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Bu Sayının Hakem Listesi *(alfabetik sıra)*
The Referees List of This Issue *(in alphabetical order)*

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ÇELİK İlhami	Selçuk Üniversitesi Veteriner Fakültesi Histoloji ve Embriyoloj Anabilim Dalı
ÇETİNKAYA Nurcan	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı
ÇOBAN Ömer	Atatürk Üniversitesi Veteriner Fakültesi Zootekni Anabilim Dalı
ÇOBAN SABUNCUOĞLU Nilüfer	Atatürk Veteriner Fakültesi Zootekni Anabilim Dalı
DAĞOĞLU Gürdal	Fırat Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı
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EFE Ercan	Kahramanmaraş Sütçü İmam Üniversitesi Ziraat Fakültesi Zootekni Bölümü
ERDOĞAN Zeynep	Mustafa Kemal Üniversitesi Veteriner Fakültesi Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı
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GÖRGÜLÜ Murat	Çukurova Üniversitesi Ziraat Fakültesi Zootekni Bölümü
GÜL Mehmet	Atatürk Üniversitesi Veteriner Fakültesi Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı
GÜLBAHAR M. Yavuz	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi Patoloji Anabilim Dalı
GÜLER Leyla	Konya Veteriner Kontrol ve Araştırma Enstitüsü
GÜRBÜZ Ümit	Selçuk Üniversitesi Veteriner Fakültesi Besin Hijyeni ve Teknoloji Anabilim Dalı
HADİMLİ Hasan Hüseyin	Selçuk Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı
HAYIRLI Armağan	Atatürk Üniversitesi Veteriner Fakültesi Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı
İMİK Halit	Atatürk Üniversitesi Veteriner Fakültesi Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı
KABAK Murat	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi Anatomi Anabilim Dalı
KALAYCIOĞLU Atilla Taner	Kafkas Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı
KAMİLOĞLU Nadide Nabil	Kafkas Üniversitesi Veteriner Fakültesi Fizyoloji Anabilim Dalı
KARACA Fikret	Mustafa Kemal Üniversitesi Veteriner Fakültesi Dölerme ve Suni Tohumlama Anabilim Dalı
KARACA Orhan	Adnan Menderes Üniversitesi Ziraat Fakültesi Zootekni Bölümü
KARAKURUM Çağrı	Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi İç Hastalıkları Anabilim Dalı
KAYAALP Tamer	Çukurova Üniversitesi Ziraat Fakültesi Zootekni Bölümü
KAYGISIZ Ali	Kahramanmaraş Sütçü İmam Üniversitesi Ziraat Fakültesi Zootekni Bölümü
KUL Oğuz	Kırıkkale Üniversitesi Vetreriner Fakültesi Patoloji Anabilim Dalı
MATUR Erdal	İstanbul Üniversitesi Veteriner Fakültesi Fizyoloji Anabilim Dalı
MUĞLALI Hakan	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi Hayvan Besleme ve Beslenme Hastalıkları Anabilim Dalı
NİSBET Cevat	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi Biyokimya Anabilim Dalı
OĞUZ Halis	Selçuk Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı
ORAL Hasan	Kafkas Üniversitesi Veteriner Fakültesi Doğum ve Jinekoloji Anabilim Dalı
ORTATATLI Mustafa	Selçuk Üniversitesi Veteriner Fakültesi Patoloji Anabilim Dalı
ÖZTÜRK Dilek	Mehmet Akif Ersoy Üniversitesi VeterinerFakültesi Mikrobiyoloji Anabilim Dalı
ÖZTÜRK Ergin	Ondokuz Mayıs Üniversitesi Ziraat Fakültesi Yemler ve Hayvan Besleme Anabilim Dalı
PAPUÇCUOĞLU Serhat	İstanbul Üniversitesi Veteriner Fakültesi Dölerme ve Suni Tohumlama Anabilim Dalı
PEKCAN Zeynep	Kırıkkale Üniversitesi Veteriner Fakültesi Cerrahi Anabilim Dalı
SEZEN Yavuz	Gebze İleri Teknoloji Enstitüsü
SÖZMEN Mahmut	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi Patoloji Anabilim Dalı
ŞEKER İbrahim	Fırat Üniversitesi Veteriner Fakültesi Zootekni Anabilim Dalı
TIPIRDAMAZ Sadettin	Selçuk Üniversitesi Veteriner Fakültesi Anatomi Anabilim Dalı

Bu Sayının Hakem Listesi *(alfabetik sıra)*
The Referees List of This Issue *(in alphabetical order)*

TİMURKAN Sema	Fırat Üniversitesi Veteriner Fakültesi Histoloji ve Embriyoloji Anabilim Dalı
TÜRKER Hakan	Abant İzzet Baysal Üniversitesi Fen Edebiyat Fakültesi Biyoloji Bölümü
TÜZER Erkut	İstanbul Üniversitesi Veteriner Fakültesi Parazitoloji Anabilim Dalı
URAL Kerem	Adnan Menderes Üniversitesi Veteriner Fakültesi İç Hastalıkları Anabilim Dalı
UZLU Erdoğan	Kafkas Üniversitesi Veteriner Fakültesi İç Hastalıkları Anabilim Dalı
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YILDIZ Hüseyin	Uludağ Üniversitesi Veteriner Fakültesi Anatomi Anabilim Dalı
YÖRÜK Mecit	Yüzüncü Yıl Üniversitesi Veteriner Fakültesi Histoloji ve Embriyoloji Anabilim Dalı

İÇİNDEKİLER
(CONTENTS)

ARAŞTIRMA MAKALELERİ
(Research Articles)

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RESEARCH ARTICLE

Seroprevalence of *Dirofilaria immitis, Ehrlichia canis* and *Borrelia burgdorferi* in Dogs in Iğdır Province, Turkey [1]

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[1] *This project was supported by the Commission for the Scientific Research Projects of Kafkas University (Project Number: 2008-VF-07)*

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Summary

In this study, by using a Snap3dx test kit, 100 dogs sera were examined. *Dirofilaria immitis* infection was detected in 40 dogs (40%) and *Ehrlichia canis* antibodies were present in just 1 dog (1%), *Borrelia burgdorferi* antibodies were not detected in the test. Twenty-two of dogs (22%) are infested with ticks. A total of 42 ticks, 9 of female and 33 of male, were collected from dogs. Ticks collected from dogs were *R. sanguineus* 76.2% (32/42) and 23.8% of them (10/42) were *Rhipicephalus spp.* Considering the prevalence of potential vectors (mosquitoes and ticks), it is concluded that dirofilariosis and ehrlichiosis cases are often encountered in Iğdır province.

Keywords: *Dirofilaria immitis, Ehrlichia canis, Borrelia burgdorferi, Dog, Iğdır*

Iğdır Yöresinde Köpeklerde *Dirofilaria immitis, Ehrlichia canis* ve *Borrelia burgdorferi*'nin Seroprevalansının Araştırılması

Özet

Bu çalışma ile Iğdır yöresinde sahipli 100 köpekten elde edilen serumlarda Snap 3dx kiti kullanılarak *Dirofilaria immitis* antijenine %40, *Ehrlichia canis* antikoruna %1 oranında rastlanmış, *Borrelia burgdorferi* antikoru ise saptanamamıştır. Köpeklerin 22'si (22%) kenelerle enfeste bulunmuştur. Köpeklerden toplanan 42 adet kenenin 9'unun dişi, 33'ünün erkek olduğu görülmüştür. Kenelerin %76.2'sinin (32/42) *R. sanguineus* ve %23.8'inin (10/42) *Rhipicephalus spp.* türü olduğu belirlenmiştir. Iğdır yöresinde potansiyel vektörlerin (sivrisinek ve kene) yaygınlığı da göz önünde bulundurulduğunda, dirofilariosis ve ehrlichiosis vakalarıyla sıklıkla karşılaşılabileceği kanısına varılmıştır.

Anahtar sözcükler: *Dirofilaria immitis, Ehrlichia canis, Borrelia burgdorferi, Köpek, Iğdır*

INTRODUCTION

Dirofilariosis, ehrlichiosis and lyme borreliosis are arthropod-borne diseases that are seen in domestic dogs as in many species of animals. Dogs infected with these diseases; can be diagnosed as characteristic symptoms, are shown non-spesific clinical appearance or even asymptomatic. Therefore, factors are needed to seen directly as well as serological methods [1].

The adults of *Dirofilaria immitis* are known as the most pathogenic species in filarial nematodes. Parasite is commonly found in the pulmonary arteries, right ventricle, *V. cava cranialis, V. hepatica,* bronchioles, interdigital cyst and abscesses, brain arteries, spinal canal and eye of dogs, other canids and humans [2-6]. While in some dogs no symptom was observed clinically, in some, dyspnea, hoarseness, fatigue, rapid breathing, cough, collapse, asphyxia, anorexia, pathological sounds in heart and lungs, different types of dermatitis, cachexia, jaundice and hemoglobinuria are seen. The vectors of *D. immitis* are genus of female *Anopheles, Aedes, Culex, Myzorhynchus, Armigeres* and *Taeniorhychus* [2-6].

Dirofilaria immitis is described for the first time in the world in a dog from Alabama in 1856 by Joseph

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Leidy. It has been reported for the first time in 1951 in Turkey [2-7]. To diagnose *D. immitis* in dogs, Thick Drop, Modified Knott, Microhematocrit-Capillary Sedimentation, Saponin Concentration, Membrane Filtration-Aside Phosphates Histochemical Staining, radiology, angiography, ultrasonography, serological techniques (Indirect Flourescent Antibody Test, Counterimmunoelectrophoresis, Latex Agglutination, Hemagglutination), Polymerase Chain Reaction (PCR), and such as Dirochek, Petchek, Snap commercial ELISA test kits are used [2-6,8-10].

In studies, in different geographical regions of the world, using different diagnostic methods the prevalence of *D. immitis* in dogs were determined between 0-73.5% [1,11-20]. In Turkey, in studies based on microscopy, necropsy and serology the prevalence of *D. immitis* in dogs were determined between 0-46.2% [3,9,21-30].

Ehrlichiosis (tropical pancytopenia) is a rickettsial disease that dogs and human are infected with vector ticks and characterized by reduction of the blood-shaped elements. The name of the disease in dogs is canine monocytic ehrlichiosis and the factor is *Ehrlichia canis*. Cases of ehrlichiosis in dogs are found especially in tropical and subtropical regions [31-34]. The vector of disease is *Rhipicephalus sanguineus* ticks. The disease is transmitted to dogs by infected ticks or blood transmission from infected dogs and seen in acute, chronic and subclinical forms [35]. In acute form; weight loss, fever, dyspnea, lympadenopathy, edema in extremity and scrotum, epistaxis, anorexia, recession, eye-nasal discharge, irritability and neurological symptoms are seen. Generally no clinical signs are observed in subclinical form [34,36,37]. Peripheral blood examination, Western Blot and ELISA techniques can be performed to diagnose the disease. However for a definitive diagnose indirect flourescent antibody test IFAT is recommended to use [34,36,38]. In Turkey, in a study 67.8% with IFAT and 57.3% with dot-ELISA seropositivity were detected [39], and also case of ehrlichiosis in dogs was reported in another region of Turkey [40]. In Aegean region of Turkey the prevalence of ehrlichiosis was detected 41.5% by nested PCR [41]. Many studies have been done about the prevalence of *E. canis* infection in various countries [13,33,42-48].

Lyme disease, especially transmitted by Ixodes genus ticks, caused by *Borrelia* genus spirochetes is a zoonotic infection [49-51]. Young dogs are more susceptible to disease and the most obvious symptom is acute polyarthritis. In chronic cases lameness may be occured. In addition to that in dogs symptoms such as fever, lymphadenopathy, anorexia may be seen [52,53]. Disease can be identified by serological methods (IFAT, ELISA, Western Blot etc.) with the help of clinical findings [51,53,54].

It has been reported that Lyme disease is one of the most common disease transmitted by ticks in Europe (2.1-53.7%), Brazil (9.7%) and North America (2.3-76.3%) [13,33,47,48,54-56]. *Borrelia burgdorferi* is the factor of disease was also isolated from vector *Ixodes ricinus* species ticks [57-60]. While in a study [53] in a dog that 2 years old, male and race of Saint Bernard, Lyme disease was found, in another study [61] the infection rate was determined as 27.75% in Turkey.

Mosquito populations are common in Iğdır province [62]. This research was carried out to determine the seroprevalence of *D. immitis*, *E. canis*, and *B. burgdorferi* in Iğdır province where potential vectors are common.

MATERIALS and METHODS

A total of 100 owned and remain outside dogs, 16 of female and 84 of male, were randomly selected. Blood samples were drawn from the cephalic vein in four different focus of Iğdır province (Baharlı, Küllük, Pirli and Söğütlü). In relation to age, 66 of the dogs were 0.5-3 years old, 22 were 4-6 years old and 12 were 7 and older dogs. In addition to that all dogs were examined for ticks, and ticks were collected from dogs which are infested.

The prevalence of *D. immitis, E. canis* and *B. burgdorferi* were simultaneously determined by using a commercial in-vitro examination kit (Snap 3dx, Idexx Lab., USA) that detects *D. immitis* antigen, *E. canis* (P30 and P30-1outer membrane proteins), and *B. burgdorferi* (C_6 peptid) antibodies in dog sera.

C_6 ELISA test can be conducted in dog sera, plasma or whole blood. The C6 synthetic peptide was conjugated to bovine serum albumin (BSA) and to horseradish peroxidase (HRP) by using standard methods. The HRP–C6 peptide conjugate was contained in a conjugate diluent containing HRP-labeled antiheartworm antibody, HRP-labeled *E. canis* peptide conjugate, nonspecific proteins, and detergents. If *Borrelia burgdorferi* and/or *E. canis* antibody or *D. immitis* antigen present in the sample, bind to the synthetic peptide–HRP conjugate and to the synthetic peptide–BSA conjugate.

C_6 ELISA test construction is shown in the diagram below. Each kit contains 8 ml *D. immitis / E. canis /B. burgdorferi* Horseradish peroxidase conjugate, transfer pipette, sample tubes and Snap device. Each Snap device contains 0.4 ml washing and 0.6 ml substrate solution. First of all specimens and kit reagents are heated at room temperature (15-25°C). The latter stages are performed according to the kit procedure as *Fig. 1*.

Statistical analysis were conducted by using Chi-squared test [63].

RESULTS

In 40 of examined 100 dogs (40%) *D. immitis* antigens were detected (an example in *Fig. 2*). A 6 years old and

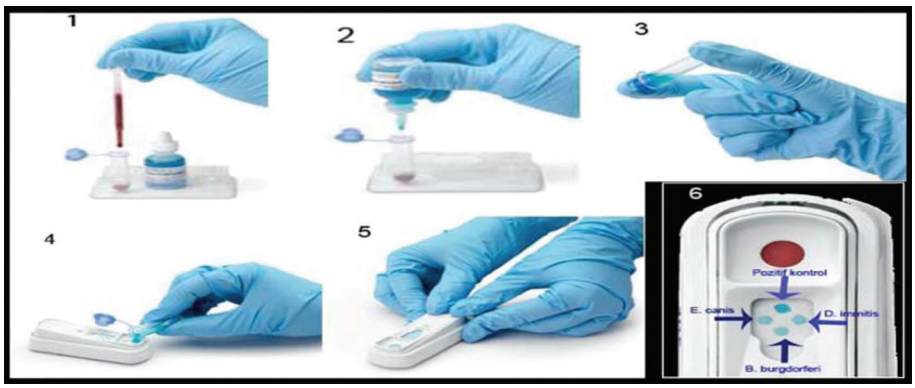

Fig 1. Procedure of Snap 3dx test

Şekil 1. Snap 3 dx testinin yapılışı

1) Three drops of suspicious sera are spotted to the sample tube with transfer pipette 2) Four drops of conjugate are spooted to the sample tube 3)The lid of sample tube is closed and mixed by inverting 3-5 times 4-) Snap device is placed horizontally on a flat surface, all of the content in sample tube is emptied to the sample well 5) Activator button is pressed when coloration in activation point starts 6) The results are evaluated in 8th min

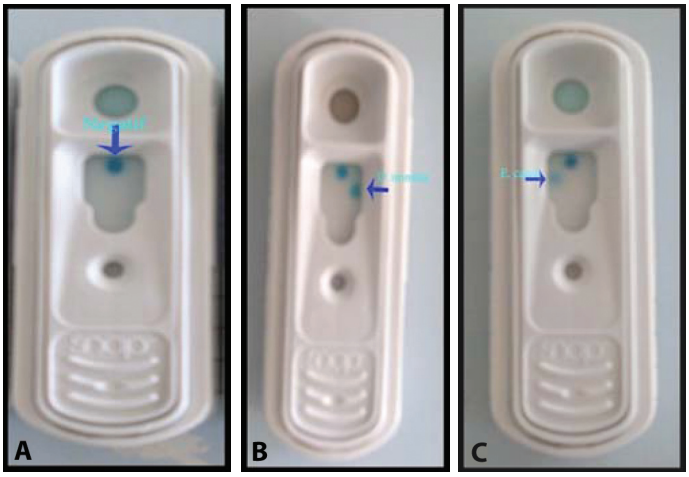

Fig 2. (ABC)- Appearance of positive and negative samples in Snap 3dx test kit

Şekil 2. (ABC)- Pozitif ve negatif örneklerin Snap 3dx test kitinde görünümü
(A: negative, B: *D. immitis* antigen, C: *E. canis* antibody)

Table 1. *The seroprevalence of D. immitis and E. canis correlated with sex and age in Iğdır province*

Tablo 1. *Iğdır yöresinde D. immitis ve E. canis seroprevalansı'nın yaş ve cinsiyet ile ilişkisi*

Dogs		*D. immitis* Antigen		*E. canis* Antibody	
		Infected/ Examined	(%)	Infected/ Examined	(%)
Sex	Female	9/16	(56.25)	0/16	(0.0)
	Male	31/84	(36.9)	1/84	(1.19)
Age	0.5-3	22/66	(33.3)	0/43	(0.0)
	4-6	13/22	(59.1)	1/45	(2.2)
	7 ≥	5/12	(41.7)	0/12	(0.0)
Total		40/100	(40.0)	1/100	(1.0)

Table 2. *Distribution of seroprevalence of D. immitis and E. canis correlated with foci in Iğdır province*

Tablo 2. *Iğdır yöresinde D. immitis ve E. canis seroprevalansı'nın odaklara göre dağılımı*

Province	*D. immitis* Antigen		*E. canis* Antibody	
	Infected/ Examined	(%)	Infected/ Examined	(%)
Küllük	14/25	(56.0)	0/25	(0.0)
Pirli	6/26	(23.1)	1/26	(3.8)
Baharlı	16/25	(64.0)	0/25	(0.0)
Söğütlü	4/24	(16.7)	0/24	(0.0)

male dog (1%) infested with *R. sanguineus* tick, and in which *E. canis* antibody was detected, while *B. burgdorferi* antibody was not determined. The seroprevalence of *D. immitis* and *E. canis* correlated with sex and age in Iğdır province were shown in *Table 1*. Foci and rates of infection in which they encountered were shown in *Table 2*.

Twenty-two of dogs (22%) are infested with ticks. A total of 42 ticks, 9 of female and 33 of male, were collected from dogs. Ticks collected from dogs were *R. sanguineus* 76.2% (32/42) and 23.8% of them (10/42) were *Rhipicephalus* spp.

DISCUSSION

In many countries of the world, many researches have been done to determine *D. immitis, E. canis* and *B. burgdorferi* in dogs by using different diagnostic techniques. Snap 3dx kit that can diagnose simultaneously these three diseases was used for this purpose. As a result of previous researches, the prevalence of *D. immitis* in dogs were determined between 0-46.2% [3,9,21-29] and *E. canis* were determined between 41.5-67.8% [39-41], while in a study [53] in a dog that 2 years old, male and race of Saint Bernard, *B. burgdorferi* was found, in another study [61] the infection rate was determined as 27.75% in Turkey. In this study, *D. immitis* infection was detected in 40 dogs (40%)

and *E. canis* antibodies were present in 1 dog (1%). But *B. burgdorferi* antibody was not determined.

In geographical regions where mosquito population is quite high and dogs are remained outside [62], the prevalences of *D. immitis* were reported in high percentages. Also, in this study the prevalence of *D. immitis* was determined highly (40%). In addition to that, it has been reported that the prevalence of *D. immitis* increased significantly together with age [11,13,23]. Also our findings seem to confirm this criterion. Because dogs between 0.5-3 age group has the infection rate as 33.3% (22/66), dogs between 4 years and older age group has the rate as 52.9% (18/34) (P=0.05).

No significant differences between the sexes were reported in some researches regarding *D. immitis* infections [12-14,28]. In this study, seropositivity was detected in 9 of 16 female (56.25%), and 31 of 84 male (36.9%) dogs. No significant differences between the sexes were observed (P>0.05).

In this study that carried out in Iğdır province, with Snap 3dx commercial ELISA kit, in only 1 of 100 dogs had *Ehrlichia canis* antibody in their sera. But *B. burgdorferi* antibody was not found in the sera. This situation can be explained by absence of *Ixodes ricinus* which is the vector of *B. burgdorferi* in dogs in Iğdır province.

In conclusion, because of Iğdır province has different geographical structure and season from region, also taking into account the population of potential vectors (mosquito and tick), arrived at an opinion that can be encountered with dirofilariosis and ehrlichiosis cases. Mosquitoes and ticks, that they are the vectors of many diseases, are common in this region. But there are not enough research about them. So, in order to determine the vector-disease relationships in all animals in this region, more detailed studies are needed.

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RESEARCH ARTICLE

Risk Factors Associated with Passive Immunity, Health, Birth Weight and Growth Performance in Lambs: III- The Relationship among Passive Immunity, Birth Weight, Gender, Birth Type, Parity, Dam's Health, and Lambing Season [1]

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Summary

This study was designed to investigate the effect of risk factors such as gender, birth type, parity, dam's health and lambing season associated with passive immunity and birth weight and to also determine interrelationship between passive immunity and birth weight. This study included 301 ewes and 347 lambs born to them on two local Akkaraman crossbred flocks in Kars. Lambs were blood sampled for serum IgG concentration at 24 hours after birth (SIgGC-24) and epidemiological parameters were recorded at birth. Parity, type of birth and gender were significantly associated with birth weight (R^2=0.339, P<0.001) on multivariable stepwise regression analysis as single born lambs (P<0.001), male (P<0.001) and lambs born to dams previously lambed (P<0.001) were significantly heavier. There was a significant (P<0.001) and positive (R^2=0.136) linear relationship between birth weight and passive immunity, but only in ill lambs during the neonatal period. In General Linear Model (GLM), lambs born with low birth weight (≤3 kg) had significantly lower SIgGC-24 than those born with medium (>3 to ≤ 4) (P<0.01) or high (>4 kg) (P<0.001) birth weight. Similarly lambs born as a twin and born to unhealthy dams had significantly lower SIgGC-24 (P<0.05). In conclusion, some farm management practices and animal characteristics were associated with birth weight and passive immunity and also birth weight had affect on passive immunity. For a productive and profitable farming, producers should take these variables into account and develop appropriate management strategies.

