği,* ***SKCA:*** *sütten kesim canlı ağırlığı,* ***OGCAA:*** *ortalama günlük canlı ağırlık artışı,* ***VK:*** *varyasyon katsayısı*

Tablo 3. *MLD ultrason ölçümleri, ortalama günlük canlı ağırlık artışı ve sütten kesim canlı ağırlıklarına ilişkin en-küçük kareler ortalamaları ve standart hataları*

Table 3. *Least squares means and standard errors for ultrasound measurements of MLD, average daily gain and weaning weight of lambs*

Irklar	N	KYK	D+KYK	KD	OGCAA	SKCA
		P=0.000	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**
KR	168	0.26±0.007	0.43±0.008	1.77±0.018	177.13±2.306	22.79±0.406
KIV	241	0.33±0.007	0.54±0.009	2.02±0.019	226.83±2.426	26.74±0.310
KM	258	0.35±0.006	0.69±0.008	2.47±0.017	284.74±2.197	38.07±0.290
Doğum Tipi		**P=0.130**	**P=0.066**	**P=0.576**	**P=0.013**	**P=0.000**
1	513	0.32±0.004	0.56±0.005	2.09±0.010	233.05±1.280	30.92±0.199
2≥	154	0.31±0.007	0.54±0.009	2.08±0.019	226.08±2.374	27.90±0.356
Cinsiyet		**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**
Erkek	334	0.28±0.005	0.52±0.006	2.04±0.013	235.27±1.669	30.73±0.185
Dişi	333	0.34±0.005	0.59±0.006	2.13±0.014	223.86±1.774	27.66±0.330
Reg Linear		**P=0.000**	**P=0.000**	**P=0.000**	**P=0.000**	
SKCA		0.007±0.001	0.01±0.001	0.03±0.002	4.320±0.232	
						P=0.000
Kuzu Yaşı						0.154±0.010
Genel	**667**	0.31±0.004	0.55±0.005	2.09±0.010	229.57±1.302	29.20±0.185

KYK: *kabuk yağı kalınlığı,* ***D+KYK:*** *deri+kabuk yağı kalınlığı,* ***KD:*** *kas derinliği,* ***SKCA:*** *sütten kesim canlı ağırlığı,* ***OGCAA:*** *ortalama günlük canlı ağırlık artışı*

Tablo 4. *Sütten kesim canlı ağırlığı ve ultrason ölçümleri arası fenotipik korelasyonlar (n=667)*

Table 4. *Correlation coefficients between ultrasound measurements and weaning weights (n=667)*

Özellikler	KYK	D+KYK	KD
D+KYK	0.850***		
KD	0.627***	0.799***	
SKCA	0.534***	0.679***	0.771***

KYK: *kabuk yağı kalınlığı,* ***D+KYK:*** *deri+kabuk yağı kalınlığı,* ***KD:*** *kas derinliği,* ***SKCA:*** *sütten kesim canlı ağırlığı*

yılarına ait değerler *Tablo 4*'te verilmiştir. Elde edilen tüm fenotipik korelasyon katsayıları pozitif yönlü ve önemli (P<0.001) bulunmuştur. En düşük korelasyon katsayısı kabuk yağı kalınlığı ve sütten kesim canlı ağırlığı arasında en yüksek katsayı ise kabuk yağı kalınlığı ve deri+kabuk yağı kalınlığı arasında bulunmuştur. Kas derinliği ile deri+kabuk yağı kalınlığı ve sütten kesim canlı ağırlığı arasındaki pozitif yönlü yüksek düzeydeki korelasyon katsayıları da ilgi çekicidir.

TARTIŞMA ve SONUÇ

Batı Anadolu ve Marmara bölgesinde yetiştiriciliği yapılan Karya, Kıvırcık ve Karacabey Merinosu ırkı koyunlar Türkiye yerli koyun populasyonu içinde küçük bir paya sahip olmalarına rağmen döl verimi ve et kalitesi bakımından önemli ırklar arasındadırlar. Bunlardan Karya yüksek döl verim özelliği yanında kuzularının hızlı gelişme özellikleri, Kıvırcık ırkı et kalitesi ve Karacabey Merinosu yapağı ve et verimi bakımından öne çıkmaktadır.

Araştırmanın hayvan materyalini oluşturan kuzularda sütten kesim canlı ağırlığı ve ultrasonik MLD özelliklerinin ölçümü sütten kesim döneminde (ort. 115 gün) gerçekleştirilmiştir. Karacabey Merinosu için elde edilen ortalama 38.07 kg'lık canlı ağırlık değeri ilgili literatürde bildirilen değerlerden belirgin derecede yüksek olup önemli bir üretim potansiyeline işaret etmektedir [16-19]. Bu durum KM ırkında uzun yıllardan beri canlı ağırlığa yönelik yürütülen yoğun seleksiyon programının doğal bir sonucu olarak ortaya çıkmıştır. Çalışmada en düşük canlı ağırlık değeri Karya ırkında gözlemlenmiştir. Elde edilen bu değer Cemal et al.[2] tarafından aynı ırkta yapılan çalışmada elde edilen değerden düşük bulunmuştur. Karya ırkında döl verimi ön plana çıkmaktadır nitekim çalışmanın yürütüldüğü işletmedeki döl verim ortalaması 1.80 civarındadır. Bu durum göz önünde bulundurulduğunda sütten kesim canlı ağırlığının diğer ırklardan düşük çıkmasının işletmede bakım yönetim uygulamalarında meydana gelen aksaklıklardan kaynaklandığı düşünülmektedir. Kıvırcık ırkı için elde edilen canlı ağırlık değerleri ilgili literatürün bir kısmı ile [1,20,21] benzerlik göstermesine rağmen Yılmaz et al.[4] tarafından Kıvırcık melezi kuzularda elde edilen değerlerden düşük, Yaralı and Karaca [22] tarafından yine Kıvırcık kuzularda yapılan çalışmadan yüksek bulunmuştur. Tüm bu farklılıklar bakım besleme yöntemlerindeki farklılıklar ile açıklanabilir. Tek doğanların ikizlerden daha yüksek canlı ağırlık değerine sahip olmaları konuyla ilgili literatürle uyum göstermektedir [1,23]. En yüksek ortalama günlük canlı ağırlık artış değeri canlı ağırlık bulgularındakine

benzer şekilde KM ırkında gerçekleşmiştir. Sezenler et al.[19] Karacabey Merinosu kuzularda 90. gün ortalama günlük canlı ağırlık artışı değerinin genel ortalamasını 273.36 g olarak bildirmiştir.

Bu çalışmada ultrason ölçümlerin gerçekleştirildiği yaş literatürde belirtilen alt ve üst limitler arasındadır [1,2,4,21,23]. Ultrason ölçümlerinin gerçekleştirildiği yaşlar bakımından ıslah programları arasında farklılıklar bulunmaktadır. Daha geç yaşta yapılan ultrason ölçümlerinde elde edilen görüntülerde dokuların ayrımı ve özellikle yağ tabakasının sınırları daha belirgin olduğu için ölçümler daha duyarlı yapılabilmektedir. Ancak, Batı Anadolu'da kuzuların sütten kesimde hemen pazarlanması daha geç yaşlarda ultrason ölçümü gerçekleştirilmesini olanaksız hale getirmektedir.

Genel ortalamalar; kabuk yağı kalınlığı için 0.31 cm, deri+kabuk yağı kalınlığı için 0.55 cm, kası derinliği için ise 2.09 cm olarak bulunmuştur. Kıvırcık ve Karya kuzularda yapılan çalışmalarda kas derinliği için benzer değerler belirtilmiştir [1,2,4]. Fernandez et al.[14] Merinoslar için elde edilen kabuk yağı kalınlığını 0.38 cm olarak bildirmişlerdir. Başka bir çalışmada ise Suffolk ve Romanov melezi kuzularda kabuk yağı kalınlıklarının 0.274 ve 0.296 cm arasında değiştiği bildirilmiştir [21].

Çalışmada etlenme ve yağlanmanın tahmininde kullanılan MLD ölçümleri ile canlı ağırlık arasında pozitif yönlü ve istatistiki olarak önemli ($P<0.001$) bulunan korelasyon katsayıları konuyla ilgili yapılan çalışmalarla [1,4,13,21] uyum içindedir. Elde edilen bulgular kas derinliğinin yüksek düzeyde canlı ağırlık ile ilişkili olduğunu göstermektedir.

Çalışma kapsamında yapılan değerlendirmelerde ırklar arasında meydana gelen istatistik farkların sürü yönetimi, özellikle besleme ve/veya otlatma ile doğum dönemi kuzuların bakım uygulamalarındaki farklılıklar yanında genetik yapı farklılıklarından da kaynaklanabileceği düşünülmektedir.

Geleneksel anlamda, Batı Anadolu'da kuzular sütten kesimle birlikte süt kuzusu olarak yüksek kasaplık değerle pazarlanmaktadır. Değiştirilmesi kısa vadede olanaksız görünen mevcut üretim sisteminde hedeflenen, yağlanma gerçekleşmeden kuzu karkasının tüketime sunulmasıdır. Bu yaklaşım sonucunda dünya standartlarının oldukça altında bir ağırlıkta karkaslar tüketime sunulmakta, ciddi üretim kayıpları şekillenmekte ve yetiştiriciler arzulanan geliri elde edememektedirler. Sütten kesimi izleyen birkaç aylık süreçte ortaya çıkacak yağlanmanın önlenmesine ve daha yüksek miktarda yağsız et üretimine yönelik etkin seleksiyon çalışmaları ile bu sorunun üstesinden gelinebilmesi mümkün görünmektedir. Et kalite özelliklerinin geliştirilmesine yönelik kimi ülkelerde uygulanan seleksiyon programlarında ultrasonik görüntüleme sistemleri önemli bir yer edinmiştir.

Gerçekleştirilen bu çalışmadan elde edilen somut bilgiler ışığında kullanılan bu üç ırktan elde edilen ultrason ölçümlerine ait bilgiler bu ırklarda yürütülen ıslah programları için seleksiyon indekslerinin oluşturulmasında önemli katkı sağlayacaktır. Etkin bir seleksiyon indeksinin oluşturulabilmesi için pedigri kayıtlarını da içeren veri tabanının genişletilerek gerekli genetik parametrelerin tahmin edilmesi gerekmektedir.

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RESEARCH ARTICLE

Determination of the Optimum Fattening Period of Tuj and Hemşin Lambs according to Different Fattening Systems

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Abstract

This study aims to determine the optimum fattening period according to various fattening systems under controlled conditions for the first time in lamb fattening in Turkey. The material of the study consists of 39 Tuj and 39 Hemşin genotype male weaned lambs that are 2.5-3 months old. The Tuj and Hemşin lambs in the study have been separated into 3 equal groups through the systematic sampling method and subject to 90-day fattening. The amount of feed in terms of consumed dry matter for 1 kg of increase in live weight of lambs in the intensive system was calculated as 5.69 kg and 5.90 respectively for Tuj and Hemşin lambs. The optimum fattening period in the intensive system for Tuj and Hemşin lambs occurred between the 70th and 84th days. The marginal cost-marginal income equality was not achieved throughout the fattening in the extensive and semi-intensive fattening periods for both genotypes. Thus, it is estimated that the optimum fattening period in the extensive and semi-intensive systems is over 90 days. The fattening period with the highest marginal income was on the 70th, 84th, and 42nd days respectively for the extensive, semi-intensive, and intensive systems for Tuj lambs and this was on the 70th, 28th, and 56th days respectively for Hemşin lambs. In conclusion, it was observed that the optimum fattening period in Tuj and Hemşin lambs was earlier in the intensive fattening period compared to other fattening periods.

Keywords: *Lamb fattening, Fattening systems, Optimum fattening period, Marginal cost, Marginal income*

Tuj ve Hemşin Kuzularında Farklı Besi Sistemlerine Göre Optimum Besi Süresinin Belirlenmesi

Özet

Bu çalışma ile kuzu besisinde Türkiye'de ilk defa kontrollü koşullar altında farklı besi sistemlerine göre optimum besi süresinin tespit edilmesi amaçlanmıştır. Araştırma materyalini Tuj ve Hemşin genotiplerinden 39'ar baş 2.5-3 aylık yaşta sütten kesilmiş erkek kuzu oluşturmuştur. Çalışmada Tuj ve Hemşin kuzular sistematik örnekleme metoduyla eşit sayıda 3 besi grubuna ayrılmış ve 90 günlük besiye tabi tutulmuştur. Entansif sistemdeki kuzular için 1 kg canlı ağırlık artışı için tüketilen kuru madde cinsinden yem miktarı Tuj ve Hemşin kuzularında sırasıyla 5.69 kg, 5.90 kg olarak hesaplanmıştır. Tuj ve Hemşin kuzularında entansif sistemdeki optimum besi süresi 70. ve 84. günler arasında gerçekleşmiştir. Her iki genotip için ekstansif ve yarı entansif besi sistemlerinde marjinal maliyet-marjinal gelir eşitliği besi süresince yakalanamamıştır. Dolayısıyla ekstansif ve yarı entansif sistemlerde optimum besi süresinin 90 günün üzerinde olduğu tahmin edilmektedir. Marjinal gelirin en yüksek olduğu besi dönemi Tuj kuzularında; ekstansif, yarı entansif ve entansif sistemleri için sırasıyla 70., 84. ve 42. gün iken, Hemşin kuzularında aynı sırasıyla 70., 28. ve 56. gün olarak saptanmıştır. Sonuç olarak; Tuj ve Hemşin kuzularında optimum besi süresinin diğer besi sistemlerine göre entansif besi sisteminde daha erken olduğu görülmüştür.

Anahtar sözcükler: *Kuzu besisi, Besi sistemleri, Optimum besi süresi, Marjinal maliyet, Marjinal gelir*

INTRODUCTION

According to the 2013 data of TURKSTAT, 18.73% of Turkey's total red meat production is provided from sheep-lamb meat. The proportion of lamb meat in the total amount of sheep and lamb meat produced is 56.11% [1]. According to the 2011 data of the Food and Agriculture Organization (FAO), sheep carcass weight is

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16.0 kg on average in Turkey, 14.6 kg on average in the European Union (EU-28), 30.8 kg on average in the United States of America, 22.4 kg on average in Australia, and 19.2 kg on average in New Zealand [2]. The reasons for the low carcasses in Turkey can be pointed out as low yield indigenous races, high rate of early lamb slaughters, and transfer of animals to slaughter after pasture fattening without being subject to intensive fattening [3,4].

The profitability and productivity of lamb fattening, sub-sector of livestock, depends on some technical and economic criteria. One or the most important economic factors contributing to the performance of lamb fattening in a profitable manner is the determination of the optimum fattening period. As is the case in other economic activities, lamb fattening is also under the effect of the "law of diminishing returns". Within the framework of this law, the daily live weight gain of animals subject to fattening gradually decrease after a certain period and after a certain point, the live weight gain obtained daily is unable to address fattening costs. Thus, the fattening activity should be completed at the point where the marginal income is equal or close to the marginal cost [5,6].

This study aims to determine the optimum fattening period for maximum profitability in the fattening of Tuj and Hemşin lambs according to various fattening systems under controlled conditions for the first time in Turkey.

MATERIAL and METHODS

This study was conducted upon the Board of Ethics Approval received through the Kafkas University Faculty of Veterinary Medicine Board of Ethics resolution no. 2011-005 of 03.03.2011. The study was conducted in the Kafkas University Faculty of Veterinary Medicine, Training, Research, and Practice Farm in 2012. The material of the study consists of 39 Tuj and 39 Hemşin male lambs that have been weaned at 2.5-3 months of age. The internal and external parasite medication was administered to lambs included in the scope of the study prior to fattening; and they were allowed to orient to the pasture and feed for a period of 10 days. Afterwards, the Tuj and Hemşin lambs were ordered according to their live weight and through the systematic sampling method, they were separated into the different fattening groups of extensive, semi-intensive, and intensive and in a way to include 26 lambs in each group, and the lambs were subject to fattening for a period of 90 days.

The lambs in extensive and semi-extensive fattening were grazed for 8 h a day in the pasture. Group feeding was performed in the feeding of the lambs in the sheep fold. In semi-intensive fattening (pasture + feed) and intensive fattening, the lambs were given concentrated feed prepared according to NRC (1985) as *ad libitum* [7]. In addition to concentrated feed, the lambs in intensive fattening were given quality roughage. The nutritional contents of the concentrated feed and roughage given to lambs have been provided in *Table 1*. Amounts of feed given to the lambs were recorded daily and the live weights of the lambs were recorded on an empty stomach in the morning in periods of 14 days and prior to being slaughtered on the 90th day. While there was constantly water in front of the animals in the intensive fattening group during the fattening process, water was provided to animals in the extensive and semi-intensive groups at least three times a day.

The sales price of concentrated feed, roughage, labor, electricity-water, pasture rent, and also carcass sales used in the calculation of marginal costs and marginal income in periods of 14 days were determined with 2012 current prices by conducting a market research in the province of Kars. General administrative expenditures, which are among marginal costs was accepted as 3% [8]. After the sheep fold amortization expense was calculated using the straight-line method, the daily amortization amount was determined. In the extensive fattening system, there are no concentrated feed, roughage, electricity-water, and sheep fold amortization expenses, in the semi-intensive fattening system, there are no roughage expenses, and in the intensive fattening system, there are no pasture lease fees.

The marginal income in the study were calculated by taking marginal live weight gain, actual cold carcass yield determined according to fattening groups, and carcass sales prices into account and in the calculation of the optimum fattening period, marginal cost and marginal income equation were utilized. Sheep fold maintenance-repair and litter floor expenses, and the secondary incomes of manure were not taken into consideration in the study as they were very low and did not reflect a significant difference between fattening groups. On the other hand, health-medication expenses were made before fattening and only once. Thus, they have not been included in the marginal cost. However, health-medication expenses have been added to the fattening material cost and included in the total cost of zero-day.

Table 1. *Nutritional contents and energy values of the concentrated feed and roughage used in the semi-intensive and intensive fattening systems*

Tablo 1. *Yarı entansif ve entansif besi sistemlerinde kullanılan konsantre ve kaba yemin besin madde içerikleri ve enerji değerleri*

Ingredient	Concentrate Feed	Roughage
Dry matter (%)	88.80	90.69
Crude protein (%)	17.12	10.35
Crude cellulose (%)	5.75	32.38
Crude fat (%)	3.54	2.00
Crude ash (%)	6.48	8.86
Metabolic energy (kcal/kg)	2710	2000

The SPSS 20.0 statistics package program was used in the study. The descriptive statistics of the data have been performed in the SPSS program and the T-Test and the One-Way Analysis of Variance were utilized in the significance check of difference between groups [9].

RESULTS

The cold carcass yield for Tuj and Hemşin lambs were respectively determined as 41.58% and 41.93% in the extensive system, 45.80% and 45.35% in the semi-intensive system, and 48.42% and 48.05% in the intensive system. The difference between fattening systems within both genotypes in terms of cold carcass yield were determined to be statistically significant (P<0.001). On the other hand, the amount of feed in terms of roughage consumed for 1 kg live weight gain was determined for lambs in the intensive system and calculated as 5.69 kg for Tuj lambs and 5.90 kg for Hemşin lambs.

The optimum fattening periods of Tuj and Hemşin lambs according to fattening periods have been respectively provided in *Table 2* and *Table 3*. When the relation between marginal cost and marginal income is examined according to fattening systems, it can be observed that marginal income are higher than marginal cost for Tuj and Hemşin lambs in the intensive system until the 70th day of fattening. However, it was determined that the marginal income dropped under the marginal cost as of the 84th day in the fattening of Tuj and Hemşin lambs in the intensive system. According to this, the optimum fattening period for Tuj and Hemşin lambs in the intensive system was between the 70th and 84th days, when the marginal cost is equal to or close to the marginal income. On the other hand, it can be observed that the marginal income did not drop under the marginal cost in the extensive and semi-intensive fattening systems throughout the 90-day fattening period for both genotypes. Thus, the designation of the optimum fattening period in these two systems was not possible in the study process.

There was no statistical difference between fattening systems for Tuj and Hemşin lambs in terms of fattening initiation live weight averages (P>0.05) and it was determined that there was a statistically significant difference between the extensive system and other fattening systems at the end of the fattening (P<0.05).

On the other hand, when the total costs of Tuj and Hemşin lambs are examined, there is no statistical difference between fattening systems until the 42nd day (P>0.05). However, it was determined that there was a statistical difference between the extensive system and other fattening systems in terms of total cost as of the 42nd day and a statistically significant difference between the semi-intensive system and intensive system as of the 84th day (P<0.001).

The fattening period with the highest marginal income in the fattening of Tuj lambs were determined to be the 70th, 84th, and 42nd days respectively for the extensive, semi-intensive, and intensive fattening systems and for the Hemşin lambs these were respectively determined to be the 70th, 28th and 56th days.

DISCUSSION

One of the most important input elements in livestock is feed consumption [5,10]. In the study, the amount of feed consumed as dry matter for 1 kg live weight gain in the intensive fattening system was determined to be lower in Tuj lambs compared to Hemşin lambs. There are studies suggesting that the amount of feed as dry matter consumed for 1 kg live weight decreases as the fattening initiation live weight decreases in fattening activities and they support the findings of the study. On the other hand, as the live weight of the animal increased, the feed conversion rate decreased throughout the fattening period [11-14].

The determination of the optimum fattening period in livestock enterprises is of utmost importance in the profitability and efficiency of fattening activities [11]. As a matter of fact, the basic objective in fattening enterprises is the maximization of profits, just like in all economic enterprises. Profit maximization in businesses can be achieved through the control of some factors inside the business besides factors outside of the business [5,13,15]. The activity of fattening is under the impact of the law of diminishing returns. Thus, when marginal cost in fattening is equal to marginal income, fattening should be finalized. This is because; at this point the maximum profit of the period will have been achieved [11,16].

In the study, the optimum fattening period of Tuj and Hemşin lambs in the intensive system was determined to be between the 70th and 84th days, when the marginal cost is equal or close to the marginal income. In the study conducted by Cinemre *et al.*[17], 10 Karayaka and 10 Sönmez X Karayaka (F_1) male 2.5 month-old lambs were subject to 91 days of intensive fattening, and the optimum fattening period was determined to be 84 days for both genotypes. On the other hand, in the study of Akdemir *et al.*[18], a total of 80 lambs in the France X İvesi, Sakız X İvesi, and İvesi X İvesi genotypes were separated into four equal groups and fed with two different concentrated feed rations. The optimum fattening period in the studies varied depending on the fattening period, genotype, utilized concentrated feed, and the fattening periods in a year at the enterprise, and it was reported to be between 49 and 63 days.

It should not be forgotten that in fattening, the optimum fattening period may vary depending on the fattening period, the age and genotype of the animal,

Table 2. *Optimum fattening periods of Tuj lambs according to the fattening system*
Tablo 2. *Tuj kuzularında besi sistemlerine göre optimum besi süreleri*

Day	Extensive Fattening System (n=13)					Semi-Intensive Fattening System (n=13)					Intensive Fattening System (n=13)				
	Average LW (kg/head)	Marginal LWG (kg)	Total Cost (TL)	Marginal Cost (TL)	Marginal Income (TL)	Average LW (kg/head)	Marginal LWG (kg)	Total Cost (TL)	Marginal Cost (TL)	Marginal Income (TL)	Average LW (kg/head)	Marginal LWG (kg)	Total Cost (TL)	Marginal Cost (TL)	Marginal Income (TL)
0	20.62a	-	2579.07a	-	-	20.55a	-	2570.43a	-	-	20.66a	-	2584.01a		-
14	21.99a	1.37a	2686.13a	107.06a	133.30a	22.77a	2.22a	2728.79a	158.36a	237.92a	22.52a	1.86a	2752.89a	168.88a	210.74a
28	24.49a	2.50a	2793.19a	107.06a	243.24a	26.15a	3.38b	2905.29a	176.50a	362.24b	24.43a	1.91a	2954.99a	202.10a	216.41a
42	24.95a	0.46a	2900.25a	107.06a	44.76a	28.88b	2.73b	3144.04b	238.74b	292.58b	28.57b	4.14c	3220.53b	265.54b	469.07c
56	26.58a	1.63a	3007.31a	107.06a	158.59a	32.09b	3.21b	3426.42b	282.38b	344.02b	32.27b	3.70b	3546.80b	326.27b	419.22b
70	29.45a	2.87a	3114.37a	107.06a	279.24a	35.86b	3.77a	3715.40b	288.98b	404.04a	36.22b	3.95a	3885.74b	338.94b	447.55a
84	30.89a	1.44a	3221.43a	107.06a	140.11a	40.52b	4.66b	4015.03b	299.63b	499.42b	38.58b	2.36a	4248.61c	**362.86c**	**267.39a**
90	31.19a	0.30a	3265.64a	44.21a	49.19a	41.22b	0.70a	4121.87b	106.85b	109.02a	40.56b	1.98b	4488.98c	**240.37c**	**224.34b**

LW: *Light Weight;* ***LWG:*** *Live Weight Gain;* ***a, b, c:*** *the difference between groups with different letters and same parameters according to the fattening systems is significant (P<0.05), the difference between groups with the same letter is not significant (P>0.05)*

Table 3. *Optimum fattening periods of Hemşin lambs according to the fattening system*
Tablo 3. *Hemşin kuzularında besi sistemlerine göre optimum besi süreleri*

Day	Extensive Fattening System (n=13)					Semi-Intensive Fattening System (n=13)					Intensive Fattening System (n=13)				
	Average LW (kg/head)	Marginal LWG (kg)	Total Cost (TL)	Marginal Cost (TL)	Marginal Income (TL)	Average LW (kg/head)	Marginal LWG (kg)	Total Cost (TL)	Marginal Cost (TL)	Marginal Income (TL)	Average LW (kg/head)	Marginal LWG (kg)	Total Cost (TL)	Marginal Cost (TL)	Marginal Income (TL)
0	22.42a	-	2801.37a	-	-	22.99a	-	2871.77a		-	22.92a	-	2863.12a	-	-
14	23.92a	1.50a	2908.43a	107.06a	147.17a	24.99a	2.00a	3031.66a	159.89a	212.24a	24.13a	1.21a	3032.78a	169.66a	136.05a
28	26.42a	2.50a	3015.49a	107.06a	245.29a	28.99b	4.00b	3209.69a	178.03a	424.48b	25.35a	1.22c	3235.66a	202.88a	137.17c
42	27.42a	1.00a	3122.55a	107.06a	98.12a	30.72b	1.73a	3449.97b	240.27b	183.59a	29.33b	3.98b	3501.98b	266.32b	447.50b
56	29.21a	1.79a	3229.61a	107.06a	175.63a	33.95b	3.23b	3733.88b	283.91b	342.76b	33.96b	4.63c	3830.68b	328.71b	520.58c
70	31.86a	2.65a	3336.67a	107.06a	260.01a	37.45b	3.50a	4024.39b	290.51b	371.42a	37.00b	3.04a	4170.41b	339.72b	341.81a
84	33.05a	1.19a	3443.73a	107.06a	116.76a	40.36b	2.91b	4325.55b	301.16b	308.81b	40.29b	3.29b	4534.05c	**363.64c**	**359.92b**
90	33.32a	0.27a	3487.94a	44.21a	46.49a	41.16b	0.80a	4432.60b	107.06b	115.90a	42.09b	1.80b	4776.83c	**242.78c**	**202.39b**

LW: *Light Weight;* ***LWG:*** *Live Weight Gain;* ***a, b, c:*** *the difference between groups with different letters and same parameters according to the fattening systems is significant (P<0.05), the difference between groups with the same letter is not significant (P>0.05)*

fattening initiation live weight, live weight gain, fattening type, feed, labor, and other elements constituting the cost [11,18].

On the other hand, it can be observed that the marginal income did not drop below or equal the marginal cost throughout the 90-day fattening period in the extensive and semi-intensive fattening systems for both genotypes, and profit maximization did not happen. When it is considered that the law of diminishing returns applies for all economic enterprises, the optimum fattening period occurring on a day after the 90th day in case fattening is continued, is an undeniable fact according to the science of economics. However, it is considered that it is actually impossible to continue the fattening of the lambs in the extensive and semi-intensive system in the manner the study was planned.

Yet, in the province of Kars, where the study was conducted, just like the most of Turkey, the pasture season starts at the end of April each year and continues until the end of July or the beginning of August depending on the amount of precipitation. Thus, the pasture season in Turkey suitable for extensive and semi-intensive fattening is a period of approximately 90 days covering the months of May, June, July [5,19]. There are many scientific studies reporting that the desired live weight gain cannot be achieved in case grazing of animals is continued after the pasture season and additional feeding is required to compensate for this [19,20].

On the other hand, it is considered that having lambs in the extensive and semi-intensive system subject to the intensive system after the pasture season until the equality of the marginal income with the marginal cost is ensured is not suitable in terms of biologic efficiency. This is because; when determining the optimum feeding period slaughter weight, market demands, and biologic efficiency should be taken into consideration [21]. Extension of the fattening period in lamb fattening causes the accumulation of fat in the carcass and as the biologic age of the lamb gets older, the form of fattening is no longer lamb fattening. This is not desired in terms of biologic efficiency. According to the quality grading system of the United States of America, fat accumulation in the lamb carcass demonstrates positive effects to a certain point for preserving the freshness and color of the carcass and preventing the increase of losses and then demonstrates adverse effects after this point [22,23]. As a matter of fact, in the study conducted by Akçapınar [24] on two groups of lambs from the Akkaraman genotype with average slaughter weights of 40 and 45 kg, it was reported that as the slaughter weight increased, the fat rate and *M. Longissimus dorsi* area of the carcass increased and in addition to this, the rate of fat-free meat in the carcass decreased.

It is considered that continuing the fattening of lambs in the semi-intensive system in an intensive manner after the pasture season caused fat accumulation in the carcass. On the other hand, it is considered that in case lambs with low weight in the extensive fattening system are subsequently subject to the intensive fattening system, as the biological ages of the animals will have become older, it will no longer be lamb fattening.

In conclusion, the necessity to assess lamb fattening in terms of not only technical criteria but also in terms of economic criteria has been set forth. For the purpose of utilizing the limited resources of lamb fattening enterprises in an effective and efficient manner and maximizing profitability, the optimum fattening period should be calculated, and a decision should be made to finalize fattening in this manner.

It was determined that there was a statistical difference between the extensive system and the other fattening systems in terms of the live weight gain throughout the 90 day fattening period of the Tuj and Hemşin lambs. On the other hand, the optimum fattening period for both genotypes in the intensive system was determined to be between the 70th and 84th days. In the semi-intensive system, the marginal income did not drop below the marginal cost throughout the fattening period for both genotypes. Furthermore, even though there was no statistical difference between the semi-intensive and intensive fattening systems at the end of the fattening period in terms of fattening finalization live weight, in the semi-intensive system the total cost was calculated to be lower at a statistically significant level. In the final analysis, it was determined that the performance of lamb fattening by lamb fattening enterprises by employing the semi-intensive system would be more rational for the lamb fattening enterprise.

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RESEARCH ARTICLE

Inhibitory Effect of L-Canavanine and L-Lysine on Arginase Activity in Sheep Spleen Tissue [1]

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Abstract

Arginase (L-arginine amidinohydrolase; (E.C.3.5.3.1) is the last enzyme of urea cycle and it catalyzes the hydrolysis of L-arginine to urea and L-ornithine in livers of animals. It helps to the excretion process of urea from the body, which is the most soluble and non-toxic form of nitrogen. Along with its essential role in urea synthesis, arginase is also found to have important roles in ornithine production for polyamine, proline and glutamate synthesis and in immune system activation. Mostly found in liver tissue, arginase enzyme is also found in non-ureolytic tissues like heart, rumen, skeletal muscle, kidney, intestine, brain, spleen, thyroid gland, salivary gland, erythrocyte, fibroblast, macrophage, mammary gland in lactation, testicle. With this study, it was aimed to assess the inhibitory effects of arginase in sheep spleen tissue on L-canavanine and L-lysine. Consequently, it was observed that L-lysine as a L-amino acid and guanidine compound L-canavanine inhibits enzyme activity non-competitively.

Keywords: *Arginase, L-lysine, L-canavanine, Sheep spleen tissue*

L-Kanavanin ve L-Lizin'in Koyun Dalak Doku Arginaz Aktivitesi Üzerine İnhibisyon Etkisi

Özet

Arginaz (L-arginin amidino hidrolaz; (E.C.3.5.3.1) üre döngüsünün son enzimi olup hayvanların karaciğerlerindeki L-arginini, üreye L-ornitine hidrolizini katalizeleyen bir enzimdir. Azotun en iyi çözünebilir ve nontoksik formu olan üre oluşumuna katkıda bulunarak vücuttan atılmasına yardımcı olur. Arginaz üre sentezindeki vazgeçilmez rolünün yanında; poliamin, prolin ve glutamat sentezi için ornitin üretimini sağlayıp, ayrıca immün sistem aktivasyonunda ve tümör biyolojisinde de rol oynadığı saptanmıştır. Arginaz enzimi en çok karaciğer dokusunda bulunmakla beraber; kalp, rumen, iskelet kası, böbrek, bağırsak, beyin, dalak, tiroit bezi, tükürük bezi, eritrosit, fibrobast, makrofaj, laktasyondaki meme bezi, testis gibi nonüreolitik dokularda da bulunmaktadır. Bu çalışma ile koyun dalak doku arginazı üzerine L-kanavanin ve L-lizinin inhibisyon etkileri incelenmesi amaçlanmıştır. Sonuç olarak, bir L-amino asit olan L-lizin ve guanidin bileşiklerinden L-kanavaninin enzimi non-kompetetif olarak inhibe ettiği görüldü.

Anahtar sözcükler: *Arginaz, L-lizin, L-kanavanin, Koyun dalak dokusu*

INTRODUCTION

Arginase enzyme (E.C.3.5.3.1), L-arginine amidinohydrolase), discovered by Kossel and Dakin in 1904, is the last enzyme of urea cycle [1]. In mammalians, there are two isoforms of arginase that hydrolyze L-arginine to ornithine and urea. Arginase I is the cytoplasmic enzyme of liver. Arginase II is found in mitochondria of extrahepatic tissues such as brain, small intestine, and macrophages [2].

Liver, in which urea cycle takes place, contains most of the arginase in the body. Arginase is also found in extrahepatic tissues with no urea synthesis such as kidney, brain, thyroid gland, salivary gland, erythrocytes, thrombocytes, macrophages, rumen, mammary gland, skeletal muscle, fibroblast, intestine, uterus and testicle [3-7]. It is known that arginase provides the necessary ornithine

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for proline, glutamate, and polyamine synthesis in these tissues with no urea cycle [8].

Arginase enzyme is the first enzyme of polyamine biosynthesis. Polyamines (putrescine, spermine, and spermidine) are important molecules for cell growth and differentiation. Ornithine, formed with the help of arginase, is then converted to putrescine by ornithine decarboxylase enzyme; and later putrescine joins in the synthesis of spermine and spermidine [9,10].

Studies showed that serum arginase activity increases in liver diseases, such as acute hepatitis, liver metastases, malignant tumors of bile duct, and cirrhosis, which cause cell destruction [11].

In different studies, it is stated that arginase is a determinant enzyme in cancer processes, because serum and tissue arginase levels increase in some cancer cases [11-13].

Serum arginase levels of patients with acute and chronic pancreatitis were found higher than control values and arginase activity was decreased after treatment [14].

Increase in erythrocyte arginase levels was found in led poisoning and diseases like pernicious anemia and thalassemia [15].

It is stated that in patients with myocardial infarction, arginase levels were increased in the first day and in the following day, these levels were a little lower than the levels of the first day, but were still higher than the control values. It was observed that in 10th day arginase levels returned back normal [16].

Ceylan *et al.*[17], investigated plasma arginase levels in the blood samples taken from asthma patients and found that arginase activities were lower than the control values.

It is found that arginase enzyme exists in some uricotelic organisms and plants [18,19].

It is observed that urea cycle enzymes decrease in low protein diet whereas urea cycle enzyme activities increase in contrast to that of the ones in low protein diet when extra protein is added [20]. It is reported that L-ornithine and L-lysine cause non-competitive inhibition over bovine lumen tissue arginase [21,22]. In view of these findings, we suppose that L-lysine and L-canavanine will inhibit the enzyme. Therefore, in this study, we aim to find the type of the inhibition. To the best knowledge of the authors, this is the first study in the literature which presents the results of kinetic effect of inhibition of L-canavanine and L-lysine on arginase level in sheep spleen tissue, which was not studied before.

MATERIAL and METHODS

Chemicals used in this study, such as L-arginine, diacetyl-monoxyme, thiosemicarbazide, sulfuric acid, HCl, L-lysine, L-canavanine, KCl were obtained from the companies Sigma, Merck and Fluka. Other chemicals used in this study were obtained from the market and in analytical purity.

Sheep spleens as study materials were obtained from Elazığ Elkas Facilities. 20 sheep from the same herd of Akkaraman race with 2 to 3 years old and with similar breeding conditions (shelter, caring and environmental conditions) and similar feeding conditions were slaughtered.

After slaughtering, the obtained spleen tissue was taken into beaker with isotonic cold NaCl solution, after cleaning out from blood and clots; and it was analyzed in iced setting out of the beaker. The rest was kept in deep freezer in -18°C for later use.

After tissue samples were cleaned out from blood and clots, they were weighed as 1 g (weight/volume) and completed to 6 ml with distilled water (dilution rate 1/6). Tissues were homogenized with Potter-Elvehjem homogenizer. The homogenates were processed in +4°C using cold centrifuge (Nuve) device for 14 min and supernatants and pllets were seperated from one another. Obtained supernatant was used as enzyme source.

Arginase activity was measured with spectrophotometry using thiosemicarbazide-diacetylmonoxyme urea (TDMU) method. This method is based on the detection of urea produced by hydrolysis of L-arginine [23].

Diacetylmonoxyme is a substance that does not directly react with urea and hydrolyzes to diacetyl and hydroxylamine in acidic setting with temperature. After diacetylmonoxyme is degraded to diacetyl, diacetylic acid condensates with urea in solution and composes a yellow compound named diazine. This yellow color is stabilized with thiosemicarbazide and Fe^{+2} ions [24].

Protein quantity in the homogenate was measured with modified Lowry [25] method. Alkaline copper tartrate reagent forms complexes with peptide bonds. When phenol reagent is added to mixture, which is processed with copper, a color of violet purple appears. This color was assessed in 650 nm in spectrophotometry.

While measuring the arginase levels, control was established by using triple tubes. For degradation of endogenous urea, 3 units of Jack-Bean urease were added to each ml of supernatant. Then, incubation was done in 37°C for 15 min [26]. Samples were diluted with 2 mM $MnCl_2$ solution with a rate of 1x40 (v/v) and held in metabolic water bath in 58°C for 13 min. Pre-incubation procedure was done the same way and samples were used as enzyme sources.

Enzyme source, which first 120 mM of L-arginine, then

200 mM of carbonate buffer were added to, was pre-incubated. Then, 0.3 ml of enzyme source was added to each tube and enzymatic reaction was initiated in shaking metabolic water bath in (pH 9.5) 37°C for 10 min. After 10 min of incubation, enzymatic reaction in each tube was stopped with 3 ml of acid mixture. Then, 2 ml of color reagent was added to each tube. Tubes were mixed with vortex, then held in boiling water bath for 10 min and color formation was implemented.

Tubes were cooled and assessed in spectrophotometry (Shimadzu UV-240) in 520 nm of wave length. Urea levels were assessed after absorbance values of zero time blanks were subtracted. These procedures were also carried out for standard study tubes and 0 to 0.6 µmol of urea standard solutions were used instead of enzyme sources and then calibration curves were drawn.

An enzyme quantity that produces 1 µmol of urea from L-arginine in 37°C in 1 h is called 1 unit of enzyme, while specific activity is stated as urea/hour/mg protein.

RESULTS

Inhibitory effect of L-lysine on sheep spleen tissue arginase activity in concentrations up to 20 mM was investigated. It was observed that 17% of enzyme activity was lost in 5 mM of L-lysine concentration, 28% of enzyme activity was lost in 10 mM of L-lysine concentration, and 36% of enzyme activity was lost in 15 mM of L-lysine concentration *(Fig. 1)*.

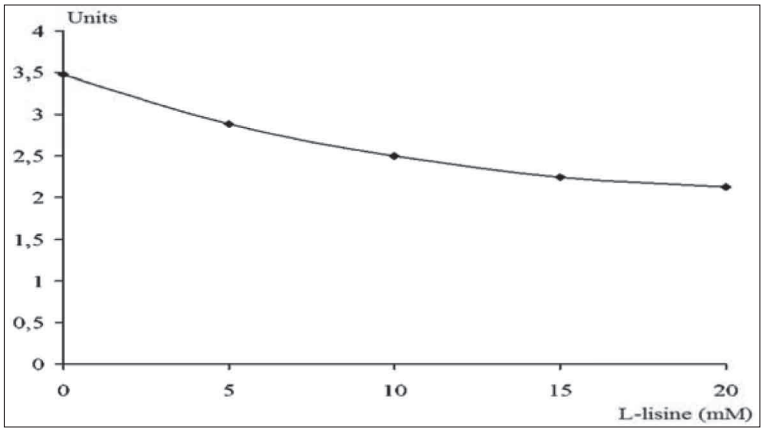
In the presence of 15 mM of L-lysine and in different L-arginine concentrations, inhibition type on sheep spleen tissue arginase activity was determined. Data was assessed with Michaelis-Menten *(Fig. 2)* and Lineweaver-Burk *(Fig. 3)* curves and inhibition type was found as non-competitive.

To assess the effect of L-canavanine on sheep spleen tissue arginase activity, 0 to 0.6 mM of L-canavanine was added to pre-incubation setting and findings were evaluated in comparison to the control. It was observed that 18% of enzyme activity was lost in 0.1 mM of canavanine concentration, 38% of enzyme activity was lost in 0.3 mM of canavanine concentration, 89% of enzyme activity was lost in 0.5 mM of canavanine concentration, and 100% of enzyme activity was lost in 0.6 mM of canavanine concentration *(Fig. 4)*.

To find out the inhibition type of L-canavanine on sheep spleen tissue arginase activity, enzyme activity was investigated in different arginine concentrations and in the presence of 0.45 mM of L-canavanine. Data was assessed with Michaelis-Menten *(Fig. 5)* and Lineweaver-Burk *(Fig. 6)* curves and it was found that L-canavanine inhibits sheep spleen tissue arginase activity non-competitively.

Fig 1. Inhibitory effect of L- lysine on sheep spleen tissue arginase activity

Şekil 1. L-lizinin koyun dalak doku arginazı üzerindeki inhibitör etkisi



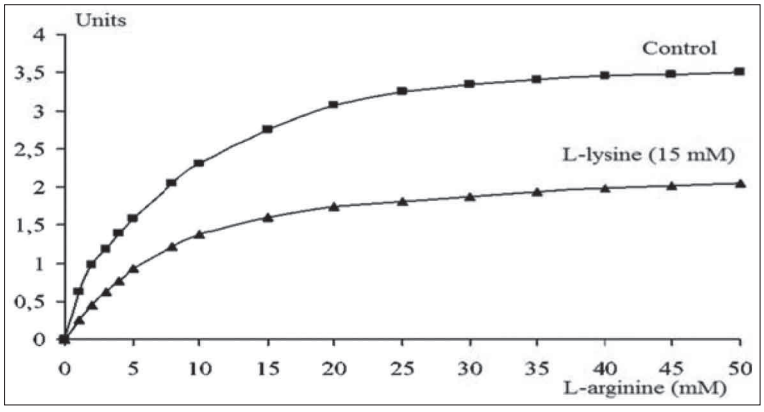


Fig 2. Presentation of inhibitory effect of L-lysine on sheep spleen tissue arginase activity with Michaelis-Menten graph

Şekil 2. L-lizinin koyun dalak doku arginazı üzerindeki inhibitör etkisinin Michaelis-Menten grafiği ile gösterilmesi

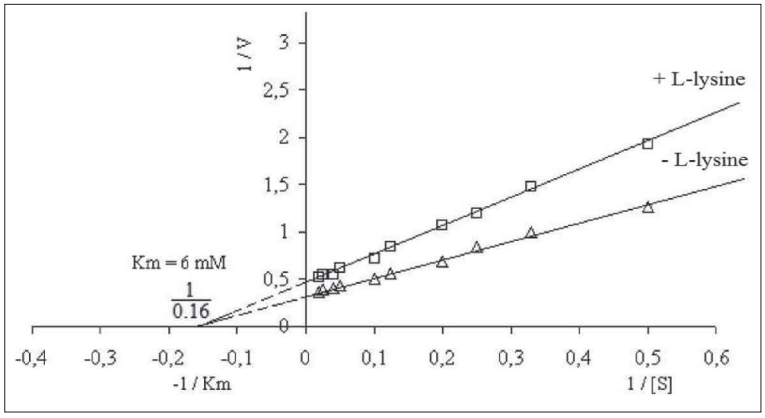
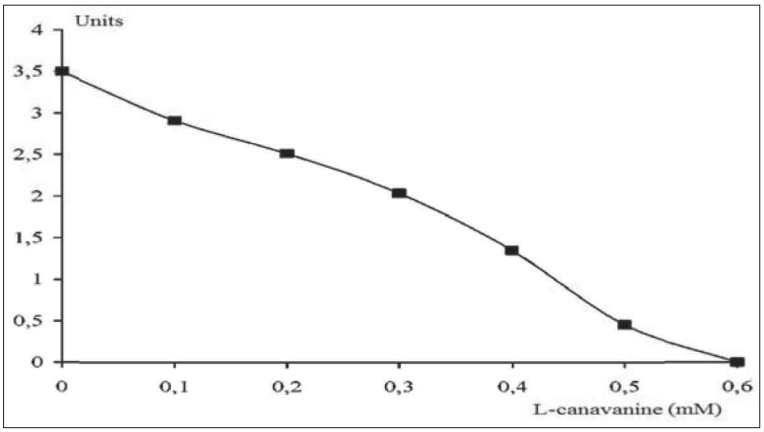


Fig 3. Assessment of effect of L-lysine on sheep spleen tissue arginase activity with Lineweaver-Burk curve

Şekil 3. L-lizinin koyun dalak dokusu arginazı üzerindeki etkisinin Lineweaver-Burk eğrisi ile gösterilmesi

Fig 4. Inhibitory effect of L-canavanine on sheep spleen tissue arginase activity

Şekil 4. L-kanavanin'in koyun dalak doku arginazı üzerindeki inhibitör etkisi



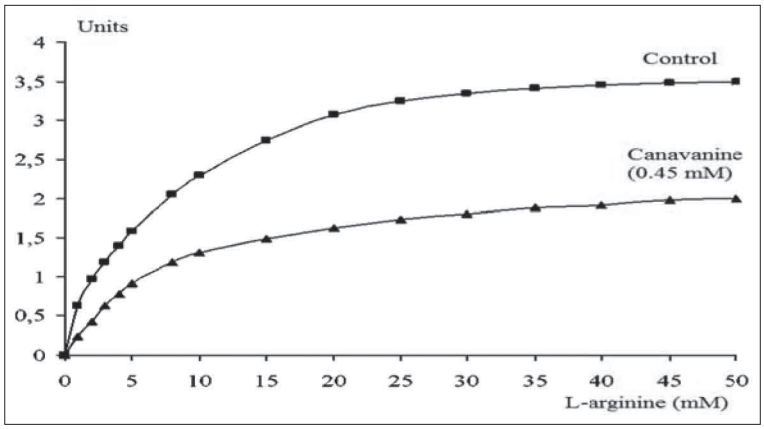
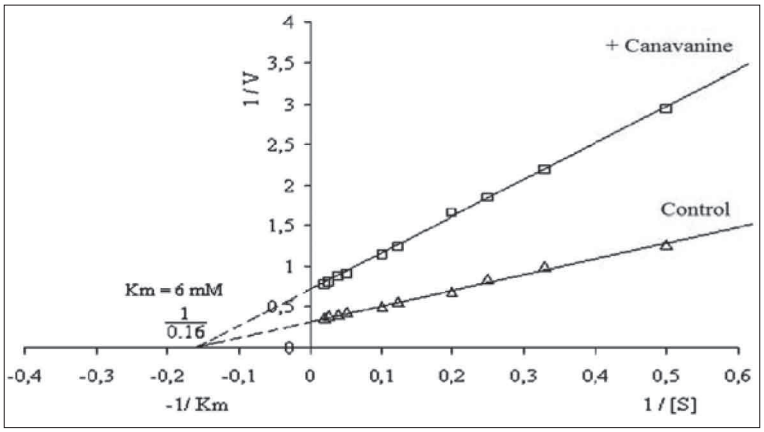


Fig 5. Presentation of inhibitory effect of L-canavanine on sheep spleen tissue arginase activity with Michaelis-Menten graph

Şekil 5. L-kanavaninin koyun dalak doku arginazı üzerindeki inhibitör etkisinin Michaelis-Menten grafiği ile gösterilmesi

Fig 6. Presentation of inhibitory effect of L-canavanine on sheep spleen tissue arginase activity with Lineweaver-Burk curve

Şekil 6. L-kanavaninin koyun dalak doku arginazı üzerindeki inhibitör etkisinin Lineweaver-Burk eğrisi ile gösterilmesi



DISCUSSION

There are a number of inhibitors of the enzyme arginase. Particularly, inhibition of arginase activity with branched chained aminoacids is found in several tissues and species [27,28].

In the studies by Muszynska [29] and Muszynska and Wojtczak [30] it was found out that ornithine and lysine competitively; valine, leucine, isoleucine and cysteine non-competitively inhibit arginase in rat liver.

It is reported that L-aminoacids such as valine, leucine, isoleucine, ornithine, proline, and cysteine inhibit arginase in bovine rumen tissue [21].

Several studies revealed that ornithine and lysine inhibit arginase in mammary gland [31] and in bovine kidney tissue [32].

Fuentes JM *et al.*[33] stated that lysine, ornithine, valine strongly and competitively inhibit arginase in rat mammary gland; while proline, isoleucine and leucine cause less inhibition.

It was seen that lysine inhibits arginase in rat liver mitochondria [34]. It was stated that lysine does the best inhibition of arginase among all amino acids and that it inhibits the enzyme in a mixed way [24].

Also, Subrahmanyam and Reddy [35] proved that lysine is a strong inhibitor of the enzyme in bovine liver and that it inhibits the enzyme competitively.

Levillain *et al.*[36] investigated inhibitory effects of lysine, proline, ornithine and glutamine in different parts of kidney tubules of ducks and stated that the most powerful inhibitor of arginase in kidney tubules is lysine and that ornithine and glutamine come after lysine, while proline had no statistically significant inhibitory power.

In studies on human erythrocyte arginase, it was stated that lysine is a competitive inhibitor and canavine is a non-competitive inhibitor [37].

In an other study [38], 0.5 mmol/kg of L-lysine was injected to children between 6 to 14 years of age; and then, increases in plasma ornithine and arginine levels and urine ammonia concentration were detected. This case was explained with the inhibition of the enzyme arginase. It was suggested that this increase in plasma ornithine was caused by inhibition of mitochondrial ornithine transport. The study concluded that lysine may inhibit both ornithine transcarbamylase activity and mitochondrial ornithine uptake, preventing the conversion of ornithine to citrulline.

In this study, inhibitory effect of L-lysine as an L-amino acid on sheep spleen tissue arginase activity was investigated and it was found that L-lysine inhibits the enzyme. Inhibition type was assessed with Michaelis-Menten and Lineweaver-Burk curves and non-competitive inhibition of the enzyme was observed.

As a substrate for purified human liver arginase, L-canavanine was used instead of L-arginine and an increase in Km from 10.5 mM to 50 mM was observed by Berüter *et al.*[39].

Non-competitive inhibition of liver arginase by canavanine was found; however, it was found that canavanine did not affect arginase in erythrocyte and uterus and it was reported that canavanine can not be used as a substrate instead of arginine [40].

In another study, Muszynska and Wojtczak [30] stated that they could not detect bovine liver arginase activity when canavanine was used, but they detected a low level of arginase activity in chicken liver.

In this study, effect of L-canavanine, as one of the guanidino compounds, on sheep spleen tissue arginase activity was investigated and non-competitive inhibition of the enzyme by L-canavanine was found.

It was suggested that due to the structural similarity between canavanine and arginase, it binds to a non-active part of the enzyme and changes its three-dimensional structure [21].

It is observed that arginase enzyme level varies with respect to nutrition and hormone level. It is reported that arginase enzyme level also varies due to protein intake diet and this variation is not enzyme kinetics, it is the increase in the amount of the enzyme molecules [20].

Studies show that, urea cycle enzymes decrease in low protein diet. When more proteins are added to the diet, it is observed that the urea cycle enzyme activities increase compared to those in low protein diet [22]. In this study, effect of L-lysine aminoacid and L-canavanine on arginase enzyme, which has a prominent role in urea cycle is studied.

To the knowledge of authors no study on kinetic properties of arginase enzyme in sheep spleen tissue has been conducted. We hope that this new data will help for future studies on this issue.

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RESEARCH ARTICLE

Anadolu Mandalarında Farklı Laktasyon Eğrisi Modellerinin Karşılaştırılması

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Özet

Bu araştırmada, farklı işletme koşullarında 2011-2013 yılları arasında yetiştirilen Anadolu mandalarına ait kontrol günü süt verim kayıtları kullanılarak sekiz farklı laktasyon eğrisi modeli karşılaştırılmıştır. Bu amaçla, laktasyon eğrisinin tanımlanmasında Wood, Cobby ve Le Du, Üssel, Parabolik Üssel, Kuadratik, Ters Polinomiyal, Logaritmik Kuadratik, Logaritmik Linear modelleri kullanılmıştır. Laktasyon eğrisini en iyi tanımlayan modeli belirlemek için belirtme (R^2) ve kalıntı standart sapma (KSS) katsayıları kriter olarak kullanılmıştır. En yüksek R^2 ve en düşük KSS değerlerini veren Logaritmik Kuadratik ve Kuadratik modellerin en iyi uyumu gösteren modeller olduğu belirlenmiştir. Sonuç olarak, Logaritmik Kuadratik veya Kuadratik modeller ile tahmin edilen parametrelerin ıslah çalışmalarında kullanılması, bu yönde yapılacak araştırmalara önemli katkı sağlayacaktır.

Anahtar sözcükler: *Laktasyon eğrileri, Anadolu mandası, Belirtme katsayısı, Kalıntı standart sapma katsayısı*

Comparison of Different Lactation Curve Models of Anatolian Buffaloes

Abstract

In this study, eight different lactation curve models were compared by using test day milk yield records belonging Anatolian Buffaloes raised in different Farm conditions between 2011 and 2013. To identify the best lactation curve models of Wood, Cobby and Le Du, Logaritmic Quadratic, Exponential, Parabolic exponential, Quadratic, Inverse Polynomial and Logaritmic Linear mathematical functions were used. The coefficient of determination (R^2) and residual standard deviation (RSD) statistics were used for determination of best fitted model in lactation curve. Logaritmic Quadratic and Quadratic functions are the best goodness of fit model as having the highest R^2 and lowest RSD coefficients. As a result, the parameters are estimated by logarithmic quadratic or quadratic models, for use in breeding programs will make an important contribution to research in this field.

Keywords: *Lactation curves, Anatolian Buffalo, Coefficient of determination and residual standard deviation*

GİRİŞ

Süt verimi hayvan ıslahı çalışmalarında üzerinde durulan ekonomik öneme sahip özellikler arasında yer almaktadır. Anadolu mandalarının süt verimleri 813 kg [1], 943.2 kg [2] ve 925 kg [3] olarak tespit edilmiştir. Süt verimi ile laktasyon süresi arasında sıkı bir ilişkinin olduğu birçok araştırıcı tarafından bildirilmektedir [3-6]. Laktasyon süt verimi ile yakından ilgili laktasyon süresi ortalaması Afyon Mandacılık Araştırma Enstitüsü'nde yerli mandalarda 220 gün, melezlerde 225 gün olarak saptanmıştır [7]. İlaslan ve ark.[8] mandalarda ortalama laktasyon süresini 224 gün olarak belirlemişlerdir. Doğum ile kuru dönem arasında geçen bir süreç olan, laktasyon süresi ırk ve sürüler arasında varyasyon göstermektedir. Söz konusu varyasyon genetik yapı ve çevresel faktörlerden kaynaklanmakta olup, çev-

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resel faktörlerin varyasyonun ortaya çıkmasındaki rolü genetik faktörlerden daha fazladır. Laktasyon eğrisi, doğum sonrasında zamanla süt veriminde gözlemlenen değişim olarak ifade edilmektedir. Süt hayvanlarının verim değerlendirilmesinde laktasyon eğrisinin şekli süt verimi ile birlikte değerlendirilen bir kriterdir [9]. Malaklama ile başlayan süt verimi doğum sonrasında maksimum süt üretim seviyesine ulaşılana kadar artar ve sonrasında kuru döneme kadar azalma trendine girer. Laktasyon eğrilerinden, günlük süt verim kayıtları ile laktasyon süt veriminin tahmini, laktasyon süreleri eşit olmayan mandaların karşılaştırılması, süt verimi düşük olan mandaların laktasyonun erken dönemlerinde ayıklanması, sürü idare ve optimum yemleme programlarının planlanmasında yararlanılabilmektedir [10]. Günümüze kadar laktasyon eğrilerinin tanımlanmasında Wood, Dhanoa, Wilmink, Cobby ve Le Du, Dave ve Ters Polinomiyal gibi matematiksel modeller yaygın olarak kullanılmıştır. Süt sığırı yetiştiriciliğinde laktasyon eğrisi ile ilgili birçok araştırma yürütülmüştür. Ancak mandalarda bu konu ile ilgili sınırlı sayıda araştırmaya rastlanılmıştır. Bu konu ile ilgili Türkiye'de [10,11] İtalya'da [12] ve Pakistan'da [13] bazı araştırmalar yapılmıştır.

Bu araştırmada, Anadolu mandalarında laktasyon eğrilerini tanımlamada kullanılan modeller karşılaştırarak, laktasyon eğrisini açıklayabilecek en iyi matematiksel modelin belirlenmesi amaçlanmıştır.

MATERYAL ve METOT

Materyal

Bu araştırmada, Anadolu mandalarının laktasyon eğrisinin tanımlanmasında Wood, Cobby ve Le Du, Üssel, Parabolik üssel, Kuadratik, Ters Polinomiyal, Logaritmik Kuadratik, Logaritmik Linear modeller olmak üzere toplam sekiz farklı model kullanılmıştır. Araştırma materyalini Tokat ili ve ilçelerinde 2011-2013 yılları arasında doğuran Anadolu mandalarına ait kontrol günü süt verim kayıtları oluşturmuştur. Bu araştırma kapsamında 536 adet 1. laktasyon, 489 adet 2. laktasyon, 436 adet 3. laktasyon, 345 adet 4. laktasyon 248 adet 5. laktasyon olmak üzere toplamda 2054 adet kontrol günü süt verim kaydı değerlendirilmiştir. Anadolu mandalarının süt verimleri Tarımsal Araştırmalar ve Politikalar Genel Müdürlüğü tarafından desteklenen Halk Elinde Manda Islahı Ülkesel projesi kapsamında elde edilmiştir. Kontrol günlerinde mandaların sabah ve akşam kilogram olarak tespit edilen süt verimleri kayıt altına alınmıştır. Araştırmanın yapıldığı bölgede manda yetiştiriciliği ekstansif koşullarda yapılmaktadır. Yetiştiriciler özellikle mera döneminde mandalara genellikle ek yemleme uygulamamakta, ancak kış aylarında elde mevcut yemlere göre (saman, kuru yonca otu, silaj vb.) ek yemleme yapmaktadırlar. Bölgede mevsim şartlarının otlatma için uygun olduğu günlerde mandalar sabah sağımından sonra meraya çıkartılmaktadır. Yetiştiricilerin çoğunluğu sağımı el ile yapmaktadırlar. Laktasyon eğrisinin tanımlanmasında en az ilk 5 kontrol verimi bilinen mandalara ait günlük süt verimleri değerlendirilmiştir [14,15].

Metot

Anadolu mandalarının laktasyon eğrilerinin belirlenmesinde Wood, Cobby ve Le Du, Üssel, Parabolik Üssel, Kuadratik, Ters Polinomiyal, Logaritmik Kuadratik, Logaritmik Linear olmak üzere sekiz farklı model kullanılmıştır. Bu çalışmada laktasyon eğrisini tanımlamada yararlanılan farklı laktasyon eğrisi modelleri *Tablo 1*'de verilmiştir. Anadolu mandalarının aylık süt kontrollerinden yararlanılarak her bir laktasyon için farklı matematik modeller ile laktasyon eğrisi modelleri tahmin edilerek, süt verimlerine ait en uygun laktasyon eğrisi belirlenmiştir.

Laktasyon eğrisi parametreleri *(a, b, c ve d)* Statistica 5.0. V [16] paket programı kullanılarak, Levenberg Marquardt iterasyon işlemi sonucu tespit edilmiştir. İterasyon yapılırken, yakınsama kriteri olarak 1.0E-8 kullanılmıştır. Araştırmada kullanılan sekiz farklı model ile belirlenen laktasyon eğrisi parametrelerinin laktasyon sırasına göre değişip değişmediğini tespit etmek amacı ile kontrol günü süt verimleri laktasyon sırasına göre gruplandırılmış 1., 2., 3., 4. ve 5. laktasyon kayıtları ayrı ayrı değerlendirilmiştir. Eşitliklerde; *Yt:* laktasyonun *t.* günündeki süt verimini (kg), *t:* malaklamadan günlük verimin (kontrol günü verimi) ölçüldüğü güne kadar geçen süreyi (gün), *e:* tabii logaritma tabanını, *exp:* matematiksel üs fonksiyonunu *a, b, c, d:* laktasyon eğrisine ait parametre tahminleri olmak üzere; *a:* eğrinin Y eksenini kestiği noktayı, *b:* laktasyonun başlangıcında eğrinin yükselmesini, *c* ve *d:* en yüksek düzeye eriştikten sonra eğrinin düşüşünü gösteren katsayıdır. Araştırmada 1., 2., 3., 4. ve 5. laktasyonlar laktasyon sırasına göre analiz edilmiştir. Laktasyon eğrilerinin uygunluğunu karşılaştırmada, belirtme katsayısı ve kalıntı standart sapma (KSS) katsayılarından faydalanılmıştır.

Tablo 1. *Laktasyon eğrilerinin tahmininde kullanılan modeller*
Table 1. *Models used to fit the lactation curve*

Modeller		**Eşitlikler**
Wood	(WD)	$Y_t = at^b \exp(-ct)$
Ters Polinomiyal	(TP)	$Y_t = t / (a + bt + ct^2)$
Cobby ve Le Du	(CD)	$Y_t = a - bt - a \exp(-ct)$
Üssel	(Ü)	$Y_t = a \exp(-ct)$
Parabolik Üssel	(PÜ)	$Y_t = a \exp(-bt + ct^2)$
Kuadratik	(K)	$Y_t = a + bt + ct^2$
Logaritmik Kuadratik	(LK)	$Y_t = a + bt + ct^2 + d \log_e (t^2)$
Logaritmik Linear	(LL)	$Y_t = a + bt + c \log_e (t)$

Y: *Kontrol günü tahmini süt verimi;* ***t:*** *Kontrol aralığı (gün);* ***a,b,c,d:*** *Modellerdeki katsayılar;* ***exp:*** *matematiksel üs fonksiyonu*

BULGULAR

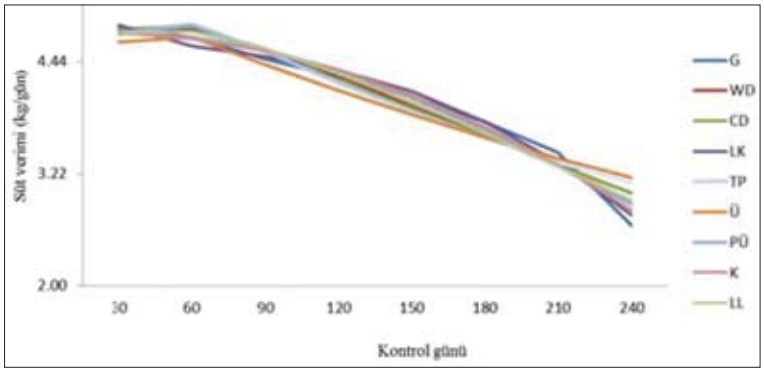
Belirtme (R^2) ve kalıntı standart sapma (KSS) katsayılarından, laktasyon eğrisini en iyi tanımlayan matematik modeli tespit etmede kriter olarak yararlanılmıştır. Araştırma sonucunda tüm laktasyon ve modellerde en yüksek R^2 ve en düşük KSS değerlerini veren Logaritmik Kuadratik ve Kuadratik modellerin en iyi uyumu gösteren modeller olduğu tespit edilmiştir. Tüm laktasyon eğrileri ile elde edilen parametreler, belirtme katsayıları (R^2) ve kalıntı standart sapma (KSS) katsayıları *Tablo 1*'de özetlenmiştir. Ayrıca, gerçek ve tahmin edilen süt verimleri ile ilgili laktasyon eğrileri ilgili şekillerde (*Şekil 1, 2, 3, 4, 5* ve *6*) verilmiştir. Bu farklılıklar dikkate alınarak, bu araştırmada beş farklı laktasyon sırası grubu için bu araştırmada incelenen sekiz farklı matematik model kontrol günü süt verimlerine uygulanarak laktasyon eğrisi parametreleri tespit edilmiştir. Farklı laktasyon eğrileri için parametre ortalamaları, standart hataları, belirtme ve kalıntı standart sapma katsayıları *Tablo 2*'de verilmiştir.

TARTIŞMA ve SONUÇ

Bu araştırmada, belirtme katsayısı [12,14,17] ve kalıntı standart sapma katsayıları [18-21] kullanılarak laktasyon süt verimlerine en iyi uyum gösteren matematik model belirlenmiştir. Anadolu mandalarının kontrol günü süt verim kayıtları kullanılarak, laktasyon eğrilerinin şekli ve bu eğrileri açıklamada gerekli olan parametreler sekiz farklı model ile tahmin edilmiş ve bu fonksiyonlardan faydalanılarak elde edilen laktasyon eğrileri karşılaştırılmıştır.