Keywords: *Lamb, Serum IgG, Passive immunity, Birth weight, Risk factors*

Kuzularda Pasif İmmünite, Sağlık, Doğum Ağırlığı ve Büyüme Performansı İle İlgili Risk Faktörleri: III- Pasif İmmünite, Doğum Ağırlığı, Cinsiyet, Doğum Tipi, Anne Sağlığı, Doğum Sayısı ve Kuzulama Sezonunun Birbiriyle İlişkisi

Özet

Bu çalışma pasif immunite ve doğum ağırlığı üzerine etkili cinsiyet, doğum tipi, kuzulama sezonu, anne doğum sayısı ve sağlığı gibi bazı risk faktörlerinin araştırılması ve ayrıca pasif immunite ve doğum ağırlığının birbiriyle olan ilişkisinin belirlenmesi amacıyla gerçekleştirildi. Çalışma Kars'ta 301 koyun ve bunlardan doğan 347 Akkaraman melezi kuzuyu içeren iki sürüde yürütüldü. Doğumdan 24. saat sonra serum IgG konsantrasyonlarını (SIgGC-24) belirlemek için kan örneği alındı ve doğumda epidemiyolojik parametreler kaydedildi. Çoklu adımsal regresyon analizine göre doğum tipi, cinsiyet ve anne doğum sayısı, doğum ağırlığını önemli seviyede ilişkili faktörler olarak belirlendi (R^2=0.339, P<0.001). Tek (P<0.001), erkek (P<0.001) ve daha önce doğum yapmış annelerden doğan (P<0.001) kuzuların doğum ağırlığı daha yüksek bulundu. Yalnızca neonatal periyotta hastalık tespit edilen kuzularda pasif immünite ve doğum ağırlığı arasında önemli (P<0.001) ve pozitif (R^2=0.136) bir linear ilişki olduğu belirlendi. Genel linear modele göre düşük doğum ağırlığı (≤3 kg) ile doğan kuzuların SIgGC-24'ları orta (>3-≤4 kg) veya yüksek (>4 kg) doğanlara göre önemli seviyede (sırasıyla P<0.01ve P<0.001) düşük belirlendi. Benzer şekilde ikiz veya hasta annelerden doğan kuzuların SIgGC-24'ları önemli seviyede (P<0.05) düşük bulundu. Sonuç olarak bazı çiftlik sevk-idare uygulamaları ve hayvan karakteristikleri doğum ağırlığı ve pasif immunite ile ilişkili bulundu ve ayrıca doğum ağırlığı pasif immunite üzerine etkiliydi. Çiftlik verim ve karlığını arttırmak için üreticilerin bu faktörleri göz önünde tutması ve uygun sevk-idare stratejileri geliştirmesi gerekir.

Anahtar sözcükler: *Kuzu, Serum IgG, Pasif İmmunite, Doğum Ağırlığı, Risk Faktörleri*

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INTRODUCTION

Morbidity and mortality are often cited as the most common problems in young lambs and it is a major cause of low productivity of sheep [1-3]. Lambs are born with hypogammaglobulinemia due to syndesmochorial placentation and must be fed colostrum as a source of immunoglobulin-G (IgG) during the neonatal period. This process is named as passive immunity, determined by measuring serum IgG concentration at 24 h after birth (SIgGC-24). Numerous studies in the past three decades correlated neonatal diseases with inadequate serum IgG, or failure of passive transfer of immunity (FPT) in animals and pointed out the importance of IgG in preventing infectious diseases of neonates [4-8]. Recent studies also reported a significantly positive relationship between SIgGC-24 and growth performance in lambs at different stages [9-12]. It is therefore, of paramount importance to investigate the underlying causes of FPT. Only a small number of studies have been conducted to clarify the effect of some environmental factors and animal characteristics on passive immunity in lambs, but these effects were not clearly elucidated [6-8,13-15].

All mammalian species have an 'optimum' birth weight that facilitates uncomplicated natural delivery and improves the rate of neonatal survival [16]. Thus, the birth weight of lambs plays an important role in achieving desirable sheep production as studies determined that birth weight is associated with enhances growth performance and decreased disease incidence [9,17-21]. The birth weight has also been associated with passive immunity [4]. Low birth weight leads to many unfavorable factors including low growth rate especially in the first three months of life, increased risk of morbidity and mortality because these lambs are physically weak to stand and suckle adequate colostrums and less viability at birth that have significant effects on lamb survival and growth performance [9,15,17,22-25]. Two greatest important contributors to problems caused by morbidity, mortality and poor growth performance are low birth weight and FPT [26].

Production and profitability of sheep farms explicitly depends on maintaining healthy lambs with desirable live weight gain. Therefore, it is of paramount importance to identify the cause of diseases and weight loss in lambs and to take appropriate measures accordingly.

The study was designed to determine the relationship between passive immunity and birth weight and also to investigate presumptive effects of gender, type of birth, lambing season, parity and dam's health on passive immunity as well as to investigate the effect of environmental factors and animal characteristics on birth weight in Akkaraman crossbred which constitutes 50% of the total sheep population in Turkey [27].

MATERIAL and METHODS

Animals, Data Collection, Farms Management

This study was carried out in two sheep farms located in the central Kars in North-Eastern Anatolia, Turkey, in 2009. All ewes and lambs were kept under identical feeding and management conditions. Management was typical of North-eastern Anatolian flocks with lambs being born in winter (December to February) or spring (March to May), and being raised intensively. At birth, the lambs were ear-tagged and registered with an individual identification number, and gender, date of birth, parity, and dam's ear tag number and type of birth were recorded for each lamb. The lambs were weighed at birth (before colostrum intake) using a bascule [CASIA DB2-150 kg (±30 g)]. After this procedure, lambs were allowed to naturally suckle their dams.

Blood Sample Collection, IgG Analysis

Blood samples were collected from all lambs by jugular venipuncture at 24±1 h. Serum was harvested by centrifugation and stored at –20°C until analysis. Passive immune status or serum IgG concentrations 24th h after birth (SIgGC-24) were measured using a commercial ELISA kits (Bio-X Competitive ELISA Kit For Ovine Blood Serum IgG Assay-BIO K 350, Bio-X Diagnostics, Belgium).

Clinical Examination

Clinical examination were performed as previously defined by the authors [2]. The health status of the lambs was monitored on farms by visits made on a daily basis during the neonatal period. Throughout the study period, ewes determined to have disease (mastitis, pneumonia, enteritis, pregnancy toxaemia etc.) were categorized as ill and recorded with their ear tag number.

Statistical Analysis

The present study included 301 Akkaraman crossbreeds and 347 lambs born to them. However, lambs whose parameters (birth weight, gender, type of birth, health status, passive immunity or serum IgG level and birth date for lambs; parity and health status of dams) were not recorded were excluded from the study thus leaving 322 lambs to be included in this study.

Study consisted of two sections. In first section, the affect of gender, type of birth, lambing season, parity and dam health on the birth weight was studied. Mean ± SD (Range) values for each parameter was calculated. Parity was categorized as 1, 2, 3 and ≥4. The Tukey HSD test was used to identify differences in birth weight in lambs grouped according to parity. Independent Samples T test was used to identify variations in birth weight according to gender, type of birth, lambing season and health of the dam. Multivariable stepwise linear regression analysis (MSRA)

was used to evaluate the association between type of birth, gender, health of dams and lambing season (considered as categorical independent variables), and parity (considered as continuous independent variables) and birth weight (continuous dependent variables). The linear regression model with the all potential independent variables considered in the study defined as follows: $Y = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 + \varepsilon$ Where Y denotes birth weight, x_1 is the type of birth (single=0, twin=1), x_2 is the gender (male=0, female=1), x_3 is the parity (1=1, 2=2, 3=3, ≥4=4), x_4 is the lambing season [Spring (March, April, May)=0, Winter (December, January, February)=1], x_5 is the health of the dam (healthy=0, ill=1), α is the y-intercept, and β_1, β_2 β_3, β_4 and β_5 are the regression coefficients and ε, indicates the random part of the theoretical model. To identify the best models in the stepwise technique, the coefficients of determination (R^2) were used. The coefficient of determination was multiplied by 100 and expressed as a percentage to indicate the total variation in Y explained by the selected independent variables.

In second section the effect of birth weight, gender, type of birth, lambing season, parity and dam's health on passive immune status or SIgGC-24. Simple regression model was used to evaluate relationship between SIgG-24 and birth weight. The methods of multivariable and simple regression have been described previously in detail [11,28]. The General Linear Model (GLM) procedure of SPSS was used to evaluate the association between some animals or management factors and SIgGC-24. The fixed effects considered in the model were: birth weight (≤ 3 kg=1, >3 to ≤ 4 kg=2, >4 kg=3), gender (female=1 and male=2), parity (1, 2, 3, ≥4), type of birth (twin=1 and single=2), health status in dams (ill=1 and healthy=2) and lambing season (winter=1, spring=2).The significant differences between fixed items were tested using Duncan test and results were expressed as least square means (LSM±SE) for the GLM procedure. The computer program SPSS (SPSS, version 16.0, SPSS Inc, Chicago, IL) was used for all analyses and values of P<0.05 were considered to be significant. The program Origin 6 was used to obtain scatter diagram illustrations (Origin 6 Copyright© 1991-1999 Microcol TM, Software, Inc) in regression analysis.

RESULTS

Risk Factors Associated with Birth Weight

Birth weight ranged from 2.260 to 5.900 g (4.037±674 g). Variations in birth weight based on various factors have been given in *Table 1*. Single born lambs were significantly (P<0.001) heavier at birth than twin born lambs. Males were significantly (P<0.001) heavier than females at birth. Lambs born to dams with any illnesses had significantly (P<0.001) lower birth weight than lambs born to healthy dams. Lambs born in winter had insignificant higher birth weight than those born in the spring season (P=0.08). Birth weight in lambs born to primiparous ewes was significantly lower than those born to second (P<0.01), third (P<0.001) and fourth or higher (P<0.001) parity ewes.

Type of birth, parity and gender had significant effect on birth weight on MSRA. These three independent variables explained 34% (R^2=0.339) of the variation in birth weight in final model. High dam's parity had lambs with great birth weight while twin and female lambs had lower birth weight (P<0.001) *(Table 2)*.

Relationship Between Birth Weight and Passive Immunity in Lambs

Relationship between SIgGC-24 and birth weight (BW), determined on simple regression model is given in *Table 3*. There was a significant (P<0.001) but weak (R^2=0.076) linear relationship between SIgGC-24 (mg/ml) and BW in lambs that contracted disease during the neonatal period. However, there was no relationship between SIgGC-24 and BW in lambs that were healthy during the neonatal period *(Table 3)*.

Table 1. *Variations in birth weight according to factors analysed in lambs*
Tablo 1. *Kuzularda analiz edilen faktörlere bağlı doğum ağırlığındaki değişiklikler*

Factor	Group	n	Birth Weight (g)
Type of Birth	Twin	84	3505±436*
	Single	238	4224±643
Sex	Male	173	4157±670*
	Female	149	3896±652
Lambing Season	Winter	98	3938±696
	Spring	224	4079±661
Dam Health	Ill	17	3442±619*
	Healthy	305	4069±662
Parity	1	54	3650±652[a]
	2	137	4048±662[b]
	3	90	4138±582[b]
	≥4	41	4284±740[b]

** P<0.001*

Table 2. *Risk factors associated with birth weight on multiple regression analyses**
Tablo 2. *Doğum ağırlığı ile ilişkili risk faktörleri üzerine adımsal regresyon modelleri**

Model	Formulas	R^2	P
Onset	BW = 4224 - (719 x type of birth)	0.220	<0.001
1	BW = 3676.9 - (781.3 x type of birth) + (238.1 x parity)	0.321	<0.001
Final	BW = 3764.8 - (759.5 x type of birth) + (233.6 x parity) - (179.5 x gender)	0.339	<0.001

** **Independents:** Type of birth, gender, lambing season, dam's parity and health,* **BW (Dependent):** *Birth weight*

Table 3. *Simple regression models between SIgGC-24 and birth weight in neonatal lambs*
Tablo 3. *Neonatal Kuzularda SIgGC-24 ve doğum ağırlığı arasındaki basit regresyon modelleri*

N	Formulas	R^2	P
322	SIgGC-24[a] = 8.024 + (0.003 x BW)	0.034	<0.01
269	SIgGC-24[b] = 21.12 + (0.001 x BW)	0.001	0.53
53	SIgGC-24[c] = (0.006 x BW) - 9.017	0.136	<0.001

[a] *General (without any evaluation of clinical examination),* [b] *healthy,* [c] *ill*
SIgGC-24: *Serum IgG concentrations 24th h after birth*

Tablo 4. *Risk factors associated with passive immunity (SIgGC-24) in lambs*
Tablo 4. *Kuzularda pasif immunite ile ilişkili (SIgGC-24) risk faktörleri*

Factor	Group	N	SIgGC-24 (mg/dl)	F	P
BW (g)	Low	27	1246±242[a]	3.17	0.04
	Medium	122	1858±167[b]		
	High	173	1837±173[b]		
Type of Birth	Twin	84	1466±168*	4.51	0.03
	Single	238	1829±173		
Gender	Male	173	1567±160	1.55	0.21
	Female	149	1727±160		
Lambing Season	Winter	98	1512±176	2.99	0.08
	Spring	224	1782±158		
Dam Healthy Status	Ill	17	1333±277*	4.55	0.03
	Healthy	305	1962±102		
Parity	1	54	1553±206	1.37	0.25
	2	137	1557±165		
	3	90	1864±192		
	≥4	41	1614±221		

SIgGC-24: *Serum IgG concentrations 24th h after birth*

Risk Factor for Passive Immunity in Lambs

Factors affecting passive immunity in lambs are presented in *Table 4*. Of the variables, birth weight, type of birth and health of the dam had a significant effect on the SIgGC-24 of the lambs. The overall least squares mean of SIgGC-24 obtained was 1.647±148 mg/dl. Twin lambs had significantly (P<0.05) lower SIgGC-24 than single lambs. Lambs born with low birth weight (≤3 kg) had significantly lower SIgGC-24 than those born with medium (>3 to ≤4) (P<0.01) or high (>4 kg) (P<0.001) birth weight. SIgGC-24 was significantly lower in lambs born to diseased dams than in lambs born to healthy dams (P<0.05). Parity and gender had no effect on SIgGC-24. A lower SIgGC-24 was determined in lambs born in the winter but this association was not significant (P=0.08).

DISCUSSION

This study presented the factors influenced birth weight and passive immunity and described the relationship between them.

Risk Factors Associated with Birth Weight

Parity, type of birth and gender were significant sources of variation for birth weight in the present study. Primiparous mothers produced lighter lambs when compared to experienced ewes as previously reported [16-21,23,24,29-33]. This may be attribute to that the reproductive organs of primiparous ewes are less developed and less able to bear large fetuses, so the dam's physiology limits the fetal size [30-33] and these ewes may be in the process development, so both the fetus and the dam might compete for nutrients thus consequently negatively influencing birth weight [20,30-32]. Contradicting results between parity and birth weight have also been reported [4,14,34-37]. In this study, lambs born to healthy dams had greater birth weight than those born to sick dams *(Table 1)*. This may be because unhealthy dams provide inadequate nutrition to the fetus, and thus negatively effected birth weight. However, the condition of the dam's health was not a significant source of variation for birth weight as there were only seventeen sick dams in this study versus 305 healthy dams.

Female lambs had lower birth weight than their male counterparts in this study. This findings is in agreement with previous studies [20-24,29,31-33,36-38]. These results have been attributed to the differences in the rate of skeletal development as well as differences in chromosomal structure during the prenatal growth period [34] as the presence of a Y-chromosome and the products of SRY gene activation have gender-specific effects on fetal growth, and therefore males apparently grow faster in utero than respective females [16,32]. However, opposite results have also been reported that birth weight did not differ between male and female lambs [4,14,18,30,39].

Single born lambs were heavier than their twin born counterparts at birth in this study. These results were in parallel with previous studies [20-22,29-34,36-39]. Birth weight declines as the litter size increases due to limited uterus and carnuncles space to gestate offspring and insufficient nutrient provided for the development of all the fetuses [16,30].

Lambs born in winter were insignificantly lighter than those born in spring as previously reported [22,32,34]. In winter, the need for nutrition and energy increases due to cold stress and the quality and amount of food sources deteriorates, so insufficient diet may affect fetal growth and consequently cause low birth weight as reported by Sušić et al.[22].

In this study the variable with the highest influence was found to be birth type, whereas parity and gender were found to have secondary influence in MSRA analyses. However, in the present study, a large proportion of the

variation in birth weight (approximately 65%) was not explained by the variation in type of birth, gender and parity, but was attributable to some other factor. These include, but are not limited to, management intervention, the farming production system, prenatal nutrition of ewes, sufficient placentation, average weight and number of cotyledons as well as dam's body condition score and live weight gain [11,16,23-26,40]. The amount of variation attributable to farm management procedures, the production system and the ewe's gestational nutrition and breed was minimized in this study because all lambs were taken from two farms that have similar management practices and reared Akkaraman crossbreeds.

Low birth weight decreases yield and profitability in sheep farms due to its negative effect on growth performance and passive immunity and consequently increased predisposition to diseases [4,6,11,17,41]. Therefore, efforts should be made to develop management practices that increase birth weight such as supplementation of ration with enough concentrated feed and protein in the third trimester [23,24,42].

Relationship Between Passive Immunity and Birth Weight

There was a significant but poor positive linear relationship between birth weight and SIgGC-24 in sick lamb in neonatal period. This may be due to the fact that lambs born with low birth weight might have not received colostrum due to physical weakness and abnormal behavior at birth, leading to weak relationship with their mother, poor suckling reflex and less vigority [19,26,40,43]. Birth weight had a significant effect on passive immunity since lambs with a birth weight of ≤3 kg had a significantly lower SIgGC-24 than did lambs with a birth weight of >3 to ≤4 or >4 kg on GLM in this study. Our study also determined that based on simple regression analysis of variables from 322 lambs without grouping them according to clinical examination, there was a positive linear relationship between birth weight and passive immunity. These results have been confirmed by previous studies [4,5,24,44]. On the other hand, we did not detect a significant relationship between birth weight and SIgGC-24 in healthy lambs during the neonatal period in our study. This is consistent with the results of other studies in healthy calves [45], lambs [11,13], and kids [46]. However, the influence of birth weight on passive transfer of IgG in neonatal ruminants has not yet been fully elucidated. Massimini et al.[11], hypothesized that the negative relationship between birth weight and the acquisition of passive immunity [48,49] in newborn lambs could be an indirect link that reflects the effects of other important physiological factors, such as duration of gestation and hormonal status at birth but the potential influences of these independent variables were not evaluated in this study.

Other Factors Effect on Passive Immunity

Gender had no effect on passive immunity in this study but SIgGC-24 of male lambs was slightly higher than females. This is similar to previous reports [6-8,12-15,44,50]. Twin lambs had lower SIgGC-24 than single lambs in the present study, which concurs with previous findings [8,44]. This may be attributed to that twin lambs have to compete for colostrums and are physically weak and with low birth weight thus being unable to suckle a sufficient amount of colostrum, leading to low IgG levels in their serum. On the other hand, it has been reported that ewes with twins have higher colostrum production than ewes with single lambs but immunoglobulin concentrations in the lambs fall significantly in proportion with the litter size [6,7].

Passive immunity is reported to be affected by the lambing season [15]. Cold-stressed newborn ruminants may have a slower rate of intestinal immunoglobulin absorption [51], and may also be reluctant to stand and suckle voluntarily [6,43,52] consequently, an increased risk of failure of passive transfer is expected in the period from December to February, which is also the coldest period of the year in Kars. Nevertheless, winter born lambs had insignificant (P=0.08) lower SIgGC-24 in our study as lambs born indoors, this might have reduced the potential impact of cold stress [50].

The level of IgG in lambs born to primiparous ewes was observed to be insignificantly lower than in those born to ewes giving birth previously in this study. These results are in line with some eralier studies in lambs [4,6,12,13,44]. Primiparous ewes may have a lower volume, concentration, or quality (low IgG level) of colostrum and poorer maternal ability than do mature ewes [7,23,43,53] and lambs born to primiparous ewes are with low birth weight and weak to stand to suckle as was the case in this study [7,23,53] On the other hand, dam's health had a significant effect on passive immunity in our study. Healthy dams produce good quality colostrum, which also influences the absorption of immunoglobulin from the intestines, and such dams also have good maternal ability [4,23,26,40,43,53] as might have been the case in our study.

In conclusion, the present study identified some important environmental factors and animal characteristics affecting passive immunity and birth weight in lambs in such details in the world and for the first time in Turkey. These results indicated that for proper lambing management, consideration should be given to maximizing the health of the flock and supplementing feed ratio of the ewes during gestation that are primiparous, carrying twin and with ill-health. Consequently, sheep farmers need to ensure that each lamb remains healthy and gains sufficient live weight in order to increase productivity and profitability.

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RESEARCH ARTICLE

Effect of *Yucca schidigera* Spraying in Different Litter Materials on Some Litter Traits and Breast Burn of Broilers at the Fifth Week of Production [1]

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Summary

This study was carried out to determine the effects of different levels of *Yucca schidigera* spraying in different litter materials on some litter traits (moisture, pH, ammonia, total colony count, number of Enterobacteriaceae and number of yeast and mold) and breast burn of broilers at the 5th week of production. A total of four hundred thirty two 1-d-old male broiler chicks (ROSS-308) were used. In this study 12 chicks were put in each pen having 170x94x90 cm (depth x length x height). Half of the litter was wood shavings, the other was rice hull. *Yucca schidigera* extract was pulverized weekly at the level of 0, 4% and 8% to each pen from the second week of the study. Litter materials and *Yucca schidigera* spraying at different level did not affect the examined litter traits and breast burns of broilers (P>0.05) at the 5th week of production.

Keywords: *Broiler, Yucca schidigera, Litter traits, Breast burn*

Farklı Altlık Materyallerine Püskürtülen *Yucca schidigera*'nın Üretimin 5. Haftasında Bazı Altlık Özellikleri ile Etçi Piliçlerin Göğüs Yanıkları Üzerine Etkisi

Özet

Bu çalışma farklı altlık materyallerine değişik dozlarda ilave edilen *Yucca schidigera*'nın üretimin 5. haftasında bazı altlık özellikleri (nem, pH, amonyak, toplam koloni sayısı, Enterobakteri ve maya-küf düzeyleri) ile etçi piliçlerde göğüs yanıkları üzerine etkisini belirlemek amacıyla yapılmıştır. Bu çalışmada toplam 432 adet bir günlük yaşta erkek civciv (ROSS 308) kullanılmıştır. Çalışmada civcivler 170x94x90 cm (genişlik x uzunluk x yükseklik) boyutlarındaki bölmelerin her birinde 12 adet olacak şekilde yerleştirilmiştir. Altlık materyali olarak bölmelerin yarısında talaş diğer yarısında pirinç kavuzu kullanılmıştır. Her altlık grubu denemenin 2. haftasından itibaren her hafta altlığa 0, %4 ve %8 *Yucca schidigera* püskürtülecek şekilde 3 eşit alt gruba ayrılmıştır. Kullanılan altlık malzemelerinin ve altlığa değişik düzeylerde *Yucca schidigera* ilavesinin üretimin 5. haftasında incelenen altlık özellikleri ile etçi piliçlerde göğüs yanıklarını etkilemediği görülmüştür (P>0.05).

Anahtar sözcükler: *Etçi piliç, Yucca schidigera, Altlık özellikleri, Göğüs yanıkları*

INTRODUCTION

Broilers are reared on the litter and they spend most of their lifetime in close contact with this. Therefore litter quality has a major effect on health and performance of broilers [1]. Many products have been used as litter. In many broiler producing areas, availability of new litter is limited by supply and price. Each litter has got advantages and disadvantages from the others. Locally available materials are usually preferred as litter for poultry [2,3]. Wood shavings

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and rice hulls have commonly been used as bedding materials [4]. There are many factors, which must be taken into account for successful litter management. These include the type of litter used, depth of the litter, floor space per bird, feeding and watering devices used, kind of flooring, ventilation system, litter amendments, and the incidence of disease that can affect litter and its fertilizer value [5].

Incorrect litter interfered directly in the appearance of breast lesions and foot-pad dermatitis. These are important sources of economic loss through downgrading and carcass condemnations as well as welfare considerations linked to the potential for associated pain and discomfort [6,7]. Litter amendment improved bird welfare and production due to better litter conditions and microorganism levels in broiler houses. Many litter additives have been used to reduce litter pH, reduce ammonia volatilization [1]. However, before this management practice can be put into widespread usage, questions concerning the environmental impact of different types of chemical amendments and their safety in broilers on commercial farms must be addressed [8]. One of the most known additives for fixing ammonia is derived from the cactus *Yucca schidigera*, and it acts by binding or converting NH_4 [9]. Yucca is currently used as dietary supplement for poultry, primarily for ammonia binding, but also to improve performances safety [10,11]. Therefore the aim of this study was to determine the effects of *Yucca schidigera* spraying in different litter materials on moisture, pH, ammonia, total colony count, number of *Enterobacteriaceae* and number of yeast and mold of litters and breast burn of broilers at the 5th week of production.