Laktasyon Eğrisi Parametreleri ve Model Uyumları

Wood modeli için farklı laktasyonlarda belirlenen laktasyonun başlangıcındaki eğrinin Y eksenini kestiği noktayı belirten a parametresi 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 5.07, 4.46, 5.74, 6.67, 5.61 ve 5.34 olarak belirlenmiştir. a parametresi en düşük değerini ikinci laktasyonda, en yüksek değerini ise dördüncü laktasyonda almıştır. Bu araştırmada Wood modeli ile belirlenen a

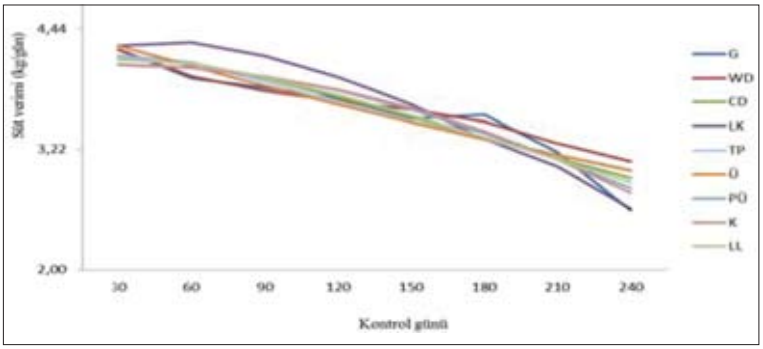


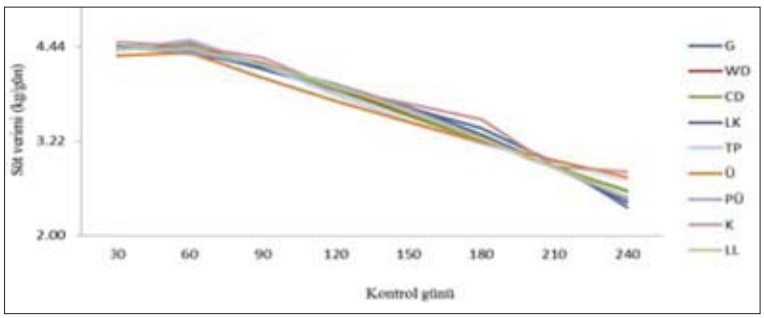
Şekil 1. Kontrol günlerine göre laktasyon eğrileri (tüm laktasyonlar)

Fig 1. Lactation curves according to test days (all lactations)

Şekil 2. Kontrol günlerine göre laktasyon eğrileri (birinci laktasyon)

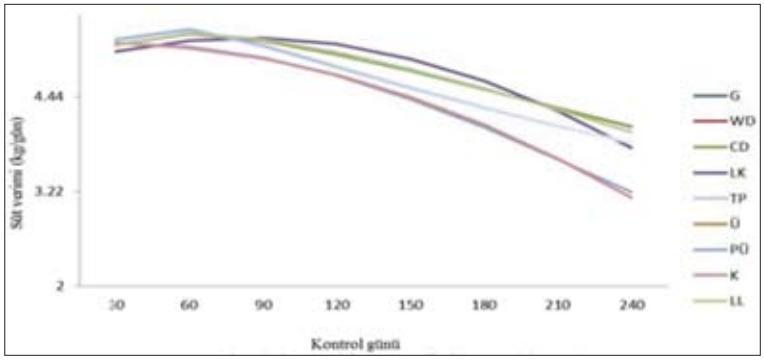
Fig 2. Lactation curves according to test days (first lactation)





Şekil 3. Kontrol günlerine göre laktasyon eğrileri (ikinci laktasyon)

Fig 3. Lactation curves according to test days (second lactation)

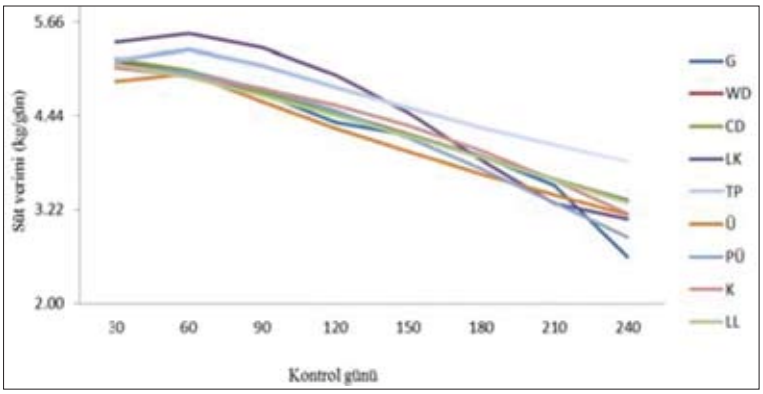


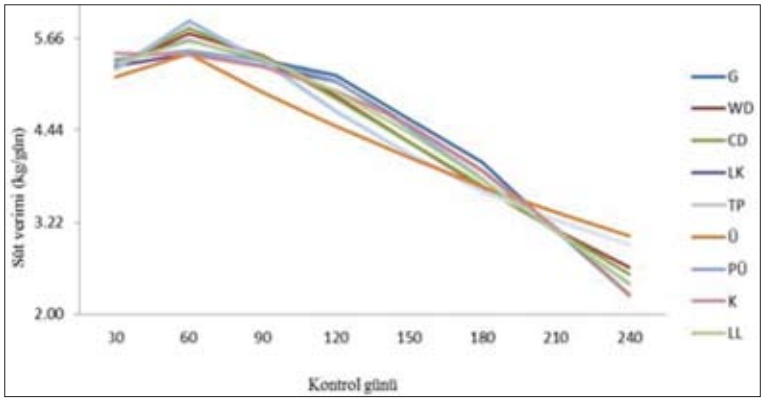
Şekil 4. Kontrol günlerine göre laktasyon eğrileri (üçüncü laktasyon)

Fig 4. Lactation curves according to test days (third lactation)

Şekil 5. Kontrol günlerine göre laktasyon eğrileri (dördüncü laktasyon)

Fig 5. Lactation curves according to test days (fourth lactation)





Şekil 6. Kontrol günlerine göre laktasyon eğrileri (beşinci laktasyon)

Fig 6. Lactation curves according to test days (fifth lactation)

parametresi Anwar ve ark.[13] Pakistan'da Nili Rawi ırkı mandalar, Barbosa ve ark.[12] Brezilya'da Murrah, Akdeniz ve Jafarabadi ırkı mandalar ve Keskin ve ark.[22] Türkiye'de Siyah Alacalar için belirlediği değerden düşük bulunmuştur. Araştırma bulgusunun Orman ve ark.[23] ilk beş laktasyon için belirlediği, (4.993, 7.66, 9.38, 7.99 ve 10.76) değerler ile uyumlu olduğu belirlenmiştir. Araştırma bulgusu yerli mandalarda yapılan bir araştırmada [11] belirlenen değerden yüksek, Jersey sığırlarda ilk yedi laktasyon için hesaplanan (13.13, 14.99, 16.37, 16.93, 16.89, 17.31, 16.62) değerlerden düşük bulunmuştur [24]. Wood modeli kullanılarak 1., 2., 3., 4., 5., 6., 7., 8., 9. ve 10. laktasyonların da olan mandalarda [25] a parametresinin 29.92-49.23 arasında değiştiği tespit edilmiştir. İtalya'da yapılan bir araştırmada ise [26] farklı yaş gruplarına ayrılan mandalara a parametresinin 0.037-0.05 arasında değiştiği belirlenmiştir. Yükselme hızını ifade eden b parametresi, 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 0.22, 0.09, 0.13, 0.45, 0.10 ve 0.19 olarak tespit edilmiştir. Bu değerler Kaygısız'ın [11] Türkiye'de yerli mandalarda ilk beş laktasyon için tespit ettiği değerlerden düşük, Aziz ve ark.'nın [25] ilk on laktasyon için belirledikleri değerler ile genel olarak uyumlu bulunmuştur. Pakistan'da Nili Rawi ırkı mandalar üzerinde yapılan bir çalışmada [13] b parametresi 0.341 olarak saptanmıştır. Ayrıca, Siyah Alaca sığırlarda yapılan bir çalışmada b parametresinin [17] 0.47 olduğu bildirilmiştir. Araştırma bulgusu Keskin ve ark.'nın [17] Siyah Alacalar için (0.47) belirlediği değerden düşük, Orman ve ark.'nın [23] Siyah Alaca sığırların ilk beş laktasyonu için, Çankaya ve ark.'nın [24] Jersey ırkı sığırların ilk yedi laktasyonu için tespit ettikleri değerler ile genel olarak uyumlu bulunmuştur. İtalya'da mandalar üzerinde yapılan bir çalışmada [26] b parametresinin 0.255-0.368 arasında değiştiği belirlenmiştir. Düşüş hızını ifade eden c parametresinin ise 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 0.14, 0.07, 0.10, 0.23, 0.09 ve 0.12 olduğu

Tablo 2. *Farklı laktasyon eğrileri için parametre ortalamaları, standart hata, R^2 ve KSS katsayıları*
Table 2. *Mean values (a, b, c and d), standard error, $R^2$and RSD of lactation parameters for different lactation curves*

Laktasyon	Modeller	a	s_x	b	s_x	c	s_x	d	s_x	R^2	KSS
Tüm laktasyonlar	WD	5.34	0.206	0.19	0.096	0.12	0.029	-	-	0.932	0.046
	CD	5.47	0.330	0.30	0.057	2.68	1.348	-	-	0.931	0.061
	LK	4.30	0.413	0.60	0.047	-0.06	0.030	-0.45	0.025	0.994	0.018
	Ü	5.36	0.244	-	-	0.06	0.010	-	-	0.877	0.070
	PÜ	4.68	0.245	-0.02	0.028	-0.01	0.003	-	-	0.959	0.027
	K	4.74	0.198	0.04	0.010	-0.03	0.012	-	-	0.970	0.020
	TP	0.05	0.032	0.13	0.030	0.02	0.004	-	-	0.898	0.070
	LL	5.21	0.146	-0.46	0.099	0.69	0.348	-	-	0.951	0.034
Birinci laktasyon	WD	5.07	0.136	0.22	0.067	0.14	0.021	-	-	0.974	0.018
	CD	5.18	0.228	0.32	0.039	2.51	0.841	-	-	0.975	0.018
	LK	4.40	0.207	0.10	0.023	-0.03	0.015	-0.06	0.013	0.994	0.004
	Ü	5.07	0.021	-	-	0.07	0.009	-	-	0.916	0.051
	PÜ	4.40	0.141	-0.018	0.017	-0.01	0.002	-	-	0.989	0.007
	K	4.49	0.087	-0.01	0.044	-0.03	0.004	-	-	0.994	0.003
	TP	0.07	0.029	0.12	0.028	0.02	0.004	-	-	0.944	0.040
	LL	4.89	0.077	-0.47	0.052	0.68	0.182	-	-	0.98	0.009
İkinci laktasyon	WD	4.46	0.245	0.09	0.134	0.07	0.040	-	-	0.719	0.072
	CD	4.45	0.277	0.18	0.051	4.61	1.013	-	-	0.654	0.072
	LK	3.09	0.543	1.26	0.625	-1.10	0.040	-0.98	0.493	0.928	0.032
	Ü	4.48	0.231	-	-	0.04	0.011	-	-	0.665	0.067
	PÜ	4.04	0.652	-0.01	0.084	-0.07	0.009	-	-	0.841	0.057
	K	4.05	0.314	0.04	0.160	-0.02	0.017	-	-	0.926	0.051
	TP	0.02	0.004	0.19	0.044	0.01	0.006	-	-	0.766	0.084
	LL	4.40	0.204	-0.28	0.139	0.35	0.484	-	-	0.816	0.065
Üçüncü laktasyon	WD	5.74	0.479	0.13	0.207	0.10	0.063	-	-	0.722	0.259
	CD	5.73	0.585	0.29	0.106	3.74	1.382	-	-	0.725	0.389
	LK	3.28	0.593	2.17	0.683	-0.17	0.044	-1.50	0.538	0.955	0.038
	Ü	5.76	0.445	-	-	0.06	0.017	-	-	0.697	0.235
	PÜ	5.06	0.778	-0.02	-0.008	0.09	0.009	-	-	0.759	0.224
	K	5.12	0.646	0.04	0.032	-0.03	0.003	-	-	0.930	0.021
	TP	0.03	0.004	0.14	0.041	0.01	0.006	-	-	0.670	0.224
	LL	5.49	0.475	-0.39	0.323	0.79	0.126	-	-	0.455	0.356
Dördüncü laktasyon	WD	6.67	0.306	0.45	0.116	0.23	0.370	-	-	0.959	0.080
	CD	7.21	0.585	0.58	0.059	1.68	0.277	-	-	0.966	0.066
	LK	5.66	0.573	-0.22	0.660	-0.04	0.022	0.34	0.052	0.985	0.035
	Ü	6.61	0.522	-	-	0.09	0.018	-	-	0.826	0.285
	PÜ	5.04	0.318	-0.08	0.035	-0.02	0.004	-	-	0.978	0.042
	K	5.32	0.248	0.20	0.126	-0.07	0.013	-	-	0.983	0.031
	TP	0.11	0.045	0.04	0.031	0.03	0.007	-	-	0.907	0.181
	LL	6.28	0.153	-0.94	0.104	0.078	0.036	-	-	0.881	0.037
Beşinci laktasyon	WD	5.61	0.503	0.10	0.220	0.09	0.670	-	-	0.680	0.288
	CD	5.60	0.608	0.28	0.110	3.64	5.84	-	-	0.687	0.282
	LK	3.82	1.631	1.57	0.878	-0.12	0.121	-1.31	0.480	0.735	0.028
	Ü	5.62	0.045	-	-	0.06	0.018	-	-	0.665	0.251
	PÜ	5.12	1.095	0.01	0.001	-0.07	0.012	-	-	0.700	0.271
	K	5.11	0.709	-0.01	0.003	0.02	0.003	-	-	0.713	0.058
	TP	0.01	0.005	0.16	0.058	0.01	0.009	-	-	0.637	0.224
	LL	5.43	0.324	0.43	0.292	0.22	0.019	-	-	0.664	0.291

WD: *Wood,* ***CD:*** *Cobby ve Le Du,* ***LK:*** *Logaritmik Kuadratik,* ***TP:*** *Ters Polinomiyal,* ***Ü:*** *Üssel,* ***PÜ:*** *Parabolik Üssel,* ***K:*** *Kuadratik,* ***LL:*** *Logaritmik Linear,* ***G:*** *Gerçek*

saptanmıştır. Bu parametre Kaygısız'ın [11] ilk altı laktasyon için, Aziz ve ark.'nın [25] ilk on laktasyon için belirledikleri değerler ile uyumlu bulunmuştur. Söz konusu parametre Pakistan'da yetiştirilen Nili Rawi ırkı mandalar için Anwar ve ark.[13] tarafından 0.038 olarak tespit edilmiştir. Benzer şekilde Siyah Alaca sığırlar üzerinde yapılan bir çalışmada da bu parametre 0.17 olarak tespit edilmiştir [22]. Araştırma bulgusu Jersey sığırlarda ilk yedi laktasyon için [24] belirlenen değerler ile genel olarak uyumlu bulunmuştur. İtalya'da mandalarda yapılan bir çalışmada [26] c parametresinin 0.005 ve 0.006 olduğu bildirilmiştir. Modellerin uyumları değerlendirildiğinde; Wood modeli ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için belirtme katsayısı ve kalıntı standart sapma katsayıları sırası ile, 0.974, 0.004; 0.719, 0.072; 0.959, 0.080; 0.680, 0.288; 0.932 ve 0.046 olarak saptanmıştır. Araştırma bulgusu Aziz ve ark.'nın [25] 1. Laktasyon için belirledikleri değerden yüksek, 2., 3., 4. ve 5 laktasyonlar için saptadığı değerlerden düşük bulunmuştur. Cobby ve Le Du modeli kullanılarak 1., 2., 3., 4., 5. ve tüm laktasyonlar için a parametresinin sırası ile, 5.18, 4.45, 5.73, 7.21, 5.60 ve 5.47 olduğu tespit edilmiştir. Bu araştırmada Cobby ve Le Du modeli ile belirlenen a parametresi Keskin ve ark.'nın [17] ilk laktasyonunda olan Siyah Alaca sığırlar için belirlediği değerden (0.25) düşük bulunmuştur. b parametresi, 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 0.32, 0.18, 0.29, 0.58, 0.28 ve 0.30 olarak belirlenmiştir. Bu parametre Keskin ve ark.'nın [17] ilk laktasyonunda olan Siyah Alaca sığırlar için belirlediği değerden (0.032) yüksek bulunmuştur. Düşüş hızını ifade eden c parametresinin ise 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 2.51, 4.61, 3.74, 1.68, 3.64 ve 2.68 olduğu tespit edilmiştir. Bu değerler Keskin ve ark.'nın [17] ilk laktasyonunda olan Siyah Alaca sığırlar için belirlediği değerden (18.37) düşük bulunmuştur. Cobby ve Le Du modeli için ilk beş ve tüm laktasyonlar belirtme katsayısı ve kalıntı standart sapma katsayıları sırası ile 0.975, 0.018; 0.654, 0.072; 0.725, 0.389; 0.966, 0.066; 0.687, 0.282 ve 0.931, 0.061 olarak tespit edilmiştir. Gerçek ve tahmin edilen laktasyon eğrileri, tüm laktasyonlar için *(Şekil 1)* birinci *(Şekil 2)*, ikinci *(Şekil 3)*, üçüncü *(Şekil 4)*, dördüncü *(Şekil 5)*, beşinci *(Şekil 6)*'de sunulmuştur.

Laktasyonun başlangıcındaki süt verimini belirten a parametresinin Logaritmik Kuadratik model kullanıldığında, 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 4.40, 3.09, 3.28, 5.66, 3.82 ve 4.30 olduğu saptanmıştır. a parametresi en düşük değerini ikinci laktasyonda, en yüksek değerini ise dördüncü laktasyonda almıştır. Gürcan ve ark.[10] tarafından yapılan bir çalışmada, ikinci laktasyonun da olan mandalar için a parametresi 3.48 olarak saptanmıştır. Araştırma bulgusu bu bildirişle benzerlik göstermektedir. Aynı model ile yükselme hızını ifade eden b parametresi 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 0.10, 1.26, 2.17, -0.22, 1.57 ve 0.60 olarak belirlenmiştir.

İkinci laktasyonunda olan Anadolu mandalarının süt verimlerinin incelendiği bir çalışmada [10] b parametresinin -0.03 olduğu tespit edilmiştir. Araştırma bulgusu bu bildirişten yüksek bulunmuştur. Düşüş hızını ifade eden c parametresi Logaritmik Kuadratik model ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, -0.03, -1.10, -0.17, -0.04, -0.12 ve -0.06 olarak belirlenmiştir. Belirlenen c parametresi Gürcan ve ark.'ın [10] ikinci laktasyon da olan mandalar için belirledikleri değere yakın bulunmuştur. 1., 2., 3., 4., 5. ve tüm laktasyonlar belirtme katsayısı ve kalıntı standart sapma katsayıları Logaritmik Kuadratik model ile 0.994, 0.004; 0.928, 0.032; 0.955, 0.038; 0.985, 0.035; 0.735, 0.028 ve 0.994, 0.018 olarak belirlenmiştir.

Eğrinin Y eksenini kestiği noktayı ifade eden a parametresi Üssel model ile, 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 5.07, 4.48, 5.76, 6.61, 5.62 ve 5.36 olarak tespit edilmiştir. a parametresi en düşük değerini ikinci laktasyonda, en yüksek değerini ise dördüncü laktasyonda almıştır. Bu model ile belirlenen a parametresi Çankaya ve ark.'nın [24] ilk yedi laktasyon için Jersey sığırlar için belirlediği değerlerden düşük bulunmuştur. Yine bu model kullanılarak düşüş hızını ifade eden c parametresi 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 0.07, 0.04, 0.06, 0.09, 0.06 ve 0.06 olarak belirlenmiştir.

Bu parametre Çankaya ve ark.'nın [24] ilk yedi laktasyon için Jersey sığırlar için saptadığı değerler ile uyumlu bulunmuştur. Üssel model ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için belirtme ve kalıntı standart sapma katsayıları sırası ile 0.916, 0.051; 0.665, 0.067; 0.697, 0.235; 0.826, 0.285; 0.665, 0.251 ve 0.877, 0.070 olarak saptanmıştır. Parabolik Üssel model ile laktasyonun başlangıcındaki süt verimini ifade eden a parametresinin, 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 4.40, 4.04, 5.06, 5.04, 5.12 ve 4.68 olduğu belirlenmiştir. a parametresi Çankaya ve ark.'nın [24] ilk yedi laktasyon için Jersey sığırlar için tespit ettiği değerlerden düşük bulunmuştur.

Yükselme hızını ifade eden b parametresi Parabolik Üssel model ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, -0.018, -0.01, -0.02, -0.08, 0.01 ve -0.02 olarak tespit edilmiştir. Araştırma bulgusu Çankaya ve ark.'nın [24] Jersey sığırlarda ilk yedi laktasyon için belirledikleri değerlerden düşük bulunmuştur. Bu model ile c parametresi ise 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, -0.01, -0.07, 0.09, -0.02, -0.07 ve -0.01 olarak saptanmıştır. c parametresi Çankaya ve ark.'nın [24] ilk yedi laktasyon için Jersey sığırlar için saptadığı değerlere yakın bulunmuştur. Parabolik Üssel model ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için belirtme ve kalıntı standart sapma katsayıları sırası ile 0.989, 0.007; 0.841, 0.057; 0.759, 0.224; 0.978, 0.042; 0.700, 0.271 ve 0.959, 0.027 olarak belirlenmiştir.

Kuadratik model kullanıldığında tespit edilen a parametresi 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 4.49, 4.05, 5.12, 5.32, 5.11 ve 4.74 olarak belirlenmiştir. Belirlenen a parametresi Gürcan ve ark.'nın [10] ikinci laktasyonunda olan Anadolu mandaları için belirlediği değerden (6.08) düşük bulunmuştur. Yükselme hızını ifade eden b parametresi 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile,

-0.01, 0.04, 0.04, 0.20, -0.01 ve 0.04 olarak tespit edilmiştir. Bu parametre Gürcan ve ark.'nın [10] ilk laktasyonunda olan Anadolu mandaları için belirlediği değerden (-0.02) yüksek bulunmuştur.

Düşüş hızını ifade eden c parametresi Kuadratik model ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, -0.03, -0.02, -0.03, -0.07, 0.02 ve -0.03 olarak saptanmıştır. Bu parametre Gürcan ve ark.'nın [10] belirlediği değerle uyumlu bulunmuştur. Kuadratik model ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için belirtme ve kalıntı standart sapma katsayıları sırası ile 0.994, 0.003; 0.926, 0.051; 0.930, 0.214; 0.983, 0.031; 0.713, 0.058 ve 0.970, 0.020 olarak tespit edilmiştir.

Laktasyon eğrisi parametrelerinin tahmininde Ters Polinomiyal modelden yararlanıldığında, laktasyonun başlangıcındaki süt verimini belirten a parametresinin 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 0.07, 0.02, 0.03, 0.11, 0.01 ve 0.05 olduğu belirlenmiştir. Belirlenen a parametresi Gürcan ve ark.'nın [10] ikinci laktasyon da olan mandalar için belirlediği değerden (0.69) düşük bulunmuştur. Aynı model ile yükselme hızını ifade eden b parametresi ilk beş laktasyon ve tüm laktasyonlar için sırası ile, 0.12, 0.19, 0.14, 0.04, 0.16 ve 0.13 olarak tespit edilmiştir. Bu parametre Gürcan ve ark.'nın [10] ikinci laktasyonunda olan mandalar için belirlediği değerden (0.096) yüksek bulunmuştur. Düşüş hızını ifade eden c parametresi ise Ters Polinomiyal model ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 0.02, 0.01, 0.01, 0.03, 0.01 ve 0.02 olarak belirlenmiştir. Bu parametre Keskin ve ark.'nın [17] ilk laktasyonunda olan Siyah Alaca sığırlar (0.0001), Gürcan ve ark.'nın [10] ikinci laktasyonunda olan mandalar için belirlediği değerlerden (0.0015) yüksek bulunmuştur.

Logaritmik linear model için, a parametresi, 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, 4.89, 4.40, 5.49, 6.28, 5.43 ve 5.21 olarak saptanmıştır. Bu parametre Gürcan ve ark.'nın [10] ikinci laktasyonunda olan Anadolu mandaları için belirlediği değerden (8.35) düşük bulunmuştur. b parametresi ise 1., 2., 3., 4., 5. ve tüm laktasyonlar için sırası ile, -0.47, -0.28, -0.39, -0.94, 0.43 ve -0.46 olarak tespit edilmiştir. Bu değerler Gürcan ve ark.'nın [10] ikinci laktasyonunda olan Anadolu mandaları için belirlediği değer ile uyumlu bulunmuştur.

Düşüş hızını ifade eden c parametresinin ise aynı model ile ilk beş laktasyon ve tüm laktasyonlar için sırası ile, 0.68, 0.35, 0.79, 0.078, 0.22 ve 0.69 olduğu belirlenmiştir. Bu parametre Gürcan ve ark.'nın [10] ikinci laktasyonunda olan Anadolu mandaları için belirlediği değerden düşük bulunmuştur. Ters Polinomiyal model ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için belirtme ve kalıntı standart sapma katsayıları sırası ile 0.944, 0.040; 0.766, 0.084; 0.670, 0.224; 0.907, 0.181; 0.637, 0.224 ve 0.898, 0.070 olarak saptanmıştır.

Bu çalışmada 1., 2., 3., 4., 5. ve tüm laktasyonlar için d parametresi sırası ile -0.06, -0.98, -1.5, 0.34, -1.31 ve -0.45 olarak tespit edilmiştir. Araştırma bulgusu, Gürcan ve ark.'nın [10] (0.97) bulgularından düşük bulunmuştur.

Bu araştırmada farklı laktasyon sıralarına göre tespit edilen a parametreleri ile ilgili değerler genel olarak literatürlerde belirtilen değerler ile farklılık göstermiştir. b, c ve d parametrelerine ait değerlerin ise genel olarak literatürler ile uyumlu olduğu belirlenmiştir. Bu durum, araştırma materyalini oluşturan mandaların süt verimleri ile diğer araştırmaların yapıldığı sürülerin süt verimlerinin farklı olmasının bir sonucu olabileceği gibi, denetim aralıklarının (gün, hafta gibi) farklı olmasından kaynaklanmış olabilir. b, c ve d parametrelerinin literatürlerle genel olarak uyumlu olması ise bu çalışmada süt verim kayıtları değerlendirilen Anadolu mandalarının laktasyon eğrilerinin tipik laktasyon eğrisi şeklinde olduğunu ifade etmektedir.

Laktasyon başlangıcındaki süt verimini ifade eden a parametresi ile ilgili en düşük değer Ters Polinomiyal, en yüksek değeri ise Wood, Cobby ve Le Du ve Üssel modeller kullanıldığında tespit edilmiştir. Yükselme hızını ifade eden b parametresi, 1., 2., 3. ve 4. laktasyonlarda Parabolik Üssel ve Logaritmik Linear modeller kullanıldığında negatif olarak tespit edilmiştir. Ayrıca, aynı parametre Kuadratik model ile 1. ve 5. laktasyonlarda da negatif olarak belirlenmiştir. Cobby ve Le Du modeli kullanıldığında düşüş hızını ifade eden c parametresi ile ilgili en yüksek değer tespit edilmiştir. Tüm laktasyonlarda Logaritmik Kuadratik, Parabolik Üssel modeller ile, birinci, ikinci ve dördüncü laktasyonlarda ise Kuadratik model kullanıldığında da b parametresi negatif olarak belirlenmiştir. Logaritmik Linear model ile 1., 2., 3., 4., 5. ve tüm laktasyonlar için belirtme ve kalıntı standart sapma katsayıları sırası ile 0.980, 0.009; 0.816, 0.065; 0.455, 0.356; 0.881, 0.037; 0.664, 0.291 ve 0.951, 0.034 olarak tespit edilmiştir. Bu çalışmada, tüm laktasyon ve modellerde en yüksek R^2 ve en düşük KSS değerler elde edildiği için Logaritmik Kuadratik ve Kuadratik modellerin en iyi uyumu gösteren modeller olduğu tespit edilmiştir. Anadolu Mandalarının kontrol günü süt verim kayıtlarından yararlanarak, laktasyon eğrisine ilişkin parametre tahminlerinin Kuadratik, Logaritmik Linear, Logaritmik Kuadratik, Linear Hiperbolik, Ters Polinomiyal, Wilmink modelleri ile yapıldığı bir çalışmada [10] model uyumlarının karşılaştırılmasında düzeltilmiş belirtme katsayısı (R^2d) kullanılmış ve Logaritmik Kuadratik modelin laktasyon eğrisini tanımlamada kullanılan en uygun model olduğu belirlenmiştir. Murrah ırkı mandalar üzerinde yapılan bir çalışmada da [27] Wood, Multiple Regresyon, Logaritmik Kuadratik ve Linear Hiperbolik modeller karşılaştırılmış ve Logaritmik Kuadratik fonksiyonun en iyi model olduğu belirlenmiştir. Benzer şekilde yetiştirici koşullarındaki mandaların laktasyon verimlerinin değerlendirildiği bir çalışmada [28] en uygun modelin Logaritmik Kuadratik model olduğu tespit edilmiştir.

Hindistan'da yapılan bir çalışmada [29] laktasyon eğrilerinin tanımlanmasında kullanılan Logaritmik Kuadratik modelin, Wood ve Ters Polinomiyal modellerinden daha iyi sonuç verdiği bildirilmiştir. Bu bildirişler araştırma bulgusu ile benzerlik göstermektedir. Araştırma bulgusu ve bu bildirişlerin aksine, Brezilya'da yetiştirilen Murrah, Akdeniz ve Jafarabadi ırkı mandaların süt verimleri kullanılarak sekiz farklı matematik model ile tahmin edilen laktasyon eğrilerinin karşılaştırıldığı bir çalışmada [12] ise seçilen modeller içinde Wood modeli en uygun model olarak tespit edilmiştir. Diğer taraftan Hindistan'da nehir mandalarının süt verimlerinin değerlendirildiği çalışmalarda [29,30] laktasyon eğrilerinin tahmin edilmesinde Kuadratik modelin kullanılmasının uygun olduğu bildirilmiştir. Prasad [31] tarafından geliştirilen modelin Saf ve melez Murrah mandalarının laktasyon eğri şeklini açıklamada çok daha iyi sonuç verdiği bildirilmiştir. Belirtme katsayısı tüm laktasyonlar için değerlendirildiğinde; Logaritmik Kuadratik modelin laktasyon eğrisindeki varyasyonun %99.4'ünü, Kuadratik modelin %97.0'ını açıkladığı belirlenmiştir. Siyah Alaca sığırlarda laktasyon eğrisinin tahminde Wood, Goodall ve Grossman modellerinin kullanıldığı bir çalışmada [9] en uygun model olarak Grossman modeli belirlenmiştir. Orhan ve Kaygısız [21], Wood, Üssel ve Parabolik Üssel fonksiyonların belirtme katsayılarını 0.626, 0.496 ve 0.611 olarak belirlemişlerdir. Söz konusu çalışmada da R^2 yüksek, hata varyansı düşük olduğu için Wood modeli en iyi model olarak seçilmiştir. İtalya'da, Catillo ve ark.[32] farklı yaş gruplarına ayırdıkları mandaların laktasyon kayıtlarını Wood, Ters Polinomiyal, Üssel, Karışık Log, Polinomiyal Regresyon modeli ile incelemişler, bu modellere ait R^2 değerlerini 3 ve daha küçük yaşlı mandalarda sırası ile 0.99, 0.97, 0.98, 0.98, 0.99 olarak tespit etmişlerdir. İtalya'da yapılan bir diğer çalışmada [32] mandalar farklı beş yaş grubuna ayrılmış, yaş gruplarına göre belirtme katsayısının 0.99 olduğu, kalıntı standart sapma katsayılarının ise 0.042-0.054 arasında değiştiği belirlenmiştir. Araştırmacılar bu sonuçlara göre araştırmada incelenen bütün matematiksel fonksiyonların laktasyon eğrisine uyumunun çok yüksek olduğunu bildirmişlerdir. Bu çalışmada belirlenen belirtme katsayısı değerleri genel olarak literatür bildirişleri ile uyumlu bulunmuştur. Model uyumlarını değerlendirmede kriter olarak kullanılan kalıntı standart sapma katsayısı ne kadar küçük olur ise, kullanılan modelin laktasyon süt verimlerine uyumu o kadar yüksek olmaktadır. Kalıntı standart sapma, modelle tahmin edilen verimle, gerçek verim arasındaki sapmanın kullanıldığı bir değerdir [10,24,33]. Bu araştırmada, tüm laktasyonlarda en yüksek R^2 ve en küçük KSS değeri Logaritmik Kuadratik ve Kuadratik model ile tahmin edilmiştir. Bu araştırma bulgusunun aksine [20], Siyah Alaca sığırlarda en düşük KSS değeri laktasyon persistensi modeli kullanıldığında tespit edilmiştir.

Sonuç olarak, Wood, Cobby ve Le Du, Üssel, Parabolik Üssel, Kuadratik, Ters Polinomiyal, Logaritmik Kuadratik, Logaritmik Linear modellerinin uyum ölçütlerine göre Anadolu mandaları için laktasyon eğrisini tanımlayan en uygun modelin Logaritmik Kuadratik ve Kuadratik modeller olduğu tespit edilmiştir. Manda yetiştiriciliğinde, mandalara ait süt ve döl verim özellikleri ile beraber laktasyon eğrisinin şekli ve eğri ile ilgili bazı temel karakteristiklerin belirlenmesi başta sürü yönetimini kolaylaştırabileceği gibi, o sürüde uygulanabilecek seleksiyondaki başarıyı olumlu yönde etkileyecektir. Genetik ve çevresel faktörlerden etkilenebilen laktasyon eğrisi ve bu eğriye ait parametreler eğri tipini belirleyebildikleri gibi mandanın laktasyon verimini de etkileyebilmektedirler. Laktasyon eğrileri, kontrol günü süt verim kayıtlarından yararlanılarak laktasyon süt veriminin tahmin edilmesinde, laktasyon süreleri eşit olmayan mandaların karşılaştırılmasında, süt verimi düşük olan mandaların laktasyonun erken dönemlerinde ayıklanmasında, sürü idaresi ve optimum yemleme programlarının planlanmasında kullanılabilmektedir. Genellikle laktasyonun erken dönemlerinde saptanan başlangıç verimi, malaklama ile ilk kontrol arasında geçen süre gibi laktasyon eğrisi ile ilgili özellikler uygulanacak seleksiyonda kriter olarak kullanılabilmektedir. Mandalara ait bazı seleksiyona esas parametreler daha erken yaşta tespit edilebildiği için verimi düşük olan mandalar daha erken bir sürede ayıklanabilmekte, sürüde generasyonlar arası süre kısalmakta, bu parametreler kullanılarak daha isabetli seleksiyon kararları alınabilmektedir. Anadolu mandalarında süt verimlerinin artırılması yönünde yürütülecek seleksiyon çalışmalarında Logaritmik Kuadratik ve Kuadratik modellerin kullanılması ile laktasyon eğrisinin şekli ve bu eğride bulunan parametreler yardımıyla kullanılan modele bağlı olarak bazı kriterler tespit edilip, yapılacak seleksiyon uygulamalarında daha yüksek isabet sağlanabilecektir.

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RESEARCH ARTICLE

The Effects of Dietary Oil Sources on Performance, Serum Corticosterone Level, Antibody Titers and IFN-γ Gene Expression in Broiler Chickens

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Abstract

The present study was performed to evaluate the effects of addition of fish oil, soybean oil and olive oil to diet on performance and immune response in broiler chickens. In a completely randomized design, 320 broiler chickens (Ross 308, 7 days old) were allocated to four dietary treatments (control and three vegetable oils), four replicates with 20 chicks per each. The results showed that body weight gain of chicks fed soybean oil was higher and feed conversion ratio was better than the other treatments in the total period (P<0.05). The gene expression of γ-interferon (IFN-γ) in spleen tissue was influenced by treatments. The mRNA level of IFN-γ was higher in chicks fed fish oil than the other groups, also antibody titers against Newcastle virus in chicks fed fish oil were higher than the other groups (P<0.05). For antibody titers against sheep red blood cell, there were no differences among treatments (P>0.05). Chicks fed fish oil had higher relative weights of bursa of Fabricius and there were no significant differences in relative weights of spleen among treatments. It was concluded that the source of oils in the diet may be affect the performance and immune response, the addition of fish oil and soybean oil to the diet resulted in significant improvement of performance and immune response in broiler chickens, respectively.

Keywords: *Oil sources, Gene expression, Immune response, Broiler chickens*

Broylerlerde Diyetteki Yağ Kaynaklarının Performans, Serum Kortikosteron Düzeyi, Antikor Titresi ve IFN-γ Gen Ekspresyonu Üzerine Etkileri

Özet

Bu çalışma diyete balık yağı, soya yağı ve zeytinyağı ilavesinin broyler tavuklarda performans ve bağışıklık cevabı üzerine etkisini araştırmak amacıyla yapılmıştır. Tamamıyla rastgele dizaynda 320 broyler tavuk (Ross 308, 7 günlük) her birinde 20 tavuk bulunan ve 4 tekrarlı olmak üzere 4 ayrı diyet grubuna (Kontrol ve üç ayrı yağ uygulaması) ayrıldı. Çalışmanın sonuçları soya yağı ile beslenen civcivlerin diğer gruplar ile karşılaştırıldığında vücut ağırlık artışlarının daha yüksek ve yem dönüşüm oranının tüm periyot içerisinde daha iyi olduğunu gösterdi (P<0.05). Dalakta γ-interferon (IFN-γ) gen ekspresyonu uygulamalar tarafından etkilenmiştir. IFN-γ mRNA düzeyi ile Newcastle virusa karşı antikor titresi balık yağı ile beslenen civcivlerde diğer gruptakilere oranla daha yüksekti (P<0.05). Koyun kırmızı kan hücrelerine karşı antikor titresi bakımından uygulama grupları arasında bir fark gözlemlenmedi (P>0.05). Balık yağı ile beslenen civcivlerin görece bursa Fabricius organ ağırlıkları daha yüksekti ve görece dalak ağırlıkları yönünden uygulama grupları arasında belirgin bir fark tespit edilmedi. Çalışmanın bulguları ışığında kullanılan yağ kaynağının performans ve bağışıklık cevabı üzerine etkisinin olabileceği ve diyete balık yağı ve soya yağı ilavesinin broyler tavuklarda sırasıyla performans ve bağışıklık cevabına önemli etkilerinin olabileceği sonucuna varılmıştır.

Anahtar sözcükler: *Yağ kaynakları, Gen ekspresyonu, Bağışıklık cevabı, Broyler*

INTRODUCTION

Lipids are mainly included in poultry diets as energy and essential fatty acids, which they cannot be synthesized in body tissues. There are evidences that feeding the broilers with diets containing oils, such as fish oil, soybean

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oil and olive oil have a many benefits [1,2]. In this regard, Dewitt et al.[3] reported that addition of fish oil, sunflower oil, and soybean oil improved feed conversion ratio. In another study, feeding the broilers with diets containing fish oil caused poorer feed conversion efficiency than the control [4]. Parmentier et al.[5] who reported that addition of soybean oil (as a source of omega-6 fatty acids) to diet increased antibody production in broilers, but other researchers [6-8] reported a decrease in antibody response against antigens. Another study [9] reported that the addition of soy oil to diet could increase serum corticosterone in broilers, which has been found to be immunosuppressive [10,11]. John et al.[12], Miles et al.[13] and Korver et al.[14] reported that addition of fish oil (as a source of omega-3 fatty acids) to diet could increase production of cytokines such as IFN-γ. In another study, Fritsche et al.[15] reported that spleen IFN- γ mRNA were lower in mice fed an omega-3 fatty acid-enriched diet compared with mice fed diets low in omega-3 fatty acid diet. Reports concerning the effects of oils on performance and immune responses are very contradictious. Moreover, in the literature, there are very few studies concerning the comparison between different sources of oils on antibody titers and gene expression of cytokines, especially IFN-γ. Therefore, this study was designed to evaluate the effects of different sources of oils on performance, serum corticosterone level, antibody titers and IFN-γ gene expression of broilers.

MATERIAL and METHODS

The study was approved by the Ethics Committee of Islamic Azad University, Science and Research Branch of Medical and Veterinary Sciences (approval date: 17.01.2013; no: 1292, AEC 3).

Animals and Diets

Three hundred and twenty broiler chicks (Ross 308, 7 days old) with the same weight (155±3 g) were separated and randomly allocated into four dietary treatments and four replicates in a completely randomized design. Birds were housed in deep litter pens (1×2 m). The relative humidity was controlled at 65% and temperature was set at 32°C on day 1 and lowered gradually to 24°C for the rest of the experiment period. Lighting schedule was 23 h light and 1 h dark. Water and feed were provided *ad libitum*. Birds were fed experimental diets from day 1 until day 42 of age in three periods: The starter (1-7 days), grower (8-28 days) and finisher (29-42 days). Diets were formulated based on the corn-soybean meal *(Table 1)*. Dietary treatments included of: 1) diet without oil as control; 2) diet with fish oil, as source of ω-3; 3) diet with soybean oil, as source of ω-6, and 4) diet with olive oil as source of ω-9. The fatty acids composition of used oils in this study was reported in *Table 2*. To reduce handling and weighting stress, average feed intake, body weight gain and feed conversion ratio of broilers in each pen were only measured at days 7 and 42 of age.

Humeral Immune Response

Blood samples were drawn from vein of two birds in each pen in day 13 of age. The blood samples were poured in tubes that had no anticoagulant and centrifuged at 1.500 × g for 15 min. Sera were collected for analysis and average antibody assessment. The titers of the anti-body against Newcastle disease were determined by hemagglutination inhibition test [16]. At day 27 of age, sheep red blood cell (SRBC) suspension (5% in sterile phosphate buffered saline) was injected in breast muscle of two birds in each pen. Seven days after each sensitization (day 34 of age), antibody titers against SRBC were measured according to Vander Zijpp and Leenstra [17] and expressed as the log 2 of the reciprocal of the highest serum dilution giving complete agglutination.

Quantification of Gene Expression by Real Time PCR

At the end of the period two birds per replicate were randomly selected, individually weighed, and killed by cervical dislocation. Their spleen were removed and immediately stored in liquid nitrogen for messenger RNA (mRNA) extraction. According to the kit Vivantis Company (Malaysia), total RNA was extracted. To convert mRNA into cDNA, Randon hexamer was used as a primer and after attachment of primer to RNA chain by reverse transcriptase cDNA synthesis according to the kit Vivantis Company (Malaysia) was done.

Primer Design and Real Time PCR

To design the primers, related studies [18,19] have reviewed and showed that all the consequences were compared to NCBI data center. Gene expression of this cytokine was analyzed by real time-PCR *(Table 3)*. In order to evaluate the samples by this method regarding the above mentioned Kit, cDNA and master mix for each sample done. The study fulfilled in 10- microliter tubs and beside each sample, a separate sample as β-actin primer prepared and were put in corbette, and according to number of cycles and temperature, diagram were drawn. Real time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). Comparison of each gene expression with its control and stimulated states was determined with the delta-delta (ΔΔ) Ct, in this method a positive result reveals an increase in the expression of the gene of interest in stimulated conditions, whereas a negative result shows a decreased expression [19].

Corticosterone Measurement and Relative Weight of Immune Organs

At the end of the period, two birds per replicate were randomly selected, individually weighed, and their blood poured in tubes with no anticoagulant and were centrifuged at 1500 × g for 15 min, sera was collected for analysis, corticosterone hormone concentration was assessed by ELISA kit (Corticosterone ELISA RE52211, IBL

Table 1. *Ingredients and chemical composition of experimental rations*[1]
Tablo 1. *Deneysel rasyonların içerikleri ve kimyasal kompozisyonları*

Ingredients (as %)	**Starter**		**Grower**		**Finisher**	
Tretments	T_1	T_2, T_3, T_4	T_1	T_2, T_3, T_4	T_1	T_2, T_3, T_4
Corn	54.82	55.45	58.81	55.84	63.6	60.61
Soybean Meal (44%)	35.6	35.69	32.5	33.07	27.7	28.27
Starch	5	0	5	0	5	0
Oil	0	2.15	0	3	0	3
DCP	1.91	1.91	1.61	1.62	1.51	1.52
$CaCO_3$	1.18	1.18	0.96	0.95	0.93	0.92
DL- Methionine	0.29	0.29	0.19	0.2	0.16	0.16
L-Lysine HCl	0.21	0.20	0.04	0.03	0.02	0.01
L-Threonine	0.11	0.11	0.03	0.03	0.01	0.01
Salt	0.27	0.27	0.32	0.33	0.32	0.33
$NaHCO_3$	0.11	0.11	0.04	0.03	0.04	0.03
Zeolite	0	3.14	0	4.4	0.21	4.64
[2]Vitamin Premix	0.25	0.25	0.25	0.25	0.25	0.25
Mineral Premix	0.25	0.25	0.25	0.25	0.25	0.25
Total	100	100	100	100	100	100
Chemical composition						
Energy (kcal/kg)	2840		2890		2940	
Crude Protein (%)	20.57		19.26		17.5	
Methionine (%)	0.59		0.48		0.43	
Met+Cys (%)	0.88		0.76		0.69	
Lysine (%)	1.22		1.06		0.89	
Threonine (%)	0.78		0.66		0.59	
Tryptophan (%)	0.22		0.21		0.19	
Arginine (%)	1.30		1.20		1.1	
Valine (%)	0.90		0.90		0.8	
Isoleusine (%)	0.90		0.88		0.8	
Calcium (%)	0.98		0.82		0.78	
Av. Phos (%)	0.46		0.41		0.39	
Sodium (%)	0.16		0.16		0.16	
Chloride (%)	0.23		0.23		0.23	
Potassium (%)	0.86		0.81		0.73	

1- *T_1: diet without oil, T_2: diet with fish oil, T_3: diet with soybean oil and T_4: diet with olive oil*
2- *Vitamin-mineral premix (each kg contained): calcium, 195 g; potassium, 70 g; sodium,18 g; magnesium, 6 g; zinc, 1.200 mg; iron, 2.000 mg; copper, 400 mg; manganese, 1.200 mg; selenium, 8 mg; cobalt, 20 mg; iodine, 40 mg; vitamin A, 200.000 IU; vitamin D_3, 80.000 IU; vitamin E,1.072 IU; vitamin K_3, 34 mg; ascorbic acid, 1.300 mg; thiamine, 35 mg; riboflavin, 135 mg; niacin, 1.340 mg; vitamin B_6, 100 mg; folic acid, 34 mg; vitamin B_{12}, 670 µg; biotin, 3.350 µg*

Gesellschaft fur Immunchemie und Immunbiologie MBH, Hamburg, Germany). Then, the birds were killed by cervical dislocation, thereafter their bursa of Fabricius, spleen and thymus were removed and their relative weights (organ weight/total weight×100) were calculated.

Statistical Analysis

All values were analyzed by one-way ANOVA using the GLM procedure of SAS software [20]. When the F-test for treatments was significant at P≤0.05 in the ANOVA table, means were compared for significant differences using the Duncan's multiple range tests [21].

RESULTS

Effects on Performance

The effects of different sources of oil on feed intake, body weight gain and feed conversion ratio of the birds are shown in *Table 4*. There were significant effects on

Table 2. *The fatty acids composition of oils (as percent)*
Tablo 2. *Yağların yağ asidi kompozisyonları (yüzde olarak)*

Fatty Acid	Fish Oil	Soybean Oil	Olive Oil
C14:0	1.94	0.48	0.02
C16:0	19.17	10.27	18.91
C18:0	4.82	3.95	4.9
C18:1n-9	22.5	22.73	72.02
C18:2n-6	3.92	56.69	3.13
C18:3n-3	1.37	5.17	0.61
C20:1n-9	2.84	0.71	0.41
C20:3n-6	4.58	-	-
C20:5n-3	13.26	-	-
C22:5n-3	3.7	-	-
C22:6n-3	21.9	-	-

Table 3. *Real-time PCR primers*
Tablo 3. *Real-time PCR primerleri*

Amplified Product		Sequence (5´- 3´)	Base	Tm (°C)	Vol
IFN-γ	F	ACACTGACAAGTCAAAGCCGC	21	61.2	281
	R	AGTCGTTCATCGGGAGCTTG	20	51.27	328
β-Actin	F	CAACACAGTGCTGTCTGGTGGTA	23	60.18	24.0
	R	ATCGTACTCCTGCTTGCTGATTCC	23	60.49	27.0

Table 4. *Effect of different sources of oil on performance of broilers at day 42 of age*
Tablo 4. *42. günde broylerlerin performansı üzerine değişik yağların etkileri*

Treatment	Feed Intake	Gain (g)	FCR
Control	3363	1558^{d}	2.15^{a}
Fish oil	3234	1713^{c}	1.88^{b}
Soybean oil	3327	1888^{a}	1.76^{c}
Olive oil	3343	1858^{b}	1.79^{b}
SEM	46.24	37.33	0.02
P-value	NS	*	*

Value in the same column with no common superscript are differ (P<0.05);
** Significant at P<0.05;* ***NS:*** *Not significant (P>0.05)*

body weight gain and feed conversion ratio among treatments at 42 of age (P<0.05). Body weight gain and feed conversion ratio were affected by the supplemented fat sources (P<0.05). Broilers fed with soybean oil had the highest body weight and the lowest level of feed conversion ratio, and these differences were significant in comparison to the other groups. By contrast there were no feed intake differences among the treatments. Although the highest feed intake observed in the birds fed control, but this difference was not significant among treatments. Also, there were no significant effect on feed conversion ratio between treatments fed fish oil and olive oil (P>0.05). Totally those birds fed soybean oil performed better than the other groups.

Table 5. *Effect of different sources of oil on relative weight of lymphoid organs at day 42 of age*
Tablo 5. *42. günde görece lenfoid organ ağırlıkları üzerine değişik yağların etkileri*

Treatment	Bursa	Thymus	Spleen
Control	0.10^{b}	0.34^{c}	0.10
Fish oil	0.19^{a}	0.52^{b}	0.15
Soybean oil	0.12^{b}	0.61^{b}	0.13
Olive oil	0.12^{b}	0.90^{a}	0.11
SEM	0.01	0.09	0.02
P-value	*	*	NS

Value in the same column with no common superscript are differ (P<0.05);
** Significant at P<0.05;* ***NS:*** *Not significant (P>0.05)*

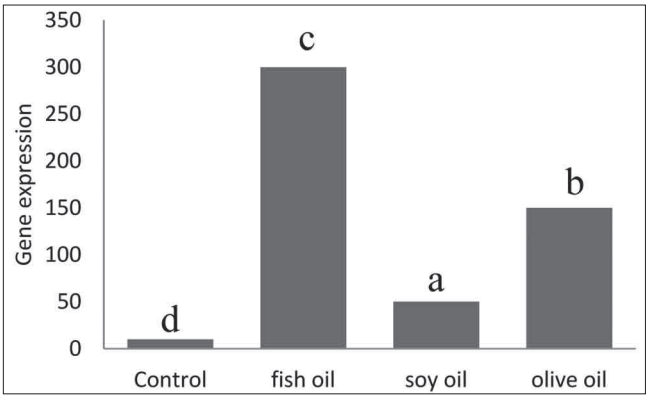

Fig 1. The effects of different sources of oil on IFN-γ gene expression
Şekil 1. IFN-γ gen ekspresyonu üzerine değişik yağların etkileri

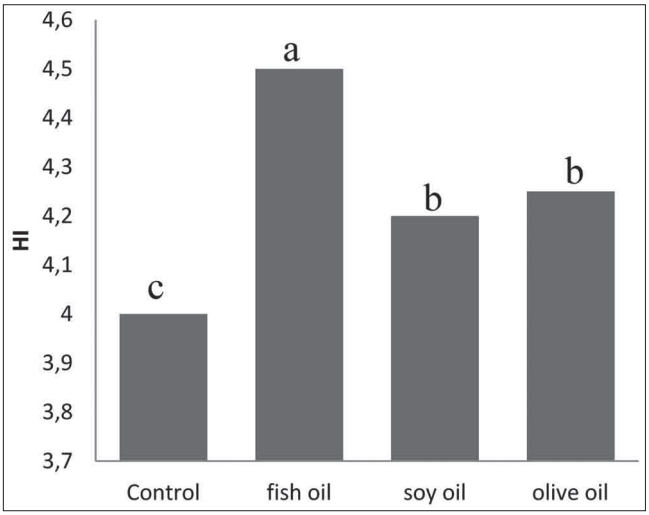

Fig 2. The effects of different sources of oil on antibody titer against hemagglutination inhibition (HI, log 2)
Şekil 2. Hemaglutinasyon inhibisyona (HI, log 2) karşı antikor titresi üzerine değişik yağların etkileri

Analysis of IFN-γ mRNA Expression

The results in *Fig. 1* show IFN-γ mRNA expression. It was influenced by oil sources in spleen tissue (P<0.05). The mean of mRNA levels of IFN-γ in birds fed fish oil increased

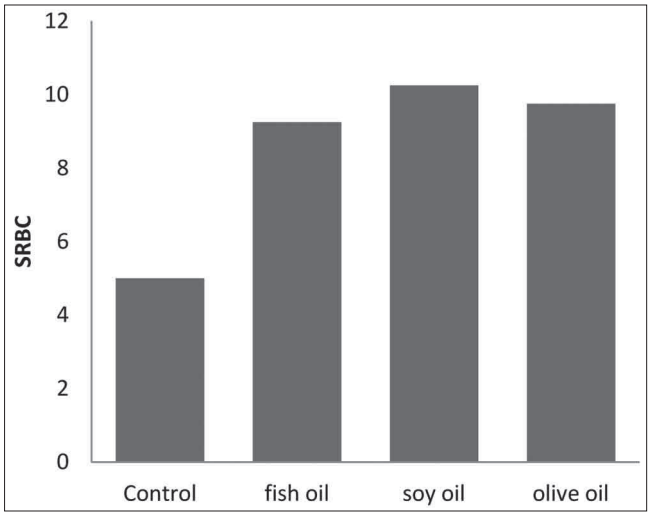

Fig 3. The effects of different sources of oil on antibody titer against sheep red blood cell (SRBC, log 2)

Şekil 3. Koyun kırmızı kan hücrelerine karşı antikor titresi (SRBC, Log 2) üzerine değişik yağların etkileri

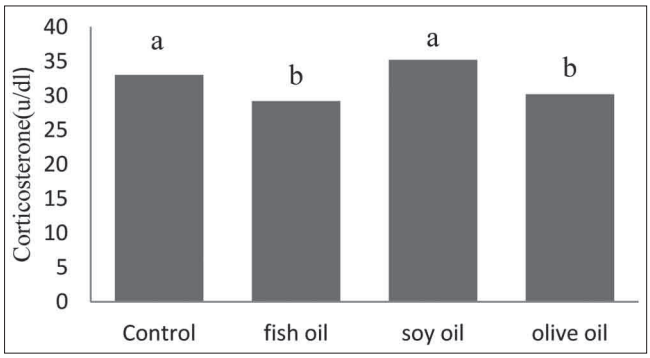

Fig 4. The effects of different sources of oil on corticosterone level

Şekil 4. Kortikostreol düzeyi üzerine değişik yağların etkileri

in comparison to other treatments. The mean of mRNA levels are notably greater in the birds fed olive oil and soybean oil compared to the control diet.

Effects on Antibody Titer Against Newcastle and SRBC

The effects of different sources of oil on antibody titer against HI and SRBC are presented in *Fig. 2* and *Fig. 3*. Antibody titers against Newcastle in chicks fed with fish oil were higher than the other groups (P<0.05). There were no significant effect on antibody titer against Newcastle between treatments fed soybean oil and olive oil (P>0.05). For antibody titers against SRBC, there were no remarkable differences among treatments (P>0.05).

Relative Lymphoid Organs Weight and Corticosterone Concentration

The effects of different sources of oil on relative lymphoid organs weight are shown in *Table 5*. The data indicate that the chicks fed fish and olive oil had the highest relative bursal and thymus weight, respectively (P<0.05). There was no significant effect among control treatment with broilers fed soybean oil and olive oil in the relative bursal weight (P>0.05). Moreover, there was no significant difference in weight of relative spleen weight among treatments (P>0.05). There were significant differences for corticosterone concentration among treatments (P<0.05). The highest corticosteroid hormone concentration was in broilers fed soybean oil and control treatment. Whereas there was no significant effect of fish oil and olive oil on corticosterone level *(Fig. 4)*.

DISCUSSION

The main purpose of the present study was to examine the effects of different sources of oil on performance, serum cortisol level, antibody titers and IFN-γ gene expression in broilers. The addition of soybean oil (as a source of omega-6) to the diet resulted in a positive effect on performance. The results showed that chicks fed diets containing soybean oil showed better performance than the other groups. Our result was consistent with the finding of some others studies that found that soybean oil improves performance [3,8]. The improved performance of broilers fed soybean oil were probably because of fatty acid composition of this oil; a long-chain n-6 fatty acid that makes it possible to increase diet digestibility and to enhance growth.

According to the results of the IFN-γ gene expression and poorly expressed genes of control treatment compared with other treatments can be argued that addition of polyunsaturated fatty acids (PUFA) in the diet can enhance IFN-γ mRNA expression significantly. These results are compatible with several other studies [14,22] that showed feeding birds with PUFA can affect lymphocyte proliferation. Among different treatments the fish oil treatments showed the highest level of gene expression, This result was agree with a study [23] that found diets enriched with fish oil increase the activity of T-helper-1. In addition, an *in vitro* study [12,24] showed that fish oil would be expected to increase production of cytokines such as interferon-gamma by decreasing production of prostaglandin E_2 (by peripheral blood mononuclear cells). Prostaglandin E_2 inhibits activity of lymphocytes. While these results were inconsistent with studies, that reported splenic IFN-γ mRNA were lower in mice fed a n-3-PUFA-enriched diet compared with low n-3-PUFA diet, indicating shift from T-helper-1 to T-helper-2 of immune response [15]. Based on these studies, we concluded that the increase of IFN-γ mRNA expression in the chickens fed fish oil diet may be attributed to enhance of innate immune cells such as T-helper-1 with reducing production of eicosanoids such as prostaglandin E_2 by peripheral macrophages [7]. Effects of oils on antibody titer against sheep red blood cells were not significant. These results were inconsistent

with studies [25,26] that reported that fish oil was shown to enhance the antibody response of chicks to sheep red blood cells than the birds that were not treated with fish oil. In antibody titer against Newcastle, we observed significant differences among treatments by adding the different sources of oil. It is concluded that by adding fish oil, immune response improved, probably because of the effects on long chain n-3 PUFA of fish oil on eicosanoid levels [13,26]. Also this result was inconsistent with the findings of Parmentier et al.[5], who reported that n-6 PUFA increased antibody production. It seems that, these discrepant resulting might be associated with the types and dose oil used. It was concluded that the addition of fish oil in the diet may be resulted to enhance antibody titer against Newcastle due to long chain n-3 PUFA metabolical function (eicosapentaenoic acid and docosahexaenoic acid).

Increasing of bursal weight could be interpreted as an indicator of increase immune activity [27]. The results of this study indicated that the addition of fish oil to the diet has a positive impact on the immune response of broilers. Previous studies have shown that decrease in the relative weight of lymphoid organs, are associated with blood corticosterone levels [9,28]. Corticosterone has been found to be immunosuppressive. Another study revealed that inclusion of soybean oil in the diet could induce significant increases in serum corticosterone level. These results agree with studies that found that on weight of bursa Fabricius [29], but it was not consistent with its effect on the weight of other organs (30). The results of this study indicated that the addition of that the addition of fish oil and soybean oil to the diet may be resulted in better improvement of immune response and performance in broiler chickens.

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RESEARCH ARTICLE

The Effect of Photoperiod Length on Performance Parameters, Carcass Characteristics and Heterophil/Lymphocyte-Ratio in Broilers

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Abstract

This study was conducted to assess the effects of photoperiod length [Continuous lighting (24L:0D): 24 Lightness:0 Darkness; Constant lighting (16L:8D): 16 Lightness:8 Darkness; Self-photoperiod (SP): 24 Lightness: free choice Darkness] on the performance, carcass characteristics and heterophil/lymphocyte ratio of male broilers grown to 42 d of age. A total of 180 Ross 308 males were exposed to one of the three lighting programmes: 16L:8D; 24L:0D and SP. In the self-photoperiod group, the percentage of animals that preferred to use the dark chamber, progressively increased throughout the trial. Starting from the 2nd week till the end of the trial, the live weights of the animals included in the 24L:0D and self-photoperiod groups were higher than those included in the 16L:8D group ($P<0.01$). The trial groups did not differ from each other for the end-trial feed consumption and feed conversion rates ($P>0.05$). No difference was detected between the groups for mortality rates or carcass percentages ($P>0.05$). The heterophil/lymphocyte ratios (H/L) were similar in the self-photoperiod and 16L:8D groups and were lower than those of the 24L:0D group. In conclusion; when the broilers allowed to regulate their own photoperiod, they had similar body weights but lower stress level than those kept under commercial lighting system.

Keywords: *Broiler, Carcass, Performance, Photoperiod, Stress*

Broilerlerde Fotoperyod Uzunluğunun Performans Parametreleri, Karkas Özellikleri ve Heterofil/Lenfosit Oranı Üzerine Etkisi

Özet

Bu çalışma, farklı fotoperiyotların [Sürekli Aydınlatma (24L:0D): 24 saat aydınlık:0 saat karanlık; Sabit Aydınlatma (16L:8D): 16 saat aydınlık, 8 saat karanlık; Self-fotoperiyot (SP): 24 saat aydınlık: istediği kadar karanlık] 42. güne kadar besi uygulanan etlik piliçlerin performans, karkas özellikleri ve heterofil/lenfosit oranları üzerine etkisini belirlemek için yapılmıştır. Bir günlük yaşta toplam 180 adet Ross 308 erkek civcivlere 24L:0D, 16L:8D ve SP şeklinde aydınlatma programı uygulanmıştır. Self-fotoperiyot grubunda bulunan piliçlerin karanlık bölmelerde zaman geçirenlerin oranı denemenin başından sonuna kadar artış göstermiştir. Denemenin ikinci haftasından deneme sonuna kadar 24L:0D ve self- fotoperiyot gruplarında bulunanların canlı ağırlıkları 16L8D grubunda olanlardan daha yüksek bulunmuştur ($P<0.01$). Deneme sonu yem tüketimleri ve yemden yararlanma oranları bakımından deneme grupları arasında fark bulunmamıştır ($P>0.05$). Ölüm oranları ve karkas parçaları bakımından da farklılık bulunmamıştır ($P>0.05$). Heterofil/Lenfosit (H/L) oranı self-fotoperiyot ve 16L:8D gruplarında benzer ve 24L:0D grubuna oranla daha düşük bulunmuştur. Sonuç olarak, broylerlere, kendi fotoperiyod dönemlerini ayarlama imkanı verildiğinde, ticari ışıklandırma şartlarında yetiştirilenler ile eşit vücut ağırlığına ulaştıkları, ancak daha az stresli oldukları belirlenmiştir.

Anahtar sözcükler: *Broiler, Karkas, Performans, Fotoperiyot, Stres*

INTRODUCTION

With the arrival of spring, migratory birds migrate to temperate regions to nest and lay their eggs in their breeding grounds [1]. It has been ascertained that, very similar to the case in mammals, in domestic poultry, the circadian rhythm is controlled by the visual suprachiasmatic nucleus and the medial suprachiasmatic nucleus [2]. In modern poultry production systems, artificial lighting programmes have found common use in increasing the

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production performance. In contrast to some studies [3,4], which showed no effect of continuous lighting on broiler performance, there are many scientific reports, which declared that the extention of the lighting period stimulated the broilers to increase feed consumption and body weight gain [5-7]. However, it has also been reported that continuous lighting programmes lead to several problems, including locomotory problems, decrease in feed conversion rate and immunosuppression, and thus, have a negative impact on animal welfare, requiring the extension of the dark period the animals are exposed to [8-11]. Besides, consumer demand and industrial operation have increased the importance of factors related to meat quality, such as the colour and pH value of meat and to the best knowledge of the authors, there is a few information in the scientific literature examining the lighting regime and meat quality relations, in the poultry production [12,13].

This study was aimed at determining the effects of three different photoperiods (24L:0D, in which the animals were withheld from the dark; 16L:8D, in which the animals were considered to satisfy their daily requirement of darkness [14], and the self-photoperiod, in which the animals self-determined to use the dark chamber) on broiler performance as well as on slaughter and carcass traits and heterophil/lymphocyte ratios parameters.

MATERIAL and METHODS

Birds and Husbandry

All experimental procedures were performed in accordance with the Turkish National Guidelines for the Care and Use of Animals for Research Purposes (Certificate of Authorisation to Experiment on Living Animals N°2014/52, The Local Ethical Committee of Ataturk University).

The trial was conducted at the Poultry Unit of the Research Farm of Ataturk University, Faculty of Veterinary Medicine. One day-old Ross 308 male broiler chicks obtained from a commercial hatchery were used in the experiment.

Experimental Design

During the first six days, the chicks were maintained in a brooder and kept under continuous lighting (24L:0D). In three different experiment windowless rooms, lighted by fluorescent, from day 7, the chicks were randomly allocated to 1.0 x 1.0 m pens, of which the floor was covered with 10 cm layer of wood shavings. The pens used for housing the broilers of in the self-photoperiod group had 1.4 x 1.0 m floor and the pens had a chamber of 0.4 x 1.0 m in size, which was separated by an opaque, light-proof, air-permeable cloth of 1.2 m height, and into which the animals had the opportunity of passing at any time. Twelve chicks were housed in each pen at a stocking density of 0.083 m^2 per broiler.

The animals included in the trial were fed on broiler chick starter diet between days 1-21 and on broiler chicken growing diet between days 22-42 *(Table 1)*. Throughout the trial period, the animals were provided with *ad libitum* feed, by using hanging tube type feeders, which allowed 9.34 cm space for each bird and water was supplied by nipples.

Treatments

The birds were exposed to one of the three lighting programmes. These were: 16L:8D (22:00-06:00, turn off); 24L:0D and 24L:self-photoperiod.

Total 180 Ross 308 males, twelve birds in each 5 replicates from 3 experiment groups were used in the study.

Light intensity was 20 lux in the open pens and 0 lux in the closed chambers. Light intensity was measured on the head level of the birds, at the center of the pens, by using a light meter (LT Lutron, model LX-105).

Calculation of the Performance Values

Body weight and feed consumption values were measured by weekly weighing (1 g sensitivity). Mortality was recorded on a daily basis. In order to assess uniformity, at the end of day 42, each animal was weighed (10 g sensitivity) individually and their weights were recorded. The proportional body weight gains of the animals, were calculated by proportioning body weight to the value measured a week before.

Rate of Broilers that Preferred the Dark Chamber

In order to determine the number of broiler chickens that preferred to remain in the dark chamber, the animals were observed twice a day (at 10:00 and 22:00) throughout the study period. The number of broilers detected in the

Table 1. *The nutrient composition of the feed used in the study*
Tablo1. *Araştırmada kullanılan yemlerin besin madde kompozisyonları*

Composition	Starter Diet	Growing Diet
Dry matter (g/kg)	880	880
Crude protein (g/kg)	240	200
Crude fiber (g/kg)	60	60
Crude ash (g/kg)	80	80
Acid-insoluble ash (g/kg)	10	10
Calcium (g/kg)	15	15
Phosphorus (g/kg)	7	6.5
Sodium (g/kg)	3	3
NaCl (g/kg)	3.5	3.5
Lysine (g/kg)	12	10
Methionine (g/kg)	5	4
Methionine + Cystine (g/kg)	9	7.5
Metabolic energy (MJ/kg)	12.90	13.40

dark chamber per day was divided by the total number of animals to calculate the percentage of animals that preferred to use the dark chamber.

Sample Collection and Measurements

At the end of the trial, on 42nd day, animals were withheld from feed at 20:00 and after 12 h, they were slaughtered.

A sample of two birds from each subgroup (total=30 birds), with weights between ± 10% of the subgroups mean were chosen. The birds were electrically stunned, bled for 120s, after severing both carotid arteries and at least one *juguler* vein. Broilers were then scalded for 30 s at 54°C, before mechanical plucking. The birds were eviscerated manually, washed, and allowed to drain for 10 min [15]. Eviscerated carcasses were tumble-chilled in ice water for 30 min and were allowed to drain for 5 min.

The carcasses were stored at 3°C for 24 h, and then dissected [16].

The carcass shrink rate was calculated using the formula given below: [17]

Carcass Shrink (%) = [1- (Cold carcass weight/Hot carcass weight)] *100

Analysis for pH

The pH values of the samples were determined using a pH meter (SCHOTT L 6880, Lab Star

The pH value was measured using a direct probe by thrusting the probe into the breast fillets. The pH values measured 24 h after slaughter.

Determination of Colour Values

A Minolta model colorimeter (Model CR-200, Minolta Corp.,) was used for the colour measurements of the breast fillet samples, with a white tile as a reference [CIE L value = lightness, CIE a value = redness, CIE b value = yellowness] [18].

Determination of the Heterophil/Lymphocyte Ratio

Blood samples were collected on day 41, from 2 randomly selected birds from each pen. Two preparations for each bird were made from the blood samples, a drop being smeared on each of 2 glass slides. The smears were stained using the May-Grünwald and Giemsa stains Lucas and Jamroz [19] , approximately 2 to 4 h after methyl alcohol fixation. One hundred leukocytes, including granular (heterophils, eosinophils and basophils) and non-granular (lymphocytes and monocytes) cells, were counted on 1 slide of each bird. For the determination of heterophil and lymphocyte counts and the heterophil/lymphocyte ratio (H/L), the average was taken of the two preparations counted for each bird.

Statistical Analysis

Data were presented as means ± standard error and subjected to one-way ANOVA using the GLM procedure of SAS version 8.02 [20]. Significant differences between the photoperiods applied were distinguished by Fisher's least significant difference (P<0.05) multiple range test.

Wilcoxon signed-rank analysis was conducted on the data from the initial comparison of the percentages of the animals that preferred to use the dark chamber.

Individual body weights were recorded at 42 d of age to assess the final BW uniformity. Pen uniformity was expressed as the CV (variation coefficient) of BW (standard deviation/mean x 100). Due to the variation coefficients calculated having determined not to display a normal distribution pattern; the non-parametric Kruskal-Wallis test was applied. The significance level of the differences observed between the groups was determined using the Mann – Whitney U Test.

RESULTS

Regulation of Photoperiod

The percentages of the animals that preferred to use the dark chamber are given in *Table 2*. Accordingly, the percentage of the animals which preferred to use the dark chamber in the morning was 21.77±1.21%; while the percentage of those, which preferred being in the dark chamber in the evening, was 21.13±1.14% (P>0.05). The number of animals that preferred to spend time in the dark chamber increased with age of the animals (P<0.001).

Performance

The differences observed between the body weights of the animals at the beginning of the trial were statistically

Table 2. *Percentage of broilers that chose the dark chamber, with respect to age (%)*

Tablo 2. *Yaşa göre karanlık bölmede zaman geçirmeyi tercih eden piliçlerin oranı (%)*

Days	RCD (%)
7 - 14	8.33^{c}
15- 21	10.71^{c}
22 - 28	24.05^{b}
29 - 35	32.26^{a}
36- 42	33.61^{a}
Total SEM	21.77 1.33
P	0.000

RCD (%): *Rate of broilers that chose the dark chamber (%);* ***a, b, c:*** *Means within a column with no common superscripts differ significantly (P<0.05);* ***SEM:*** *Standard error of means*

Table 3. *The impact of three different photoperiod treatments on body weight (g) and relative growth rates (wt/wt) between days 7 and 42*
Tablo 3. *Yedinci-42. günler arasında üç farklı muamelenin canlı ağırlık (g) ve relatif büyüme oranları üzerine etkisi (wt/wt)*

Days	Body Weight (g)					Relative Growth Rates *(wt/wt)*				
	SP	24L:0D	16L:8D	SEM	P	SP	24L:0D	16L:8D	SEM	P
7 d	140	141	143.0	1.19	0.081					
14 d	351[a]	350[a]	316.2[b]	5.70	0.001	2.52[a]	2.49[a]	2.19[b]	0.03	0.000
21 d	765[a]	770[a]	697[b]	14.45	0.006	2.18	2.20	2.21	0.04	0.863
28 d	1197[a]	1186[a]	1118[b]	12.13	0.001	1.56	1.54	1.61	0.02	0.085
35 d	1840[a]	1827[a]	1720[b]	22.69	0.005	1.54	1.54	1.54	0.02	0.992
42 d	2499[a]	2530[a]	2395[b]	21.51	0.002	1.36	1.39	1.39	0.02	0.426

SP: *Self-photoperiod;* ***a.b:*** *Means within a row with no common superscripts differ significantly (P<0.05);* ***SEM:*** *Standard error of means*

Table 4. *The impact of three different photoperiod treatments on cumulative feed consumption (g/bird) and feed conversion rate (g/g) between days 7 and 42*
Tablo 4. *Yedinci-42. günler arasında üç farklı muamelenin yem tüketimi (g/piliç) ve yemden yaralanma oranları üzerine etkisi (g/g)*

Days	Daily Feed Consumption During Different Periods (g/bird)					Feed Efficiency (g feed consumption/g gain)				
	SP	24L:0D	16L:8D	SEM	P	SP	24L:0D	16L:8D	SEM	P
7 - 14	257	244	230	12.46	0.338	1.21	1.17	1.35	0.08	0.294
7 - 21	897	951	922	15.49	0.083	1.45[b]	1.52[b]	1.68[a]	0.05	0.019
7 - 28	1594	1675	1657	43.55	0.414	1.52	1.60	1.71	0.05	0.085
7 - 35	2765	2873	2698	47.75	0.067	1.63	1.70	1.72	0.03	0.198
7- 42	4225	4340	4117	63.896	0.086	1.79	1.82	1.83	0.03	0.601

SP: *Self-photoperiod;* ***a.b:*** *Means within a row with no common superscripts differ significantly (P<0.05);* ***SEM:*** *Standard error of means*

Table 5. *The mean and median CV (variation coefficient) values of the birds at 42nd days of the trial*
Tablo 5. *Deneme gruplarına ait CV (varyasyon katsayısı) ortalama ve medyan değerleri*

Treatment	Homogeneity (mean ± SE)	Median
SP	9.24±1.28[ab]	8.09
24L:0D	7.38±0.79[b]	7.02
16L:8D	11.73±1.20[a]	10.61
P	0.038	

SP: *Self-photoperiod;* ***a.b:*** *Means within a row with no common superscripts differ significantly (P<0.05);* ***SE:*** *Standard error*

insignificant (P>0.05). In the following weeks, the effect of photoperiod on daily body weight values was found to be statistically significant (P<0.01). From week 2 until the end of the trial, higher and relatively similar daily body weights were measured for the self-photoperiod and 24 L groups, and the values pertaining to these two groups were lower than those of the 16 L group *(Table 3)*.

Cumulative feed consumptions and Feed conversion rates are shown in *Table 4*. The length of the light period does not affect cumulative feed consumption. Differences between the trial groups for Feed conversation rate were found to be statistically insignificant (P>0.05).

Uniformity was determined to be lowest in the 16L:8D group and highest in the 24L:0D group. The differences between the trial groups were statistically significant (P=0.038) *(Table 5)*.

Carcass Yields

The results of the analysis of variance for slaughter and carcass characteristics are presented in *Table 6*. The influence of the length of the light period on slaughter traits, excluding blood and feathers, was found to be statistically insignificant (P>0.05).

It was determined that the length of the light period had no effect on carcass percentages.

The ultimate pH values measured at 24 h post-mortem are given in *Table 6*.

The color parameters, the L* and a* values, determined for the three trial groups, were observed not to differ statistically, yet the yellowness values of the self-photoperiod and 24 L groups were lower than those of the 16 L group *(Table 6)*.

Carcass shrink percentages in the 16L:8D had the highest (1.14) level, where 24L:0D and self-photoperiod groups were determined as 0.93 and 0.94, respectively (P=0.028). The comparison of the trial groups demonstrated only the 16L:8D group and the self-photoperiod group to statistically differ from each other (P=0.002). The multi-

Table 6. *Slaughter, carcass, pH and colour traits of the broilers included in the three trial groups*
Tablo 6. *Üç farklı deneme grubunda bulunan piliçlere ait kesim, karkas, pH ve renk özellikleri*

Slaughter Characteristic						Carcass Characteristic					
Parameters	**SP**	**24L:0D**	**16L:8D**	**SEM**	**P**	**Parameters**	**SP**	**24L:0D**	**16L:8D**	**SEM**	**P**
BWBS (g)[1]	2544[a]	2407[ab]	2336[b]	48.4	0.017						
HCY (%)[2]	75.1	74.0	74.9	0.43	0.195	Neck (%)	5.61	5.68	5.46	0.19	0.704
Blood (%)	4.01[ab]	4.24[a]	3.54[b]	0.17	0.026	Tail (%)	1.07	1.14	1.18	0.07	0.546
Feather (%)	4.94[ab]	5.43[a]	4.84[b]	0.17	0.049	Wing (%)	11.3	11.2	11.4	0.19	0.724
Head (%)	2.60	2.58	2.73	0.09	0.481	Whole Legs (%)	39.8	39.1	39.5	0.59	0.731
Offals (%)	4.86	4.98	5.16	0.16	0.445	Whole Breast (%)	42.2	42.9	42.5	0.58	0.705
Gizzard (%)	2.11	2.23	2.24	0.12	0.714	pHu[3]	6.12[ab]	6.14[b]	6.07[a]	0.02	0.041
Liver (%)	1.76	1.83	1.96	0.07	0.108	CIE L value[4]	52.6	54.4	52.5	0.64	0.071
Heart (%)	0.59	0.54	0.62	0.03	0.248	CIE a value[5]	5.04	3.17	3.64	1.10	0.456
Feet (%)	4.05	4.16	3.95	0.09	0.262	CIE b value[6]	5.95[a]	6.37[a]	7.76[b]	0.32	0.000

SP: *Self-photoperiod;* ***a.b:*** *Means within a row with no common superscripts differ significantly (P<0.05);* ***SEM:*** *Standard error of means; [1] BWBS; g: Body weight before slaughter; [2] HCY, %: Hot carcass yield; [3] pHu: ultimate pH; the pH at 24 h post-mortem; [4] CIE L: lightness; [5]: CIE a: redness; [6] CIE b: yellowness*

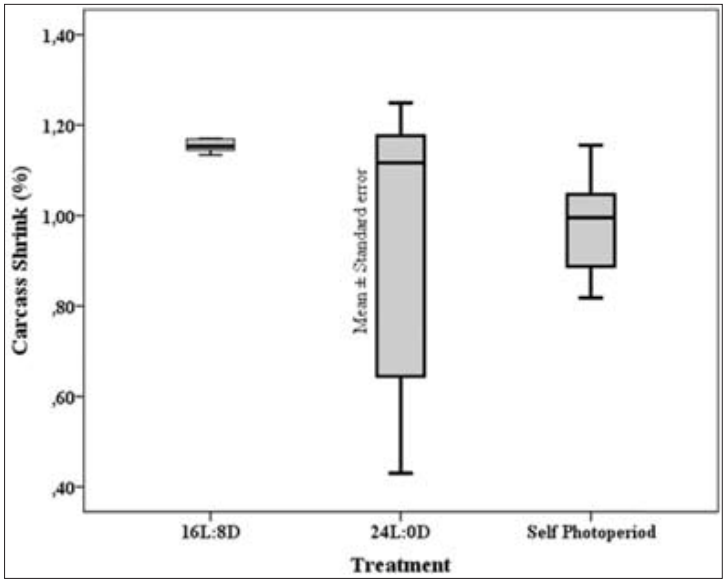

Fig 1. Carcass Shrink (%) values belonging to the groups

Şekil 1. Gruplara ait sıcak/soğuk karkas fire (%) değerleri

Table 7. *The heterophil/lymphocyte ratios and mortality rates of the broiler chickens included in the three trial groups*
Tablo 7. *Üç farklı deneme grubunda bulunan piliçlere ait heterofil/lenfosit ve ölüm oranları*

Treatment	HLR	Mortality %
Self	0.40[b]	0.00
24L:0D	0.94[a]	0.00
16L:8D	0.52[b]	3.30
SEM	0.07	0.013
P	0.000	0.134

SP: *Self-photoperiod;* ***HLR:*** *heterophil/lymphocyte ratios;* ***a,b:*** *Means within a column with no common superscripts differ significantly (P<0.05);* ***SEM:*** *Standard error of means*

faceted comparison of the other groups did not reveal the presence of any statistically significant difference *(Fig 1)*.

Stress and Mortality

The Heterophil/lymphocyte ratio of the self-photoperiod and 16 L groups were found to be similar, whilst the ratio of the 24 L group differed *(Table 7)*.

In the self-photoperiod and 24L:0D groups, the survival rate was 100%, and in the 16L:8D group, this rate was 96.70%. This difference was found to be statistically insignificant.

DISCUSSION

Regulation of Photoperiod

In conventional broiler production systems, for animals that reach a final body weight less than 2.5 kg, it is

suggested that the lighting program be adjusted as 23L:1D between days 0-7, as 20L:4D from day 8 till at least 3 days before slaughter, and as 23L:1D at least three days before slaughter [21]. According to the criteria applied in the European Union, in lighting programmes adjusted to a 24-h cycle, there needs to be 6 h dark period per day, and 4 h of this period should allow for the exposure of animals to continuous darkness [22]. Savory and Duncan [23] reported that broilers preferred to spend approximately 9% of the day in the dark. In the present study, it was observed that the percentage of animals that preferred to use the dark chamber increased from 8.33% during the first 7-14 days post-hatching, to 33.61% at the end of the trial. The percentages of animals preferred to use dark chamber was seemed to increase linearly with the age.

Performance

In the present study, from the second week of the trial, the chickens included in the self-photoperiod group achieved body weights similar to those of the 24L:0D group and higher than those of the 16L:8D group. At the end of the trial, the relative growth rate of the birds were similar in all groups. This result may have arisen from the animals having spent longer periods of time in the dark chamber during the last few days before slaughter. In a study, in which male broilers were exposed to three different lighting schedules, Rozenboim et al.[3] reported that the groups exposed to 23L:1D and 16L:8D did not display any statistically significant difference for the final body weights measured on day 42. Similarly, it has been indicated that lighting schedules of 20L:4D and 16L:8D did not produce any statistically significant difference for broiler body weights [4].