MATERIAL and METHODS

This study was approved by Ankara University Animal Care and Use Committee (2010/100/366). A total of 432 1-d-old male broiler chicks (ROSS-308) obtained from a commercial hatchery (Beypiliç, Bolu, Turkey). The trial design with 2 litter and 3 *Yucca schidigera* treatments with 6 replication pens (n=12 chicks). Half of the pens contained the wood shavings (8 kg/pen) and the other half of the pens contained rice hulls (8 kg/pen) as a litter material. Each litter material groups were divided into 3 *Yucca schidigera* groups; control (no treatment of *Yucca schidigera*), 100 ml of 4% *Yucca schidigera* and 100 ml of 8% *Yucca schidigera*. Liquid *Yucca schidigera* extract used in this experiment was DK Sarsaponin liquid (Ekol Gıda, İstanbul, Turkey). DK Sarsaponin liquid is a pure, natural extract of the Mahave Yucca plant. It contains 50% total soluble Yucca and 12% saponins. In this trial 4% (4 ml *Yucca schidigera* with 96 ml water) and 8% (8 ml Yucca schidigera with 92 ml water) solutions were prepared using DK Sarsaponin liquid. 100 mililitres of these prepared solutions were applied by spraying onto the litter surface of each pen (1.6 m^2) litter groups using a small hand pump weekly (once a week) from the second week to the fifth week in *Yucca schidigera* groups. Therefore every week 0.4 and 0.8 g saponin and totally from the second to the fifth week 1.6 g and 3.2 g were added to each pen of 4% and 8% *Yucca schidigera* groups.

During the first week each pen was equipped with one chick drinker and one chick feeder and the other weeks each pen was equipped with two nipples and one hanging suspended feeder. Feed as mash form and water were provided *ad libitum* during the experiment. Birds were fed with a starter diet (222.8 g/kg crude protein and 3136 kcal/kg ME) from 1 to 21 day of age and a grower diet (220.2 g/kg crude protein and 3200 kcal/kg ME) from 22 to 35 d of age.

A litter sample was collected from five sites (4 corner and 1 central samples), then mixed and 100 g sub sample from mixed sample was taken in a plastic bag at 35 day of age from pen to determine the pH, moisture, ammonia and microbiological analyses. To determine litter pH, 20 g of litter sample was mixed with 30 ml of sterile distilled water. Then pH was measured [12] with pH meter (Selecta, pH-2004, Barcelona, Spain). 2 g litter sample of each pen were dried in an oven at 105°C for 8 h to determine moisture content of samples [13]. Litter ammonia-N of each pen was determined using the spectrophotometer [14]. Analyses were made in three parallel. For microbiological analyses, initial suspensions were prepared with 10 g sample and 90 ml Peptone Salt Diluent (Merck, Darmstadt, Germany) using stomacher (Masticator, IUL, Barcelona, Spain). Additional tenfold dilutions were made by Peptone Salt Diluent [15]. 1 ml of initial suspension and dilutions were transferred in two sterile plates and 10 ml melted Plate Count Agar (Merck) was then added per plate. The plates were incubated at 37°C for 48 h. After incubation colonies on plates were counted and total aerobic counts were calculated as cfu/g [16]. 1 ml of initial suspension and dilutions were transferred in two sterile plates and 10 ml melted Violet Red Bile Glucose Agar (Merck) was poured into each plate. After solidification of the mixture additional 15 ml Violet Red Bile Glucose Agar was added per plate to achieve semi-aerobic condition. The plates were incubated at 37°C for 24 h. After incubation, presumptive pink-red colonies were subcultured on Nutrient Agar (Merck) and confirmed by means of tests for fermentation of glucose and presence of oxidase. The numbers of *Enterobacteriaceae* as cfu/g of the samples were calculated from the number of confirmed typical colonies per plate [17]. 0.1 ml of initial suspension and dilutions were surface plated on two Dichloran Rose Bengal Chloramphenicol Agar (Merck) plates. After incubation at 25°C for 5 days colonies on plates were counted and yeast and mould counts were calculated as cfu/g [18].

All broilers of each pen were examined for breast burn at 35 days of age. The breast burn scoring system was a 2-point visual ranking system, where a score of 0 indicated

breast with no lesions present and a score of 1 indicated breast with lesions [19].

Data were tested for distribution normality and homogeneity of variance. Data set showed normality and a two-way ANOVA was used to determine the differences between litter and *Yucca schidigera* groups as well as their interactions with respect to moisture, ammonia, pH, total colony count, number of *Enterobacteriaceae* and number of yeast-mold. Comparisons among means were made by Duncan's multiple range test. Multiple logistic regression analysis was used to explore the effect of litter and *Yucca schidigera* groups in breast burn of broilers. Odds ratios (AOR) and 95% confidence intervals (CI) were estimated. A value of $P<0.05$ was considered statistically significant [20].

RESULTS

In this study moisture content of litter was 32.9% in wood shavings group and 30.9% in rice hull group and ammonia-N content of litter was 5.7 g/kg in wood shavings group and 5.7 g/kg in rice hull group at the 5th week of production ($P>0.05$). Moisture contents of litter were 32.0%, 32.3% and 31.4%, and ammonia-N contents of litter were 6.1 g/kg, 5.7 g/kg and 5.4 g/kg in the groups of 0, 4 and 8% *Yucca schidigera* spraying in litters, respectively and these differences among them were not statistically significant ($P>0.05$).

pH and microorganism levels in groups of *Yucca schidigera* spraying in the different litter materials were not statistically different in *Table 1*. Percentage of breast burn as a 2 point scale was 4.3% and 3.8% of broilers reared on wood shavings and rice hull and they were 3.6, 3.6 and 5.0% of broilers reared on 0, 4 and 8% spraying in litters, respectively *(Table 2)*. There were no statistically significant differences in breast burn of broilers among examined group.

DISCUSSION

In this study wood shavings or rice hull as a litter material had no statistically significant influence on moisture of litter at the 5th week of production. Similarly, Sarıca and Cam [21] reported that wood shavings and rice hull groups gave similar results in terms of litter moisture at the 5th week of production. In their study moisture content of litter was 33.5% in wood shavings group and 36.9% in rice hull group. However, Oğan [22] found moisture level at 42 days of age 28.9% and 39.07% in wood shavings and rice hull groups. Meluzzi et al.[23] reported that litter moisture was changed by season and moisture content of litter as rice hull was found to be 38.7% and 25.3% in winter and summer, respectively. Villagra et al.[4] showed that moisture content of wood shavings was 18% at 35 days of age. The reported moisture contents of same litters vary widely because of attributing to the several factors such as stocking density, feeding and watering devices used, kind of flooring, ventilation system, slaughter age and season [5,23].

Table 1. *Effect of Yucca schidigera (YS) spraying in different litter materials on litter characteristics at the 5th week of production*
Tablo 1. *Farklı altlık materyallerine püskürtülen Yucca schidigera'nın (YS) üretimin 5. haftasında altlık özelliklerine etkisi*

Litter Material	Level of YS	Moisture (%)	Ammonia-N (g/kg)	pH	TCC (log_{10} cfu/g litter)	NE (log_{10} cfu/g litter)	NYM (log_{10} cfu/g litter)
Wood shavings	0	32.7	6.1	7.5	7.3	6.5	6.3
	4	33.2	5.8	7.3	7.2	6.1	5.9
	8	32.8	5.2	7.0	7.4	6.4	6.2
Rice hull	0	31.4	6.0	7.4	7.3	6.4	6.0
	4	31.4	5.5	8.1	7.1	6.6	6.5
	8	29.9	5.7	7.9	7.2	6.5	6.0
Main effect							
Wood shavings		32.9	5.7	7.2	7.3	6.4	6.1
Rice hull		30.9	5.7	7.8	7.2	6.5	6.1
0% spraying of YS		32.0	6.1	7.4	7.3	6.4	6.1
4% spraying of YS		32.3	5.7	7.7	7.2	6.4	6.2
8% spraying of YS		31.4	5.4	7.4	7.3	6.5	6.1
Pool SEM		0.626	0.228	0.157	0.055	0.083	0.078
P							
Litter		NS	NS	NS	NS	NS	NS
Level of YS		NS	NS	NS	NS	NS	NS
Litter X Level of YS		NS	NS	NS	NS	NS	NS

TCC: *Total colony count,* ***NE:*** *Number of Enterobacteriaceae,* ***NYM:*** *Number of yeast and mold,* ***NS:*** *Non significant, n=6*

Table 2. *Effect of Yucca schidigera (YS) spraying in different litter materials on breast burn of broilers at the 5th week of production*

Tablo 2. *Farklı altlık materyallerine püskürtülen Yucca schidigera'nın (YS) üretimin 5. haftasında etçi piliçlerde göğüs yanıklarına etkisi*

Group	Breast Burn (%)	Odds Ratio	95% C.I		P
			Lower	Upper	
Wood shavings	4.3	1			
Rice hull	3.8	0.888	0.336	2.350	NS
0% spraying of YS	3.6	1			
4% spraying of YS	3.6	0.992	0.281	3.507	NS
8% spraying of YS	5.0	1.400	0.433	4.523	NS

NS: *Non significant, n=72*

Ammonia is an abundant pollutant in broiler houses and it may have a great impact on poultry welfare [24]. Ammonia is formed during the decomposition of uric acid and the efficiency of this conversion is directly related to the level of litter moisture [25]. Due to the similar moisture between the litter materials; there were no statistically significant in ammonia-N levels of wood shavings and rice hull at the 5th week of production.

Hence, litter amendments are suggested to improve litter conditions and keep NH_3 levels in check [1]. But it is important that the production cost and their safety for bird health and environment. *Yucca schidigera* contains high levels of saponin steroids that bind the ammonia. Yucca extracts are used as animal feed additives and in crop production safely [10,26]. Therefore, it does not affect negatively on broiler health and environment. We thought that it may be added to the poultry litter to bind the ammonia in litter during the production. However, in this study it is observed that moisture and ammonia levels of litter, in groups of *Yucca schidigera* spraying in litters were not statistically different. *Yucca schidigera* spraying in litters during production in examined dose (4% and 8%) did not change these properties. We did not find any study about Yucca extract spraying in poultry litter. Only, it was added to the swine manure, mariculture and shrimp farming. Panetta et al.[27] added the Yucca extract to the swine manure at 0, 7.4 and 14.9 mg/L to determine the effects of a nitrogen-binding agent on ammonia emission potential. In their study headspace ammonia-N concentration was affected by the different doses of Yucca extract. Santacruz-Reyes and Chien [28] showed that *Yucca schidigera* concentration of 18 mg/L was effective solution for ammonia reduction in seawater and mariculture.

In this study it was showed that pH, total colony count, number of *Enterobacteriaceae* and number of yeast-mold levels in groups of *Yucca schidigera* spraying in the different litters were not statistically different at the 5th week of production. pH levels in examined groups ranged from 7.0 to 8.1 in examined groups at the 5th week. It was found that total colony count, number of *Enterobacteriaceae* and number of yeast-mold ranged from 7.1 to 7.4 $\log_{10}$ cfu/g, 6.1 to 6.6 $\log_{10}$ cfu/g and 5.9 to 6.5 $\log_{10}$ cfu/g, respectively in examined groups at the 5th week. Terzich et al.[29] indicated that total bacteria counts range from a minimum of $1.72x10^7$ cfu/g to a maximum of $8.80 x10^{11}$ cfu/g in poultry litter taken from different examined region. Martin et al.[30] reported the results of total bacteria counts between $1.2x10^3$ cfu/g and $8.4x10^3$ cfu/g.

Choi and Moore [31] reported that some litter amendments resulted in the greatest reduction in NH_3 emissions and the greatest litter nitrogen contents. Choi and Moore [31] also reported that ammonia nitrogen was about 1.3-2.3 g/kg in control litter and was about 1.0-7.1 g/kg in the litters with various amendments. One of the most important factors that can affect ammonia volatilization is litter pH [31]. Reece et al.[32] suggested that very little ammonia was released from litter having pH below 7, whereas more ammonia was rapidly released from litter having pH above 8. Carr et al.[33] also reported that ammonia release from litter increased as litter pH increased. However in this present study moisture levels and pH levels of litters in all groups were less than 32.9% and 8.08, respectively. Because of these reasons in the present study ammonia nitrogen levels and microbial load in litter may not be affected in the present study.

Good litter management is vital to maintain animal welfare including the absence of contact dermatitis. However, this management is related to litter material [34]. Bray and Lynn [35] and Haslam et al.[36] concluded that breast burn is primarily affected by litter type and quality. In this study, multiple logistic regression analysis revealed similar rates of breast burn with wood shavings and rice hull as a litter and different levels of *Yucca schidigera* ("4" vs "0" and "8" vs "0"), because similar results were observed in our study in which moisture and ammonia levels of examined groups. Sarıca and Cam [21] reported that breast blister scores as a 5 point scale of broilers were reared on the wood shavings and rice hull were 1.8 and 1.4, respectively and this was not statistically different. Moisture levels above 35% in litter resulted negative effects on bird health such as pododermatitis [37], folliculitis and necrotic enteritis [38]. However in the present study moisture levels of litters in all groups were less than 32.9%.

As a conclusion, wood shavings and rice hull as a litter material and *Yucca schidigera* spraying in these litters at the level of 4 and 8% from the second week to the fifth week of production did not affect the moisture and ammonia of the litter and breast burn of broilers at the 5th of production. The effect of *Yucca schidigera* could be seen in bad litter conditions depend on stocking density. Further studies will be done in negative poultry housing conditions and also with different applications such as application time, way and dose of *Yucca schidigera* spraying in litters.

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RESEARCH ARTICLE

Bazı Ötücü Kuşlarda (Aves: Passeriformes) Bulunan Bit (Phthiraptera; Ischnocera, Amblycera) Türleri

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Özet

Bu araştırma Türkiye ve Yunanistan'ın Midilli Adası'nda yayılış gösteren bazı ötücü kuşlardaki (Passeriformes) bit türlerini belirlemek amacıyla 2005–2009 yılları arasında yapılmıştır. Türkiye'deki yedi farklı yöreden ve Midilli Adası'ndan yakalanan 204 ötücü kuş örneği bit yönünden incelenmiştir. İncelenen 12 aile, 29 türe ait 204 kuş örneğinin beşi (%2.45) bitlerle enfeste bulunmuş ve dört bit türü; *Brueelia jacobi* (Eichler, 1951); *Philopterus sittae* Fedorenko, 1978; *Philopterus vernus* (Zlotorzycka, 1964) ve *Philopterus* spp. tespit edilmiştir. Bunlardan *Philopterus vernus* Midilli Adası'ndan, diğer türlerin tamamı ise bu makaleyle Türkiye'den ilk kez bildirilmektedir.

Anahtar sözcükler: *Brueelia jacobi, Philopterus sittae, Philopterus vernus, Philopterus spp.*

Chewing Lice (Phthiraptera; Ischnocera, Amblycera) Species Found on Some Songbirds (Aves: Passeriformes)

Summary

This study was performed to detect of the louse species found on some song birds (Passeriformes) between the dates of 2005–2009. Two hundreds and four song bird specimens in 29 species belong to 12 families captured in seven different localities in Turkey and Lesbos island (Greece) were examined for louse specimens. Five of them (2.45%) were found to be infested with louse and detected four species; *Brueelia jacobi* (Eichler, 1951); *Philopterus sittae* Fedorenko, 1978; *Philopterus vernus* (Zlotorzycka, 1964) and *Philopterus* spp.. *Philopterus vernus* from Lesbos island an the all of other species are reported for the first time in Turkey with this paper.

Keywords: *Brueelia jacobi, Philopterus sittae, Philopterus vernus, Philopterus spp.*

GİRİŞ

Tüm dünyada yaklaşık 10500 kuş türü bulunmakta [1] ve bu türlerden 500'e yakını Türkiye'de görülmektedir [2,3]. Türkiye'de görülen kuşlar 20 takım altında toplanmış olup, Passeriformes (Ötücü kuşlar) takımı 25 familya, 70 civarında cins ve 200'ün üzerinde tür ile temsil edilmektedir. Kanatlı hayvanlarda görülen bit türleri çiğneyici-ezici tipte ağız yapısına sahip olup, konaklarının kıl ve tüylerini yiyerek veya bazen de deri bütünlüğünü bozarak, kan emerek kuşlara zarar verirler. Kanatlı hayvanlarda görülen bitler Phthiraptera takımı, Ischnocera ve Amblycera alt takımlarında yer alırlar. Bu takımda bulunan 6.000'den fazla türün büyük bir kısmı kanatlı hayvanlarda görülmektedir [4]. Değişik ülkelerde, ötücü kuşlarda bulunan bit türleri üzerine yapılmış çalışmalar bulunmakla birlikte [5-8], Türkiye'deki ötücü kuşlarda görülen bit türlerinin belirlen-mesi üzerine yapılan çalışma sayısı oldukça az olup, bu araştırmalar az sayıdaki kuş üzerinde yapılmıştır [9-13]. Sığırcıklarda *(Sturnus vulgaris)* bulunan bit türlerinin belirlenmesi amacıyla yapılan bir araştırmada *Myrsidea cucullaris* (Nitzsch, 1818), *Brueelia nebulosa* (Burmeister, 1838), *Brueelia sp.* ve *Sturnidoecus sturni* (Schrank, 1776) olmak üzere dört bit türüne rastlandığı ifade edilmiştir [10]. Konya'da yapılan bir çalışmada, Leş kargası, Ev kırlangıcı ve Serçelerden oluşan 16 ötücü kuş örneğinin bit yönünden incelendiği, fakat hiçbirisinde bite rastlanmadığı kaydedilmiştir [9]. Kars'ta, Kuyucuk Gölü'nde yakalanan 22 cinse ait 51

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ötücü kuştan 11 (%21.57)'inin bitlerle enfeste olduğu belirlenmiş, *Brueelia cruciata* (Burmeister, 1838), *Menacanthus chrysophaeus* (Kellogg, 1896), *Menacanthus pusillus* (Nitzsch, 1866), *Myrsidea rustica* (Giebel, 1874) ve *Penenirmus rarus* (Zlotorzycka, 1976) olmak üzere beş tür saptanmıştır [14]. Kars'ta, Aras nehrindeki kuşların bit türlerinin belirlenmesi üzerine yapılan bir çalışmada ise bir Karatavuktan nimf dönemine ait iki adet bit toplanmış ve bu bitler *Menacanthus* spp. olarak teşhis edilmiştir. Konya ve Eskişehir'de 2008-2010 yılları arasında bazı yabani kanatlıların bitlerini belirlemek amacıyla yapılan bir çalışmada, yedi cinste yer alan sekiz ötücü kuş örneği bit yönünden incelenmiş, fakat hiç birisinde bite rastlanmamıştır [12]. Çanakkale'de avlanan dört Karatavukta, *Ricinus elongatus* (Olfers, 1816) ve *Brueelia merulensis* (Denny, 1842) olmak üzere iki tür saptanmış *Ricinus* cinsi ile birlikte her iki tür de Türkiye'den ilk kez bildirilmiştir [13]. Bu araştırma Türkiye'nin yedi farklı bölgesinden ve Midilli Adası'ndan yakalanan ötücü kuş türleri üzerindeki bit türlerini belirlemek amacıyla yapılmıştır.

MATERYAL ve METOT

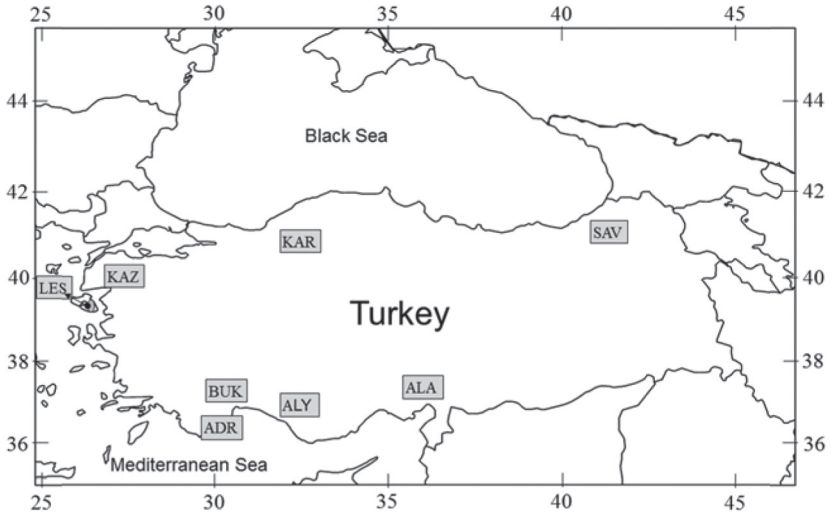
Bu araştırma 2005 - 2009 yıllarında Aladağlar (ALA; 34 örnek), Alanya (ALY; 5 örnek), Lütfi Büyük Yıldırım Araştırma Ormanı (BUK; 58 örnek), Adrasan (ADR; 5 örnek), Kazdağları (KAZ; 32 örnek), Kartalkaya (KAR; 24 örnek), Şavşat (ŞAV; 13 örnek) ile Midilli Adası'nda (LES; 33 örnek) kuşların üreme dönemleri olan Mart-Haziran ayları arasında yapılmıştır *(Fig. 1)*. Kuşların yakalanmasında 78 m (2x12, 2x10, 2x8, ve 3x6 m)'lik Japon ağları kullanılmıştır. Kuşlar yakalanıp, ektoparazit yönünden incelendikten sonra tekrar doğaya bırakılmışlardır. Kuşların üzerlerinden toplanan bit örnekleri, içinde %70 alkol bulunan küçük cam tüplerde saklanmış ve sonuçlar protokol defterine kaydedilmiştir. Toplanan bit örnekleri Selçuk Üniversitesi Veteriner Fakültesi Parazitoloji Anabilim Dalı laboratuarına getirilmiş ve %10 Potasyum hidroksit (KOH) içerisinde bir gün süreyle saydamlaştırılmıştır. Daha sonra, bir gün süreyle distile suda bekletilmiş, birer gün süreyle %70, 80, 90 ve %99 alkol serilerinde tutulduktan sonra Kanada balsam ile lam üzerine ayrı ayrı yapıştırılmış ve kuruyana kadar etüvde bekletilmişlerdir. Hazırlanan preparatlar ışık mikroskobunda incelenmiş ve ilgili kaynaklara [15-17] göre teşhis edilmişlerdir.

BULGULAR

Araştırma süresince 12 ailede yer alan 29 kuş türüne ait 204 ötücü kuş yakalanmış, yakalanan türlerin hangi aile ve cinste yer aldıkları ve enfestasyon durumları *Tablo 1*'de gösterilmiştir. Bu tabloda da görüleceği üzere 204 kuştan sadece iki aile ve iki cinsteki üç kuş türünde bitlere rastlanmış, diğer aile, cins ve türlerde bit saptanamamıştır. Araştırmada 75 bireyle en çok Anadolu sıvacısı *(Sitta krueperi)* incelenmiş, fakat sadece BUK'ten yakalanan iki örnekte (%2.66) bite tesadüf edilmiştir. Her iki kuştan toplanan dokuz bit örneğinin hepsi *Philopterus sittae* Fedorenko, 1978 (4 ♀♀, 4 ♂♂, 1 nimf) *(Fig. 2)* olarak teşhis edilmiştir. Turdidae ailesinde sadece *Turdus* cinsinde yer alan *Turdus merula* (Karatavuk) ve *Turdus viscivorus* (Ökse ardıcı)'da bit saptanmış, incelenen beş karatavuktan birisinde, Ökse ardıcının ise her ikisinde de bite rastlanmıştır. Midilli Adası'ndan yakalanan iki Ökse ardıcından toplanan yedi bitin tamamı *Philopterus vernus* (Zlotorzycka, 1964) (4 ♀♀, 2 ♂ ♂, 1 nimf) *(Fig. 3)*, Kartalkaya'da yakalanan Karatavuktan toplanan dört bitin ikisi *Brueelia jacobi* (Eichler, 1951) (2 ♀♀) *(Fig. 4)*, diğer ikisi ise *Philopterus* spp. (2 nimf) *(Fig. 5)* olarak teşhis edilmiştir.

TARTIŞMA ve SONUÇ

Her ne kadar son zamanlarda Türkiye'de, yabani kuşların bitleri üzerine yapılan çalışma sayısı giderek artmakta ise de, Türkiye'de bulunan yaklaşık 500 kuş türünden çoğu ya bit yönünden incelenmemiş ya da incelenen kuşlarda herhangi bir bit türüne rastlanmamıştır. Bununla birlikte, özellikle son yıllarda yapılan çalışmalar sonucu [9-13,18,19] Türkiye'deki evcil ve yabani kanatlılarda saptanan bit türü sayısı 100'ü geçmiştir.



Şekil 1. Kuş örneklerinin yakalandığı bölgeler. Aladağlar (ALA), Alanya (ALY), Lütfi Büyük Yıldırım Araştırma Ormanı (BUK), Adrasan (ADR), Kazdağları (KAZ), Kartalkaya (KAR), Şavşat (SAV), Midilli Adası (LES)

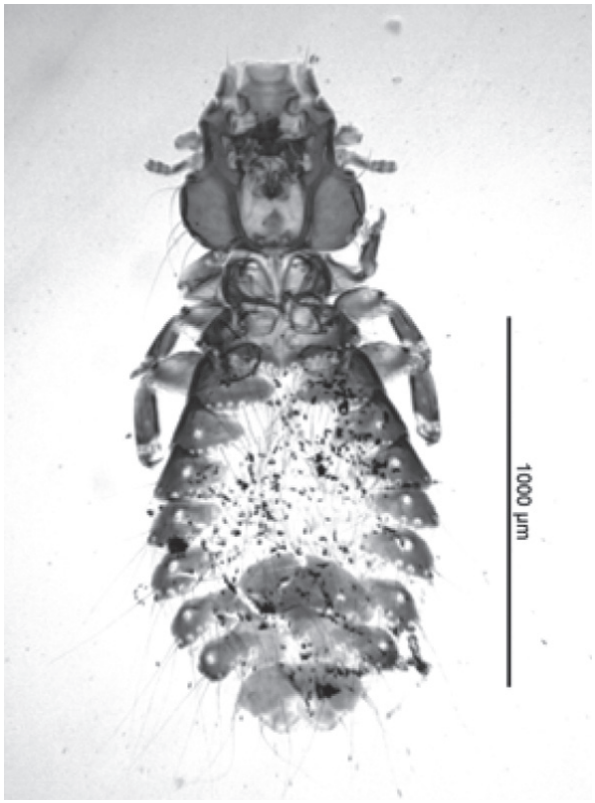
Fig 1. Bird specimens captured regions: Aladağlar (ALA), Alanya (ALY), Lütfi Büyük Yıldırım Research Forest (BUK), Adrasan (ADR), Kazdağları (KAZ), Kartalkaya (KAR), Şavşat (SAV), Midilli Island (LES)

Tablo 1. *İncelenen ötücü kuş türleri, enfestasyon durumları ve tespit edilen bit türleri*
Table 1. *Studied bird species infestation rates and louse species*

Familya	Cins	Kuş Türü	Latince	İngilizce	Toplam*	Enfeste Kuş Sayısı	Bit Türü
Aegithalidae	*Aegithalos*	Uzunkuyruklu Baştankara	*A. caudatus*	Long-tailed Tit	7	-	
Certhiidae	*Certhia*	Bahçe Tırmaşıkkuşu	*C. brachydactyla*	Short-toed Treecreeper	4	-	
		Orman Tırmaşıkkuşu	*C. familiaris*	Treecreeper	2	-	
Emberizidae	*Emberiza*	Bahçe kirazkuşu	*E. cirlus*	Cirl Bunting	1	-	
Fringillidae	*Carduelis*	Saka	*C. carduelis*	Goldfinch	4	-	
		Florya	*C. chloris*	Greenfinch	6	-	
	Fringilla	İspinoz	*F. coelebs*	Chaffinch	22	-	
	Serinus	Küçük iskete	*S. serinus*	Serin	4	-	
Muscicapidae	*Muscicapa*	Benekli sinekkapan	*M. striata*	Spotted Flycatcher	3	-	
Paridae	*Parus*	Çam baştankarası	*P. ater*	Coal Tit	15	-	
		Mavi baştankara	*P. caeruleus*	Blue Tit	9	-	
		Akyanaklı baştankara	*P. lugubris*	Sombre Tit	2	-	
		Büyük baştankara	*P. major*	Great Tit	15	-	
		Kayın baştankarası	*P. palustris*	Marsh Tit	1	-	
Passeridae	*Passer*	Serçe	*P. domesticus*	House Sparrow	1	-	
Prunellidae	*Prunella*	Dağ bülbülü	*P. modularis*	Dunnock	1	-	
Sittidae	*Sitta*	Anadolu sıvacısı	*S. krueperi*	Krüper's Nuthatch	75	2	*Philopterus sittae*
Sylviidae	*Phylloscopus*	Orman söğütbülbülü	*P. sibilatrix*	Wood Warbler	1	-	
		Söğütbülbülü	*P. trochilus*	Willow Warbler	1	-	
	Regulus	Çalıkuşu	*R. regulus*	Goldcrest	3	-	
	Sylvia	Karabaşlı ötleğen	*S. atricapilla*	Blackcap	1	-	
		Küçük Akgerdanlı Ötleğen	*S. curruca*	Lesser Whitethroat	1	-	
		Maskeli ötleğen	*S. melonocephala*	Sardinian Warbler	2	-	
Troglodytidae	*Troglodytes*	Çitkuşu	*T. troglodytes*	Wren	1	-	
Turdidae	*Oenanthe*	Karakulaklı Kuyrukkakan	*O. hispanica*	Black-eared Wheatear	4	-	
	Phoenicurus	Kızılkuyruk	*P. phoenicurus*	Redstart	9	-	
	Turdus	Karatavuk	*T. merula*	Blackbird	5	1	*Brueelia jacobi Philopterus spp.***
		Öter ardıç	*T. philomelos*	Song Thrush	2	-	
		Ökse ardıcı	*T. viscivorus*	Mistle Thursh	2	2	*P. vernus*
Toplam					204	5	

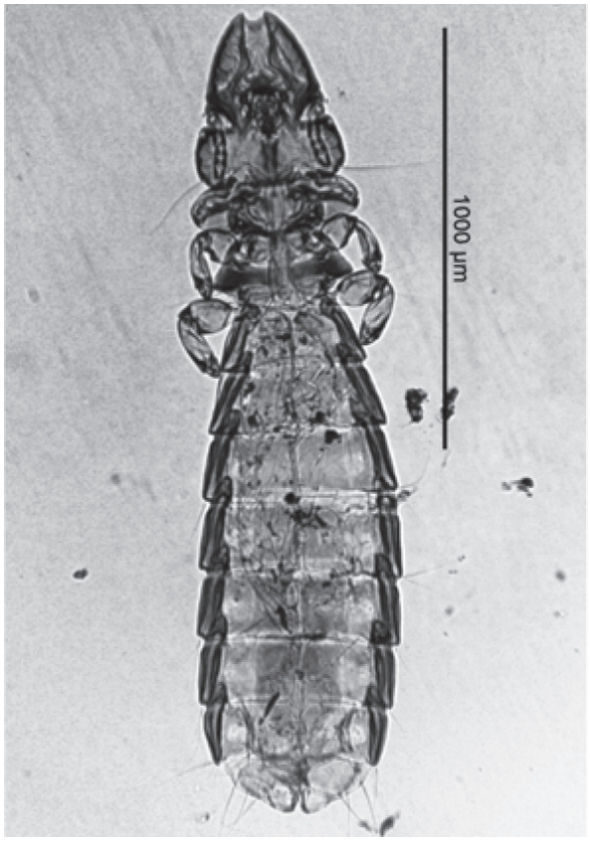
** Araziden yakalanan toplam kuş sayısı; ** Nimf*

Doğa Koruma ve Milli Parklar Genel Müdürlüğü'nün ilgili mevzuatları ve/veya doğadan kuş örneği bulmanın zorluğu nedeniyle bugüne kadar yapılan çalışmalarda incelenen kuş türü ve örneği sayısı genel olarak düşük olmuştur. Yapılan çalışmalardan birinde 27 sığırcık incelenirken [10], başka bir çalışmada Serçe *(Passer domesticus)*, Leş kargası *(Corvus corone)* ve Ev kırlangıcından *(Delichon urbica)* oluşan 16 örnek [9], bir diğerinde ise 7 ötücü kuş türüne ait 7 örnek [12] bit yönünden muayene edilmiştir. Aras nehri kuşlarında yapılan bir çalışmada; 16 ötücü kuş türüne ait 73 birey [11], Kars'ın Kuyucuk Gölü'nde yapılan bir çalışmada ise 10 aile, 16 cinste yer alan 22 kuş türüne ait 51 ötücü kuş [14] bit yönünden incelenmiştir. Karatavuklar üzerinde yapılan bir çalışmanın materyalini ise dört kuş örneği oluşturmuştur [13]. Bu çalışmalar sonucu; sığırcıklarda *(Sturnus vulgaris) M. cucullaris, B. nebulosa*, *Brueelia sp.* ve *S. sturni*'ye [10], Boğmaklı toygar *(Melanocorypha calandra),* Sarı kuyruksallayan *(Motacilla flava)* ve Dağ incirkuşunda *(Anthus spinoletta) M. pusillus*'a, Bataklık kirazkuşunda *(Emberiza schoeniclus) M. chrysophaeus*'a, Kır kırlangıcında *(Hirundo rustica) M. rustica*'ya, Kızılsırtlı örümcekkuşunda *(Lanius collurio) B. cruciata*'ya, Orman söğütbülbülünde

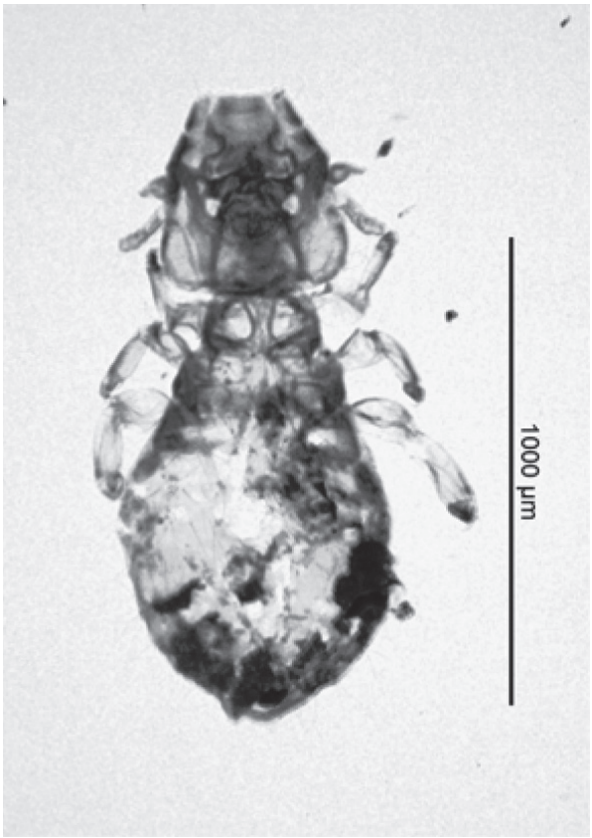


Şekil 2. *Philopterus sittae*, dişi, orijinal
Fig 2. *Philopterus sittae*, female, original

Şekil 3. *Philopterus vernus*, dişi, orijinal
Fig 3. *Philopterus vernus*, female, original



Şekil 4. *Brueelia jacobi*, dişi, orijinal
Fig 4. *Brueelia jacobi*, female, original



Şekil 5. *Philopterus* spp, nimf, orijinal
Fig 5. *Philopterus* spp, nymph, original

(Phylloscopus sibilatrix) ise *P. rarus*'a [14], Karatavukta *(Turdus merula) Menacanthus* sp.[11], *R. elongatus* ve *B. merulensis*'e rastlanmıştır [13]. Diğer taraftan, Leş kargası, Ev kırlangıcı ve Serçeden oluşan 16 ötücü kuş örneği [9] ile, Bülbül *(Luscinia megarhynchos)*, Çaprazgaga *(Loxia curvirostra)*, Kanarya *(Serinus canaria)*, Mavi baştankara *(Parus caeruleus)*, Maskeli ötleğen *(Sylvia melanocephala)*, Saka *(Carduelis carduelis)* ve Serçeden oluşan sekiz ötücü kuş örneğinin hiçbirisinde bite rastlanmadığı ifade edilmiştir [11,12]. Türkiye'de, daha önce yapılan araştırmalarda ötücü kuşlarda bit enfestasyonuna genel olarak düşük oranlarda rastlandığı ifade edilmiştir [14]. Enfestasyon oranının sığırcıklarda %14.81 [10], Kars'ın Kuyucuk Gölü'nde yakalanan ötücü kuşlarda ise %21.57 [14] olduğu bildirilmiş, fakat bazı çalışmalarda [9,12] incelenen ötücü kuşların hiç birisinde bite rastlanmamıştır. Özellikle Doğu Avrupa ülkelerinde yapılan çalışmalarda ötücü kuşlarda birçok bit türüne rastlanmış ve yeni türler tanımlanmış olmasına rağmen [5-8], bu araştırmada incelenen 204 ötücü kuştan sadece beşi (%2.45) bitlerle enfeste bulunmuş ve dört bit türü; *B. jacobi, P. sittae*, *P. vernus* ve *Philopterus* spp. tespit edilmiştir. Bu araştırmada enfestasyon oranının çok düşük çıkmasının muhtemel nedenleri Dik ve ark.[14] tarafından daha önce tartışılmıştır.

Bu araştırmada incelenen Aegithalidae, Certhiidae, Prunellidae ve Sittidae aileleri, *Aegithalos*, *Certhia, Muscipapa, Prunella, Sitta, Regulus, Oenanthe* ve *Turdus* cinslerinde yer alan 18 kuş türü; Uzunkuyruklu Baştankara, Bahçe Tırmaşıkkuşu, Orman Tırmaşıkkuşu, Bahçe Kirazkuşu, Florya, Küçük İskete, Benekli Sinekkapan, Çam Baştankarası, Akyanaklı Baştankara, Kayın Baştankarası, Dağ Bülbülü, Anadolu Sıvacısı, Orman Söğütbülbülü, Söğüt Bülbülü, Çalıkuşu, Küçük Akgerdanlı Ötleğen, Karakulaklı Kuyrukakan ve Öter Ardıç Türkiye'den ve Ökse Ardıcı ise Midilli Adası'ndan bu araştırmayla ilk kez bit yönünden muayene edilmiş olup bunlardan sadece Anadolu Sıvacısı ile Ökse Ardıcında bit saptanmıştır. Daha önceki çalışmalarda incelen 10 ötücü kuş türünden sadece Karatavukta bite tesadüf edilmiş, diğer türlerde ise bit tespit edilememiştir.

İncelenen kuş aileleri esas alındığında, Sittidae ailesi 75 örnekle en çok örnekle ilk sırayı almış, onu 42 örnekle Paridae, 36 örnekle Fringillidae ve 22 örnekle Turdidae aileleri takip etmiştir. Sittidae ailesinde, *Sitta* cinsinde bulunan *S. krueperi* ile Turdidae ailesinde, *Turdus* cinsinde bulunan *T. merula* ve *T. viscivorus* dışındaki aile ve cinslerde yer alan kuş türlerinin hiçbirisinde bit tespit edilememiştir. Birey sayısı esas alındığında; ilk sırayı 75 örnekle Anadolu sıvacısı *(S. krueperi)* almış, onu 22 örnekle İspinoz *(Fringilla coelebs)*, 15'er örnekle Büyük baştankara *(Parus major)* ve Çam baştankarası *(Parus ater)* takip etmiş, diğer türler ise daha az sayılarda yakalanmıştır. Bunlardan sadece iki Anadolu sıvacısı bitlerle enfeste bulunmuş, her ikisinde de *P. sittae*'ye rastlanmış, diğerlerinde ise bit saptanamamıştır.

Fedorenko [17] Sıvacıkuşundan *(Sitta europea) P. sittae*'yi tanımlamıştır. Price ve ark.[4] ise Anadolu sıvacısından, o güne kadar herhangi bir bit türünün kaydedilmediğini bildirmişlerdir. Türkiye'de, bugüne kadar yapılan çalışmalarda herhangi bir Sıvacıkuşu *(Sitta europea)* veya Anadolu sıvacısının *(S. krueperi)* bit yönünden incelendiğine dair bir kayıt bulunamamıştır. Bu araştırmada, Anadolu sıvacısından toplanan 5 dişi ve 4 erkek *Philopterus* örneğinin morfolojik özelliklerinin Fedorenko [17] tarafından tanımlanan *P.sittae* ile uyumluluk gösterdiği belirlenmiş ve bu tür hem Türkiye'den hem de Anadolu sıvacısından ilk kez bildirilmektedir.

Ökse Ardıcında *(Turdus viscivorus)* görülen *Philopterus* türleri ile ilgili fazla yayına rastlanmamıştır. Zlotorzycka [16] ötücü kuşlarda görülen ve Philopterinae alt ailesinde yer alan bit türleri üzerine yaptığı araştırmada, Ökse ardıcından *P. vernus*'u *(Docophorulus vernus)* tanımlamış ve Eichler tarafından Ökse ardıcından 1951 yılında tanımlanan *Docophorulus merulae* ile Denny tarafından karatavuktan tanımlanan *Docophorus merulae*'nin bu türün sinonimi olduğunu bildirmiştir. Gerçi, Zlotorzycka [16] makalesinde *P. vernus*'un morfolojik özellikleri hakkında ayrıntılı bilgi vermemiş, sadece bu türe ait bazı ölçümlerle erkek genitaliasının çizimini vermiştir. Bu araştırmada incelenen örnekler morfolojik özellikleri bakımından Zlotorzycka'nın [16] verdiği ölçümlerle benzerlik göstermektedir. Erkek örneklerin genitaliaları da verilen şekildeki ile uyumlu olup, buna bağlı olarak *P. vernus* olarak teşhis edilmişlerdir.

Price ve ark.[4] o güne kadar karatavuklarda *Brueelia amsel* (Eichler, 1951), *Brueelia jacobi* Eichler, 1951, *Brueelia merulensis* (Denny, 1842), *Brueelia oudhensis* Ansari, 1956, *Menacanthus eurysternus* (Burmeister, 1838), *Myrsidea thoracica* (Giebel, 1874), *Philopterus turdi* (Denny, 1842) ve *Ricinus elongatus* (Olfers, 1816) olmak üzere sekiz bit türünün saptandığını kaydetmişlerdir. Eichler [20], yaptığı bir çalışmada *Brueelia merulensis (Docophorulus merulae)*'in karatavuklarda görüldüğünü bildirmiş ve karatavuklardan *Brueelia amsel (Allobrüelia amsel)* ile *Brueelia jacobi* (*Brüelia jacobi*)'yi tanımlamıştır. Bununla birlikte, Eichler (1951) bu türler hakkında yeterli bilgi vermemiştir Ansari [15] *Brueelia* cinsindeki türlerle ilgili karşılaştırmalı herhangi bir çalışmanın yapılmadığını belirtmiş, karatavuklarda görülen *Brueelia merulensis* (Denny, 1842), *Brueelia amsel* (Eichler, 1951) ve *B. jacobi* (Eichler, 1951) hakkında geniş bilgi vermiş, ayrıca *Brueelia oudhensis*'i tanımlamıştır. Türkiye'de karatavuklarda görülen bit türleri üzerine yapılmış çok fazla çalışma bulunmamaktadır. Yapılan bir çalışmada [13], Kars'ta, Aras nehri çevresinde yakalanan bir karatavukta *Menacanthus* sp.'ye rastlandığı bildirilmiştir. Diğer bir çalışmada ise [13] Türkiye'deki karatavuklardan *Ricinus elongatus* ve *Brueelia merulensis*'in varlıkları ilk kez bildirilmiş ve karatavuklarda görülen *Brueelia* türlerinin bazı morfolojik özellikleri tartışılmıştır. Bu araştırmada incelenen beş karatavuğun birisi bitlerle enfeste bulunmuş, bu kuştan toplanan bitlerden ikisi *B. jacobi* olarak teşhis

edilmiş, fakat diğer *Brueelia* türlerine ise rastlanmamıştır. Her ne kadar Price ve ark.[4] karatavuklarda *Philopterus* cinsinden sadece *Philopterus turdi*'nin bulunduğunu kaydetmişlerse de bu araştırmada enfeste karatavuktan toplanan üç *Philopterus* örneğinden ikisi nimf döneminde olduklarından, tek dişi örnek de başının kopmuş olmasından dolayı tür seviyesinde teşhis edilememiştir. Böylece; bu araştırmayla *Philopterus* spp'nin ve *B. jacobi* nin karatavuklardaki varlıkları bu araştırmayla Türkiye'den ilk kez bildirilmiştir.

Sonuç olarak; bu araştırmada incelenen 204 kuş örneğinin beşi bitlerle enfeste bulunmuş, 29 kuş türünden sadece Anadolu sıvacısı, Karatavuk ve Ökse ardıcında bite rastlanmıştır. Enfeste kuşlarda dört tür; *B. jacobi, P. sittae, P. vernus* ve *Philopterus* spp. tespit edilmiş olup, bu araştırmayla bu türlerden *P. vernus* Midilli Adası'ndan, diğer türlerin tamamı ise Türkiye'den ilk kez bildirilmektedir. Buna ilaveten, bu araştırma ile *P. sittae* tüm dünyada Anadolu sıvacısından ilk kez rapor edilmektedir.

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RESEARCH ARTICLE

Investigation on the Polymorphism of Some Loci by Using PCR-RFLP in Japanese Quails (*Coturnix coturnix japonica*) Raised in Different Locations of Turkey [1]

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Summary

The aim of this study was to investigate the restriction fragment length polymorphism (RFLP) of some loci on chromosomes 1 (CJA1) and 3 (CJA3) in Japanese quails (*Coturnix coturnix japonica*) raised in Turkey. With this study population genetics parameters were estimated in order to select individuals for establishing a reference population, which would be used for studies on recombination frequency. Fertilized eggs obtained from flocks raised in six different provinces of Turkey were incubated for 120 h and 191 embryos were collected. From the tissue samples of the embryos, DNA was isolated by using DNA isolation kits. Particular regions of *SEMA3E, IFR1, HAL, LOC396025, UGP2, LOC396192, TLX* and *BMP5* loci were amplified with specifically designed primers for each locus by using PCR technique. The PCR products were cut with an appropriate restriction enzyme for each locus and analysed by using agarose gel electrophoresis. Presence of different alleles, allele and genotype frequencies, heterozygosities and genetic distances were estimated. Out of the eight loci studied, polymorphism was found for the *SEMA3E* and *TLX* loci on 1. and 3. chromosomes, respectively, while five loci were found to be monomorphic and one locus (*HAL*) could not be amplified by PCR. The populations studied were found to be mostly in Hardy-Weinberg equilibrium. The results indicated that the *SEMA3E* and *TLX* loci can be used for studying recombination frequencies in the populations included into the study.

Keywords: *Japanese quail, RFLP, Polymorphism, Recombination*

Türkiye'deki Japon Bıldırcınlarında (*Coturnix coturnix japonica*) Bazı Lokuslardaki Polimorfizmin PCR-RFLP Yöntemi İle Araştırılması

Özet

Bu çalışmanın amacı Japon bıldırcınlarında (*Coturnix coturnix japonica*) 1. (CJA1) ve 3. (CJA3) kromozom üzerinde bulunan bazı genlerdeki kesim bölgesi polimorfizminin (RFLP) araştırılmasıdır. Bu çalışma ile rekombinasyon oranları ile ilgili araştırma amacıyla kullanılacak bir popülasyonu oluşturacak bireylerin seçimine temel teşkil edecek olan popülasyon genetiği parametrelerinin ortaya konması amaçlanmıştır. Türkiyenin altı farklı ilindeki işletmelerden elde edilen döllü bıldırcın yumurtaları 37°C'de 120 saat süreyle inkube edildikten sonra 191 adet embriyodan doku örneği alınmıştır. Alınan doku örneklerinden özel kitler yardımıyla DNA izolasyonu yapılmıştır. *SEMA3E, IFR1, HAL, LOC396025, UGP2, LOC396192, TLX* ve *BMP5* lokuslarının belirli bölgeleri özel olarak dizayn edilmiş olan primerler yardımıyla ve PCR işlemi ile çoğaltılmıştır. Elde edilen PCR ürünleri uygun bir restririksiyon enzimi ile kesilmiş ve agaroz jel elektroforezi yardımıyla ayrımlanmıştır. İncelenen popülasyonlarda farklı allellerin varlığı, genotip ve allel frekansları, heterozigotluk dereceleri ve genetik mesafeler hesaplanmıştır. Çalışmada kullanılan sekiz lokustan 1. ve 3. kromozomlar üzerinde bulunan *SEMA3E* ve *TLX* lokuslarında polimorfizm tespit edilmiş, beş lokus monomorfik olarak bulunmuş bir lokusta (*HAL*) ise PCR işlemi ile çoğaltma yapılamamıştır. Çalışılan popülasyonların büyük oranda Hardy-Weinberg dengesinde oldukları gözlenmiştir. Araştırmanın sonuçları *SEMA3E* ve *TLX* lokuslarının çalışılan popülasyonlarda rekombinasyon oranlarının araştırılması amacıyla kullanılabileceğini göstermiştir.