It is suggested that broiler chickens modify their feeding behaviour according to the lighting programme they are exposed to [24,25]. Lewis et al.[25] suggested that, the extension of the photoperiod length during the period up to day 21 increased feed consumption, while between days 22-35, photoperiods longer than 6 hours resulted in similar feed consumption rates.

In another study, the extension of the scotoperiod during days 7-32 reduced feed consumption, increased feed conversation ratio but all groups had consumed equal amounts of feed [26]. Scott [27] reported that the application of a lighting schedule of 23L:1D during the first 10 days post-hatching enabled optimum feed consumption in broilers, thus uniform body weights and intestinal development. Feed restriction in the early growth period has been reported to result in decreased final body weights and daily body weight gain, but has been indicated not to alter feed conversion rates [28]. This result is in contrast wiht the other finding Ayasan et al.[29] which indicate that feed restriction had significant effects on feed intake and feed conversion ratio. Similar to the findings of Lippens et al.[28] during the first week of the present trial, the 16L:8D group was determined to have consumed feed 10.6% less than the self-photoperiod group and 5.9% less than the 24L:0D group. This was considered to have resulted in an effect, similar to the feed restriction in the early growth period.

It should be noted that decline in uniformity in the body weights of broiler chickens results in the decrease of the retail market value of the end product.

In contrast to our findings, Griffin et al.[30] stated that increased photoperiod reduced the uniformity, however Lien et al.[31] declared no effect of photoperiod on uniformity. According to present study, the delayed growth in the 16L:8D group may be attributed to the decrease in uniformity.

Carcass Yields

Hot carcass percentage were not affected by photoperiod length in the present study. This is in line with the findings of Coban et al.[9] in male quails and Downs et al.[32], who was also found no significant differences between carcass yields of broilers housed under different lighting programmes.

In the present study, the internal organ weights of the animals in all groups were similar. In male quails, Coban et al.[9], also reported that internal organ percentages were not affected by photoperiod.

In this study, similar to body weight gain, feather development was also influenced by photoperiod length. The potential muscle development of animals depends on the formation of muscle fibrils in the prenatal period and the hypertrophy of these fibrils in the postnatal period. Berri et al.[33], upon examining the cross-sections of the fibrils of the major pectoral muscle and assessing breast meat quality traits, determined that the correlation coefficient calculated for phenotype was statistically significant and positive while the correlation coefficient calculated for drip loss was statistically significant and negative. In present study, in the 16L: 8D group, the carcass shrink rate (%) was higher and the pH value was lower than other groups. It was considered that the animals in this group had small fibril diameter due to lower body weight gain.

In contrast to present findings, Renden et al.[10], compared the effect of 23L:1D and 16L:8D; and noted that in the 23 h lighting group, breast meat of the carcasses increased; while the animals exposed to short lighting period, the rate of whole legs of the carcasses were higher than the ones, exposed to long photoperiod. Lewis et al.[25] indicated that continuous lighting increased the percentage of breast meat. Similarly, Lien et al.[31] confirmed that the extension of the light period from 16 h to 23 h increased the percentage of the whole

breast. In the present study, in contrast to previous results, whole breast and leg percentages were not affected by photoperiod.

Stress and Mortality

Gross and Siegel [34] suggested that, in birds, the heterophil/lymphocyte should be used for detecting long-term environmental effects. In response to increased environmental stress, the heterophil/lymphocyte ratio increases.

In the present study, the heterophil/lymphocyte ratios of the self-photoperiod and 16L:8D groups were lower than those of the 24L:0D group. Similarly, Coban et al.[9], recorded lower H/L ratio in self photoperiod group than quails exposed to continuous lighting. Campo et al.[8] was in line with our findings and stated that continuous lighting programmes induced the stress in broiler chickens, thereby, resulting in the increase of the heterophil/lymphocyte ratio. However, Lien et al.[31] reported that the heterophil/lymphocyte ratios of the two groups exposed to short and long photoperiod were equal.

It has been noted that rapid growth rates achieved in the early stages of life through the application of long lighting programmes bring about increased mortality [11,26,35,36]. In the present study, mortality rates were similar in all groups, in agreement with the results of Lien et al.[31], indicating no significant effect of lighting on mortality.

According to the present study, it is concluded that; (I) If given an opportunity to prefer to use a dark chamber in the pen, the broiler chickens do choose. (II) When allowed to prefer their dark period by instinct (self photoperiod group), the broiler chickens reached higher final body than 16L:8D hours lighting, as recommended by EU. (III) As indicated by H/L ratio, it seemed that the stress levels were equal in both '16-hours lighting' and 'self photoperiod' groups and H/L ratio was highest in '24 hours' lighted chickens. (IV) Except for feather and blood ratio of the bird, carcass and slaughter parameters were not affected by photoperiod.

According to the present study, 'self photoperiod method' is seemed to have a potential to be a choice/profit for poultry production and may be applied on some other species (e.g. laboratory animals) in the future. Further studies are needed to understand the physiological mechanism and welfare status of the animals, exposed to self photoperiod conditions.

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RESEARCH ARTICLE

Bovine Hypodermosis in North-Central Algeria: Prevalence, Intensity of Infection and Risk Factors

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Abstract

From January to June 2009 the biggest livestock market and five bovine farms of Bejaia department (Northcentral Algeria) were visited in order to undertake an epidemiological survey on warble fly infestation (WFI). A total of 3.442 and 226 bovines were clinically examined in both market and farms, respectively. The prevalence was higher in farms (18.1%) than in the animal market (3.7%). On the contrary, the intensity of infection was highest in the market (12.61±7.78 vs 7.5±4.15). The husbandry system exerts a significant effect on the prevalence of WFI, being the prevalence highest under extensive management. However, cattle age, sex and breed did not exert a significant effect on WFI prevalence. Breed was the only factor influencing the intensity of infection; the Montbeliarde breed was the most heavily infested as compared to the crossbreed and the local breed.

Keywords: *Bovine hypodermosis, Prevalence, Intensity, Risk factors, North-central Algeria*

Kuzey-Orta Cezayir'de Sığır Hypodermosis: Yaygınlık, Enfeksiyon Yoğunluğu ve Risk Faktörleri

Özet

Büvelek sineği enfestasyonu (WFI) üzerine bir epidemiyolojik araştırma gerçekleştirmek üzere Ocak-Haziran 2009 döneminde Bejaia Bölgesi'nin (Kuzey-Orta Cezayir) en büyük hayvan pazarı ve beş sığır çiftliği ziyaret edildi. Pazar ve marketlerde sırası ile toplam 3.442 ve 226 sığır klinik olarak muayene edildi. Prevalansın çiftliklerde (%18.1) hayvan pazarına gore (%3.7) daha yüksek olduğu belirlendi. Fakat, enfeksiyonun şiddetinin pazarda (12.61±7.78 vs 7.5±4.15) en yüksek olarak gözlemlendi. Ekstansif yetiştiricilikte en yüksek yaygınlıklta olan WFI prevalansı üzerine yetiştirme sistemi önemli bir etki göstermektedir. Ancak, sığır yaş, cinsiyet ve ırk özelliklerinin WFI yaygınlığı üzerinde önemli bir etki göstermediği belirlendi. Enfeksiyon yoğunluğunu etkileyen tek faktor olarak ırk özelliği bulundu; melez ve yerli ırk ile karşılaştırıldığında Montbeliarde ırkının en ağır enfestasyona maruz kaldığı gözlemlendi.

Anahtar sözcükler: *Sığır hypodermosis, Prevalans, Yoğunluk, Risk faktörleri, Kuzey-orta Cezayir*

INTRODUCTION

Cattle hypodermosis is a myiasis caused by larvae of *Hypoderma bovis* and *Hypoderma lineatum* (Diptera: Oestridae) and characterized by the presence of warbles under the skin of infested animals. Hypodermosis may also infect humans accidentally [1].

Bovine hypodermosis is still widely distributed all over the Northern hemisphere [2] where has been noticed in more than 50 countries from North America, Europe, Africa and Asia; nevertheless, warble fly infestation (WFI) has been occasionally found in the south of Equator, Argentina, Chile and in South of Africa as a consequence of cattle importation [3].

These parasites have an annual synchronized biological cycle, from summer to the following spring. The first endo-

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parasitic stage migrates through the deep connective tissues of their hosts. In spring, larvae reach the dorsal subcutaneous tissue and molt into the second and third larval stages, producing swellings on the skin along the back called warbles. Mature third larval stages fall on the ground and pupate at the end of spring; one month later the adult stages emerge. These flies are unable to feed and consequently, have a short life of one week; they spread the disease by laying their eggs on the hair of the cattle. *Hypoderma* spp. cause economic losses by reducing milk production (10-15%) and affecting weight gain (20%), welfare, bovine immune defense mechanisms and the leather industry (more than 10 warbles represent 50% devaluation of hides) [4].

In Algeria little data exist about WFI [5]. In the North East of the country several aspects of this disease had been explored in the 90s by Benakhla *et al.*[6,7], but the knowledge must be updated. Unfortunately, in Algeria, like in so many countries where warble flies are present, cattle breeders are confronting with severe economic losses in the absence of a warble management program.

The present study was carried out to determine the prevalence of WFI, its intensity and the risk factors involved in the incidence of this myiasis in northcentral Algeria.

MATERIAL and METHODS

Study Area

The study was conducted in the department of Bejaia *(Fig. 1)*, located in North-central Algeria (36º45´N5º04´E). This department belongs to the Tellian Atlas and has a superficies of about 3223.48 km^2. Climate is Mediterranean one with hot/dry summers and wet/fresh winters.

Animals

In order to determine the prevalence and the intensity of WFI, the biggest livestock market of Bejaia, located in Sidi aich city, was fortnightly visited, from January to June 2009. This market receives bovines from different departments from northern Algeria. A total of 3.442 animals were examined visually and by palpation of the dorsal area to register the presence and the number of warbles.

In addition, to establish the risk factors involved in the occurrence of bovine hypodermosis, five cattle farms with different husbandry systems (intensive, semi-extensive or extensive) were monthly visited during the same period of time, with a total of 226 animals examined (32 males and 194 females). Sex, breed, age and husbandry system were

Fig 1. Location of Bejaia department (North-central Algeria)

Şekil 1. Bejaia Bölgesi'nin konumu (Kuzey-Orta Cezayir)

taken into account as risk factors. Three categories of age were considered: animals younger than two years (n=51), between two and five years (n=93) and bovines older than five years (n=82). Four breeds were taken in account in our study: local breed (n=97), Prim Holstein breed (n=14), Montbeliarde breed (n=39) and Crossbreed (n=76).

Naturally emerged larvae (n=29) were collected and preserved in ethanol 70° and subsequently identified by using the morphological keys as described by James [8] and Zumpt [9].

Statistical Analysis

Three parametric statistical tests were used in the studying the risk factors involved in WFI. Pearson's Chi-squared test was used to study the effect of husbandry system, age, breed and sex on the prevalence. Fisher's exact test (2-sided) was exploited for the same goal as precedent. Chi-squared automatic interaction detector (CHAID) was used after chi-squared test to classify results in homogeneous groups. For the examination analysis of the intensity of infection, a unifactorial association analysis (ANOVA) was applied on the 42 positive animals detected in the farms sampled.

Statistical analyses were done using R statistical package (R v.2.15.2; 2012-10-26). CHAID algorithm was performed with Answer Tree 3.1 (SPSS Inc., Chicago, IL USA) [10].

RESULTS

Prevalence and Intensity of Infestation

Table 1 represents the monthly evolution of the prevalence by *Hypoderma* spp. recorded in the animal market. The inspection of the animals and the warble counting enabled us to note that on 3.442 inspected animals 129 were found to be warbled, which represented an overall prevalence of 3.7%. In the present study, the prevalence in the animal market increased progressively until April (7.1%); after this month the number of infested animals began to descend until June. No animal was found infested after the first week of June. The mean intensity of infestation in the animal market was 12.6±7.78; the highest intensity (13.1±7.35) was recorded in March.

Analysis of Risk Factors

Table 2 shows the prevalence and intensity of infestation in the five studied farms, along with the management system in each of them. The overall prevalence in the farms was 18.1%, with an intensity of infestation of 7.5±4.15. The prevalence is higher on cattle under extensive and semi-extensive husbandry systems. Pearson's Chi-squared test revealed a significant effect of management system on the WFI ($\chi 2$= 19.555; Fisher's exact test P<0.001). The CHAID algorithm divided population studied in three groups,

Table1. *Monthly evolution of the prevalence and the intensity of infestation in Bejaia livestock market (Northcentral Algeria)*
Tablo 1. Aylık yaygınlık gelişimi ve Bejaia hayvan pazarında enfestasyon yoğunluğu (Kuzey-Orta Cezayir)

Month	Number of Examined Animals	Number of Infested Animals	Prevalence (%)	Total Number of Warbles	Intensity of Infestation
January	150	4	2.7	35	8.7±3.09
February	507	10	2.0	49	4.9±2.51
March	791	46	5.9	604	13.1±6.20
April	678	48	7.1	306	6.4±3.24
May	674	18	2.7	84	4.7±2.47
June	642	3	0.5	4	1.3±0.58
TOTAL	3442	129	3.7	1082	12.6±7.78

Table 2. *Prevalence and intensity of infestation in the farms according to the management system*
Tablo 2. *Çiftliklerde yönetim sistemine göre yaygınlık ve enfestasyon yoğunluğu*

Farm	Management System	Number of Examined Animals	Prevalence	Intensity of Infection
Farm 1	Semi-extensive	162	21 (12.9%)	5±2.28
Farm 2	Semi-extensive	15	7 (46.6%)	13±3.21
Farm 3	Intensive	19	0 (0%)	0
Farm 4	Extensive	16	9 (56.2%)	6.6±2.82
Farm 5	Semi-extensive	14	5 (35.7%)	12±3.03
TOTAL		226	18.1	7.5±4.1

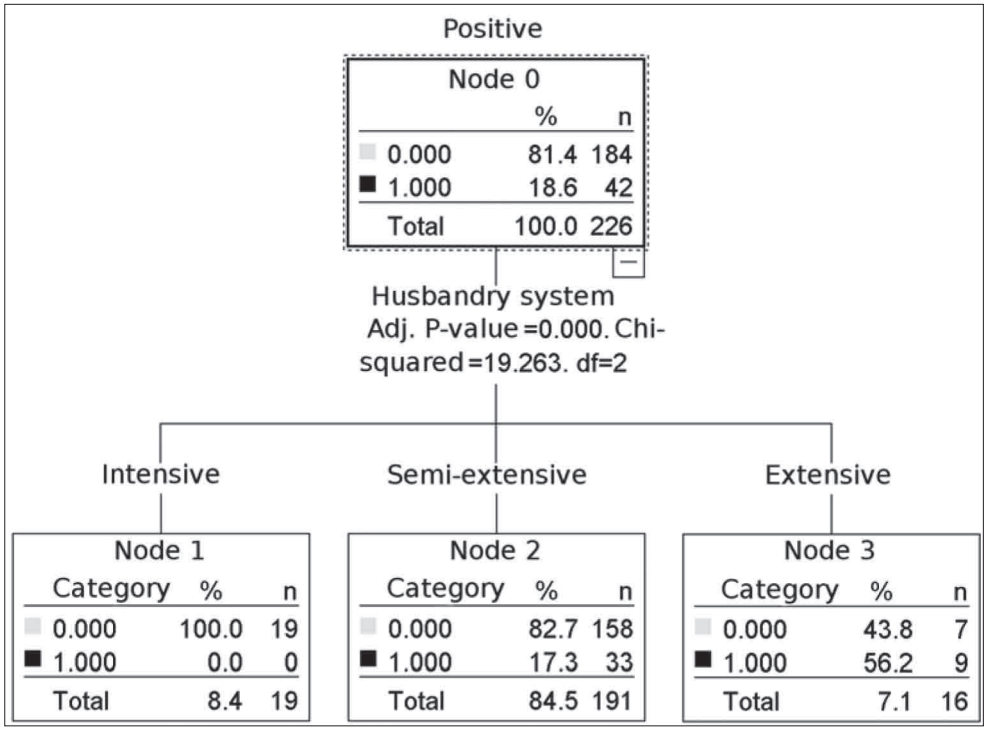

Fig 2. Classification tree produced by the CHAID algorithm when considering different factors

Şekil 2. Farklı faktörler göz önüne alındığında CHAID algoritması tarafından üretilen sınıflandırma ağacı

intensive, semiextensive and extensive management, in ascending prevalence order *(Fig. 2)*. No significant association between WFI prevalence and age, breed or sex was detected.

Likewise, one analysis of variance (ANOVAs) did not detect significant effect of the sex of the animals or management system on the intensity of infestation. However, the precedent parametric test showed a very significant association between breed and intensity of infestation (F=10.96; *P*=0.002). With Tukey multiple comparisons of means, the Montbeliarde breed was detected as the most heavily infested or the most sensitive to WFI. Besides, the effect of farm on intensity of infestation was very significant (F=20.97; P<0.001). The Tukey test shows two homogeneous groups (farms 1 and 4, and farms 2 and 5). Curiously, there was no effect of age on the parasitic burden.

Out of the 29 identified larvae, 16 (55%) was found to belong to *H. lineatum* and 13 (45%) to *H. bovis*.

DISCUSSION

The results of the present study provide initial baseline data on epidemiological assessment of the disease in northern Algeria, which is necessary before the implementation of any control and eradication program.

No animal was found infested after the beginning of June. This result did not fully agree with those of a previous survey done in an Algerian slaughterhouse [5], in which nodules were found from beginning of October until the end of April. Those differences could be explained by the change in weather conditions from one year to another. Indeed, *Hypoderma* sp. biology, as occurs with most of insects, is noticeably conditioned by weather conditions; climate directly influences the development of free stages of the parasite, affecting the chronology of this myiasis, specially the time of appearance of warbles in the back, emergence of the prepupal grubs, pupation on the ground and adult emergence, and the intensity of infestation [11].

The higher prevalence in farms versus animal market could be due to the fact that the major part of bovines came from different departments from northern Algeria where the climate and the management system are different.

The rates of infestation found in our study were lower than those recorded by Benakhla et al.[7] in the Northeast of Algeria, and in our opinion, this fact could be mainly explained by the widely use of avermectins in Bejaia department by cattle breeders. It has been demonstrated simultaneously, in North America [12] and in France [13], that ivermectin was highly effective even, at very low dosages (0.2 mg/per kg weight) against the endo-parasitic stages of *Hypoderma* spp. In addition, it is usual to notice a wide variation in the prevalence of WFI among different parts of the world and even within the same country [14]. This variation in the rate of prevalence of WFI in different areas might be due to the differences in the environmental conditions (topography of the land, season, humidity, temperature, rain fall, wind velocity) affecting the development of the warble flies [15].

In our survey, *H. bovis* (45%) and *H. lineatum* (55%)

were found infesting cattle, although there was a slight predominance of *H. lineatum*. This statement agrees with previous investigations [5]. It is crucial to identify the distribution of the two species, firstly because *H. bovis* is currently believed to affect only cattle living in the Northern countries, and secondly because the impact of *H. bovis* on animal welfare and health is more important than *H. lineatum* due to the fact that if treatments are not carried out promptly, when first stage larvae are still in the peri-rachidian channel, paralysis of the hind quarters may occur. This finding is of relevance for the correct use of drugs against hypodermosis [1].

The management system was found to exert an effect on the prevalence of bovine hypodermosis, since cattle at pasture have more chances to contact with warble flies than those kept in stables. This result agrees with several previous studies [11,16]. Ahmed *et al.*[17] observed that the major risk factor for hypodermosis positivity is the free grazing practices.

Differences in parasitic burden among breeds showed a very consistent pattern in the present study, the Montbeliarde breed seemed to be the most heavily infested. This would suggest that there was apparent breed preference on the part of the ovipositing flies. Certain breeds have been shown to harbor greater numbers of grubs [6,18]. According to Charbon and Pfister [19] the differences are a result of differences in skin thickness which affect larval survival. However, Panadero *et al.*[11] considered that the difference between breeds is mainly caused by differences in the husbandry system. In our case, this difference might be due to the fact that the Monbeliarde is a dairy breed and dairy cows in Algeria spend long time in pasture. It is well known that daily duration of pasturage affect directly the parasitic burden.

Curiously, no significant difference among age has been found. However, it is important to point out that a certain degree of resistance against the infestations by *Hypoderma* is acquired after repeated exposures of the animal to the parasite [20-22]. Therefore, if an old animal had been never exposed to the parasite, it would have the same sensibility as a younger one. Indeed, some authors such as Ahmed *et al.*[23] found no differences in the intensity of infestation with respect to different factors studied including age, breed, etc, except for previous exposure to the parasite, so animals that had presented warbles in previous years showed less warbles than those no previously infested. It is concluded that management system is the most important factor affecting the prevalence of bovine hypodermosis.

There was no significant difference of intensities respect to the sex of the animals. Other authors found that the males were more heavily infested than females [14,24] because most males are kept tied at the stables, so they are less exposed to the parasite than females.

Finally, the possibility of an underestimated prevalence of WFI mostly in the animal market cannot be ruled out as it was based on direct examination. Further studies by using more sensitive techniques like ELISA [22] are needed for an early diagnose of bovine hypodermosis before the larvae reach the back and subsequently to avoid economic losses by implementing an early efficient treatment against warble fly infestation.

Conflict of Interest

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

The Neuroprotective Effect of Caffeic Acid Phenethyl Ester on Global Ischemia-Reperfusion Injury in Rat Brains [1]

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Abstract

The aim of this study was to investigate the neuroprotective effects of caffeic acid phenethyl ester (CAPE) on phosphodiesterase 4 (PDE4) mRNA isoenzymes, oxidant and antioxidant defence in ischemia/reperfusion (I/R) injured rat brains. Twenty-one rats were randomly divided into three equal groups: sham-control, ischemia/reperfusion (I/R) and I/R+CAPE. Rats in sham-control group underwent only surgical intervention without bilateral common *carotid artery* occlusion. Ischemia/reperfusion was induced by bilateral common *carotid artery* occlusion with atraumatic clips for 30 min, followed by artery reopening. The I/R+CAPE group was subjected to the same surgical procedure as I/R group, but CAPE was administered intraperitoneally at the dose of 15 μmol kg^{-1} twice, 1 h before occlusion and at 12th h of reperfusion. The rats were sacrificed 24 h after I/R. The cAMP concentration was analyzed by ELISA and PDE4 isozyme mRNA transcriptions were evaluated by qRT-PCR methodology in the brain cortex. Ischemia-induced NO production was significantly attenuated by CAPE in the cerebral cortex. CAPE significantly enhanced GSH-Px activity, while SOD, CAT and XO activities non-significantly changed, as compared to the I/R group. CAPE significantly decreased PDE4A and PDE4B transcripts, without changing cAMP levels compared to I/R group. Ischemia-induced neurologic deficit scores were reduced by CAPE. These results suggest that CAPE slightly modulates the antioxidant defense system and NO release in rat brain during global cerebral ischemia/reperfusion injury. In addition, CAPE treatments produce the neuroprotective effect by reducing the levels of some PDE4 transcriptions.

Keywords: *CAPE, Brain, Ischemia/reperfusion, Antioxidant activity, cAMP-phosphodiesterase 4, Neuroprotective effect, Rat*

Rat Beyinlerinde Global İskemi-Reperfüzyon Hasarı Üzerine Kafeik Asit Fenetil Esterin Nöroprotektif Etkisi

Özet

Bu çalışma iskemi-reperfüzyon (I/R) hasarlı rat beyinlerinde fosfodiesteraz 4 (PDE4) mRNA izoenzimleri, oksidant ve antioksidant savunma sistemi üzerine kafeik asit fenetil ester (KAFE)'in nöroprotektif etkilerini araştırmak amacıyla yapıldı. Yirmi bir adet rat rastgele üç eşit gruba ayrıldı. Sham-kontrol, iskemi/reperfüzyon (I/R) ve I/R+KAFE. Sham-kontrol grubundaki ratlara bilateral common *carotid arter* oklüzyonu yapılmaksızın sadece cerrahi müdahalede bulunuldu. İskemi/reperfüzyon (I/R) bilateral *common carotid* arterlerin atravmatik klempler ile 30 dakika oklüzyonu ve takiben arter klempleri açılarak reperfüzyonu ile sağlandı. I/R+KAFE grubu I/R grubu ile aynı cerrahi usüle tabi tutuldu fakat oklüzyondan 1 saat önce ve reperfüzyondan 12 saat sonra iki defa 15 μmol kg^{-1} dozunda intraperitoneal KAFE verildi. Ratlar iskemi/reperfüzyondan 24 saat sonra sakrifiye edildi. Beyin korteksindeki cAMP düzeyi ELISA ile, PDE4 mRNA izoenzim transkripsiyonları ise qRT-PCR ile değerlendirildi. KAFE iskemi ile uyarılan beyin korteksindeki NO üretimini önemli oranda azalttı. I/R grubu ile karşılaştırıldığında SOD, CAT ve XO aktivitelerini KAFE anlamlı düzeyde değiştirmezken, GSH-Px aktivitesini önemli oranda arttırdı. KAFE cAMP düzeyini değiştirmeksizin PDE4A ve PDE4B düzeyini önemli oranda azalttı. İskemi ile uyarılan nörolojik hasar skorları KAFE tarafından azaltıldı. Bu sonuçlar KAFE'nin global beyin iskemi/reperfüzyon hasarı sırasında rat beyinlerinde antioksidant savunma sistemini ve NO salınımını hafifce dengelediğini önerir. Ayrıca KAFE bazı PDE4 izoenzim düzeylerini azaltarak nöroprotektif etki sağlar.

Anahtar sözcükler: *KAFE, Beyin, İskemi/reperfüzyon, Antioksidant aktivite, cAMP-fosfodiesteraz 4, Nöroprotektif etki, Rat*

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INTRODUCTION

Reactive oxygen radicals (ROS) are likely participants in the pathogenesis of cerebral ischemia-reperfusion (I/R) injury. Transient focal and global cerebral I/R triggers a plethora of cellular and molecular events that promotes neuronal cell death in several regions of the brain due to glutamate excitotoxicity, oxidative stress, inflammation and apoptosis [1-4]. Studies show that nitric oxide (NO) has beneficial properties to I/R injury including increase of blood flow produced by cerebral vasodilatation and inhibition of inflammation [1,5]. NO is also a free radical and initiates various pathophysiological events by reaction with superoxide anion to form peroxinitrite, on the contrary of its protective effects in various models of I/R injury [1,5,6].

Mammalian cyclic nucleotide phosphodiesterases (PDEs) are composed of 21 genes and are categorized into 11 families based on sequence homology, enzymatic properties, and sensitivity to inhibitors, the enzymes that hydrolyze and inactivate cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) [7]. As PDE4 is the major cAMP-hydrolysing family in many cells types, it represents a promising therapeutic target. The PDE4 family is composed of four subfamilies (PDE4A, PDE4B, PDE4C and PDE4D) encoded by different gene loci, and each of them has been shown to produce several mRNAs by alternative splicing [8]. Most recently, it has demonstrated that rolipram, selective PDE4 inhibitor, attenuates memory deficits produced by global brain ischemia [9].

Caffeic acid phenethyl ester (CAPE), an active component of propolis, has been shown to possess anti-inflammatory, immunomodulatory, anticarcinogenic, and antioxidant properties [1,2,10,11]. It has also been shown that CAPE treatment significantly reduces the infarction size and neuronal damage in ischemia-induced brain injury and suppresses cerebral lipid peroxidation [1,2,11]. To date, no studies have been reported that how CAPE treatment affects the levels of cAMP and PDE4s after transient global brain ischemia. For this purpose, the present study was designed to evaluate the neuroprotective effects of CAPE on PDE4 mRNA isoenzymes, oxidant and antioxidant defence in transient global cerebral ischemia rat model.

MATERIAL and METHODS

Transient Global Cerebral Ischemia in Rats

Experimental procedures were approved by Mustafa Kemal University, Veterinary Faculty Ethics Committee for the use and care of laboratory animals (25.04.2007, no:09). Experiments were performed on adult male wistar rats weighing 250-275 g. They were given free access to food and tap water. All animals were maintained under a controlled temperature (21±1°C) and humidity (55-60%) throughout the experiment. Twenty-one healthy rats were randomly divided into three equal groups: Sham-control, ischemia/reperfusion (I/R), I/R+CAPE. The experimental and surgical procedures in groups were performed as in the following: All rats were anesthetized intraperitoneally with xylazyn hydrochloride (4-5 mg/kg, Rompun, Bayer, Turkey) and ketamine HCl (40-50 mg/kg, Alfamine, Egevet, Turkey) and placed on heat blanket during surgical operation. Sham-control (sham-operated) group: Rats underwent only surgical intervention without bilateral common *carotid artery* (BCCA) occlusion. Briefly, the right and left common *carotid arteries* (CCAs) were isolated through a ventral midline cervical incision and separated carefully from vagosympathetic nerve. Ischemia-reperfusion (I/R) group: The common *carotid arteries* (CCAs) were isolated through a ventral midline cervical incision and separated carefully from vagosympathetic nerve by microsurgical procedures. Transient global cerebral ischemia was achieved by temporarily occluded (30 min) CCAs using atraumatic aneurysm clips and the opening in the skin was closed with wound clips. Reperfusion was achieved by declamping the arteries after 30 min. The surgery line was routinely closed. The rats in the sham-control and I/R groups were received intraperitoneally dimethyl sulfoxide (DMSO) at the dose of 15 µmol kg^{-1} twice, 1 h before occlusion and at 12 h of reperfusion. Ischemia-reperfusion + CAPE (I/R + CAPE) group: The rats in this group were subjected to ischemia/reperfusion with the same procedure in I/R group as mentioned above and treated with CAPE (Sigma, Germany). CAPE was dissolved in sterile DMSO (Sigma, Germany) and administered intraperitoneally at the dose of 15 µmol kg^{-1} twice, 1 h before occlusion and at 12 h of reperfusion. The CAPE doses used were chosen on the basis of previous experiments [2,11]. Body temperature was maintained at approximately 35.2±0.4°C with a heating pad until the animal had recovered from surgery. The rats were sacrificed 24 h after reperfusion with the anesthetic procedure reported above. Venous blood samples (5 ml) were taken by cardiac puncture into tubes with EDTA. Plasma was separated by centrifugation at 3.000 rpm for 15 min and was stored at -20°C until use. Immediately after taking blood specimen, brain was carefully removed, washed with ice-cold physiological saline and were rapidly stored at -20°C until analyses.

Assessments of Neurologic Deficit and Behavior

A neurologic evaluation was performed 24 h after the onset of the experiments by an investigator blinded to the study groups, using a neurologic deficit score (NDS) as described previously, with several modifications [3,12-14]. The four categories of reactions and the functions with associated tested were: the general behavioral deficit, cranial nerve reflexes, sensory-motor deficit, balance and coordination. The presence or absence of the appropriate reaction was scored. See *Table 1* for the exact procedure used. The NDS could range from 0 to 100, an NDS of 0 reflects normal brain function and an NDS of 100 reflects brain death.

Tissue Homogenization

Tissue samples were homogenized in a PBS buffer (pH 7.0) containing complete protease inhibitor mixture (Sigma, Germany). Homogenates were centrifuged at 4°C, 15.000 rpm for 10 min and the soluble fraction was retained. Protein concentrations of supernatants were measured by the method of Bradford [15] using bovine serum albumin as a standard.

The Analysis of Oxidant/Antioxidant Stress Markers

Lipid peroxidation levels were assessed by measuring malondialdehyde (MDA) concentration in tissues (µmol/mg protein) [16]. The method was based on thiobarbituric acid (TBA) reactivity. 2.5 mL of 20% trichloroacetic acid was added to the 0.5 mL of plasma and then 1 mL of 0.675% TBA was added. The coupling of lipid peroxide with TBA was carried out by heating at 95°C water bath for 30 min. After cooling in cold water, the resulting chromogen was extracted with 4.0 mL of n-butyl alcohol by vigorous shaking. Separating of the organic phase was facilitated by centrifugation at 3.000 rpm for 10 min and its absorbance was determined at 535 nm by spectrophotometer.

Catalase (CAT) activity was measured according to the method of Luck [17]. One unit of CAT activity was defined as the amount of enzyme required to decompose 1 mol of H_2O_2 in 1 min in a tube containing 2.95 mL of a freshly prepared 30% H_2O_2 in phosphate buffer (pH 7.0), 50 µl of tissue supernatant or plasma were added. The rate of decomposition of H_2O_2 was measured spectrophotometrically at 240 nm for 1 min. Using the reaction time (Δt) of the absorbance ($A1$ and $A2$), the following equation was generated to calculate the rate constant (k): $k = (2.3/\Delta t)(\log A1/A2)$. The enzyme activity was expressed as k/mg protein in tissues. Total superoxide dismutase (SOD) activity in the homogenates was determined according to the method of Sun and colleagues [18]. The method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generation. The enzyme activity was measured in the ethanol phase of the lysate after addition of 1.0 ml ethanol/chloroform mixture (5/3, v/v) to the same amount of sample and the tubes were centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in NBT reduction rate. SOD activity was expressed as U/mg protein in tissues. Nitric oxide (NO) concentration in plasma (µmol/L) and tissue (µmol/mg protein) samples were determined indirectly by measuring the nitrite levels based on Griess reaction [19]. Samples were firstly deproteinized with 75 mmol zinc sulphate. Total nitrite was determined by spectrophotometer at 545 nm after conversion of nitrate to nitrite by copperized cadmium granules. Xanthine oxidase (XO) activity was measured as the rate of uric acid production when xanthine was incubated with tissue homogenates (U/g protein) [20]. Glutathione peroxidase (GSH-Px) activity was detected in the tissue homogenates by a kinetic method using a commercial kit (RANSEL by Randox Lab. UK). GSH-Px

Table 1. *Neurologic deficit scores (NDS) for rats with global cerebral ischemia-reperfusion*
Tablo 1. *Global beyin iskemi-reperfüzyonlu ratlarda nörolojik hasar skorları*

Neurologic Evaluations	Function Studied	Grades and Points
1. General behavioral deficits	Consciousness	Explore spontaneously (0)
		Sleepy (5)
		Epileptic seizures (10)
		No attempt (comatose) (20)
	Respirations	Normal (0) Abnormal (10)
	Feeding	Normal (0) Absent (5)
	Water intake	Normal (0) Absent (5)
2. Cranial nerve reflexes	Vision (follows hand)	Present (0) Absent (5)
	Corneal reflex	Present (0) Absent (5)
	Whisker movement	Present (0) Absent (5)
	Hearing (turning to clapped hands)	Present (0) Absent (5)
3. Sensory motor deficits	Leg and tail movements	Normal (0) Paralysis (20)
4. Balance and coordination	Ability to walk	Present (0) Absent (5)
	Righting reflex	Present (0) Absent (5)
	Climbing the wall of a wire cage	Normal (0) Weakness of one limb (5) Weakness of more than one limb (10)
Total score		100

activity was expressed as U/mg protein. The intracellular cAMP concentration was determined in the brain homogenates (Cayman Chemical, USA) using enzyme-linked immuno-sorbent assay (ELISA).

RNA Isolation and Real-Time QRT-PCR Analyses

Transcription levels of samples were performed by a qRT-PCR system (CFX96 Touch™-USA). Total RNA from tissues was extracted using TRIZOL reagent (Sigma, USA) according to the manufacturer's instructions. The rat primer sets (Thermo Electron Corporation, Germany) used for PCR reactions are given in *Table 2*. B-Actin was used as endogenous control, and each sample was normalized on the basis of its β-actin content. Cycling conditions included reverse transcription at 42°C for 30 min, incubation at 94°C for 30 s and 40 cycles of 94°C for 10 s [a denature temperature of PCR profile at 95°C for 5 s, according to the manufacturer's instructions (SYBR Green Quantitative RT-PCR Kit, Sigma)] and 60°C for 10 s for annealing and 72°C for 30 s extension step. The cycle number required to achieve a definite fluorescence signal (crossing point, CP) was calculated by the second derivative maximum method (CFX Manager™ software, qbase^{PLUS}).

Statistics

Statistical analyses were accomplished with the use of the SPSS computer program (version 13.0). All data were expressed as mean±S.E. The differences between groups in biochemical, molecular and neurological deficit scores were evaluated using one-way analysis of variance (ANOVA) with Tukey's tests for post hoc comparisons. *P* values less than 0.05 were considered statistically significant.

RESULTS

CAPE Attenuates Ischemia-Induced Neurologic Deficit Scores

In the current study, three rats died as total, ischemia/reperfusion (I/R) group: 2, and I/R + CAPE group: 1. No rat died in the sham-control group. Ischemia-induced neurologic deficit scores were significantly higher than the sham-operated rats (67.4±12.3, 18.5±1.3, respectively, *Fig. 1*, P<0.01). CAPE administration was able to attenuate ischemia-induced neurologic deficit scores 24 h after I/R (46.4±12.1, 67.4±12.3, respectively, *Fig. 1*, P>0.05).

CAPE Slightly Modulates the Oxidant and Antioxidant Defense System

Transient global cerebral ischemia caused to significant increase in NO levels in brain homogenates, and this elevation was significantly inhibited by CAPE treatment (P<0.05, *Table 3*). On the other hand, NO production was decreased in plasma samples of occluded animal, but CAPE reserved plasma NO suppression (P<0.05, *Table 3*). CAPE non-significantly decreased ischemia-induced cerebral cortex MDA concentration *(Table 3)*. The activity of cerebral cortex XO significantly increased in the ischemic group (P<0.05, *Table 3*), but CAPE non-significantly decreased this elevation. Significant increase in cerebral cortex SOD activity in the ischemia group (P<0.01, *Table 3*) was not effectively changed by CAPE. While there was no difference in the cerebral cortex CAT activity between the groups, CAPE significantly prevented the reduction in GSH-Px activity caused by ischemia (P<0.05, *Table 3*).

Table 2. *List of primer sequences used for RT-PCR analyses*
Tablo 2. *RT-PCR analizi için kullanılan primer dizileri*

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
PDE4A	GCG GGA CCT AGC TGA AGA AAT TCC	CAG GGT GAG TCC ACA TCG TGG
PDE4B	CAG CTC ATG ACC CAG ATA AGT GG	GTC TGC ACA AGT GTA CCA TGT TGC G
PDE4C	ACT GAG TCT GCG CAG GAT GG	CAC TCC TCT TCC TCT GCT CTC CTC
PDE4D	CCC TCT TGA CTG TTA TCA TGC ACA CC	GAT CCT ACA TCA TGT ATT GCA CTG GC
β-actin	CAT CGT CAC CAA CTG GGA CGA C	CGT GGC CAT CTC TTG CTC GAA G

Table 3. *Effects of CAPE administration (15 µmol kg^{-1}) on the antioxidant-oxidant enzyme activities and cAMP levels in cerebral cortex, and NO in plasma after 24 h ischemia-reperfusion injury*
Tablo 3. *İskemi-reperfüzyon hasarından 24 saat sonra plasma NO ile cerebral cortex antioksidant-oxidant enzim aktiviteleri ve cAMP düzeyleri üzerine KAFE (15 µmol kg^{-1})' in etkileri*

Groups	MDA (µmol/mg protein)	NO (µmol/mg protein)	NO (µmol/L plasma)	SOD (U/mg protein)	CAT (k/mg protein)	GSH-Px (U/mg protein)	XO (U/g protein)	cAMP (pmol/mg protein)
Sham-control	0.49±0.04	2.80±0.83	7.5±1.63	0.26±0.01	0.191±0.02	10.8±0.93	0.54±0.04	2.13±0.11
I/R	0.67±0.06	5.04±0.48*	2.1±0.86‡	0.53±0.04†	0.186±0.02	6.9 ±0.29‡	1.44±0.19$^{\#}$	1.98±0.14
I/R+CAPE	0.57±0.14	3.02±0.77	9.6±1.27	0.55±0.06$^{\#}$	0.219±0.04	10.6±1.22	1.32±0.19*	1.94±0.08

*Data were presented as mean ± S.E. from six rats in each group. * P<0.05, $^{\#}$ P<0.05, † P<0.01 vs sham-control group, ‡ P<0.05 vs sham-control and I/R+CAPE group,* ***I/R:*** *Ischemia-reperfusion*

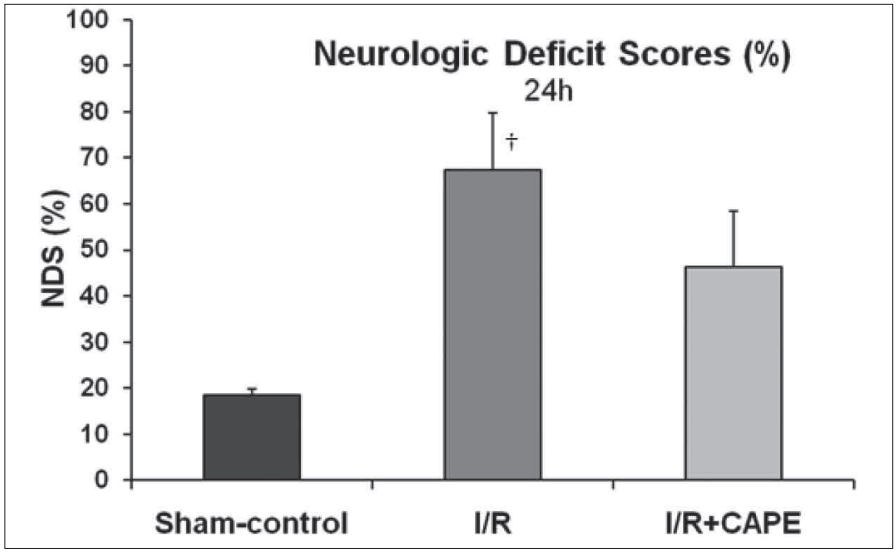

Fig 1. Effects of CAPE on neurologic deficit scores after 24 h ischemia-reperfusion injury. Ischemia-stimulated neurologic deficit scores were attenuated by CAPE. Data are presented as mean ± S.E. from seven rats in each group. † P<0.01 *vs* sham-control group. I/R: ischemia/reperfusion

Şekil 1. İskemi-reperfüzyondan 24 saat sonra nörolojik hasar skorları üzerine KAFE'in etkisi. İskemi ile uyarılan nörolojik hasar skorları KAFE tarafından azaltıldı. Değerler herbir gruptaki 7 rat'ın ortalama ± standart hatası olarak verildi. † P<0.01, sham-kontrol grubu ile karşılaştırıldığında. I/R: iskemi/reperfüzyon

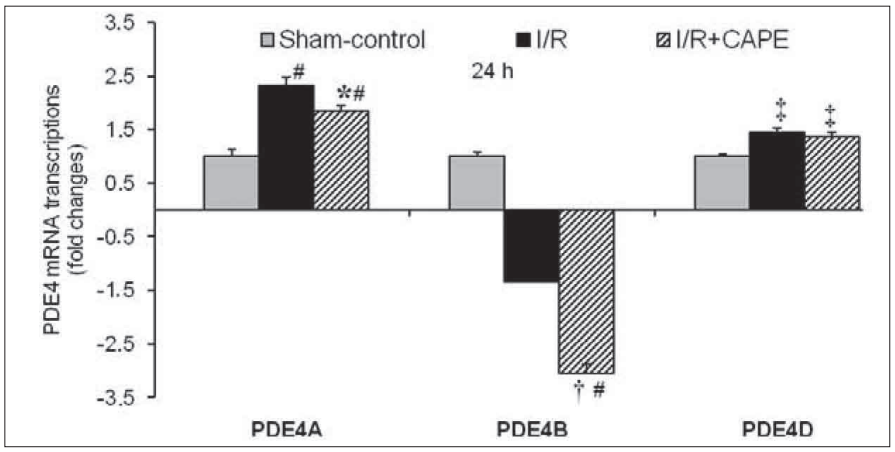

Fig 2. The fold change values of PDE4A, PDE4B and PDE4D mRNA transcriptions in qRT-PCR analyses after 24 h ischemia-reperfusion injury.

Global cerebral I/R compared to sham-control PDE4A and PDE4D transcriptions were elevated by 2.31 and 1.44-folds after 24 h, respectively. PDE4B transcription was decreased by 2.34-fold. CAPE compared to I/R significantly decreased PDE4A and PDE4B transcriptions by 1.25 and 2.26-folds, respectively. Non-significant decrease of PDE4D transcription was detected after 24 h. Neither global cerebral I/R nor CAPE unchanged PDE4C transcription in the rat cerebral cortex (data not shown)

Data were presented as mean ± S.E. from six rats in each group. * P<0.05, † P<0.001 *vs* I/R group; ‡ P<0.05, # P<0.001, *vs* sham-control group; I/R: Ischemia/reperfusion

Şekil 2. İskemi-reperfüzyon hasarından 24 saat sonra qRT-PCR analizinde PDE4A, PDE4B ve PDE4D mRNA kat değişim değerleri.

Sham-kontrol ile karşılaştırıldığında global beyin I/R, PDE4A ve PDE4D transkripsiyonlarını 24 saat sonra söylendiği sıra ile 2.31 ve 1.44 kat arttırdı. PDE4B transkripsiyonu 2.34 kat azaltıldı. I/R ile karşılaştırıldığında KAFE, PDE4A ve PDE4B transkripsiyonlarını söylendiği sıra ile 1.25 ve 2.26 kat önemli oranda azalttı. PDE4D transkripsiyonu 24 saat sonra önemsiz oranda azaldı. Global I/R ve KAFE rat beyin korteksinde PDE4C transkripsiyonunu değiştirmedi (veri gösterilmedi).

Değerler herbir gruptaki 6 rat'ın ortalama ± standart hatası olarak verildi. * P<0.05, † P<0.001, I/R grubu ile karşılaştırıldığında; ‡ P<0.05, # P<0.001, sham-kontrol grubu ile karşılaştırıldığında; I/R: İskemi/reperfüzyon

CAPE Inhibits Global Cerebral Ischemia-Induced Increases in PDE4 mRNA Expression

Ischemia-induced cerebral cortex cAMP levels were not changed by CAPE treatment *(Table 3)*. Global cerebral I/R compared to sham-control raised PDE4A ($P<0.001$) and PDE4D ($P<0.05$) mRNA transcrips by 2.31 and 1.44-folds in rat cerebral cortex 24 after I/R, respectively *(Fig. 2)*, and also decreased PDE4B by 2.34-fold (*Fig. 2*, $P<0.001$). Whereas, CAPE treatment significantly decreased PDE4A ($P<0.05$) and PDE4B ($P<0.001$) expressions compared to I/R by 1.25 and 2.26-folds 24 h after I/R, respectively *(Fig. 2)*. CAPE has also non-significantly decreased PDE4D transcription by 1.05-fold. Neither ischemia-reperfusion nor CAPE unchanged *the* PDE4C mRNA transcription in the rat cerebral cortex (data not shown).

DISCUSSION

So far, CAPE treatment has not been reported how it affects the levels of cAMP and PDE4 transcripts. We found that the global cerebral ischemia/reperfusion (I/R) and CAPE treatment non-significantly decreased in cerebral cortex cAMP levels after 24 h reperfusion *(Table 3)*. Our cAMP *findings* are partially *consistent* with those of Choi et al.[21] 24 h after I/R. As similar to that reported in an earlier study [22], CAPE treatment may influence the cAMP levels by increasing activity of cAMP-responsive element binding protein, and thus it may inhibit ischemia-induced oxidative stress and inflammation. A recent study [9] has demonstrated that cerebral ischemia led to increases in activity of PDE, primarily PDE4. However, it is still unknown whether specific PDE4 subtypes are differentially expressed after global cerebral I/R injury in rat cerebral cortex. This study firstly explains that the cerebral I/R significantly raised PDE4A and PDE4D expressions in the rat cerebral cortex at 24 h reperfusion, and also PDE4B mRNA was decreased *(Fig. 2)*. Accordingly, a more recent study reports increased PDE4D expression following global cerebral ischemia [23]. In addition, despite different experimental models, our data are in agreement with PDE4A increases newly reported by traumatic brain injury [24]. The phosphodiesterase (PDE4) is the predominant PDE isozyme in various leukocytes and plays an important role in the regulation of inflammatory cell activation [7]. Consistent with our data previous studies reported that the brain PDE4A, PDE4B and PDE4D mRNA transcripts are highly expressed, whereas PDE4C is absent [25,26]. Furthermore, previous studies observed that chronic antidepressant treatment increased PDE4A and PDE4B gene expression in rat cerebral cortex, but PDE4D gene expressions were unchanged [25,27,28]. Additionally, our results firstly shown that CAPE treatment significantly decreased PDE4A and PDE4B transcripts 24 h after I/R compared to I/R *(Fig. 2)*. However, PDE4D transcription was not significantly changed *(Fig. 2)*. The current study clearly suggests that CAPE treatments produce the neuroprotective effect by reducing the levels of some PDE4 isosyme transcriptions with a mechanism similar to phosphodiesterase inhibitors, and also may be useful for the treatment of cerebral ischemia.

The present study clearly showed that the cerebral cortex NO production was stimulated 24 h after global cerebral I/R *(Table 3)*, and this was inhibited by CAPE treatment. *CAPE,* a structural derivative of *flavonoids,* possesses its antioxidant properties by inhibiting the gene expressions and/or catalytic activity of certain free radical producing enzymes such as NOS [10,29]. In consistent with the NO sera data presented here *(Table 3)*, Tsai and colleagues [1] noted that pretreatment with CAPE increased NO bioavailability in plasma at 24 h of reperfusion in rats subjected to focal and global brain ischemia. CAPE is able to inhibit the reaction of NO with superoxide anion to prevent the formation of peroxinitrites which is more toxic oxidant than either NO or superoxide anion alone [1]. It was previously reported that NO has protective effects during ischemic injury, although in a narrow concentration range, overproduction may facilitate or mediate neurotoxicity [5,6,30]. Indeed, NO could act with a dual action either protective or pro-oxidant [6]. This dual effect of NO in cerebral ischemic injury has been suggested that the effects depend on the stage of evolution of tissue damage and NOS isoforms. Immediately after induction of ischemia, NO is synthesized by endothelial NOS (eNOS) and neuronal NOS (nNOS), but later times after ischemia NO is synthesized by iNOS [5]. As previously reported [5,6], we suggest that an increase in NO production may be due to stimulation of all NOS subtypes expression in the brain. In addition, the increase in NO bioavailability induced by CAPE is attributed to its strong free radical scavenging ability in lipophilic environments.

Xanthine oxidase (XO) is an important oxidant enzyme which catalyzes the reduction of O_2 initiating to the formation of superoxide anion and H_2O_2. In the present study, ischemia-stimulated increases in the XO activity could be reduced by CAPE *(Table 3)*. A few studies have reported similar results in the XO activity in relation to I/R injury, which is time and dose dependent manner [1,11]. Similar to our study, it is demonstrated that ischemic circumstances lead to the accumulation of hypoxanthine and stimulates XO activity [29]. The activity of GSH-Px, which detoxifies H_2O_2 while oxidizing reduced GSH to oxidized GSSG, was depressed during I/R [4]. In the present study, cerebral cortex GSH-Px activity significantly decreased after 24 h ischemia. However, the decrease in the GSH-Px activity was prevented by CAPE treatment *(Table 3)*. The curative effect of CAPE on GSH-Px activity could be due to scavenging ROS produced during oxidative stress [31]. Superoxide dismutase (SOD) is an antioxidant enzyme which plays key role to convert the superoxide anion to less toxic compound H_2O_2 and molecular oxygen. Toyoda and Lee [32] showed that SOD activity enhanced in some

ischemic regions of brain parallel to this study *(Table 3)*. Horakova and colleagues [33] have reported a reduction in GSH-Px activity, but an increase in the SOD activity in rat brain ischemia model induced by the ligation of the common carotid artery for 260 min and followed by a reperfusion of 10 min. Another study [34] reported that MnSOD increased in hippocampus 24, 48 and 72 h after ischemia, coincident with the marked reduction in the activity of glutathione-related enzymes. In the presented study, CAPE exhibited a slight antioxidant effect with regarding to cerebral cortex SOD and CAT enzyme activities *(Table 3)*. Our CAT results are in agreement with the study of Mishra and colleagues [35]. It has also been explained the stimulation of antioxidant enzymes in brain I/R as the transient substrate induction [36]. Therefore, it may be explained that free radicals produced in moderate ischemia were not intense enough to affect the catalase activity which is kept in peroxisomes.

The neurological evaluations shown that CAPE treatment could attenuate ischemia-induced cerebral neurologic deficit scores *(Fig. 1)*. This finding is in agreement with our previous study showing that CAPE reduces the infarction percentage and neurological damage against focal permanent middle cerebral artery occlusion [11]. In addition to its antioxidant properties, this neuroprotective effect is supported further by the decrease of PDE4 isoforms in this current study. Therefore, we propose that CAPE plays a protective role for therapy against neuronal death after transient BCCA occlusion with its preconditioning and therapeutic effects.

In conclusion, these results suggest that CAPE administrations slightly modulate the antioxidant defense system and NO release in rat brain during peracute global cerebral ischemia-reperfusion injury. In addition, CAPE treatment produces the neuroprotective effect by reducing the levels of some PDE4 isosyme transcriptions.

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RESEARCH ARTICLE

Genotypic and Pathogenic Characterization of Newcastle Disease Viruses Isolated from Domestic Ducks in China [1]

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Abstract

In order to determine the prevalence of Newcaste Disease Virus (NDV) in ducks in Guangdong province of China, 10 NDVs were isolated in domestic ducks. Eight isolates were pathogenic as determined by MDT, ICPI and cleavage site of F protein. The F genes of 10 isolates were sequenced, all the genes and their deduced amino acids were compared with 32 reference strains. 5 isolates were clustered in genotype VII (VIId), 3 isolates were classified as genotype IX and 2 isolates were classified as genotype. The subgenotype VIId isolates possessed the motif 112R-R-Q-K-R-F117, and the genotype IX isolates possessed the motif 112R-R-Q-R-R-F117. The 8 NDV isolates exhibited a high ICPI, they were classified as velogenic type of NDVs and were collected between the years of 2006 and 2010. The genotype I isolates possessing the motif 112G-K-Q-G-R-L117 were collected in 2005, they were exhibited a low ICPI and were classified as lentogenic type of NDVs. The NDVs of subgenotype VII are now dominant and have been implicated in most of the recent ND outbreaks in duck farms in Guangdong province. These findings provide data on genetics and molecular evolution of NDV in ducks in China and emphasize importance of NDV surveillance for improving of strategies for the control of the disease.

Keywords: *Newcastle Disease Virus, Duck, Genotype, Pathogenicity*

Çin'de Evcil Ördeklerden İzole Edilen Newcastle Hastalığı Viruslarının Genotipik ve Patojenik Karakterizasyonu

Özet

Çin'in Guangdong Bölgesindeki ördeklerde Newcastle Hastalığı Virusu (NDV) prevalansını belirlemek amacıyla evcil ördeklerden 10 NDV izole edildi. MDT, ICPI ve F proteininin ayrılma yeri dikkate alınarak sekiz izolatın patojenik olduğu belirlendi. 10 izolatın F genlerinin sekansları yapıldı, tüm genler ve onlara ait amino asitler 32 referans türleri ile karşılaştırıldı. 5 izolat genotip VII'de toplanırken (VIId), 3 izolat genotip IX ve 2 izolat genotip olarak sınıflandırıldı. Subgenotip VIID izolatları 112R-R-Q-K-R-F117 motifine sahipken genotip IX izolatları 112R-R-Q-R-R-F117 motifi gösterdi. 8 NDV izolatı yüksek ICPI sergilerken bunlar NDV'nin velojenik tipi olarak sınıflandırıldı ve bu örnekler 2006 ile 2010 yılları arasında toplandı. 112G-K-Q-G-R-L117 motifine sahip olan genotip I izolatları 2005 yılında toplandı. Bunlar düşük ICPI gösterdi ve NDV'nin lentojenik tipi olarak sınıflandırıldı. Subgenotip VII'nin NDV'leri şimdi dominant olup Guangdong Bölgesindeki ördek çiftliklerinde son zamanlardaki çoğu ND salgınlarında belirlenmiştir. Bu bulgular Çin'de ördeklerdeki NDV'ün genetik ve moleküler gelişimi hakkında bilgi vermekte ve hastalığın kontrol stratejileri hakkında NDV takibinin önemini ortaya koymaktadır.

Anahtar sözcükler: *Newcastle Hastalığı Virusu, Ördek, Genotip, Patojenite*

INTRODUCTION

Newcastle disease (ND), which is caused by ND virus (NDV), is one of the most serious diseases affecting the commercial poultry industry around the world and has caused significant economic loss. NDV belongs to the

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Avulavirus genus within the family Paramyxoviridae and is designated avian paramyxovirus type 1(APMV-1), one of 12 identified APMVs serotypes [1,2]. The virus is enveloped with negative-stranded RNA genome of approximately 15 kb that encoding six structural proteins, including the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), an attachment protein, the hemagglutinin-neuraminidase, and a large polymerase protein (L). Additionally, two non-structural proteins, V and W are derived from P gene by a process called RNA editing respectively [3-6].

NDV strains can be classified into two major classes (class I and II) on base of genetic and antigenic analyses. The class I (1-9 genotypes) viruses are distributed world-wide in wild birds and chickens, and the class II (IX genotypes) viruses include most virulent viruses, some avirulent viruses and vaccine viruses [7,8]. The NDVs currently circulating worldwide exhibit multiple lineages and highly diversive geneticity [6,9].

ND has been classified as an Office International des Epizootics (OIE) "List A" disease [1]. Although NDV isolates are of a single serotype, and numerous live and inactivated vaccines have been developed to control the disease, naturally occurring pathogenicities from avirulent (lentogenic) to mildly virulent (mesogenic) and highly virulent (velogenic) are still in a wide range [9]. Highly virulent strains of NDV are not only found in poultry in China, but also circulate in a large number of countries worldwide. In addition, more avian species are naturally or experimentally susceptible to NDV, and some NDV strains are now becoming particularly virulent in avian species, e.g. duck, which had generally been resistant to clinical disease with virulent NDV infections [10].

The incidences of ND outbreaks in domestic ducks have gradually increased in different regions since the 1990s. It becomes more difficult to prevent and control the disease when NDV is circulating in different avian species [11-13]. In China, many isolates have been isolated from domestic ducks in recent years. Most of isolates are lentogenic and have not lead to great losses to duck farms. Occasionally when a velogenic strain is isolated, it is particularly urgent to evaluate potential risk to domestic ducks [14,15]. While there have insufficient knowledge of pathogenic characterizations and molecular analyses of NDV isolated from domestic ducks in China, and we also have limited knowledge of the relationship between the Chinese representative NDV isolated from chickens and the strains isolated recently from domestic ducks in China. Therefore, continuous surveillance of domestic ducks may help better understand the NDVs circulating in China.

In this study, to clarify the genotypic and pathogenic characterization, 10 NDVs were isolated from outbreaks in domestic ducks in China from 2005-2010, the full-length F gene of the isolates was amplified by RT-PCR and sequenced. F protein cleavage site sequences were analyzed. Molecular genetic analysis was performed to compare the evolution epidemiology and pathotype of these genes with those of reference strains published in GenBank.

MATERIAL and METHODS

Virus Isolation and Biological Characterization

Between the years of 2005 and 2010, ND-suspected field samples from different duck farms were collected. The viruses were isolated and purified by the limiting dilution method in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE) using standard procedures. The virus identity was confirmed by haemagglutination-inhibition (HI) assay with polyclonal chicken antiserum to NDV.

Pathogenicity Test

Intra-cerebral pathogenicity index (ICPI) tests in 1 day-old chicks were done as previously described. Initial pathotyping of the isolates involved virus inoculation of 9-day-old embryonated SPF chicken eggs to determined the mean death time (MDT) for the embryos. All tests were performed using the standard procedures devised to distinguish these viruses [1].

RNA Isolation and RT-PCR

Viral RNA was extracted directly from the allantoic fluid of the NDV-inoculated embryos with using HP total RNA purification kit (Omiga, US). The sense primer was F-A (5'-GCCATTGCYAAATACAATCC-3'), and the sequence S1-R (5'- GGCTCCTCTKACCG -TTCTAC-3') was used as antisense primer. The expected size of a PCR-amplified fragment is 1993 bp in length. Briefly, the PCR was performed in 50 uL reaction mixture. The PCR reactions were subjected to 30 cycles consisting of denaturation for 1 min at 94°C, annealing for 30 s at 53°C, and extension for 90 s at 72°C followed by a final extension cycle at 72°C for 8 min.

Cloning and Sequencing of PCR Products

RT-PCR products were analyzed on 1% agarose gels and sequenced after cloning into the pMD18-T (Takara, Dalian, China). F nucleotide in each NDV isolate was sequenced in the forward and reverse directions at least five times and the consensus sequence was determined.

Analysis of Nucleotide and Deduced Amino Acid Sequences

32 NDVs were chosed as reference strains which including current vaccine strains such as LaSota and Clone 30, classical viruses and typical prevailing strains isolated in China and other counties. The reference strains were classified as genotyes I-IX, and at least 2 strains were chosed every genotype. The detailed information and Genbank

accession numbers of NDV reference strains were shown in *Table 1*. Nucleotide and deduced amino acid sequences of full-length F genes of 10 NDV isolates and reference strains were aligned using MEGALIGN program in DNAStar software in this study. In all NDV stains (including 10 isolates and reference strains), the deduced amino acid residues (1-125) of F genes were aligned and compared, and the variation of amino acid residues (including F cleavage sites) were analyzed.

Phylogenetic Analysis

Comparative analysis of part of F aa residues (1-125) in all NDVs obtained in this study (including 10 isolates and 32 references strains). A phylogenetic tree was constructed using MEGA4.1 software (Molecular Evolutionary Genetics Analysis, version 4.0) by Neighbor-Joining method. The evolutionary distances were computed by Pair wise Distance method using the Maximum Composite Likelihood Model.

RESULTS

Virus Isolation and Identification

All 10 NDV strains were isolated from ND-suspected field samples from ducks of different breed and age in

Table 1.*The NDV preference strains and their accession numbers used for phylogenetic analysis*
Tablo 1. *NDV referans tipleri ve patojenite analizi için erişim numaraları*

NDV Isolates	Genotype	Host	Accession Number	Cleavage Site	Country
PHY-MLV42	I	Chicken	DQ097394	GKQGRL	Hungary
Ulster-67	I	Chicken	AY562991	GKQGRL	Ireland
V4	I	Chicken	AF217084	GKQGRL	Australia
B1	II	Chicken	AF375823	GRQGRL	American
Clone30	II	Chicken	Y18898	GRQGRL	American
LaSota	II	Chicken	AY845400	GRQGRL	China
AUS-Victoria-32	III	\	M21881	RRQKRF	Australia
Mukteswar	III	Chicken	EF201805	RRQRRF	N
Herts-33	IV	Chicken	AY741404	RRQRRF	England
Italien	IV	Chicken	EU293914	RRQRRF	Italien
Largo/71	V	Pet birds	AY562990	RRQKRF	American
44083/93	V	Anhinga	AY562986	RRQKRF	American
211472/02	V	Gamefowl	AY562987	RRQKRF	American
Argentian-97	VI	Pigeon	AY734536	RRQKRF	Argentina
Belgium248VB	VI	Pigeon	EF026584	RRQKRF	Belgium
Pigeon-1	VI	Pigeon	AJ880277	RRQKRF	Hungary
Dove Italy	VI	Dove	AY562989	RRQKRF	Italy
FP1-02	VIId	Duck	FJ872531	RRQKRF	China
JSD0812	VIId	Duck	GQ849007	RRQKRF	China
GM	VIId	Chicken	DQ486859	RRQKRF	China
SD09	VIId	Duck	HQ317395	RRQKRF	China
SDWF02	VIId	Duck	HM188399	RRQKRF	China
SF02	VIId	Goose	NC_005036	RRQKRF	China
ZJ	VIId	Goose	AF431744	RRQKRF	China
TW2000	VIIe	Chicken	AF358786	RRQKRF	China/Taiwan
UAE-AE232-96	VIIb	Chicken	AF109884	KRQRRF	Britain
D-83-95	VIIa	\	AF001118	RRQKRF	Hungary
TW-84C	VIIc	\	AF083965	RRQKRF	China/Taiwan
AF2240	VIII	\	AF048763	RRQKRF	Malaysia
F48E9	IX	Chicken	AY508514(F)	RRQRRF	China
JS-1-97	IX	Chicken	FJ436305	RRQRRF	China
FJ-1-85	IX	Chicken	FJ436304	RRQRRF	China

Note: *GKQGRL, Gly-Lys-Gln-Gly-Arg-Leu; GRQGRL, Gly- Arg -Gln-Gly-Arg-Leu, RRQKRF; Arg - Arg -Gln- Lys -Arg-Leu; RRQRRF, Arg - Arg -Gln- Arg -Arg-Leu; KRQRRF, Lys - Arg -Gln- Arg -Arg-Leu*

2005-2010 in Guangdong province *(Table 2)*. The isolates were characterized by HI assays, and conformed by genomic sequencing and the nucleotides BLASTn analysis.

Pathogenicity Analysis

Table 2 presents the initial biological characterizations of 10 NDV isolates, including ICPI and MDT. 8 Guangdong field isolates of NDV with MDT of 45.8-58.8 h and with ICPI of 1.7-1.96 were classified as velogenic NDVs, 2 isolates were classified as lentogenic NDVs with MDT beyond 168 h and with ICPI of 0.15 and 0.21. The results were consistent with cleavage site motifs. *Table 2* also lists the initial biological characterizations of the 10 NDV isolates.

Phylogenetic Analysis

A phylogenetic tree was constructed based on the nt sequences of the full-length F gene in all the 10 field isolates of NDV and the corresponding region of the other 32 NDV strains retrieved from GenBank. The field isolates and reference strains were classified as genotyes I-IX. 5 field isolates were classified into genotype VII and all these NDVs were further subclassified into subgenotype VIId (Duck/CH/GD/SD-06, Duck/CH/GD/SD-06 II, Duck/CH/GD/SD-09, Duck/CH/GD/JY-08, Duck/CH/GD/SS-10). 3 isolates were classified into genotype IX (Duck/CH/GD/FS-06, Duck/CH/GD/YF-09, Duck/CH/GD/NH-10) and 2 isolates (Duck/CH/GD/SH, Duck/CH/GD/SZ-05) were classified into genotype I *(Fig. 1)*.

The Divergence of nt and aa Sequences

The nt and deduced aa sequences of the 10 isolates were compared. The nt and aa sequences data of 32 NDV reference strains obtained from the GenBank database *(Table 1)* were used for comparison. The results of sequence analysis showed that the total length of F gene is 1617 bp, the homology of nt and aa sequences between 10 isolates and 32 reference strains were 83.5%-99.8% and 86.5%-99.5%, respectively. Further analysis was also carried out. 5 isolates (Duck/CH/GD/SD-06, Duck/CH/GD/SD-06, Duck/CH/GD/JY-08, Duck/CH/GD/SD-09, Duck/CH/GD/SS-10) showed great nt and aa identities (96.9-98.8% and 96.8-98.9% respectively) with the velogenic NDV GM strain (Accession number DQ486859, genotype VII). 3 isolates (Duck/CH/GD/FS-06, Duck/CH/GD/YF-09, Duck/CH/GD/NH-10) were highly nt and aa sequences similar (99.5-99.7% and 99.1-99.3%, respectively) to NDV F48E9 strain (Accession number AY508514, genotype IX). 10 isolates had homology of 83.5-89.2% and 86.6-92.6% respectively at nt and aa level with strain LaSota (Accession number AY845400, genotype II), the common vaccine strain used in China. Of all the 10 isolates, 2 strains (Duck/CH/GD/SH-05, Duck/CH/GD/SZ-05) shared greater nt and aa identities (88.9-89.2% and 92.4-92.6%, respectively) with LaSota than other 8 NDV strains *(Fig. 2)*.

Amino acid Variation of the F Proteins of Recent NDV Isolates

Proteolytic cleavage site motifs (residues 112-117) of F protein in the 10 isolates were analyzed. The F cleavage sites in 5 NDV strains isolated in 2008, 2009 and 2010 (Duck/CH/GD/SD-06, Duck/CH/GD/SD-06 II, Duck/CH/GD/SD-09, Duck/CH/GD/JY-08, Duck/CH/GD/SS-10) possessed aa sequence ^{112}R-R-Q-K-R-F^{117}. The motif is commonly found in strains that are highly virulent in chickens, especially in genotype VII viruses. The 3 NDVs (Duck/CH/GD/FS-06, Duck/CH/GD/YF-09, Duck/CH/GD/NH-10) isolated in 2006, 2009 and 2010 exhibited the sequence motif ^{112} R-R-Q-R-R-F ^{117}, which is another common motif in the other virulent NDVs including strain F48E9 (a genotype IX virus). Additionally, 2 NDV strains (Duck/CH/GD/SH-05, Duck/CH/GD/SZ-05) isolated in 2005 were shown to have a lentogenic motif (^{112}G-K-Q-G-R-L^{117}) composed of 2 basic amino acids at the F cleavage site *(Table 2)*.

Table 3 shows the aa residues 1-125 of F protein of different genotypes. An important aa residue substitution at the highly conserved region of Q114K was noted in the five isolates (Duck/CH/GD/SD-06 I, Duck/CH/GD/SD-06 II, Duck/CH/GD/SD-09, Duck/CH/GD/JY-08, Duck/CH/

Table 2. *Details of the 10 NDV isolates investigated in this study*
Tablo 2. *Bu çalışmada araştırılan 10 NDV izolatın detayları*

Strain	City	Year of Isolation	MDT/h	ICPI	Cleavage Site	Genotype
Duck/CH/GD/SH-05	Sihui	2005	>168h	0.15	GRQGRL	I
Duck/CH/GD/SZ-05	Shenzhen	2005	>168h	0.21	GKQGRL	I
Duck/CH/GD/SD-06 I	Shunde	2006	51.75h	1.94	RRQKRF	VII
Duck/CH/GD/SD-06 II	Shunde	2006	54.86h	1.78	RRQKRF	VII
Duck/CH/GD/FS-06	Foshan	2006	48h	1.95	RRQRRF	IX
Duck/CH/GD/JY-08	Jieyang	2008	45.8	1.96	RRQKRF	VII
Duck/CH/GD/YF-09	Yunfu	2009	57.6	1.93	RRQRRF	IX
Duck/CH/GD/SD-09	Shunde	2009	56.4	1.95	RRQKRF	VII
Duck/CH/GD/SS-10	Sanshui	2010	51.6	1.89	RRQKRF	IX
Duck/CH/GD/NH-10	Nanhai	2010	58.8	1.7	RRQRRF	VII

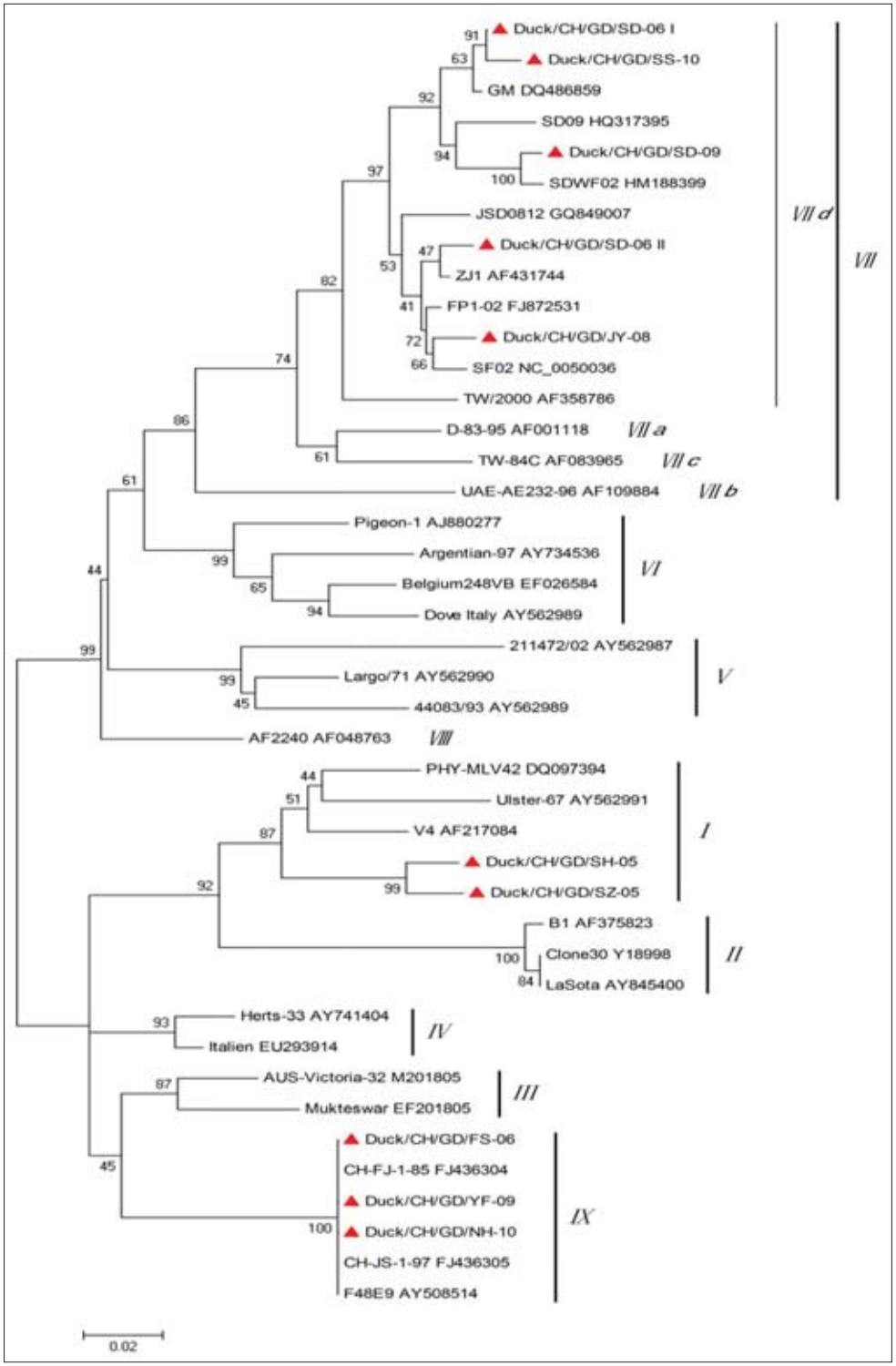

Fig 1. Phylogenetic tree of the nt sequences of NDV strains on a variable portion (nt1-374) of the F gene. (▲) The recent Chinese NDV strained were isolated in domestic ducks in this study in 2005-2010. Accession number and other background information of the reference strains are in *Table 1* and *Table 2*

Şekil 1. F geninin değişken kısmındaki (nt1-374) NDV'nin nt sekansının filogenetik ağacı. (▲) Bu çalışma kapsamında güncel Çin NDV türleri 2005-2010 yılları arasında evcil ördeklerden izole edildi. Referans türlerinin erişim numarası ve diğer bilgileri *Tablo 1* ve *Tablo 2*'de yer almaktadır

GD/SS-10) of subgenotype VIId of NDVs. The aa residue changed at position 3 (S to F) in 3 isolates and at position 24 (S to G) in 2 isolates in the 5 isolates mentioned above. It is interesting to note that one aa residue is unique to 3 isolates of genotype IX at aa position 3 (S to P). One aa residue is changed at amino acid position 24 (S to C) to 2 isolates of genotype. These mutations may alter the pathogenicity and antigenicity of recent Guangdong NDVs isolated in domestic ducks *(Table 3)*.