Anahtar sözcükler: *Japon bıldırcını, RFLP, Polimorfizm, Rekombinasyon*

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INTRODUCTION

Recombination due to cross over is controlled by many enzyme and factors [1]. Recombination frequency between two loci on the same chromosome varies depending on the distance between two loci considered, as well as between sexes or families [2-4].

In some cases, it may be necessary that an allele of a locus is linked to a particular allele of another locus on the same homologous chromosome in coupling phase. This can only be achieved via recombination, if the alleles considered are on different homologous chromosomes in repulsion phase. On the other hand, the probability of recombination between closely linked loci is very low. In this case it takes a long time or a large number of individuals should be examined, in order to obtain a particular combination of alleles linked on the same chromosome. However some drugs may elevate recombination rate. Kunz et al.[5] have shown that 5-floro-deoxy uridinmonophosphate (FdUMP) increase mitotic recombination in yeast *(Saccharomyces cereviciae)* by inhibiting the enzyme thimidine synthase. Silber et al.[6] have reported that fluorodeoxyuridine (FUdR) and aminopterine elevates meiotic recombination in *Drosophyla melanogaster*, by inhibiting dihydrofolate reductase enzyme.

In order to estimate recombination frequency it is necessary to know whether an allele is of maternal or paternal origin. The most convenient way for this is back crossing of F1 individuals, which have been obtained by crossing two different homozygous lines with one of the parental lines. Therefore polymorphic loci and homozygous lines for the loci considered are necessary.

Japanese quail *(Coturnix coturnix japonica)* is an ideal experimental animal species due to its small body size, lower cost of care and high reproduction rate. Therefore Japanese quails have been widely used for studies of different purposes [7-9]. Since Japanese quail is closely related to chickens, it is an ideal model organism as well [10,11].

The aim of this study was to investigate the variability of eight loci located on chromosomes 1 (CJA1) and 3 (CJA3) of Japanese quails raised in different provinces of Turkey by using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) method. It was aimed to estimate some population genetics parameters in order to select individuals for establishing a reference population, which would be used for studies on recombination frequency.

MATERIAL and METHODS

Sample Collection and DNA Isolation

Fertilized Japanese quail eggs were purchased from six different provinces of Turkey, and incubated at 37°C and 70% relative humidity for 120 h. Following the incubation the eggs were stored at 4°C for 24 h, and the embryos were taken into microfuge tubes containig 96% ethanol and stored at -20°C until DNA isolation. A total of 191 embryos were obtained from Gaziantep (n= 40), Konya (n= 33), Manisa (n= 16), Mersin (n= 23), Afyon (n= 39) and Eskişehir (n= 40) provinces. From the embryos, DNA was isolated by using DNA isolation kits according to instructions of the manufacturer (Fermentas, Vilnius, Lithuania).

Selection of the loci and Genotyping of the Samples

The loci included into the study and sequences of the primers used for polymerase chain reaction (PCR) were shown in *Table 1*. The loci included into the study were selected based on the chromosome map constructed by Sasazaki et al.[11]. The primers used for amplification of the loci were designed based on the sequences submitted to the GenBank by Sasazaki et al.[11], by using Primer-Blast program *(http://www.ncbi.nlm.nih.gov)*.

PCR was carried out in 25 µL reaction volume. The reaction mix consisted of 0.4 µM of each primers, 200 µM dNTPs each, 2 mM $MgCl_2$, 1.25 U DNA polymerase (Thermo Scientific, Espoo, Finland) and 2.5 µL of 10X reaction buffer containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl and 0.1% Triton X-100. The amplification protocol consisted of an initial denaturing step of 94°C for 5 min, followed by 10 cycles of 94°C for 30 s, 60°C for 30 s decreasing 1°C in each cycle and 72°C for 30 s and 25 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s. In the final cycle the extension step was carried out at 72°C for 10 min

A 10 µL of PCR product was digested by using the restriction enzyme reported by Sasazaki et al.[11] according to the instructions of the manufacturer (Fermentas, Vilnius, Lithuania). The restriction enzymes and reaction conditions used were shown in *Table 2*. Control of the PCR and digestion products was performed by electrophoresis on a 2% agarose gel stained with ethidium bromide.

Data Analysis

Observed and expected genotype frequencies were compared by chi-square test [12]. Inbreeding coefficients (F-values) in each population and genetic distances due to inbreeding were estimated according to Weir and Cockerham [13]. Genetic distances between the populations were estimated according to Nei's formula (D_{SA}) [14]. Significance of the differentiation between populations was tested according to Raymond and Rousset [15]. Software packages of GENEPOP Version 3.1 [16], GenAlEx.6 [17], ARLEQUIN 3.5 [18] and POPGENE 1.31 [19] were used for the analysis of the data.

The study was carried out with the permission of Harran University Animal Experimentation Local Ethics Committee [Approval No: 2010/04 (1/2)].

Table 1. *The loci included into the study and sequences of the primers used.*
Tablo 1. *Araştırmaya dahil edilen lokuslar ve kullanılan primerlerin baz dizisi*

Locus	Chromosomal Location (cM)	*Primer Sequence (5'-3')*	Accession Number
SEMA3E	CJA1 (70.5)	Forward-ATACTCCAGCTGAGTGGGGA	AB250305
		Reverse-CAGAAGTATGAGGGAGATCAG	
IFR1	CJA1 (88.8)	Forward-AGTGTGCAGCCTTTTAGTGATGAAG	AB250306
		Reverse-TGAAGGGAGGCTGTAGTGAG	
HAL	CJA1 (106.8)	Forward-AATCACCACAGGCTTTGGGA	AB250307
		Reverse-TTCCACTGTAGCCCTTTGCG	
UGP2	CJA3 (12.5)	Forward-TTGGTGTGTGTGCTTCAGAG	AB250322
		Reverse-CCAGTCTGCATTGCCTAAC	
LOC396025	CJA3 (24.6)	Forward-TGGTGACCAGCACCAAAGC	AB250321
		Reverse-TTCCACTGTAGCCCTTTGCG	
LOC396192	CJA3 (157.4)	Forward-AGTGGTTATTGCCTGTGGTT	AB250325
		Reverse-AGGAGTAGTAAGTGAAGCCTG	
TLX	CJA3 (165.4)	Forward-ACACTAGGAACATAATGGGCT	AB250326
		Reverse-TCACTGTGGCGTTTCAGATT	
BMP5	CJA3 (171.6)	Forward-ACTGATCATAAGCGTGCCCT	AB250327
		Reverse-CCAGACGCTTACACTGTGC	

Table 2. *Restriction enzymes and reaction conditions used for each loci*
Tablo 2. *Restriksiyon enzimleri ve her bir lokus için kullanılan reaksiyon şartları*

Locus	Enzyme	Amount (Unit)	Incubation (°C/h)	Inactivation	Buffer
SEMA3E	*Hae* III	5	37/12	80°C /20 min.	R
IFR1	*Hin*6I	5	37/12	65°C/ 20 min.	Tango
HAL	*Nco*I	5	37/12	65°C/ 20 min.	Tango
LOC396025	*Mse*I	2.5	65/12	0.5 M EDTA	R
UGP2	*Hpa*I	5	37/12	65°C/ 20 min.	B
LOC396192	*Mse*I	2.5	65/12	0.5 M EDTA	R
TLX	*Pst*I	2.5	37/12	0.5 M EDTA	O
BMP5	*Tai*I	5	65/12	0.5 M EDTA	R

RESULTS

The loci included into the study, except for the *HAL* locus, were successfully amplified by using the primer pairs designed. Length of the PCR products of different loci varied from 356 to 630 *(Table 3)*. After cutting with the appropriate enzymes, one to five different fragments for each locus were observed *(Table 3)*. Restriction products of some samples for each locus were shown in *Fig 1*. Polymorphism was observed only for the *SEMA3E* and *TLX* loci. For the *SEMA3E* locus three different alleles and four genotypes were observed, while two different alleles and three genotypes were found for the *TLX* locus. Allele and genotype frequencies of *SEMA3E* and *TLX* loci in each population were shown in *Table 4* and *5*, respectively.

Number of alleles, heterozygosities and inbreeding coefficients of *SEMA3E* and *TLX* loci in each population were given in *Table 6*. Deviation from the equilibrium was observed only for *TLX* locus in Manisa population ($P<0.05$). When the *SEMA3E* and *TLX* loci were considered together, significant genotypic differentiations were observed

Table 3. *Fragment lengths of PCR and restriction products.*
Tablo 3. *PCR ve restriksiyon ürünlerinin fragman uzunlukları*

Locus	Length of PCR Product (bp)	Cut	Lengths of Restriction Fragments (bp)
SEMA3E	412	+	412, 362+50, 335+77
IFR1	630	+	390+240
HAL	-	-	-
LOC396025	385	+	252+83+50
UGP2	476	-	476
LOC396192	363	+	264+78+21
TLX	546	+	546, 404+142
BMP5	356	+	281+75

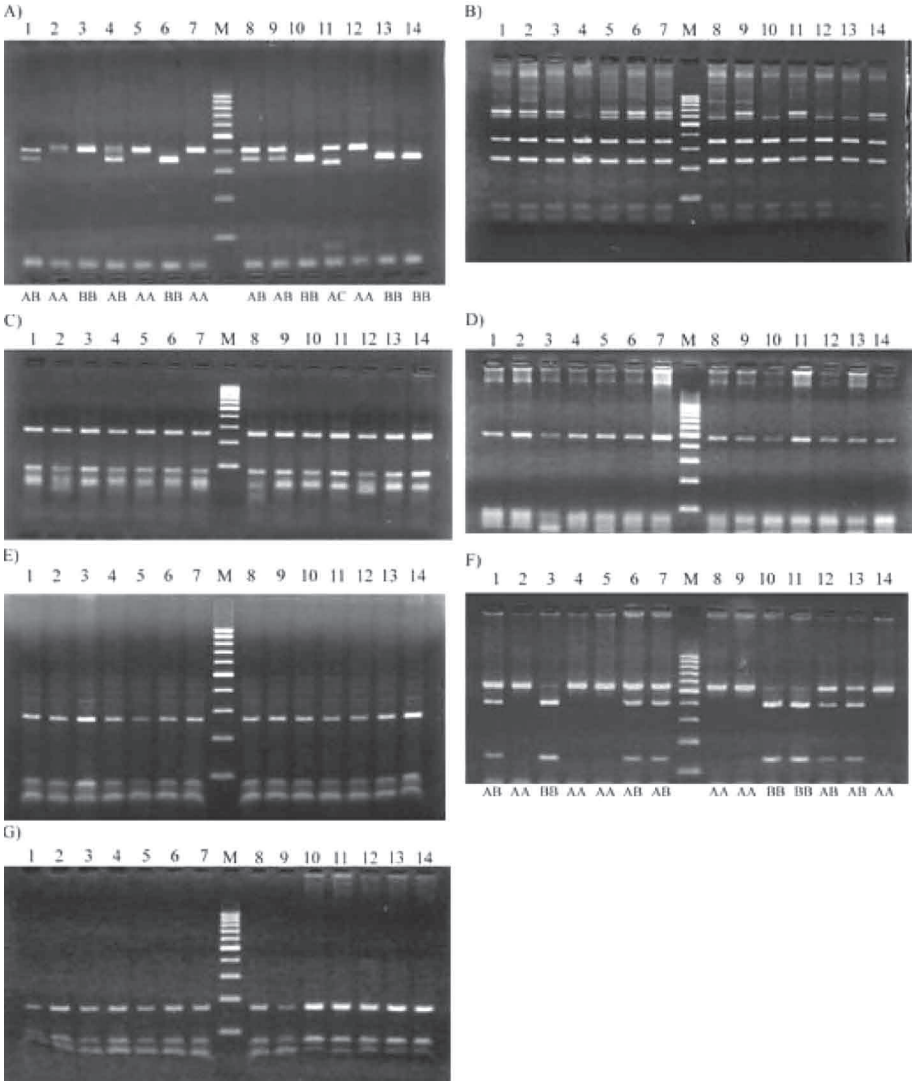

Fig 1. Restriction products of some samples on 2% agarose gel. **A)** *SEMA3E*; **B)** *IFRI*; **C)** *LOC396025*; **D)** *UPG2*; **E)** *LOC396192*; **F)** *TLX*; **G)** *BMP5*. Lines 1-14: Samples; M: Molecular size standard (100 bp ladder). The genotypes of the samples assigned for *SEMA3E* and *TLX* loci were shown at the bottom of the related picture

Şekil 1. Bazı örneklere ait restriksiyon ürünlerinin %2'lik agaroz jeldeki görünümü. **A)** *SEMA3E*; **B)** *IFRI*; **C)** *LOC396025*; **D)** *UPG2*; **E)** *LOC396192*; **F)** *TLX*; **G)** *BMP5*. 1-14: Örnekler; M: Moleküler standart (100 bç merdiven). Örneklerin *SEMA3E* ve *TLX* lokusları açısından genotipleri ilgili resmin altında gösterilmiştir

Table 4. *Allele frequencies at the SEMA3E and TLX loci in each population*
Tablo 4. *Her bir populasyondaki SEMA3E ve TLX lokuslarına ait allel frekansları*

Locus	Allel	Gaziantep (n=40)	Mersin (n=23)	Konya (n=33)	Manisa (n=16)	Eskişehir (n=40)	Afyon (n=39)	All (N=191)
SEMA3E	A	0.625	0.674	0.652	0.469	0.738	0.769	0.675
	B	0.363	0.326	0.318	0.531	0.263	0.231	0.317
	C	0.013	0.000	0.030	0.000	0.000	0.000	0.008
TLX	A	0.550	0.391	0.606	0.563	0.488	0.654	0.550
	B	0.450	0.609	0.394	0.438	0.513	0.346	0.450

between Mersin and Afyon ($P<0.05$), Manisa and Eskişehir ($P<0.05$) as well as Afyon ($P<0.01$) populations. The lowest genetic distance was observed between Gaziantep and Konya populations, while the highest genetic distance was observed between Manisa and Afyon populations.

DISCUSSION

The loci in this study were selected due to their location on the chromosomes, by considering the map reported by Sasazaki et al.[11]. The loci should be closely linked to each other so that a recombination could be detected and double recombinations did not occur at a high frequency. In addition the loci *SEMA3E, HAL* and *IFR1* on CJA1 as well as *LOC396025, TLX* and *BMP5* on CJA3 were selected, in order to assess an interference between the loci. The functional properties of the loci were not considered.

All the loci, except for *HAL* locus, were successfully amplified by using the designed primer pairs and PCR method. Except for the *UGP2* locus, PCR products of *IFR1*,

Table 5. *Genotype frequencies at the SEMA3E and TLX loci in each population*
Tablo 5. *Her bir populasyonda SEMA3E ve TLX lokuslarına ait genotip frekansları*

Locus	Genotype	Gaziantep	Mersin	Konya	Manisa	Eskişehir	Afyon	All
SEMA3E	AA	0.325	0.522	0.424	0.125	0.600	0.564	0.450
	AB	0.575	0.304	0.394	0.687	0.275	0.410	0.424
	AC	0.025	0.000	0.061	0.000	0.000	0.000	0.016
	BB	0.075	0.174	0.121	0.188	0.125	0.026	0.110
	BC	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	CC	0.000	0.000	0.000	0.000	0.000	0.000	0.000
TLX	AA	0.275	0.174	0.424	0.438	0.250	0.410	0.320
	AB	0.550	0.435	0.364	0.250	0.475	0.487	0.450
	BB	0.175	0.391	0.212	0.312	0.275	0.103	0.230

Table 6. *Number of alleles, heterozygosities and inbreeding coefficients for SEMA3E and TLX loci in each population*
Tablo 6. *Her bir populasyonda SEMA3E ve TLX lokuslarına ait allel sayıları, heterozigotluk derecesi ve kanyakınlı/ğı katsayısı*

Population	Locus	Na	Ho	He	UHe	F
Gaziantep	*SEMA3E*	3	0.600	0.478	0.484	-0.256
	TLX	2	0.550	0.495	0.501	-0.111
Mersin	*SEMA3E*	2	0.304	0.440	0.449	0.308
	TLX	2	0.435	0.476	0.487	0.087
Konya	*SEMA3E*	3	0.455	0.473	0.481	0.040
	TLX	2	0.364	0.478	0.485	0.238
Manisa	*SEMA3E*	2	0.688	0.498	0.514	-0.380
	TLX	2	0.250	0.492	0.508	0.492
Eskişehir	*SEMA3E*	2	0.275	0.387	0.392	0.290
	TLX	2	0.475	0.500	0.506	0.049
Afyon	*SEMA3E*	2	0.410	0.355	0.360	-0.156
	TLX	2	0.487	0.453	0.459	-0.076
All	*SEMA3E*	3	0.440	0.443	0.445	0.008
	TLX	2	0.450	0.495	0.496	0.090

Na: *Number of alleles;* ***Ho:*** *Observed heterozygosity;* ***He:*** *Expected heterozygosity;* ***UHe:*** *Unbiased expected heterozygosity;* ***F:*** *Inbreeding coefficient*

LOC396025, *LOC396192*, *BMP5*, *SEMA3E* and *TLX* loci were cut with the restriction enzymes *(Table 3)* at specific sites. PCR products of *UGP2* locus were not cutable with *Hpa*I. This indicated that there was no restriction site for this enzyme on target region.

When the PCR products of the *IFR1*, *LOC396025*, *LOC39619* and *BMP5* loci were cut with the respective enzyme, specific banding patterns for each locus were observed (*Table 3* and *Fig. 1B, C, D, E* and *G,* respectively). However no variability between individuals for the banding patterns of these loci was observed. Therefore the data for these loci were not included into the estimation of population genetics parameters.

When restriction products of *SEMA3E* locus was examined five different bands were observed, suggesting the presence of two polymorphic restriction sites on different position of the PCR products (*Table 3* and *Fig. 1A*). On the other hand three different bands were observed for *TLX* locus (*Table 3* and *Fig. 1F*), indicating the presence of a polymorphic restriction site for *Pst*I enzyme.

To our knowledge there was no report on the polymorphism of the loci used in the present study in other populations of Japanese quails. Although Sasazaki et al.[11] have mapped these loci on the CJA1 and CJA3, they have reported no data on allele frequencies or heterozygosities. Therefore the results of the present study were compared with those reported on other loci or species.

Various genetic markers, such as microsatellites [7,20,21], amplified fragment length polymorphism (AFLP) [22,23] or PCR and restriction fragment length polymorphism (RFLP) [11] have been used to establish a linkage map in Japanese quails. Since by using PCR-RFLP method the presence or absence of a restriction site is detected, two alleles are expected at a particular site [24-26]. However, if there are two

polymorphic sites within the target region, more than two alleles can be found [27-29].

Allele frequencies in various studies, in which the same method has been used as in this study, have varied among populations from 0.000 to 1.000 [26,27]. Therefore allele frequencies observed in this study was in accordance with those reported in the literature.

A deviation from Hardy-Weinberg equilibrium was observed only in Manisa population for *TLX* locus ($P<0.05$). The deviation was due to an excess of homozygotes, as could be seen from the F value. On the other hand, an excess of heterozyotes was found for *SEMA3E*. This suggested that the deviation from the equilibrium might be due to the small sample size from this population.

In order to study recombination frequencies, test individuals should be heterozygous for the two loci considered, which should be polymorphic as well. In this study only *SEMA3E* and *TLX* loci located on CJA1 and CJA3, respectively, were polymorphic and thereby suitable for studying recombination frequency in the Japanese quail populations studied. Therefore further loci or other marker systems, such as microsatellites will be necessary. Individuals, which will be selected for establishing a reference population, should be obtained from the genetically more distant populations, such as Manisa and Afyon populations.

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RESEARCH ARTICLE

Overektomili Sıçanlarda Alendronatın Böbrek Dokusu Üzerindeki Etkileri

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Özet

Alendronat kemik rezorpsiyonunun selektif inhibitörü olan bir aminobifosfonat olup özellikle postmenopozal dönemde görülen osteoporozun tedavisinde kullanılmaktadır. Postmenopozda ortaya çıkan değişikliklerin yaşam kalitesini etkilediği bilinmektedir. Bu çalışmada overektomili sıçanlar üzerinde alendronat tedavisinin zamana bağlı olarak böbrekteki oksidan durum üzerinde etkisi incelenmiştir. Overektomi yapılan sıçanlara 2, 4 ve 8 hafta alendronat (0.3 mg/kg) intravenöz uygulanmıştır. Serumda ürik asit ve kreatinin düzeyleri, böbrek dokusunda malondialdehid (MDA) ve glutatyon (GSH) düzeyi, katalaz (CAT), süperoksid dismutaz (SOD) ve miyeloperoksidaz (MPO) aktivitesine bakılmıştır. Overektomili sıçanlarda serum kreatinin ve ürik asit düzeylerinin yükseldiği, alendronat tedavisi ile zaman bağlı olarak azaldığı görülmüştür. Böbrek MDA düzeyleri ve MPO aktivitesinin hem overektomi grubunda hem de alendronat tedavisi alan gruplarda yükseldiği görülmüştür. Buna karşın overektomili grupta böbrek GSH, SOD ve CAT düzeylerinin azaldığı, alendronat tedavisi ile zamana bağlı olarak arttığı gözlenmiştir. Sonuç olarak kemik rezorbsiyonun görüldüğü overektomide böbrek dokusunda bazı oksidatif değişiklikler meydana geldi ve alendronat tedavisinin zamana bağlı olarak antioksidan kapasiteyi arttırdığı bu çalışmada gösterilmiştir.

Anahtar sözcükler: *Alendronat, Oksidatif stres, Overektomi, Bifosfonat*

Effects of Alendronate on Kidney Tissue of Ovariectomized Rats

Summary

Alendronate is an aminophosphanate, selective inhibitory of bone resorbtion, used for treatment of osteoporosis in postmenopause. It is known that changes occurring in postmenopausal period effect the quality of life. In this study, effect of the alendronate treatment time on renal oxidative status was investigated in ovariectomized rats. Alendronate was intravenous administered at 0.3 mg/kg dosage on weeks 2, 4 and 8. Serum creatinine and uric acid levels and kidney malondialdehyde (MDA), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) and myeloperoxidase (MPO) level and activities were measured. Serum creatinine and uric acid levels increased in depending on the time of alendronate treatment on ovariectomized rats. Kidney MDA level and MPO activity increased in both ovariectomized and alendronate groups. Kidney GSH level and SOD and CAT activities decreased in the ovariectomized rats and these levels increased in depending on time of alendronate treatment. As a result, some oxidative changes occurred in the kidney tissue of ovariectomized rats with bone resorption and alendronate treatment increased the antioxidant capacity in depending on the time.

Keywords: *Alendronate, Oxidative stress, Ovariectomy, Biphosphanate*

GİRİŞ

Günümüzde osteoporoz tedavisinde birçok ajan kullanılmaktadır. Özellikle hormon replesman tedavisi hem osteoporozun önlenmesinde hem de tedavisinde kullanılır. Bunun yanı sıra hormon replesman tedavisi ile birlikte veya tek başına bifosfanatların da oldukça etkili olduğu bilinmektedir. Bifosfonatlar, kemik metabolizmasına güçlü etkileri olan pirofosfatın sentetik analoglarıdır. Başlıca etkiyi kemik metabolizması üzerine doğrudan kemik yıkımından sorumlu osteoklastik aktiviteyi azaltarak gösterirler. Bifosfonatlar dolaylı olarak da osteoblastik aktiviteyi de azaltırlar ve kalsiyumun tutulumunu sağlarlar [1]. Bifosfanatlar içerisinde en sık kullanılanlar arasında bulunan alendronat nitrojen içeren osteoporoz tedavisinde etkinliği ispatlanmış antirezorptif ajanlardan biridir [2]. Alendronat

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amino-bifosfanat grubunun bir bileşiği olup nitrojen içermeyen diğer bifosfonatlara göre 10-100 kat daha fazla rezorbsiyonu inhibe etme gücüne sahiptir [3]. Yapılan çalışmalarla post menopozal osteoporozda alendronat tedavisinin etkin olduğu ve bazı ilaçlar ile kombinasyonunda etkili bir tedavi rejimi olabileceği gösterilmiştir [4]. Ayrıca nitrojen içeren bifosfanatların kanserde gerçekleşen kemik metastazı üzerinde de etkili olduğu bildirilmektedir [5]. Bifosfanatların oksidatif stresi arttırarak antioksidan etkiyi azalttığı bildirilirken [6], bazı çalışmalarda da antioksidan sistemler üzerinde farklı etkiler gösterdiği öne sürmektedir [7]. Bu çalışma ile overektomili sıçanların böbrek dokusunda alendronatın oksidan ve antioksidan denge üzerinde etkili olup olmadığının araştırılması amaçlanmıştır.