DISCUSSION

The first ND outbreak was recorded in poultry in 1926. Since then, four major epizootics occurred in the world causing considerable economic losses to poultry industry [16,17]. In China, outbreak of Newcastle disease (ND) was first reported in poultry in 1928, the standard strain F48E9 was isolated in 1948 for the first time. There often, ND became endemic in chicken flocks and large-

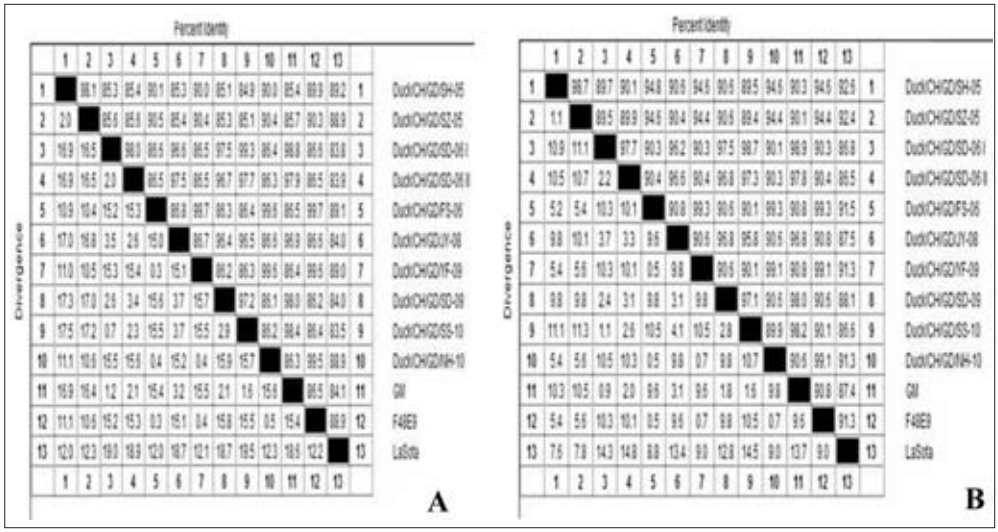

Fig 2. A- Analysis of nucleotide sequences (nt 1-375) between the 10 isolates and 3 reference strain (GM, LaSota and F48E9), **B-** Analysis of amino acid sequences (aa 1-125) between the 10 isolates and 3 reference strains (GM, LaSota and F48E9)

Şekil 2. A- 10 izolat ile 3 referans türü (GM, LaSota and F48E9), arasında nükleotid sekanslarının analizleri (nt 1-375), **B-** 10 izolat ile 3 referans türü (GM, LaSota and F48E9), arasında amino asit sekanslarının (aa 1-125) analizi

scale ND outbreaks were controlled with the wide spread use of ND vaccines. However, ND is still recognized as a major disease of poultry, for the disease is enzootic in some areas and non-typical ND infections occur frequently [18-20].

In the past twenty years, more and more hosts were found susceptible to NDVs. At present, over 200 avian species are naturally or experimentally susceptible to NDVs, e.g. duck, geese, green-winged teal and wood duck. Wild aquatic bird species are considered the natural reservoir of NDVs, some reports have revealed that wild aquatic birds may play an important role in the evolution of NDV [8,21]. The birds mostly harbor lentogenic NDVs, the strains apparently have the potential to become velogenic after transmission and circulation, and the virulence of these viruses has increasing trends. In ducks, many NDVs have been isolated in different country in recent years, studies on genetic diversity among the isolates revealed that most of the NDVs belong to class I with low virulence, occasionally a velogenic strain is isolated [15,22-24]. Due to the lack of surveillance, little is known about distribution, pathogenic and molecular characterizations of NDV in domestic ducks.

This study presents the characterization of NDVs isolated from domestic ducks in Guangdong province of China. It highlights the importance of domestic ducks in the epizootiology of ND. It has been reported that the virulence of NDV is associated with ICPI and the cleavage site in the F protein. ICPI of 0.7 or greater in 1 day-old chicken or presence of three basic amino acids (R or K) at the F protein cleavage site between aa residues 113 and 116 indicate the virulent form of NDV. In this study, we determined the virulence of 10 isolates on base of the criteria. Our results showed that the F cleavage sites in 5 NDV isolates possessed a virulent amino acid sequence 112R-R-Q-K-R-F117. 3 isolates exhibited another virulent sequence motif 112 R-R-Q-R-R-F117, the 8 field isolates in current study exhibited MDT of 45.8-58.8 h in embryonated chicken eggs and ICPI of 1.7-1.96 were classified as velogenic NDVs. The rest of 2 NDV isolates were shown to have a lentogenic motif (112G-K-Q-G-R-L117) composed of 2 basic amino acids at the F cleavage site, they were classified as lentogenic NDVs also with MDT beyond 168 h and with ICPI of 0.15 and 0.21. The finding indicate that the domestic ducks can harbour virulent strains of NDV and that it consequently may constitute a serious threat to the commercial duck farms. Further analysis showed that some aa residues in F protein of different genotypes changed. These mutations may alter the pathogenicity and antigenicity of recent Guangdong NDVs isolated in domestic ducks [25,26].

Sequence comparison and phylogenetic analysis of the 8 NDV isolates were used to predict the genotypes and to determine the origin of NDV outbreaks. Our results showed that 5 isolates belong to genotype VIId, and indicated great nt and aa identities (96.9-98.8% and 96.8-98.9%, respectively) with the velogenic strain GM. 3 isolates are genotype IX, shared high nt and aa similarities (99.1-99.7% and 99.1-99.3%, respectively) to F48E9. Two strains belonged to genotype I had a higher homology than 8 isolates mentioned above at nt and aa level with strain LaSota (genotype II). These results are not in agreement with some studies reporting the detection of lentogenic NDVs in wild birds and domestic ducks [7,8,22,24,27,28]. But the results are consistent with some other reports with the detection of velogenic NDVs in wild birds and domestic ducks, for example, Zhang et al.[15], reported subgenotype VIId of NDV as a predominant genotype spread in waterfowl in China and Yang et al.[29] discovered the presence of VII genotype NDVs in wild in Serbia.

In China, the most commonly used live vaccine LaSota and Clone-30 belong to genotype II, while in the past decade, the predominant NDV strains were genotype VII viruses in China. The prevailing field NDV strains have significant differences from the current vaccine strains in their biology, serology and genetics, which might

Table 3. *Amino acid variation of F protein (aa 1-125) of different genotypes*
Tablo 3. *Değişik genotiplerin F proteininin (aa 1-125) amino asit varyasyonu*

Majority Sequences	**Positions**																								
	3	**4**	**5**	**8**	**9**	**11**	**16**	**17**	**19**	**20**	**22**	**24**	**26**	**30**	**52**	**71**	**101**	**104**	**106**	**107**	**112**	**115**	**117**	**121**	**124**
	S	**K**	**P**	**R**	**I**	**A**	**I**	**T**	**I**	**M**	**I**	**S**	**I**	**S**	**I**	**K**	**R**	**G**	**V**	**T**	**R**	**K**	**F**	**I**	**S**
Ulster-67	-	R	S	-	-	V	T	V	V	A	E	-	V	-	-	-	-	E	-	-	G	G	L	-	G
PHY-MLV42	P	R	S	-	T	-	T	V	L	V	A	-	V	-	-	-	-	E	-	-	G	G	L	-	G
V4	-	R	S	-	-	V	T	V	V		A	-	V	-	-	-	-	E	-	-	G	G	L	-	G
Duck/CH/GD/SH-05	-	R	S	-	-	V	T	V	V	A	A	C	V	-	-	-	-	E	-	-	G	G	L	-	G
Duck/CH/GD/SZ-05	-	R	S	-	-	V	T	V	V	A	A	C	V	-	-	-	-	E	-	-	G	G	L	-	G
Clone30	-	-	-	K	N	-	T	I	V	A	V	-	-	N	-	-	-	E	-	-	G	G	L	-	G
B1	-	R	-	K	N	-	T	I	V	A	V	-	-	N	-	-	-	E	-	-	G	G	L	-	G
LaSota	-	R	-	K	N	-	T	I	V	A	V	-	-	N	-	-	-	E	-	-	G	G	L	-	G
AUS-Victoria-32	P	R	S	-	-	I	T	I	-	A	A	-	V	-	-	-	-	E	-	-	-	-	-	-	-
Mukteswar	P	R	S	-	-	V	T	I	-	T	A	-	V	-	-	-	-	E	-	-	-	R	-	-	-
Italien	-	R	S	-	-	V	-	I	-	A	T	-	-	-	-	-	-	E	-	-	-	R	-	-	-
Herts-33	-	R	S	-	-	V	-	I	-	V	T	-	-	-	-	-	-	E	-	-	-	R	-	-	-
211472/02	-	-	-	W	-	V	-	-	T	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
44083/93	-	R	-	-	L	V	T	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
Largo/71	-	-	-	-	L	V	-	-	-	T	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-
Dove Italy	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-
Belgium248VB	-	-	-	-	-	-	-	-	-	T	V	-	-	-	-	-	-	-	-	S	-	-	-	-	-
Argentian-97	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-
Pigeon-1	-	-	-	-	-	V	-	-	T	T	-	-	-	-	-	-	-	-	-	S	G	-	-	-	-
D-83-95	-	-	-	-	T	V	-	-	-	-	-	-	-	-	-	-	K	-	-	S	-	-	-	V	-
UAE-AE232-96	-	-	-	-	-	-	S	N	V	-	-	-	-	-	-	-	-	E	-	S	K	R	-	V	-
TW-84C	-	-	S	G	S	V	-	-	-	-	-	-	-	-	-	-	K	-	-	S	-	-	-	V	-
JSD0812	-	-	-	-	-	-	-	-	T	-	-	G	-	-	V	R	K	-	-	S	-	-	-	V	-
ZJ	-	-	L	-	-	-	-	-	-	-	-	G	-	-	V	R	K	-	-	S	-	-	-	V	-
TW2000	-	R	S	-	-	-	-	-	-	-	-	-	-	-	V	-	K	-	-	S	-	-	-	V	-
SF02	-	R	-	-	-	-	-	-	-	-	-	G	-	-	V	R	K	-	-	S	-	-	-	V	-
SDWF02	-	-	-	W	-	-	-	-	-	-	-	-	-	-	V	R	K	-	-	S	-	-	-	V	G
SD09	-	-	-	-	-	-	-	-	A	-	-	-	-	-	V	R	K	-	-	S	-	-	-	V	-
GM	F	-	-	-	-	-	-	-	-	-	-	-	-	-	V	R	K	-	-	S	-	-	-	V	-
FP1-02	-	-	-	-	-	-	-	-	-	-	-	G	-	-	V	R	K	-	-	S	-	-	-	V	-
Duck/CH/GD/SD-06 I	F	-	-	-	-	-	-	-	-	-	-	-	-	-	V	R	K	-	-	S	-	-	-	V	-
Duck/CH/GD/SD-06 II	F	-	L	-	-	-	-	-	-	-	-	G	-	-	V	R	K	-	-	S	-	-	-	V	-
Duck/CH/GD/JY-08	-	-	-	-	-	-	V	-	-	-	-	G	-	-	V	R	K	-	-	S	-	-	-	V	-
Duck/CH/GD/SD-09	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	R	K	-	-	S	-	-	-	V	G
Duck/CH/GD/SS-10	F	-	-	-	-	-	-	-	-	-	-	-	-	-	V	R	K	-	-	S	-	-	-	V	-
AF2240	-	-	S	-	-	T	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-
F48E9	-	-	S	N	V	-	T	V	-	A	A	-	V	N	-	-	-	E	A	-	-	R	-	-	-
JS-1-97	-	-	S	N	V	-	T	V	-	A	A	-	V	N	-	-	-	E	A	-	-	R	-	-	-
FJ-1-85	-	-	S	N	V	-	T	V	-	A	A	-	V	N	-	-	-	E	A	-	-	R	-	-	-
Duck/CH/GD/FS-06	P	-	S	N	V	-	T	V	-	A	A	-	V	N	-	-	-	E	A	-	-	R	-	-	-
Duck/CH/GD/YF-09	P	-	S	N	V	-	T	V	-	A	A	-	V	N	-	-	-	E	A	-	-	R	-	-	-
Duck/CH/GD/NH-10	P	-	S	N	V	-	T	V	-	A	A	-	V	N	-	-	-	E	A	-	-	R	-	-	-

Only sequences that differ from the majority sequences are shown. Strains in bold are isolated and characterized in the present study. The representative strains are from published data and their source reference is presented in Table 1

be considered as major reasons for the ND outbreaks, and brought enormous pressure for NDV control and prevention.

In summary, we have demonstrated that more than one genotype of NDVs are circulating in the domestic ducks in Guangdong province of China, and NDV isolates of subgenotype VIId are predominant strains. It is of particular importance to characterize epidemic strains in domestic ducks and identify new candidates for vaccine upgrades which are efficacious and provide adequate cross protection.

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RESEARCH ARTICLE

Mutagenecity of Enniatin A1 and B1 Mycotoxins in Ames Salmonella Microsome Test [1]

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Abstract

Mycotoxins are secondary metabolites produced by microfungi like *Aspergillus, Penicillium, Fusarium, Alternaria*. The enniatins are cyclic peptide mycotoxins and have produced by several strains of *Fusarium* sp. They have got biological activities like acting as enzyme inhibitors, antifungal and antibacterial agents. In this research the mutagenic effects of enniatin A1 and B1 were investigated with Ames assay. Ames is a test system which can detect mutations at celluler level. In this test *Salmonella typhimurium* TA98 and *Salmonella typhimurium* TA100, both in the presence and absence of S9 metabolic activation was used. Five different concentrations of Enniatin A1 and B1 (12.5 µM, 25 µM, 50 µM, 100 µM, 200 µM) were exposed to these strains. In all test strains (*Salmonella typhimurium* TA98 and *Salmonella typhimurium* TA100) Enniatin A1 and B1 did not show mutagenic effect.

Keywords: *Ames test, Enniatin A1, Enniatin B1, Salmonella typhimurium*

Enniatin A1 ve B1 Mikotoksinlerinin Ames Salmonella Mikrozom Testi ile Mutajenitesi

Özet

Mikotoksinler *Aspergillus, Penicillium, Fusarium, Alternaria* gibi bazı mikrofunguslar tarafından üretilen sekonder metabolitlerdir. Enniatinler birkaç *Fusarium* suşu tarafından üretilir ve siklik peptid mikotoksinlerdir. Enniatinler antifungal, antibakteriyel ajan ve enzim inhibitörü olarak hareket etmek gibi biyolojik aktivitelere sahiptir. Bu çalışmada Enniatin A1 ve B1'in mutajenik etkileri Ames testiyle araştırıldı. Ames hücresel seviyedeki mutasyonları belirleyen bir test sistemidir. Bu testte *Salmonella typhimurium* TA98 ve *Salmonella typhimurium* TA100 suşları metabolik enzim olan S9'lu ortamda ve S9'suz ortamda kullanıldı. Enniatin A1 ve B1'in 5 farklı konsantrasyonu (12.5 µM, 25 µM, 50 µM, 100 µM, 200 µM) bu suşlara uygulandı. Bütün test suşlarında(*Salmonella typhimurium* TA98 ve *Salmonella typhimurium* TA100) Enniatin A1 ve B1 mutajenik etki göstermedi.

Anahtar sözcükler: *Ames testi, Enniatin A1, Enniatin B1, Salmonella typhimurium*

INTRODUCTION

Enniatins are fungal metabolites produced by several species of Fusarium, and were first isolated in 1947 by Gäumann et al.[1,2]. Enniatins are members of a family of fungal N-methylated cyclic hexadepsipeptides and consist of three residues of D-2-hydroxyisovaleric acid alternating with three of N-methyl branched-chain L-amino acids such as valine, leucine, or isoleucine. Enniatin A contains only N-methyl isoleucine residues, whereas other enniatin homologues contain other patterns of branched-chain amino acids [3]. Enniatins are non-ribosomal, cyclic hexadepsipeptides with general cation chelating, ionophore [3], and they have got biological activities like acting as enzyme inhibitors, e.g. acyl-CoA-cholesterol-acyl transferase [4] and cyclic nucleotide phosphodiesterase [5], antifungal and antibacterial agents, and immunomodulatory substances [6-8]. They are also anthelmintic, cytotoxic and phytotoxic activities [2,9-11]. Although they can accumulate in Fusarium-infected grain, enniatins have not been associated with any animal disease outbreaks nor been shown to cause disease in experimental animals [3], but Wätjen et al.[5] said

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that Enniatins have important impact on human health and they can cause outbreaks in humans and animals [12]. They are also known as phytotoxins and are associated with plant diseases characterized by wilt and necrosis [13]. Enniatins exert potent cytotoxic activity against cancer cell lines [5,14], and can act also as potent inhibitors of an ABC membrane transporter related to multidrug-resistance [15]. These properties make them candidate compounds for further development as drugs, particularly as potent cytotoxic effects against several cancer cell lines [2,16].

The aim of this study was to investigate the mutagenic effects of enniatin A1 and B1 using Ames test. *Salmonella typhimurium* TA98 and TA100 was used both in the presence and absence of a liver microsome (S9).

MATERIAL and METHODS

Mutagenic effects of Enniatin A1 and B1 were investigated with Ames test system. The Ames test was performed with or without S9 mix using the incorporation method of exposure in accordance to the work of Maron and Ames [17].

Sample Preparation and Doses

Enniatin A1 and B1 were disolved in the Dimethyl Sulfoxide (DMSO). Each of them stored in DMSO for best to best dissolve. The stock was stored room temperature. Non-toxic doses of the compounds for standard test strains was determined and the five non-cytotoxic dose (200-100-50-25-12,5 µM) of each substances were studied.

The Bacterial Strains

The tester strains used were histidine requiring *Salmonella typhimurium* TA98 and TA100. The culture stocks were stored at -80°C. While the TA98 strains were used for determining the frameshift, TA100 was used to determine the base pair exchange [18]. The tester strain was freshly prepared by pre-culturing for 12 h at 37°C in nutrient broth No. 2 and suspension was used for the assay. To carry out experiments healthy, strains have been checked at regular intervals whether the strains have the original mutations.

Procedure in Presence of Liver Microsome (S9)

100 µL of the test solution for each concentration, 300 µL biotin/histidine solution and 100 µL of a cell suspension from an overnight culture ((1-2) × 10^9 cells/mL) were added to 3 mL of top agar (kept at 45°C) and vortexed the mixture. Experiments with S9, 500 µL of S9 mix was added. The entire mixture was transferred on the minimal agar plate. Samples were tested on triplicate plates in independent parallel experiments. The plates were incubated at 37°C for 48-72 h and then bacterial colonies on each plate were counted.

Positive Control

100 µL 2 Amino flourene, 300 µL biotin/histidine solution and 100 µL of a cell suspension from an overnight culture ((1-2) × 10^9 cells/mL) were added to 3 mL of top agar (kept at 45°C) and vortexed the mixture. Experiments with S9, 500 µL of S9 mix was added. 2AF was used as positive controls with metabolic activation. The entire mixture was transferred on the minimal agar plate. Samples were tested on triplicate plates in independent parallel experiments. The plates were incubated at 37°C for 48-72 h and then bacterial colonies on each plate were counted.

Solvent Control

100 µL of dimethylsulfoxide (DMSO), 300 µL biotin/histidine solution and 100 µL of a cell suspension from an overnight culture ((1-2) × 10^9 cells/mL) were added to 3 mL of top agar (kept at 45°C) and vortexed the mixture. Experiments with S9, 500 µL of S9 mix was added. The entire mixture was transferred on the minimal agar plate. Samples were tested on triplicate plates in independent parallel experiments. The plates were incubated at 37°C for 48-72 h and then bacterial colonies on each plate were counted.

Spontaneous Control

300 µL biotin/histidine solution and 100 µL of a cell suspension from an overnight culture [(1-2) × 10^9 cells/mL] were added to 3 mL of top agar (kept at 45°C) and vortexed the mixture. Experiments with S9 500 µL of S9 mix was added. The entire mixture was transferred on the minimal agar plate. Samples were tested on triplicate plates in independent parallel experiments. The plates were incubated at 37°C for 48-72 h and then bacterial colonies on each plate were counted.

Procedure in Absence of Liver Extract (S9)

All the steps in this stage are the same as previous part. But, here, liver microsome extract (S9) was not used. 4-nitrophenyldiamine was used as a positive control for TA98, Sodium Azide was used for TA100.

RESULTS

Mutagenecity Enniatin A1 and B1were investigated with Ames test. In the Ames assay *S. typhimurium* TA98 and *S. typhimurium* TA100 strains were used: Strain TA98 detects frame-shift mutations whereas TA100 detects base-pair substitutions. The test results of Enniatin A1 and B1 are summarised in *Table 1* and *Table 2*. Analyzing test results, all doses of Enniatin A1 and B1, in the presence and absence of S9 enzyme was determined to be non-mutagenic. Results were compared with the number of colonies of spontaneous control group.

Table 1. *Results of mutagenecity of Enniatin A1 with Ames test in different S. typhimurium strains (TA98 and TA100) in the presence or absence of S9-mix*

Tablo 1. *Enniatin A1'in Ames testi ile farklı S. typhimurium (TA98 ve TA100) suşlarında S9 enzimi varlığında ve yokluğunda mutajenite sonuçları*

Strain	TA98		TA100	
	-S9	+S9	-S9	+S9
Spontaneous control	34±3	29±5	152±12	252±8
Solvent control (DMSO)	30±2	35±5	149±6	270±20
200 µM Enniatin A1	33±3	28±4	146±4	281±4
100 µM Enniatin A1	38±8	29±5	141±11	257±11
50 µM Enniatin A1	37±4	39±4	144±20	271±13
25 µM Enniatin A1	37±7	29±10	153±16	243±26
12,5 µM Enniatin A1	32±5	28±6	176±11	247±20
Positive control				
4 Nitro-o-phenilenediamine	1235±35	-	-	-
Sodium azide	-	-	1720±30	-
2 Aminoflourene	-	1240±20	-	2327±16

Table 2. *Results of mutagenecity of Enniatin B1 with Ames test in different S. typhimurium strains (TA98 and TA100) in the presence or absence of S9-mix*

Tablo 2. *Enniatin B1'in Ames testi ile farklı S. typhimurium (TA98 ve TA100) suşlarında S9 enzimi varlığında ve yokluğunda mutajenite sonuçları*

Strain	TA98		TA100	
	-S9	+S9	-S9	+S9
Spontaneous control	34±3	29±5	152±12	252±8
Solvent control (DMSO)	30±2	35±5	149±6	270±20
200 µM Enniatin B1	36±5	34±3	136±10	243±24
100 µM Enniatin B1	34±3	36±3	150±18	245±22
50 µM Enniatin B1	33±5	28±2	183±13	275±16
25 µM Enniatin B1	33±6	34±3	167±20	267±10
12,5 µM Enniatin B1	34±6	29±3	160±15	240±5
Positive control				
4 Nitro-o-phenilenediamine	1235±35	-	-	-
Sodium azide	-	-	1720±30	-
2 Aminoflourene	-	1240±20	-	2327±16

DISCUSSION

Enniatins are non-ribosomal, cyclic hexadepsipeptides with general cation chelating ionophore and antibiotic activities [3]. Enniatins have got various biological activities [5,14,15]. These properties make them candidate compounds for further development as drugs [2,16]. For these reasons; evaluating the mutagenic, cytotoxic, antimicrobial effects of enniatin A1 and B1 is important.

Studies on the mutagenic effects of enniatins are found of limitedly. Behm et al.[16], investigated mutagenic effects of Enniatin B with *Salmonella* Ames test system. They used *S. typhymurium* TA98, 100, 102 and 104 strains with five different concentrations (100 nM, 1 µM, 10 µM, 30 µM, 100 µM). No mutagenecity of Enniatin B was detected in their research [16]. Our results support the findings of Behm et al.[16]. Studies of the cytotoxic effects of enniatins have been evaluated by MTT assay by Lu et al.[12]. They reported that ENs cytotoxicity depend on their concentrations, and also on their combination with other mycotoxins [12]. The effect of EN A_1, B, and B_1 on cell viability determinated in rat hepatoma (H4IIE), human hepatoma (HepG2), and rat glioma (C6) cell lines using the tetrazolium salt (MTT) assay. The enniatins showed a moderate toxicity in C6 glioma and HepG2 hepatoma cells [5].

Antimicrobial effect of Enniatin were studied against *Escherichia coli*, *Enterococcus faecium*, *Salmonella enterica*, *Shigella dysenteriae*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium perfringens*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* by Meca et al.[19]. Enniatin B was found no toxic, at tested concentrations on the strains *S. aureus* CECT 240, *E. coli* CECT 4782, and *S. dysenteriae* CECT 584 but all the others strains tested showed inhibitory activity dependent on the quantity utilized [19].

In conclusion the Ames test showed us that Enniatin A1 and B1 did not posses mutagenic activity. Further mutagenicity tests should perform to reach whole decision about their safety.

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RESEARCH ARTICLE

Clinical and Electrophysiological Mapping of Nerve Root Injury Following Trauma of Brachial Plexus: A Retrospective Study in 23 Dogs and 42 Cats

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Abstract

Objective of this study is to report mapping of nerve root injury with clinical and electrophysiological examination in cats and dogs following trauma of brachial plexus (TBP). Medical record of 65 patients (23 dogs and 42 cats) with brachial plexus injury without any fracture between July 2009 and January 2014 were reviewed. Needle electromyography, motor nerve conduction and/or sensory nerve conduction and somatosensory evoked potentials (SEP) of the forelimb were studied. Injured area was examined at necropsy in 3 cases (1 dog, 2 cats), and in 11 cases (4 dogs, 7 cats) during surgery. Assessment of cutaneous zone innervation revealed caudal brachial plexus lesion in 40 cases (29 cat and 11 dogs) and complete brachial plexus lesions in 25 cases (13 cats, 12 dogs). Nociception was also absent at the denervated cutaneous zone in all cases. Complete avulsion of isolated radial nerve roots was diagnosed in 11 cats and 5 dogs, and injury of isolated radial nerve roots was diagnosed in 4 dogs and 11 cats. Rest of the cases had complete avulsion (17 cats, 14 dogs) or injury (3 cats) of radial nerve roots in addition to other nerves' of the brachial plexus. In conclusion, Radial nerve roots prone to TBP, but other nerve roots of brachial plexus can also be affected. Electrophysiological assessment of TBP should be carried out as an ancillary diagnostic tool for determining affected nerves and type of lesion for radial nerve.

Keywords: *Brachial plexus injury, Trauma, Electrophysiology, Dog, Cat*

Travmatik Brakial Pleksus Yaralanmalarının Klinik ve Elektrofizyolojik Olarak Değerlendirilmesi: 23 Köpek ve 42 Kedide Retrospektiv Bir Çalışma

Özet

Bu çalışmanın amacı kedi ve köpeklerde brakial pleksus travmasını takiben oluşan sinir kökü hasarının klinik ve elektrofizyolojik muayene ile haritalanmasını bildirmektir. Çalışmada herhangi bir kırığa bağlı olmaksızın gelişen brakial pleksus yaralanması ile Haziran 2009 ve Ocak 2014 arasında kliniğimize başvuran 65 hastanın (23 köpek, 42 kedi) medikal kayıtları incelendi. Elektrofizyolojik olarak iğne elektromyografi, motor sinir iletimi ve/veya sensorik sinir iletimi ve somatosensorik uyandırılmış potansiyeller çalışıldı. Hasarlı bölge üç hastada (1 köpek, 2 kedi) nekropside, 11 hastada ise (4 köpek, 7 kedi) cerrahi girişim sırasında incelendi. Kutanöz innervasyon alanlarının değerlendirilmesi sonucunda 40 olguda (29 kedi, 11 köpek) kaudal ve 26 olguda (14 kedi, 12 köpek) total brakial pleksus yaralanması belirlendi. Ayrıca bütün olgularda denerve kutanöz alanlarda nosisepsiyon yoktu. On bir kedi ve 5 köpekte izole radial sinir köklerinin total avulzivonu belirlendi. Dört köpek ve 11 kedide izole radial sinir kökü hasarı tespit edildi. Geriye kalan vakalarda radial sinir ile birlikte diğer brakial pleksus sinirlerinin total avulziyonu (18 kedi, 14 köpek) ya da hasarı (3 kedi) belirlendi. Sonuç olarak, brachial pleksusu oluşturan sinirlerden radial sinir travmatik olarak en çok yaralanan olsa da diğer sinirlerin de yaralanması söz konusudur. Travmatik brakial pleksus lezyonlarının değerlendirilmesinde elektrofizyolojik muayene, lezyonun tipinin ve etkilenen sinirin belirlenmesi açısından yararlı bir tanı aracı olarak göz önünde bulundurulmalıdır.

Anahtar sözcükler: *Brakial pleksus hasarı, Elektrofizyoloji, Travma, Köpek, Kedi*

INTRODUCTION

Traumatic brachial plexus injuries are explained as traction injury, which occurs while the limb is abducted severely from the body, and the entire shoulder mechanism is driven away from its normal position [1,2]. Brachial plexus

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injuries are the most common neurological disorder of the forelimb in small animals [1,3]. They can occur separately, or can be accompanied by fractures of the humerus or Horner's syndrome [4].

The dorsal and ventral rootlets of the C6-T2 spinal cord segments form the spinal nerves from which the ventral branches arise and interweaving of them lead to the formation of the brachial plexus. Individual peripheral nerves coming from brachial plexus descend distally to innervate the muscles of the forelimb. The dorsal branches that arise from spinal nerves innervate paraspinal muscles and skin [2,5]. Following trauma different nerves of the brachial plexus can be affected in different degrees, and the combination of these multiple injuries causes varying degree of deficits effecting forelimb muscles [3]. The prognosis for most brachial plexus injuries is poor for functional return of the limb [6].

Recent development in imaging technology provide to diagnosis brachial plexus nerve roots lesion in human, but limited number of study published in dogs [7]. Ultrasonography is another valuable diagnostic tool in the setting of suspected brachial plexus lesion especially a mass or traumatic lesion in humans [8]. However, the functional integrity of brachial plexus injury can be evaluated through neurological and electrophysiological examination. Cutaneous sensation can be used as a clinical tool in localizing peripheral nerve lesions. Autonomous zones are those sensory areas of the skin supplied only by particular nerve lesions. The clinician should be familiar with these autonomous zones. With brachial plexus injuries, there may be inconsistency in patterns of sensory versus motor deficits as the ventral nerve roots appear to be more susceptible to damage than the dorsal roots [6]. Reports for electrophysiological examinations of dogs and cats are limited in number [9]. The objective of this study was to report mapping of nerve root injury with clinical and electrophysiological examination of traumatic brachial plexus injury in dogs and cats.

MATERIAL and METHODS

Medical records of Ankara University Faculty of Veterinary Medicine Department of Surgery were searched between July 2009 and January 2014 for the cases presented with the clinical signs of brachial plexus injury without any fracture and had electrophysiological examination. Breed, age, sex, aetiology and the time passed since trauma were recorded.

Neurological examination findings were retrospectively analysed to record clinical signs of injury to radial, median-ulnar, musculocutaneous and suprascapular nerves. Each nerve involved was diagnosed by its characteristic clinical findings. Radiological examination was also carried out to diagnose fracture and/or luxation of the forelimb. The distribution of sensory loss in an affected limb has great localizing value because lesions can be pinpointed to a particular nerve or within two to three spinal cord segments. The total area innervated by a particular cutaneous nerve is termed "cutaneous area". The cutaneous area includes a peripheral overlap zone innervated by other cutaneous nerves and a central autonomous zone innervated solely by that nerve. These zones can be detected clinically using a method termed the "two-step pinch technique" [6]. The motor deficits associated with brachial plexus injury were accepted as the major clinical signs for determining affected nerve. According to the cutaneous sensation and affected nerves, brachial plexus injury was further classified as cranial (C6-C7 nerve roots - loss of shoulder movement and elbow flexion), caudal (C8-T2 nerve roots - elbow flexion are spared) or complete (C6-T2 nerve roots - loss of extension and flexion in all joints) lesion [1].

Electrophysiological assessment was carried out under general anesthesia. This was achieved using xylazine hydrochloride (2 mg/kg IM, Alfazyne®, Izmir, Turkey) and ketamine hydrochloride (15 mg/kg, IM, Alfamine®, Izmir, Turkey). All of the electrophysiological assessments were performed after the 5th day from the injury.

Stainless steel disposable monopolar needle electrodes (diameter: 0.3 mm and length: 12 mm) were used for all recordings and stimulations. The recording electrodes were attached to a 5-channel electromyography - evoked potentials (EMG/EP) system (Medelec, Oxford).

Electromyography: In all cases digital extensor muscles, triceps brachii muscles, biceps brachii muscles, superficial and/or deep digital flexor muscles, supraspinatus muscle, infraspinatus muscle, deltoid muscle and paraspinal muscles were assessed for presence of denervation potentials (fibrillation potentials, positive sharp waves and complex repetitive discharges).

Motor nerve conduction studies: This was carried out for radial and ulnar nerves. Frequency limits for recording were 10 Hz-2 KHz, sweep duration was 10 msec. A rectangular 0.1 ms duration stimulus at supramaximal intensity was used and at least 3 consecutive, repeatable compound muscle action potentials (CMAP) were recorded. The distal stimulation point of the radial nerve was at the flexor angle of the elbow. A cathode electrode was inserted near the radial nerve, just lateral to the cephalic vein and an anode was inserted subcutaneously about 1 cm laterally. The proximal stimulation point of the radial nerve was at the mid-portion of the humerus. Active electrode was inserted sub facially over the common extensor digitalis muscle, and the reference electrode was inserted subcutaneously 1-2 cm laterally to the cathode electrode. The distal stimulation point of the ulnar nerve was at the medial aspect of the elbow joint and just caudally to the epicondylar crest of the humerus. The cathode electrode was inserted subcutaneously over the olecranon. The

proximal stimulation point of the ulnar nerve was at the medial aspect of proximal third of humerus and just cranial to brachial arteries. Recording was carried out from the digital flexor muscles. Ground electrode was inserted between stimulating and recording electrodes.

Sensory nerve conduction studies: This was carried out for radial and ulnar nerves. The stimulation point for the radial nerve was from its superficial branch over the carpal joint. Recording was from the radial nerve over the craniolateral elbow joint and the reference electrode 1-2 cm laterally to the radial nerve. The stimulation point for the ulnar nerve was from its dorsal branch over the metacarpal bones and recording was from the same point as for distal ulnar nerve stimulation point for motor nerve conduction study. The ground electrode was inserted between stimulating and recording electrodes. Presence of sensory nerve action potentials (SNAP) with profound denervation in related muscles was accepted as an indicator of dorsal root avulsion.

Somatosensory Evoked Potential (SEP): SEP was performed in radial nerve SNAP recorded cases. The radial nerve was stimulated as described before for motor nerve conduction studies, and SEP was recorded from the scalp and 250 responses were averaged. Presence of the potential was accepted as an indicator of dorsal root integrity, and absence was accepted as preganglionic loss of integrity.

Results of the neurological examination and electrophysiological assessment were compared.

RESULTS

Twenty-three dogs and 42 cats matched the inclusion criteria. All animals had forelimb paresis or paralysis without fracture or luxation of the forelimb. Dog breeds presented were mainly medium to large size breeds. (Anatolian Sheep dog (n=5), Doberman (n=1), Rottweiler (n=1), German Sheperd dog (n=1), Golden Retriever (n=2), Afghan Hound (n=1) and mixed breed (n=12, minimum 15 kg). Cat breeds were mainly domestic shot haired except two Angora cat and three Van cat. The mean age was 2.68 years (5 months–9 years) for dogs and 1.78 years (1 months-8 years) for cats. The sex dispersion was 14 male, 9 female for dogs, and 24 male, 18 female for cats. The etiology of the brachial plexus injury was traffic accidents (n=13 dogs, n=8 cats), falling from heights (n=2 cat), fighting (n=1 dog), and unknown causes (n=9 dog, n=32 cats). All the cases were outdoor pets.

The mean time between trauma and presentation was 12 days (5-60 days). The left fore limb was involved in 17 dogs (73.9%) and the right fore limb in 6 dogs, and the left fore limb in 31 cats (73.8%) and the right fore limb in 11 cats. Two cats were presenting Horner's syndrome in addition to brachial plexus injury.

Cutaneous sensation mapping were suggestive of complete brachial plexus trauma in 13 cats and 12 dogs (38.46%), and caudal brachial plexus trauma in 29 cats and 11 dogs (61.54%). Nociception was also absent at the area without cutaneous twitches. Cranial brachial plexus trauma was no seen in any case.

The affected nerves based on needle EMG in dogs were; isolated radial nerve (n=10, 43.47%), radial, ulnar -median, suprascapular and musculocutaneous nerves (n=8) and radial, ulnar - median nerves (n=2), and radial, ulnar - median, musculocutaneous nerves (n=3) *(Fig. 1a-1b)*. The radial nerve was unexcitable after stimulating from the distal point in 17 dogs, while in the remaining 6 cases, the motor nerve conduction velocity was lower than 40 m/s.

The affected nerves in cats were; isolated radial nerve (n=22, 52.38%), and radial, ulnar - median nerves (n=7, 16.66%), and radial, ulnar - median, suprascapular nerves (n=2), and radial, musculocutaneous (n=2), and radial, ulnar-median, suprascapular, musculocutaneous nerves (n=9). The deltoid muscle was not evaluated in cats because of its small size. The radial nerve was unexcitable after stimulating the distal point in 25 cats (62.5%), while means motor nerve conduction velocity were under the reference values in the rest. Radial SNAP was recorded in six dogs and 15 cats, while SEP was reliable and repeatable in 3 of dogs and 12 of cats.

The type of lesion for radial nerve, identified by electrophysiological examination was avulsion of the isolated radial nerve roots in 11 cats and 5 dogs *(Fig. 2)*. Isolated radial nerve injury or avulsion of some of its roots was diagnosed in 11 cats and 4 dogs *(Fig. 3)*. Rest of the cases had avulsion (17 cats, 14 dogs) or injury (3 cats) of radial nerve concomitant to other nerves of the forelimb.

The C6-8 and T1 nerve roots found to be avulsed in the post mortem examination of 2 euthanized cats. However just C7 and C8 nerve roots were avulsed in one cat. Post mortem examination of one of the dogs revealed that all the nerve roots of brachial plexus were avulsed *(Fig. 4)*. The lower part of the brachial plexus was exposed surgically in 11 cases (4 dogs, 7 cats). Among these cases, avulsed stump of probably C7 and C8 nerve roots was seen in 2 dogs and 4 cats. There was no abnormality in the exposed area in the rest of cases.

When the clinical signs were compared to the affected nerve which was diagnosed by the needle EMG, cranial brachial plexus trauma was not seen individually in both methods and the results was at the same line.

DISCUSSION

In this study, the involved nerves and preganglionic or postganglionic injury for radial nerve roots was depicted by electrophysiological evaluation. Most of the cases had

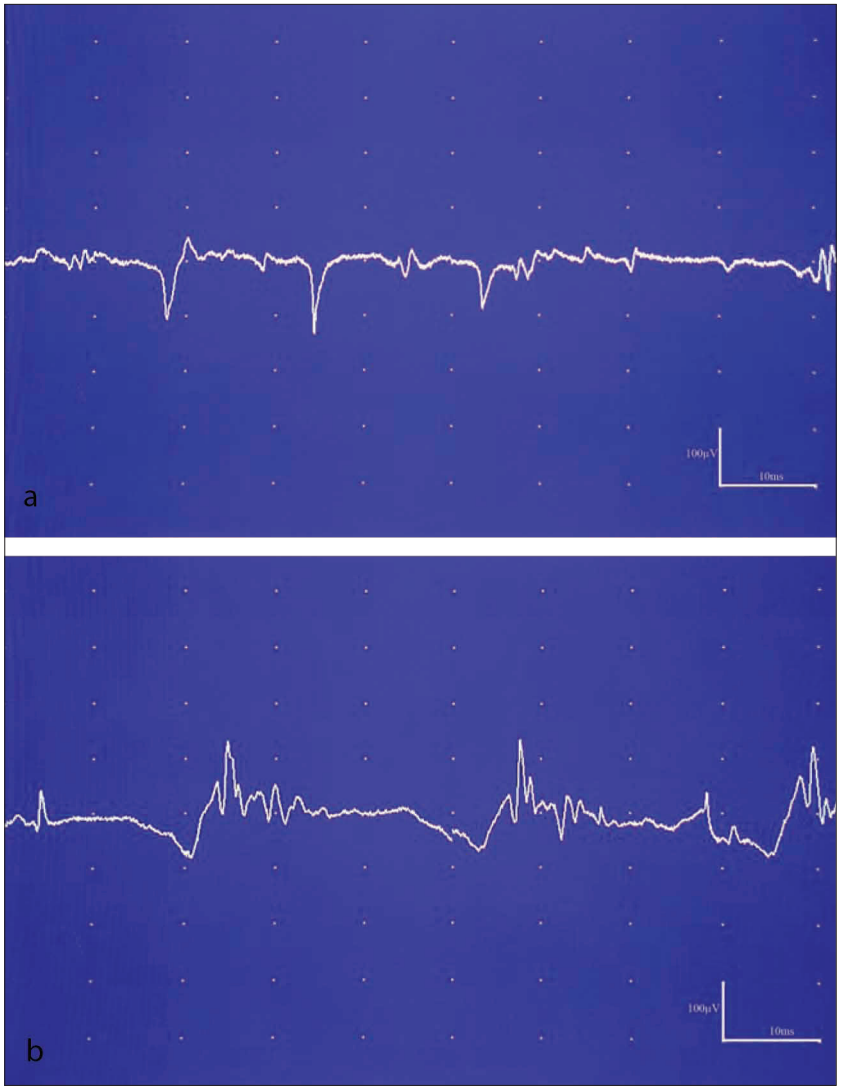
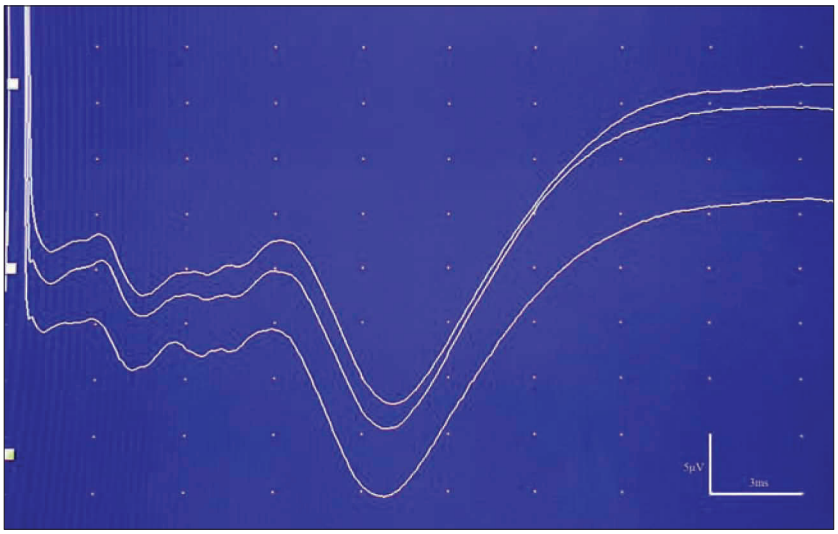


Fig 1. a- Abnormal spontaneous activity, in the form of positive sharp waves in extensor digitorum communis muscle in a dog; **b-** Abnormal spontaneous activity in the form of mainly fibrillation potentials in triceps brachii muscle in a dog

Şekil 1. a- Bir köpekte ekstensor digitorum kommunis kasında ağırlıklı olarak pozitif keskin dalgalardan oluşan anormal spontan aktivite, **b-** Bir köpekte triseps brachii kasında ağırlıklı olarak fibrilasyon potansiyellerinden oluşan anormal spontan aktivite

Fig 2. In SEP study, triphasic reproducible potentials following stimulation of radial nerve and recording from scalp in cats with radial nerve injury

Şekil 2. Radial sinir hasarı olan bir kedide yapılan SEP çalışmasında, radial sinirin uyarılmasıyla oluşan ve kafadan kaydedilen trifazik tekrarlanabilir potansiyeller



avulsion-type (preganglionic) injuries of the radial nerve in both dogs and cats, and there was no other case with isolated nerve injury of forelimbs except for the radial nerve based on clinical and needle EMG results of this study.

Traumatic brachial plexus injury is a traction injury, which usually results from traffic accidents [10]. Although unknown causes and traffic accidents were cited in equal numbers in the present study. The cases presented as

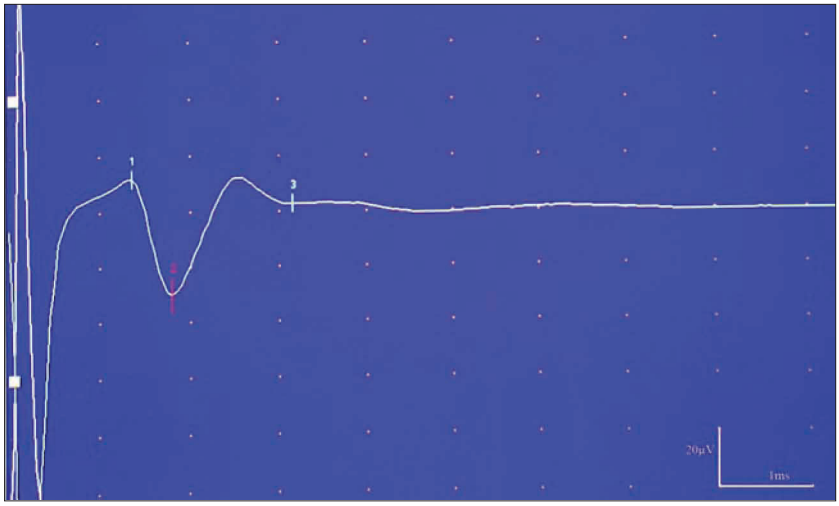


Fig 3. Sensory nerve action potential following stimulation from superficial branch of radial nerve over the carpal joint and recording from the nerve over the craniolateral elbow joint in a dog

Şekil 3. Bir köpekte karpal eklem üzerinden geçen radial sinirin süperfisiyal dalının uyarımı sonucu oluşan ve kraniyolateral dirsek eklemi üzerindeki sinirden kaydedilen sensorik sinir aksiyon potansiyeli

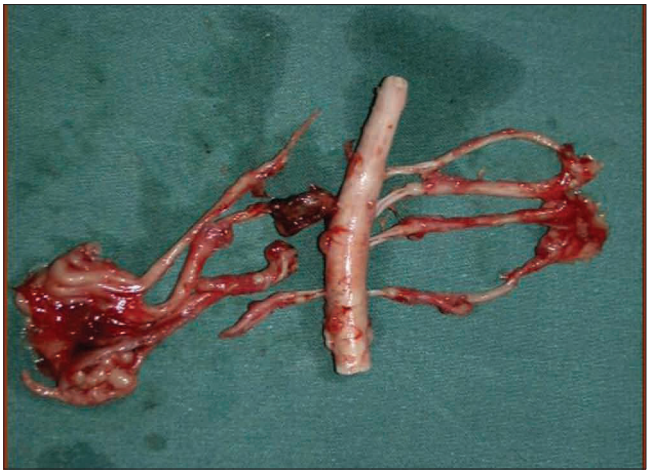


Fig 4. Macroscopic appearance of root avulsions in dog.
Şekil 4. Köpekte sinir kökü avulziyonunun makroskopik görünümü

unknown causes were identified as their forelimbs could not support their body weight and all of them were kept outdoors. It is not wrong to assume some sort of trauma (traffic accidents or falling from height) could be cause behind the initial trauma. All of the dog cases in this retrospective study were medium to large sized dogs. This indicates a relationship between body weight and traumatic injury to brachial plexus. We speculate that the force responsible for injury is greater in larger dogs. The body side involved in TBP was predominantly (73.84%) the left in both dogs and cats. However, this interesting finding is likely being coincidental; the authors think it should be investigated in more detail.

Spinal nerve roots are more susceptible to traction injury. This is likely because they contain less connective tissue than the extra-vertebral neural structures. This is possibly why the nerve roots are often avulsed from the spinal cord intradurally rather than the more peripheral portions of the nerves [1,11]. Caudal and complete avulsions are more common than cranial avulsions and cause more severe clinical signs [12]. They both cause paralysis of the triceps brachii muscle, so the animal cannot extend the elbow or bear weight on the affected limb. In this case series the incidence of caudal brachial plexus injuries was higher (69.04%) than complete brachial injury in cats, however caudal brachial plexus injury was 47.82% in dogs. Cranial avulsion was not diagnosed in the current retrospective cases. These differences between both species should be followed in more detail. The absence of cranial lesions can be explained by the concentrated force in this area is weaker than the caudal area, or the differences between roots of two area (eg. Amount of fibrose tissue around the roots).

The affected radial nerve was seen as isolated or associated with other nerves in both dogs and cats in the current study. There was no other isolated nerve involvement of the forelimb in any case. These findings indicate that radial nerve which innervates the main weight bearing muscles of the fore limb is dominant clinically and electro physiologically. However multiple nerve injuries are common because they share some of the nerve root. When the associated nerve was evaluated, the ulnar median and musculocutaneous nerve was affected most commonly. These findings are important for prognosis and also to justify the treatment procedure like tendon transposition for restoration of phalangeal extension.

Electrophysiological assessment of traumatic injury of the brachial plexus is the most reliable method in human and veterinary medicine [13,14]. Electrophysiological findings are found to be more characteristic for the axonal degeneration that occurs after the 5th day, and most authors prefer to evaluate 5-10 days after trauma. The ventral nerve roots are most often involved in humans [15]. Electrophysiological examination was performed after 5 days in all cases in the current study. The radial nerve was found to be unexcitable during standard nerve conduction studies in 40 cases. This finding represents axonal degeneration. Sensory nerve conduction studies are

the first choice to determine whether the injury site is preganglionic or post-ganglionic [13,15,16]. When the damage to the sensory fibers is proximal to the dorsal root ganglia, the distal sensory fibers are viable, thus producing normal sensory nerve action potentials in the forelimb. In contrast, if the injury is to the nerve forming the plexus, Wallerian degeneration of axons distal to the injury occurs and no sensory nerve action potential would be detectable. Somatosensory evoked potentials can also be used to estimate pre-ganglionic or postganglionic involvement of brachial plexus roots in humans [17,18]. However, in un excitable nerve SEP cannot be recorded reliably. In this study, the type of injury was elucidated by standard sensory nerve conduction studies, but in cases where there was doubt; SEP was studied to clarify root avulsion or brachial plexus lesion. The presence or absence of SNAP and/or SEP was found to be valuable as an indicator of the type of lesion. Decrease in nerve conduction velocity relative to the previously reported reference values was interpreted as partial involvement of the nerve [16,19,20].

EMG may be helpful in assessing the functional integrity of brachial plexus in determining the extent and severity of brachial plexus lesion and in distinguishing such a lesion from nerve root or peripheral nerve pathology [13]. In the current study, paralysis of involved nerves of the extremity was diagnosed with needle EMG. Identification of denervation activity in a muscle was accepted as an indicator of paralysis of its nerve. Spontaneous denervation activity was found in all animals that underwent needle EMG examination. The results were in agreement with the clinical examination results.

Management methods and prognosis differ for brachial plexus injury depending on the nerve involved and the type and the level of the lesions present (pre–postganglionic) [21]. One of the most important roles of electromyography in traumatic brachial plexus injury is to clarify whether nerve root avulsion or brachial plexus injury is present [15]. However anatomical confirmation is the best way to make clear conclusions. In this case series avulsed roots were seen in 8/11 cases that were surgically explored. Even though in 3 cases there was no abnormality at the brachial plexus after exposing surgically, in both clinically and electrophysiologically diagnosed complete brachial plexus injury.

In conclusion, large size dogs have a predisposition for traumatic brachial plexus injury and the nerve roots of the radial nerve are affected predominantly in both dogs and cats. Before planning treatment for traumatic brachial plexus injury, the possibility of involvement of associated nerves in addition to the radial nerve and also the fact that avulsion-type injuries are dominant in dogs and cats should be kept in mind. In electrophysiological evaluation (EMG, sensory and motor nerve conduction studies and SEP) can be suggested to identify the nerves involved and the type of injury.

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RESEARCH ARTICLE

Hayvancılık Alanında Bir Veri Madenciliği Uygulaması: Japon Bıldırcını Yumurtalarında Döllülüğe Etki Eden Bazı Faktörlerin Belirlenmesi

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Özet

Bu çalışmanın amacı, Japon bıldırcını yumurtalarının döllülük üzerine etkisi olan mevsim, seleksiyon ve yerleşim sıklığı faktörlerine göre veri madenciliği yöntemi ile sınıflandırılması ve bu faktörlerin etkisinin belirlenmesidir. Çalışmada seleksiyon yapılmış bir hattan ve rastgele çiftleştirilmiş bir kontrol hattından 3 farklı mevsimde (Yaz, Kış ve Sonbahar) elde edilen 180 dişi bıldırcın kullanılmıştır. İki farklı tip kafeste barındırılan (160-240 cm2/bıldırcın) bıldırcınlardan 12 haftalık yaşta bir hafta boyunca toplanan 1141 kuluçkalık yumurta çalışmanın materyalini oluşturmuştur. Araştırmada kullanılan sınıflandırma algoritmaları sırasıyla YSA, RBF Network, Naive Bayes, KStar, ve Ridor algoritmalarıdır. Söz konusu bu algoritmalara göre oluşturulan modellerin karşılaştırmasında Kappa istatistiği, Ortalama Mutlak Hata (OMH), Ortalama Hata Karekök (OHK), Göreli Mutlak Hata (GMH) ve Göreli Hata Karekök (GHK) performans kriterleri kullanılmıştır. Analizler sonucunda, yapılan karşılaştırmada performans kriter değerleri sırasıyla OMH: 0.002, OHK: 0.05, GMH: %1.07, GHK: %14.50 ve Kappa: 0.98 olan Ridor algoritmasına göre oluşturulan modelin en az hata ile sınıflandırma yaptığı görülmüştür. Yapılan bu çalışma ile %99.73 doğru sınıflandırma başarısı ile bıldırcın yumurtalarının genel olarak %85'inin döllü, %15'nin ise üreme kapasitelerinin düşük olduğu tespit edilmiştir.

Anahtar sözcükler: *Bıldırcın, Sınıflandırma, Üreme, Veri madenciliği, WEKA*

A Data Mining Application in Animal Breeding: Determination of Some Factors in Japanese Quail Eggs Affecting Fertility

Abstract

The purpose of this study, classification with data mining methods according to the factors of season, selection, and frequency of settlement which have an effect on fertility in Japanese quail eggs, and is to determine the effect of these factors. In this study, 180 female quails in three different seasons (summer, winter and autumn) which were obtained from a selection line and a control line were used. 1141 hatching eggs collected from quails which were hosted on two different types of cages (160-240 cm2/quail) during a week at 12 weeks of age have formed the material of study. Classification algorithms used in the study are YSA, RBF Network, Naive Bayes, KStar, and Ridor algorythms, respectively. In the comparison of the models formed according to these algorithms, Kappa statistic, Mean Absolute Deviation (MAD), Mean Square Root Error (MSE), Relative Absolute Error (RAE), Relative Square Root Error (RSE) performance criteria were used. As a result of analysis, it has been seen in the comparison made that the model formed according to Ridor algorithm that has MAD: 0.002, MSE: 0.05, RAE: 1.07%, RSE: 14.50% and Kappa: 0.98 performance criteria values, respectively, has made the classification with minimum error. With this study conducted, it was determined that 85% of the quail eggs fertile and 15% of them has low reproduction capacity with the accurate classification success of 99.73%.

Keywords: *Classification, Data mining, Reproduction, Quail, WEKA*

GİRİŞ

Veri madenciliği günümüzde hızla ilerleyen bir bilgi teknolojisi olmakla birlikte büyük veri setlerinin içerisinde saklı kalmış önemli bilginin açığa çıkarılması için uygulanan bir yöntem olarak tanımlanmaktadır. Söz konusu

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yöntem veri tabanı bakış açısı, makine öğrenimi ve istatistiksel bakış açısı gibi üç farklı unsurun birleşimiyle uygulanmaktadır. Veri madenciliğinin temelleri 1990'lı yıllarda ortaya konulmuş ve bazı bilgisayar teknikleri sayesinde veri üzerinden örüntü ve model sıralamaları üreten bir veri tabanından bilgi keşfi süreci olarak tanımlanmıştır [1]. Literatürde yer alan veri madenciliği tanımlarında belirtilen ortak amaç, çok fazla sayıdaki bilginin bir ambarda muhafaza edilmesi ve bu bilgiler arasından öz bilginin keşfedilmesidir. Veri madenciliğinde veri işlemede izlenen işlem adımları sırasıyla, verinin temizlenmesi, işe yarar bilgilerin seçimi (önişleme), işlenmiş verilerinin dönüşümü ve veri madenciliği uygulamalarıyla örüntü tanımlama ile yorumlama sayesinde anlamlı bilgilere ulaşılması şeklinde sıralanmaktadır.

Hayvancılık alanında kullanımı oldukça yeni olan veri madenciliği yöntemi daha çok sınıflandırma ve tahmin amaçlı yapılan çalışmalarda kullanılmıştır. Britanya'da sığır sürülerinde biyogüvenlik konusunda çalışan Ortiz-Pelaez ve Pfeiffer [2], hastalık risklerine göre hayvan popülasyonlarını sınıflandırmak amacıyla bir veri madenciliği çalışması yapmışlardır. Söz konusu çalışmada veri madenciliği yöntemlerinden lojistik regresyon, sınıflandırma ağacı ve faktör analiz yöntemleri kullanılmıştır. Sonuç olarak, yoğun yetiştiricilik yapılan alanlarda hastalık riskinin daha yüksek olduğu ve yetiştirime sıklığı ile hastalık riski arasında pozitif yönlü ilişki bulunduğu belirlenmiştir. Kamphuis ve ark.[3], klinik mastitis'in tespitinde yaygın kullanılan bir veri madenciliği algoritması olan karar ağacı yönteminden yararlanmışlar ve yöntemin otomatik sağım sistemlerindeki modellerle benzerlik gösterdiği bilgisine ulaşmışlardır.

Kanatlı hayvanların damızlık sürülerinde ekonomik üretim için kuluçkadan çıkan civcivlerin sayıca çok olması istenmektedir. Elde edilen civciv sayısını etkileyen faktörlerden en önemlilerinden birisi döllülüktür [4]. Bu özellik, genetik ve çevresel faktörlerin etkisi ile şekillenmekte ve döllü-dölsüz şeklinde binomial bir dağılış göstermektedir. Döllülük özelliğinin kalıtım derecesi düşük seviyede olduğundan, söz konusu özelliğin ıslahında daha çok çevrenin iyileştirilmesi üzerinde durulmaktadır [5]. Kanatlı hayvanlarda döllülüğü etkileyen çevresel etmenler; dişi-erkek oranı, damızlık hayvanların yaşı, ağırlığı, sağlığı ile yumurtaların depolanma süresi ve depolama sıcaklığı, uygulanan bakım-yönetim ve besleme koşulları olarak sıralanmaktadır [6].

Bu çalışmada iki farklı hattaki Japon bıldırcınlarından farklı mevsim ve yerleşim sıklıklarında elde edilen kuluçkalık yumurtalarda tespit edilen döllülük özelliği üzerinde durulmuş, mevsim, yerleşim sıklığı ve genotip faktörlerinin döllülük üzerindeki etkileri veri madenciliği yöntemi ile araştırılmıştır.

Araştırmada veri madenciliğinde yaygın olarak kullanılan YSA, RBF Network, Naive Bayes, KStar, ve Ridor gibi sınıflandırma algoritmalarından yararlanılmış ve en iyi sınıflandırma algoritmasına göre oluşturulan model aracılığıyla yumurtaların döllülük durumları sınıflandırılmıştır. Ayrıca, döllü - dölsüz sınıfların oluşturulmasında en etkili olan faktörün varlığı çeşitli istatistiksel yöntemlerle araştırılmış ve karşılaştırmalı olarak incelenmiştir.

MATERYAL ve METOT

Materyal

Araştırma materyalini Akdeniz Üniversitesi bünyesinde barındırılan seleksiyon yapılmış bir hattan ve rastgele çiftleştirilmiş bir kontrol hattından 3 farklı mevsimde (Yaz, Kış ve Sonbahar) elde edilen 30'ar dişi bıldırcından 12 haftalık yaşta bir hafta boyunca toplanan 1141 kuluçkalık yumurta oluşturmuştur. Araştırmada dört kuşak boyunca 4. hafta yüksek canlı ağırlığına göre fenotipik kitle seleksiyonu yapılmış bir hat ve bunun paralelinde dört kuşak boyunca şansa bağlı çiftleştirilen bir kontrol hattına ait bıldırcınlar kullanılmıştır. Seleksiyonda fenotiplere göre en yüksek canlı ağırlığa sahip dişilerin %30'u, erkeklerin %10'u damızlık olarak seçilmiştir. Seleksiyonla her iki hattın nasıl elde edildiğine dair detaylı bilgi Narinc ve Aksoy [7] tarafından bildirilmiştir. Denemede üç mevsimde kullanılan toplam 6 grupta erkek-dişi oranı 1:3 olup; damızlıklar %20 ham protein, 2800 kcal/kg metabolik enerji içeren karma yem ile beslenmiştir [8]. Bıldırcınlara yem ve su ad-libitum olarak sağlanmış, deneme süresince günlük 16 saatlik aydınlatma uygulanmıştır. Bıldırcınlar iki farklı tip kafeste barındırılmış ve yarısına 160 cm^2/bıldırcın diğer yarısına da 240 cm^2/bıldırcın yerleşim sıklığı sağlanmıştır. Haftalık olarak toplanan yumurtalar kuluçka makinesine konulmuş ve 18 günlük kuluçka sonrasında yumurtalar döllü-dölsüz olarak kayıt edilmiştir.

Metot

Veri madenciliğinde verilerden bilgiye ulaşmak için çeşitli metotlar kullanılmaktadır. Bu metotlarda birçok algoritmanın mevcut olmasından dolayı hangisinin optimum sonuç verdiğini bulmak amaçlı bir çok çalışma yapılmıştır. Yapılan bu çalışmaların her birinde ise farklı sonuçlar elde edilmiştir. Bunun nedeninin ise kullanılan veri kaynağı, veriler üzerinde yapılan dönüşümler, önişleme ve algoritma parametrelerinin seçiminden kaynaklandığı görülmüştür [9].

Çalışmada kuluçkalık bıldırcın yumurtalarında döllülük durumunu belirlemek üzere Weka (Waikato Environment for Knowledge Analysis) 3.4.5 sürümünde yer alan sınıflama algoritmalarından sırasıyla YSA, RBF Network, Naive Bayes, KStar, ve Ridor algoritmaları kullanılmıştır [10]. Veriler, öncelikle veri madenciliği sürecinde belirtilen işlem basamaklarına göre analiz için uygun hale getirilmiştir. Verilerin işlenmesinden önce yapılan bu hazırlık aşamasında, öncelikle analiz için uygun veriler seçilmiş, yapılan uygun

kodlama ile de bu verilerin ön işlemesi ve dönüştürülmesi sağlanmıştır. Söz konusu bu algoritmalar veri setine sırasıyla uygulanmış ve algoritmaların doğruluk derecelerinin karşılaştırılmasında sırasıyla, Kappa istatistiği, Ortalama Mutlak Hata (OMH), Ortalama Hata Karekök (OHK), Göreli Mutlak Hata (GMH) ve Göreli Hata Karekök (GHK) değerleri kullanılmıştır. Söz konusu eşitlikler ve eşitliklerde yer alan terimler aşağıda verilmiştir [11].

$$K = \frac{P_G - P_B}{1 - P_B}, \qquad P_G = \frac{\sum_i G_i}{n}, \qquad P_B = \frac{\sum_i R_i C_i}{n^2},$$

$$OHK = \sqrt{\frac{\sum_{i=1}^{n} (\bar{y}_i - y_i)^2}{n}}, \qquad OMH = \frac{\sum_{i=1}^{n} \left| y_i - \bar{y}_i \right|}{n},$$

$$GMH(\%) = \frac{\sum_{i=}^{n} \left| \frac{(y_i - \bar{y}_i)}{y_i} \right|}{n} x100,$$

P_G ve P_B sırasıyla gözlenen ve beklenen olasılıkları, G_i: i-inci satır ve sütundaki gözlenen frekansı, R_i: i-inci satırdaki toplam frekansı, C_i: i-inci sütundaki toplam frekansı ve N:Toplam gözlem sayısını, $\bar{y}$: i-inci sınıfın tahmin değeri ve y_i: i-inci sınıfın gerçek değerini göstermektedir [12,13].

Weka programında oluşan sınıfların değerlendirilmesi için Ki kare test istatistiği kullanılmıştır. Sonuçlar frekans ve yüzde olarak belirtilmiştir. Ki kare istatistiğinin hesaplanmasında SPSS 15.0 programında yararlanılmıştır [14].

BULGULAR

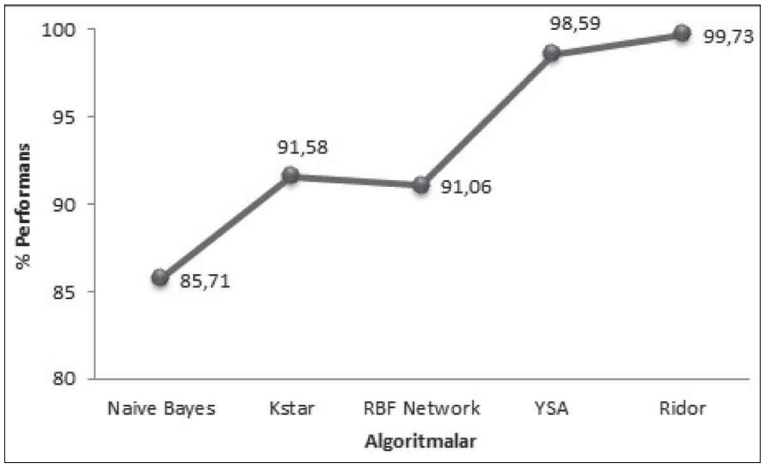
Kuluçkalık yumurtaların döllülük (döllü-dölsüz) durumlarını sınıflandırmak amacıyla veri madenciliği algoritmalarına göre oluşturulan modellere ait Kappa istatistiği, Ortalama Mutlak Hata (OMH), Ortalama Hata Karekök (OHK), Göreli Mutlak Hata (GMH) ve Göreli Hata Karekök (GHK) kriterlerinin değerleri *Tablo 1*'de verilmiştir. Algoritmalara göre yumurtaların döllülük durumlarının doğru sınıflandırma başarı yüzde değerleri %85.71 ile %99.73 arasında elde edilmiştir *(Şekil 1)*. Söz konusu modellerin diğer başarı performansları karşılaştırıldığında ise Ridor algoritmasına göre oluşturulan modelin ortalama mutlak hata OMH: 0.002, OHK: 0.05, GMH: %1.07 ve GHK %14.50 değeri ile en düşük hata değerlerine sahip olduğu görülmektedir *(Tablo 1)*. Sınıflandırma başarısı en iyi olarak

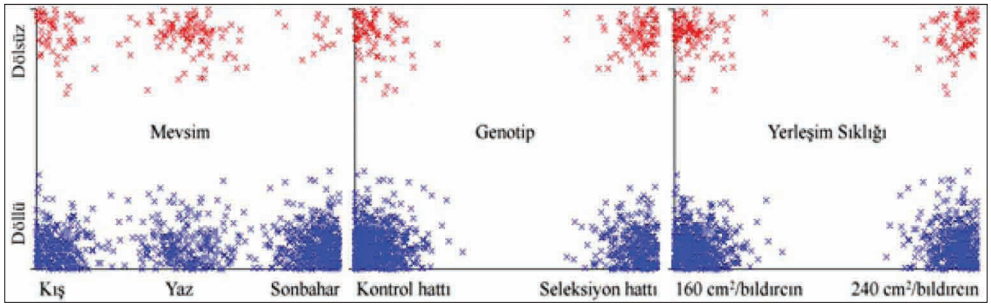
Tablo 1. *Sınıflandırma algoritmaları ve doğruluk oranları*
Table 1. *Classification algorithms and accuracy rates*

Doğruluk Ölçütleri	Naive Bayes	KSTAR	YSA	RBF Network	Ridor
Doğru Olarak Sınıflandırılan Örnek Sayısı	978	1045	1125	1039	1138
Yanlış Olarak Sınıflandırılan Örnek Sayısı	163	96	16	102	3
Doğru Sınıflandırılan Örnek %	85.71	91.58	98.59	91.06	99.73
Yanlış Sınıflandırılan Örnek %	14.28	8.41	1.40	8.93	0.26
Kappa İstatistiği	0.00	0.55	0.94	0.51	0.98
Ortalama Mutlak Hata (OMH)	0.22	0.12	0.02	0.15	0.002
Ortalama Hata Karekök (OHK)	0.33	0.21	0.11	0.26	0.05
Göreli Mutlak Hata (GMH) %	93.20	49.96	11.69	61.10	1.07
Göreli Hata Karekök (GHK) %	97.11	60.91	33.51	76.79	14.50

Şekil 1. Veri madenciliği algoritmalarının doğru sınıflandırmadaki performansları

Fig 1. The performances of selected algorithms of data mining





Şekil 2. Ridor algoritmasına göre her bir etki faktörü için döllülük özelliğine ait dağılımlar

Fig 2. Distributions of fertility trait according to the Rider algorithm for each impact factor

Tablo 2. *Etki faktörlerine ait gözlem sayıları ve Ki kare test istatistiği sonuçları (n, %)*

Table 2. *The number of observations of the impact factors and Chi-square test statistic results (n, %)*

Etki Faktörü	Döllü f, (%)	Dölsüz f, (%)	Toplam f, (%)	P Değeri
Mevsim				
Kış	280 (%24.5)	48 (%4.2)	328 %28.7	***
Sonbahar	326 (%28.6)	22 (%1.9)	354 %30.5	
Yaz	372 (%32.6)	93 (%8.2)	465 %40.8	
Genotip				
Kontrol	638 (%55.9)	90 (%7.9)	%63.8	**
Seleksiyon	340 (%29.8)	73 (%6.4)	%36.2	
Yerleşim sıklığı				
240 cm²	568 (%48.9)	66(%5.8)	%54.7	***
160 cm²	410 (%36.8)	97 (%8.5)	%45.7	

*** P:0.01; *** P<0.001*

belirlenen bu algoritmaya göre oluşan modelin Kappa istatistiği 0.98 değeri ile uyum derecesi bakımından çok iyi olarak tanımlanmaktadır.

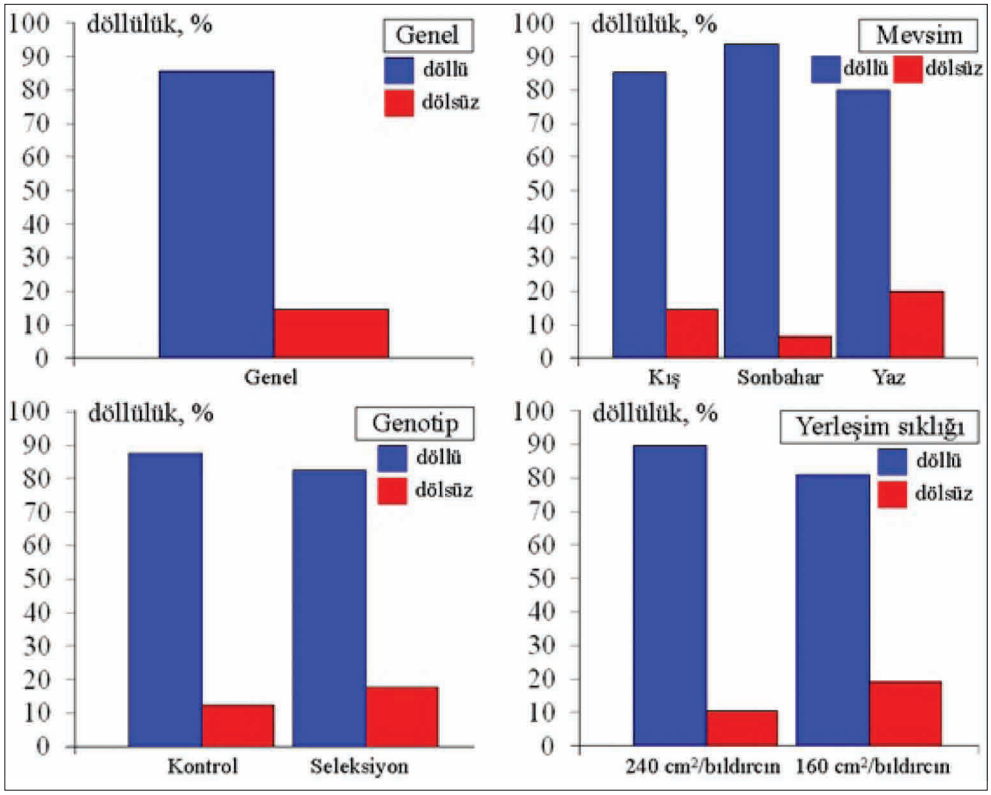
Ridor algoritmasına göre yapılan analiz sonucunda gerçekte 978 adet yumurtanın döllülük durumu "var" olarak doğru, 3 tanesi "yok" olarak hatalı bir şekilde sınıflandırırken, 163 adet yumurta ise döllük durumu "yok" olarak doğru bir şekilde sınıflandırmıştır. Buna göre toplamda 3 adet yumurtanın döllülük durumu bu algoritma ile hatalı sınıflandırmış, geriye kalan 1138 tane yumurta %99.73 başarı oranı ile doğru sınıflandırmıştır *(Tablo 1)*. Ridor sınıflandırma algoritmasına göre yumurtaların döllü-dölsüz olarak sınıflandırılmasında etkili olan genotip, mevsim ve yerleşim sıklığı faktörlerinin etkileri Weka programından yararlanılarak çizilen grafikler aracılığıyla incelenmiş ve *Şekil 2*'de sunulmuştur. Her bir faktöre göre çizilen grafiklerde, mavi renkler ile gösterilen döllülük durumunun kırmızı renk ile gösterilen dölsüz olanlara nazaran daha fazla olduğu görülmektedir. Ayrıca bu renklerin her bir mevsim (kış, yaz ve sonbahar), yerleşim sıklığı (160-240 cm²/bıldırcın) ve genotip (kontrol-seleksiyon sürüsü) içinde kümelenmesi birbirinden farklılık göstermektedir *(Şekil 2)*. Veri madenciliğinin Ridor algoritmasına göre elde edilen bu grafiklerden yumurtaların döllü ve dölsüz sınıflardaki frekans dağılımları ki kare test istatistiği ile araştırılmış ve sonuçlar *Tablo 2*'de verilmiştir. Araştırmaya konu olan ve döllülük üzerine etkileri araştırılan mevsim, genotip ve yerleşim sıklığı gruplarına ait döllülük oranları ise *Şekil 3*'te sunulmuştur.

TARTIŞMA ve SONUÇ

Araştırmada kuluçkalık yumurtaların döllülük özelliğine göre (döllü-dölsüz) sınıflandırılması için veri madenciliğinde yer alan farklı algoritmalarından yararlanılmış ve doğru sınıflandırmalarındaki yüzde başarı oranları karşılaştırmalı olarak incelenmiştir *(Tablo 1)*. Yapılan karşılaştırma sonucunda Ridor algoritmasının en az hata ile daha başarılı sonuçlar ürettiği görülmüştür *(Şekil 1)*. Bu algoritmaya göre oluşturulan model ile yapılan sınıflandırma sonucunda, farklı mevsim ve yerleşim sıklıklarında yetiştirilen iki hattan Japon bıldırcınlarına ait kuluçkalık yumurtalarda döllülük oranı %85.71 olduğu belirlenmiştir *(Tablo 1)*.

Japon bıldırcını rasyonlarında Yucca bitkisi tozu kullanımının üreme performansına etkisini araştıran Ayaşan [15], deneme gruplarında döllülük oranının %85.09 ve %85.42 olduğunu, gruplar arasında anlamlı bir farklılık olmadığını bildirmiştir. Ana yaşı, baba yaşı ve yumurta ağırlık sınıflarının döllülük oranını etkilediğini ortaya koyan Sarı ve ark.[16], söz konusu çalışmalarında döllülük oranının %85.32-93.72 aralığında olduğunu bildirmişlerdir. Benzer bir çalışmada da [17] yumurta ağırlığı, depolama sıcaklığı ve depolama süresi faktörlerinin bıldırcın yumurtalarında döllülük oranını etkilediği tespit edilmiş ve döllülük oranlarının %73.28-86.31 arasında değerler aldığı bildirilmiştir. Bu çalışmada saptanan döllülük ortalaması (%85.71), her üç çalışmanın sonuçlarıyla uyumlu bulunurken, Japon bıldırcınlarında döllülük özelliğinin araştırıldığı birçok çalışmada [18-20] saptanan değerlerle (%79.3-94.8) de uyumlu olmuştur. .

Mevsim, genotip ve yerleşim sıklıklarında oluşan döllü-dölsüzlüğün Ki kare analizi ile incelenmesi sonucunda ise dölsüz yumurta oranları bakımından en yüksek değerin (%8.2) yaz mevsiminde meydana geldiği, bu mevsimde elde edilen her 5 kuluçkalık yumurtadan birinin dölsüz olduğu ortaya çıkmıştır *(Tablo 2)*. Oysa, kış ve sonbahar mevsimlerinde elde edilen kuluçkalık yumurtalarda döllülük oranları sırasıyla %85.36 ve %93.67 olarak tespit edilmiştir *(Şekil 3)*. Bu durum, Renaudeau ve ark.[21] tarafından



Şekil 3. Etki faktörlerine ait döllülük oranları

Fig 3. Fertility rates of impact factors

bildirilen yüksek yaz sıcaklarının çiftlik hayvanlarının üreme yeteneği ve döllülük özelliği üzerine olumsuz etkilere sahip olduğu görüşünü desteklemektedir. Bıldırcınlar kullanılarak gerçekleştirilen çalışmalar sonucunda yerleşim sıklığının döllülük özelliği üzerinde etkili bir çevresel unsur olduğunu bildirilmiştir [6,22]. Benzer bulgular bu araştırmada da saptanmış, yerleşim sıklığının döllülük özelliği üzerinde etkisi anlamlı bulunmuş *(Tablo 2)* ve geniş bir yerleşim sıklığına (160 cm^2/bıldırcın) sahip olan bıldırcınların yumurtalarında döllülük oranı %89.59 olarak bulunurken, bıldırcın başına 160 cm^2 alanda yetiştirilen bıldırcınlarda bu oranın %80.87'ye gerilediği ortaya konulmuştur *(Şekil 3)*. Anthony ve ark.[23] tarafından gerçekleştirilen araştırma sonuçlarına göre, Japon bıldırcınlarında dördüncü hafta canlı ağırlığını arttırmak için gerçekleştirilen seleksiyon uygulamasının döllülük özelliği üzerine negatif etkisi bulunmaktadır. Benzer sonuca bu çalışmada da rastlanmıştır. Seleksiyon ile canlı ağırlığı artırılmış hattan elde edilen kuluçkalık yumurtalarda döllülük oranı %82.32 olarak saptanmış, buna karşın herhangi bir seleksiyon uygulanmayan kontrol sürüsünde ise aynı özellik %87.64 olarak bulunmuştur.

Bu çalışmada mevsim, genotip ve yerleşim sıklığı faktörlerinin Japon bıldırcınlarından elde edilen kuluçkalık yumurtalarda saptanan dölllük özelliğine etkilerinin ölçülmesi için bir veri madenciliği uygulaması gerçekleştirilmiştir. Bugüne kadar ekonomi, sanayi ve sağlık alanında yoğun bir şekilde kullanılan, ancak tarım alanında nadir uygulamaları bulunan veri madenciliğinin hayvancılık alanında toplanan verilerle de kolaylıkla uygulanabileceği ortaya konulmuştur. Özellikle büyük veri setleri ile çalışılan hayvan ıslahı alanında veri madenciliği uygulamaları ile verinin işlenmesi aşamasında oldukça kolaylık sağlanabileceği düşünülmektedir.

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RESEARCH ARTICLE

Limbal Stem Cells in Dogs and Cats Their Identification Culture and Differentiation into Keratinocytes

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Abstract

Limbal epithelial stem cells are the source of regeneration of the corneal epithelium in normal and diseased conditions and it is proven that they are located at the area known as limbus. Maintaining avascularity and clearity of the cornea is a crucial role in homeostasis and integrity of the eye. This study focuses on cats and dogs limbal derived mesenchymal stem cells (LMSCs) differentiation potentials into keratinocytes for developing a culture model for corneal diseases. Limbus-derived mesenchymal stem cells were obtained from the limbus of terminated fetuses of the pregnant dogs (45 d) and cats (35 d). Explant cultures were allowed to grow in the culture medium untill 3rd passage. LMSCs differentiated into keratinocytes and allowed to grow into the decelularized amniotic membrane (AM) until three layer formation is maintained. Characterization was carried out by transition of cells from all three germ layers (osteogenic, chondrogenic and adipogenic). Histology and immunohistochemistry was carried out with cytokeratin 19 (CK 19) and pancytokeratin detected in the cornea. Three lineage differentiation and keratinocyte transformation was succesfully maintained. Immunohistochemically LMSCs were confirmed for the cell surface markers and cells were positive for CD90, CD44 and CD49 and negative for CD45 and CD11b\c. LMSCs are potentially the native cells of the cornea and reconstruction of the cornea with these cells will have a better outcome.

Keywords: *Cat, Dog, Cornea, Keratinocytes, Limbal mesencymal stem cell (LMSC)*

Kedi ve Köpeklerde Limbal Kök Hücrelerin Kültürü ve Keratinositlere Farklılaştırılması

Özet

Limbal epitel kök hücrelerin limbus olarak bilinen bölgede bulundukları kanıtlanmış olup normal ve hastalık durumlarında kornea epitelinin yenilenmesinden sorumludurlar. Korneanın damarsızlığının ve saydamlığının devamı, gözün homeostazisinin ve bütünlüğünün korunmasında önemli role sahiptir. Bu çalışmada kedi ve köpeklerin korneal hastalıklarının tedavisinde kulanılmak üzere keratinositlere farklılaştırılan limbal kaynaklı mezenkimal kök hücrelerin (LMSCs) farklılaşma potansiyelleri ve kültür özellikleri üzerinde durulmaktadır. Limbus kaynaklı mezenkimal kök hücreler gebe köpek (45 gün) ve kedilerin (35 gün) istenmeyen gebeliklerinin sonlandırılması ile elde edilen fötuslardan elde edilmiştir. Eksplant kültürler, kültür ortamında üçüncü pasaja kadar büyümeye bırakılmıştır. Keratinositlere farklılaştırılmış LMSCs desellülerize amniyotik membran (AM) üzerinde üç katman oluşumu sağlanana kadar üremeye bırakılmıştır. Hücrelerin karakterizasyonu her üç germ tabakasına (osteojenik, kondrojenik ve adipojenik) hücrelerin geçişi ile sağlanmıştır. Histoloji ve immonohistokimyasal analizler gerçekleştirilmiş ve korneada sitokeratin 19 (CK 19) ile pansitokeratinin pozitifliği gösterilmiştir. Keratinositlere ve her üç germ tabakasına dönüşüm başarı ile gerçekleştirilmiştir. Immunohistokimyasal olarak LMSC'ler hücre yüzey işaretleyicileri tespit edilmiş ve CD90, CD44 ve CD49 için pozitiflik, CD45 ve CD11b\c için ise negatiflikleri belirlenmiştir. LMSC'ler kornea dokusunun kendi hücreleri olması sebebiyle, ileriki çalışmalarda bu hücreler kullanılarak kornea onarımından daha iyi sonuçlar alınacağı düşünülmektedir.

Anahtar sözcükler: *Kedi, Köpek, Keratinosit, Kornea, Limbal mezenkimal kök hücreler (LMSC)*

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INTRODUCTION

Many eye diseases such as abnormal growth or disorders of the corneal epithelium are observed in veterinary ophthalmology. Among them, the most common are corneal ulcers, neurotrophic ulcers, feline corneal necrosis (FKN), keratoconjunctivitis sicca (KCS), Uber-Reiters Syndrome, Feline herpesvirus type-1 (FHV-1) [1].

The use of stem cells in veterinary medicine is limited. Limbal stem cells found within the basal layer of the limbal epithelium are responsible for continious renewing the entire corneal epithelium and ensures the integrity of the eye [2-4]. The XYZ hypothesis clearly explains the epithelial cell renewal in the eye. X (proliferation of basal cells) + Y (proliferation and centripedal migration of limbal cells) = Z (epithelial cell loss from the surface). Under normal conditions the cells that are lost from the corneal epithelium are renewed by epithelial cells derived from the limbal stem cells. When limbus area is damaged, limbal stem cell deficiency related disorders can be seen. These are as follows; corneal ingrowth of conjunctival epithelium, neovascularization, scarring, chronic inflammation, pain and reduced vision [3,5,6]. In cases of unilateral limbal stem cell deficiency limbal autograft can be harvested from the healthy eye. when limbal stem cell deficiency is bilateral a limbal allograft from a living relative or cadaver donor can be applied. Unfortunately corneal allografts may couse some problems like patient rejection of the graft or avalibility of the graft in the time it is needed. As an alternative to limbal grafting corneal stem cell theraphy or exvivo expanded limbal epithelial cells can be used and these cells can preserve the structural and functional integrity of the cornea. The use of limbal stem cells in corneal defects are very effective, although not yet available [6]. The use of limbal stem cells in the treatment of diseases by *in vitro* cultivation is considered.

Compared to bone marrow derived stem cell limbal mesenchymal stem cells are the native cells of the eye and will ensure a better corneal healing when used in cornea. Limbal mesenchymal stem cells differentiation into keratocytes and use of these cells in the corneal diseases is believed to show a better outcome.

The aim of this study is to focus limbal derived mesenchymal stem cell culture characteristics and optimization of the cell culture conditions and to show their differentiation capacity into keratocytes in cats and dogs. In this way, we are intending to treat primarily corneal diseases and then stem cell deficiency related diseases. In future therapeutic use of limbal-derived mesenchymal cells is aimed to clinically diagnosed corneal ulcer, Uber-Reiters syndrome, FKN, KCS and FHV-1 patients.

MATERIAL and METHODS

Fetus samples were collected from the bitches of both cats and dogs while termination of the unwanted pregnancies during the ovariohysterectomy operation. Samples were used with the permission of the Board of Ethics in Animal Experiments of Ankara University (20.01.2014/53184147-50.04.04/3800). Samples taken from fetuses were transferred to the laboratory as fresh. Transferred newborn dogs and cats eyes were placed into the Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Belgium).

Limbus region was determined and divided into small pieces. The removed tissue was divided into segments. The explants were performed by the method of cultivation, and then seeded in T25 flasks and incubated at 37°C in a humidified atmosphere 5% CO_2.77% DMEM (Lonza, Belgium), 20% fetal bovine serum (Lonza, Belgium) 2% L-Glutamine (Lonza, Belgium) 1% Penicillin, streptomycin, amphotericin (Biological Industries, Israel) composed medium was added. Medium was replaced every 2-3 days and nonadherent cells were discarded. At aproximately 70% confluence, adherent LSCs were passaged at split ratio of 1:2 using 0.25% trypsin in PBS. The cells were grown untill the 3rd passage.

All placental samples were obtained under sterile conditions after elective caesarean delivery from the same bitches. To separate the amniotic membrane from the whole placenta, the amniotic membrane was peeled off from the chorionic membrane mechanically. Under sterile conditions, the collected amniotic membranes were rinsed with normal saline (0.9%) several times. Generally, amniotic membrane (AM) composed of epithelial monolayer and an avascular stroma. Under a laminar flow hood, the placenta was first washed free of blood clots with sterile saline and later in 4%, 8%, and 10% dimethylsulphoxide (DMSO) and phosphate buffered saline (PBS) for 5 min each. The membrane was then flattened onto a nitrocellulose paper, with the epithelium/basement membrane surface up. The amniotic membrane was then cut into 5-5 cm pieces. Each of them was placed in a sterile vial containing 10% DMSO medium. The vials were frozen at -70°C. The membrane was defrosted immediately before use by warming the container to room temperature for 10 minutes, and rinsed three times in saline. Limbal derived MSCs (grown into 3rd passage) growth medium was was replaced by keratinocyte differention medium. Limbal derived MSCs differentiation into keratinocytes were as follows: medium change was carried out 3 days intervals and the differentiation was maintained in 14 days the differentiation was ended in total 21 days. Before seeding onto the amniotic membrane the cells were stained with Kodak invivo fluorescent stain to allow the folllow of the cells in vivo by Kodak equipment. Initially the AM was prapared then the cells were seeded and allowed to

differentiate into keratinocytes. Keratinocyte seeding onto the AM was carried out in six well plate and $5x10^4$ cells were seeded in total. The second layer of cells were seeded onto the first layer and allowed to grow for 4-6 days with 2 days intervals medium change. The third layer of cells were seeded onto the second layer and allowed to grow for 4-6 days with 2 days interval medium change. This three layer tissue formation maintained in 30 days and at the end of 30 days the medium is replaced with formol and fixed. Three layer of cells on the both side of the membrane could be visualised and this sandwich structure is embedded into parafine. The tissue block is stained with hematoxylin-eosin and immunohistochemically surface markers are visualised.

The LMSCs were seeded at a density of $5x10^4$ cells\cm^2 in six-well plates at P3 and differentiated into adipogenic, chondrogenic or osteogenic differentiation medium for 21 days as previously described [7,8]. The differentiation of cells into the adipogenic, osteogenic, and chondrogenic lineages was determined using different histological staining techniques. Von Kossa staining demonstrated deposition of minerals in osteogenic cultures. Adipogenic differentiation was evaluated using Oil red O to reveal lipid droplet accumulation in the cell cytoplasm and Alcian blue (pH 2.5) staining was used to reveal chondrogenic differentiation based on the production of a ground substance matrix. Each passage was photographed and in each passage in days 1, 3, and 6 photography was documented as well. In all these staining methods, the nuclei were counterstained with Mayer's hematoxylin and the stained cells were examined under a light microscope.

For cytokeratin 19 (CK19) and pancytokeratin staining, frozen section tissue slides were fixed in cold methanol for 30 min, followed by two PBS washes. After being blocked in 5% skim milk at room temperature for 1 h, slides were incubated with a mouse antibody against human/mouse CK19 (1:500, Millipore, Billerica, MA, USA), followed by incubation with DyLight 488-conjugated goat anti-mouse IgG (1:500, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA), or DyLight 594-conjugated goat anti-rabbit IgG (1:500, Jackson ImmunoResearch Laboratories, Inc.) at room temperature for 30 min. Nuclei were then stained with hematoxylene eosin and samples were assessed under a fluorescence microscope (Leica Microsystem).

RESULTS

The third passage cat and dog LSCs were grown in T25 culture flasks and showed fibroblast-like morphology. At the beginning of the culture period, dog cells showed better proliferation than cat LSCs. But overall, throughout the culture period, the confluence of the cells was similar (10-12 days). By the end of the first week, both the cat and dog LSCs displayed interconnection and monolayer confluence was observed.

The cultured LMSCs typically showed colony forming unit fıbroblast CFU-U morphology both in cat and dog cultures *(Fig. 1)*. LMSCs were cultured in normal medium and the expression of a panel of cell surface markers were studied. Analysis of LMSCs cultured in normal medium confirmed the cell surface markers for mesenchymal stem cells. At P3 95% of cells were positive for CD90, CD44 and CD49 and negative for CD45 and CD11b\c.

The ability of LMCSs to differentiate into adipogenic, osteogenic and chondrogenic lineages were assesed. It is observed that LMSCs differentiated into all three lineages when cultured in a normal medium. In adipogenic cultures the Oil red O stained fat vacuoles were observed throughout the culture *(Fig. 2)*. Chondrogenesis was observed with Alcian blue staining of glycosaminoglycans. Deep staining

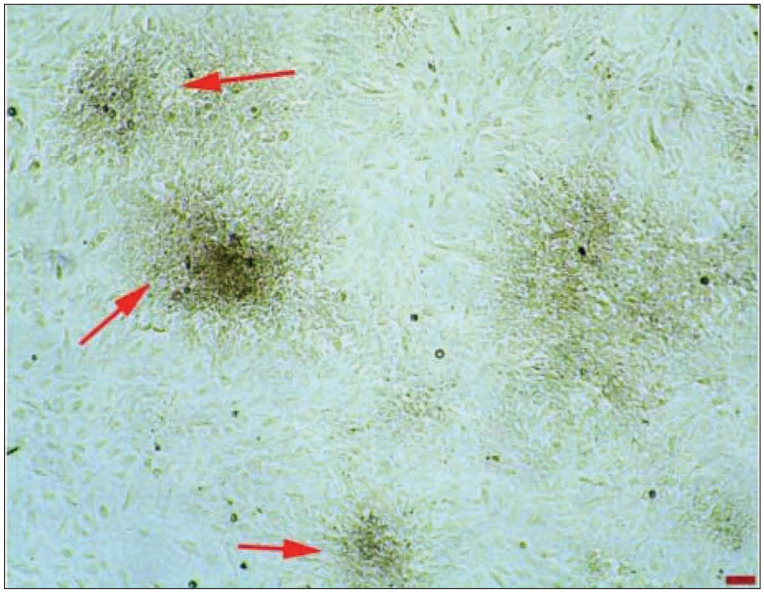


Fig 1. The cultured LMSCs showing colony forming unit fıbroblast (CFU-U) morphology both in cat and dog cultures

Şekil 1. Kedi ve köpeklerde kültüre edilmiş LMSC'lerde koloni forming fibroblast (CFU-F) yapılarının gözlenmesi

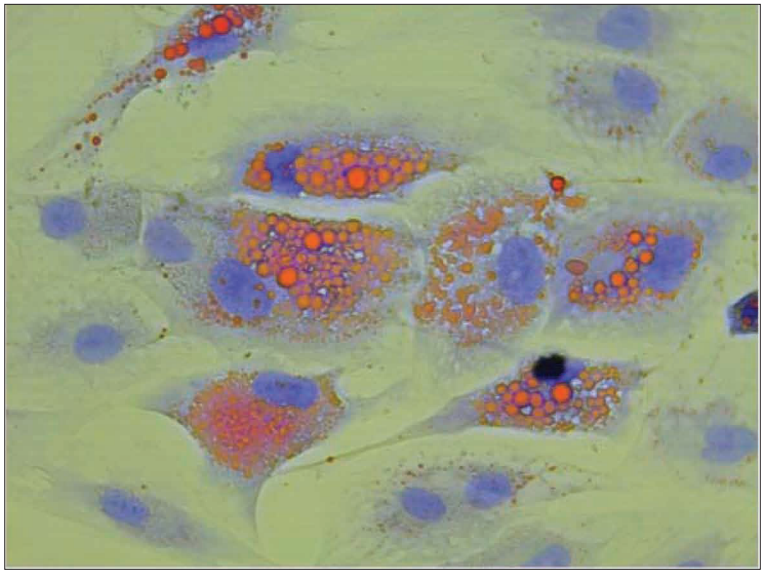
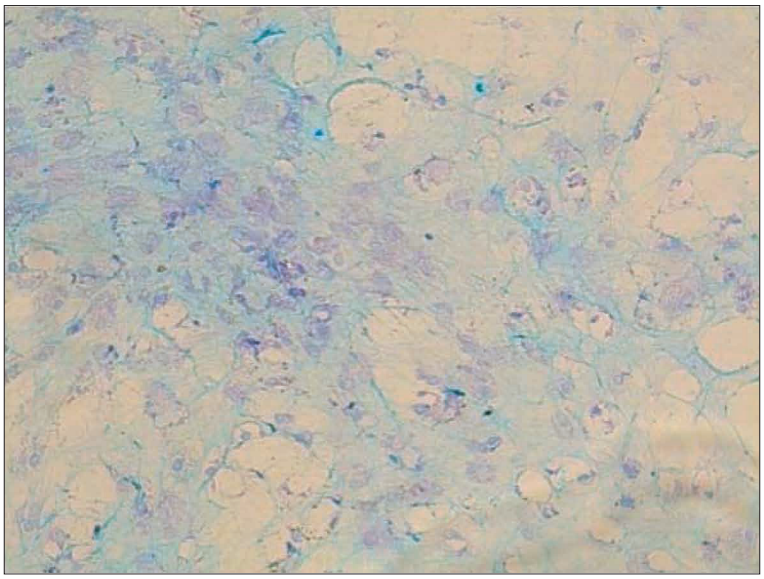


Fig 2. Adipogenic differentiation with Oil red O stain showing fat vacuoles throughout the culture

Şekil 2. Adipogenezin göstergesi olan Oil red O bayama ile yağ damlacıklarının kültürde yaygın olarak gözlenmesi

Fig 3. Chondrogenic differentiation with Alcian Blue showing glycosaminoglycans

Şekil 3. Kondrojenik farklılaşmada glikozaminoglikanların Alcian Blue ile boyanması



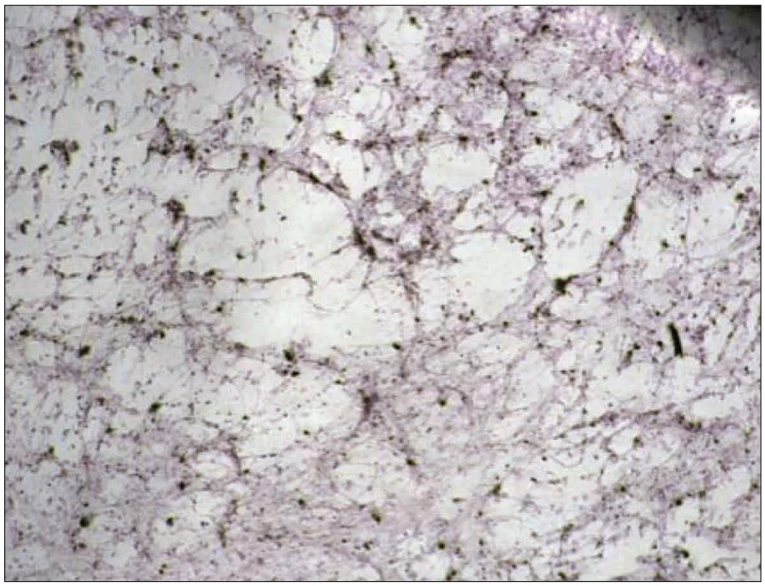


Fig 4. Osteogenic differentiation with Von kossa showing calcium deposition in cultures

Şekil 4. Osteojenik farklılaşmada kalsiyum birikimlerinin Von Kossa ile boyanması

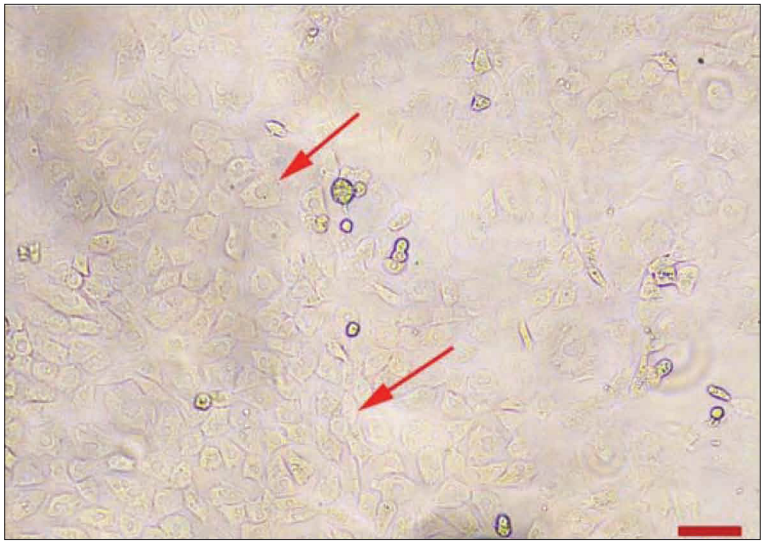


Fig 5. The LMSCs differentiation into keratinocytes

Şekil 5. Limbal kaynaklı mezenkimal kök hücrelerin keratinositlere dönüşümü

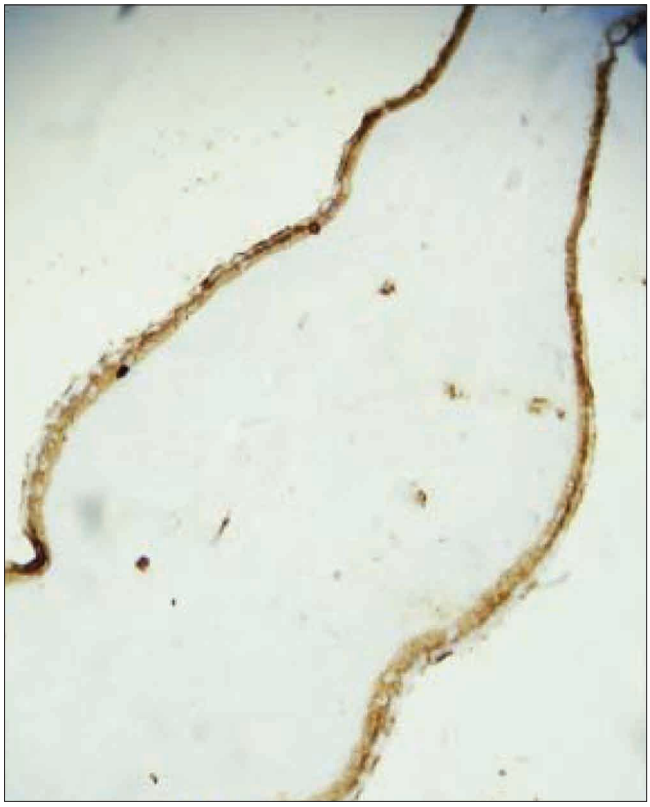


Fig 6. Positivity of CK19 and pancytokeratin bilaterally on amniotic membrane

Şekil 6. CK 19 ve pansitokeratinin amniyotik membran üzerinde çift taraflı pozitifliği

of chondrogenic nodules can be seen in cultures *(Fig. 3)*. Osteogenic differentiation was shown by Von Kossa staining of calcium deposition in cultures. Also individual bone nodules can be seen in the cultures *(Fig. 4)*.

The LMSCs were put through the procedure for keratinocyte differentiation *(Fig. 5)* and subsequently analysed by using flow cytometry for CK19 and pancytokeratin markers of progenitor and diffrentiated corneal epithelium respectively. The high positivity of CK19 and pancytokeratin was observed in both sides of the amniotic membrane with a multilayer manner *(Fig. 6)*.

DISCUSSION

The placenta is comprised three-layer structure of the amnion, chorion and decidua. The roles of the placenta are to provide nutrients and oxygen, which are essential for fetal survival and development. The amnion is a thin, nonvascular membrane, which has a two-layer structure: an epithelial monolayer and a stromal layer [9]. In particular, the amnion is the sac that binds the fetus and constructs the environment. The amniotic membrane has clinical applications in covering wounds and burn lesions and ocular surface reconstruction [10]. Recently, canine stem cells have been studied for use in cell therapy [11,12]. However, they have been confined to limited stem cell sources. Most of the cells were isolated from canine adipose-derived tissue, umbilical cord blood or bone marrow [13-15]. Therefore, limbal mesenchymal stem cells provided from the fotal tissue seems to be a new cell source for corneal reconstruction. Beyond this, these cells are the native cells of the eye itself and it is thought that with the use of these cells a better corneal healing and more transparent cornea could be obtained.

Systemic transplantation of a high dose of MSCs reduces induced inflammatory damage to the cornea by secretion of TNF-α-stimulated gene/protein 6 with minimal engraftment [16], which shows that systemic administration is not an efficient way to deliver stem cells to the cornea, a physiologically avascular tissue. However, the abundant paracrine effect of MSCs contributes to inhibition of inflammation. It has been reported that BM-MSCs expanded on an amniotic membrane can reconstruct cornea by inhibiting inflammation and angiogenesis [17]. Alternatively, application of stem cells with the help of

the amniotic membrane provides stem cells with direct contact with the corneal epithelium and stroma on a damaged cornea.

Wound healing is essential for tissue regeneration. Corneal transparency determines visual acuity. Corneal tissues are physiologically composed of the corneal epithelium, stroma and endothelium. Clarity of the cornea depends on an intact corneal epithelium, tight organisation of epithelial cells, constant water content, and regular arrangement of keratocytes and keratocyte produced extracellular matrix in the stroma [18-20]. For the future applications, when LMSCs differentiated into keratinocytes are applied to the corneal wound, it is believed that it will reduce the time needed for healing of the corneal opacity.

In our previous study, we found that cell-cell interactions between stem cells were crucial for cell signalling [21]. Recently, application of autologous MSCs to the bottom of a corneal ulcer for a persistent sterile corneal epithelial defect has been reported [22]. The corneal stroma is connective tissue maintained by keratocytes [23]. Administration of stem cells to the cornea with an epithelial defect provides a diffuse distribution of cells in the stroma, and which favors direct stem cells-keratocyte interactions. Furthermore application of keratocytes with the help of amniotic membrane will result with a better corneal healing and transparent cornea. At the same time it will reduce the time of these cells to turn into keratinocytes.

Initially it is believed that anti inflammatory effect of stem cells will inhibit the cellular response and at a late stage promotion of the differentiation of stem cells into keratinocytes will have a high impact corneal transparency. Disorders like corneal ulcers, neurotrophic ulcers, feline corneal necrosis (FKN), keratoconjunctivitis sicca (KCS), Uber-Reiters Syndrome, Feline herpesvirus type-1 (FHV-1) associated disorders can be cured with cultured limbal epithelial stem cells. Furthermore the fetal limbal stem cell cultivation and differentiation into keratinocytes is a simple and a reliable method of delivering stem cells to cornea for corneal tissue regeneration. Once it is produced it can safely and effectively be used in the same species and also be stored for subsequent applications.

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RESEARCH ARTICLE

Investigation of Mast Cell Distribution in the Ovine Oviduct During Oestral and Luteal Phases of the Oestrous Cycles [1]

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[1] *This study had been presented at XII. National Congress of Histology and Embryology (27-30 May 2014, Ankara - Turkey)*

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Abstract

Mast cells are heterogeneous cell populations that play significant roles in many organs and systems and involve various physiological processes. We aimed to evaluate mast cells in the ovine oviduct mucosa by means of their staining and ultrastructural characteristics. The ovine oviduct samples of Akkaraman breed were collected from the slaughterhouse and they are categorized as luteal and oestral phases. They were fixed either with 10% formalin or IFAA and stained with Toluidine blue and Alcian blue and Safranin O (Ab/SO). Mast cells were located near blood vessels and basal membrane. Compared to 10% formalin fixed tissues, the number of mast cells were higher in IFAA fixed tissues (P=0.003). Importantly all mast cells Ab(+) and SO(-) so that they were categorized as mucosal type. The number of mast cells did not differ between luteal and oestral phases (P>0.05). However, there were significant differences among different regions of the oviduct with a less count in the isthmus regions (P=0.006). Transmission electron microscopy revealed that the oviduct mast cells contained two types of granules: an electron lucent, electron dense. Some electron lucent granules contained an eccentrically located crystal-like structure. The significance of less mast cell counts in the isthmus and the eccentrically located single crystal-like structure should be further investigated in future studies.

Keywords: *Mast cell, Oviduct, Ovine, Sexual cycle, Electron microscopy*

Östral ve Luteal Dönemlerdeki Koyunların Oviduktunda Mast Hücrelerinin İncelenmesi

Özet

Mast hücreleri birçok organ ve sistemdeki çeşitli fizyolojik süreçlerde önemli rolleri bulunan heterojen hücre topluluğudur. Bu çalışmada koyun ovidukt mukozasındaki mast hücrelerini boyanma özellikleri ve ince yapı düzeyinde incelemeyi amaçladık. Mezbahanede kesim sırasında östral ve luteal dönemleri Akkaraman koyunlarının oviduct örnekleri alındı. Alınan örnekler %10 formol ve IFAA ile tespit edilerek Toluidin blue ve Alcian blue-Safranin O (Ab/SO) ile boyandı. Mast hücrelerinin, kan damarlarının ve ovidukt epitelinin bazal membranına yakın olarak yerleştiği görüldü. IFAA ile tespit edilen dokulardaki mast hücre sayısının %10 formolle tespit edilen dokulara göre daha fazla olduğu belirlendi (P=0.003). Dikkat çekici olarak mast hücrelerinin tamamının Ab(+) ve SO(-) olduğu görüldü. Luteal ve östral dönemler arasında mast hücre sayısı yönünden fark bulunamadı (P>0.05). Fakat ovidukt bölgeleri arasında istatistiksel olarak önemli fark vardı ve mast hücre sayısı istmusta daha azdı (P=0.006). Elektron mikroskobik incelemelerde mast hücrelerinin elektron açık ve elektron koyu olmak üzere iki tip granül içerdiği ortaya konuldu. Bazı elektron açık granüllerde eksantrik yerleşimli elektron koyu kristal benzeri bir yapının varlığı gözlendi. İstmusta mast hücrelerinin daha az sayıda görülmesinin ve granüllerdeki eksantrik yerleşimli kristal benzeri yapının daha sonraki çalışmalarda araştırılması önemlidir.

Anahtar sözcükler: *Mast hücresi, Ovidukt, Koyun, Seksüel siklus, Elektron mikroskop*

INTRODUCTION

Mast cells are connective tissue cells that contain numerous basophilic granules in the cytoplasm and exhibit strong metachromasia due to heparin and highly sulfated proteoglycans present in their granules [1].

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Taking origin from the bone marrow, mast cells circulate in the blood stream without presence of granules and subsequently migrate to connective tissue sites and then differentiate to mature types that begin synthesis of specific granules [2]. Based on staining feature, size, and location, mast cells are classified, especially in rodents, into two groups: atypical mucosal mast cells (MMC) and typical connective tissue cells (CTMC) [3]. While MMCs are found especially in the lamina propria of the gastrointestinal (GI) tract and respiratory canal, CTMCs are found in the peritoneum, skin, and submucosa of the GI tract [4]. Mast cells can also be classified based on protease contents of the granules. The first type only contains tryptase such that MMCs are categorized in this category. The second type contains chymase carboxypeptidase and catepsin in addition to tryptase such that CTMCs are categorized in this category. The third type contains chymase and carboxy peptidase [5]. Such proteases directly affect tissues in which they are located [6]. Under influence of various mediators released into vicinity of connective tissue, mast cells can differentiate to each type, from MMC to CTMC and CTMC to MMC [4].

Mast cells are commonly found in connective tissues of the several organs and they play critical roles in hypersensitivity reactions and bacterial inflammations [4]. In addition, mast cells have critical roles in angiogenesis, inflammation and tissue regeneration [7]. Located near vasculature, numerous mast cells are found in skin and mucosa. In addition their critical roles in innate immunity, they also have roles in required immune response [8]. Among the mast cell granule contents are histamine and heparin, which are known to induce vascularization and endothelial cell proliferation [4].

In the reproductive tract, mast cells contribute to cellular immune response and to formation of the anti-bacterial barrier [7]. It is known that heparin is physiologically important for sperm capasitation [9]. It has been proclaimed that histamine inhibits cytotoxic lymphocyte activity [10]. In turn, reproductive hormones estrogen and progesterone can activate mast cells through receptors [11]. In response to estrodiol, mast degranulate and release a variety of bioactive substance. For instance, an *in vitro* study showed that estrodiol increases histamine release from rat mast cells [7]. Histamine increases the capillary permeability in the ovarium during ovulation. Increase in blood flow and vascular leakage result in edema in the oviduct [12].

Hormonal changes during sexual cycle may influence mast cell metabolism as they respond to sexual hormones. The mast cell involvement in physiology of the female reproductive tract and hormonal influence on mast cells as well as cascade of events following mast cell degranulation are still of a scientific interest [11]. The oviduct is an important region of the reproductive tract for being the fertilization sites. Mast cells are considered as important components of the reproductive physiology as bioactive molecules contained in granules directly involved in several physiological events such as vasodilatation and vasoconstrictions, which are very common during the sexual cycle. In support to this notions, histamine and 5-hydroxytriptamine (5-HT), commonly found in mast cells granules, are present in high concentration in the rat oviduct [12]. The sheep is an economically important domestic animal and, thus, we aimed to investigate mast cell distribution and ultrastructural characteristic of the ovine mast cells present in the oviduct.

MATERIAL and METHODS

The oviduct samples were collected from the Kazan Slaughterhouse. The ovine oviduct samples were collected from 14 sheep of Akkaraman breed, 7 samples representing oestral phase and 7 samples representing luteal phase of the sexual cycle. The phase of the sexual cycle was determined based on macroscopic evaluation of the ovarium [13] and RIA [14] test to determine progesterone concentration on blood samples collected at slaughter.

For light microscopic evaluation, the oviduct samples were further divided into three parts: fimbria, ampulla and isthmus. Such subregions were divided into two pieces and one piece was fixed in 10% formalin and the other was fixed in isotonic formaldehyte acetic acid (IFAA). Following routine histological procedure, all samples were embedded in paraffin blocks [3]. From the paraffin blocks, 5 µm thick two consecutive sections were cut for Toluidine blue and Safranin O staining. Such a double consecutive sectioning was repeated with a 30 µm interval for 10 times. One section was stained with 0.5% Toluidine blue (pH 4) prepared in Mc Ilvaine's citric acid disodium phosphate buffer and the other section was stained with Alcian blue/ Safranin O (Ab/SO) [15,16]. On a same slide, an IFAA fixed and a 10% formalin fixed sample were placed and stained. For staining positive control, the rat intestine and rat skin were used for MMC and CTMC, respectively.

For Transmission Electron Microscopy, a modified Karnovsky's method was followed [17,18]. Briefly, tissues were pre-fixed in gluteraldehyte-paraformaldehyte (pH 7.4) for 24 h, washed in cocdylate buffered for 3 h, and further fixed in 1% osmic acid for 2 h. Tissues were then kept in 0.5% uranyl acetate for 2 h, in graded alcohol, and propylene oxide and then embedded in Araldite M. The 300-400 Angstrom thick sections cut from these blocks were contrasted according to the Veneable and Coggeshall [19] methods and evaluated using the Carl Zeiss EM 9S-2 model transmission electron microscopy.

Mast cells in the lamina propria of the oviduct were counted in the Toluidine blue stained sections according to previously used methods [18,20]. Briefly, using an 100 -square ocular micrometer (eye piece graticule), mast cells were counted in per unit at the 40x objective. For each

section, 10 randomly selected areas were counted. Then all counted data of per unit area were converted into the number of mast cells in 1 mm^2 area.

Data processing was performed with the SPSS 15.0 (SPSS, Inc., Chicago, IL, USA). The normality of all data was assessed by Shapiro-Wilk Test. To compare the IFAA fixation with formol fixation, Mann Whitney U Test was used. The difference between oestral and luteal phase within the same oviduct region were assessed with either Mann Whitney U Test or Student T-test. The numerical distribution (mm^2) of mast cells with %10 formol fixation in the various regions of the oviduct during the luteal and oestral phases was evaluated by Kruskall-Wallis analysis of variance. *Post hoc* comparisons were performed using *Mann-Whitney U test with Bonferroni corrected*. Other parameters were analyzed by One-way analysis of variance (ANOVA). When the *F* values were significant, Duncan's Multiple Range Test was performed. *P* values less than 0.05 were considered as significant for all statistical calculations; however, in *Mann Whitney U test with Bonferroni corrected*, P value less than 0.016 was considered as significant.

RESULTS

Mast cells were generally localized to vessel surroundings and near the basal membrane *(Fig. 1A)*. Mast cells in Toluidine blue stained sections were observed oval in shape with metachromatically stained cytoplasmic granules and easily distinguishable nucleus *(Fig. 1B)*. Metachromasia was more prominent in the IFAA fixed tissues.

Mast cells were present in various tunics of the oviduct; however, we only counted those in the lamina propria. In general, IFAA fixed oviduct samples exhibited a higher number of mast cells compared to formalin

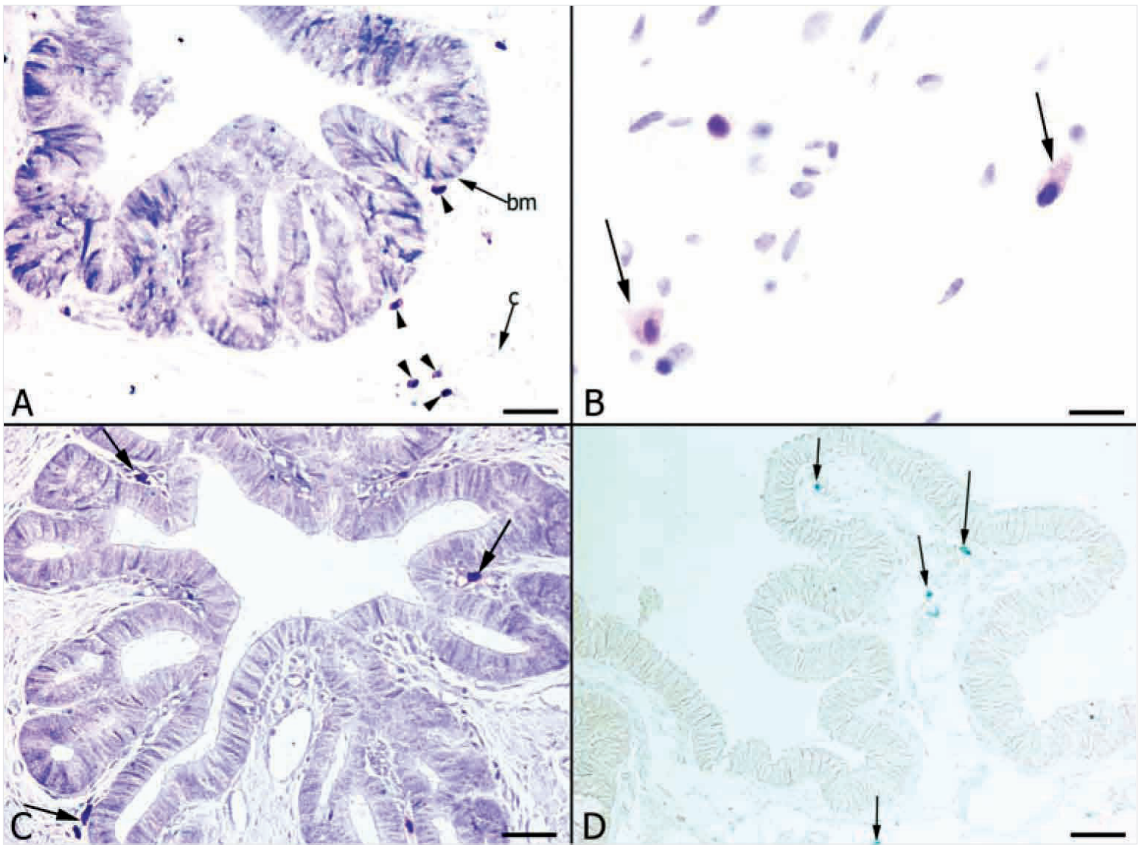

Fig 1. Light microscopic views of the ovine oviduct mast cells. **A-** Mast cells *(arrow heads)* are located around capillaries (c) and near the basal membrane (bm) as exemplifies in this ampulla region, **B-** Ovine oviduct mast cells *(arrows)* in higher magnification obtained in the fimbria, **C-** In the isthmus, the number of mast cells *(arrows)* is limited, but well illustrated in IFAA fixed samples, **D-** As exemplified this section of the fimbria, all oviduct mast cells in alcian blue/safranin O staining (Ab/SO) are Ab(+) and SO(-) A: oestral phase, IFAA fixation, B: luteal phase, 10% formalin, C: oestral phase, IFAA fixation, and D: oestral and 10% formalin fixation. Toluidine blue staining (A, B and C) and Alcian blue/Safranin O staining (D). Bar=120 µm in A and C, 40 µm in B and 160 µm in D

Şekil 1. Koyun ovidukt mast hücrelerinin ışık mikroskopik görüntüsü. **A-** Mast hücreleri *(ok başları)* kapillerlerin çevresinde (c) ve bazal membran (bm) yakınlarında görüldü, ampulla, **B-** Koyun ovidukt mast hücrelerinin *(oklar)* daha yüksek büyütmedeki görüntüsü, fimbriya, **C-** İstmusta mast hücre sayısı *(oklar)* sınırlıydı, ancak IFAA ile tespit edilen dokularda daha iyi belirlendiler, **D-** Resimdeki fimbriya bölgesinde görüldüğü gibi tüm ovidukt mast hücreleri Ab(+) ve SO (-) boyandı. A: östral dönem, IFAA tespiti, B: luteal dönem, %10 formol tespiti, C: östral dönem, IFAA tespiti, D: östral dönem, %10 formol tespiti. Toluidine blue boyaması (A, B ve C) and Alcian blue/Safranin O boyaması (D). Bar A ve C'de 120 µm, B'de 40µm ve D'de 160 µm

fixed oviduct samples, both in oestral and luteal phase (P=0.003) *(Table 1)*. No statistically significant difference was detected between oestral and luteal phase within the same oviduct regions and same fixative (P>0.05). The number of mast cells per counted area in different regions of the oviduct for luteal and oestral phases was presented in *Table 2* and *Table 3*.

In formalin fixed samples *(Table 2)*, there were significant differences among different regions of the oviduct both luteal and estral phases (P=0.006). The isthmus has significantly less mast cell counts compared to the other regions *(Fig. 1C)*. The ampulla tends to have a higher number of mast cells compared to the fimbria, but difference is not significant (P>0.05).

Table 1. *The numerical distribution (mm²) of mast cells in the ovine oviduct segments during sexual cycle (oestral and luteal phases): comparison of IFAA and 10% formalin fixation*

Tablo 1. *Seksüel siklusta (östral ve luteal dönem) ovidukt bölümlerindeki (mm²) mast hücrelerinin sayısal dağılımı: IFAA ve %10 formolde karşılaştırılması*

Parameter	IFAA Fixation	10% Formol Fixation	P value
Relative number of mast cells	88±9.11*	50.67±6.47	0.003

*The data were expressed as mean ± standart error. * There was a significantly difference between the groups within the same row*

Table 2. *The numerical distribution (mm²) of mast cells in the various regions of 10% formol fixed ovine oviduct samples collected during the luteal and oestral phases*

Tablo 2. *%10 formalinle tespit edilmiş östral ve luteal dönemlerdeki koyun ovidukt örneklerinin farklı bölgelerindeki (mm²) mast hücrelerinin sayısal dağılımı*

Phase of the Oestrous Cycle	n	Ampulla	Isthmus	Fimbria	P value
Luteal phase	7	82.29±11.80[a]	11.43±2.95[b]	61.71±15.78[a]	0.006
Oestral phase	7	82.29±15.39[a]	11.43±11.43[b]	54.86±9.14[a]	0.006

Data were analyzed with Kruskal Wallis test. Data were given as mean ± standard error. Mean values within the same row with different superscripts letters ([a, b]) are significantly different

Table 3. *The numerical distribution (mm²) of mast cells in the various regions of IFAA fixed ovine oviduct samples collected during the luteal and estral phases*

Tablo 3. *IFAA ile tespit edilmiş östral ve luteal dönemlerdeki koyun ovidukt örneklerinin farklı bölgelerindeki (mm²) mast hücresinin sayısal dağılımı*

Phase of the Oestrous Cycle	n	Ampulla	Isthmus	Fimbria	P value
Luteal phase	7	134.86±24.90[a]	29.71±6.47[b]	105.14±18.76[a]	0.002
Oestral phase	7	102.86±21.48	57.14±19.08	98.29±19.26	NS

The data were analyzed with One way ANOVA. Data were given as mean ±standart error. Mean values within the same row with different superscript letters ([a,b]) are significantly different, NS: Not significant

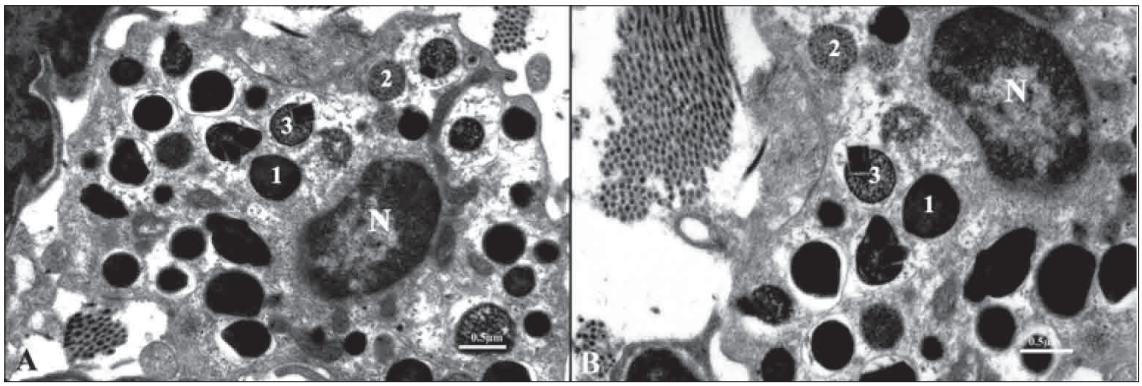

Fig 2. Transmission electron microscopic features of the ovine oviduct mast cells. **A-** Mast cells have an electron dense (1) and an electron lucent (2) granules. Some electron lucent granules have an eccentrically located crystal-like structure (3). **B-** The higher magnification of the mast cell granules. Luteal phase, isthmus (A,B)

Şekil 2. Koyun ovidukt mast hücrelerinin elektron mikroskobik özellikleri. **A-** Mast hücreleri elektron koyu (1) ve elektron açık (2) granüller içermektedir. Bazı elektron açık granüller eksantrik yerleşimli kristal benzeri yapı taşımaktadır (3). **B-** Mast hücre granüllerinin daha yüksek büyütmedeki görüntüsü. Luteal dönem, istmus (A,B)

In IFAA fixed samples *(Table 3)*, there were significant differences among different regions of the oviduct during the luteal phase (P=0.002). Similar to formalin fixed tissues of the luteal phase, the isthmus has significantly less mast cell counts compared to the other regions and the ampulla tends to have a higher number of mast cells compared to the fimbria, but difference is not significant (P>0.05). On the other hand, the regional differences during oestral phase was insignificant although the isthmus considerably less mast cell counts (P>0.05).

In Ab/SO stained tissues, mast cells Ab(+) and SO(-) in all regions of the oviduct in both oestral and luteal phase samples *(Fig. 1C)*.

In transmission electron microscopic evaluation, the oviduct mast cells exhibited two types of cytoplasmic membrane bound granules: electron dense, electron lucent granules *(Fig. 2A,2B)*. Some electron lucent granules had an eccentrically located crystal structure *(Fig. 2A,2B)*.

DISCUSSION

Mast cells execute numerous biological activities due to a wide range of bioactive molecules they contain in granules [4]. Mast cells with different types of granules may locate in different regions of the body. Based on their fixation and histochemical staining characteristics, mast cells were classified especially in rodents into two categories: MMC and CTMC [3]. The MMC granules contain chondroitin sulfate and little histamine contents; on the other hand, the CTMC granules contain heparin and higher amount of histamine content. MMCs are resistant to metachromatic staining when fixed in formalin based fixatives [4,21]. They can better preserve staining features in Carnoy's fixatives and fixatives containing acetic acid and low concentrated formalin and they are best stained with cationic dyes [6]. Due to formalin sensitivity of MMCs, Toluidine blue can stain well both MMCs and CTMCs in IFAA fixed tissues [3]. Mast cells studies conducted on the bovine uterus [22] and canine skin [23] indicated that the mast cell counts in IFAA fixed tissues were higher. Similarly, the results the present study indicated that the IFAA fixed ovine oviduct of Akkaraman breed had a higher number of mast cells per counter area in all regions. Thus, we also think that ovine mast cells exhibited some sensitivity to formalin. In the present study, we presumed that both types of mast cells (MMC and CTMC) were stained with toluidin blue in IFAA fixed ovine oviduct tissues. However, in AB/Safranin O staining, another staining technique used in differentiation between MMC and CTMC [16], we did not find any evidence regarding CTMC presence as all samples were Safranin O negative. It has been used to observe mast cell heterogeneity in various species. For instance, in bovine oviduct and ovarium [18,24] and uterus [22] mast cells in AB/Safranin O staining were AB(+) and SO(-). Similarly, the AB/SO combined technique to determine mast cell heterogeneity in the goat reproductive tract resulted in AB(+) and SO(-) [25]. Mast cells in the ovine respiratory system were AB(+) reactive [26]. In the present study, mast cells in the ovine oviduct were determined as AB(+) and SO(-). Such staining characteristics in the present study indicate that mast cells in the ovine oviduct are MMCs and CTMCs are either absent or limited in number. Furthermore, the ovine oviduct was similar to the bovine and caprine reproductive systems by means of mast cell staining feature. In the mean time, one should not forget that each mast cell type interchanges phenotypically to another type [4]. In our study, IFAA fixation did not affect AB/SO staining as all mast cells in formalin and IFAA fixed tissues were AB(+) and SO(-).

The oviduct is an import part of the genital tract as it is the place for fertilization and fosters embryo during embryogenesis [27]. As reported by two previous studies [10,24], the number of mast cells increases in the bovine oviduct mucosa especially in the isthmus during the luteal phase of the cycle. On the other hand, the number of mast cells increases in the bovine ovarium during the oestral phase compared to luteal phase [18]. Another study [22] reported that the number of mast cells in the cow endometrium increased during the luteal phase of the cycle. In the present study, the number mast cells in the lamina propria of the ovine oviduct did not change significantly between luteal and oestral phases of the cycle. However, there were significant differences among the oviduct regions. The isthmus had a less number of mast cell counts. As the isthmus serves as a sperm reservoir prior to fertilization, the significance of less mast cell counts in the isthmus should be investigated with respect to sperm deposition and microphysiology of the region.

Electron microscopic studies revealed that mast cells in the bovine endometrium [22], oviduct [24] and ovarium [18] contain two types of granules, one of which contains thin particular granules and the other one contains homogenous granules. Similar types of granules have been reported in mast cells found in the ovine respiratory system [26]. In addition to these two types, a third type has been defined, an intermediate type, between the former two types by means of electron density [26]. We classified the ovine oviduct mast cell granules into two categories: an electron dense and electron lucent. Notably, an eccentrically located electron dense crystal-like structure with well define edges was located in some of the electron lucent granules. It is known that the electron density and physical characteristics of the granule contents of mast cells granules is related to biochemical properties of mast cell granules, in which a number of bioactive molecules are entrapped [26]. Crystals with different shapes have been reported on human mast cells [28]. The significance of the eccentrically located crystal-like structure in the ovine mucosal mast cells should be further investigated.

In conclusion, mast cells in the lamina propria of the oviduct are classified as MMCs since they are AB(+) and SO(-). They are generally located near blood vessels. Mast cells are also found near the basal membrane. The number of mast cells in the lamina propria of the oviduct is not different between luteal and oestral phases. However, there are significant differences among different regions of the oviduct with a less count in the isthmus regions, significance of which should be investigated. The oviduct mast cells contain two types of granules: an electron lucent, electron dense. Some electron lucent granules contain an eccentrically located crystal-like structure. Such crystal-like structure should be further investigated for their content and biological significance.

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RESEARCH ARTICLE

The Rapid Analyses of Cardiac Troponins in Dogs with Dilated Cardiomyopathy, Distemper or Parvoviral Infection [1]

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Abstract

In this study, concentrations of cardiac troponins (cTnI and cTnT) and using a cassette kit which is a qualitative assay were used to investigate the diagnosis of myocardial damages due to different diseases in dogs. Study groups were composed of dogs with distemper infection (DI) (Group I, n=8), parvoviral infection (PVI) (Group II, n=18) and dilated cardiomyopathy (DCM) (Group III, n=18). Healthy dogs were included for each group as control. Levels of troponins (cTnI, cTnT) were determined by commercially available ELISA kits and cassette kits were used for qualitative assays. The mean concentrations of cTnI in Groups I, II and III were 2.26±0.41 ng/ml, 2.41±0.98 ng/ml and 5.076±1.32 ng/ml, respectively. Positive cTnI and cTnT expressions were determined in groups as 5/8 (62.5%) and 4/8 (50%) in group I; 5/18 (27.7%) and 5/18 (27.7%) in group II and 13/18 (72.2%) and 6/18 (33.3%) in group III, respectively. In healthy control dogs, cTnI and cTnT were determined to be negative. The cTnI concentrations were more reliable cardiac marker in dogs with cardiomyopathy. Moreover, cTnI and cTnT casette kits used in this study may be valuable diagnostic tools to diagnose the myocardial damage in dogs with DI, PVI and DCM for the small animal practioners.

Keywords: *Cardiac troponin, Cardiomyopathy, Distemper, Parvoviral infection, Dog*

Dilate Kardiyomiyopati, Distemper ya da Parvoviral İnfeksiyonlu Köpeklerde Kardiyak Troponinin Hızlı Analizi

Özet

Bu çalışmada, köpeklerdeki farklı hastalıklara bağlı miyokardial dejenerasyonların teşhisinde kardiyak troponin (cTnI, cTnT) konsantrasyonları ve kalitatif analiz yapan kaset kitlerinin kullanımı araştırıldı. Çalışma grupları distemper enfeksiyonlu (DE) (Group I, n=8), parvoviral enfeksiyonlu (PVE) (Group II, n=18) ve dilate kardiyomiyopatili (Group III, n=18) köpeklerden oluşturuldu. Her bir grubun kontrolü için sağlıklı köpekler çalışmaya dahil edildi. Ticari ELISA kitleri ile troponin (cTnI, cTnT) düzeyleri belirlendi ve kalitatif analizler için kaset kitler kullanıldı. Grup I, II ve III'de ortalama cTnI düzeyleri sırasıyla 2.26±0.41 ng/ml, 2.41±0.98 ng/ml ve 5.076±1.32 ng/ml idi. Pozitif cTnI ve cTnT varlığı da sırasıyla grup I'de 5/8 (62.5%) ve 4/8 (50%); grup II'de 5/18 (27.7%) ve 5/18 (27.7%), ve grup III'te 13/18 (72.2%) ve 6/18 (33.3%) olarak belirlendi. Sağlıklı kontrol köpeklerinde cTnI ve cTnT testlerinin negatif olduğu belirlendi. Kardiyomiyopatili köpeklerde kalp biyobelirteçleri daha güvenilirdi. Ayrıca cTnI ve cTnT kaset kitlerinin küçük hayvan pratisyenleri için; DE, PVE ve kardiyomiyopatili köpeklerde miyokarditisi belirlemek için faydalı bir teşhis aracı olabileceği belirlendi.

Anahtar sözcükler: *Kardiyak troponin, Kardiyomiyopati, Distemper, Parvoviral enfeksiyon, Köpek*

INTRODUCTION

Cardiovascular diseases in dogs are commonly encountered by pet veterinarians. Cardiac valve diseases, cardiac arrhythmias and myocardiopathies are among the most common causes of heart failure in dogs [1].

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Damage of the myocardium for a variety of reasons such as infections, toxications, nutritional deficiencies and idiopathic disorders also occurs in dogs. One of the mechanisms of damage of the myocardium is acute inflammation in the myocardial wall due to thromboembolic diseases caused by bacterial, viral or other parasitic microorganisms [2]. Myocardial damage may also occur due to bacteremia, sepsis, pericarditis or endocarditis. It is difficult to determine the presence of acute myocardial damage and in many occasions it can not be diagnosed [1-3].

Myocardium damage may be classified according to etiopathogenesis [1-3]. *ßeta*hemolytic *Streptococcus, Staphylococcus, Neosporacaninum, Distemper, Parvovirus, Dirofilaria imminitis*, micotic factors, various drugs (Adriamycin, Isoprenalin) and tumors can cause myocardial damages in dogs [1,2]. Physical examination, electrocardiography, echocardiography and analysis of serum biochemical parameters are important in the clinical evaluation of myocardial diseases in dogs. The levels of myoglobin, creatin kinase - myocardial band (CK-MB), lactate dehydrogenase (LDH) and aspartat aminotransferase (AST) enzyme activities are commonly used in the diagnosis of myocardial diseases. The levels of these biomarkers can be used alone or together with to evaluate patients with cardiac injury and they are the most important indicators of cardiac cell death or necrosis in humans or animals [4].

Cardiac troponins (cTn) can also be important indicators of myocardial damage since they leak out of the myocardial cells under inflammatory conditions [4-7]. The troponin complex consists of a group of proteins. There are three different types of troponins and they are classified according to their functions: cardiac troponin I (cTnI), cardiac troponin T (cTnT) and cardiac troponin C (cTnC). Troponin is important in mediating the interaction between actin and myosin in the sarcomere. When there is a damage of the myocardium, the levels of these enzymes increase in the circulation. As a result of acute myocardial syndrome and necrosis, cTnT and cTnI are released into circulation [4,8]. The kits and assays that used to detect the levels of the troponins in humans have also been used in dogs [9-13]. In recent publications, the concentration of cTnI has been investigated in different canine cardiovascular diseases, including congenital cardiac diseases [12,14,15], acquired cardiac heart diseases such as arrhythmogenic right ventricular dysplasia [16], pericardial effusion [15,17,18], cardiac contusion [19], experimental infarction [20], cardiovascular injury, pacing, cardiotoxic drugs with positive and inotropic effects [21] neoplasia [22] dogs with dilated cardiomyopathy [23] and parvoviral enteritis [24]. A previous study also showed to use cTnI to evaluate the efficacy of antiarrhythmia treatment in dogs [25].

However, to the best of our knowledge, the clinical use of troponin cassette kits and cTnT concentration values have not been established in dogs with distemper and parvovirus infections.

In this preliminary study, our goal was to determine the expression of cTnI and cTnT by using qualitative immune chromatographic cassette kits and to measure the serum concentrations of cTnI and cTnT by quantitative ELISA methods in dogs with dilated cardiomyopathy (DCM), parvovirus infection (PVI) and distemper. With the aim of diagnosis of myocardial injury in clinical practice, accuracy of quick cTn kits was investigated. In addition, we wished to determine the advantages and disadvantages of using either cTnT or cTnI to determine myocardial injury in dogs with cardiomyopathy.

MATERIAL and METHODS

Animals

The study group consisted of dogs of various ages and breeds that had different cardiac disorders with cardiac and/or non-cardiac causes. Dogs with DCM, Distemper and PVI were provided from the clinic of the Faculty of Veterinary Medicine of Erciyes University between 2007 and 2009. These dogs were grouped according to their diagnosis as follows: Dogs with Distemper infection (Group I, n=8), dogs with Parvoviral infection (Group II, n=18) and dogs with Dilated Cardiomyopathy (Group III, n=18). The control group consisted of healthy mixed breed dogs (40 male, 29 female) from the Kayseri Municipal dog shelter. The control group was divided into 4 groups according to age: 1-6 months (n= 17), 6-12 months (n= 10), 1-5 years (n= 21) and 5-10 years (n= 21). The general appearance, hair coat, lymph nodes, mucosa (mouth, conjunctive, vaginal), body temperature, heart rate, respiratory rate were examined. Regular clinical examinations of all systems were performed. To determine presence of parvovirus and distemper infections, canine parvovirus casette test kit 10 (Orgenics, Israel) and canine distemper casette test kit (Orgenics, Israel) were utilized.

Cardiac Examinations and Sample Collection

Rightlaterolateral (L/L) dorsoventral (D/V) radiographs, and echocardiographic and electrocardiographic (ECG) records were taken from dogs with cardiologic disorders. Referance values were assessed by the animal's body weight. Cases of DCM also have been identified as outside of FS 25-40%. In dogs with distemper or PVI only ECG findings were recorded.

P and T waves amplitudes, duration of P, T and QRS complex, PQ and QT interval were determined on the ECG recording. Transmission gel applied by shaving the hairs on the 4^{th}-6^{th} intercostal area for echocardiographic examinations. The contraction of the heart muscle and valves were evaluated with 2-D echocardiography. The interventricular septum thickness (IVS) left ventricular

diameter (LVD), left ventricular posterior wall thickness (LVW) and LA/Ao ratio were measured by M-mode echocardiography in systolic and diastolic phases separately. From these values, The fractional shortening (FS%) and the ejection fractions (EF%) were calculated automatically.

Blood samples (2 mL) were collected into a tube containing heparin for the troponin cassette test. Four mL of blood was also collected in tubes without anti-coagulants for the biochemical analyses. To obtain serum samples, blood samples were centrifuged at 3.000 rpm for 15 min. In addition, 2 mL of blood samples were collected in EDTA containing vacuum tubes for hematological examination.

The Results of cTnI and cTnT Cassette Tests, cTnI and cTnT Concentrations and Selected Biochemical Parameters

In order to determine the presence of heart-derived troponin I (cTnI) in the blood of the dogs, Card-I kit Combo test kits (BioMarket, Finland) were used. For determination of troponin-T (cTnT) in the blood Trop-T Sensitive Rapid Assay (Roche, Germany) were employed. Clinical examination, echocardiography, radiography and ECG tests on the dogs were all done on the same day and all results were recorded. The concentration of cardiac TnI was determined using an ELISA reader (Bio-Tek ELX 50; USA). cTnI analysis was performed using a commercial ELISA kit containing 96-well plates which were coated with a monoclonal antibody according to manufacturer's recommended protocol (Multiscan Spectrum Thermo, Finlandiya). Quantitative assessment of cardiac TnT was done at the Kayseri Research and Teaching Hospital Emergency laboratory using Troponin T Stat Elecsys 04660307 (Lot No: 15263501, Roche) kits with an electrochemiluminescence immunoassay technique using a Cobas e 411 device. Some selected biochemical parameters such as; aspartate transaminase (AST), lactate dehydrogenase (LDH) and creatinine kinase (CK), were measured with spectrophotometric commercial kits (Biolabo, France) in serum samples**.**

Statistical Analysis

Statistical analysis was performed using One-way ANOVA followed by Dunnett's t-test for comparison the patient and control groups and a Tukey test was used to compare cTn positive and negatives within the groups. Statistical significance was considered to be $P<0.05$. The results are expressed as means ± standard deviations (SD).

RESULTS

Findings of Serum Biochemical Analysis

Apart from CK, LDH and AST no other significant changes in biochemical parameters were detected *(Table 1)*. Particularly in group III, the mean CK and LDH parameters were found to be increased statistically significantly compare to other groups. Based on this, the CK and LDH activities in cTn I positive dogs were significantly higher when compared to both the cTn negative dogs and the control group ($P<0.001$). The mean AST activity in the cTn positive group was also found to be significantly higher than the cTn negative and control groups ($P<0.001$).

Cardiologic Findings in Group I and II

The elongation of the R wave length, the expansion of the T wave amplitude, P-wave amplitude, sinus tachycardia and sinus arrhythmia findings were determined in ECG examinations in group I. Sinus Tachycardia, arrhythmia and P wave abnormalities were also observed in dogs with Parvovirus infection.

Cardiologic Findings in Group III

In right L/L and D/V radiographs of dogs with DCM,

Table 1. *Mean levels of selected biochemical parameters in cardiac Troponin kit positive and negative dogs. Results are present as mean±SD*
Tablo 1. *Gruplarda kardiyak Troponin kit negatif ve pozitif olan köpeklerin diğer biyokimyasal bulguları. Sonuçlar ortalama±SD olarak verildi*

Groups	Casette Kit Result	LDH (IU/L)	CK (IU/L)	AST (IU/L)
Group I	Tn (+) n=5	21.87±11.50	27.21±12.12	4.33±3.99 [a]
	Control n=15	28.51±13.33	30.88±10.97	0.55±0.31 [b]
Group II	Tn (+) n=5	44.96±17.35	22.635±7.95	2.11±1.20 [c]
	Control n=12	38.72±18.89	22.61±15.31	0.70±0.58 [d]
Group III	Tn (+) n=13	89.94±27.58 [a]	500.02±125.14 [a]	21.05±1.21 [e]
	Control n=42	35.78±41.30 [b]	27.76±7.90 [b]	0.84±0.95 [f]

Different letters in the same column represent statistical difference. P value is P<0.001

generalized or ventricular expansion of the heart, dorsal deviation of the trachea, steepen of the posterior border, loss of details in the thorax, pleural effusion, and diffuse radiopacity in the thorax were observed (n=18). In dogs with right heart dilatation (6 dogs); pouch formations on the dorsal posterior border of myocardium and pulmonary venous congestion were observed. Arrhythmia and LA/Ao ratio were >2 in M-mode graphs in all dogs. In the right parasternal short axis view of the interventricular septum (IVS) and posterior wall (PW), the reduction in the wall thickness, increase in values of left ventricular space (LVID) were observed in echocardiography in all dogs of group III.

Doppler echocardiographic examinations determined in sufficiency of mitral and tricuspid valves with varying degrees. In addition, above 2 mm pericardial fluid was observed in the 2-D ultrasound in 8 dogs.

Results of cTnI and cTnT Measurements

The cTnI and cTnT concentrations of cTn positive animals in each group are given in *Table 2*. In all three groups, the cTnI concentrations were found to be statistically significantly higher in the cTn positive cases than in the cTnI negative cases and the control group. The mean cTnT concentrations were not statistically significantly different in all groups. Mean concentrations of cTnT positive cases were higher than those of cTnI negative cases in the group I and III but were not statistically significant.

Results of cTn Casette Tests

A total of 44 sick dogs admitted to the clinics of Veterinary Hospital between 2007 and 2009 and diagnosed as Distemper infection, Parvovirus infection and dilated cardiomyopathy were included in this study. Positive cTnI and cTnT expressions were determined in groups as 5/8 (62.5%) and 4/8 (50%) in group I; 5/18 (27.7%) and 5/18 (27.7%) in group II and 13/18 (72.2%) and 6/18 (33.3%) in group III, respectively *(Table 3)*. In healthy control dogs, cTnI and cTnT were negative. Cardiac troponins were determined within 30 min after admission to the clinic of Internal Medicine. cTn kits results with the number of cTn positive animals are given in the *Table 1*. Pictures of cTn kits used in the study are shown in *Fig. 1, 2,* and *3*.