MATERYAL ve METOT

Çalışmada 42 adet ortalama ağırlığı 302.21±30.88 g olan wistar albino sıçan kullanıldı. Çalışma Marmara Üniversitesi Deney Hayvanları Uygulama ve Araştırma Merkezinde 45.2005.MAR nolu etik kurul onayı alınarak yapıldı. Hayvanlar standart kafeslerde standart laboratuvar yemiyle beslendiler, su ve yem kısıtlaması yapılmadı. Oda ışığı 12 saat aydınlık 12 saat karanlık, sıcaklık 22±2°C ve nem oranı %50±10 olacak şekilde ayarlandı.

Overektomi

Overektomize gruplara ait sıçanlara 40 mg/kg g ketamin hidroklorid (Ketasol*, Richter Pharma, Avusturya) AG ve 10 mg/kg xylazine hidroklorid (Rompun* Bayer, İstanbul, Türkiye) intramusküler uygulandı ve hayvanlar genel anesteziye alınıp dorso-ventral olarak diseksiyon tahtasına sabitlendi. Abdominal bölgede merkez 1 cm bir deri insizyonu yapılarak ve bağ dokusu ayrılarak periton boşluğuna ulaşıldı. Ovaryumlar, insizyonun uterus tüpü ile birleştiği yerden ligatüre edilerek kesildi ve uterus tekrar eski yerine yerleştirildi. Çalışmada sıçanlar 7 gruba ayrılmış ve her bir grup 6 adet sıçandan oluşmaktadır. Gruplar kontrol, overektomi (OV) ve overektomi + Alendronat (OV + AL) olarak oluşturuldu, alendronat uygulaması 2, 4 ve 8 hafta periyodlarda gerçekleştirildi. Alendronatın osteoporoz tedavisinde önerilen günlük dozu baz alınarak sıçanlara alendronat 0.3 mg/kg olacak şekilde intravenöz olarak verildi. Aynı zamanda kontrol grubunu oluşturan sağlıklı ve overektomili sıçanlara da placebo olarak serum fizyolojik verilerek aynı koşullarda bekletildi.

Kan ve Doku Örnekleri

Bütün gruplardaki hayvanlar, son uygulamadan 24 saat sonra genel anestezi altında 40 mg/kg ketamin hidroklorid (Ketasol*, Richter Pharma, Avusturya) AG ve 10 mg/kg xylazine hidroklorid (Rompun* Bayer, İstanbul, Türkiye) kullanılarak kardiyak delme işlemi ile sakrifiye edildikten sonra kan ve böbrek dokusu örnekleri alındı. Kan örnekleri +4°C'de 2.500 rpm'de 5 dak. santrifüj edilerek serum ayrıldı. Serumda kreatinin ve ürik asit seviyelerine bakıldı. Böbrek dokuları tüm homojenatta 1:10 (w/v) bulunacak şekilde homojenizatörde %1.15 KCl içeren tampon ile buz içerisinde homojenize edildi. Homojenattan 5000 rpm'de, +4°C'de 15 dak. santrifüj yapılarak süpernatant elde edildi. MDA, GSH düzeyleri ile MPO, SOD ve CAT aktiviteleri belirlendi.

Serumda Kreatinin ve Ürik Asit Ölçümü

Serumda kreatinin ölçümü alkali koşullarda kreatinin ve pikrik asitin kompleks oluşturmasına dayanan Jaffe [8] metoduna göre yapıldı. Kompleksin renk yoğunluğunun absorbansı 520 nm'de spektrofotometrede ölçüldü, sonuçlar kreatinin standart grafiği kullanılarak hesaplandı. Ürik asitin ürikaz enziminin katalizörlüğünde allantoin ve hidrojen peroksite dönüşmesi sonrasında oluşan kromojen bileşiğin rengin 520 nm de ölçülmesine dayanan ürik asit ticari kiti (URI-10100, BT) kullanılarak spektrofotometrede (Shimadzu UV 1800, Japan) tespit edildi.

Oksidatif Stres ve Antioksidan Parametrelerin Ölçümü

- *MDA ve GSH Ölçümü:* Dokuda MDA için Beuge yöntemine göre yapıldı [9]. 1:10 doku homejenatı üzerine eşit hacimde %15 w/v trikoloroasetik asit, %0.375 w/v tiyobarbütürik asit ve 0.25 mol/L hidroklorik asit içeren çözeltisi eklendi ve karıştırıldı. Karışım 30 dak. kaynar su banyosunda bekletildikten sonra 10 dakika 3000 rpm'de santrifüj edildikten sonra 535 nm de okundu. GSH tayini için modifiye Ellman yöntemi kullanıldı [10]. Homojenattan elde edilen 0.5 ml süpernatant üzerinde 2 ml of Ellman ayıracı eklenerek absorbansı 412 nm de ölçüldü.

- *MPO, CAT ve SOD Aktivite Ölçümü:* MPO aktivitesi için Hillegass yöntemi kullanıldı [11]. Doku örnekleri %0.5 HETAB içeren 50 mM fosfat tamponunda homojenize edildi. Örnekler 20 mg/ml o-dianisidin hidroklorür ve 20 mM H_2O_2 içeren tampon ile karıştırıldıktan 3 dak. sonra 460 nm'de ölçüm yapıldı. CAT aktivitesi Aebi yöntemi kullanılarak UV/visible spektrofotometrede (Shimadzu UV 1800, Japan) ölçüldü [12]. Bu yönteme göre H_2O_2'nin H_2O ve O_2'ye dönüşümünü sırasında oluşan absorbans azalması 240 nm de takip edildi. SOD aktivitesi Mylroie yöntemine göre yapıldı [13]. 50 µl örnek üzerine 2 ml tampon (50 mM fosfat tamponu içerisinde 0.1 mM EDTA, 0.39 mM riboflavin, 6 mM o-dianisidin) eklenir. 20W floresan ışık önünde 37°C'de 8 dakikadaki absorbansı 460 nm'de ELISA okuyucusunda (Epoch Biotek, USA) ölçüldü. Protein tayini için Bradford yöntemi kullanıldı [14].

İstatistiksel Analizler

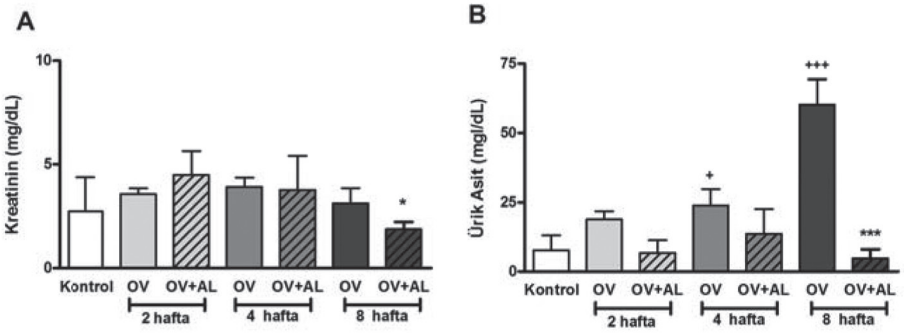
İstatistiksel hesaplamalar SPSS 13.0 (SPSS Inc., Chicago, IL, ABD) yazılımı kullanılarak yapıldı. Tek örneklemeli Kolmogorov-Smirnov testi ile verilerin normal dağılıma uyup uymadığı incelendi. Tüm gruplarda normal dağılım olması durumunda, grup içi değerlendirmeler one-way

Anova testi kullanılarak yapıldı. İstatistiksel anlamlılık sınırı P<0.05 olarak kabul edildi. Değerler ortalama±standart sapma (ort.±SS) olarak verildi.

BULGULAR

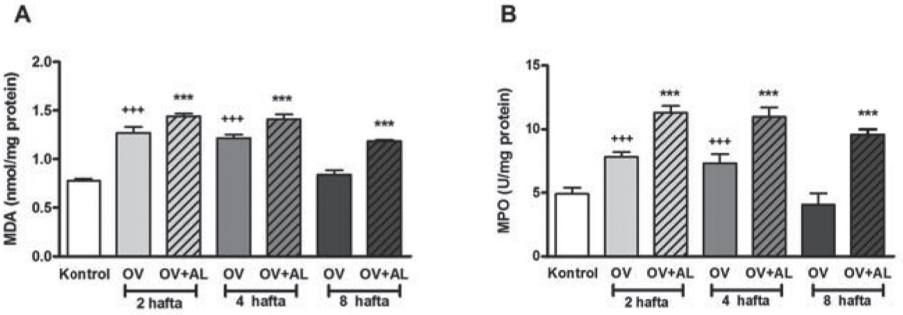
Serum kreatinin düzeylerinin zamana bağlı olarak OV grubunda kontrol grubuna göre yükseldiği görülmüş fakat bu artış anlamlı bulunamamıştır *(Şekil 1A)*. 8 haftalık sonuçlara bakıldığı zaman OV+AL grubunda kreatinin düzeylerinin OV grubuna göre anlamlı olarak azaldığı görülmektedir (P<0.05). *Şekil 1B*'de görüldüğü gibi serum ürik asit düzeyleri zamana bağlı olarak 4 hafta (P<0.05) ve 8 haftalık (P<0.001) OV gruplarında kontrol grubuna göre anlamlı olarak artış göstermiştir. 8 haftalık alendronat uygulaması olan OV+AL grubu ile OV grubu karşılaştırıldığında serum ürik asit seviyeleri anlamlı derecede azalmıştır (P<0.001).

Oksidatif stres göstergelerinden biri olan MDA seviyeleri böbreklerde 2 ve 4 haftalık OV gruplarında kontrol grubuna göre artış göstermiştir (P<0.001). Alendronat uygulaması ile 2, 4 ve 8 haftalık bütün OV+AL grupları kendi OV grubu ile karşılaştırıldığında MDA seviyelerinin anlamlı derecede arttığı görülmektedir (*Şekil 2A*, P<0.001). Aynı zamanda MPO düzeylerine bakıldığında 2 ve 4 haftalık OV gruplarında kontrol grubuna göre anlamlı derecede artış görül-



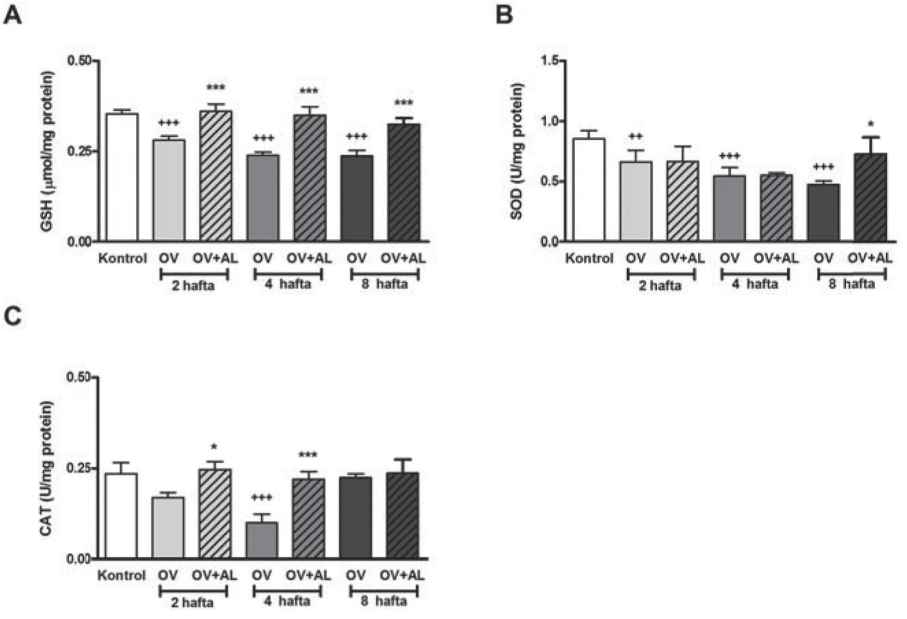
Şekil 1. Serum parametrelerindeki değişiklikler, **A)** Serum kreatinin seviyeleri, **B)** Serum ürik asit seviyeleri (+: kontrol grubu ile karşılaştırıldığında, ***:** OV grubu ile karşılaştırıldığında)

Fig 1. Changes in serum parameters, **A)** Serum creatinine levels,**B)** Serum uric acid levels (+: compared to control group, ***:** compared to OV group)



Şekil 2. Böbrek dokusunda oksidatif stres değişiklikleri, **A)** Böbrek MDA seviyeleri, **B)** Böbrek MPO seviyeleri (+: kontrol grubu ile karşılaştırıldığına, *: OV grubu ile karşılaştırıldığında)

Fig 2. Oxidative stres changes in kidney tissue, **A)** Kidney MDA levels, **B)** Kidney MPO levels (+: compared to control group, *: compared to OV group)



Şekil 3. Böbrek dokusu antioksidan kapasite değişiklikleri, **A)** Böbrek GSH seviyeleri, **B)** Böbrek SOD seviyeleri, **C)** Böbrek CAT düzeyleri (+: kontrol grubu ile karşılaştırıldığına, ***:** OV grubu ile karşılaştırıldığında)

Fig 3. Antioxidant capacity changes in kidney tissue, **A)** Kidney GSH levels, **B)** Kidney SOD levels, **C)** Kidney CAT levels (+: compared to control group, ***:** compared to OV group)

mektedir (P<0.001). OV+AL gruplarında da alendronat tedavisinin ardından OV gruplarına göre 2, 4 ve 8 haftalık gruplarda anlamlı bir artış görülmektedir (*Şekil 2B*, P<0.001).

Antioksidan durumun belirteçleri GSH, SOD ve CAT aktivitesi *Şekil 3*'te gösterilmektedir. Böbrek GSH düzeyleri her üç grubun OV gruplarında kontrol grubuna göre anlamlı bir düşüş göstermektedir (P<0.001). Bu GSH düzeylerindeki düşüş alendronat uygulanması ile her üç grubun OV+AL gruplarında OV gruplarına göre artış göstermektedir (*Şekil 3A*, P<0.001). SOD aktivite seviyelerinin OV grupları ile kontrol grubu karşılaştırıldığında 2 haftalık grupta azaldığı (P<0.01), 4 ve 8 haftalık grupta bu azalışın daha fazla olduğu görülmektedir (P<0.001). Alendronat uygulanan gruplarda böbrek SOD düzeylerinin 8 haftalık uygulamada OV+AL grubu ile OV grubu karşılaştırıldığında yükseldiği görülmektedir. SOD düzeylerinde 2 ve 4 haftalık alendronat uygulanan gruplarda anlamlı değişiklikler gözlenmemiştir (*Şekil 3B*, P<0.05). CAT aktivite düzeylerinin OV gruplarında kontrol grubuna göre azaldığı fakat 4 haftalık OV grubunda anlamlı olarak değiştiği görülmektedir (P<0.001). Alendronat uygulanması ile OV+AL grupları OV grupları ile karşılaştırıldığında CAT aktivitesinde artış görülmekle birlikte 2 hafta (P<0.05) ve 4 haftalık (P<0.05) uygulamalarda anlamlı değişiklik bulunmuştur *(Şekil 3C)*.

TARTIŞMA ve SONUÇ

Alendronat özellikle postmenopozal dönemde görülen osteoporozun tedavisinde kullanılan osteoklasta bağımlı kemik rezorpsiyonunun selektif inhibitörü olan bir aminobifosfonattır [15]. Bifosfonatlar insan vücudunda böbrekler tarafından elimine edilir. Hayvanlarda yüksek dozlarda verilen bifosfonatların renal fonksiyonlar üzerinde çeşitli yan etkilerinin olduğu bildirilir. Bifosfonatların postmenopozal osteoporozda böbrek fonksiyonları üzerinde yan etkisinin olmadığı ile ilgili görüşlerde mevcuttur [16].

Alendronat uygulamasının serumda bazı parametrelerde değişiklik yarattığı yapılan çalışmalarla gösterilmiştir [17]. Çalışmamızda renal fonksiyonlardaki değişikliği görmek için serum kreatinin ve serum ürik asit değerlerini inceledik. Çalışmamızda 8 hafta alendronat uygulanması ile serum kreatinin düzeylerinin azaldığı görülmektedir. Serum kalsiyum/kreatinin düzeyleri böbrek fonksiyon testlerinin yanı sıra osteoporoz tanısında kullanılan belirteçlerdir. Kovac ve ark.[18], böbrek transplantasyonu yapılan hastalarda kemik kaybının önlenmesinde alendronatın etkili olduğunu ve alendronatın serum kreatinin düzeylerini uzun süreli tedavide azalttığını bildirmişlerdir. Buna karşın Shahbazian ve ark.[19], postmenopozlu hastalarla yaptıkları çalışmada oral olarak kullanılan günlük 10 mg alendronatın serum kreatinin ve kalsiyum düzeyleri üzerinde etkili olmadığını göstermişlerdir. Postmenopoz döneminde serum ürik asit seviyelerinin arttığı ve renal fonksiyonlar üzerinde olumsuz etkileri olduğu yapılan çalışmalarla bildirilmektedir. Postmenaopoz dönemde uygulanan hormon replasman tedavisi ile bu etkinin azaldığı öne sürülmektedir [20,21]. Çalışmamızda overektomili sıçanlarda serum ürik asit düzeylerinin arttığını ve alendronat tedavisi ile düştüğünü gözlemledik. Jamal ve ark.[22], alendronat kullanımının ileri yaşlardaki kadınlarda renal fonksiyonlar üzerine yaptıkları çalışmada kreatinin klirensi ve serum kreatinin düzeyleri ile ilgili olumlu bulgular elde etmişlerdir. Post menopozda azalan kreatinin klirensinin ve artan serum kreatinin düzeylerinin alendronat tedavisi ile düzeldiğini ve alendronat tedavisinin güvenli ve yan etkisinin az olduğunu öne sürmektedirler

Bifosfanatların oksidatif stres üzerinde etkili olduğu ve antioksidan sistemler üzerinde farklı etkiler gösterdiği bildirilmektedir [6,7]. Reaktif oksijen türleri (ROT) birçok biyolojik reaksiyonlarda yer almakla birlikte bazı hastalıkların patogenezinde önemli rol oynamaktadırlar [23]. ROT' lar hücre membranı, genetik materyaller ve enzimatik yollar üzerine etki ederek doku hasarına neden olabilirler. Serbest radikaller ile antioksidan dengenin bozulması durumunda hücreler oksidatif hasara karşı birtakım enzimatik ve enzimatik olmayan sistemleri harekete geçirirler [24,25]. Serbest radikaller aracılığında hücre membranının stabilitesinin bozulması ile lipid peroksidasyonun gerçekleşmesi sonucu meydana gelen MDA, oksidatif stresin belirlenmesinde önemli bir belirteç olarak kullanılır. MPO nötrofillerden salınan bir enzim olup, nötrofil infiltrasyonunun göstergesi olarak kabul edilir. Nötrofiller serbest oksijen radikallerinin potansiyel bir kaynağıdır ve özellikle inflamatuar hastalıklarda meydana gelen doku hasarında rol oynarlar [26]. Hücreler için SOD, CAT ve GSH önemli savunma mekanizmalarının temelini oluştururlar. Hücre içinde mitokondride yer alan SOD süperoksit radikalini hidrojen peroksite dönüştüren antioksidan bir enzimdir. CAT ise hücrelerde peroksizomlarda bulunan hidroksil radikallerinin oluşumunu önleyerek hidrojen peroksiti suya ayrıştıran bir enzimidir. Glutamik asit, sistein ve glisin aminoasitlerinden meydana gelen suda çözünür bir antioksidan ve indirgeyici ajan olan GSH serbest radikaller ve peroksitlerle reaksiyona girerek hücreleri oksidatif hasara karşı korur. GSH hücresel savunma sistemlerinde rol oynayarak oksidatif hasarlarda başlıca koruyucu mekanizmayı oluşturur. Çeşitli uyarılarla oluşan doku hasarlarında GSH düzeylerinde azalma olduğu bildirilmektedir [25,27].

Postmenopoz döneminde oksidatif streste artış olduğu ve antioksidan denge üzerinde büyük değişikliklerin olduğu klinik çalışmalarda bildirilmektedir [23]. Yapılan çalışmalarda postmenopoz döneminin taklit edildiği overektomi oluşturulan sıçanlarda oksidatif stresin arttığı SOD, CAT, GPx gibi antioksidan enzimlerin azaldığı gösterilmiştir [28-30]. Çalışmamızda overektomide SOD ve CAT aktivitesinin düştüğünü uzun süre alendronat uygulaması ile bu aktivitenin arttığını gözlemledik. Muthusami ve ark.[31], overektomili sıçanların femur dokusunda MDA seviyelerinin normal sıçanlara göre arttığını, SOD ve GPx seviye-

lerinin düştüğünü göstermişlerdir. Biz çalışmamızda overektomide ve alendronat uygulanmasının MDA ve MPO düzeylerinin arttırdığını belirledik. Alendronatın yüksek dozlarda toksik etki yaratarak oksidatif stresi arttırdığı da ayrıca bildirilmiştir [32]. Yalın ve ark.[33], overektomili sıçanlarda alendronat uygulamasının hepatik antioksidan enzimler üzerine etkisi konusunda yaptıkları çalışmada alendronat uygulanması ile karaciğer MDA ve SOD seviyelerini yükseldiğini bildirmişlerdir. Alendronat uygulamasının antioksidan sistemler üzerindeki etkisi ile ilgili çok fazla çalışmaya rastlanamamıştır. Konyalıoğlu ve ark.[34], overektomize sıçanlarda beyin, kalp ve karaciğer dokusunda GSH düzeylerinin azaldığını göstermişlerdir. Çalışmamızda overektomi gruplarında GSH düzeylerinin azaldığını ve alendronat tedavisi ile arttığını bulduk. Literatürle bu konuda paralellik göstermekle birlikte alendronatın GSH düzeyleri üzerinde etkili olduğu ile ilgili yapılan ilk çalışmadır. Benghuzzi ve ark. tarafından overektomili sıçanlarda 4 hafta alendronat tedavisi ile renal fonksiyonlarda meydana gelen hasarın düzeltilebileceği vurgulanmıştır [35]. Zinnuroglu ve ark.[7], postmenopozal osteoporozda görülen oksidatif strese karşı bifosfonat kullanımının etkili olduğunu bildirmişlerdir.

Sonuç olarak, overektomi ile meydana gelen kemik rezorbsiyonunda böbrek dokusu üzerinde bazı oksidatif değişiklikler üzerinde alendronat tedavisinin zamana bağlı olarak antioksidan kapasiteyi arttırdığı tespit edilmiştir. Klinik açıdan post menopozal dönemde alendronat kullanımı osteoporoz riskini azaltabileceği gibi böbrek fonksiyonlarının korunmasında da önemlidir.

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RESEARCH ARTICLE

Effect of Short Photoperiod on Some Growth Traits in Sprague Dawley Rats [1]

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Summary

The effect of short photoperiod on some growth traits in outbred Sprague Dawley rats was examined. The pups were assigned to two photoperiod groups: long/routin (12 h light/dark: control) and short (9 h light/15 h dark: experiment) lighting, since, in nature, photoperiod may decrease to 9 hours in winters, in subtropical regions. At 15 weeks of ages, body weight, weight of heart, liver, spleen, lungs, kidneys, gastear, adrenal glands, testis and ovaries of the rats from experimental group were compared to those in control group. Body growth, weight of heart, liver and spleen and large intestine length in control and experimental rats were not significantly different (P>0.05). No differences were observed in testis and ovary weights between control and experiment groups, statistically. Rats exposed to short photoperiod had lower lung, kidney, gastear, adrenal gland and intestine weight than those of control rats (P<0.01 and P<0.05). In outbred Sprague Dawley rats, short photoperiod had no effect on body growth and reproduction organs and it is concluded that, there may be a chance for some strains of outbred Sprague Dawley rats to be kept under short photoperiod conditions, without reducing body weight and reproductive organ growth.