Table 2. *Mean cardiac Troponin I and Troponin T concentrations in dogs with distemper infection (group I), parvovirus infection (group II) and dilated cardiomyopathy (group III). Results are present as mean±SD*

Tablo 2. *Distemper, Parvoviral enfeksiyonlu ve dilate kardiyomyopatili köpeklerde ortalama kardiyak Troponin I ve Troponin T konsantrasyonları. Sonuçlar ortalama±SD olarak verildi*

Groups	Casette Kit Results	cTnIng/ml Mean ±SD	cTnTng/ml Mean ±SD
Group I	Tn (+) n= 5	2.26±0.41 [a]	0.15±0.24
	Control n=15	0.27±0.13 [b]	0.01±0.00
	P value	0.025	NS
Group II	Tn (+) n= 5	2.41±0.98 [a]	0.01±0.00
	Control n=12	0.09±0.03 [b]	0.01±0.00
	P value	0.001	NS
Group III	Tn (+) n= 13	5.076±1.32 [a]	0.08±0.06
	Control n=42	0.18±0.09 [b]	0.01±0.00
	P value	0.001	NS

P value was determined between cTn (+) and cTn (-), and was set at P<0.05. Different letters (a, b, c) in each group show statistical difference. NS, not significant

Table 3. *Cardiac Troponin cassette kit findings in dogs with distemper infection (group I), parvovirus infection (group II) and dilated cardiomyopathy (group II)*

Tablo 3. *Distemper (group I), Parvoviral enfeksiyonlu (grup II) ve dilate kardiyomiyopatili (grup III) köpeklerde kardiyak troponin kaset kit bulguları*

Groups	n	cTnI		cTnT	
		+ (%)	-	+ (%)	-
Group I	8	5 (62.5)	3	4 (50)	4
Group II	18	5 (27.7)	13	5 (27.7)	13
Group III	18	13 (72.2)	5	6 (33.3)	12

Numbers describes proportionof positivity or negativity determined by the cassette kits. cTnI: cardiac Troponin I, cTnT: cardiac Troponin T

DISCUSSION

In the present study, commercial cassette kits were successfully used to assess myocardial damage in dogs. To the best of our knowledge, the usage of these cassette kits has not been reported in dogs before. As the sequence of amino acids in these proteins is very much alike in different species [26], it was possible to diagnose myocardial disorders in dogs by kits used in humans. Additionally these cTn kits have been validated for use in the cow [27], calf [28,29] and lambs [30]. Recently, an high-sensitivity immunoassay (direct chemiluminometric method) has been described in clinically affected dogs [12,13]. But these

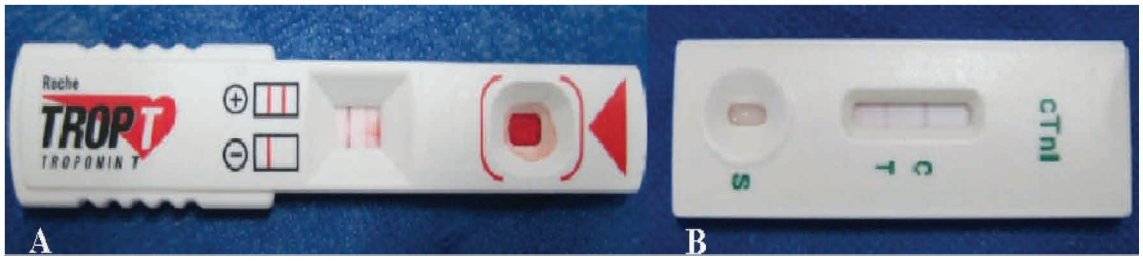

Fig 1. A terrier presented with bloody diarrhea and bloody vomit (hemorrhagic gastroenteritis), **A-** cTnT positive; **B-** cTnI positive *(double line on reading window)*

Şekil 1. Kanlı ishal ve kanlı kusma ile başvuran 14 yaşındaki terrier (hemorajik gastroenteritis), **A-** cTnT Pozitif; **B-** cTnI pozitif *(okuma penceresi üzerindeki çift çizgi)*

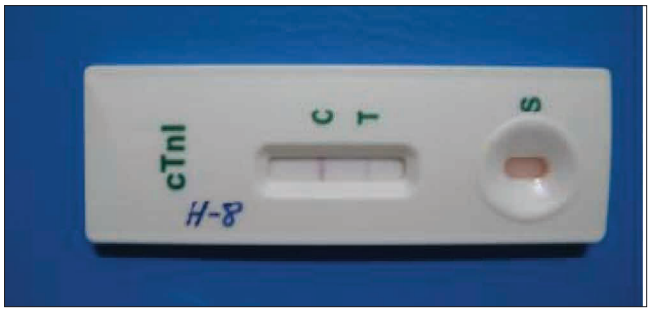

Fig 2. Positive cTnI test in a dog with distemper

Şekil 2. Distemperli bir köpekte pozitif cTnI test

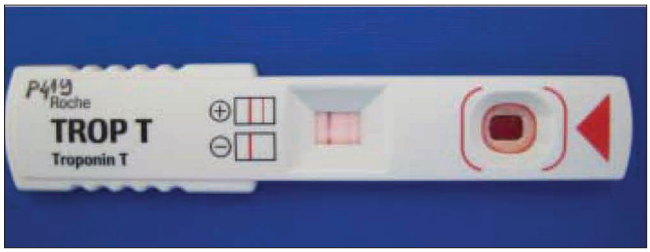

Fig 3. Positive cTnT results obtained from a dog with respiratory difficulties and fatigue symptoms due to mild DCM

Şekil 3. Hafif DCM nedeni ile solunum güçlüğü ve yorgunluk semptomu gösteren bir köpekten elde edilen pozitif cTnT sonuçları

assay process different from the process reported in this study.

In this preliminary study, positivity rates for both cTnI and cTnT casette kits were determined in dogs with distemper infection parvoviral infection and DCM. Positive cTnI rates were higher than those of cTnT in group I and III, however, cTnT were determined as similar rates in group II *(Table 3)*. These data suggest that cardiac cTnT may be positive in more acute cases such as parvoviral infection (group II). Although the concentration of cTn I has been reported to be increased in pericardial effusions [17], in our study the three cases with defined diastolic heart failure and pericardial effusion were negative for cTn I and cTn T based on the results from the casette kits. Since the clinical symptoms were mild, it is possible that in these cases, the myocardium was not yet fully affected.

Acccording to the casette kits results of our study, where result of cTn I cassette kits was higher than those of cTnT in all groups, we speculate that cTnI kit is more sensitive marker than cTnT kits in dogs with myocardial damage *(Table 3)*. In previous studies with healthy dogs [14,15,17,18,31], dogs with myxomatous mitral valve disease, arrhythmia [12,13,25] and dogs with congenital heart failure [12,16], the concentrations of myocardium troponins and especially cTnI were determined [30].

It was reported that the life expectancy of the dogs was longer when the troponin concentrations were low and that the life expectancy was drastically reduced when the troponin concentrations increased [13,24]. The low troponin levels indicate that there is no distinct, active cardiomyocyte death. The dogs that survived cardiac diseases and lived for 1, 2 or 3 years had cTnI concentrations of 0.18 ng/mL, 0.07 ng/mL and 0.05 ng/mL, respectively. In this study, it was determined that positive cassette cTnI kit results compatible with high cTnI concentrations *(Table 2)*.

Cardiac TnI results of this study were in agreement with those of Kocaturk et al.[24]. They postulated that high cTnI levels were likely to contribute to increasing mortality rate and shortening survival length in dogs with parvoviral enteritis. O'Brien et al.[21] compared the cTnI in tissues from different species using a new immunoassay method with the goal of determining human cTnI. Their data showed that cTnI is a perfect candidate to be a biomarker of cardiac injury in mammals. In our study, we also used the diagnostic systems that were developed for human. In healthy dogs, the plasma cTnI levels were determined to be between 0.03 ng/mL and 0.07 ng/mL (average 0.02 ng/mL). Guglielmini et al.[33] found that average cTnI was 0.10 ng/mL (range 0.10-0.17 ng/mL) in healthy dogs. The mean cTnI concentrations of the 1-6 months, 6-12 months and 1-5 years old dogs were 0.09±0.03 ng/mL, 0.27±0.13 ng/mL and 0.18±0.09 ng/mL, respectively, in the present study. These findings were consisted with previous studies [33,34]. There were no significant differences in dogs with different ages. It seems that age might not influence to these parameters. A cTnI level of approximately 1 ng/mL and lower were determined to be normal for healthy dogs.

However, the normal cTnI levels in our study were close to the results obtained by Fonfara et al.[32] where dogs without distinct cardiac disease had a cTnI concentration of 0.21-0.26 ng/mL. The higher values reported in previous studies might be due to methodology and the use of different animals. In a study where the cTnI concentrations of dogs with cardiac diseases were determined using three different analyzers, it was shown that the values obtained by each method were similar but were impossible to compare with each other [35].

The reliability of cTnT concentrations was interpreted as quite low in the present study. As mean cTnT levels were very close to those of control, group I, group II and III. We supposed that cTnT analyses had an insufficient sensitivity in the present study. Moreover, Apple et al.[36] reported that cTnI assays providing a measurable signal in the absence of a cTnT signal in rat, dog and monkey. Additionally, cTnI is more sensitive than cTnT and its values 3-7-fold higher than from cTnT with any given heart damage [37].

In our study, the mean LDH levels in all groups were found to either be within the limits or lower than the normal range. On the other hand, the CK activity was found to be significantly higher in dogs with cardiomyopathy using the cassette kits for troponin (500.02±125.14 IU/L) compared to the control data from the same group (27.76±7.90 IU/L) and to other groups. This difference was interpreted as the evidence of cardiac damage in the present study which similarly reported by Jurlander et al.[4]. In addition, this study showed that cTnI positive dogs had increased CK activity that parallels with an increase in the cTnI concentration and these parameters can be used as specific markers for diagnosis of myocardium damage in dogs. Our results were in agreement with a study that showed cTnI is important and highly specific serological marker [38].

When the cTnI, CK and LDH values compared, it was found that they were not correlated. The cTnI concentrations were significantly higher in the cTnI positive cases compared to cTnI negative cases, whereas there were no distinct differences in the CK and LDH values in these groups. Furthermore, the lacks of tissue specifity and sensitivity and short half time in blood stream of above enzymes have major limitation factors [37] for the diagnosis. Due to this reason, analysis of serum cTnI is more sensitive in diagnosing myocardial damages than the other conventional biochemical markers such as CK and LDH.

A usual cardiovascular system examination includes: disease history, physical examination, complete blood count, serum biochemical analysis, ECG and chest radiographs [39]. In our study, dogs with cardiac diseases were subjected to all the tests listed above, their clinical findings and mean LDH, CK and AST results *(Table 1)* were consistent with the literature [3]. These parameters could be used to differentiate between cardiac and non-cardiac patients, but the cTn parameters were more potent biomarkers for the diagnosis of myocardial diseases. In addition, the results from the cassette cTn kits made diagnosis stronger along with evidence obtained from the physical examination, electrocardiography, echocardiography and biochemical analyses.

In conclusion, expression of cTnI with practice kits and analysis of cTnI concentrations are sensitive determinant of both myocardial damage due to parvoviral myocarditis and distemper and dilated cardiomyopathy in dogs. The concentration of cTnI and cTn cassette kits can be utilized by small animal practioners for the diagnosis of developing myocardial defects. Additionally due to the rapid diagnosis of myocardiopathies with cassette kits without the need for sophisticated laboratory techniques, these quick kits are an important helper diagnostic tool for small animal practioners. Cardiac troponin analyzes should not be disregarded in assessing the prognosis of abovementioned diseases. These cassette kits may also be beneficial to evaluate mortality rate in veterinary emergency and critical care medicine.

Conflict of Intereststatement

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

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RESEARCH ARTICLE

Hepatoprotective Effects of B-1,3-(D)-Glucan on Bortezomib-Induced Liver Damage in Rats

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Abstract

The aim of this study was to evaluate the effects of β -1,3-(D)-glucan as an antioxidant and tissue protective agent and study the biochemical, histopathologic, and immunohistochemical effects of first therapeutic proteasome inhibitor bortezomib on the liver for treating relapsed multiple myeloma. The experiment included 36 adult male rats, which were divided into four treatment groups: control (healthy); bortezomib-treated; β-1,3-D-glucan-treated; and bortezomib + β-1,3-(D)-glucan-treated. Each group was subdivided into two subgroups based on time of sacrifice (48 or 72 h). After the experiments, superoxide dismutase (SOD) activity and lipid peroxidation (LPO) amounts were determined, and immunohistochemical and histopathological changes were examined in all rat liver tissues. β -1,3-(D)-Glucan treatment normalized changes of LPO and stimulated an over activity of endogenous SOD. The results of the histopathologic parameters showed that treatment with β -1,3-(D)-Glucan in the bortezomib group ameliorated the development of non-specific reactive hepatitis (NSRH) and Kupffer cell activation via NF-kB. Administration of β -1,3-(D)-Glucan is effective in reversing tissue damage induced by bortezomib in rat livers.

Keywords: *Bortezomib, β-1,3-(D)-glucan, Oxidative stress, Liver, Histology*

Sıçanlarda Bortezomib İndüklü Karaciğer Hasarında B-1,3-(D)-Glukan'ın Hepatoprotektif Etkileri

Özet

Bu çalışmanın amacı, relaps multiple miyelom tedavi etmek için kullanılan ilk terapötik proteazom inhibitörü olan bortezomibin karaciğer üzerine immunohistokimyasal, histopatolojik ve biyokimyasal etkilerini araştırmak ve bir antioksidant ve doku koruyucu ajan olarak B-1,3-(D)-glukanın etkilerini değerlendirmekdi. Deney; kontrol (sağlıklı), bortezomib ile tedavi, B-1,3-(D)-glukan ile tedavi ve bortezomib + B-1,3-(D)-glukan ile tedavi olmak üzere dört tedavi grubuna bölünen 36 yetişkin erkek sıçan içerdi. Her bir grup sakrifikasyon zamanına (48 veya 72 saat) göre iki alt gruba ayrıldı. Deneylerin bitiminden sonra, süperoksit dismutaz (SOD) aktivitesi ve lipid peroksidasyon (LPO) miktarları ölçüldü ve tüm sıçan karaciğer dokularında immünohistokimyasal ve histopatolojik değişiklikler incelendi. B-1,3-(D)-glukan ile tedavi LPO değişikliğini normalize etti ve endojen SOD aktivitesi aşırı uyardı. Histopatolojik parametrelerin sonuçları, bortezomib grubunda B-1,3-(D)-glukan ile tedavi NF-kB yoluyla Kupffer hücre aktivasyonunu ve non-spesifik reaktif hepatit (NSRH) gelişimini regüle ettiğini gösterdi. B-1,3-(D)-glukan uygulaması, sıçan karaciğerinde bortezomibin neden olduğu geri döndürülebilir doku hasarında efektifdir.

Anahtar sözcükler: *Bortezomib, β-1,3-(D)-glucan, Oksidatif stress, Karaciğer, Histoloji*

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INTRODUCTION

Proteasome is a multicatalytic protein complex and the major non-lysosomal system for intracellular protein degradation in the eukaryotic cells. Proteasomes work in concert with the marking protein ubiquitin and generate the ubiquitin-proteasome pathway. This pathway is responsible for the controlled degradation of a wide range of proteins, including cellular regulators that control processes such as the cell cycle, apoptosis, inflammation, cell migration, angiogenesis, and transcription [1]. Many studies have shown that the proteasome system can act as a cellular defense mechanism because it prevents the accumulation of aggregation misfolded/oxidized proteins generated by post-translational modification errors or oxidative stress [2].

Bortezomib, a dipeptidyl boronic acid, is the first reversible 26S proteasome inhibitor used for the treatment of multiple myeloma (MM) in humans [3]. The initial target for bortezomib use in MM was the blocking of pathways of nuclear factor-kappa B (NF-kB) by limiting proteasomal degradation of inhibitor of kappa B alpha (IkBα). Although the first studies argued that bortezomib could inhibit all pathways of NF-kB, later studies have shown that it activates only the canonical pathway in neoplastic cells of MM [4]. Bortezomib was also found to be effective on NF-kB pathways in cancer types other than MM [5]. The drug has been shown beneficial in animal-model studies of autoimmune diseases such as myasthenia gravis, psoriasis, arthritis, and autoimmune encephalomyelitis [6].

Therapeutic effectiveness of anti-cancer drugs is associated with severe side effects due to their toxicity [7]. Bortezomib is one of the more widely used anti-cancer drugs for a number of cancers and is metabolized in the liver, which can develop drug toxicity due to these metabolisms [8]. A few bortezomib-based studies on the liver have shown that the 26S ubiquitin-proteasome pathway may play an etiologic role in the development of liver disorders, especially endoplasmic reticulum stress, insulin resistance, alcoholic liver disease, and lipid metabolism [9]. Earlier, an experimental study on rates revealed that the therapeutic dose of bortezomib and its deboronated metabolites M1 and M2 dealkylated to form M3 and M4 could causes liver toxicity. This study also showed a decrease in cytochrome P450 content and activity and an important increase in palmitoyl-CoA activity in *ex vivo* analyses of rat liver samples [10]. However, this present research has not found studies about oxidant and antioxidant parameters in bortezomib-induced liver toxicity. Many studies, though, have demonstrated that oxidative stress is responsiple for chemotherapeutic drug-induced liver toxicology [11]. In mitigation of chemotherapy side effects, some antioxidant drugs or agents with anti-cancer effects have been determined helpful depending on the reducing effect on oxidative stress [12]. Many studies have shown that beta-1,3-D-glucan, an antioxidant and anti-cancer agent, has protective antioxidant activity against chemotherapy-induced liver toxicity [13].

B-1,3-(D)-Glucanis a long chain polymer of D glucose from the cell wall of baker's yeast (*Saccharomyces cerevisiae*), fungi, and plants. Many experimental studies have demonstrated its pharmacological properties, especially its immunomodulator, antioxidant, and anti-tumor effects [14]. The immunomodulator effects of B-1,3-(D)-Glucan are on innate immune cells andare enlisted for immune system reinforcement in humans [15]. B-1,3-(D)-Glucan acts as an agonist on dectin-1 and complement receptor 3 (CR3) on the surface of innate immune cells [16]. Recent studies indicate that B-1,3-(D)-Glucan also promotes activation of CR3 in granulocytes and destroys iC3b-opsonized tumor cells via granulocyte bound-CR3 [17]. In addition, B-1,3-(D)-Glucan is a potent antioxidant that has been reported to prevent oxidative damage in liver and renal ischemia/reperfusion injury [18].

This present research aims to determine whether an antioxidant such as B-1,3-(D)-Glucan could provide a protective effect against bortezomib-induced liver damage, using both stereological, histopathological, and biochemical methods.

MATERIAL and METHODS

Animals

The animals were housed in facilities and the experiments conducted in accordance with international guidelines, and the studies were approved by the Institutional Animal Care and Use committee of Ataturk University. This study used 36 adult male Sprague-Dawley rats (230-250 g) from the Ataturk University Experimental Animal Laboratory (ATADEM-Approval No: 2013-03/96).

Chemicals

Bortezomib (Velcade®) was purchased as a lyophilized powder (Velcade; Janssen-Cilag, Beerse, Belgium) and dissolved in a sterile saline solution at final concentration. β-1,3-D-glucan was purchased from Sigma-Aldrich (Steinheim, Germany). All chemicals for laboratory experimentation were purchased from Sigma-Aldrich (Germany).

Experimental Design

Four groups (control; bortezomib-treated; β-1,3-D-glucan-treated; and bortezomib + β-1,3-D-glucan-treated) were formed for the research study. Bortezomib, β-1,3-D-glucan, and bortezomib + β-1,3-D-glucan groups were subdivided into two subgroups of six rats each based on time of sacrifice: 48 or 72 h after drug administration. The rats in the bortezomib and bortezomib + β-1,3-D-glucan groups were injected subcutaneously (sc) once

with 0.2 mg/kg of bortezomib on the first day of the study [19]. The rats in the bortezomib group were not given any treatment after the bortezomib injection until sacrifice. The rats in the bortezomib + β-1,3-D-glucan group were injected intraperitoneally (ip) with 75 mg/kg of β-1,3-D-glucan every day after bortezomib injection until sacrifice. The rats in the β-1,3-D-glucan group were injected ip with 75 mg/kg of β-1,3-D-glucan every day until sacrifice [13].

All six groups were sacrificed with an overdose of a general anesthetic (thiopental sodium, 50 mg/kg). The livers were then quickly removed from the rats and washed in ice-cold saline. Half of the tissues were transferred to a biochemistry laboratory and kept at −80°C for biochemical analyses, while the other half were fixed in a 10% formalin solution for histopathological analyses.

Histopathological Analyses

Preparation of Liver Tissues for Histopathology: The livers were fixed in 10% formaldehyde, dehydrated in a graded alcohol series, embedded in paraffin wax, and sectioned using a Leica RM2125RT microtome (Leica Microsystems, Wetzlar, Germany). In this study, 4 µm thick sections from paraffin blocks were obtained using a systematic randomized sampling method (stereological method) for immunohistochemical and histopathologic examinations.

Histopathological Examination of the Liver: Sections 4-µm thick for histopathological examinations were stained with H&E and periodic acid-Schiff (PAS). All livers were examined by light microscopy for histopathological evaluation of the following parameters: H&E staining for sinusoidal expansion; inflammatory cell infiltrates; sinusoidal expansion; hyperthrophic degeneration; necrotic cell deaths, apoptotic cell deaths.

Biochemical Analyses

Preparation of Liver Tissues for Biochemical Analysis: Rat livers were kept at -80°C for biochemical investigation. To prepare the tissue homogenates, the liver tissues were ground with liquid nitrogen in a mortar, and 0.5 g was weighed for each group and treated with 4.5 mL of an appropriate buffer. This mixture was homogenized on ice using an IKA® Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany) for 15 min. Homogenates were filtered and centrifuged using a refrigerator centrifuge at 4°C. The supernatants were then used to determine enzymatic activities. All assays were carried out at room temperature. To prepare the tissue homogenates, tissues were ground with liquid nitrogen in a mortar. The ground tissues (0.5 g each) were then treated with 4.5 mL of the appropriate buffer. The mixtures were homogenized on ice using an Ultra-Turrax homogenizer for 15 min.

SOD Activity: SOD activity was measured according to Sun et al.[20]. The estimation was based on the generation of O_2^- produced by xanthine and xanthine oxidase, which react with nitro blue tetrazolium (NTB) to form formazan dye. SOD activity was then measured at 560 nm by the degree of inhibition of this reaction, and was expressed as millimole per minute per milligram of tissue (mmol/min/mg tissue).

LPO Determination: The level of gastric LPO was determined by estimating MDA using the thiobarbituric acid test [21]. The rat livers were promptly excised and rinsed with cold saline. The livers were weighed and homogenized in 10 mL of 100 g/L KCl. The homogenate (0.5 mL) was added with a solution containing 0.2 mL of 80 g/L sodium laurylsulfate; 1.5 mL of 200 g/L acetic acid; 1.5 mL of 8 g/L 2-thiobarbiturate; and 0.3 mL of distilled water. The mixture was incubated at 98°C for 1 h. Upon cooling, 5 mL of n-Butanol:pyridine (15:l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 1875 × g. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane, and recovery was over 90%. The results were expressed as nanomol MDA per gram of tissue (nmol/g tissue).

Immunohistochemical Analysis

In addition to the histopathological analyses, activity in Kupffer cells was detected by immunohistochemical staining of NF-kB protein (p65). Immunohistochemical staining for NF-kB protein was performed by an automated method on the VENTANA BenchMark GX System (Ventana Medical Systems, Inc.) with an ultraView Universal DAB Detection Kit on 4-µ sections from a representative block in each rat. After deparaffinization to water, the antigenic determinant sites for NF-kB were unmasked in citrate buffer with steam for 60 min. The primary antibody used for NF-kB, an IgG1 class mouse monoclonal directed against the p65 (F-6) relA component of the NF-kB complex (Santa Cruz sc8008, CA), was used at a dilution of 1:80 for 32 min at 37°C. The slides were then incubated with the diluted antibody, followed by application of ultraView Universal DAB detection kit (Ventana Medical Systems, Inc.). DAB was used as a chromogen and hematoxylin as a counter stain. Similarly, processed sections from human prostate cancer were used as positive controls for p65 (RelA) immunostaining, respectively. The specificity of staining was confirmed by the inclusion of negative control slides processed in the absence of primary antibody on tissue from the same animal.

In liver tissue, the numerical density of nuclear immunoreactivity for NF-kB of Kupffer cells was evaluated according to stereological analyses.In this study, unbiased counting frame and fractionator methods to estimate numerical density of NF-kB nuclear localization in the Kupffer cells were used. Each glass microscope slide was sampled using the fractionator principle of the stereology

software (Stereo Investigator® version 8.0, Micro Bright-Field, Colchester, Vermont, USA). NF-kB-positive nuclei were counted by using a 63x Leica Plan Apo objective (NA = 1.40), which allowed accurate recognition. The coefficient of error (CE) for the estimations was the last calculated value. The generally accepted highest limit of CE is 5% (22).

Data Analysis

The statistical analysis of all parameters was performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using the IBM® SPSS software package, version 19.00 (SPSS Inc., Chicago, IL). Statistical significance was considered $P<0.05$. All the results were expressed as mean±standard error of the mean (SE) for the six rats in each group.

RESULTS

Histopathological Results for Liver Toxicity

All zones (periportal, midzonal, and centrilobular) of liver acinus in the control and β-1,3-D-glucan-treated groups exhibited a typical appearance (*Fig. 1A* and *B, Table* 1). Liver tissues in the bortezomib and bortezomib + β-1,3-D-glucan-treated groups showed histopathological changes, such as cell degeneration, inflammatory cell infiltrates and foci, necrotic, and apoptotic cells (*Fig. 2A, B, C,* and *D, Table 1*). While these histopathological changes were at severe level at hour 72 (*Fig. 2B* and *Table 1*), they were at moderate level at hour 48 in the bortezomib-treated groups (*Fig. 2A* and *Table 1*). Inflammatory cell types were generally lymphocytes and macrophages, which organized both aggregates (foci) and diffuse, and were situated in the all zones of liver acinus (*Fig. 2A* and *B, Table 1*). Necrotic and degenerative cells (hypertrophic and abnormal membrane counters) were dense and focal with inflammatory responses. In addition, Councilman bodies (apoptotic cell death) and hypertrophic Kupffer cells (tissue macrophages) were observed within parenchyma in these groups (*Fig. 2A* and *B, Table 1*).

Bortezomib induced these histopathological changes, which decreased via β-1,3-D-glucan treatments at both hours 48 and 72 (*Fig. 2C* and *D, Table 1*). The liver tissues

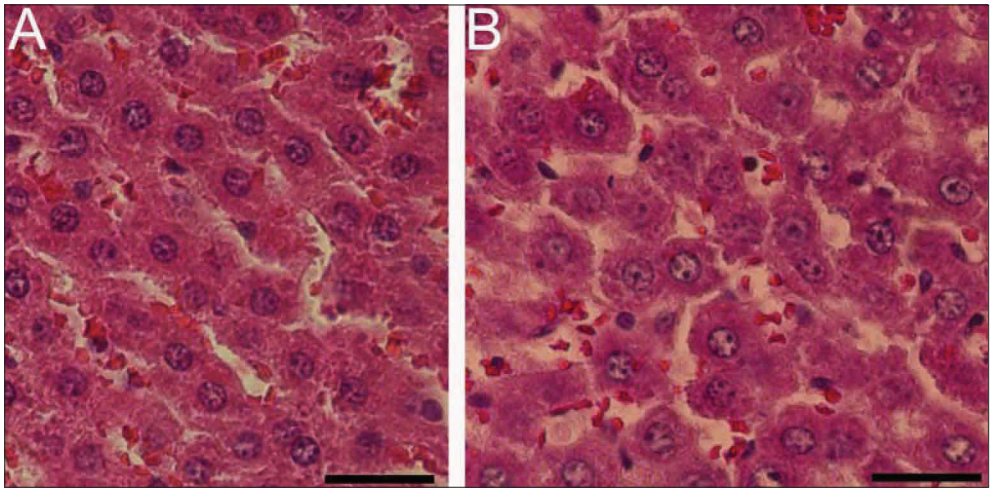


Fig 1. The normal appearance of organized plates of hepatocytes, which are separated by sinusoidal capillaries in control group **(A)** and β-1,3-D-glucan group **(B)**; Scale bars in A, B, 30 µm

Şekil 1. Kontrol **(A)** ve β-1,3-D-glukan **(B)** grubunda sinüzoidal kapillerle ayrılan hepatositlerin organize kordonların normal görünümleri. A, B 'deki ölçek barlar, 30 um

Table 1. *Histopathological score for rat liver tissues treated singly and in combination with bortezomib or β-1,3-D-glucan*
Tablo 1. *Tek başına ve kombinasyon halinde bortezomib ya da β-1,3-D-Glukan ile tedavi edilen sıçan karaciğer dokuları için histopatolojik skor*

Data	Group					
	Control	β-1,3-D-glucan	Bortezomib 48th h	Bortezomib 72th h	Bortezomib+β-1,3-D-glucan 48th h	Bortezomib+β-1,3-D-glucan 72th h
Hypertrophic hepatocyte degeneration	-	-	++++	++++	++	++
Sinusoidal expansion	-	-	+++	++++	+	++
Necrotic cell deaths	-	-	+++	++++	+	++
Inflammatory cell foci or infiltrates	-	-	+++	++++	-	+
Hypertrophic Kupffer cells	-	-	+++	++++	-	+

- none; + minimal; ++ mild; +++ moderate; ++++ severe

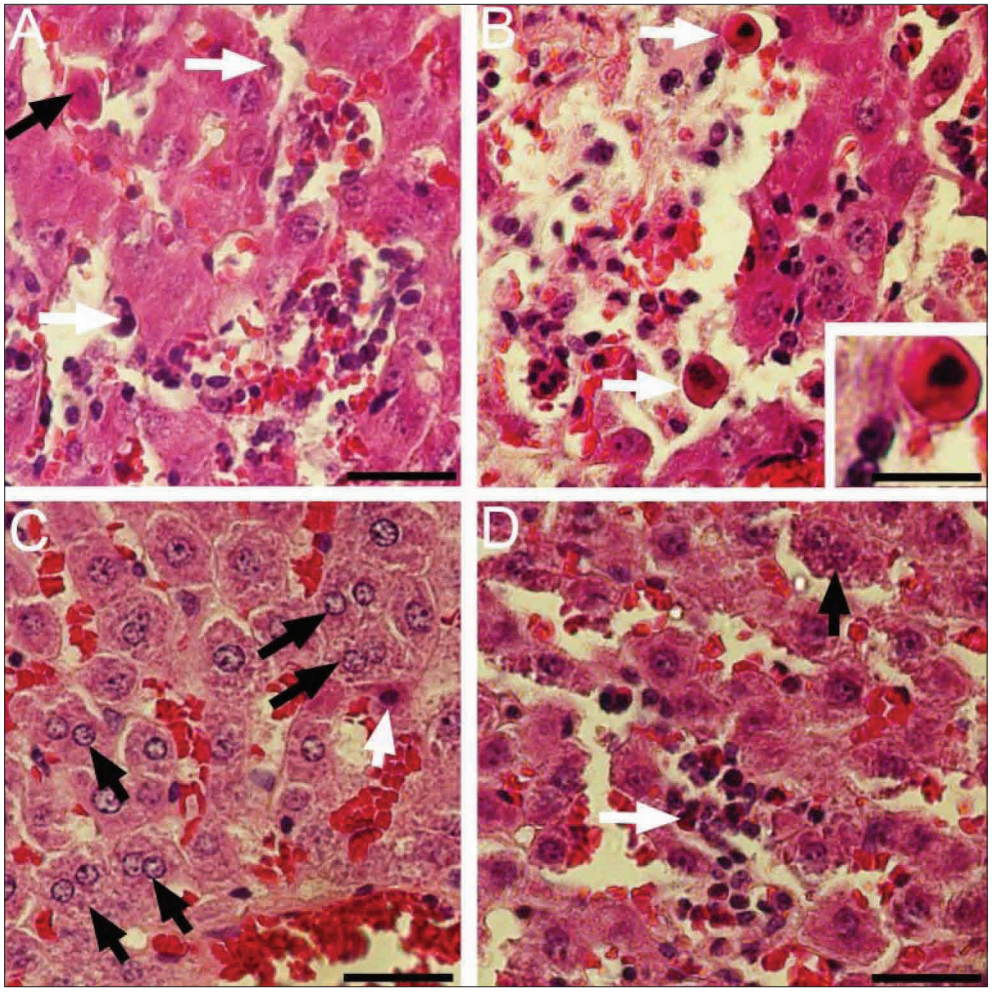


Fig 2. A- Irregular thickened hepatocyte plates with inflammatory cell infiltrates, Councilman bodies *(black arrow)* and hypertrophic Kupffer cells *(white arrows)* in parenchyma of livers from bortezomib-treated (hour 48) group, **B-** Severe inflammatory cell infiltrates with hepatocytes debris, Councilman bodies *(small square)* in parenchyma of livers from bortezomib-treated (hour 72) group, **C-** Necrosis in hepatocytes *(white arrow)* and moderate level hypertrophic and binucleate hepatocytes in midzonal and centrilobular zones of the liver tissue from bortezomib + β-1,3-D-glucan-treated (hour 48) group, **D-** Degenerative and binucleate *(black arrow)* hepatocytes and migration and adhesions of inflammatory cells (mono and polymorphonuclear leukocytes) within sinusoids in livers from Bortezomib + β-1,3-D-glucan-treated (hour 72) group. Scale bars in A, B, C, and D, 30 µm

Şekil 2. A- Bortezomib ile tedavi (saat 48) grubunda karaciğer parankimasında hipertrofik Kupffer hücreleri *(beyaz oklar)*, Councilman cisimcikleri *(siyah ok)* ve inflamatuvar hücre infiltrasyonları ile düzensiz kalınlaşmış hepatosit kordonları, **B-** Bortezomib ile tedavi (saat 72) grubunda karaciğer parankimasında Councilman cisimcikleri *(küçük kare)* ve hepatosit debrisleri ile şiddetli inflamatuvar hücre infiltrasyonları **C-** Bortezomib+ β-1,3-D-glukan (saat 48) ile tedavi grubunun karaciğer dokusunun midzonal ve sentrilobuler bölgelerinde orta düzeyde hipertrofik ve iki nükleuslu hepatositler ve hepatositlerde nekrozis *(beyaz ok)*, **D-** Bortezomib + β-1,3-D-glukan (saat 72) ile tedavi grubunun karaciğerlerinde sinüzoidler içinde inflamatuvar hücrelerin (mono ve polimorfonükleer lökositler) adhesyonu ve göçü ve dejeneratif ve iki nükleuslu hepatositler *(siyah oklar)*. A, B, C ve D 'deki ölçek barlar, 30 um

of the bortezomib + β-1,3-D-glucan-treated group at hour 72 had no Councilman bodies, only mild level necrotic and degenerative cells within midzonal and centrilobular zones, and minimal level inflammatory areas (mono and polymorphonuclear leukocytes) within and around sinusoids, minimal level hypertrophic Kupffer cells (*Fig. 2D* and *Table 1*). The bortezomib + β-1,3-D-glucan-treated group at hour 48 showed no Councilman bodies or inflammatory cells and had minimal level necrotic and mild level degenerative cells, moderate level binucleate hepatocytes within midzonal and centrilobular zones, and normal appearance Kupffer cells. The general liver histological structures of the Bortezomib + β-1,3-D-glucan-treated group at hour 48 were close to the control group (*Fig. 2C* and *Table 1*).

Biochemical Results for Oxidant and Antioxidant Parameters

The lipid peroxidation amounts (as indicators of oxidative stress of livers) for the treatment and control

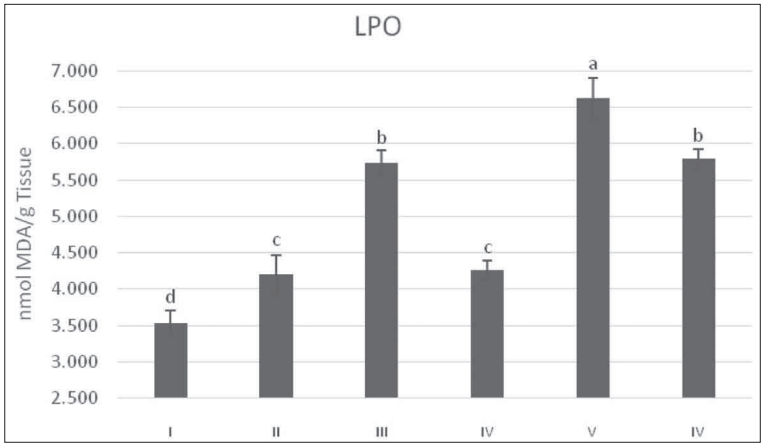
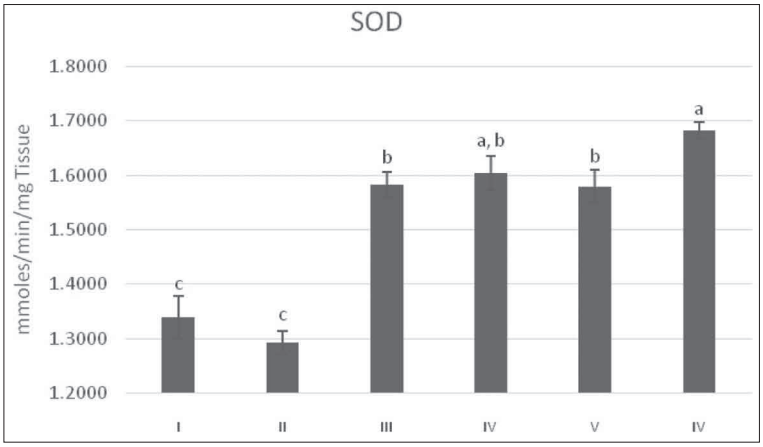


Fig 3. Effects of bortezomib, β-1,3-D-glucan, and bortezomib + β-1,3-D-glucan treatments in levels of lipid peroxidation (LPO) in rat livers (mean ± S.E.M.) [I; control, II; β-1,3-D-glucan, III; bortezomib (hour 48), IV; bortezomib + β-1,3-D-glucan (hour 48), V; bortezomib (hour 72), VI; bortezomib + β-1,3-D-glucan (hour 72)].

Şekil 3. Bortezomib, β-1,3-D-glukan, ve bortezomib + β-1,3-D-glukan tedavilerinin sıçan karaciğerlerinde lipid peroksidayon (LPO) seviyelerine etkileri (ortalama ± S.E.M.) [I; kontrol, II; β-1,3-D-glukan, III; bortezomib (saat 48), IV; bortezomib + β-1,3-D-glukan (saat 48), V; bortezomib (saat 72), VI; bortezomib + β-1,3-D-glukan (saat 72)]

Fig 4. Effects of bortezomib, β-1,3-D-glucan, and bortezomib + β-1,3-D-glucan treatments in activity changes of superoxide dismutase (SOD) in rat livers (mean ± S.E.M.) [I; control, II; β-1,3-D-glucan, III; bortezomib (hour 48), IV; bortezomib+β-1,3-D-glucan (hour 48), V; bortezomib (hour 72), VI; bortezomib + β-1,3-D-glucan (hour 72)]

Şekil 4. Bortezomib, β-1,3-D-glukan, and bortezomib + β-1,3-D-glukan tedavilerinin sıçan karaciğerlerinde süperoksit dismutaz (SOD) aktivite değişimlerine etkileri (ortalama ± S.E.M.) [I; kontrol, II; β-1,3-D-glukan, III; bortezomib (saat 48), IV; bortezomib + β-1,3-D-glukan (saat 48), V; bortezomib (saat 72), VI; bortezomib + β-1,3-D-glukan (saat 72)]



groups are shown in *Fig. 3*. Hepatic MDA levels in the bortezomib treatment groups (hours 48 and 72) with the increasing influence of time-dependence was significantly higher than the control group (P<0.05). Administration of β-1,3-D-glucan significantly reduced the tissue MDA levels (26.7%) at hours 48 (25.7%) and 72 in hepatic MDA levels increased by bortezomib treatment.

The hepatic SOD enzyme activities for all treatment and control groups were measured to understand the behavior of the antioxidant defense mechanism and were shown in *Fig. 4*. In both bortezomib treatment groups (hours 48 and 72), SOD activity was higher than the control group (P<0.05). Administration of β-1,3-D-glucan significantly elevated the tissue SOD activity at hours 48 and 72 in hepatic SOD activity comparing all groups.

Immunohistochemistry Results for p65 (RelA) Activity in Kupffer Cells

Kupffer cells are specialized macrophages located in the walls of the sinusoids and play an important role in late-phase hepatotoxicity. Early-phase hepatic injury-induced (xenobiotics dependent) Kupffer cells produce cytokines and growth factors via canonical NF-κB pathway. Activated Kupffer cells increase other inflammatory cell migration from the liver microcirculation to the parenchyma and lead to hepatic cell death by uncontrolled inflammation. NF-κB activation is measured by p65 (NF-κB subunit) migration to the nucleus in Kupffer cells. Thus, this study investigated the numerical density of nuclear p65 localization of Kupffer cells in liver tissues of the control and treatment groups using immunohistochemical staining and stereological methods *(Fig. 5)*. The numerical density of nuclear p65 (RelA) for treatment and control groups are shown in *Fig. 6*.

The immunohistochemistry results showed no statistically significant differences between the control group and β-1,3-D-glucan,Bortezomib + β-1,3-D-glucan (hour 48) groups in numerical density of p65, as determined by stereological examination of the liver tissues (P>0.05). However, this data was high at hour 48 and very high at hour 72 after bortezomib treatments (P<0.05). Bortezomib treatment-induced increased nuclear p65 localization was decreased via β-1,3-D-glucan treatments at hours 48 and 72 (P<0.05).

DISCUSSION

Hepatotoxicity associated with chemotherapeutic agents is of interest to clinicians and researchers. The chemotherapeutic agents are among the more commonly used drugs associated with hepatotoxic effects ranging from acute to chronic damage, hepatitis, steatosis

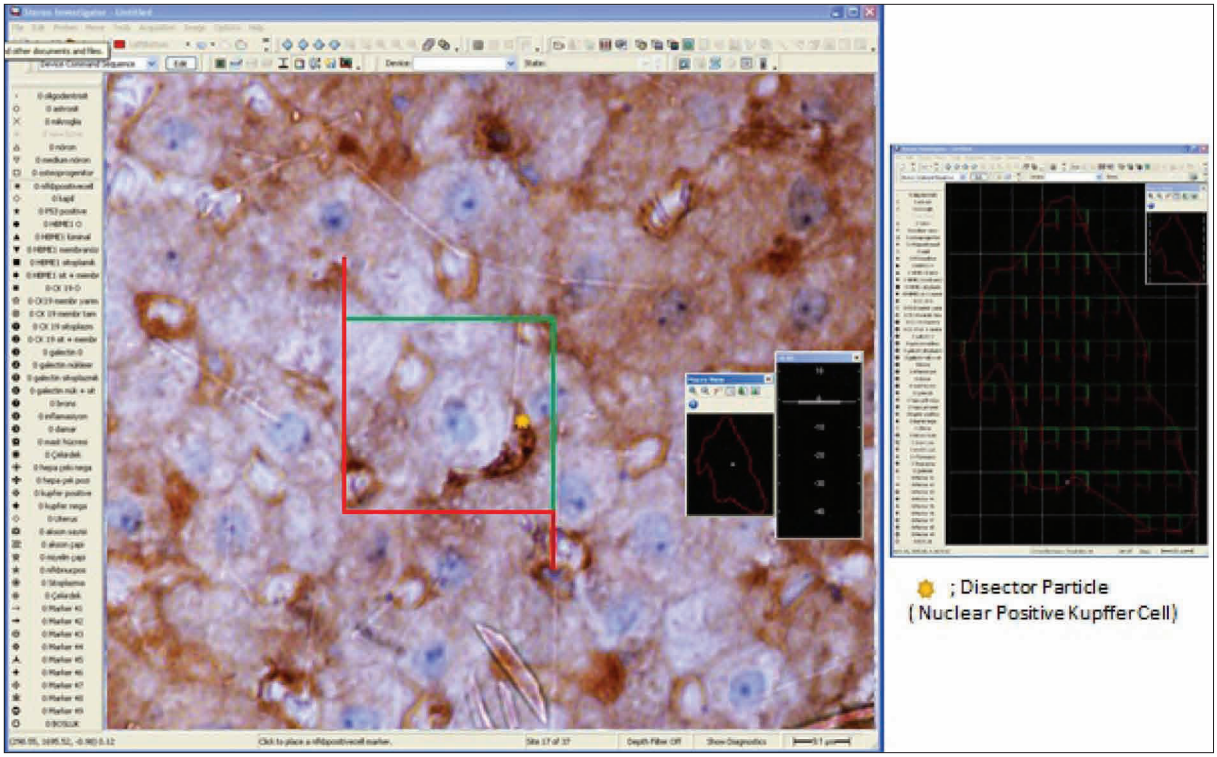


Fig 5. Unbiased counting frames used for numerical calculation of nuclear p65 localization by fractionator method (63×). Nuclear p65 hitting inclusion lines were calculated [Bortezomib + β-1,3-D-glucan group (hour 48)]

Şekil 5. Parçalama yöntemi (63 ×) tarafından nüklear p65 lokalizasyonu sayısal hesaplaması için kullanılan tarafsız sayım çerçeveleri. Dahil hatlara isabet eden nüklear p65 hesaplandı [Bortezomib + β-1,3-D-glukan grubu (saat 48)]

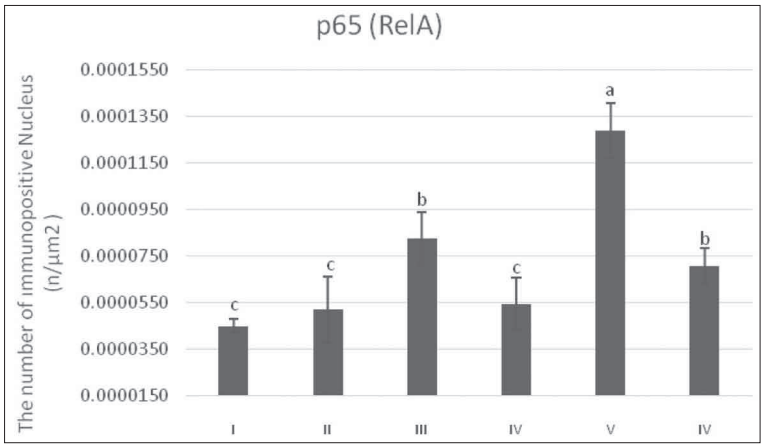


Fig 6. Effects of Bortezomib, β-1,3-D-glucan, and Bortezomib + β-1,3-D-glucan treatments on changes in nuclear p65 localization in Kupffer cells (mean ± S.E.M.) [I; control, II; β-1,3-D-glucan, III; bortezomib (hour 48), IV; bortezomib + β-1,3-D-glucan (hour 48), V; bortezomib (hour 72), VI; bortezomib + β-1,3-D-glucan (hour 72)]

Şekil 6. Bortezomib, β-1,3-D-glukan, and bortezomib + β-1,3-D-glukan tedavilerinin sıçan karaciğerlerinde Kupffer nüklear p65 lokalizasyon değişimlerine etkileri (ortalama ± S.E.M.) [I; kontrol, II; β-1,3-D-glukan, III; bortezomib (saat 48), IV; bortezomib + β-1,3-D-glukan (saat 48), V; bortezomib (saat 72), VI; bortezomib + β-1,3-D-glukan (saat 72)]

cholestasis and granuloma [23]. Bortezomib (PS-341 or Velcade®) is a modified dipeptidyl boronic acid 26S proteasome inhibitor for the treatment of multiple myeloma which is the second most common hematological cancer (after non-Hodgkin's lymphoma) [24]. Although Hideshima et al.[25] reported bortezomib is 1.000-times more effective for triggering apoptosis on myeloma cells than normal plasma cells. There is limited information about its effects on other normal cells because of new drug marketing. Phase studies have shown that the drug had tolerable adverse effects, such as peripheral neuropathy, gastrointestinal symptoms, thrombocytopenia, and hypotension in non-target organs [26]. However, a few case reports clinically determined that bortezomib increased liver enzymes (aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase) levels [27].

According to literature, bortezomib-induced hepatotoxicity has been rarely characterized and or studied. This research evaluated the time-dependent histopathological, biochemical, and immunohistochemical changes of liver tissues in rats given bortezomib. The first observation was that nonspecific reactive hepatitis (NSRH) or focal hepatitis was histologically observed in the liver tissue of rats given bortezomib alone. NSRH is an entity characterized by the presence of Kupffer cell

mobilization, portal inflammatory infiltration, and focal periportal necroinflammatory areas (generally mononuclear cells) with acidophilic cells (Councilman bodies) through the liver parenchyma [28]. Drug-induced NSRH is rare and determined in some drugs such as naproxen, aspirin, and paclitaxel [29]. This present study is the first to report that bortezomib causes drug-induced NSRH. According to these findings, liver tissues 48 h after bortezomib treatment showed a large number of focal necrotic areas with prominent inflammatory responses in all zones of the liver acinus. Inflammatory responses were associated with the relationship between activated (hypertrophic) Kupffer cells and mononuclear cells. This study also showed wide sinusoidal expansion and hypertrophic changes in hepatocytes in liver parenchyma (without focal necroinflammatory areas) in this group. By hour 72, bortezomib-treated livers had noticeably greater numbers and sizes of focal inflammatory cell infiltrations, necrotic areas, and other degenerative changes than those observed at 48 hours.

In summary, this study demonstrated that bortezomib treatment-induced prominent liver degeneration was apparent by hour 48 and gradually increased until hour 72. Drug-induced liver toxicity is a common cause of liver injury and generally occurs two pathophysiological processes. In the first pathway, drugs and their metabolites with possible toxic effects directly affect the biochemistry of the cell primarily due to increased oxidative stress and changes of intracellular signaling pathways associated with apoptotic or necrotic cell death in hepatocytes (mainly), endothelial cells, and cholangiocytes. In the second pathway, these also caused immune response in inflammatory cells and triggered cellular damage and apoptotic or necrotic cell death in parenchymal cells [30]. As a result, these two pathways are related to the occurrence of inflammatory and oxidative products in the liver [31]. Oxidative stress is often implicated in various deleterious processes resulting from an imbalance between pro-oxidants (reactive oxygen species [ROS] and/or reactive nitrogen species [RNS]) and antioxidants in favor of the pro-oxidants [32]. ROS are formed through oxidative processes within the cell but can be produced at elevated rates under pathophysiological conditions. The excessive ROS may attack polyunsaturated fatty acids of the cellular membrane and initiate lipid peroxidation within the cell, which results in the formation of Malondialdehyde (MDA). Malondialdehyde, a reactive aldehyde, is the major product of lipid peroxidation and widely used to indicate LPO level [33]. Antioxidant reactions inhibit the oxidation of cellular compounds via antioxidant enzymes and molecules such as SOD, CAT, GPx, and GR. SOD is the first line in the defense process against superoxide radicals produced by oxidative reactions and is a significant parameter to evaluate cellular antioxidant activity [34]. In the biochemical analysis the terms of SOD and LPO values, this study revealed that bortezomib treatment elevated SOD and LPO values at hours 48 and 72, increasing with time. However, the increase in the LPO values was higher than the increase in the SOD values of livers in the bortezomib groups compared to other groups. This finding suggested that oxidant-antioxidant balance is broken in favor of the oxidants in cells, or insufficient levels of antioxidants and oxidative stress occur. This elevation is probably related to elevated endogenous pro-oxidants associated with first pathway and increased exogenous pro-oxidants associated with second pathway (resident macrophages [Kupffer cells] and infiltrating phagocytes) according to this study's histopathologic findings.

Although bortezomib is a potent apoptotic activator via proteasome inhibition, this research showed that it caused necrotic (hypertrophic changes) and necrotic cell deaths via oxidative stress in focal necroinflammatory and other areas in rat livers. In a preclinical *in vivo* toxicity study in rats, an increase in liver enlargement (35%) caused by the induction of increased peroxisomal acyl-CoA oxidase (60%) activity and after a repeat dose of bortezomib was observed [10]. This data overlaps to hypertrophic changes of hepatocytes in the present study, and increasing LPO amounts may be related to increased peroxisomal acyl-CoA oxidase activity and other oxidases by induced bortezomib treatments. LPO values above vital limits may break down membrane integrity, causing necrotic death [35]. In addition, apoptotic cell deaths (Councilman bodies) were only observed in the focal necroinflammatory areas as well as necrotic cell deaths. Apoptotic cell deaths could result from inflammatory responses of mononuclear cells [36]. Other than direct effects of bortezomib and its metabolites because it was only observed in necroinflammatory areas. It was shown that Kupffer cells have an important role in the initiation and maintenance of this immune response in necroinflammatory areas because of the time-dependent increase in hypertrophic changes and p65 activity. Activated canonical p65 pathway-dependent increased proinflammatory cytokine secretion, such as tumour-necrosis factor (TNF) and interleukin-6 (IL-6) in Kupffer cells induces the activation and migration of mononuclear cells [37,38] toward necroinflammatory areas. Furthermore, endothelial cells were shown to have a role in endothelial dilatation-dependent this migration.

In conventional therapies, cancer patients often receive complementary medical treatment to prolong survival and reduce toxicity in non-target organs. It's necessary that the substances used in complementary therapy have protective effects as well as anti-cancer properties [39]. β-1,3-D-glucan has been shown in Japanese medicine, experimental studies, and clinical trials to enhance the effectiveness of chemotherapeutic agents on the neoplastic cells and have protective effects on non-target organs [40-42]. Therefore, this present experiment

examined the effects of β-1,3-D-glucan. Whether β-1,3-D-glucan had adverse effects on the liver compared to the control group was first examined. Delaney et al.[43] reported that β-1,3-D-glucan had no toxic effects on animals. Similarly, this study did not reveal toxic effects in parenchyma or Kupffer cells according to immunohistochemical and histopathologic analyses. However, LPO values showed an increase in physiological levels. Next, this study examined the effectiveness of β-1,3-D-glucan as a protective agent against liver damage induced by bortezomib. β-1,3-D-glucan significantly eliminated bortezomib-induced NSRH findings at both 48 and 72 hours. However, some histopathologic changes were observed in both groups. Diffuse hypertrophic hepatocyte degeneration (an irreversible cell damage), non-focal necrotic deaths and sinusoidal dilatation were observed in the middle-middle, minimal-middle, and minimal-middle levels at 48 and 72 hours respectively. Hypertrophic Kupffer cells and non-focal inflammatory cell infiltrates were also observed at minimal levels at 72 h.

In summary, these findings showed that cellular degeneration and inflammatory response were significantly decreased in both groups. These findings were also parallel with decreasing values of LPO, an important indicator of cellular damage and reduced Rel A activity as an indicator of inflammatory processes. According to our results, β-1,3-D-glucan decreased LPO levels by increasing defense mechanisms such as the activity of SOD enzymes. This may indicate the presence or trend of increasing amounts of superoxide radicals, which could be linked with an enhanced SOD activity [44]. Indeed, the increased SOD activity would also protect tissues from oxidative stress, which reveals that the accumulation of superoxide anion radicals might be responsible for an increased LPO [45]. Descending necrotic deaths and decreased LPO values probably must remain below the threshold value required for the activation of Kupffer cells, so it can cause an inflammatory response is not generated. In addition, β-1,3-D-glucan increased activity on other cellular antioxidative enzymes, and its free radical scavenging activity [46-49] might contribute to the reduction of oxidative stress and cellular degeneration and eliminate NSRH findings. In addition, in this group, absence of apoptotic death, these deaths did not occur with the direct effect of bortezomib and again seems to be associated with the inflammatory process.

To knowledge, this present study is the first to show that, based on biochemical, immunohistochemical, and histopathological findings, the administration of bortezomib causes increased tissue damage and NSRH in rat livers, and β-1,3-D-glucan may decrease the increasingly toxic effects of bortezomib by regulating Kupffer cell activation and suppressing oxidative stress leading to NSRH. In light of this information, β-1,3-D-glucan may be used as a supportive agent for reducing the side effects of bortezomib therapy.

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RESEARCH ARTICLE

Yumurtacı Tavuklarda *Salmonella* İzolatlarının Tanısı ve Tiplendirilmesi [1] [2]

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Özet

Çalışmada, farklı yetiştirme dönemlerindeki yumurtacı tavuklarda *Salmonella* aranmasında farklı metotların değerlendirilmesi, elde edilen izolatların serotiplendirilmesi, ribotiplendirilmesi ve antibiyotiplendirilmesi ile *Salmonella* yaygınlığının belirlenmesi amaçlandı. Bu amaçla, 58 adet ticari yumurtacı tavuk kümesinden yetiştirmenin 15., 25. ve 40. haftalık dönemlerinde alınan toplam 174 adet drag svap örneği, *Salmonella* varlığı açısından standart kültür metodu olan ISO (ISO 6579:2002/Amd 1:2007 - ISO) ve 2 farklı gerçek zamanlı polimeraz zincir reaksiyonu (rPCR) sistemi (Light Cycler SYBR Green I rPCR - LCSGIrPCR ve DuPont BAX Q7 rPCR - BAXrPCR) kullanılarak analiz edildi. İncelenen 58 kümesin 17 adedi (%29.31), 174 adet drag svap örneğinin 15. ve 25. haftalık dönemlerde 6'şar (%3.44 ve %3.44), 40. haftada ise 10 adedi (%5.74) olmak üzere toplam 22'si (%12.62) her 3 metotla *Salmonella* yönünden pozitif olarak bulundu. Elde edilen 22 adet *Salmonella* izolatının 20'sinin (%90.90) *Salmonella enterica* subsp. *enterica* olup 9'unun Enteritidis (SE) (%40.9), 7'sinin Infantis (SI) (%31.8), 1'inin Hadar (%4.5), 1'inin Montevideo (%4.5), 1'inin Colombo (%4.5) ve 1'inin de Spartel (%4.5) serovarı olduğu, 2'sinin (%9.10) ise *Salmonella enterica* subsp. *arizonae* ile *Salmonella enterica* subsp. *houtenae* alttürlerine ait olduğu belirlendi. Ayrıca izolatların test edilen 24 antibiyotiğin 23'üne karşı direnç oluşturduğu, en yüksek direnç oranının Ampicillin (%100), Neomycin (%100), Penicillin G (%100) ve Erythromycin'e (%95.45) karşı olduğu saptandı. Çalışmanın sonucunda, yumurtacı tavuklarda *Salmonella*'nın hızlı deteksiyonunda LCSGIrPCR ve BAXrPCR sistemlerinin ISO metodunu destekleyici olarak kullanılabileceği, incelenen tüm yetiştirme dönemlerinde *Salmonella enterica* subsp. *enterica* varlığının yüksek olduğu belirlendi.

Anahtar sözcükler: *Salmonella, Yumurtacı tavuk, Drag svap, ISO, Real-time PCR, Serotiplendirme, Ribotiplendirme, Antibiyotiplendirme*

Detection of *Salmonella* from Layer Flocks and Typing of the Isolates

Abstract

This study aimed to evaluate different detection methods in determining *Salmonella* prevalence in layers of various rearing periods, and to serotype, ribotype and antibiotype the isolates. For this, 174 drag swab samples taken from 58 commercial layer flocks of 15, 25, and 40 week age were analyzed for *Salmonella* by ISO standard culture method (ISO 6579:2002/Amd 1:2007 - ISO) and by 2 real time polymerase chain reaction (rPCR) systems (Light Cycler SYBR Green I rPCR - LCSGIrPCR and DuPont BAX Q7 rPCR - BAXrPCR). Seventeen out of 58 (29.31%) flock samples, and 6 of the 15th and 25th weeks' (3.44% and 3.44%), and 10 (5.74%) of the 40th week's drag swab samples were *Salmonella* positive by all 3 methods. Twenty out of 22 *Salmonella* isolates (90.90%) were *Salmonella enterica* subsp. *enterica*, including serovars of 9 Enteritidis (SE) (40.9%), 7 Infantis (SI) (31.8%), 1 Hadar (4.5%), 1 Montevideo (4.5%), 1 Colombo (4.5%), 1 Spartel (4.5%), and 2 subspecies (9.10%) as *Salmonella enterica* subsp. *arizonae* and *Salmonella enterica* subsp. *houtenae*. Also isolates showed resistance to 23 of 24 antibiotics, with the highest resistances to Ampicillin (100%), Neomycin (100%), Penicillin G (100%) and Erythromycin (95.45%). Results indicate that both LCSGIrPCR and BAXrPCR systems can be used to complement ISO method in rapid detection of *Salmonella* from layer chickens, and that there is high prevalence of *Salmonella enterica* subsp. *enterica* in all rearing periods examined.

Keywords: *Salmonella, Layer chicken, Drag swab, ISO, Real-time PCR, Serotyping, Ribotyping, Antibiotyping*

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GİRİŞ

Ülkemizde ve dünyada kanatlı endüstrisinde önemli bakteriyel zoonoz hastalıklardan biri olan salmonellozis, yumurtacı tavuk yetiştiriciliğinde de hem ekonomik kayıplara neden olarak hem de kontamine ürün tüketimine bağlı oluşabilecek gıda zehirlenmeleri ile insan sağlığını olumsuz etkileyerek problem olmaya devam etmektedir [1].

Salmonella varlığının standart kültür metotları ile tespiti 5-11 gün sürmekte [2] bu nedenle etkenin özellikle yetiştirme dönemlerinde erken belirlenebilmesi ve gerekli önlemlerin alınabilmesi için daha kısa sürede sonuç veren hızlı ve ekonomik farklı deteksiyon sistemleri de kullanılmaktadır [3,4]. Kanatlı hayvanlara ait farklı örnek tiplerinde *Salmonella* aranması çalışmalarında, LightCycler SYBR Green I gerçek zamanlı polimeraz zincir reaksiyonu (LCSGIrPCR) sisteminin birçok kez güvenli olarak kullanıldığı bildirilmektedir [4-6]. *Salmonella* teşhisinde bir diğer gerçek zamanlı PCR sistemi olarak DNA'ya bağlanan boyalara alternatif bir sistem ile kullanıma hazır hedef-spesifik problu tabletleri içeren DuPont BAX *Salmonella* sisteminin (BAXrPCR) de çeşitli gıda, su, çevresel örnekler ve fekal örneklerde başarıyla kullanıldığı rapor edilmektedir [7-12].

Salmonellaların tiplendirilmesi, epidemiyolojik yönden kontrol önlemlerinin alınabilmesi açısından önem taşımaktadır. Klasik serotiplendirme, uygun laboratuvar şartları, uzmanlık, malzeme ve zaman gerektirdiği için sadece referans laboratuvarlarda gerçekleştirilebilmektedir. Ribotiplendirme bazı çalışmalarda, genotiplendirmede altın standart yöntem olarak kabul edilen Pulsed Field Gel Electrophoresis (PFGE) testi kadar duyarlı bulunamasa da, PFGE testinden çok daha kısa sürede sonuç vermesi, uzman personel ve laboratuvar şartlarına gereksinim göstermeyen kapalı otomotize bir sistem olması ile bir defada birçok örneğin çalışılmasını gerektiren epidemiyolojik çalışmalarda alternatif olması yönünden yaygın kullanım alanı bulmaktadır [13,14]. *Salmonella* tiplendirilmesinde kullanılan antibiyotiplendirme ile ilgili olarak son yıllarda yapılan çalışmalarda özellikle hayvan ve insan sağlığı açısından önem taşıyan antibiyotiklere karşı etkenin çoklu direnç geliştirdiği bildirilmektedir [15-17].

Bu çalışmayla, farklı yetiştirme dönemlerinde yumurtacı tavuklara ait drag svap örneklerinde *Salmonella* aranmasında ISO metodu ile LCSGIrPCR ve BAXrPCR sistemleri değerlendirilecek, elde edilen izolatların serotiplendirilmesi, ribotiplendirilmesi ve antibiyotiplendirilmesi ile yetiştiriciliğin yoğun olarak yapıldığı bölgelerde sağlıklı görünen yumurtacı tavuklardaki *Salmonella* yaygınlığı belirlenecektir.

MATERYAL ve METOT

Çalışma, Uludağ Üniversitesi Tıp Fakültesi Araştırma Etik Kurulu onayı (Karar No: 2011-02/04) ile gerçekleştirildi.

Salmonella Standart Suşları

Antibiyotiplendirme dışında tüm analizlerde *Salmonella enterica* subsp. *enterica* serovar Enteritidis 64K (M.Y. Popoff, Institut Pasteur, 28 rue du Dr Roux, 75015 Paris Cedex 15, France) ve *Salmonella enterica* subsp. *enterica* serovar Typhimurium NCTC 12416 (Türkiye Halk Sağlığı Kurumu, Ankara) pozitif kontrol olarak kullanıldı.

Örnek

2013-2014 yılları arasında, Afyon, Balıkesir, Bursa, İzmir, Karaman, Konya, Manisa illerinde bulunan 58 farklı işletmeye ait (klinik semptom gözlenmeyen) yumurtacı tavuk kümeslerinden yetiştirmenin 3 farklı döneminde (15., 25. ve 40. haftalarda) 2'şer adet olmak üzere toplam 348 adet drag svap örneği alınıp soğuk zincir koşullarında laboratuvara getirilerek 2 saat içerisinde analize alındı [18].

Kültür

Herbir kümese ait 2 drag svap örneği (ortalama 300 g) öncelikle steril polietilen bir poşet içerisinde dışarıdan elle masaj yapılarak homojenize edildi. Homojenize edilen bu örnekten steril olarak 25 g alındı ve ön zenginleştirme amacı ile içerisinde 225 ml Buffered Peptone Water ISO (BPW-ISO, Oxoid, CM1049B) bulunan steril polietilen poşette 37°C'de 18 saat inkübe edildi. İnkübasyon sonunda rPCR çalışmalarında kullanılmak üzere 1 ml örnek alınarak -20°C'de saklandı. Ön zenginleştirme aşaması sonrasındaki tüm izolasyon işlemleri ISO metoduna göre uygulandı [19]. Biyokimyasal identifikasyon ise API 20E (bioMerieux, Marcy L'Etoile 20120, France) kullanılarak gerçekleştirildi.

Serotiplendirme ve Ribotiplendirme

O- ve H- grup antijen reaksiyonları temel alınarak, White- Kauffmann-Le Minor Şeması [20] ve Guibourdenche ve ark.'na göre [21] ticari antiserumlar (Becton Dickinson, USA) kullanılarak gerçekleştirildi. Ribotiplendirme otomatize Riboprinter sistemi (Dupont Qualicon, Wilmington, DE, USA) ile kullanım kılavuzunda belirtildiği şekilde uygulandı [22].

Antibiyotiplendirme

Salmonella izolatlarının antibiyotik direnç profilleri disk agar difüzyon metodu ile belirlenerek Clinical and Laboratory Standards Institute (CLSI)'a göre değerlendirildi [23]. Test edilen 24 antibiyotik ve konsantrasyonları: Amikacin (AK, 30 µg, Oxoid CT0107B), Amoxycillin (AML, 10 µg, Oxoid CT0161B), Ampicillin (AMP, 10 µg, Oxoid CT0003B), Azithromycin (AZM, 15 µg, Oxoid CT0906B), Cefoxitin (FOX, 30 µg, Oxoid CT0119B), Ceftriaxone (CRO, 30 µg, Oxoid CT0417B), Cephazolin (KZ, 30 µg, Oxoid CT0011B), Chloramphenicol (C, 30 µg, Oxoid CT0013B), Ciprofloxacin (CIP, 5 µg, Oxoid CT0425B), Colistin (CT, 10 µg, Oxoid CT0017B), Doxycycline hydrochloride (DO, 30 µg, Oxoid CT0018B), Enrofloxacin (ENR, 5 µg, Oxoid

CT0639B), Erythromycin (E, 15 µg, Oxoid CT0020B), Forfenicol (FFC, 30 µg, Oxoid CT1754B), Gentamicin (CN, 10 µg, Oxoid CT0024B), Nalidixic acid (NA, 30 µg, Oxoid CT0031B), Neomycin (N, 30 µg, Oxoid CT0033B), Ofloxacin (OFX, 5 µg, Oxoid CT0446B), Oxytetracycline (OT, 30 µg, Oxoid CT0041B), Penicillin G (P, 10 units, Oxoid CT0043B), Streptomycin (S, 10 µg, Oxoid CT0047B), Sulphamethoxazole/Trimethoprim 19:1 (SXT, 25 µg, Oxoid CT0052B), Sulphonamides Compound (S3, 300 µg, Oxoid CT0059B), Tetracycline (TE, 10 µg, Oxoid CT0053B). Kontrol amaçlı olarak CLSI'da belirtilen *E. coli* (ATCC 25922) ve *S. aureus* (ATCC 25923) standart suşları kullanıldı [23].

Primerler

LCSGIrPCR'da *Salmonella inv*A-spesifik primerler [4,24], BAXrPCR'da ise DuPont BAX System PCR Assay for *Salmonella* 2 kiti (Part D14368501, DuPont, USA) PCR reagent tabletleri içeriğindeki primerler kullanıldı.

Templeyt Hazırlama ve rPCR

LCSGIrPCR'da kaynatma metoduyla [4] hazırlanan templeyt DNA, BAXrPCR'da ise DuPont BAX System PCR Assay for *Salmonella* 2 kitinde (Part D14368501, DuPont, USA) belirtilen şekilde elde edilen DNA kullanıldı. LCSGIrPCR parametrelerinin uygulanması ve sonuç değerlendirilmesi Temelli ve ark.'na göre [4], BAXrPCR parametrelerinin uygulanması ve sonuç değerlendirmesi ise DuPont BAX System PCR Assay for *Salmonella* 2 kit (Part D14368501, DuPont, USA) bilgileri ile BAX sistem kullanım kılavuzunda belirtilen şekilde yapıldı.

İstatistik

SPSS Windows (SPSS Inc. USA)'un 20.0 versiyonu [25] kullanılarak yapıldı. Farklı yetiştirme dönemlerine ait örneklerin *Salmonella* pozitiflik oranları üzerindeki etkisini belirlemek için Ki-Kare testi uygulandı. Değerler arasındaki olası farklılığın önem derecesi P<0.05 düzeyinde incelendi.

BULGULAR

Kültür

İncelenen toplam 58 kümesin 17 adedinin (%29.31), farklı yetiştirme dönemlerinde alınan 174 adet örneğin 15. ve 25. haftalık dönemlerde 6'şar adedi (%3.44 ve %3.44), 40. haftada ise 10 adedi (%5.74) olmak üzere toplam 22 adedi (%12.62) *Salmonella* yönünden pozitif olarak bulundu *(Tablo 1)*.

Serotiplendirme ve Ribotiplendirme

Yirmi iki adet *Salmonella* izolatının serotiplendirilmesi ve ribotiplendirilmesi sonrasında 20'sinin (%90.90) *Salmonella enterica* subsp. *enterica* olup 9'unun Enteritidis (SE) (%40.9), 7'sinin Infantis (SI) (%31.8), 1'inin Hadar (%4.5), 1'inin Montevideo (%4.5), 1'inin Colombo (%4.5) ve 1'inin de Spartel (%4.5) serovarı olduğu, 2'sinin (%9.10) ise *Salmonella enterica* subsp. *arizonae* ile *Salmonella enterica* subsp. *houtenae* alttürlerine ait olduğu belirlendi *(Tablo 1)*.

Antibiyotiplendirme

İzolatların tümünde test edilen 24 antibiyotikten Amikacin haricinde 23'üne karşı direnç bulundu. En yüksek direnç oranının Ampicillin (%100), Neomycin (%100), Penicillin G (%100) ve Erythromycin'e (%95.45) karşı olduğu, test edilen diğer antibiyotiklerde bulunan direnç oranlarının ise azalan sırayla OT (%72.72), DO, CN, NA (%50), TE (%45.45), S3 (%40.90), ENR, FFC, S, SXT (%36.36), AZM, C (%31.81), FOX, KZ, CT (%22.72), CRO, CIP, OFX (%9.09) olduğu saptandı. Çoklu direnç (MDR) yönünden 22 izolattan 2'si en az 4 (AMP/E/N/P), 1'i en çok 18 adet antibiyotiğe karşı dirençli bulundu *(Tablo 1)*.

rPCR

LCSGIrPCR ve BAXrPCR sistemlerinin sonuçlarının kültür metodunda elde edilen sonuçlar ile aynı olduğu belirlendi *(Tablo 1)*.

İstatistiksel Bulgular

Farklı yetiştirme dönemlerine ait *Salmonella* pozitif bulunan örnekler arasındaki farklılığın istatistiksel olarak önemli olmadığı saptandı (P>0.05).

TARTIŞMA ve SONUÇ

Bu çalışmada, yumurta tavukçuluğunun yoğun olduğu bölgelerde sağlıklı görünen sürülerde farklı metot ve sistemler ile *Salmonella* yaygınlığı değerlendirilerek elde edilen *Salmonella* izolatlarının alttür/serotipleri ile antibiyotik direnç profilleri belirlenmiştir.