Keywords: *Sprague dawley, Photoperiod, Growth*

Sprague Dawley Ratlarda Kısaltılmış Işık Süresinin Bazı Büyüme Özelliklerine Etkisi

Özet

Outbred Sprague Dawley ratlarda, kısaltılmış ışık süresinin bazı büyüme özelliklerine etkisi incelendi. Yavrular 12 sa aydınlık/karanlık (kontrol) ve doğal şartlarda, kış mevsiminde, subtropik bölgelerde gerçekleşen kısa ışık süresi kadar (9 sa aydınlık/15 sa karanlık: muamele) olmak üzere iki fotoperyot şartına maruz bırakıldılar. Muamele grubu ratlarda, 15 haftalık yaşta, canlı ağırlık, kalp, karaciğer, dalak, akciğer, böbrek, mide, adren, testis, ovaryum, kalın ve ince bağırsakların ölçümleri, kontrol grubu ile karşılaştırıldı. Vücut ağırlığı artışı ile kalp, karaciğer, dalak ağırlık ve kalın bağırsak uzunlukları, kontrol ve muamele grupları arasında farklılık göstermedi (P>0.05). Kontrol ve muamele grupları arasında testis ve ovaryum ağırlıkları arasında da istatistiksel olarak bir farklılık gözlenmedi. Kısa fotoperyota maruz bırakılan ratların akciğer, böbrek, mide, adren ve ince bağırsakları, kontrol grubuna göre daha düşük ağırlıklarda belirlendi (P<0.01 ve P<0.05). Kısa fotoperiyodun, outbred Sprague Dawley ratlarda, vücut ve üreme organlarının ağırlığı üzerinde etkisi bulunmadı ve bazı outbred Sprague Dawley rat soylarının, vücut ve üreme organlarında ağırlık kaybı olmaksızın kısa fotoperyot şartlarında da yetiştirilebilme şansı olabildiği sonucuna varıldı.

Anahtar sözcükler: *: Sprague Dawley, Fotoperyot, Büyüme*

INTRODUCTION

Laboratory rats are bred in micro environments, where physiological needs are optimally supplied. Photoperiod is one of the biological needs of rats, and in common, 12 h light: 12 h dark lighting regime is used. However, in natural conditions, it is known that, even the light period is less than 9 h, rats continue their biological activities, including reproduction [1].

Photoperiod regulates melatonin level, epiphysis gland and organ development [2] and the respond varies according to breed, family and strain of the rats [3]. In Fischer *(F344)* rats, which are exposed to short photoperiod, puberta longened, feed intake and cellular growth decreased [4]. Feed intake did not differ in Zucker rats in both short and long photoperiod [5].

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It is reported that, youngers are more sensitive to photoperiod than olders, in most rodent species [6]. Pups, exposed to short lighting (6 h, daily) had less growth rate but similar feed intake, when compared to those, subjected to long (12 h) photoperiod [7].

In *F344* rats that exposed to short lighting after birth, had similar feed intake and body growth but less testis volume than the animals, kept in 12 h lighting conditions [8].

In scientific literature, very limited number of researchs, related to organ development of rodents, which were subjected to various lighting environments, is available. Short photoperiod did not effect spleen weight but inhibited testis and uterus growth in the Marsh rats [9] and short lighting regime also had a suppressive effect on adrenal growth in Wistar rats [10]. In Mongolian gerbil, uterus, testis and body weight, as weel as, feed intake were significantly affected by various environmental factors, including lighting [11]. In the male deer mice *(Peromyscus maniculatus*), short photoperiod significantly inhibited reproduction organ growth [12]. Reproduction organs of *Prairie voles* males were negatively affected by short lighting period, and females had lower uterus and ovarium weights when compared to those, kept in long photoperiod condition [13].

In mice, it is noticed that gonads were reduced in weight, when exposed to short lighting [14], but according to another research, body and testis weight were not affected by lighting methods [15]. Short photoperiod (8 h) resulted in a decrease of testis measures, feed intake, body weight, puberta period in F344 rats [8].

To the best of the authors knowledge, there is scarce knowledge in scientific literature, on the the relation between photoperiod and growth in Sprague dawley rats, one of the common used rat breed.

Energy sources of the World are not enough to meet the demands of the humanity. Energy saving methods have been in the news of both developed and developing countries, in recent years. For scientific aims, billions of rodents are still kept in artificial lighted environments, by using a serious amount of electrical energy.

The aim of present study was to compare the effect of short (9 h) and long/routin photoperiod (12 h) lighting on the growth traits of Sprague dawley pups from birth to the weeks that they reached to 200 g body weight and examine the possibility of keeping Sprague dawley rats during shortened photoperiod, just as in 'natural'conditions, in winters of subtropical regions and so the possibility of energy saving.

MATERIAL and METHODS

The trial protocol was approved by the ethics committee of Ataturk University, Turkey, (number: ATA-28.11.2008 /84). Twenty out bred Sprague dawley females, 6 months of ages, in the second partrition, specific patogen free, at similar weights were divided into 2 groups. Mean weight of females were 224.1±14.6 gr for control (long/routin photoperiod: 12 h light: 12 h dark) group, 232±12.6 gr for experiment (short photoperiod: 9 h light: 15 h dark) and the females were mated and kept in individual cages (47x35x20 cm), 21±2°C and 55±5% relative humidity room conditions. White color lighting was supplied, the light intensity was 150 lux, and equally distibuted on the cage floor [1].

Pups were weighed after birth, 7th, 14th and 21st of birth day, and after preweaned on 21st day, the pups were seperated according to sex and transferred to the cages. The cages were 50x30x30 cm, 10 pups/cage. Feed consumption were recorded, weekly. At the end of 15th week, 10 females-10 males were randomly chosen from control and experiment cages and were starved for 12 h, anaesthetised and the abdomens were incised, *Aorta abdominalis* was cut and bleeded. The ventricles of heart were palped the residual blood was removed and the heart was weighed. The spleen, liver, kidney, ovary, testis, adrenal gland, gastear and lung were removed and weighted (CAS, Model: ME–410). Content of the large and small intestines were removed, intestines were weighed and length of the intestines were measured by a ruler. The statistical methods were:

Model was used to analyse of birth-preweaned period, 0-3 weeks

Model was used for 4-15. weeks

Model was for organs, ovary-testis analyse

I. Model $yij = \mu + C + bi + eij$

II. Model $yijk = \mu + bi + sj + (bs)ij + eijk$

III. Model $yij = \mu + bi + eij$

C: Kovariance (numbers of pups),

bi: Effect of experiment (photoperiod),

sj: Effect of sex,

(bs)ij: Interaction (experiment and sex),

SPSS 9.0 statistical programme, General Linear Model (GLM) procedure was performed.

RESULTS

Mean weight values and variance analyse results for birth-preweaning period are presented in *Table 1*. Short photoperiod had not significant effect on body weight in pups, until preweaning at the end of 3. weeks of ages ($P>0.05$).

Control group (37 rats) consumed 52.32 kg; experiment group (39 rats) 53.12 kg, after preweaned period (4-15. weeks). Body weight were similar in both groups between 4. and 9. weeks ($P>0.05$), *(Table 2a)*. Body weight of the male and female rats was significantly different between 10-15

weeks (P<0.01), *(Table 2b)*.

At the end of trial, at 15th week, both groups were statistically similar in weight (P>0.05), *(Table 3)*. The weight of spleen, heart, liver and length of large intestine were also similar in control and experiment groups (P>0.05), *(Table 3)*.

The mean weight of gastear, kidneys, adrenal glands, intestines and length of small intestines of long photoperiod group were significantly higher than those of exposed to short photoperiod (P<0.05 and P<0.01), *(Table 3)*. At the end of trial, at 15th week, both groups were statistically equal in testis and ovary weight (P>0.05), *(Table 4)*.

Table 1. *Mean and standard error (X±Sx) and variance analyse results of body weight between birth-preweaned [0-3 weeks] periods of pups*

Tablo 1. *Doğum-sütten kesim arası [0-3 hafta] yavruların canlı ağırlık ortalamaları ve standart hataları (X±Sx) ile varyans analiz sonuçları*

Weeks	Group	N	X±Sx	P
1	Control	78	6.18±0.43	NS
	Experiment	61	7.33±0.43	
2	Control	55	13.86±1.01	NS
	Experiment	51	14.56±0.93	
3	Control	41	26.52±2.01	NS
	Experiment	50	23.68±1.87	

NS: Non-significant

DISCUSSION

Short photoperiod inhibited growth in hamsters [16,17] but stimulated in *Prairie voles* [13]. In wistar pups, exposed to 6 h lighting, body weight was lower than those of controls [7]. Six hours lighting is not natural for the World's photoperiod system and it may not be appropriate for the growth of the wistars.

F344 rats did not reduced body weight and feed intake when exposed to short lighting [8]. Similar result is obtained in the present research and the body weight of Sprague dawley rats, until 15th week and the growth was not affected by the 9 h lighting regime. Some rodent strains were recorded not to be very sensitive to photoperiod [18], however, some researchers declared that the photoperiod had a significant effect on growth of the laboratory rodents [19].

F344 and Brown Norway rats reacted to 8 h lighting by reducing feed intake and body weight, where as body weight of Harlan Sprague dawleys decreased about 5-10% [3]. It is recorded that, if *F344* rats, which were exposed to short photoperiod after a long photoperiod term, their growth traits were negatively effected, but if they were exposed to short lighting regime just after birth, the lighting time had no negative effect on the growth traits [19].

In present study, growth traits of Sprague Dawley rats were not affected by experiment but sex (P<0.05 ve

Table 2a. *Mean and standard error (X±Sx) and variance analyse results of body weight after preweaned [4-9 weeks] period of rats*

Tablo 2a. *Sütten kesim sonrası [4-9. hafta] ratların canlı ağırlık ortalamaları ve standart hataları (X±Sx) ile varyans analiz sonuçları*

Groups	Sex	4. Weeks	5. Weeks	6. Weeks	7. Weeks	8. Weeks	9. Weeks
Control	M	44.19±1.4	64.00±5.0	95.53±6.8	136.97±8.1	161.83±10.6	179.88±11.0
	F	41.52±1.4	57.95±5.0	84.97±6.8	108.76±8.1	119.23±10.6	130.60±11.0
Experiment	M	41.73±1.7	59.93±6.1	87.05±8.3	118.08±9.9	136.63±12.9	150.51±13.4
	F	40.24±1.6	62.86±5.5	87.55±7.4	111.77±8.8	131.17±11.6	140.82±12.0
Sex		NS	NS	NS	NS	NS	*
Experiment		NS	NS	NS	NS	NS	NS
Sex x Experiment		NS	NS	NS	NS	NS	NS

NS: *Non-significant, * P<0.05*

Table 2b. *Mean and standard error (X±Sx) and variance analyse results of body weight after preweaned [10-15 weeks] periods of rats*

Table 2b. *Sütten kesim sonrası [10-15. hafta] ratların canlı ağırlık ortalamaları ve standart hataları (X±Sx) ile varyans analiz sonuçları*

Experiment Groups	Sex	10. Weeks	11. Weeks	12. Weeks	13. Weeks	14. Weeks	15. Weeks
Control	F	190.53±13.3	211.92±11.2	230.29±12.7	241.39±13.2	256.86±15.4	278.06±16.1
	M	157.50±11.9	160.30±10.0	169.70±11.3	175.11±11.8	180.59±13.7	184.07±14.4
Experiment	F	190.53±13.3	211.92±11.2	230.29±12.7	241.39±13.2	256.86±15.4	278.06±16.1
	M	157.50±11.9	160.30±10.0	169.70±11.3	175.11±11.8	180.59±13.7	184.07±14.4
Sex		**	**	**	**	**	**
Experiment		NS	NS	NS	NS	NS	NS
Sex x Experiment		NS	NS	NS	NS	NS	NS

NS: *Non-significant, * P<0.05*

Table 3. *Mean and standard error (X±Sx) and variance analyse results of body weight and organ weight*

Tablo 3. *Canlı ağırlık, organ ağırlık ortalamaları ve standart hataları (X±Sx) ile varyans analiz sonuçları*

Traits	Control	Experiment	P
Body weight (g)	223.84±2.84	215.90±2.84	NS
Spleen (g)	0.50±0.02	0.44±0.02	NS
Heart (g)	0.87±0.04	0.78±0.04	NS
Liver (g)	7.21±0.25	7.79±0.25	NS
Kidneys (g)	2.07±0.05	1.90±0.05	*
Adrenal glands (g)	0.13±0.01	0.09±0.01	**
Gastear (g)	1.74±0.08	1.49±0.08	*
Large intestine (cm)	15.40±0.47	13.95±0.47	NS
Small intestine (cm)	99.00±1.50	91.05±1.50	**
Intestine total (g)	14.18±0.43	13.17±0.43	**
Lungs (g)	1.78±0.08	1.44±0.08	**

NS: *Non-significant, * P<0.05, ** P<0.01*

Table 4. *Mean and standard error (X±Sx) and variance analyse results of ovary and testis weight*

Tablo 4. *Ovaryum ve testis ağırlık ortalamaları, standart hataları (X±Sx) ile varyans analiz sonuçları*

Reproductive Organ	Group	N	X±Sx	P
Ovary (g)	Control	10	0.10±0.01	NS
	Experiment	10	0.25±0.08	
Testis (g)	Control	10	2.57±0.09	NS
	Experiment	10	2.47±0.09	

NS: *Non-significant*

P<0.01). Males had significantly higher weight than females and the result was previously confirmed by Poyraz [1].

In the marsh rice rat *(Oryzomys palustris)* and Siberian hamsters, short photoperiod was reported to be ineffective on spleen growth [20]. In the present study, spleen, heart and liver weight of rats, also length of large intestines were not affected by 9 h lighting regime. Rats, subjected to 12 h light: 12 h dark photoperiod, had higher adrenal glands than those exposed to 9 h lighting, the present results were in accordance with those reported for Wistar rats [10]. Adrenal glands were noticed to be most sentisitive tissue to the photoperiod in Sprague dawley and Wistar rats [10].

In scientific literature the researches, examining the relation between photoperiod and organ weight are very limited. It is thought that, the results may be present the reference values, for better understanding the photoperiodic response of Sprague dawley out bred rats.

Testis and ovary weight of rats were not affected significantly by short photoperiod lighting (P>0.05), *(Table 4)*, including pubertal period. It is reported that, 12, 14 and 16 h lighting were not effected the ovary weights of *Oryzomys palustris* [20].

In mice, it is declared that testis weight was not effected by short photoperiod [15] but another research determined a reduction in gonads of females [14]. In marsh rice rats, testis, ovary and uterus growth were significantly and negativelyl affected by short photoperiod [9]. Similar results were declared for also deer mice (*Peromyscus maniculatus*) and some hamster strains [12,21].

Reproductive organ growth were inhibited by short photoperiod in *Prairie voles* males, but fertilization was not impressed both in males and females [13]. Before puberta, *F344* rats, subjected to short (8 h) had similar reproduction organ weight when compared to the ones, kept in long photoperiod regime (16 h) [8]. Presents results stated that, weights of testis and ovary in Sprague Dawley rats were not impressed by 9 h photoperiod, however, some of the strains of Harlan Sprague dawleys, like ACI, BUF and PVG males had lower (5-20%) testis weight than those kept in long photoperiod lighting [3].

When compared to previous results, it is noticed that reaction of the rodents to photoperiod may significantly differ, according to the species, breed, strain, family of the animals. It is conluded that, there may be a possibility for reducing routin the artificial lighting (12 L: 12 D) up to 25% in rat breeding systems, without a decrease in body, testis and ovary weight. If supported by advance researches, lighting periods may be reduced/adapted to the flocks, species, strains of the animals, so that there will be a chance to save the electrical energy of the World.

It is also thought that, breeding/keeping procedures of the laboratory animals should be re-arranged according to the breed, strains and family of the animals and lighting needs of rodents should be discussed for each species.

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RESEARCH ARTICLE

Interaction Between Phenylbutazone and Thiopental Sodium in Female Stray Dogs: The Effect on the Recovery from Anesthesia

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Summary

Anesthesia often involves administration of several drugs from different classes. Drug–drug interactions may affect the duration of action of anesthetic agents. The purpose of this research was to investigate interaction between phenylbutazone and thiopental sodium in dogs. Twenty-six female stray dogs were randomly divided into two groups. Each group subdivided into two subgroups <18kg and ≥18kg. Equivalent doses of thiopental sodium were intravenously administered following the injection of 0.9% normal saline in control group and phenylbutazone via the same route in experimental group. After anesthesia, time intervals needed for the return of palpebral reflex, opening of eyes, tongue movement, stretching of limbs, head and neck movements, sitting position, trying to stand, imbalanced walking and normal walking were recorded. Results showed the average time periods in the experimental group were generally more than those in the control group. It was shown the duration of the above chronological parameters were significantly higher in heavier dogs compared to the lighter. Females generally have a larger proportion of body fat content than males. This may cause a higher volume of distribution and a longer elimination half-life for thiopental in female dogs. This interaction with phenylbutazone may be due to an increase in the unbound form of thiopental sodium and its quicker distribution into the brain and a longer duration of anesthesia.

Keywords: *Drug interaction, Thiopental sodium, Phenylbutazone, Anesthesia, Dog*

Kısırlaştırılmış Dişi Köpeklerde Fenilbutazon ve Thiopental Sodyum Etkileşimi: Anesteziden Çıkışa Etkisi

Özet

Aneztezi çoğu zaman birden fazla değişik sınıftan ilaçların uygulanmasını içerir. İlaçlar arası etkileşim anestezik maddenin etki süresini etkileyebilir. Bu çalışmanın amacı köpeklerde fenilbutazon ve thiopental sodyumun etkileşimini araştırmaktır. Yirmi altı kısırlaştırılmış dişi kopek rastgele iki gruba ayrıldı. Her bir grup <18kg ve ≥18kg olmak üzere iki altgruba ayrıldı. Eşit dozlarda olmak üzere thiopental sodyum control grubundakilere intravenöz %0.9 tuzlu su verilmesinin ardından, çalışma grubundakilere ise aynı yolla fenilbutazon verilmesini takiben intravenöz olarak uygulandı. Anesteziyi takiben göz kapağı refleksi, gözünü açma, dilini hareket ettirme, ayaklarını uzatma, kafa ve boyunu hareket ettirme oturma pozisyonunu sağlama, kalkmaya çalışma, dengesiz yürüme ve norma yürüme için geçen süreler kaydedildi. Sonuçlar çalışma grubundaki hayvanlarda kaydedilen ortalama sürelerin genel olarak control grubundaki hayvanlardan daha uzun olduğu gösterdi. Yukarıda belirtilen parametrelerde sürelerin daha ağır köpeklerde hafif olanlara oranla önemli derecede daha uzun olduğu gözlemlendi. Dişiler genellikle erkeklere oranla daha fazla vücut yağına sahiptir. Bu durum, dişilerde daha fazla miktarda yayılmaya ve thiopentalin yarılanma ömrü için daha uzun zamana ihtiyaç doğurabilir. Fenilbutazon ile etkileşim, thiopental sodyumun bağsız formundaki artışa ve onun daha hızlı olarak beyine yayılmasına ve daha uzun anestezi süresine bağlı olabilir.

Anahtar sözcükler: *İlaç etkileşimi, Thiopental sodyum, Fenilbutazon, Anestezi, Köpek*

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INTRODUCTION

Anesthesiaisan important subject in veterinary medicine and is used for a wide range of circumstances in animals due to animals' unwillingness to cooperate with certain diagnostic or therapeutic procedures [1]. Amonganimals, cats and dogs are frequently anesthetized for surgical procedures [1]. Anesthesia often involves administration of several agents belonging to different classes of drugs [1]. In addition, many patients should take a number of drugs related to their surgical condition or for other medical situations [1-3]. Unexpected events may occur when the drugs are administered simultaneously. Thus, there is a considerable potential for drug interactions to occur, some of which may be potentially harmful to the patients. This may be accompanied by cardiovascular and respiratory depression and a delay in recovery for hours or even days [2,3].

Sleep may be prolonged in patients with increased central nervous system responses to depressant drugs, e.g., patients who are hypothermic; or factors affecting redistribution and metabolism of drugs, e.g., patients who have hepatic or renal dysfunction [1]. Patient's gender, age, weight and drug interaction may be important factors influencing recovery from general anesthesia [2-4]. Nowadays, medications having minimal side effects and with short recovery period are mostly used to induce anesthesia [5].

Animal studies and controlled studies in human volunteers suggest that there are differences in the speed of recovery from various anesthetics [6]. Thiopental sodium is an intravenous short-acting thiobarbiturate with rapid onset of action [7].

Phenylbutazone is indicated for the relief of musculo-skeletal inflammation and mild to moderate somatic or visceral pain particularly in post-operative pain in dogs, horses, and cattle [8]. In certain situations, thiopental may be used concurrently with phenylbutazone. Therefore, the aim of the present study was to examine the possible interactions between phenylbutazone and thiopental in dogs as it may change various post-anesthetic signs.

MATERIAL and METHODS

Thiopental sodium (0.5 g vials; Sanduz, Austria, Lot No. 150566), phenylbutazone (Vetanyl 20%, RayhanDaroo, Tehran, Iran), normal saline (sodium chloride 0.9%, Martyr Judge Serum Company, Tabriz, Iran) were purchased from local suppliers. Twenty-six female stray dogs of one to two year old weighing 8 to 23 kg were used. Twenty six dogs were divided into two groups (control and experimental) and divided into two subgroups (6 dogs < 18 kg and 7 dogs ≥ 18 kg). After a physical check-up of all animals,blood samples were taken and were tested for CBC, Hb and PCV and for several biochemical parameters (total protein, ALT and AST) in order to ensure about their health. The first group received 0.1 ml/kg normal saline five minutes before administration of thiopental sodium (5%) with a dosage of 17 mg/kg. Dogs in the second group were injected phenylbutazone (20%, 0.1 ml/kg) five minutes prior to anesthesia (induced by the same dosage of sodium thiopental). After injection of sodium thiopental, the times required for the return of various parameters such as palpebral reflex (PR), tongue movement (TM), stretching of limbs (SL), opening of eyes (OE), head and neck movement (HNM), sitting position (SP), trying to stand (TTS), imbalanced walking (IW), and normal walking (NW) were recorded.

Analysis of Data

All results are expressed as mean ± SEM and were analyzed using one-way ANOVA followed by Tukey's test. P value of < 0.05 was considered statistically significant.

The study was approved by the AnimalEthics Committee of the Iranian laboratory animal ethic frameworks under the referencecode IAEC 1-12/2.

RESULTS

Biochemical and hematological parameters were depicted in *Table 1*. Mostly, the measured values are within the normal ranges reported so far [9-11]. The durations for various chronological parameters following anesthesia induced by thiopental in female subjects in both control and experimental groups are illustrated in *Table 2*. The average times for the return of PR and OE in the control group were around 4 and 12 min, respectively. The corresponding valuesin the experimental group were approximately 15 and 25 min *(Table 2)*. The average times required for the TM and SL in the control group were around 12 and 12 min, but were recorded to be approximately 24 and 24 min in the experimental group *(Table 2)*. The average times for HNM and SP were found to be approximately 15 and 21 min in the control group, while corresponding values of 31 and 41 min were recorded in the experimental group *(Table 2)*. Times required for TTS and IW in the control

Table 1. *Hematology and blood biochemical parameters of female dogs*
Tablo 1. *Dişi köpeklerin hematoloji ve kan biyokimyası parametreleri*

Parameters	Observed Values	
	Mean±s.e.m. (n=26)	Range
Body weights (kg)	16.7±0.7	12-30
WBC (x 10^3 cells/µL)	12.5±0.8	4.8-15.4
RBC (x 10^6 cells/µL)	6.9±0.5	4.2-12.3
Hb (g/dL)	11.0±0.4	8.6-14.3
PCV (%)	35.2±2.5	21-60
Total Protein (g/dL)	5.7±0.2	4.3-6.9
ALT (IU/L)	23.5±1.4	12.9-39.1
AST (IU/L)	20.3±1.3	10.8-30.5

group were around 24 and 32 min, but were found to be around 43 and 50 min in the other group *(Table 2)*. Finally, the average time required for NW was about 52 and 67 min in the control and experimental groups, respectively *(Table 2)*. Various parameters related to the return of the anesthesia in female dogs in control and experimental groups with respect to their body weights are shown in *Table 3*. Average times for various parameters including PR, OE, SL, TM, HNM, SP, TTS, IW and NW were around 4, 11, 10, 10, 13, 18, 20, 27 and 58 min in the control group weighting less than 18 kg body weight, but the corresponding values in the experimental group were obtained to be 11, 23, 23, 21, 28, 42, 43, 49 and 67 min *(Table 3)*. The above values for dogs weighing ≥18 kg were respectively found to be 4, 11, 13, 13, 16, 24, 26, 36 and 47 min in the control group. However, the corresponding values in the experimental group were recorded to be 19, 26, 25, 27, 34, 40, 43, 51 and 66 min, respectively *(Table 3)*.