İncelenmiş olan kümeslerin %29.31'inde *Salmonella* tespit edilmiş olup bu sonuç yumurtacı tavuk kümeslerinde *Salmonella* varlığını araştıran Eyigör ve ark.'nın [26] sonucu (%27.7) ile uyumlu iken Ata ve Aydın'ın [27] sonucundan (%12) yüksek bulunmuştur. Alınan drag svap örneklerindeki %12.62'lik *Salmonella* oranının ise benzer örnek tipi kullanılan çalışmalardan Eyigor ve ark.[26] (%11.1) ile Pitesky ve ark.'nın [28] (%11.9) bulgularına paralel, Eyigor ve ark.'nın [6] bir diğer çalışmalarında elde ettikleri bulgudan (%4) yüksek, Zhang ve ark.'nın [29] sonucundan (%18) düşük olduğu saptanmıştır. Çalışmamızda, 3 farklı yetiştirme döneminde uygulanan drag svab örneklemelerinde 15. ve 25. haftalık dönemlerde daha düşük (%3.44), 40. haftada ise daha yüksek (%5.74) oranda *Salmonella* tespit edilmiş olmasına rağmen yapılan istatistiksel analiz sonrasında bu farkın önemli olmadığı saptanmıştır (P>0.05). Bulgularımıza benzer bir şekilde, Mahmud ve ark.[17] tarafından *Salmonella* prevalansının yetiştirmenin 20-25. haftalarına

Tablo 1. *Drag svap örneklerine (n = 174) ait kültür ve rPCR sonuçları ile Salmonella serotipleri ve antibiyotik direnç profilleri*
Table 1. *Culture and rPCR results of drag swab samples (n = 174) and Salmonella serotypes and antibiotic resistance profiles*

İşletme No	Örnek Pozitif			Hafta Pozitif			Serovar /Alttür	Antibiyotik Direnç Profili																								
	ISO	rPCR		15	25	40		AK	AML	AMP	AZM	FOX	CRO	KZ	C	CIP	CT	DO	ENR	E	FFC	CN	NA	N	OFX	OT	P	S	SXT	S3	TE	Toplam Direnç
		BAX	LC																													
1	+	+	+	+	-	-	Hadar		+	+				+				+		+			+	+		+	+				+	10
2	+	+	+	-	-	+	Spartel		+	+							+			+	+	+	+	+		+	+			+		11
3	+	+	+	-	+	-	Enteritidis			+										+	+	+		+		+	+		+			8
4, 5	-	-	-	-	-	-	-																									
6	+	+	+	-	+	-	Enteritidis			+			+	+						+		+	+	+			+					8
7	-	-	-	-	-	-	-																									
8	+	+	+	-	-	+	Infantis		+	+		+			+			+		+			+	+	+	+	+		+	+	+	14
9-11	-	-	-	-	-	-	-																									
12	+	+	+	-	+	-	arizonae		+	+	+			+					+	+		+		+			+					9
13-25	-	-	-	-	-	-	-																									
26	+	+	+	+[1]	-	+[2]	[1]Enteritidis		+	+										+		+		+			+	+				7
							[2]Enteritidis		+	+								+		+		+	+	+		+	+				+	10
27-31	-	-	-	-	-	-	-																									
32	+	+	+	-	-	+	Colombo		+	+	+	+		+				+	+	+	+	+		+		+	+			+	+	15
33	+	+	+	-	+[1]	+[2]	[1]Infantis		+	+	+	+			+	+		+	+	+	+	+	+	+		+	+	+		+	+	18
							[2]Montevideo		+	+							+	+	+	+	+	+		+		+	+		+			12
34	+	+	+	+	-	-	Enteritidis			+										+				+			+					4
35	+	+	+	-	+[1]	+[2]	[1]Infantis		+	+	+					+		+	+	+	+	+	+	+		+	+	+	+	+	+	17
							[2]Infantis			+	+	+	+		+			+		+			+	+		+	+	+	+	+	+	15
36-42	-	-	-	-	-	-	-																									
43	+	+	+	+[1]	+[2]	-	[1]houtenae		+	+							+							+		+	+	+				7
							[2]Enteritidis		+	+										+	+			+		+	+		+			8
44, 45	-	-	-	-	-	-	-																									
46	+	+	+	+[1]	-	+[2]	[1]Infantis			+	+	+		+	+			+	+	+			+	+	+	+	+	+		+	+	16
							[2]Enteritidis			+										+				+			+					4
47	+	+	+	+	0	0	Enteritidis			+										+				+		+	+					5
48	-	-	-	-	-	-	-																									
49	+	+	+	-	-	+	Enteritidis		+	+							+		+	+	+			+		+	+		+			10
50-53	-	-	-	-	-	-	-																									
54	+	+	+	-	-	+	Infantis		+	+						+	+	+	+	+		+	+	+		+	+	+	+	+	+	16
55, 56	-	-	-	-	-	-	-																									
57	+	+	+	-	-	+	Infantis		+	+	+					+		+	+		+			+	+			+	+		+	+	13
58	-	-	-	-	-	-	-																									
Toplam	17	17	17	6	6	10	22	0	15	**22**	7	5	2	5	7	2	5	11	8	21	8	11	11	**22**	2	16	**22**	8	8	9	10	

AK, *Amikacin;* ***AML,*** *Amoxycillin;* ***AMP,*** *Ampicillin;* ***AZM,*** *Azithromycin;* ***FOX,*** *Cefoxitin;* ***CRO,*** *Ceftriaxone;* ***KZ,*** *Cephazolin;* ***C,*** *Chloramphenicol;* ***CIP,*** *Ciprofloxacin;* ***CT,*** *Colistin;* ***DO, D****oxycycline hydrochloride;* ***ENR,*** *Enrofloxacin;* ***E,*** *Erythromycin;* ***FFC,*** *Forfenicol;* ***CN,*** *Gentamicin;* ***NA,*** *Nalidixic acid;* ***N,*** *Neomycin;* ***OFX,*** *Ofloxacin;* ***OT,*** *Oxytetracycline;* ***P,*** *Penicillin G;* ***S,*** *Streptomycin;* ***SXT,*** *Sulphamethoxazole/Trimethoprim 19:1;* ***S3,*** *Sulphonamides Compound;* ***TE,*** *Tetracycline*

göre 40. ve sonrasındaki haftalarda daha yüksek olduğu ancak bu farkın istatistiksel olarak önemli olmadığı rapor edilmiştir. Pitesky ve ark.[28], *Salmonella* insidansında yumurta tavuklarında ilerleyen haftalarda artış olduğunu ve dönemler arasındaki bu farkın istatistiksel olarak önemli olduğunu bildirmişlerdir. Diğer bir çalışmada ise Li ve ark.[15] yetiştirmenin erken dönemlerinde (18. hafta) en yüksek oranda *Salmonella* prevalansı elde ettiklerini ancak bu farkın istatistiksel olarak önemli olmadığını belirtmişlerdir. Bulgularımız ile diğer çalışmaların sonuçları arasındaki farklılıkların (1) örnekleme stratejisi (sadece rastgele örnekleme, programlı rastgele örnekleme ile belirlenen periyot/yaştaki tavuklardan belirli sıklıkla ve sayıda yapılan örnekleme), (2) örnekleme yapılan bölge(ler) ve mevsim, (3) tavuk ırkı, yetiştirme ve kümes özellikleri, (4) örnek tipi (drag svap, kloakal svap, barsak örneği, çevre örneği), (5) kullanılan test metodu (metot/sistem farklılıkları ile bunların duyarlılık, özgünlük, örneğe uyumluluğu) gibi değişkenlerin tekil ve/veya kombine etkileşimlerinden kaynaklandığı düşünülmektedir.

Çalışmada kullanılan iki farklı hızlı deteksiyon sisteminin (LCSGIrPCR ve BAXrPCR) standart kültür metodu (ISO)'na paralel olarak, 174 örneğin 22 (%12.62)'sinde *Salmonella* pozitif sonuç verdiği tespit edilmiştir. Yumurtacı tavuklarda drag svap örneklerinde *Salmonella*'nın hızlı deteksiyonunda standart kültürü destekleyici olarak LCSGIrPCR'ın test edildiği ve sonuç olarak bu sistemin kültür ile bire bir uyumlu olduğunu bildiren çalışmalar [6,26] bulgularımızı destekleyici niteliktedir. Bunun yanında çalışmamızla benzerlik gösteren Sommer ve ark.[12] tarafından rPCR ve BAXrPCR sistemleri ile ISO standart kültür metodunun kullanımlarının değerlendirildiği bir çalışmada, yumurtacı kümeslerden alınan bot svap örneklerinden aynı oranda, yüksek duyarlılık ve özgünlük ile *Salmonella* tespit edildiği bildirilmiştir. Ayrıca, Eriksson ve Aspan [3] çalışmalarının sonucunda, fekal örneklerden *Salmonella* tespitinde BAXrPCR'ın ISO kültür metodundan sonra ikinci en iyi performansı gösterdiğini rapor etmişlerdir.

Çalışmamızda elde edilen izolatların tiplendirilmesi sonrasında, en yüksek oranda SE (%40.9) ve SI (%31.8) olduğu belirlenmiştir. Ülkemizde ve dünyada SE'in en dominant serovar olduğunu gösteren birçok çalışma bulunmaktadır [4,6,26,29,30]. 2012 yılında Lapuz ve ark.'nın [31] yumurtacı tavuklarda SE'i ve SI'i sırasıyla en yüksek (%7.14) ve ikinci en yüksek (%1.07) serovar olarak belirttikleri çalışma bulgularımıza paralellik gösterirken, *S.* serovar Kentucky'nin %62 oranında dominant serovar olarak rapor edildiği [15] ve *S.* serovar Infantis'in %11.1 ile en dominant serovar olarak bildirildiği [32] farklı çalışmalar da bulunmaktadır. Bulgularımız SE'in uygulanan aşılama programlarına rağmen ülkemizde halen yumurtacı tavuk kümeslerinde var olan en dominant serovar olduğunu göstermesi yönünden önem arz etmektedir.

İzole ettiğimiz salmonellaların tümünün Ampicillin (%100), Neomycin (%100), Penicillin G (%100) ve Erythromycin'e (%95.45) karşı dirençli olduğu bulunmuştur. Salmonellaların antibiyotik dirençliliği ile ilgili kanatlı hayvanlarda yapılmış birçok çalışma bulunmakla birlikte özellikle çalışmamızda kullanılan örnek tipi temel alınarak bir değerlendirme yapıldığında, 2012 yılında Temelli ve ark.[16] tarafından yumurtacı tavuklardan elde edilen *Salmonella* izolatlarının Neomycin, Penicillin G ve Erythromycin'e karşı olan direnç bakımından bulgularımıza paralel ancak Ampicillin dirençliliği açısından bulgularımızdan farklı olarak düşük bulunduğu rapor edilmiştir. Aynı yılda Mahmud ve ark.'nın [17] yumurtacı tavuk kökenli *Salmonella* izolatlarında yaptıkları antibiyotiplendirme sonrasında, sırasıyla Penicillin G'ye ve Ampicillin'e karşı buldukları %100 ve %99'luk direnç oranları da bulgularımıza paralellik göstermektedir.

Çalışmada test edilen 24 antibiyotikten Amikacin haricinde 23'üne karşı izolatların tümünde direnç oluştuğu belirlenmiştir. Benzer şekilde Temelli ve ark.[16] tarafından yapılan bir çalışmada Amikacin'e karşı düşük (%7.6) oranda bir direnç gözlendiğini rapor edilmiştir. Çoklu direnç yönünden 22 izolattan 2'si en az 4 (AMP/E/N/P), 1'i en çok 18 adet antibiyotiğe karşı dirençli bulunmuş olup bu antibiyotiklerin (aminoglikozid grubundan Neomycin, makrolid grubundan Erythromycin, penicillin grubundan Ampicillin ve Penicillin G) yapılan diğer çalışmalarda [16,17] da MDR profillerinde temel olarak yer aldığı tespit edilmiştir. Bu durumun, adı geçen bu antibiyotiklerin insan ve hayvan hekimliğinde bakteriyel infeksiyonlarda primer tedavi amacıyla sıklıkla ve yanlış olarak uygulanması ile ilişkili olduğu düşünülmektedir.

Çalışmanın sonucunda, yumurta tavukçuluğunun yoğun olduğu bölgelerde *Salmonella*'nın hızlı deteksiyonunda LCSGIrPCR ve BAXrPCR sistemlerinin ISO metodunu destekleyici olarak kullanılabileceği, tüm yetiştirme dönemlerinde *Salmonella* varlığının yüksek olduğu belirlenmiştir. Ayrıca *Salmonella* izolatlarının büyük bir kısmının SE olduğu ve tamamına yakınının çoklu antibiyotik dirençliliği gösterdiği ortaya konulmuştur.

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RESEARCH ARTICLE

Effect of Processing on PCR Detection of Animal Species in Meat Products

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Abstract

Although most consumers are sensitive about the origin of the meat they consume, adulteration of meat products is not uncommon. For this reason, the development of reliable methods for animal species identification in meat products is an important research priority for food scientists. Species-specific protein- and DNA- identification methods generally used for this purpose. ELISA and protein electrophoresis are used for protein, PCR is used for species-specific DNA identification. Because DNA is known to be more resistant to processing than protein, PCR methods are generally considered as more sensitive for processed foods. However, processing conditions may also degrade DNA resulting in decreased DNA quality and yield. In this study, the individual and combined effects of heat treatment and low pH on the identification of animal species in meat products by PCR were evaluated. Beef sausage mixtures containing two different amounts of meat from a secondary species (either poultry, pork, or horse) were prepared and were subjected to heat treatment (65°C, 85°C, and 121°C) and pH adjustment (5.2 and 6.2). PCR screening for the four animal species was performed using DNA extracts of these meat samples. The results showed that, the combined effect of high temperature and low pH significantly affects the detection limit of the PCR method. Nevertheless, even low levels of adulteration can still be detected fallowing heat treatment.

Keywords: *Animal species identification, PCR, Heat processing, DNA degradation, Low pH*

Proses Koşullarının Et Ürünlerinde Hayvan Türünün PCR İle Teşhisine Etkisi

Özet

Birçok tüketicinin tükettiği etin orijini konusunda hassasiyetinin bulunmasına karşın, et ürünlerinde yapılan hileler oldukça sıktır. Bu sebeple, çeşitli hayvan türlerinin et ürünlerinde tespit edilmesinde kullanılacak güvenilir metotların geliştirilmesi, gıda bilimcilerinin öncelikli çalışma konularındandır. Bu amaçla, türe spesifik protein yada DNA'nın tanımlanması yöntemleri kullanılır. Protein tanımlanması için ELISA ve protein elektroforezi kullanılırken, türe spesifik DNA tanımlanmasında PCR yönteminden yararlanılır. DNA'nın proses koşullarına daha dayanıklı olduğu bilindiğinden, PCR işlem görmüş ürünlerde daha hassas olarak kabul edilmektedir. Buna karşın, proses koşulları DNA üzerine de yıkımlandırıcı etki göstererek, DNA kalite ve miktarını zayıflatabilir ve böylece PCR metotlarının işlenmiş ürünlerde kullanımını sınırlandırabilir. Bu çalışma ile ısıl işlem ve düşük pH'nın bağımsız ve kombine etkisinin et ürünlerinde hayvan türünün PCR ile teşhisine olan etkisini incelenmiştir. Bu amaçla, iki farklı seviyede ikincil bir türe ait (tavuk, domuz yada at) et katılmış olan deneysel sığır sosis karışımları yaygın olarak kullanılan proses şartlarını temsil etmek üzere ısıl işleme (65°C, 85°C, and 121°C) ve pH ayarlamasına (5.2 and 6.2) tabi tutuldu. Bu karışımların DNA ektraktlarının, PCR ile tür tayini testleri gerçekleştirildi. Elde edilen sonuçlar, düşük pH ve ısıl işlemin kombine etkisinin tespit limiti üzerine oldukça önemli etkisi olduğunu, ancak düşük miktarlardaki karışımın tespit edilmesinin mümkün olduğunu gösterdi.

Anahtar sözcükler: *Hayvan türü tayini, PCR, Isıl işlem, DNA yıkımlanması, Düşük pH*

INTRODUCTION

The composition of food is a major concern of consumers today. In the case of adulterated meat product consumption, several factors including economic, food safety (allergy) and moral reasons (religious belief), trigger

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such apprehensions. Among these concerns, consumers are most sensitive because of religious factors and do not tolerate even trace amounts of adulteration of meat products with forbidden meats like pork. Nevertheless, the results of several studies and market cases have shown that meat products are frequently adulterated with forbidden/cheaper meats that undeclared on the product [1-4], and for this reason, there is an increased demand for effective methods for the identification of foreign animal species in meat products [5].

Several methods of species identification in meat products have been employed in a number of studies [6-9], and in most cases these methods are based on either protein- or DNA-analysis [5,8]. Species identification methods based on protein analysis include immunologic techniques such as ELISA, as well as electrophoretic methods [2,6,7,10].

However, species identification is often required for heat-treated meat products and heat processing can cause denaturation of proteins, thus limiting the sensitivity of protein-based tests [5,8,11,12]. On the other hand, due to its stability and resistance to degradation, most analytical methods today use DNA [5,8,13]. Moreover, conventional- and real time- PCR are frequently used because of the sensitivity and specificity of these methods [11,14-16]. The results of several studies have shown that these methods are also reliable for the analysis of heat-treated meat [12,17]; however, processing conditions have been shown to have an effect on DNA fragment size and thus on the sensitivity of PCR detection [11,18]. In this study, we aimed to evaluate the individual and combined effects of pH and heat treatment on the identification of certain animal species in meat samples using PCR.

MATERIAL and METHODS

Preparation of Experimental Meat Samples

Beef, poultry, domestic pork, and horse meat were used to prepare experimental sausage mixtures for use in this study. Beef sausage mixture was prepared from 73% beef, 15% beef fat, 0.03% $NaNO_3$, 0.5% paprika, 1% black pepper, 1% cumin, 3% salt, 0.5% mixed spices, 5% potato starch, and 1% sugar. To this mixture, selected amounts of poultry, pork, or horse meat were added to give final concentrations of 0.1 and 0.5% of a secondary animal species in the meat mixture. The mixtures were finely blended, after which each mixture was divided into two groups. The pH of one group was adjusted to both 5.2, and that of the second group to 6.2 by the addition of 0.5 N either lactic acid or NaOH. Following pH adjustment, each group was further divided into three subgroups. One was heated to 65°C, another to 85°C (internal temperature, 15 min heating) and the third subgroup was autoclaved at 121°C for 15 min.

DNA Extraction

DNA was extracted and purified in duplicate from all sausage mixtures as well as from raw meat samples of each species used for testing the primer specificities. DNA extraction was carried out using a NucleoSpin™ Tissue DNA isolation kit (MACHEREY-NAGEL GmbH and Co., Germany) according to the manufacturer's instructions. Extracted DNA was quantified by measuring UV absorption at 260 nm using a Spektonic Aquamate spectrometer (ThermoSpectronic, UK).

PCR Primers and PCR Conditions

Species-specific primers for horse, pork, poultry, and beef DNA were used. The sequences and origins of each primer set are summarized in *Table 1*. All PCR reactions were carried out using a Bioneer Thermocyler (Bioneer Corporation, South Korea). PCRs of poultry, horse, and pork primers were performed as multiplex-PCR reactions, while beef PCR reactions were performed separately as simplex reactions. Multiplex PCR amplification reactions were performed in total volumes of 50 µl containing 50 ng genomic DNA, 25 µl of the PCR mastermix 2^X (Fermentas, Turkey) and 3, 4, and 5 pmol of poultry, pork, and horse primer sets, respectively. Simplex PCR amplification reactions were performed in total volumes of 25 µl containing 50 ng genomic DNA, 12.5 µl of the PCR mastermix 2^X (Fermentas, Turkey) and 2.5 pmol of beef primer set.

Table 1. *Summary of the primer pairs used in the study*
Tablo 1. *Çalışmada kullanılan primer çiftlerinin özeti*

Target Gene	Primer	Sequences (5' → 3')	Length of PCR product	Reference
Horse	Horse	CTCAgATTCACTCgACgAgggTAg	439 bp	[19]
	SIM	gACCTCCCAgCTCCATCAAACATCTCATCTTgATgAAA		
Pork	Pork1	CTA CAT AAg AAT ATC CAC CAC A	290 bp	[3]
	Pork2	ACA TTg Tgg gAT CTT CTA ggT		
Poultry	Poultry1	TgA gAA CTA CgA gCA CAA AC	183 bp	[3]
	Poultry2	ggg CTA TTg AgC TCA CTg TT		
Beef	Beef	CTAgAAAAgTgTAAgACCCgTAATATAAg	274 bp	[19]
	SIM	gACCTCCCAgCTCCATCAAACATCTCATCTTgATgAAA		

The amplification protocol used for multiplex PCR reactions was as fallows:10 min at 94°C; amplification for 30 s at 94°C, 60 s at 60°C and 60 s at 72°C for 35 cycles; and final extension for 5 min at 72°C. For simplex reactions the protocol used consisted of 10 min at 94°C; amplification for 30 s at 94°C, 30 s at 60°C and 30 s at 72°C for 35 cycles; and final extension for 5 min at 72°C [3,19].

Agarose Gel Electrophoresis

The PCR products were electrophoresed through a 2% agarose gel, containing 0.05% ethidium bromide. As a size reference, a 100 bp DNA ladder (Generuler, Fermentas, Turkey) was used. Visualization of the agarose gels was performed using a UV transilluminator, and the images were captured using DNR Minibis Pro analysis software (St. Paul, USA).

RESULTS

DNA Yield

The DNA concentration in extracts was calculated by using the 260 nm absorbance values for evaluation of the effects of processing conditions on DNA quantity. The overall average DNA concentrations were 30.8 ng/µl and 27.9 ng/µl for pH 5.2 and pH 6.2 sausages, respectively. The detailed concentrations of the samples are given in *Table 2*. The results did not showed a significant difference in relation with the processing conditions.

Species Detection

DNA extractions were carried out in duplicate for all experimental sausage batches, and PCR reactions were then repeated to obtain four amplification results for each sample. The results of the replicated amplifications are summarized in *Table 3*.

For quality control purposes, in each PCR reaction, a no template control (sterile MILLI Q water) was include (negative control), and a mixture of DNA extracted from raw meat samples of all four species was used as positive controls.

According to these results presence of all three secondary species could be detected in both concentrations (0.1 or 0.5%) after 65°C heating in both pH 5.2 and 6.2 sausage mixtures. The agar gel electrophoresis results of 65°C heated meat mixtures containing 0.1% foreign species meat are given in *Fig. 1*. The results were similar for 85°C heated sausage mixtures. The agar gel electrophoresis results of 85°C heated meat mixtures containing 0.1% foreign species meat are given in *Fig. 2*. However the results of 121°C heated mixtures different form the above results and detection could not be possible in pH 5.2 sausage mixtures while it was possible for pH 6.2 sausage mixtures *(Fig. 3)*.

The band intensities of bands corresponds to samples containing 0.1% secondary meat species were indistinct compare to samples containing 0.5% secondary meat

Table 2. *Absorbance values and concentration of the DNA extracts*
Tablo 2. *DNA ekstraktlarının absorbans değerleri ve konsantrasyonları*

pH	Heat	Absorbance	DNA Concentration
5.2	65°C	0.024	30.2
	85°C	0.029	35.2
	121°C	0.022	27.0
Mean		0.025	30.8
6.2	65°C	0.017	21.0
	85°C	0.022	27.5
	121°C	0.028	35.3
Mean		0.022	27.9

** absorbance values and concentrations are means of all 0.1 and 0.5% samples*

Table 3. *PCR screening results of experimental samples determined with primer pairs for poultry, horse and pork*
Tablo 3. *At, domuz ve kanatlı eti için primer çiftleri ile incelenen deneyesel örneklerin PCR tarama sonuçları*

Species mixture	pH	Heat	Horse*	Pork*	Poultry*
0.1%	5.2	65°C	4/4	4/4	4/4
		85°C	4/4	4/4	4/4
		121°C	0/4	0/4	0/4
	6.2	65°C	4/4	4/4	4/4
		85°C	4/4	4/4	4/4
		121°C	4/4	4/4	4/4
0.5%	5.2	65°C	4/4	4/4	4/4
		85°C	4/4	4/4	4/4
		121°C	0/4	0/4	0/4
	6.2	65°C	4/4	4/4	4/4
		85°C	4/4	4/4	4/4
		121°C	4/4	4/4	4/4

** number of the positive results in all 4 replicated amplifications*

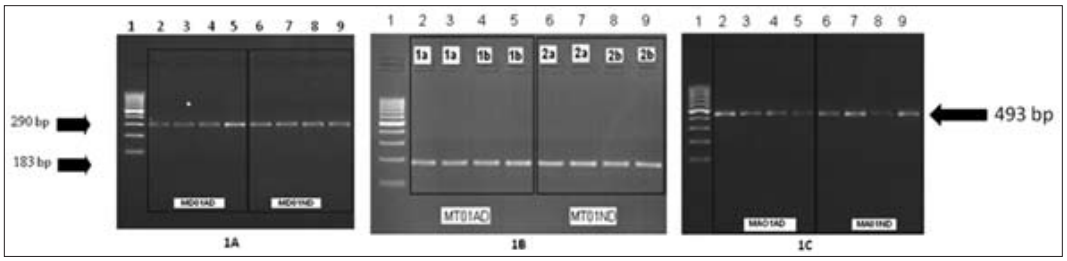

Fig 1. Multiplex PCR results of 65°C heated 0.1% samples
A- Pork (Lane-1:100 bp DNA ladder, Lane 2-5:pH 5.2, Lane 6-9:pH 6.2), **B-** Poultry (Lane-1:100 bp DNA ladder, Lane 2-5:pH 5.2, Lane 6-9:pH 6.2), *C-* Horse (Lane-1:100 bp DNA ladder, Lane 2-5:pH 5.2, Lane 6-9:pH 6.2)

Şekil 1. 65°C'de ısıtılmış %0.1 örneklerin multipleks PCR sonuçları
A- Domuz (Sıra-1:100 bp DNA marker, Sıra 2-5:pH 5.2, Lane 6-9:pH 6.2), **B-** anatlı (Sıra-1:100 bp DNA marker, Sıra 2-5:pH 5.2, Sıra 6-9:pH 6.2), *C-* At (Sıra-1:100 bp DNA marker, Sıra 2-5:pH 5.2, Sıra 6-9:pH 6.2)

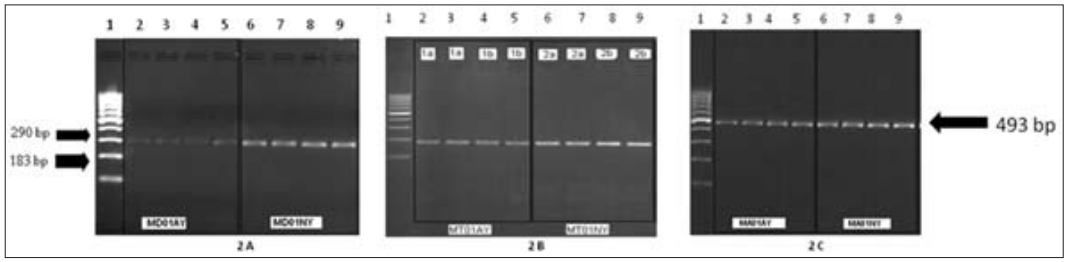

Fig 2. Multiplex PCR results of 85°C heated 0.1% samples
A- Pork (Lane-1:100 bp DNA ladder, Lane 2-5: pH 5.2, Lane 6-9: pH 6.2), **B-** Poultry (Lane-1: 100 bp DNA ladder, Lane 2-5: pH 5.2, Lane 6-9: pH 6.2), **C-** Horse (Lane-1: 100 bp DNA ladder, Lane 2-5:pH 5.2, Lane 6-9: pH 6.2)

Şekil 2. 85°C'de ısıtılmış %0.1 örneklerin multipleks PCR sonuçları
A- Domuz (Sıra-1:100 bp DNA marker, Sıra 2-5: pH 5.2, Sıra 6-9: pH 6.2), **B-** Kanatlı (Sıra-1: 100 bp DNA marker, Sıra 2-5: pH 5.2, Sıra 6-9: pH 6.2), **C-** At (Lane-1: 100 bp DNA marker, Sıra 2-5:pH 5.2, Sıra 6-9: pH 6.2)

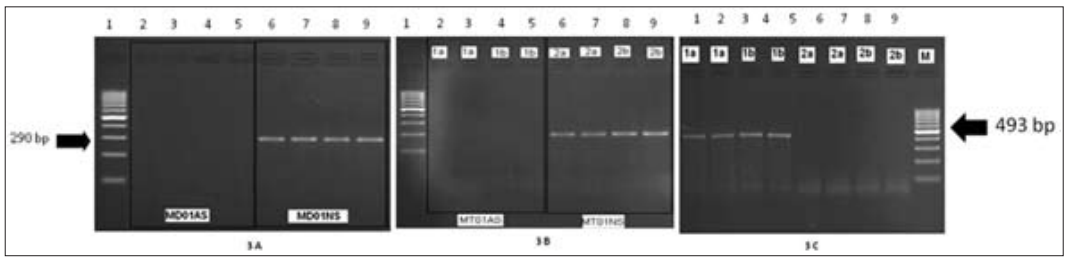

Fig 3. Multiplex PCR results of 121°C heated 0.1% samples
A- Pork (Lane-1: 100 bp DNA ladder, Lane 2-5: pH 5.2, Lane 6-9: pH 6.2), **B-** Poultry (Lane-1:100 bp DNA ladder, Lane 2-5:pH 5.2, Lane 6-9:pH 6.2), **C-** Horse (Lane-1:100 bp DNA ladder, Lane 2-5:pH 6.2, Lane 6-9:pH 5.2)

Şekil 3. 121°C ısıtılmış %0.1 örneklerin multipleks PCR sonuçları
A- Domuz (Sıra 1: 100 bp DNA marker, Sıra 2-5: pH 5.2, Sıra 6-9: pH 6.2), **B-** Kanatlı (Sıra 1:100 bp DNA marker, Sıra 2-5:pH 5.2, Sıra 6-9: pH 6.2), **C-** At (Sıra 1:100 bp DNA marker, Sıra 2-5:pH 6.2, Sıra 6-9:pH 5.2)

species. The results of sausage mixtures containing pork meat are given in *Fig. 4*.

The presence of beef could be possible in all samples regardless of the PH and heat treatment *(Fig. 5)*. However band intensities of pH 5.2 sausages were weaker compare to pH 6.2 samples.

DISCUSSION

The results of several studies have shown that heat processing strongly effects the detectability of species-specific DNA in meat products and thus significantly decreases the sensitivity of PCR [11,12,17,19]. Furthermore, the results of a study by Bauer et al.[20] showed that the combined effect of low pH and heat is stronger than the individual effects of conditions on PCR detection of Genetically Modified DNA in food products. The effects of a similar set of conditions on the detectability of species-specific DNA in meat products was assessed in the present study.

The first and important step of PCR screening is to extract sufficient amount of detectable DNA [21]. Therefore, the amount of DNA extracted from experimental sausages was screened by measuring the absorbance at 260 nm in

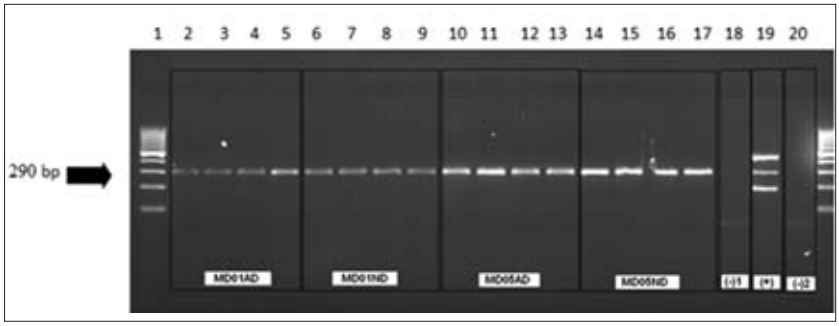

Fig 4. Multiplex PCR results of 65°C heated pork samples (Lane-1:100 bp DNA ladder, Lane 2-5: 0.1% pH 5.2, Lane 6-9:0.1%, pH 6.2, Lane 10-13:0.5%, pH 5.2, Lane 14-17: 0.5% pH 6.2, Lane 18: Ext negative control, Lane 19: meat mixture positive control, Lane-20: PCR negative control)

Şekil 4. 65°C'de ısıtılmış domuz sosis örneklerinin multipleks PCR sonuçları (Sıra-1:100 bp DNA marker, Sıra 2-5: %0.1 pH 5.2, Sıra 6-9:%0.1, pH 6.2, Sıra 10-13: %0.5, pH 5.2, Sıra 14-17: %0.5 pH 6.2, Sıra 18: Ektraksiyon negatif kontrol, Sıra 19: et karışımı pozitif kontrol, Sıra 20: PCR negatif kontrol)

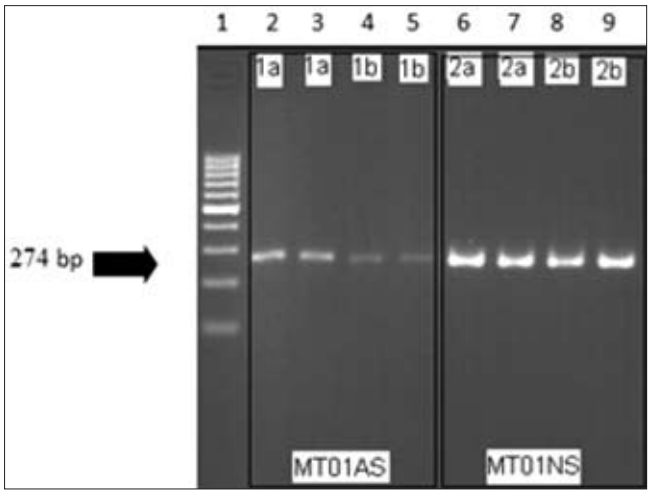

Fig 5. Simplex (beef) PCR results of 121°C heated samples (Lane-1:100 bp DNA ladder, Lane 2-5:pH 5.2, Lane 6-9:pH 6.2)

Şekil 5. 121°C sısıtlmış örneklerin simpleks (sığır) PCR sonuçları (Sıra-1:100 bp DNA marker, Sıra 2-5:pH 5.2, Sıra 6-9:pH 6.2)

order to determine the effect of temperature and pH. The results were not found to differ significantly between low- and high- pH meat samples or between batches subjected to different temperatures.

The results of the PCR testing performed on DNA extracts are given *Table 3* and the PCR amplification products of heat-processed experimental sausage samples visualized on agarose gels are shown in *Fig. 1, 2, 3, 4* and *5*. The results of the multiplex PCR reactions carried out on sausage meat prepared from binary meat mixtures showed that the meat species of all three secondary species can be detected after heating at 65 and 85°C in both 0.1 and 0.5% mixtures regardless of the pH (*Fig. 1* and *2*). These findings are in keeping with those reported by Kesmen et al.[12], which showed that 0.1% of foreign meat in sausage mixture was detectable after heating at 72°C. Despite a combination of low pH and high temperature being applied in our study, the detection limit of PCR method, like that in the study by Kesmen et al.[12], was low (0.1%).

As shown in *Fig. 4*, agarose gel bands corresponding to samples containing smaller amounts of foreign meat (0.1%) were indistinct compared to those corresponding to samples containing larger amounts of foreign meat (0.5%). Band intensities of PCR products on agarose gels are known to be proportional to DNA concentration [22], and thus although these observed differences cannot be used for quantitative purposes, they demonstrates the negative effect of processing on the amplification of DNA by PCR.

The multiplex PCR experiments carried out in this study showed that even 0.1% of foreign species meat is detectable in samples subjected to heating at 121°C (15 min, pH 6.2). These results differ from those reported by Kesmen et al.[12], which showed that the limit of detection of foreign meat mixture was increased to 0.5% when the mixture was heated at 120°C for 30 min; however, this difference may accounted for by the difference in heat exposure time (15 min *vs* 30 min). In another study by Arslan et al.[17] it was shown, like our study, that PCR detection of beef was possible for autoclaved beef meat samples. In another study which horse meat was cooked at 120ºC [19], findings differed from those reported here and by Arslan et al.[17]: horse DNA was not detectable fallowing autoclaving process despite the samples consisting of 100% horse meat compared with the 0.1% horse meat used in our study. Arslan et al.[17], attribute the discrepancy between findings of the two studies to difference in target DNA fragment size. The primers used in our study, however target fragment equal in size to those targeted in the study by Matsuagana et al.[19] and thus the difference is more likely to result from the different extraction methods used in the two studies. Hird et al.[13] have shown that autoclaving and canning processes strongly affects the DNA fragmentation. Fragmented DNA is not extracted with the same efficiency as intact DNA by some DNA extraction methods; and because it is more difficult to precipitate short DNA fragments compared with larger ones, this is particularly true for methods including a DNA precipitation step. The extraction method used in our study is a spin column-based method, while that used in the study by Matsuagana et al.[19] is precipitation-based method.

PCR experiments in this study showed differences for meat samples adjusted to different pHs, specifically between low pH (pH 5.2), 121°C heat-treated samples and pH 6.2, 121°C heat-treated samples. Foreign species present in meat samples at either 0.1 or 0.5% were not detectable in low pH (5.2) samples subjected to heating

at 121ºC, while they were detectable in pH 6.2 samples *(Fig. 3)*. Kesmen et al.[22] previously showed that 0.1% of foreign species in a meat mixture is detectable in fermented sausages. The final pH of the fermented meat was not reported; however, the pH of the meat is likely to have been low due to 15 days ripening process applied. Considering the results of Kesmen et al.[22] (low pH-no heat treatment) and our results for pH 6.2 autoclaved meat samples showed that the effect of combined processing conditions (low pH and high temperature) is stronger than the conditions applied individually. Similarly, Pascoal et al.[1] reported that the combined effect of heat and high pressure might result in stronger DNA degradation.

In the case of simplex PCR experiments for the detection of beef in meat samples, detection was possible in all samples irrespective of the processing conditions applied *(Fig. 5)*. This finding can most likely be attributed to the high concentration of beef present in the final sample product. As shown in *Fig. 5*, the agarose gel bands corresponding to pH 5.2 samples that were autoclaved were significantly weaker than those corresponding to pH 6.2 samples subjected to autoclaving, demonstrating that the degrading effect of 121°C heat treatment on DNA was more pronounced in samples subjected to low pH.

Certain cases, the low limit of detection of PCR methods can be considered a disadvantage in the identification of foreign animal species in meat products: accidental contamination across processes can result false- positive identification of meat adulteration. However, the low limit of detection associated with PCR is required in instances in which even trace amounts of foreign animal species are unacceptable, such as in cases where certain species are not tolerated for religious reasons.

In conclusion, our results demonstrated that PCR identification of foreign animal species represented at low levels in meat products is possible even after severe heat processing. Food additives commonly used in meat production did not interfere with the results of this study. It was also shown that the combined effect of pH and high temperature on DNA integrity was stronger than the effect of either condition individually, and the limit of detection of the PCR method used was significantly increased by these conditions. During industrial meat processing, various conditions are applied in combination. Considering the likelihood that the combination of such processes negatively affects the detectability of foreign animal species in meat products as demonstrated in this study, we recommend that method validation studies for PCR detection of foreign animal species should be performed for different types of meat products subjected to different processing conditions. We also recommend that further studies on animal species quantification methods be conducted, especially in the case of meat from animal species, which are of economical concern such as poultry.

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RESEARCH ARTICLE

The Effects on Follicular Dynamics Caused by Changing the Application Time of PGF2α and GnRh in the Cosynch Protocol Administered in Montofon Cows with Estrus Stimulated by Presynchronization [1] [2]

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Abstract

The objective of this study was to investigate how follicular dynamics are affected by changing the initial injection times of PGF2α and GnRH in the Cosynch protocol in Montofon cattle that have been administered presynchronization. Initially, all cattle whose corpus luteum was identified were administered an injection of PGF2α. Six days after estrus was identified, the Cosynch protocol was started. In keeping with this, Group I (GI; n=9) was administered GnRH on day 0 and PGF2α 7 days later. Fifty-six h later, artificial insemination was performed and GnRH was injected. In the second group of cows (GII; n=10), PGF2α was administered in the Cosynch protocol 8 days after the first GnRH injection. Fifty-six h later, artificial insemination was performed and GnRH was injected. Development of the follicle and the corpus luteum was monitored each day with ultrasonography. On days 6, 7 and 8 of the procedure, a statistically significant difference in the follicle diameters was identified between the groups ($P<0.05$). No significant difference between the groups was identified in the dominant follicle diameter during artificial insemination ($P>0.05$). In conclusion, we determined that for cattle administered the Cosynch protocol on the 6th day of the sexual cycle, extending the application time of PGF2α by one day in did not affect the size of the Graafian follicle.

Keywords: *Cow, Corpus luteum, Cosynch, Follicular dynamics*

Presenkronizasyon İle Östrusu Uyarılan Montofon Irkı İneklerde Seksüel Siklusun Erken Luteal Döneminde Uygulanan Cosynch Protokolünde PGF2α ve GnRH Uygulama Zamanının Değiştirilmesinin Folliküler Dinamik Üzerine Etkisi

Özet

Bu çalışma ile presenkronizasyon oluşturulan Montofon ırkı ineklerde Cosynch protokolündeki ilk GnRH ile PGF2α uygulanma arası zamanının değiştirilmesinin folliküler dinamikleri üzerine etkisini araştırmak amaçlanmıştır. İlk olarak corpus luteum belirlenen tüm ineklere tek doz PGF2α enjekte edildi. Östrus tespitinden sonraki 6. gününde Cosynch protokolüne başlandı. Bu doğrultuda Grup 1'de (GI; n=9) 0. gün GnRH, 7 gün sonra PGF2α enjekte edildi. Bu uygulamadan 56 saat sonra suni tohumlama yapıldı ve GnRH enjekte edildi. İkinci gruptaki ineklerde (GII; n=10) ise Cosynch protokolündeki PGF2α, ilk GnRH enjeksiyonundan 8 gün sonra uygulandı. Bu uygulamadan 56 saat sonra suni tohumlama yapıldı ve GnRH enjekte edildi. Follikül ve corpus luteum gelişimi ultrasonografi ile günlük takip edildi. Uygulamanın 6, 7 ve 8. günlerinde gruplar arasında follikül çaplarında istatistiksel olarak önemli bir fark belirlendi ($P<0.05$). Suni tohumlama sırasında dominant follikül çapı büyüklüğünde gruplar arasında fark belirlenmedi ($P>0.05$). Sonuç olarak, seksüel siklusun 6. gününde Cosynch protokolü uygulanan ineklerde PGF2α uygulama zamanının bir gün uzatılmasının graaf follikülünün büyüklüğünü etkilemediği belirlendi.

Anahtar sözcükler: *İnek, Corpus luteum, Cosynch, Folliküler dinamik*

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INTRODUCTION

For cattle to be able to calve every year, it is very important that signs of estrus be regularly monitored and to identify the best time for artificial insemination (AI) accordingly. Reproductive performance is limited on most farms because they fail to identify the signs of estrus more than 50% of the time. The rate of identifying estrus is low because the external signs of estrus are short and infrequent, as well as the fact that protocols for identifying estrus are limited [1]. Pursley et al.[2] developed the Ovsynch protocol to eliminate these problems in cattle. The Ovsynch protocol ensures follicular development and maturation of follicles during luteolysis prior to ovulation [2,3]. With the first GnRH injection, the existing dominant follicles start ovulation and approximately 2 days later a new wave of follicles begins [4]. At this time, one follicle gains dominance and the other large follicles (subordinate) undergo atresia. This separation occurs 6 days after the first GnRH injection [5,6]. In the Cosynch program, which is a modified form of the Ovsych program, AI is performed at the same time as the second GnRH injection. A number of studies performed by researchers on cattle have reported pregnancy rates of 22.6%-58.5% with the Ovsynch and Cosynch synchronization programs [7-11]. It has been observed that changing the time of the second GnRH injection in the Ovsynch and Cosynch synchronization protocols has an effect on the pregnancy rates for lactating milk cows. In addition, it has been determined that pregnancy rates are higher in cows administered Ovsynch 56 (second GnRH injection administered 56 h after the $PGF_{2\alpha}$ injection). Furthermore, it has been reported that cows administered Cosynch 48 (AI performed with the second GnRH injection 48 h after the $PGF_{2\alpha}$ injection) had higher pregnancy rates than those administered Cosynch 72 (AI performed with the second GnRH injection 72 h after the $PGF_{2\alpha}$ injection) [7]. Pursley et al.[2] achieved the highest pregnancy rate in the Ovsynch protocol with insemination performed 16 h after the second GnRH injection. Similarly, an increase in pregnancy rates was achieved by administering the Ovsynch protocol after 12-14 days in cows receiving $PGF_{2\alpha}$ treatment at an interval of 14 days [7].

The objective of this study was to examine Montofon cows whose estrus was stimulated with presynchronization and investigate the effect on follicular dynamics starting the Cosynch synchronization program in the early luteal phase (the 6th day) of the sexual cycle and administering the $PGF_{2\alpha}$ injection in the Cosynch protocol 8 days after the first GnRH injection.

MATERIAL and METHODS

Animals

This study was approved by the Kafkas University Local Ethics Committee for Animal Experiments, Kars, Turkey (KAÜ-HADYEK; 2010/04-09).

This study was performed on 19 clinically and gynecologically healthy Montofon cows who were at least 45 days postpartum and were fed on dry grass and concentrate feed (minimum of 88% dry content, minimum of 16% crude protein, maximum of 14% crude cellulose, maximum of 9% crude ash, maximum of 1% HCl acid-insoluble ash, approximately 1.6% calcium, 0.4% phosphor, 0.4% sodium, 1% NaCl and 2.500 kCal/kg of metabolic energy). Rectal and ultrasonography examinations (Titan®, Sonosite, USA, 5 MHz) were used on all cows to verify that uterine involution was complete in postpartum period. After the corpus luteum was identified in the cow during the ultrasonographic examination, $PGF_{2\alpha}$ (2 ml, IM, 0.075 mg, D-Cloprostenol, Dalmazin®, Vetaş, Turkey) was administered and an electronic heat detection device (DEC®, IVM, France) able to identify the start time of estrus with an accuracy of 2 h was attached to the sacrum in order to identify signs of estrus. The day that estrus began was recorded as day 0 of the sexual cycle and the Cosynch protocol was started on day 6 (in the early luteal phase of the estrus cycle). After starting the Cosynch protocol, ultrasonographic measurements were made every day on both groups of cows to determine the sizes of the follicles and corpus luteum on the ovary. Measurements were made after identifying the follicle or CL that had the largest diameter on the screen. Daily ultrasonographic examinations were continued from the beginning of the Cosynch protocol until fertilization occurred. Furthermore, 6 days after AI, ultrasonography was used to investigate the presence of the corpus luteum to determine whether or not ovulation had occurred. In addition, body condition scoring (BCS) was measured for the groups from the beginning of the study using a 5-point system with quarter-point increments [12].

Study Procedure

Group I (GI): This group of cows (n=9; average age of 5.0±0.5 years; BCS: 3.2±0.3; average milk production: 8.1±0.9 L) was administered a single dose of $PGF_{2\alpha}$ for the purpose of presynchronization. The Cosynch protocol was started on the sixth day of the cycle after signs of estrus were identified in a given cow. According to this protocol, GnRH (2 mL, IM, 25 µg, Lecirelin acetate, Dalmarelin®, Vetaş, Turkey) was administered on day 0. Seven days later, $PGF_{2\alpha}$ (2 mL, IM, 0.075 mg, D-Cloprostenol, Dalmazin®, Vetaş, Turkey) was administered, followed by AI and a GnRH injection 56 h after that *(Fig. 1)*.

Group II (GII): This group of cows (n=10; average age of 5.0±0.5 years; BCS: 3.1±0.3; average milk production: 8.8±0.9 L was administered a single dose of $PGF_{2\alpha}$ for the purpose of presynchronization. As in GI, the Cosynch protocol was started on the sixth day of the cycle after estrus symptoms were identified in a given cow. In contrast

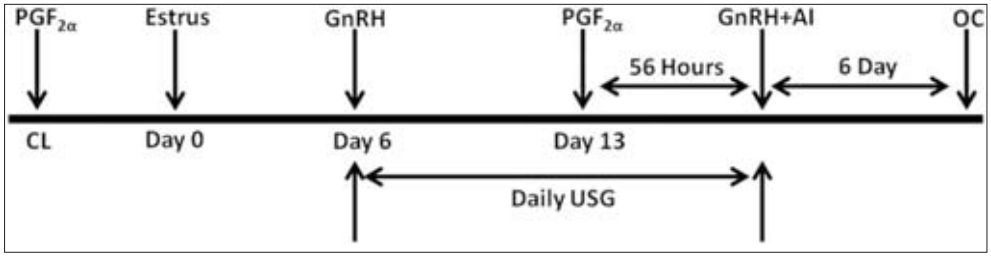

Fig 1. GI application protocol (**CL:** Corpus luteum, **OC:** Ovulation control, **USG:** Ultrasonography)

Şekil 1. GI uygulama protokolü (**CL:** Corpus luteum, *OC:* Ovulasyon kontrolü, **USG:** Ultrasonografi)

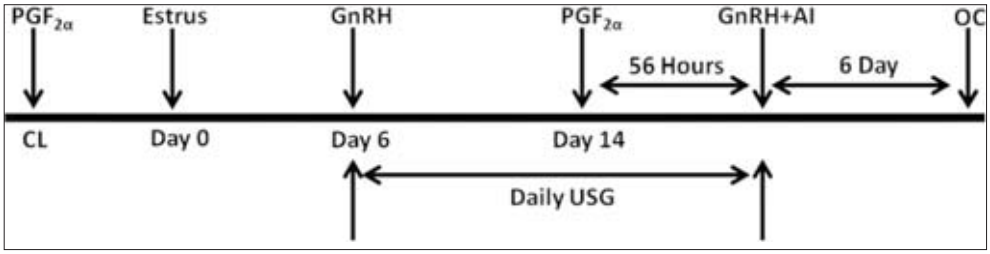

Fig 2. GII application protocol (**CL:** Corpus luteum, **OC:** Ovulation control, **USG:** Ultrasonography)

Şekil 2. GII uygulama protokolü (**CL:** Corpus luteum, **OC:** Ovulasyon kontrolü, **USG:** Ultrasonografi)

to the first group, however, this group was administered $PGF_{2\alpha}$ 8 days after the GnRH injection. Fifty-six hours after prostaglandin $F_{2\alpha}$ was administered, AI was performed and GnRH was administered. In this group, it was not possible to perform AI on one animal because it developed severe diarrhea *(Fig. 2)*.

Statistical Analysis

Statistical analysis of the findings obtained in the study was performed using the SPSS 16.0 statistical program. One-way ANOVA was used in the statistical analysis of follicle sizes, corpus luteum diameters, BCS, age and milk production for the groups. Chi-square was used for statistical analysis of the ovulation rate and accessory corpus luteum development rate. P<0.05 was considered to be statistically significant.

RESULTS

Dominant follicle sizes in the cows during the first application of GnRH in the Cosynch protocol were found to be 12.3±3.3 mm in GI and 12.4±2.2 mm in GII. At that same time, the size of the corpus luteum (CL) was observed to be 20.4±3.2 mm in GI and 20.5±3.8 mm in GII. No statistically significant difference was found between the groups (P>0.05) with regard to the sizes of the dominant follicle and CL during the first administration of GnRH in the Cosynch protocol. For both GI and GII, development of an accessory CL was not identified in 2 cows. No statistically significant difference was found between the groups (P>0.05) with regard to the sizes of the dominant follicle and CL during administration of $PGF_{2\alpha}$ in the Cosynch protocol (day 7 for GI and day 8 for GII). In addition, no statistically significant difference was found between the groups with regard to the follicle size in measurements performed 24 h after $PGF_{2\alpha}$ was administered in GI and GII (14.2±1.0 and 13.0±2.0; P>0.05; *Table 1*).

It was determined that there was no statistically significant difference in GI's follicle size as compared with GII as of day 4 of the study (P=0.06). However, follicle diameters were found to be statistically different between the groups on days 6, 7 and 8 (*Fig. 3*; P<0.01).

Fig. 4 shows the follicle sizes measured in the daily ultrasonographic examinations of the ovary after the Cosynch protocol was started (day 0) in GI and GII, while *Fig. 5* shows the CL diameters. In our study, we determined that the subordinate follicles began to undergo atresia after

Table 1. *Follicle, CL diameters and ovulation in the groups on different days*
Tablo 1. *Grupların farklı günlerdeki follükül ve CL çapları ve ovulasyon oranları*

Parameters	Group I Mean ± SD	Group II Mean ± SD	P
Size of dominant follicle during the first administration of GnRH (mm)	12.3±3.3	12.4±2.2	P>0.05
Size of CL during the first administration of GnRH (mm)	20.4±3.2	20.5±3.8	P>0.05
Rate of developing an accessory CL (%)	77.7 (7/9)	88.8 (8/10)	P>0.05
Size of dominant follicle during administration of $PGF_{2\alpha}$ in the Cosynch protocol (mm)	13.3±1.3	12.0±2.2	P>0.05
Size of CL during the administration of $PGF_{2\alpha}$ in the Cosynch protocol (mm)	23.2±5.1	22.4±3.7	P>0.05
Size of the Graafian follicle during artificial insemination (mm)	14.4±1.2	13.5±1.9	P>0.05
Ovulation rate 6 days after artificial insemination (%)	75 (7/9)	100 (9/9)	P>0.05

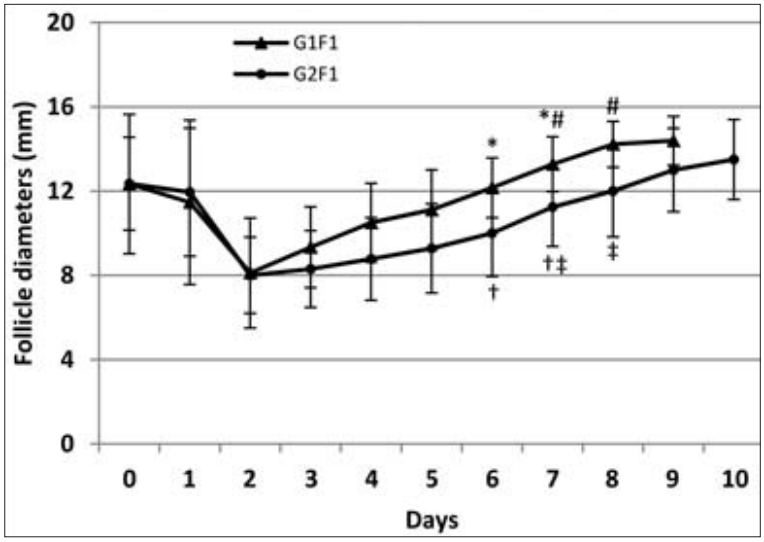

Fig 3. Dominant follicle diameters measured in GI and GII during the period lasting from the beginning of the Cosynch protocol until the time artificial insemination was performed (**F1:** Dominant follicle); * : #, † : ‡ = P<0.05; * : †, *# : †‡, # : ‡ = P<0.01

Şekil 3. GI ve GII'de Cosynch protokolü başlangıcından suni tohumlama yapılıncaya kadar geçen sürede ölçülen dominant follikül çapları (**F1:** Dominant follikül) * : #, † : ‡ = P<0.05; * : †, *# : †‡, # : ‡ = P<0.01

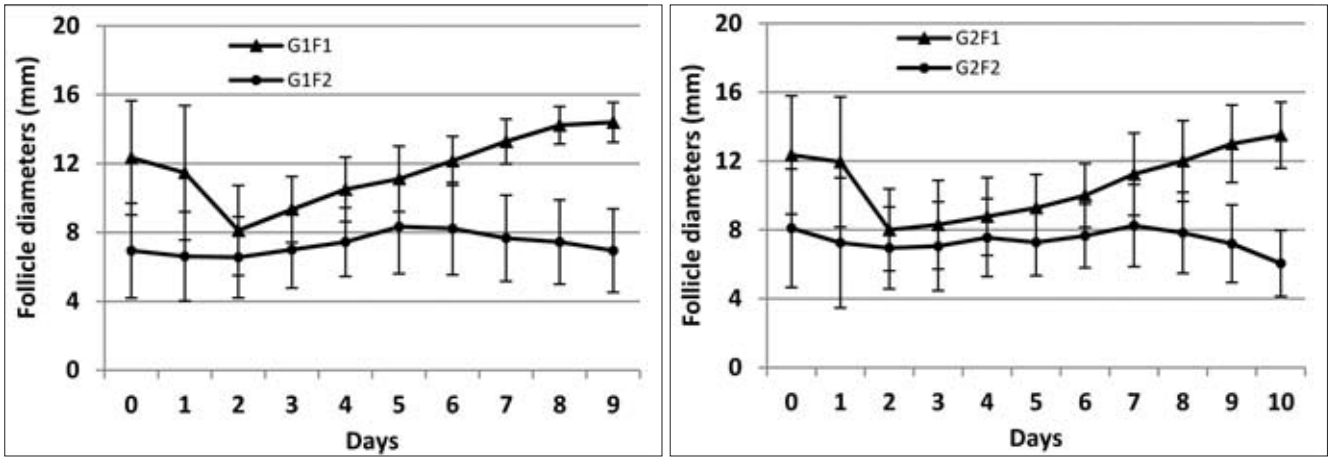

Fig 4. Follicle development in GI and GII during the period lasting from the beginning of the Cosynch protocol until the time artificial insemination was performed (**F1:** Dominant follicle, **F2:** Subordinate follicle)

Şekil 4. GI ve GII'de Cosynch protokolü başlangıcından suni tohumlama yapılıncaya kadar geçen sürede folliküllerin gelişimi (**F1:** Dominant follikül, **F2:** Subordinate follikül)

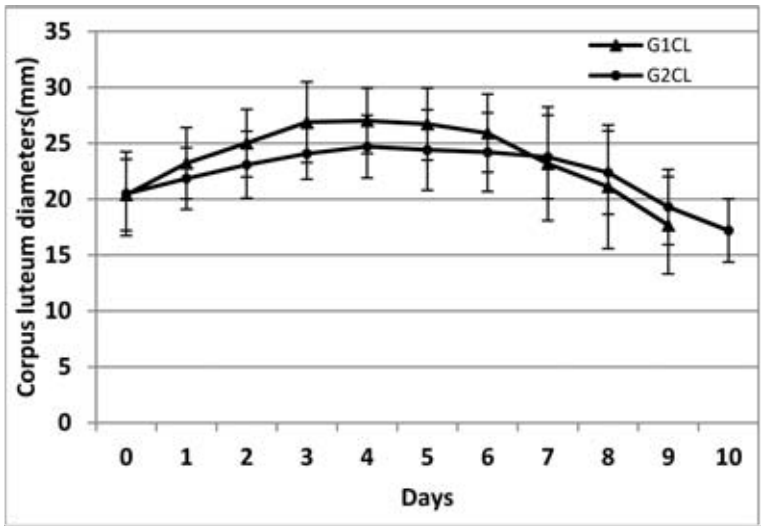

Fig 5. Corpus luteum development in GI and GII during the period lasting from the beginning of the Cosynch protocol until the time artificial insemination was performed (**CL:** Corpus luteum)

Şekil 5. GI ve GII'de Cosynch protokolü başlangıcından suni tohumlama yapılıncaya kadar geçen sürede corpus luteum gelişimi (**CL:** Corpus luteum)

day 6 of the protocol in GI (8.2±2.7 mm) and after day 7 in GII (8.3±2.4 mm) *(Fig. 4)*. Measurements taken on these days did not reveal any statistically significant difference between the subordinate follicle diameters (P>0.05).

DISCUSSION

The response to administering GnRH in cows varies depending on the development phase of the follicle. Ovulation occurs 100% of the time from a follicle that is developing with GnRH, 33% of the time from a follicle in the static phase and 1% of the time from a follicle that is in the regression phase [2,13]. It has been reported that the pregnancy rate is higher when

the Ovsynch protocol is administered between days 5-12 of the sexual cycle in dairy cows. Starting the Ovsynch protocol in the late luteal stage may cause estrus before the second GnRH injection as well as premature CL regression. As a result, an abnormal CL may develop from the ovulating follicle, producing a small amount of progesterone, which can cause a reduction in pregnancy rates [3]. In our study, we started the Cosynch protocol on day 6 of the cycle in order to achieve the best follicle for ovulation.

Follicular waves are optimally synchronized for some cows in the Ovsynch protocol, but they are not sufficiently synchronized for other cows. Insufficient development of follicular waves depends on the initial response to GnRH. Differences in the dominant follicle's development stage when the second GnRH is administered causes negative results [14]. Presynchronization with $PGF_{2\alpha}$ prior to starting the Cosynch protocol has been observed to increase the pregnancy rate in multiparous cows [8]. Studies on cows that have been administered presynchronization have reported follicle diameters of 15.5 mm [15] and 14.4 mm [16] during the first GnRH injection administered in the Ovsynch protocol. Furthermore, the ovulation rate was found to be 69.5% after this procedure [15]. In our study, we found the dominant follicle to be 12.3 mm in GI during the first GnRH injection administered in the Cosynch protocol and 12.4 mm for GII in which $PGF_{2\alpha}$ was administered at a different time. Furthermore, the ovulation rate after administering GnRH was found to be 77.7% and 88.8%, respectively ($P>0.05$). In our study, measurements taken on days 6, 7 and 8 revealed that dominant follicle diameters were larger in Group I than Group II and we found that there was a statistically significant difference between these follicles. In one study performed on heifers, the $PGF_{2\alpha}$ injection time was changed (7 or 8 days after the first GnRH injection) and progesterone was administered during this time period. In that study, follicle diameter was found to be 13.5 mm before ovulation and the ovulation rate was reported to be 100% in the heifers in all groups [17]. In addition, preovulatory follicle diameters of 14.0 mm [15] and 15.7 mm [16] have been reported during the second GnRH injection administered in the Ovsynch protocol with presynchronization. Furthermore, the ovulation rate was found to be 96.2% after the second GnRH injection [15]. In our study, we also found the preovulatory follicle diameter to be 14.4 mm and 13.5 mm, respectively, during the second GnRH injection in both groups. We found the ovulation rate to be 75% in Group I and 100% in Group II. Our findings are similar to the results of the aforementioned study. Assey et al.[18] found the average corpus luteum diameters to be 20.8±0.1 mm at day 7 after $PGF_{2\alpha}$ injection in cows. Similarly in mentioned study, we found the corpus luteum diameters to be 23.2±5.1 mm in Group I and 22.4±3.7 mm in Group II. Rastegarnia et al.[19] found the average corpus luteum diameters to be 12.5±0.29 mm in river buffalo while administering $PGF_{2\alpha}$ in the protocol. Based on this data, we found the corpus luteum diameters to be larger when $PGF_{2\alpha}$ was administered. Similar to our findings, Bulbul et al.[20] found no effect on the diameter of CL following initiation of the Ovsynch protocol between on day 0 and 8 of sexual cycle in Montofon cows.

In a study performed on cows that were administered the Cosynch protocol, follicle size at ovulation when administering GnRH 56 h after the $PGF_{2\alpha}$ injection was found to be smaller than Cosynch 72 and larger than Cosynch 48 [7]. Ovulation of the maturing dominant follicle is stimulated with the first GnRH injection and a new follicular wave is produced within approximately 2 days [4]. In both groups in our study, the existing dominant follicles ovulated with the first GnRH injection, followed by a new follicular wave 2 days later. Studies on follicle dynamics have observed a significant difference in growth of the dominant follicle and the subordinate follicle by 6 days after the GnRH injection. It has been reported that in the following days, the subordinate follicle will undergo atresia while the dominant follicle will develop even further for ovulation. It has been observed that the subordinate follicle grows approximately 8 mm and then becomes smaller, in contrast to the dominant follicle [5,6]. Similarly, our study also found that the subordinate follicles in both groups grew approximately 8 mm and then became smaller. Furthermore, these follicles began to undergo atresia on day 6 in Group I and on day 7 in Group II.

In conclusion, our study determined that changing the injection time of $PGF_{2\alpha}$ after the first GnRH injection in the Cosynch protocol does not affect the follicle size at ovulation.

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SHORT COMMUNICATION

Examination of *Escherichia coli* O157:H7 and some Virulence Genes in Marketed Minced Meat Samples

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Abstract

In this study, the presence of *Escherichia coli (E. coli)* O157:H7 was investigated in minced meat samples. *E. coli* O157:H7 was detected in six (7.5%) of the minced meat samples. Examination of the positive isolates for the presence of virulence genes; *stx1* was determined in one isolate, *stx2* in three isolates and both *stx1* and *stx2* were found in one isolate. Also eae genes were observed in all the positive isolates.

Keywords: *E. coli O157:H7, Minced meat, PCR*

Tüketime Sunulan Kıymalarda *Escherichia coli* O157:H7'nin ve Bazı Virülens Genlerinin Araştırılması

Özet

Bu çalışmada, tüketime sunulan kıyma örneklerinin *Escherichia coli (E. coli)* O157:H7 yönünden incelenmesi amaçlandı. Kıyma örneklerinin 6'sında (%7.5) *E. coli* O157:H7 tespit edildi. Bu kıyma izolatları, virülens genleri bakımından incelendiğinde bir tanesinde *stx1*, üç tanesinde *stx2* ve bir tanesinde hem *stx1* hem de *stx2* geni tespit edildi. Ayrıca izolatların tümünde eae genlerinin varlığı gözlendi.

Anahtar sözcükler: *E. coli O157:H7, Kıyma, PCR*

INTRODUCTION

Shigatoxin producing *Escherichia coli* (STEC) strains are included in the significant foodborne pathogens and O157 is known as the most shigatoxin (stx) releasing serotype [1]. This serotype cause severe diseases in humans such as hemorrhagic colitis (HC), thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS) characterized with hemolytic anemia, thrombo-cytopenia and renal failure [2].

E. coli O157 was firstly reported in the United States in 1982 and gave rise to serious outbreaks in many countries in decades [3]. It has been reported that most of the O157 infections in the US were raised from foods and most of these infections were resulted from consumption of contaminated foods including ground beef [4].

Cattle are considered as the main reservoir of the agent and agents in digestive tract cause contamination of meat and meat products during slaughtering. On the other hand, contamination come up through the environmental route or while dressing [5]. Besides this, the pathogen can enter the production chain in storehouses, butchers, markets, restaurants etc. and pose risk for public health [6].

E. coli O157 has some virulence factors such as flagellar antigen H7, (*fliCh7*) shigatoxins (*stx*), and intimin (*eae*). *Stx* has a similar structure with *Shigella dysenteriae* type 1 toxin and inhibits protein synthesis and cause cell death due to affecting ribosomal RNA [5]. Additionally, the agents adhere to intestinal epithelial cells with *eae* adhesion and cause various diseases in humans [2].

Detection of STEC O157 strains in meat and stool samples by conventional methods is time consuming, owing to the existence of other bacterial species in samples. On the other hand, investigating the *E. coli* O157 and its virulence genes can be carried out in a short time by Polymerase Chain Reaction (PCR) tests.

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The aim of this study was to isolate *E. coli* O157 from minced meat samples obtained from restaurants, grilled meatball restaurants and butchers and to investigate the presence of shigatoxin (stx_1 and stx_2), intimin (*eae*), O157 (O157 *rfbE*), and H7 antigen (*fliCh7*) genes by PCR.

MATERIAL and METHODS

Sample Collection

A total of 80 minced meat samples were collected from butchers, restaurants and grilled meatball restaurants in Elazig province between December 2009 and November 2010. Approximately 50 g minced meat sample were put into sterile stomacher bags and transferred to the laboratory within 2 h in cold chain conditions. All the samples were analyzed on same day in the laboratories of Department of Microbiology, Faculty of Veterinary Medicine, University of Fırat.

Isolation

Ten grams of minced meat sample was put into sterile bags and treated with 100 ml enrichment broth that composed of modified tryptone soy broth (mTSB) (CM0989, Oxoid)] containing 20 mg/l novobiocin (SR0181, Oxoid). The samples were homogenized in mTSB broth using a stomacher (Bag mixer, Interscience, France) for 2 min. Liquid part of sample was transferred to erlenmayer flask and left for incubation at 41.5°C for 24 h for preenrichment. After incubation, pre-enriched samples were plated onto CT-SMAC (sorbitol MacConkey's agar [SMAC; CM0981, Oxoid] containing 0.05 mg/L Cefixime and 2.5 mg/L tellurite [SR0172; Oxoid]) both directly and with Immuno Magnetic Separation (IMS) and incubated at 37°C for 24 h.

Samples were subjected to IMS, using dynabeads anti-*E. coli* O157 (Dynal Biotech, Oslo, Norway), as described by the manufacturer. The pellet was resuspended in 50 µL of distilled water and used for cultivation. Forty microliters of the samples from IMS and a loopful (5 mm diameter) from pre-enrichment broths were plated onto CT-SMAC and incubated at 37°C for 24 h.

Non-sorbitol-fermenting β-glucuronidase negative (pale) colonies were selected for the detection of O157 (*rfbE*) and virulence genes by PCR.

DNA Extraction and PCR

A few suspicious colonies grown on CT-SMAC medium were transferred into microcentrifuge tube and homogenized with 300 µl sterile distilled water. DNA extraction method and PCR amplification protocol was performed according to our previous study [7], and the presence of O157 *rfbE*, *fliCh7*, stx_1, stx_2, and *eae*, genes were investigated in sorbitol negative isolates by PCR with specific primer pairs *(Table 1)*.

The amplified products were detected by ethidium bromide (0.5 µg/ml) staining after electrophoresis at 80 V for two hours in 1.5% agarose gel. A reference strain of *E. coli* O157:H7 (ATCC 43895) was included as positive control and distilled water was used as a negative control at all steps of the assay *(Fig. 1)*.

RESULTS

Twenty five of minced meat samples cultured on CT-SMAC medium were found to be negative for sorbitol and β-glucuronidase. Of the 25 isolates, 11 were obtained from direct plating and 14 were by IMS method. Isolates determined by direct plating and IMS were different, none of them was detected with both (direct plating or IMS) methods. In the O157-specific PCR analysis *E. coli* O157 was identified in six of IMS isolates but none of the direct plating isolates were found to be positive for *E. coli* O157 *(Fig. 1)*.

All O157 strains were examined for the existence of stx_1, stx_2, *fliCh7* and *eae* virulence genes by PCR assays. The stx_1 gene was detected in only one and stx_2 was in three of isolates. One of the isolates possessed both stx_1 and

Table 1. *Primer sequences and lengths of PCR amplification products*
Tablo 1. *PCR amplifikasyon ürünlerinin primer sekansları ve uzunlukları*

Gene	Primer	Oligonucleotide Sequence (5'-3')	Fragment size (bp)	Reference
stx_1	VT1-A VT1-B	CGCTGAATGTCATTCGCTCTGC CGTGGTATAGCTACTGTCACC	302	[2]
stx_2	VT2-A VT2-B	CTTCGGTATCCTATTCCCGG CTGCTGTGACAGTGACAAAACGC	516	[2]
O157 *rfbE*	O157-AF O157-AR	AAGATTGCGCTGAAGCCTTTG CATTGGCATCGTGTGGACAG	497	[8]
fliCh7	H7-F H7-R	GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC	625	[9]
eae	eaeAF eaeAR	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	384	[10]

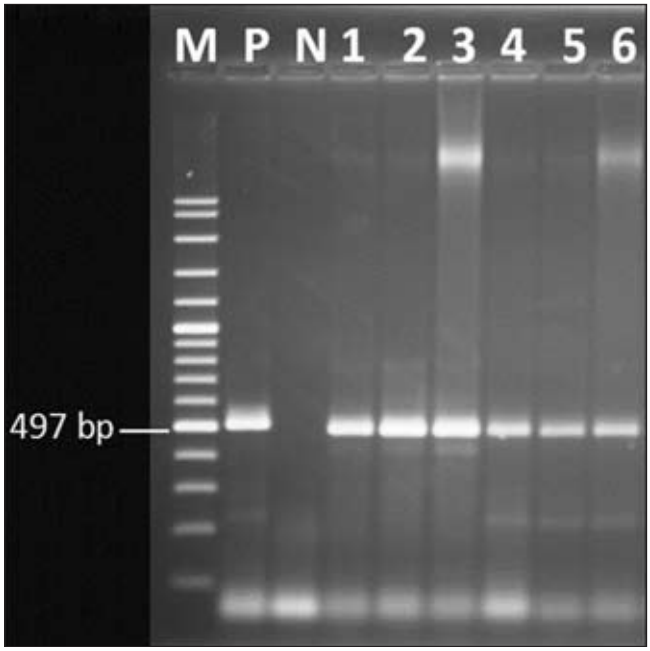

Fig 1. *Escherichia coli* O157 specific polymerase chain reaction products of isolates obtained from minced meat samples. **M:** Marker, **P:** Positive control, **N:** Negative control, 1**, 2, 3, 4, 5, 6:** Positive isolates

Şekil 1. Kıyma örneklerinden elde edilen izolatların *Escherichia coli* O157 spesifik polimeraz zincir reaksiyonu ürünleri. **M:** Marker, **P:** Pozitif kontrol, **N:** Negatif kontrol, **1, 2, 3, 4, 5, 6:** Pozitif izolatlar

products, in Turkey [7,12]. In other studies performed in different countries, the prevalence of *E. coli* O157 was reported to vary from 0.2% to 15% [1,5]. High isolation rate such as 73% has been reported in South Africa [13].

In the present study, *E. coli* O157:H7 was detected in 7.5% (6/80) of minced meat samples by both conventional and molecular methods. This proportion is in parallel with previous studies except the researchers conducted in South Africa. The differences between the results may be due to cultivation method, sample size and resource, geographical region, season and degree of dispersion of *E. coli* O157: H7 infections in the region [14].

Immunomagnetic separation (IMS) is an easy, rapid and reliable assay, which has been widely used for epidemiological studies of *E. coli* O157:H7 and has provided to accomplish efficient recovering microorganisms from heterogeneous samples [15]. In the current study higher *E. coli* O157:H7 isolation rate was obtained by IMS, although all of the direct plated samples were found to be negative. This conclusion suggests that combination of conventional culture method and PCR assay are more advantageous than single cultivation methods for identification of *E. coli* O157:H7 serotype.

It has been reported that the virulence factors such as

Table 2. *Distribution of virulence genes of Escherichia coli O157 isolated from minced meat samples*
Tablo 2. *Kıyma örneklerinden elde edilen Escherichia coli O157 izolatlarının virülens genlerinin dağılımı*

Numbers of Positive Isolates	Number of Samples (n)	Number of Samples Positive by PCR	O157 *rfbE*	*fliCh7*	*eae*	stx_1	stx_2	stx_1 + stx_2
1	80	6 (%7.5)	+	+	+	-	-	-
2			+	+	+	-	-	-
3			+	+	+	-	-	-
4			+	+	+	-	+	-
5			+	+	+	-	+	-
6			+	+	+	+	+	+
Total	80	6	6	6	6	1	3	1

stx_2. Neither stx_1 nor stx_2 genes were determined in three of isolates. All of the *E. coli* O157 isolates were positive for *fliCh7* (H7) and *eae* genes *(Table 2)*.

DISCUSSION

The most important source of *E. coli* O157 originated infections in humans is foods and animal products have a significant role. Raw or undercooked meats may pose the agent. Although *E. coli* O157 has been found in animals such as cattle, sheep, pigs, and goats, studies indicated that poultry may also carry it and pose health risk to humans [11].

The prevalence of *E. coli* O157 was reported to vary between 0-6% in studies conducted on meat and meat

stx_1, stx_2 and intimin are frequently associated with HC and HUS in humans [10]. In this study, multiplex PCR results showed that 16.6% and 50% of *E. coli* O157:H7 isolates were found to be positive for stx_1 and stx_2 respectively and pose a severe health risk to humans. Studies conducted on cattle and cattle meats were declared high amount of stx_2 gene presence in *E. coli* O157 serotype [2]. Also all the *E. coli* O157:H7 isolates in the present study were found to possess intimin that leads to diarrhea (and encourage HC) in humans by an attaching and effacing (A/E) ability. This result was in parallel with previous studies [2].

In conclusion, this study revealed that some of the marketed minced meats were contaminated with *E. coli* O157:H7. All the isolates were determined to possess the *eae* gene and 50% of them were found to contain at

least one of the stx_1 or stx_2, which are important in the development of HC and HUS conditions. The consumption of these meats may have potential risk for human health. Taking hygienic precautions and complying with Hazard Analysis and Critical Control Point (HACCP) and Good Manufacturing Practice (GMP) requirements in all steps of processing meat and meat products are important to prevent the *E. coli* O157:H7 infections in humans.

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CASE REPORT

Feline Infectious Peritonitis with Distinct Ocular Involvement in A Cat in Turkey

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Abstract

An approximately 2-year old domestic short hair female cat weighting 2.750g was presented to Firat University Veterinary Teaching Hospital with a history of depression, anorexia and weight loss for the last 15 days. Clinical examination revealed incoordination, hyperaesthesia, circling, head tilt, posterior paraparesis, absence of the pupillary reflex in both eyes, opisthotonus and anisocoria. Blood samples were examined for hematological, RT-PCR and biochemical analyses. The cat died without any improvement of clinical findings although symptomatic treatment was made. Necropsy revealed that distinct ocular lesions such as hyphema and thick proteinous exudate accumulation in vitreous in both eyes. Microscopically; there was pyogranulomatous vasculitis in meninges, sclera, corpus ciliare choroid, retina was partially detached in the right eye with retinal epithelial hypertrophy. The kidneys contained focal subcapsular, gray to yellow raised nodules varying from 3 to 10 mm in diameter, and there was discrete fatty degeneration in the liver. The RT-PCR revealed that RNA for FCoV was positive in the blood sample. As a result; the non-effusive form of FIP with distinct ocular involvement was diagnosed through clinical, pathological and polymerase chain reaction (PCR) findings.

Keywords: *Anisocoria, Cat, Eye, Feline infectious peritonitis, Hyphema*

Türkiye'de Bir Kedide Göz Lezyonlarıyla Belirgin Feline Enfeksiyöz Peritonitis Olgusu

Özet

Fırat Üniversitesi Hayvan Hastanesi'ne onbeş gündür süren depresyon, anoreksi ve canlı ağırlık kaybı şikayetiyle yaklaşık 2 yaşında ve 2.750 g ağırlığında, evcil, kısa tüylü dişi bir kedi getirildi. Klinik muayenede inkoordinasyon, hiperestezi, dönme, başı eğme, posterior paraparesis, her iki gözde pupilla refleksinin olmayışı, opistotonus ve anizokori belirlendi. Hematolojik, biyokimyasal ve RT-PCR analizleri için kan örnekleri alındı. Semptomatik tedaviye rağmen klinik bulgularda hiçbir iyileşme olmaksızın kedi öldü. Nekropside, her iki gözde vitreusta yoğun protein tabiatında eksudat birikimi ve hifem'le belirgin oküler lezyonlar ortaya konuldu. Mikroskobik olarak; meninkslerde, sklerada ve korpus silyar koroidde pyogranulomatoz vaskülitis gözlenirken sağ gözde ise retina, kısmen ayrılmış olarak epitelyal hipertrofili şekilde belirlendi. Böbrekler fokal subkapsular 3-10 mm çapına kadar değişen gri-sarı nodülller içeriyordu ve karaciğerde farklı yerlerde yağ dejenerasyonu vardı. Kan örneğinde RT-PCR ile FCoV nükleik asiti pozitif olarak tespit edildi. Sonuç olarak; klinik, patolojik ve RT-PCR bulgularına göre göz lezyonlarıyla belirgin non-efüzif feline infeksiyöz peritonitis tanısı konuldu.

Anahtar sözcükler: *Anizokori, Kedi, Göz, Feline infeksiyöz peritonitis, Hifem*

INTRODUCTION

Feline infectious peritonitis (FIP) is a fatal, immune-mediated disease triggered by infection with a feline coronavirus (FCoV) [1]. FCoV strains are subdivided into two distinct biotypes, feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). Infection with FECV is highly common and specific antibodies are present in up to 90% of cats in catteries and in up to 50% of those in single-cat households. However, only about 5% of FECV-infected cats develop FIP in a cattery environment [2]. Based on widely accepted *in vivo* mutation theory, FIP

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arises by mutated enteric FECV in infected cat [3]. The average age for development of FIP is between 6 months to 2 years old [2]. Two major forms of FIP, an effusive and a non-effusive form, are recognized. In some cases of non-effusive FIP, body effusion can develop at the terminal stage of the diseases and become a mixed-type of FIP upon necropsy [1].

Cats with a strong humoral immunity and a weak or absent cell-mediated immune response against FIPV develop a persistent viremia and effusive FIP. Effusive disease results from widespread deposition of immune complexes in blood vessels and complement activation leading to vessel damage, vasculitis and leakage of serum and protein into body cavities [2,4]. Cats with partial cell-mediated immune responses along with humoral immunity develop the more chronic non-effusive form of FIP, which is characterized by immune mediated (delayed hypersensitivity-like) granulomatous, frequently perivascular, lesions in abdominal viscera, lungs, brain and eyes [2,4,5]. Ocular involvement is common, leading to a variety of changes such as iris color, dyscoria or anisocoria secondary to uveitis, sudden loss of the vision and hyphaema [6,7]. Although FIP cases were described in totally four cats [8-10] and two captive lions [11,12], there is no case report adressing ocular changes of FIP in Turkey. The aim of this report is to present clinical and pathological findings of a non-effusive FIP case with distinct ocular involvement.

CASE HISTORY

An approximately 2-year old weighting 2.750 g domestic short hair female cat was presented to Firat University Veterinary Teaching Hospital The history indicated that the cat had been adopted from street by the owner 6 month ago and she had depression, anorexia and weight loss for the last 15 days. Her vaccinations were current for feline herpesvirus-1 and feline calicivirus and feline parvovirus.

Clinical examination revealed depressed appetite, inco-ordination, hyperesthesia, circling, head tilt, posterior paraparesis, absence of the pupillary reflex, opisthotonus and anisocoria (mydriasis in the right eye and myosis in the left eye). Rectal temperature, heart and respiratory rates were 39.6ºC, 160 beats per minute and 60 breaths per minute, respectively. Abdominal ultrasounography was performed.

Following physical and ultrasonographic examination, blood samples were collected for hematological and biochemical analyses. The serum was separated by centrifugation at 2.000 g at 4ºC for 10 min and stored at -20ºC until use. Red blood cell (RBC) and white blood cell (WBC) count, hemoglobin (Hb) level, and packed cell volume (PCV), measurements were obtained by using manual methods. Serum alanin amino transferase (ALT), aspartate aminotransferase (AST), total protein (TP), total bilirubin (TB), direct bilirubin (DB), blood urea nitrogen (BUN), creatinine (CRSC), albumin (ALB) and globulin (GLB) levels were determined with a biochemistry analizator (Dimension ARX, Dade Behring). Following physical examination, blood samples were obtained for complete blood cell count (CBC), serum biochemistry (Dimension ARX, Dade Behring) and serological analyses.

Abdominal ultrasound revealed no significant finding. CBC was unremarkable including RBC: 7.3 10^6/µL, Hb: 11 g/dL, PCV 35% and WBC: 5.8 10^3/µL. Serum biochemistry analyses showed moderately elevated AST activity (108 U/L), however ALT (23 U/L), TP (7.3 g/L), TB (0.3 µmol/L), DB (0.1 µmol/L) BUN (28 mmol/L), CRSC (0.9 µmol/L) were in normal ranges. ALB was slightly under the lower limit of normal range and GLB was near the upper limit of normal range (ALB 2.5 g/dL, GLB 4.8 g/dL) [13].

Based on history and clinical findings (mild pyrexia, weight loss, dullness, depressed appetite, hyperaesthesia, circling, head tilt, opisthotonus, posterior paraperesis, absence of the pupillary reflex, uveitis, anisocoria and hyphaema), tentative diagnosis of FIP was made. Although symptomatic treatment including oral (prednisolone) and topical steroids (prednisolone acetate 1%) together with fluid therapy was instituted, the patient's condition worsened and died in following day and, subsequently complete necropsy was performed on the cat with histologic evaluation of tissues. Complete necropsy was performed and samples of kidney, eyes, liver, spleen, tongue, brain, intestines and lungs were fixed in 10% formalin and embedded in paraffin wax. Five µm sections were stained with hematoxyline and eosin (H-E) for histological examination.

A reverse transcriptase (RT)-PCR was performed in order to detect RNA of FCoV in the blood and brain tissue samples as described previously by Simons et al.[14].

Hyphyma which is more prominet in left eye, cloudy appearence of anterior chamber and bilateral pupillary dilatation *(Fig. 1A)*. Macroscopically; upon vertical sectioning of the globes, there was severe hyphema and thick proteinous fluid accumulation in vitreous of the both eyes *(Fig. 1B)*. The kidneys contained focal sub-capsular, gray to yellow raised nodules varying from 3 to 10 mm in diameter. There was discrete fatty change in the liver. The alimentary track had no abnormalities, nor spleen, heart and brain. The results of RT-PCR revealed that FCoV was positive in the blood sample *(Fig. 1C)*. For RT-PCR reactions, the primers were chosen from the highly conserved M gene sequence of the FCoV genome. As a control to check the efficiency of the RNA isolation from the blood samples and the subsequent reverse transcriptase reaction, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH gene). After PCR, The DNA bands

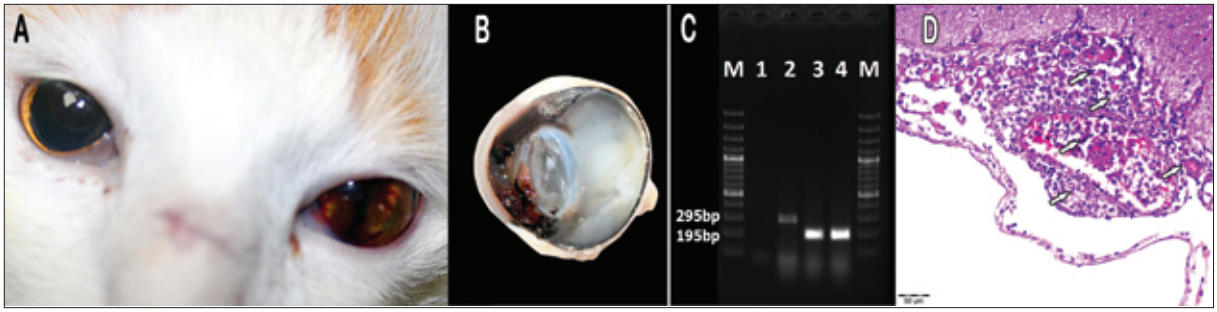

Fig 1. A- Hyphyma which is more prominet in left eye, cloudy appearence of anterior chamber and bilateral pupillary dilatation, **B-** Accumulation of proteinous exudate in vitreous and hemorrhage in anterior chamber, **C-** The RT-PCR results using primer pairs for FCoV M gene and GAPDH gene region (for FIPV 295bp, 195 bpfor GAPDH). M: 100 bp DNA ladder (Fermentas, Lithuania) Line 1: Brain tissue (FIPV), Lane 2: Leukocyte sample (FIPV), Lane 3: Brain tissue (GAPDH gene), Lane 4: Leukocyte sample (GAPDH), **D-** Vasculitis in cerebral meninges containing mixed inflammatory infiltrate in inner and outer surface of vessels *(arrows)*

Şekil 1. A- Sol gözde daha belrgin bir hifem, ön göz kamerasının bulanık görünümü ve çift taraflı pupilla dilatasyonu, **B-** Vitreusta proteinöz eksudat birikimi ve anteriyor kamarada kanama, **C-** FCoV M geni ve GAPDH gen bölgesi için kullanılan primer eşleşmelerine göre RT-PCR sonuçları (FIPV 295bp, 195 bpfor GAPDH). M: 100 bp DNA basamağı (Fermentas, Litvanya) Sıra 1: Beyin dokusu (FIPV), Sıra 2: Lökosit örneği (FIPV), Sıra 3: Beyin dokusu (GAPDH), Sıra 4: Lökosit örneği (GAPDH), **D-** Damarların iç ve dış yüzeyinde miks yangısal infiltrat içeren vaskülitis *(oklar)*

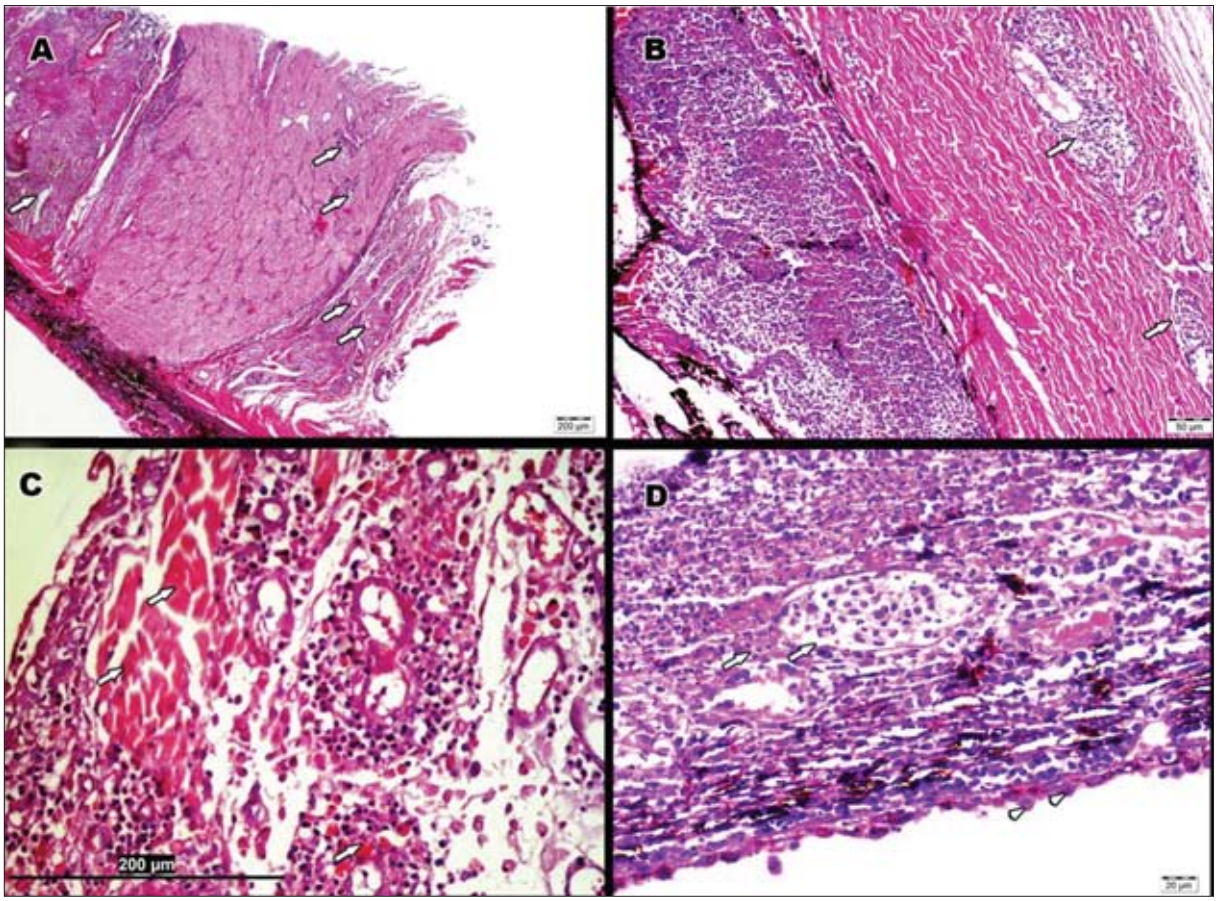

Fig 2. A- Optic nerve meningitis *(arrow)* and optic neuritis characterized by perivascular infiltration *(arrows)*, **B-** Pyogranulomatous inflammatory reaction in corpus cilliare and perivascular infiltrations in scleara *(arrows)*, **C-** Necrotic changes in muscle *(arrows)* and perivascular lymphoplasmacytic infiltrations, **D-** Vasculitis *(arrows)*, interstitial lymphohistiocytic infiltration in choroidea and hypertrophic retinal pigment epithelium *(arrow head)*

Şekil 2. A- Perivasküler infiltrasyon ile belirgin optik sinir meningitis *(ok)* ve optik nöritis *(oklar)*, **B-** Korpus siliare'de pyogranulomatoz inflamatuar reaksiyon ve sklerada perivasküler infiltrasyon, **C-** Kas liflerinde nekrotik değişimler *(oklar)* ve perivasküler lenfoplazmasitik infiltrasyonlar, **D-** Vaskülitis *(oklar)*, koroidde intersitisyel lenfohistiositik infiltrasyon ve retinal pigment epitelinde hipertrofi *(ok başı)*

were observed under UV light and photographic records were made. 195 bp band were positive reaction control provided by the Feline GAPDH gene. 295 bp band were positive as feline coronavirus. Microscopic changes were limited to the meninges, optic nerves, eyes, and kidneys. Most of these changes were characterized by mixed inflammatory reaction together with vasculitis and perivascular infiltration. Meninges showed foci of pyogranulomatous to lymphohistiocytic infiltration and vasculitis *(Fig. 1D)*. Perivascular inflammatory reaction was more prominent in orbital meninges and optic nerve *(Fig. 2A)*. There was a pyogranulomatous panuveitis and anterior uveitis in the left and right eye, respectively. The interstitial and perivascular inflammatory infiltrate contained neutrophils, lymphocytes and foamy macrophages with a higher neutrophilic component in iris, ciliary process, ciliary body and sclera *(Fig. 2B)*. Pyogranulomatous vasculitis by mixed inflammatory infiltrate was limited to the iris and sclera in the right eye. There was mild edema, focal mild perivascular lymphohistiocytic infiltrations and few hemosiderine containing macrophages in iridial stroma in the left eye. There was moderately fibrino-purulent exudate accumulation with considerable foamy macrophages in vitreous in both eyes. Rhabdomyocytes showed moderate to severe degenerative to necrotic changes and perivascular infiltrations of pure lympho-plasmacytic cells *(Fig. 2C)*. The choroid was expanded by mixed cellular infiltration, mild pigment incontinence and showed vasculitis *(Fig. 2D)*. Retina was partially detached due to subretinal exudate in non-tapetal region. The other lesions included mild corneal and conjunctival edema. Renal changes consisted of focal pyogranulomatous nephritis characterized by infiltration of cell mixture including lympho-histiocytes and neutrophiles in renal cortex and rarely medulla. Pyogranulomas were non-necrotic and there were diffuse hydropic changes in renal tubules and multifocal hyaline casts in tubular lumen.

DISCUSSION

As there is no gold standard for diagnosis of FIP and the lesions are diverse and highly variable, it is always challenge to make histo-pathological diagnosis. However, in the present case the diagnosis was based on histopathology and PCR [6]. Consistent with the earlier reports, clinical signs including mild pyrexia, weight loss, dullness, depressed appetite were determined in the present case. Mild pyrexia had probably caused to tachycardia and tachypnea. Signs referable to spinal cord involvement, such as incoordination, hyperaesthesia, circling, head tilt, posterior paraperesis were also present [2,5]. As the eye is the last effected organ in FIP and the cat could die before it's involvement, the ocular pathology might not be observed in most cases. In the present case, involvement of the organs other than eye was mild. Ocular lesions were bilateral and included protein rich fluid accumulation in ocular compartments, uveitis, vasculitis, pure lymphoplasmacytic infiltrations and rhabdomyositis were fairly characteristic and almost pathognomonic for FIP [2,6,7]. Ocular involvement is relatively common in dry form, leading to a variety of changes, such as iris colour, anisocoria secondary to anterior uveitis, sudden loss of the vision and hyphaema [6,7]. These ocular signs completely coincided with the findings determined in this case. Although there are some reports indicating ocular lesions of FIP in the worldwide, ocular lesions in FIP have not been reported in Turkey.

A very common laboratory finding in cats with FIP is an increase in total serum protein concentration caused by a rise in globulins [15]. This is found in about 50% of cats with effusion and 70% of cats without effusion [1]. Albumin level remains normal or falls slightly [16]. Low albumin level is usually associated with protein loss caused by glomerulopathy secondary to immune complex deposition or by extravasation of protein-rich fluid during vasculitis [17]. GLB level increases, possibly through stimulation of B cells by interleukin-6, which is produced as part of the disease process [16]. In the present case, although serum total protein was in the reference ranges, ALB was slightly under the lower limit of normal range and GLB was near the upper limit of normal range. Other laboratory parameters (liver enzymes, bilirubin, urea, creatinine) can be variably elevated depending on the degree and localization of organ involvement, however they are generally not diagnostic [17]. In the present case, those values were in the reference ranges out of slightly increased AST level. Slightly increased AST level was probably associated with discrete fatty change. In this study, the non-effusive FIP was diagnosed through history, clinical, pathological and polymerase chain reaction (PCR) findings.

Collectively; FIP should be considered in differential diagnosis of diseases showing similar findings in cats. Distinct ocular lesions in combination with the other findings may be useful criteria in the diagnosis of FIP.

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CASE REPORT

High-level Mupirocin Resistance in a *Staphylococcus pseudintermedius* Strain from Canine Origin [1]

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Abstract

Mupirocin is an important antibacterial agent used in both humans and animals topically, especially against nasal carriage and decolonization of methicillin-resistant *Staphylococcus pseudintermedius*. In this report, a *S. pseudintermedius* strain was isolated from a dog with pyoderma. Molecular identification of this strain was performed by digestion of *pta* gene with *MboI* enzyme. High-level mupirocin resistance was determined by agar dilution method and amplification of *ileS*-2 gene. Sequence analysis of 16S rRNA and *ileS*-2 gene of S. pseudintermedius was also performed and both sequences were accessed in GenBank. This is the first report of a high-level mupirocin resistant *S. pseudintermedius* strain from a dog with pyoderma in Turkey.

Keywords: *Antibiotic resistance, Dog, Mupirocin, Staphylococcus pseudintermedius*

Köpek Kökenli Bir *Staphylococcus pseudintermedius* Suşunda Yüksek Seviye Mupirosin Direnci Olgusu

Özet

Mupirosin insan ve hayvanlarda özellikle metisilin dirençli *Staphylococcus pseudintermedius*'un dekolonizasyonuna ve burun taşıyıcılığına karşı kullanılan önemli bir antibakteriyel ajandır. Bu çalışmada, piyodermalı bir köpekten *S. pseudintermedius* izole edildi. Bu suşun moleküler teşhisi için *pta* geni *MboI* enzimi ile kesildi. Yüksek seviye mupirosin direnci agar dilusyon metodu ve *ileS*-2 geninin çoğaltılmasıyla ortaya kondu. *S. pseudintermedius*'un 16S rRNA ve *ileS*-2 genlerinin dizi analizi yapılarak her iki genin sekansları GenBank'a kayıt ettirildi. Bu rapor, Türkiye'de piyodermalı bir köpekten izole edilen ilk yüksek seviye mupirosin dirençli S. pseudintermedius olgusunu bildirmektedir.

Anahtar sözcükler: *Antibiyotik direnci, Köpek, Mupirosin, Staphylococcus pseudintermedius*

INTRODUCTION

Staphylococcus pseudintermedius is an important, opportunistic, coagulase-positive staphylococcus species that is frequently isolated from skins of healthy dogs and dogs with cutaneous infections particularly canine pyoderma. Canine pyoderma is generally treated with antibacterial shampoos and topical or systemic antibiotics. Nevertheless, inaccurate or ampiric misuse of antibiotics are the leading factors in development of bacterial antibiotic resistance. Recently, multidrug-resistant *S. pseudintermedius* (MDRSP) to different antibiotic classes and methicillin-resistant *S. pseudintermedius* (MRSP) is described in detail [1,2]. Acquired antibiotic resistance in *S. pseudintermedius,* challenges the treatment of infected animals and concerns public health closely, since it is thought to have a zoonotic potential. In 2006, Van Hoovels et al.[3] demonstrated the first zoonotic description of *S. pseudintermedius*.

Penicillins, cephalosporins, tetracyclines, macrolides, fusidic acid, chloramphenicol, aminoglycosides and fluoroquinolones are used in the treatment of canine pyoderma frequently. Beside this, mupirocin is also an

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important agent used in both humans and animals topically, especially against nasal carriage and decolonization of methicillin-resistant *Staphylococcus aureus* (MRSA) and MRSP [4]. Mupirocin resistance in *S. aureus* is classified in categories according to MIC values. Low-level mupirocin resistance MICs are between 8-256 mg/L, whereas MICs above or equal to 512 mg/L referred to high-level mupirocin resistance. High-level resistance to mupirocin in *Staphylococci* generally acquired by horizontal gene transfer, especially those carry *ileS*-2 gene on conjugative plasmids [5]. We here report a high-level mupirocin resistant *S. pseudintermedius* strain from a dog with pyoderma for the first time in Turkey.

CASE HISTORY

Swap and skin samples taken from a 5 year old, female Rottweiler, clinically diagnosed as pyoderma was accepted for microbiological investigation. Skin swap samples, skin scrapings and hair from the edge of lesions were taken for bacteriological and fungal examination. Fungal culture was found to be negative.

Swap samples were cultured on blood agar containing 5-7% ovine blood and incubated aerobically at 37°C for 24 h. After incubation, suspected colonies were Gram stained and treated with catalase, coagulase and DNAse tests. Microbact Staphylococcal 12S Identification System (Oxoid MB 1561) was used to identify coagulase-positive staphylococci. For further phenotypic identification, β-galactosidase, resistance to polymyxin B and acetoin production were also tested.

Molecular identification of *S. pseudintermedius* was carried out by PCR-restriction fragment length polymorphism method of Bannoehr et al.[6], and genomic DNA was extracted by a modified phenol-chloroform extraction method as described previously by Ardic et al.[7]. A 320 bp fragment of *pta* gene was amplified in a 50 µl total volume with 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA Polymerase (Fermentas, Lithuania), 200 µM each dNTPs, 5 µl PCR reaction buffer (1×), 4 µl template DNA and 0.2 µM of each primer (pta_f1, AAA GAC AAA CTT TCA GGT AA, and pta_r1, GCA TAA ACA AGC ATT GTA CCG). DNA amplification was performed with the following thermal cycling conditions: an initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min with final extension at 72°C for 7 min. Amplified products were digested enzymatically with 5 U *Mbo*I for 2 h at 37°C. Two fragments (213 bp and 107 bp) of *pta* gene confirming *S. pseudintermedius* identification were detected after the digested products were resolved by 2% agarose gel electrophoresis.

Agar dilution method (MIC value) and PCR assay (*ileS*-2 gene) was used to determine the high-level mupirocin resistance in *S. pseudintermedius*. Serial dilution of mupirocin beginning with a concentration of 1024 mg/L in Mueller-Hinton agar was prepared according to Clinical and Laboratory Standards Institute guidelines [8]. Mupirocin MIC value of *S. pseudintermedius* isolate was found as 512 mg/L. *Staphylococcus aureus* ATCC 29213 was served as quality control strain in all tests.

In order to verify high-level mupirocin resistance genotypically, *ileS*-2 gene was amplified according to the PCR method of Anthony et al.[9]. Primers (mupA, TAT ATT ATG CGA TGG AAG GTT GG, and mupB, AAT AAA ATC AGC TGG AAA GTG TTG) each with a concentration of 50 pmol were used in a 50 µl PCR mixture that contains; reaction buffer (1×) (50 mmol/L KCl, 10 mmol/L Tris-HCl; pH 9.0), 2.5 mM $MgCl_2$, 200 µM each dNTPs, 1.5 U of *Taq* DNA Polymerase (Fermentas, Lithuania) and 5 µl template DNA. Same amplification conditions were used as mentioned above in identification of *S. pseudintermedius* by PCR-RFLP. After agarose gel electrophoresis, an amplicon with a size of 458 bp was visualized by UV transilluminator. A high-level mupirocin resistant, *ileS*-2 gene positive *S. aureus* strain obtained from culture collection of Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey was used as positive control. Sequence analysis of 16S rRNA and *ileS*-2 gene of *S. pseudintermedius* was performed by Sanger sequencing method on an Applied Biosystems 3130 Genetic Analyzer, using standard protocols. Both sequences were included in GenBank with the corresponding accession numbers KC561085 for *ileS*-2 and KC561086 for 16S rRNA, respectively.

Staphylococcus pseudintermedius was identified by both phenotypic and molecular methods as described above. Beside this high-level mupirocin-resistant *S. pseudintermedius* with a MIC value of 512 mg/L was found. High-level mupirocin resistance was also confirmed by the amplification of *ileS*-2 gene genotypically.

DISCUSSION

Zoonotic potential and acquisition of resistance to different antibiotics increased the interest in *S. pseudintermedius* recently. Although new studies are being published on MDRSP and MRSP, there are few reports about mupirocin resistance in *S. pseudintermedius* isolated from dogs. Loeffler et al.[10], investigated mupirocin and fucidic acid resistance in coagulase-positive staphylococcal isolates from dogs and cats. High-level mupirocin-resistant *S. pseudintermedius* strain could not be detected and the results (89.7% of all MICs ≤0.25 mg/L) were found to be compatible with previous studies. Authors concluded their research that both mupirocin and fucidic acid could be used in treatment of superficial staphylococcal infections and decolonization of multi-resistant strains of *S. aureus* and *S. pseudintermedius*.

High-level mupirocin-resistance in a *S. pseudintermedius* isolate from a dog with canine pyoderma was reported for the first time with this study in Turkey. This is an important result, since, mupirocin is known as an important antimicrobial agent used in eliminating nasal carriage and treatment of MRSA, MRSP, and MDRSP strains in animals and humans. Hurdle et al.[11], reported that conjugative transfer of mupirocin resistance between different *Staphylococcus* species and acquisition of high-level mupirocin resistance could occur during treatment with mupirocin. Regarding zoonotic potential of the agent and likelihood of transfer of antibiotic resistance amongst staphylococci and/or other competent microorganisms, it can be concluded that comprehensive research and studies should be performed on prevalence of mupirocin resistance in staphylococci of animal origin.

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CASE REPORT

Doppler Evaluation of Fetal and Feto-Maternal Vessels During Dystocia in Cats: Four Cases [1]

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Abstract

Doppler ultrasonography has been becoming an essential tool in veterinary medicine, especially in theriogenology. It has being widely used for the evaluation of fetal well-being in pathologic pregnancies in human medicine. The paper highlights the usage of Doppler ultrasonography in veterinary obstetrical pathologies with a review of literature of human and veterinary medicine. Four cats with dystocia and their fetuses were evaluated for fetal and maternal hemodynamics. As a conclusion, flow velocity of the fetal and feto-maternal vessels can inform us of the existence of the pathology related to gestation and also can help choosing the method of treatment in veterinary medicine.

Keywords: *Cat, Dystocia, Doppler ultrasonography, Feto-maternal vessel*

Kedilerde Güç Doğum Sırasında Fötal ve Föto-Maternal Damarların Doppler Değerlendirilmesi: Dört Olgu

Özet

Doppler ultrasonografi, başta teriyojenoloji olmak üzere veteriner hekimlikte sıklıkla başvurulan yöntemlerden biri olmaya başlamıştır. İnsanlarda özellikle patolojik gebeliklerde fötal iyilik halinin değerlendirilmesi amacıyla yaygın olarak kullanılmaktadır. Bu makalede, tıp ve veteriner hekimliğinde yer alan literatürlerin derlenmesiyle beraber, veteriner obstetrik patolojilerde Doppler ultrasonografi kullanımının aydınlatılması amaçlanmıştır. Güç doğum şikayeti bulunan dört adet kedi ve bu kedilere ait fötuslarda fötal ve maternal hemodinamik değerlendirilmiştir. Sonuç olarak, fötal ve föto-maternal damarlara ait akım hızlarının gebelik patolojilerinin belirlenmesinde kullanılabileceği ve güç doğum olgularında tercih edilecek tedavi metodunun belirlenmesinde yardımcı olabileceği düşünülmüştür.

Anahtar sözcükler: *Kedi, Güç doğum, Doppler ultrasonografi, Föto-maternal damar*

INTRODUCTION

Doppler ultrasonography (D-USG) is a non-invasive technique which enables us to follow the physiologic and physiopathologic differences in the circulation between mother and fetus. However D-USG alone is not sufficient for the evaluation of fetal well-being, but this technique enables the doctor to diagnose fetal distress earlier than other tests [1]. Obstetric D-USG gives the doctor a reliable opportunity to review maternal and fetal hemodynamics by investigating the vessels like the umbilical artery and vein, uteroplacental arteries, fetal thoracic aorta, fetal caudal vena cava and fetal cerebral artery [2,3]. Abnormal vascular placental development in fetal and/or maternal compartments may be an indicator of intrauterine growth restriction, fetal distress and early pregnancy failure [4,5].

Both in medical science and veterinary gynaecology, using D-USG with the aim of monitoring pregnancy and research is becoming increasingly widespread. There are

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limited number of studies [3,6,7] about feline fetal D-USG. This is the first report of fetal D-USG in cats with dystocia. The present paper contains the evaluation of maternal and fetal haemodynamic characters of four cats diagnosed with dystocia.

CASE HISTORIES

The presented four cases were referred to the Department of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, Istanbul University, Turkey. The patient's information was documented in *Table 1*. After a detailed anamnesis and physical examination, existence of the fetal heartbeat of each fetus was checked with B-mode real-time ultrasonography (MyLab 5-Vet ESAOTE®, Genova, Italy) using a microconvex probe of 5 MHz. Immediately after the recording of a fetal heart rate, pulsed-wave Doppler (PWD) sonography was performed to the most caudal fetus. First, the vessel was visualized using Color-Doppler mode and subsequently the PWD sonography mode of the device was turned on. The *angle* of *insonation* was approximately 60° in all examinations. Pulsatility (PI) and resistance index (RI) of umbilical artery, uteroplacental artery, fetal thoracic aorta and fetal inferior vena cava were recorded and the waveform of each vessel was assessed qualitatively.

The cat and her kittens in Case 1 and the kittens in Case 2 and 4 died within 24 h of the operation while the other cats and kittens in Case 3 recovered. The fetal heart rates and Doppler findings are presented in *Table 1*.

There was a high resistance in the umbilical artery of Case 1 and 4 and in the fetal aorta of Case 1 and 2. PI values of fetal vena cava were high in Case 1, 2 and 4.

According to qualitative Doppler waveform analysis, a decreased "a" wave during atrial systole was detected in the inferior vena cava of Case 2 *(Fig. 1)*. Also decreased end diastolic flow in fetal aorta was clearly seen in the same fetus *(Fig. 2)*.

DISCUSSION

There are many techniques of Doppler analysis. However, analyzing the pulsatilty of the Doppler waveform is one of the widely accepted approaches in clinical use.

Table 1. *Case information, fetal heart rates and Doppler indices of the cats*
Tablo 1. *Olgulara ilişkin bilgiler, fötuslara ait Doppler bulguları ve kalp atım sayıları*

Information	**Case 1**		**Case 2**		**Case 3**		**Case 4**	
Age	4-year-old		4-year-old		3-year-old		6-year-old	
Breed	Mixed-breed		Mixed-breed		Siamese		Persian	
History	The cat had been struck by a car fifteen days ago		Vaginal bleeding which had begun 3h before		Lower respiratory tract infection		Given birth to a kitten 10 h before. The second and dead kitten was taken out from the birth canal 5 h later	
Clinical findings	Haemorrhagic vaginal discharge, paraplegia, dyspnea, 35.7°C		Haemorrhagic vaginal discharge, general condition was good, 37.7°C		Severe dyspnea, muco-purulent nasal discharge, 39.7°C		General condition was good, 37°C	
Gestational age (after mating)	58th day		62nd day		58th day		63rd day	
Hematological findings	Severe anemia (RBC 3.1×10^{12}/l and HCT 22%), Leukocytosis (WBC 25.6×10^{9}/l)		In normal range		Leukocytosis (WBC 23.7×10^{9}/l)		In normal range	
Treatment	warm crystalloid (25 ml/kg NaCl 0.9%, Mediflex®, Eczacıbaşı-Baxter) and colloid (5ml/kg Gelofusine®, Irengun) i.v.		-		Amoxicillin and clavulanic acid (8.75 mg/kg SC, Synulox®, Pfiezer) and O_2 treatment for three days		-	
Operation	c-section		c-section		c-section (after 3 day medical treatment)		c-section	
Fetal heart rate (bpm)	150		125		225		189	
	PI	**RI**	**PI**	**RI**	**PI**	**RI**	**PI**	**RI**
A. umbilicalis	1.45	0.74	1.19	0.75	1.08	0.69	1.47	0.75
Fetal aorta	2.81	0.90	2.64	0.91	1.73	0.81	2.68	0.89
Fetal vena cava	1.31	0.77	1.46	0.85	0.45	0.38	1.47	0.84
Uteroplacental artery	0.45	0.36	0.31	0.27	0.59	0.44	0.65	0.49

PI: *Pulsatility,* ***RI:*** *Resistance index*

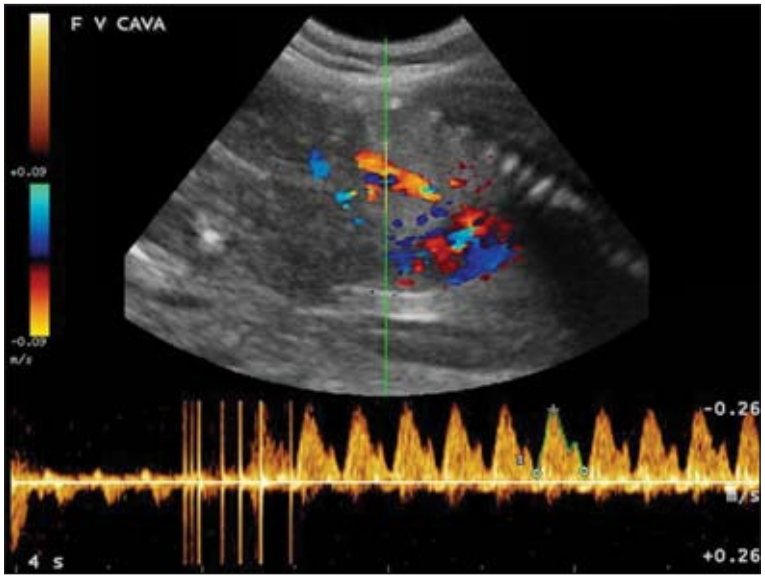

Fig 1. A decreased "a" wave during atrial systole in the inferior vena cava of Case 2

Şekil 1. Olgu 2'nin inferior vena kavasında atrial sistol boyunca şekillenen azalan "a" dalgası

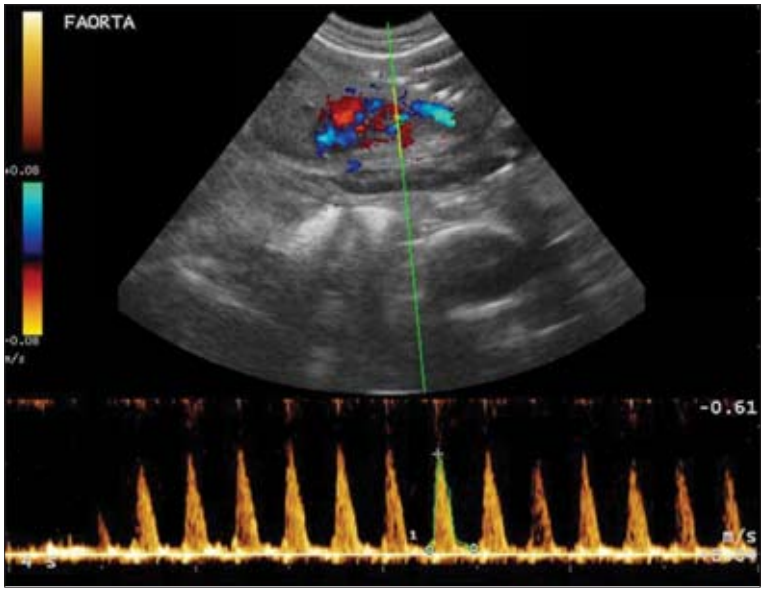

Fig 2. A decreased end diastolic flow in fetal aorta of Case 2

Şekil 2. Olgu 2'ye ait fetal aortada şekillenen "azalan diyastol sonu akımı"

A Doppler index is calculated as a ratio and is, therefore, virtually angle-independent [8].

Normal fetal heart rate in kittens is 193-263bpm. The cases in which the fetal heart rates are below these levels suggest developing hypoxia and fetal distress [9]. A tentative diagnosis of hypoxia and fetal distress was given in all cases except case 3. Progressive hypoxia causes a decreased, reversed or absent end diastolic flow in thoracic aorta [2]. Indeed, a decreased end diastolic flow in fetal aorta was detected in Case 2.

Three major pathologies are described in the umbilical artery blood flow in human medicine. They are high PI and RI values; absent end diastolic flow and reversed end diastolic flow [2]. In recent studies of veterinary practice [3,6,7] it has been reported that the PI and RI of the umbilical artery progressively decrease and reach the lowest level at delivery. The PI and RI values of the umbilical artery at the prenatal period were found in the range of 1.05-1.39 and 0.70-0.75 respectively in these studies. When compared to these results, the umbilical artery RI values were in the same range as in the other research, whereas PI values in Case 1 and 4 were a little higher than the others. The high resistance in the umbilical artery of these cases may be a result of fetal distress or hypoxia. Another important pathology in the umbilical cord is the pulsation in the umbilical vein. This finding is a manifestation of a serious fetal decompensation or fetal heart failure [2,8]. However, no abnormal finding was seen in the umbilical vein of our cases.

A normal utero-placental transport is essential for the developing fetus. Otherwise, fetal growth retardation may occur and end-diastolic velocity in the descending aorta may be reduced, reflecting a change from low arterial downstream impedance to high arterial downstream impedance at fetal trunk and placental level. Consequently, pulsatility index values will be raised [10,11]. Onen et al.[10]

have stated that high PI values of fetal aorta are strongly correlated with poor pregnancy outcome, fetal distress and a high c-section rate. In the presented cases, PI values of fetal aorta were found higher in Case 1, 2 and 4 compared to the findings of Scotti et al.[3]. The increased PI value of these cases may indicate fetal distress and intrauterine fetal growth restriction. However, all of the kittens in these cases died within 24 hours of c-section.

The Doppler indices from fetal venous vessels give more reliable information about fetal acidosis than indices from arterial vessels [12]. Since the oxygenated blood comes from the placenta to the fetal heart via the inferior vena cava, alterations in the pattern of vena cava flow is an indicator of fetal condition [13]. Rizzo et al.[12] have concluded that the inferior vena cava has a greater efficiency when compared to the ductus venosus. Furthermore, ductus venosus flow is greatly influenced by other vasoactive factors [14]. Therefore, the inferior vena cava was chosen for the Doppler measurements in the presented cases. The flow velocity waveform of the inferior vena cava displays a biphasic flow profile with two peaks during ventricular systole and diastole. In contrast to the ductus venosus waveform, absence or retrograde flow may occur during atrial contraction [13,15]. A decreased "a" wave during atrial systole was detected in the inferior vena cava of Case 2. However, this condition was not identified as a pathologic condition. Mori et al.[16] reported two different abnormal waveforms in the inferior vena cava of intrauterine growth retarded fetuses: one with a high-pulsatile pattern and the other with a slight and low-pulsatile pattern. The authors explained this abnormal pattern as reduced ventricular filling caused by impaired contractility and reduced ventricular output. However, no abnormal pattern was found in the inferior vena cava of the presented cases.

The D-USG measurement of the uteroplacental blood-stream is an important tool for evaluating materno-fetal circulation [17]. Scotti et al.[3] have reported that PI and RI values of uteroplacental arteries decrease significantly as the pregnancy progresses. In spite of this, Pereira et al.[7] have indicated that PI and RI of uteroplacental arteries were invariable throughout pregnancy, except for a sudden increase of PI on Day 63. The values of PI and RI of uteroplacental arteries in Case 3 were similar to those observed by Scotti et al.[3], while the other cases were dissimilar. The higher indices in Case 1, 2 and 4 may be identified with the low perfusion in the uteroplacental sites of these cases. It is known that the higher the resistance (RI) the lower the perfusion; in addition, the increasing PI values indicate decreasing perfusion of the tissue [18,19].

The Doppler ultrasonography is routinely used in human medicine to recognize several obstetrical pathologies. Similarly, assessing the number of fetal heart-beats and flow velocity of the fetal, feto-maternal vessels can inform us of the existence of the pathology related to gestation and also can help choosing the method of treatment in veterinary medicine. This issue, however, requires more studies and more statistical analysis.

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REVIEW

Uterine Infections in Cows and Effect on Reproductive Performance

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Abstract

Uterine infections affect the hypothalamus, hypophysis, ovaries and uterus. These infections inhibit the development of dominant follicles, prevent both the release of the luteinizing hormone (LH) and ovulation, reduce the size of the corpus luteum (CL), and decrease progesterone production. As these infections reduce fertility or cause infertility, they result in major economic losses. The diagnosis of uterine infections by means of various techniques and the detection of the severity of the infection enable the selection of the most appropriate treatment method and the estimation of the success rate that may be achieved with the application of the particular treatment method selected. This review aims to provide researchers and veterinary practitioners with practical information on the prevalence of postpartum uterine infections and currently applied diagnostic and treatment methods.

Keywords: *Cow, Uterine diseases , Reproductive performance*

İneklerde Uterus Enfeksiyonları ve Üreme Performansı Üzerine Etkisi

Özet

Uterus enfeksiyonları hipotalamus, hipofiz, ovaryum ve uterus üzerine etki eder. Bu enfeksiyonlar; dominant follikül gelişimini baskılar, luteinize edici hormon (LH) salınımını ve ovulasyonu engeller, korpus luteumun (CL) daha küçük ve progesteron üretiminin daha az olmasına neden olur. Fertilitenin düşmesine veya infertiliteye neden olarak ciddi ekonomik kayıplar oluşturur. Uterus enfeksiyonlarının çeşitli yöntemlerle teşhisi ve enfeksiyon şiddetinin belirlenmesi, uygun tedavi yönteminin belirlenmesine ve tedavi yöntemi başarısının tahminine olanak sağlar. Sunulan derleme ile postpartum uterus hastalıklarının yaygınlığı, güncel teşhis ve tedavi yöntemleri hakkında araştırmacılara ve veteriner hekimlere pratik bilgiler sunulmaya çalışıldı.

Anahtar sözcükler: *İnek, Uterus enfeksiyonları, Üreme performansı*

INTRODUCTION

The parturition period is characterized by high risk. During this period, the risk of the development of microbial uterine infections increases, due to several reasons including the possible physical damage of the birth canal, and retained placenta. The occurrence of a negative energy balance in this period further increases this risk. Uterine infections have a negative impact on animal welfare and reproductive performance, and lead to major economic losses [1]. This review aims to provide detailed literature information on the incidence, pathogenesis, classification and diagnosis of uterine infections, their effects on fertility, economic impact and available treatment options.

THE DEFENCE SYSTEM OF THE UTERUS

Prior to parturition, pathogenic microorganisms are not found in the uterus. The elimination of the mechanical protective barriers of the uterus with parturition enables the access of microorganisms to the organ [1]. For the bacterial contamination of the uterus to occur, pathogens must firstly pass the anatomical barriers of the uterus (the sphincter of the vulva and the cervix uteri). The microorganisms, which manage to pass the first barrier, are later tried to be eliminated by the regional and systemic

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defence mechanisms of the uterus. In uterine infections, the inflammatory cells, which first infiltrate to the site of infection, are the neutrophil leukocytes [2]. Apart from neutrophil leukocytes, lymphocytes also play a major role in uterine immunology. Pathogens, which are opsonized by the humoral defence system, are phagocytized by neutrophil leukocytes. The endometrium constitutes the first line of defence against bacteria, owing to the Toll-like receptors (TLRs) it bears and the antimicrobial proteins it secretes. The TLRs are capable of recognizing the molecules of bacterial pathogens (lipopolysaccharides, lipoteichoic acid and the deoxyribonucleic acid of the bacteria), and upon recognition, secrete prostaglandin E_2 (PGE_2) and antimicrobial proteins from uterine epithelial cells [3]. These proteins are secreted locally by the tumor necrosis factor, interleukin-6 and interleukin-8, following the recognition of the pathogens by the TLRs [4], and lead to the secretion of acute-phase proteins from the liver. Acute-phase proteins are molecules, which are effective in the elimination of uterine infections [5].

THE INCIDENCE OF METRITIS AND ENDOMETRITIS

The incidence of clinical metritis in cows and heifers has been reported as 18.6% and 30%, respectively [5]. Sheldon et al.[6] have determined the incidence of clinical metritis during the first two weeks of the postpartum period as 25-40%. It has been indicated that the incidence of clinical metritis at herd level ranges between 5% and 26%, and is on average 17% [7-9]. Furthermore, the incidence of subclinical endometritis has been reported to range from 19% to 74%, and to be 53% on average [10,11]. The large differences observed between the incidences determined in previous studies have been attributed to the diagnostic methods employed, the method used for the classification of uterine infections, the postpartum period in which the uterine infection was diagnosed, the general traits of the bovine animals included in previous research, their parity, and the management of the herds included in previous studies [12].

PATHOGENESIS OF UTERINE INFECTIONS

Following parturition, in 80 to 100% of animals, the uterus becomes contaminated with bacteria [13]. Uterine infections develop upon the establishment of pathogenic microorganisms onto the mucosa, their colonisation and penetration of the epithelial layer, and the production of toxins by these microorganisms [14]. The development of uterine infections depends on the immune status of the animal, the species and number of pathogenic microorganisms contaminating the uterus, and several other environmental and individual factors [1,2]. Different opinions exist on the impact of nutrition on the development of uterine infections. The increase in insulin resistance, decrease in feed consumption, ocurrence of a negative energy balance, and loss of body weight in animals in advanced gestation lead to the weakening of the defence system [15]. It has been ascertained that the rate of uterine bacterial contamination is above 90% during the first few days after calving [12], and decreases to 78% on day 30 postpartum, to 50% on day 45 postpartum, and to 9% by day 60 postpartum [16,17].

The most common bacterium encountered in uterine infections during the first few days of the postpartum period is *Escherichia coli*. After the first week postpartum, *Fusobacterium necrophorum* and *Trueperella pyogenes* also become involved in uterine infections [18,19]. Certain bacteria, including *Campylobacter fetus* and *Trichomonas fetus*, are specific to cases of endometritis [20]. Nevertheless, bacteria of the genera *Clostridia, Pepticoccus* and *Peptostreptococcus,* as well as several unidentified *Gram positive* and *Gram negative anaerobic bacteria* and aerobic and facultative anaerobic pathogens such as *alpha-haemolytic streptococci, Arcanobacterium pyogenes* and *Escherichia coli* may also cause endometritis [2,16,17,20]. Uterine infections are most commonly observed following twin births, dystocia, surgical interventions employed to aid in cases of dystocia, retained placenta, stillbirth, prolapsus uteri, artificial insemination, copulation, and the use of intrauterine irritants [1,2,14].

In the postpartum period, the puerperal discharge (remains of the fetal membranes, endometrial sloughing, fetal fluids, etc.) constitutes an ideal environment for microbial growth. The elimination of the puerperal discharge from the uterus reduces the bacterial load of the organ [21]. Furthermore, during the postpartum period, the oestrogen level increases in cattle. Oestrogen increases the blood supply and enables the infiltration of white blood cells, including neutrophil leukocytes, to the uterus. This hormone also induces the secretion of vaginal mucus. This mucus enables the reduction and discharge of the bacterial load of the uterus [2]. Oestrogen also increases the number of oxytocin receptors in the uterus and the susceptibility of the myometrium to contractions. Muscle contractions both accelerate uterine involution and decrease the bacterial load of the uterus by enabling the elimination of bacteria in the puerperal discharge [22]. Previous studies have suggested that ovulation in the early postpartum period has no effect on the incidence of subclinical metritis [23].

CLASSIFICATION AND DIAGNOSIS OF UTERUS INFECTIONS

Metritis is defined as the inflammation of the superficial and deep layers of the uterus. Depending on the severity

of metritis, degeneration and leukocyte infiltration may be observed in some or all layers of the uterus [14]. Acute puerperal metritis occurs during the first two weeks after parturition (mostly during days 4-10 postpartum). Animals suffering from acute puerperal metritis, in general, present with toxaemia, septicaemia and pyemia. Cases of acute puerperal metritis are characterized by an enlarged uterus with a a tapered wall, reddish brown coloured and malodorous vaginal discharge, and systemic signs including fever (40-41°C), increased heart and respiratory rates, anorexia, diarrhoea and shock [2,13]. Clinical metritis is defined as a uterine infection, which develops until day 21 postpartum with no systemic disorder, but displays purulent or mucopurulent discharge [24]. Clinical endometritis develops as from the third week postpartum. In such cases, the uterus is filled with a purulent or mucopurulent content, and if the cervix uteri is open, a vaginal discharge of a character the same with that of the uterine content may be observed [8,25]. Subclinical endometritis is the inflammation of the uterus, which does not present with any of the signs observed in clinical endometritis, but causes reproductive disorders [26]. The excessive infiltration of neutrophil leukocytes is observed in subclinical cases [14]. Subclinical endometritis is reported to develop between days 35-60 postpartum in 35 to 50% of animals, and to prolong the parturition-conception interval and to reduce fertility [10,26]. Pyometra is characterized by the accumulation of a purulent exudate in the uterus and the persistence of the corpus luteum (CL) [2,27]. If the ovulation occurs before the elimination of the microorganisms in the uterus, then the CL is maintained [16,20]. During the period in which the uterus is under the influence of progesterone, generally the cervix uteri is tightly closed and the purulent exudate begins to accumulate in the cornu uteri. In some cases, a small amount of purulent vaginal discharge may be observed [2]. Pyometra is very rarely associated with systemic findings [20]. In general, pyometra progresses into chronic endometritis [2].

Puerperal metritis presents with both local and systemic clinical findings. Fever is common and both the heart rate and respiratory rate are altered. A malodorous reddish coloured uterine discharge is observed [2]. Animals with clinical metritis do not present with any impairment of the general condition. An enlarged and incompletely involuted uterus [14] and a purulent or mucopurulent vulvar discharge are observed [25]. The method most commonly used in the diagnosis of clinical endometritis is vaginal examination. This method is based on an evaluation of the colour and purulency of the discharge [5,14]. Clean vaginal discharge containing transparent mucus is scored as 0, while discharge with a greater amount of transparent mucus but a low level of pus is scored as 1. Discharges containing white or yellow pus at a level lower than 50% are scored as 2, while discharges containing yellow and white pus at a level greater than 50%, and which may sometimes also contain blood, are scored as 3 [9,14]. The scoring of the vaginal discharge according to its odour is as follows: odourless=0, malodorous or putrified=1 [5].

The ultrasonographic measurement of the diameter of the cornu uteri and cervix uteri, the quantity of fluid in the lumen of the uterus, and the thickness of the endometrium provide insight on the presence of endometritis and its severity [26,28]. Animals with a cervix uteri diameter greater than 7.5 cm on postpartum day 20 are considered to suffer from clinical endometritis [9]. Bacterial isolation can be performed with endometrial swabs and endometrial biopsy samples taken during the postpartum period [25,29]. If no colony growth is observed the score is considered as 0, while the growth of less than 10 colonies is scored as 1. The growth of 10 to 100 colonies is scored as 2, the growth of 101 to 500 colonies as 3, and the score of the growth of more than 500 colonies is 4 [5]. Another method used for the diagnosis of endometritis is biopsy sampling [30]. Endometrial cytology (the lavage and cytobrushing of the uterus) has a very important place in the diagnosis of endometritis [14,31]. Subclinical cases of endometritis are best diagnosed based on the evaluation of neutrophil leukocyte infiltration by cytological examination [14,25,28]. The percentage of neutrophil leukocytes and the neutrophil leukocyte/lymphocyte ratio increase in postpartum uterine infections [32]. Cows determined to have a neutrophil leukocyte percentage >18% between postpartum days 20-33 [26], >10% between postpartum days 34-47 [14], and >5% between postpartum days 40-60 are considered to have developed subclinical endometritis [10]. In recent years, it has become possible to diagnose uterine infections by the polymerase chain reaction (PCR) technique [33], and based on the levels of acute-phase proteins (creatine kinase, haptoglobulin, α_1-acid glycoprotein, non-esterified fatty acid (NEFA), β-hydroxy butyric acid (BHBA) and nitric oxide levels) [34-37]. The reduction of dry matter intake by pregnant animals during advanced gestation results in the mobilisation of NEFA from fat tissue. The body levels of NEFA and BHBA increase [38]. Decreased levels of dry matter intake and increased NEFA levels inhibit the immune system and cows become prone to infection [38,39]. Hammon et al.[39] determined that serum NEFA and BHBA levels increased significantly in animals with subclinical endometritis and puerperal metritis, when compared to healthy animals. Although BHBA levels have been reported not to be correlated with the bactericidal capacity of polymorphonuclear leukocytes [39], literature reports are available, which suggest ketone bodies to induce adverse effects on the migration of polymorphonuclear leukocytes (PMN) and to damage the structure of PMN prior to their migration [40]. It has been indicated that the risk of the development of metritis is three-fold higher in animals with high BHBA levels during the first two weeks after parturition [41]. Walsh et al.[42] indicated that animals with increased BHBA levels presented with reduced conception rates after the first postpartum insemination, and suggested that increased BHBA levels led to a lower

number of conceived cows by postpartum day 140, and to a prolongation of the parturition-conception interval. In the past few years, the reagent test strips have also found use in the diagnosis of subclinical endometritis [37]. These test strips provide information on the hydrogen ion (pH level), leukocyte esterase, and protein levels of the uterine lavage fluid [43]. The level of leukocyte esterase is an indicator of the presence of white blood cells in the lavage fluid. The principle of measurement is based on the formation of a purple colour as a result of the reaction of indoxyl carbonic acid with neutrophil esterase. The intensity of the colour varies with the number of leukocytes involved in the reaction [44]. Animals with endometritis have been determined to present with increased pH values and increased leukocyte esterase and protein levels. It has been ascertained that the correlation of endometritis with the pH value is stronger than that with the leukocyte esterase level or protein level [43]. The diagnosis of pyometra is based on the ultrasonographic detection of a CL on the ovarian surface, fluid accumulation of heterogenic ecogenicity in the uterus lumen and uterine enlargement. Furthermore, although the cervix uteri is physiologically closed, in some cases the accumulation of pus may be observed in the vagina [14].

EFFECT OF UTERINE INFECTIONS ON OVARIAN FUNCTIONS AND FERTILITY

Uterine infections may cause the formation of chronic endometrial scars, the narrowing of the oviduct, and the adherence of the bursa to the ovaries (at an approximate rate of 2%). Although uterine infections have been suggested not to have any impact on the formation of a new follicular wave or on the peripheral level of the follicle stimulating hormone (FSH) [6], they have been detected to have effect on the hypothalamus and the hypophysis [45]. Once absorbed from the uterus lumen, endotoxins released from the wall of Gram negative bacteria pass into the peripheral blood circulation, and prevent the secretion of the gonadotropin releasing hormone (GnRH) from the hypothalamus and the luteal hormone (LH) from the hypophysis. These toxins also reduce the susceptibility of the hypophysis to the secretion of endocrine and exocrine GnRH [1,46,47]. Lipopolysaccharides secreted by Gram negative bacteria, inhibit the transcription of steroidogenic enzymes, including 17β-hydroxylase/17,20-lyase and P450 aromatase, and thereby, inhibit follicular activity [48]. In this context, it has been determined that, in animals in which the uterus is exposed to a high level of bacterial contamination following parturition, the growth of the first dominant follicle slows down, the oestrogen level decreases, when compared to healthy animals a smaller CL develops following ovulation, the production of the progesterone hormone decreases and the development of ovarian cysts increases [49,50]. On the contrary, Strüve et al.[51] suggested that the effect of metritis on luteal activity was temporary and limited to the first oestrus cycle after parturition, and indicated that no alteration occurred either in the size of the CL or in the progesterone levels in the following postpartum oestrus cycles.

Another disorder caused by uterine infections in endocrine functions is the impairment of the luteolytic mechanism. It has been determined that, in uterine infections, due to the damage of the endometrium, inadequacy of the secretion of PGF_2 alpha is observed [17,20], while the secretion of luteotropic PGE_2 from the uterine epithelial cells and stromal cells is induced as a result of the direct effect of bacterial toxins on these cells. It has been detected that PGE_2 leads to the persistence of the CL, and thus to the continued production of progesterone from the CL. In this event, the onset of oestrus is prolonged and the uterine immune mechanism, known to be susceptible to oestrogenic effect, proves to be insufficient [36,39].

Since genital infections cause the disorder of ovarian and uterine functions, the parturition-first insemination and parturition -conception intervals are prolonged [49,52]. Sandals et al.[53] indicated that clinical endometritis prolonged the parturition-conception interval for a period of 9 days. In another study, it was determined that the parturition-conception interval was 14 days longer in animals diagnosed with endometritis, in comparison to healthy animals [7]. Barlund et al.[28] suggested that the parturition-conception interval was 24 days longer in animals suffering from endometritis, when compared to healthy animals. Similarly, Goshen et al.[54] determined this interval to be 25 days longer in animals with endometritis. In a research conducted by Kasimanickam et al.[26], it was ascertained that the rate of pregnancy of animals with endometritis (41%) was lower than that of healthy animals (51%). Similarly, Goshen et al.[54] determined that the preganancy rate of animals with clinical metritis was 20% lower than that of healthy animals. Furthermore, LeBlanc et al.[9] detected a reduction of 27% in the total pregnancy rate due to chronic endometritis.

TREATMENT OPTIONS FOR UTERINE INFECTIONS

Postpartum uterine infections are treated with antibiotics, hormones or their combinations [55]. The use of these therapeutic agents is aimed at the elimination of pathogens from the uterus, the induction of the uterine immune system, and the elimination of the adverse effects of inflammation products on fertility [8,12].

Antibiotics and PGF_2 alpha analogues are used for the parenteral treatment of uterine infections [8]. The most commonly used antibiotic is ceftiofur hydrochloride, which is a member of the family of third-generation broad

spectrum cephalosporins [56]. In severe cases of uterine infection, the antibiotic treatment is combined with intravenous fluid treatment and the administration of anti-inflammatory agents [55].

Prostaglandin F_2 alpha has been shown to be effective in the treatment of uterine infections [57]. The administration of exogenous PGF_2 alpha both induces the secretion of endogenous PGF_2 alpha from the uterus and enables the development of the immune functions [27]. It has been reported that the use of PGF_2 alpha and its analogues during dioestrus resulted in approximately 90% of the cows showing oestrus [58]. Each oestrus enables the self-cleansing of the uterus by means of physiological leukocytosis and increased uterine motility [59]. Oestrogen increases uterine contractions, mucus production and leukocyte infiltration to the uterus [2,22]. Another systemic method used for the treatment of uterine infections is homeopathy. Homeopathic substances enable the organism to stimulate itself and trigger the onset of the treatment process [60]. The administration of *Tarantula cubensis* extract, a homeopathic substance, to animals in the early postpartum period, has been demonstrated to both decrease the incidence of retentio secundinarum and accelerate uterine involution [61]. Similarly, the last period of pregnancy levamisole treatment (2.5 mg/kg) has been reported to accelerate uterine involution [62]. Some reports indicate that trace elements have effect on the immune functions [63,64]. On the other hand, Machado et al.[65] reported that some trace elements (zinc, copper, manganese and selenium) did not alter leukocyte activity.

In cases of chronic endometritis and subclinical endometritis, intrauterine treatment is preferred. Intra-uterine treatment methods aim to maintain a high concentration of therapeutics in the endometrium. Thereby, it is ensured that the therapeutics penetrate at a limited level to the deeper layers of the uterus or to the other genitalia [8]. Mostly, the antibiotics of choice for intrauterine treatment are chlortetracycline [54] and ceftiofur hydrochloride [66]. Furthermore, peroxyacetic acid [67,68], meta-cresol sulfonic acid (Lotagen®) [69], ozone [70], mixtures prepared from healing herbs (AV/RMI/45) [71] and *Pelargonium sidoides* extract (EPs 7630) [72], proteolytic enzymes including chemotrypsin, trypsin and papain [73], liquid paraffin [74], hyperimmune sera produced against *Arcanobacter pyogenes* and *Escherichia coli* [75], formo-sulphathiazole, the phytotherapeutic EucaComp® [76] and hypertonic dextrose solution (50%) are also used for intrauterine treatment [77].

It has been demonstrated that treatment (peroxyacetic acid, Lotagen® and PGF_2 alpha) do not alter the levels of C-reactive protein, plasma lipopolysaccharides, and serum creatinase aspartate aminotransferase, which are all important criteria used in the diagnosis of endometritis [67,78,79].

Apart from intrauterine treatment methods, in recent years, intravaginal bacteriophages have also found use against *Esherichia coli*, which is a major cause of uterine infections, with an aim to prevent the development of infections in the postpartum period. Although having been demonstrated to show beneficial effects when used *in vitro* [80], the use of intravaginal bacteriophages has been proven to be ineffective *in vivo* [81].

ECONOMIC IMPLICATIONS OF UTERINE INFECTIONS

Uterine infections not only reduce fertility and cause infertility, but also adversely affect animal welfare [45]. Uterine infections cause major economic losses as a result of several reasons, including among others, reduced reproductive performance, decreased milk yield, the culling of infected animals from the herd, and the prolongation of the parturition-first insemination and parturition-conception intervals [49,52,82]. Taking into consideration all possible reproductive disorders, Overton and Fetrow [83] calculated the loss arising from the culling of animals from the herd up to day 60 postpartum, due to metritis, as 85 USD per case, the loss for reduced milk yield as 83 USD, and the total loss as 109 USD. It is estimated that the annual cost of metritis ranges between 329 USD and 386 USD per case. The economic implications of endometritis have not been investigated in detail in Turkey before. In a previous study conducted in the United Kingdom between the years 1998 and 1999, the daily cost of the prolongation of the parturition-conception interval, due to endometritis, to the holding was reported as 3 Euros [84]. In another research conducted in the United Kingdom, the direct and indirect costs of uterine infections at country level were reported to exceed 16 million Euros per year [85].

RECOMMENDATIONS

The peripartum period is of critical importance for uterine health and fertility. The increased growth rate of the foetus in the advanced stages of gestation and the start of milk production after parturition increase the energy requirements of the animal. This requires the feeding of animals with good quality roughage and high-energy feed. The ideal body condition score for bovine animals in this period is indicated as 3.00-3.75. A negative energy balance triggered by poor nutrition after parturition affects the restart of the oestrus cycle, the normalisation of the sexual cycle, and the success achieved with artificial insemination. The cleanliness of the calving paddocks, and compliance with disinfection and hygiene rules during interventions to aid birth and dystocia, have an important role in reducing the risk of uterine infections. Regular controls during the postpartum period enable the monitoring of the uterine involution process, the differentiation of

physiological and pathological changes and the treatment of infected animals without delay. Furthermore, the access of the veterinary practitioner to the records kept on the farm and to information on the previous parturition of the cow, the diseases the animal has undergone, whether the animal has given brith to twins, the oestrus intervals, findings of the last gestational examination and previous inseminations of the animal, as well as the oestrus and ovulation synchronization protocols and treatments the animal has been subjected to, all shed light on the reproductive problems of the animal and aid in diagnosis and treatment.

CONCLUSION

This review provides an assessment of the current approaches to uterine infections. Light uterine inflammation is generally eliminated by means of the immune system of the organism. Uterine infections not able to be eliminated by the defence system of the body affect the reproductive performance. Although the economic implications of uterine infections are not able to be precisely measured, these infections are well known to have negative effects of varying degree on both the reproductive performance and yields. With the acquisition of increased information on the correlation between uterine infections, the immune system and reproductive performance, it has become ever more important to develop protection and treatment strategies for infertility caused by uterine infections.

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LETTER to EDITOR

Overlooked Infection in a Dog with Degenerative Discospondylitis and Hip Joint Osteoarthritis: Ruminant Originated Brucellosis

(Dejeneratif Diskospondilitis ve Koksafemoral Osteoartritisli bir Köpekte Gözden Kaçan Enfeksiyon: Ruminant Kökenli Brusellozis)

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Dear Editor,

Canine degenerative osteoarthritis and discospondylitis are serious disorders of locomotor system with many etiologic factors contributing to pathogenesis [1,2]. Canine brucellosis caused by *Brucella canis* is one of these factors and enlargement of lymph nodes, uveitis, osteomyelitis, polyarthritis, glomerulonephritis, pyogranulomatous dermatitis, epididymitis, orchitis, scrotal dermatitis (sometimes scrotal necrosis) are other clinical signs of this infection [3-6]. This letter is written to underline the possibility of canine brucellosis caused by other *Brucella* species, and to share an interesting case of dog brucellosis originated from ruminants with colleagues.

A 4½ years old male Labrador dog was brought to the clinic with complaints of reluctance to walk; inability to jump into a car; growling when his back was touched and lethargy. As it was learned from the patient's story, the complaints were become apparent during past 6 months, but since two years he does not want to sit after walking due to swelling and pain in the testicles, and therefore he had been castrated. On physical examination, body temperature, pulse and respiratory rates were within normal values and pain was recorded on palpation of the left hip joint and lumbar region. When raised on hind legs, it was noted that he could not tolerate this position more than 30 seconds. Laboratory examination revealed that WBC count (19.57 x10^9/L), % values of basophils and neutrophils were increased and % value of lymphocytes was decreased. Serum CRP level was quite high (119 mg/L). On radiographic examination, signs of degenerative osteoarthritis were observed in right hip joint *(Fig. 1A* and *B)* and lumbar-coccygeal vertebrae *(Fig. 1C* and *D)*. A *Brucella* infection had aroused suspicion due to the orchitis and castration story. His serum sample gave negative result for *Brucella canis* with an immunochromatographic test kit (Brucella IC®, Biopronix, Agrolabo SpA, Italy), but it was positive with Rose Bengal plate test (SeroLam®, Seromed, Istanbul) which detects IgG and IgM formed against smooth *Brucella* species. According to the test results, dog was thought to be infected with one of these bacteria species: *Brucella melitensis* (sheep and goats), *Brucella abortus* (cattle) or *Brucella suis* (swine). While this development has been shared with the owner, it was learned that the dog had run around in neighboring goat farm since his puppyhood. Isolation of specific bacteria was not able because synovial fluid sampling of the relevant joints was not possible. Based on the serological tests results and owner's last information, the dog's disease was considered as ruminant originated brucellosis, and concerned pathologies (orchitis, diskospondylitis and degenerative right hip osteoarthritis) were thought to be related to this overlooked and chronic infection. Dog was treated for 2 months with doxycycline (2 times a day 300 mg, oral route, Monodoks® 100 mg capsules, Deva, Istanbul) and streptomycin sulfate (1 g IM and in 1, 3, 5 and 8th weeks for 7 days, Streptomycin® vial, IE ULAGAY, Istanbul). After 1 month of therapy, blood serum CRP level was found in normal levels (9 mg/L). By future phone calls with the owner, it was learned that the dog can swim, get in the car, play ball and has no more back pain.

The presented case teaches us 3 important lessons: **1-** Dogs with epididymitis, orchitis, scrotal dermatitis, uveitis, osteoarthritis or discospondylitis may be candidates of canine brucellosis. **2-** During serological examinations, we must not only refer with *Brucella canis* specific tests. **3-** Serological tests for *B. melitensis*, *B. abortus* or *B. suis* infections must absolutely be in diagnostic protocols particularly for dogs whose close contact with farm animals or have the possibility of eating farm animals' placenta or drinking their milk.

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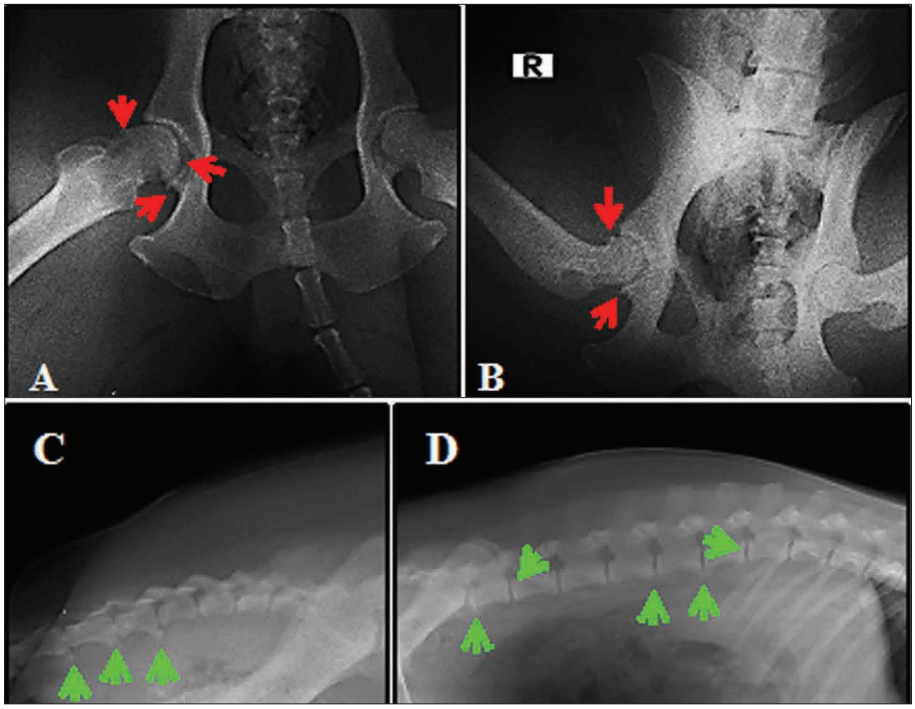

Fig 1. Radiographic appearance of right hip joint (A) and (B) with degenerative osteoarthritic lesions (red arrows). Similar lesions *(green arrows)* in coccygeal (C) and lumbar (D) vertebrae and joints.

Şekil 1. Sağ kalça ekleminde (A) ve (B) dejeneratif osteoartritis'e işaret eden lezyonlar (kırmızı oklar) ile koksigeal (C) ve lumbar (D) vertebral kemik ve eklemlerdeki benzer lezyonların *(yeşil oklar)* radyografik görünüşü.

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YAZIM KURALLARI

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Başvuru sırasında yazarlar yazıda yer alacak şekilleri (13 X 18 cm boyutlarından büyük olmamalı) online makale gönderme sistemine yüklemelidirler. Yazının kabul edilmesi durumunda tüm yazarlarca imzalanmış *Telif Hakkı Devir Sözleşmesi* editörlüğe gönderilmelidir.
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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) 10 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 4 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.
Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 2 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.
Gözlem, 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 kaynaklardan oluşmalı ve 4 sayfayı geçmemelidir.
Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 1 sayfayı geçmemelidir.
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Çeviri, makalenin orijinal formatı dikkate alınarak hazırlanmalıdır.
Yazarla ilgili kişisel ve kuruma ait bilgiler ana metin dosyasına değil, on-line başvuru sırasında sistemdeki ilgili yerlere unvan belirtilmeksizin eklenmelidir.
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.
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