Table 2. *Comparison of times (minutes) required for the return of various parameters following anesthesia induced by sodium thiopental in female dogs*

Tablo 2. *Sodyum thiopental ile anestezi edilen dişi köpeklerde kaydedilen (dakika olarak) çeşitli parametrelerin karşılaştırılması*

Parameters	Groups (body weight)	
	I[1] (17.3±1.2 kg)	II[2] (16.2±0.7 kg)
Palpebral reflex	4.2±0.6	15.2±3.8*
Opening of eyes	11.5±2.0	25.0±3.9*
Tongue movement	12.2±1.8	24.5±3.5*
Stretching of limbs	12.5±1.7	24.3±3.4*
Head and neck movement	14.9±1.7	31.2±4.6*
Sitting position	21.7±2.1	41.4±5.0*
Trying to stand	23.9±2.7	43.0±5.5*
Imbalanced walking	32.8±5.4	50.3±5.6*
Normal walking	52.3±6.0	67.1±6.4

*[1] Normal saline (0.1 ml kg^{-1}) 5 min before the injection of sodium thiopental (17 mg kg^{-1}), [2] Phenylbutazone (20 mg kg^{-1}) 5 min before the injection of sodium thiopental (17 mg kg^{-1}), [3] Mean ± s.e.m. (n=13), * Statistically different (P<0.05) from the corresponding value in the control group*

DISCUSSION

Anesthesia is used for a wider range of circumstances in animals than in people, due to animals' unwillingness to cooperate with certain diagnostic or therapeutic procedures. Selection of an anesthetic protocol with minimum complications is quite necessary and should be based on scientific evidences. For example, drug–drug interaction can lead to an increase or a decrease in the anesthetic requirement or may prolong duration of action of anesthetic agents [12,13]. Non-steroidal anti-inflammatory drugs (NSAIDs) have the potential for both pharmacokinetic and pharmacodynamic interactions with several anesthetic agents through interfering with their plasma protein binding and/or their analgesic effects [14]. The interaction between thiopental sodium (a drug that is used for the induction of general anesthesia) and phenylbutazone (among NSAIDs) was studied in the present study.

Ghoneim et al.[8] showed in a study that uremic and sulfonamide-pretreated rats had significantly higher levels of ^{14}C in their brain and heart and more free thiopental was present in their plasma at each time than did control animals. They concluded that reduced protein binding of thiopental sodium leads to accelerated distribution and increased drug concentrations in the brain and heart. According to our study, dogs in the control group had faster

Table 3. *Comparison of times (minutes) required for the return of various parameters following anesthesia induced by sodium thiopental in female dogs weighing <18 kg or ≥18 kg*

Tablo 3. *Sodyum thiopental ile anestezi edilen <18 kg veya ≥18 kg dişi köpeklerde kaydedilen (dakika olarak) çeşitli parametrelerin karşılaştırılması*

Parameters	Groups			
	I[1]		II[2]	
	Body Weights (kg)		Body Weights (kg)	
	<18 14.3±0.6[3] (n=6)	≥18 19.2±1.7 (n=7)	<18 14.2±0.5 (n=6)	≥18 18.4±0.8 +(n=7)
Palpebral reflex	4.6±0.8	3.9±0.9	11.4±3.1*	19.5±7.3**
Opening of eyes	11.0±0.6	11.8±3.2	23.7±5.2*	26.5±6.3**
Tongue movement	10.0±1.2	13.6±2.8	21.7±4.6*	27.8±5.3*
Stretching of limbs	10.6±0.9	13.8±2.7	23.6±5.1*	25.2±4.9*
Head and neck movement	13.0±1.2	16.1±2.7	28.6±6.9*	34.2±6.3*
Sitting position	18.2±1.2	23.9±3.1	42.4±8.2*	40.2±6.0*
Trying to stand	20.6±3.4	26.3±3.6	42.9±8.4*	43.2±7.6*
Imbalanced walking	27.8±3.8	36.4±8.2	49.0±8.8*	51.8±7.4*
Normal walking	58.0±7.8	47.5±8.5	67.6±11.0	66.5±6.7*

[1] Normal saline 5 min before the injection of sodium thiopental (n=13), [2] Phenylbutazone 5 min before the injection of sodium thiopental (n=13), [3] Mean ± s.e.m. (n=13), Statistically different (P<0.05; ** P<0.01) from the corresponding values in the control group*

recovery times than dogs in the experimental group and the differences in the chronological parameters measured were mostly significant (P<0.05). This may lead to drug interaction between thiopental and phenylbutazone and cause to increase free-form of thiopental.The increased concentration of thiopental can be attributed to the interaction between phenylbutazon and thiopental.

Yu et al.[15] claim that sulfadimethoxine causes displacement of thiopental from plasma proteins which significantly increases the free fraction of thiopental. This result may explain the significant increase in V_{ss} and the decrease of both beta and intrinsic clearances. Phenylbutazone given during the pre-surgical period has been reported to increase the intensity and duration of thiamylal anesthesia in horses [16]. A possible mechanism of competitive plasma protein binding has been suggested in this respect.

In obese patients, thiopental has an increased V_d and a longer elimination half-life ($t_{1/2}$), but Cl values are unchanged. It was stated in as long ago as 1969 that thiopental dosage should be based on lean body mass (LBM) [17]. For lipid-soluble drugs, such as opioids and benzodiazepines, the volume of distribution is generally larger in women, but for water-soluble drugs, such as muscle relaxants, it is smaller [18].

On the basis of the above evidences and findings of the present study (the average times required for various chronological parameters were mostly less when thiopental was injected alone compared to those anesthetized following phenylbutazone administration, and the fact that this variation is more pronounced in the heavier dogs) may support this hypothesis that the interaction between these two drugs is most probably due to competition for protein binding sites. A higher concentration of the unbound form of thiopental will increase distribution of the drug to the tissues, particularly to the fat deposits and brain due to the lipid soluble nature of barbiturates. Redistribution of thiopental will extend the duration of action of the drug and, therefore, the duration for recovery period will increase in dogs receiving phenylbutazone before induction of anesthesia by sodium thiopental. This is in contrast to the usual expectation of similar interactions between less lipid soluble drugs which leads to increased unbound form of drug. The consequence of itis greater pharmacological activity andless duration of action.

In conclusion, interaction between phenylbutazone and thiopental sodium can increase the unbound form of thiopental which has pharmacokinetics impact on the duration of action of the anesthetic drug. Considering the drug interaction can increase free form and Vdof thiopentalwhich is a slower redistribution phase when the drug is taken up by body fat and partitioned out of the CNS. Multitude of factors that may influence recovery, body weight effect appears to be strong one. Female dogs have generally a larger proportion of body fat, redistribution of the drug from adipose tissue to brain will take longer and recovery time will be higher in the heavier female dogs.

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RESEARCH ARTICLE

The Effects of Gemfibrozil and Ovariectomy on the Peroxisome Proliferator Activated Receptors (PPARs) in Mice with Experimentally Induced Obesity [1]

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Summary

Effects of gemfibrozil and ovariectomy on Peroxisome Proliferator Activated Receptors (PPARs) activity were studied in obese mice. Ovariectomised and sham operated mice were fed with high fat diet and low fat diet for 15 weeks. Furthermore, gemfibrozil (100 mg/kg) was orally with high fat and low fat diets to the ovariectomised and sham operated mice. Body weights significantly increased in ovariectomised mice compared to sham operated mice ($P<0.001$) while gemfibrozil supplementation prevented such increase. Steatosis was as well more pronounced in the livers of ovariectomised mice fed with high fat diet compared to sham operated mice. However, liver steatosis was not seen in gemfibrozil supplemented high fat diet fed mice. On the other hand gemfibrozil supplementation slightly increased the liver weights. High fat diet significantly increased liver AST and ALT enzyme levels and serum total cholesterol, triglyceride and VLDL levels while gemfibrozil lowered these parameters. Immunohistochemically, while tissue PPAR-α and PPAR-γ expressions were affected by ovariectomy, diet and gemfibrozil, PPAR-β expression unchanged. While high fat diet incremented liver PPAR-α and PPAR-γ expressions their levels were reduced following ovariectomy operation. Only high fat diet and only gemfibrozil application increased PPAR-α expression level. PPAR-α expression levels were higher in gemfibrozil and high fat diet combined groups compared to all other groups. In conclusion, gemfibrozil reduced abdominal and hepatic fat deposition in mice with high fat diet and ovariectomy.

Keywords: *Gemfibrozil, Ovariectomy, PPAR-α, PPAR-β, PPAR-γ, High fat diet*

Deneysel Obezite Oluşturulan Farelerde Ovarioektomi Operasyonu ve Gemfibrozilin Peroksizom Proliferatörleri ile Aktive Olan Reseptörler (PPARs) ve Obezite Üzerine Etkilerinin Araştırılması

Özet

Ovarioektomi uygulanan farelerde deneysel olarak oluşturulan obezitenin peroksizom proliferatörleri ile aktive olan reseptörler (PPARs) üzerine etkisi araştırılmıştır. Bu amaçla ovarioektomi ve sham uygulanan fareler yağlı diyet ve düşük yağlı diyetle 15 hafta süre ile beslendi. Ayrıca, ovarioektomi ve sham uygulanan farelere farklı diyetlerle beraber 100 mg/kg dozunda oral gemfibrozil uygulandı. Çalışma sonunda ovarioektomi operasyonu uygulanan farelerde sham uygulanan farelere göre istatistiksel olarak anlamlı ($P<0.001$) canlı ağırlık artışı şekillenirken gemfibrozil uygulaması bu canlı ağırlık artışını engelledi. Yüksek yağlı diyet uygulanan farelerde, ovarioektomi uygulanan grupta, sham uygulanan farelerin karaciğerlerine göre daha belirgin bir steatozis tablosu görüldü. Diğer yandan yüksek yağlı diyet ve gemfibrozil uygulanan gruptaki fare karaciğerlerinde steatozis görülmedi. Buna karşın gemfibrozil uygulaması karaciğer ağırlığında hafif şiddette artışa neden oldu. Yüksek yağlı diyet uygulaması karaciğer AST ve ALT enzim seviyeleri ile plazma toplam kolesterol, trigliserid ve VLDL seviyelerinde artışa neden olurken gemfibrozil uygulaması bu parametrelerde iyileşme ile sonuçlandı. İmmunohistokimyasal olarak dokulardaki PPAR-α ve PPAR-γ ekspresyonu ovarioektomi operasyonu, diyet ve gemfibrozil uygulamasından etkilenirken PPAR-β ekspresyonu etkilenmedi. Yağlı diyet uygulamasında PPAR-α ve PPAR-γ ekspresyonu artarken ovarioektomi operasyonunda azaldı. Yalnızca yağlı diyet ve yalnızca gemfibrozil uygulanan gruplarda PPAR-α ekspresyonunda artış şekillendi. Yağlı diyet ile gemfibrozilin beraber uygulandığı gruplarda ise PPAR-α ekspresyonu diğer tüm gruplara göre daha fazla arttı. Çalışma sonunda gemfibrozil uygulamasının yüksek yağlı diyet ve ovarioektomi oluşturulan farelerde abdominal ve hepatik yağlanmanın azalmasında olumlu etkileri olduğu görüldü.

Anahtar sözcükler: *Gemfibrozil, Ovarioektomi, PPAR-α, PPAR-β, PPAR-γ, Yağlı diyet*

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INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the family of ligand-inducible nuclear receptors. Three isotypes called PPAR-α, -β, and - γ have been identified in lower vertebrates and mammals [1,2]. While PPAR-α is expressed in the liver, heart and kidneys PPAR-β is mainly present in the adipose tissue, skin and brain, and PPAR-γ is present in adipose tissue, large intestines, heart, kidneys, pancreas and spleen [3-5]. PPAR-γ is activated by prostaglandin J_2 whereas PPAR-α is activated by leucotrien B_4 [6,7]. PPARs are known to regulate glucose metabolism, energy balance and body weight. These effects are partially related to beta-oxidation of fatty acids leading to fatty acid degradation in the liver [8,9].

Activation of PPAR-α can occur as a result of treatment with hypolipidemic fibrate class of drugs (fenofibrate, gem-fibrozil etc) [3,9]. Fibrates organize a series of genes responsible for lipid and lipoprotein synthesis. Activated PPAR ligands induce heterodimerization with retinoid X receptor, and the subsequent interaction with steroid receptor co-activators, followed by binding to PPAR response elements [3,10]. Fibrates regulate energy homeostasis. A diet rich in energy results elevated plasma triglyceride and cholesterol levels. Consequently, high levels of triglycerides present in the circulation cause lipocyte hypertrophy and hyperplasia [11-13]. Therefore, it was suggested that obesity could be prevented by fibrates that have the ability to reduce plasma triglyceride and fatty acids levels [11,12].

Studies in humans and laboratory animals indicate that estrogen has a significant role in the adipose tissue regulation [14,15]. It has been reported that ovariectomy increases adiposity in rodents, and administration of estrogen derivatives reduce fat deposition in these animals [16]. Similarly, following menopause total body fat increases in women and of estrogen-replacement therapy attenuates the accumulation of fat in postmenopausal woman [15]. D'Eon et al.[17] reported in their ovariectomized mice model of menopause, estrogen replacement up-regulates PPAR-δ mRNA expression. Estrogen replacement also up-regulates PPAR-α gene expression [18] and increases lipid peroxidation in genetically PPAR-α deficient mice [19].

In the present study, the effects of gemfibrozil administration for the treatment of lipid metabolism disorders due to decreased estrogen levels was investigated in ovariectomized rodent model of menopause. Furthermore, the effects of various treatment and intervention methodologies including high fat diet, gemfibrozil treatment and ovariectomy in the tissue distribution and expression levels of PPARs were investigated by immunohistochemistry.

MATERIAL and METHODS

Animals and Husbandry

The experiment and study design were approved by the Experimental Ethics Committee, Kafkas University, Kars, Turkey. All animals were maintained in accordance to university policies. The material was consisted of 80 female (12-14 weeks old, 27±2.33 g bw) Swiss albino mice supplied from Ataturk University. The animals were housed in a well-ventilated, temperature-controlled room (23±2ºC), at 55% relative humidity under a 12 h light/dark cycle until the end of experiment, which lasted 15 weeks.

Eighty mice were initially divided equally into two main groups. Fourty mice in the first group were further subdivided into four groups (Group 1, 2, 3 and 4; *n*=10). Mice in these groups were ovariectomized by surgery performed from the median line. Mice from other main part were also subdivided into four groups (Group 5, 6, 7 and 8; *n*=10) and they were only sham operated from the median line.

Diet Preparation

Low fat diet (2.500 Kcal metabolic energy and 4.5% crude fat) were given *ad libitum* to the groups 3, 4, 7, and 8 througout the experiment. High fat diet (16.03 g margarine containing 80% vegetable fat melted at 50ºC and mixed with 83.97 g rodent chow containing 4.5% crude fat) were given *ad libitum* to the groups 1, 2, 5, and 6. The final composition of the high fat diet was calculated to posses 3109 Kcal metabolic energy and 15% fat.

Experimental Design and pathological examinations

Following 20 days of convalescence period, ovariectomized mice were divided into 4 groups (*n*=10; Group 1, 2, 3, and 4). Mice in Group 1 were given only high fat diet (15% fat) for 15 weeks. Group 2 received high fat diet plus gemfibrozil (Lopid®, Pfizer). Group 3 was fed with low fat diet (4.5% fat) plus gemfibrozil. Mice in the Group 4 were fed only with low fat diet (4.5% fat) for 15 weeks. Sham operated mice were also allocated in 4 groups (*n*=10; Group 5, 6, 7, and 8) and these groups were also received high fat diet, high fat diet plus gemfibrozil, low fat diet plus gemfibrozil and only low fat diet, respectively. Gemfibrozil was given orally (100 mg/kg). During the experimental period, animals were monitored three times a day, 30 minutes each and, weighed weekly.

At the end of the 15th, week mice were euthanized by cervical dislocation. Before sacrifice blood samples were collected from the abdominal aorta and necropsy was performed. Tissue samples were fixed in 10% neutral buffered formalin. After fixation, the sections were embedded in paraffin wax, sectioned at 4-6 µm, and stained with haematoxylin-eosin (H&E). Toluidine blue was stained in semi thin sections of liver to demonstrate fat globules.

Immunohistochemistry and Evaluation of Immunostaining

Tissue sections were labelled immunohistochemically by the streptavidin-biotin-peroxidase complex (ABC) technique for detection of the polyclonal rabbit anti-PPAR-α (1/100; sc-9000; Santa Cruz, California, USA), polyclonal goat anti-PPAR-β (1/100; sc-1987; Santa Cruz), and monoclonal mouse anti PPAR-γ (1/100; sc-7273; Santa Cruz) markers.

PPAR-α, -β and -γ immunoreactivities were evaluated on a semiquantitative grading scheme on which the number of positive cells in a certain area were counted. All sections were scanned at low magnification and analysis was commenced from the area where the staining intensity was the highest. Every sample was analysed under a light microscope with a 10x ocular (10x10 grid mounted) and an x 40 objective from a total of 10 different area (area of total analysis 0.025 mm^2). Furthermore, the staining intensity of relevant markers was also assessed as follows: (0) none; (1) weak; (2) moderate; (3) intense immunolabelling.

Serum Assays

Serum aspartat aminotransferase (AST) and alanine aminotransferase activities and total cholesterol, triglyceride and very low density lipoprotein (VLDL) concentrations were measured spectrophotometrically (Spectramax®; Plus 384) with use of commercial kits (Spinreact®, Spain).

Statistical Analysis

For the statistical analysis, differences between the groups were tested by analysis of variance (ANOVA) and the Tukey test using SPSS for Windows version 10.0.

RESULTS

Body Weight Changes and Gross Pathological Findings

Weekly average body weight changes were shown in detail in *Table 1*. Ovariectomy, the feed consumption and gemfibrozil administration were effective for body weight changes. Group 1 and 5, significant increases in body weight (mean; Group 1 and Group 5 was 30.74%: 21.92%) were observed. In Group 3 and 7, body weights were significantly decreased (median, Group 3: - 12.63%, and Group 7: -14.36%). In Group 2 and 6 body weight gain was 1.02%, and 0.28%, respectively. The body weight gain rate in sham operated mice in Group 4 was 2.74% while it was 6.5% in ovariectomized mice in Group 8.

Most remarkable gross finding was an evident increase of abdominal adipose tissue in the only high fat diet given groups (Group 1 and 5). On the other hand gemfibrozil administration significantly reduced abdominal fat content in groups 2, 3, 6, and 7. Differences between organ weights and their statistical significance were summarised in *Table 2*. Organ weight to body weight ratio showed that high fat diet and gemfibrozil administration significantly increased liver weights. Abdominal adipose tissue significantly increased in ovariectomy and high fat diet groups. Gemfibrozil administration significantly reduced abdominal fat contents of mice.

Table 1. *Weekly average body weights (g) changes and statistical significance between the groups*
Tablo 1. *Haftalık canlı ağırlık değişimleri ve gruplar arası istatistiksel farklılıklar*

Weeks	Groups								P
	1	2	3	4	5	6	7	8	
1	26.6±1.8	27.9±1.6	27.6±3.1	26.9±2.0	26.9±2.8	26.3±1.8	26.9±2.5	26.9±2.2	NS
2	27.5±1.8	28.7±1.7	28.4±3.3	27.6±2.3	28.1±2.8	28.4±2.1	28.1±2.8	27.8±1.9	NS
3	28.2±2.0	28.4±1.5	27.3±3.3	28.1±2.4	28.3±3.2	27.9±1.7	27.0±3.0	27.9±1.8	NS
4	28.8±2.0	27.6±1.1	27.0±3.5	27.6±2.9	28.9±3.3	27.0±2.1	27.9±4.4	27.4±1.9	NS
5	28.8±1.6	27.1±1.2	26.2±3.1	26.8±2,8	29.3±3.6	26.8±1.9	26.3±3.1	27.6±1.7	NS
6	$29.9±1.9^{A}$	$27.0±1.3^{ABC}$	$25.2±3.3^{BC}$	$27.6±2.7^{AB}$	$29.9±3.6^{A}$	$27.1±1.7^{AB}$	$24.6±2.7^{C}$	$27.3±2.1^{AB}$	P<0.001
7	$30.4±1.9^{A}$	$27.2±1.4^{BC}$	$26.2±3.3^{C}$	$27.8±2.8^{ABC}$	$29.8±4.1^{AB}$	$27.4±1.9^{ABC}$	$26.4±3.0^{C}$	$26.6±1.6^{BC}$	P<0.017
8	$31.1±1.6^{A}$	$27.4±1.1^{BC}$	$24.6±3.5^{C}$	$27.5±2.4^{BC}$	$30.0±3.9^{AB}$	$27.3±1.8^{BC}$	$25.3±2.9^{C}$	$26.5±2.0^{C}$	P<0.001
9	$32.1±1,7^{A}$	$27.3±1.3^{BCD}$	$25.8±3,1^{CD}$	$27.7±2.7^{BC}$	$30.5±3.2^{AB}$	$27.2±1.8^{BCD}$	$25.0±3.0^{D}$	$26.8±2.2^{CD}$	P<0.001
10	$32.3±2.2^{A}$	$27.7±1.3^{BC}$	$24.0±3.1^{D}$	$27.9±2.8^{BC}$	$30.8±3.4^{AB}$	$27.4±2.8^{BC}$	$25.4±3.0^{CD}$	$27.4±2.3^{BC}$	P<0.001
11	$33.3±2.1^{A}$	$27.5±1.3^{B}$	$24.3±2.8^{C}$	$27.7±2.7^{B}$	$31.3±3.5^{A}$	$27.4±2.5^{B}$	$24.4±3.0^{C}$	$27.9±2.1^{B}$	P<0.001
12	$34.0±2.1^{A}$	$27.0±1.4^{C}$	$24.3±2.8^{D}$	$27.2±2.6^{C}$	$31.6±3.1^{B}$	$26.9±1.9^{C}$	$23.2±3.1^{D}$	$27.3±2.4^{C}$	P<0.001
13	$34.7±1.4^{A}$	$27.7±1.6^{C}$	$23.3±2.4^{D}$	$27.8±2.5^{C}$	$32.0±2.8^{B}$	$26.8±2.2^{C}$	$23.5±2.8^{D}$	$27.6±2.4^{C}$	P<0.001
14	$34.7±2.1^{A}$	$27.9±1.8^{C}$	$24.2±2.6^{D}$	$28.3±3.1^{C}$	$32.4±3.2^{B}$	$26.4±1.4^{C}$	$23.2±2.7^{D}$	$28.0±2.5^{C}$	P<0.001
15	$34.8±2.3^{A}$	$27.9±1.9^{C}$	$24.1±2.2^{DE}$	$28.6±3.1^{C}$	$32.8±2.8^{B}$	$26.5±2.1^{CD}$	$23.0±2.8^{E}$	$27.6±2.4^{C}$	P<0.001

Values were presented as mean ± SE. Means denoted with different superscripts within the same column are statistically significant, ***NS:*** *Not Significant*

Table 2. *Between the groups organ weights (g) and the statistical significance of the differences*
Tablo 2. *Gruplar arasında, organ ağırlıklarındaki (g) farklılıklar ve istatistiksel önemi*

Groups	Organ Weights (g)						
	Liver	Kidneys	Spleen	Heart	Lungs	Brain	Abdominal Adipose Tissue
1	1.910±0.36^{A}	0.422±0.09	0.198±0.08^{A}	0.145±0.03	0.351±0.05^{AB}	0.452±0.03^{A}	0.882±0.158^{A}
2	1.793±0.24^{AB}	0.382±0.06	0.126±0.04^{B}	0.135±0.03	0.329±0.06^{ABC}	0.393±0.03^{D}	0.350±0.104^{D}
3	1.658±0.23^{BC}	0.400±0.12	0.165±0.05^{A}	0.153±0.05	0.383±0.16^{BC}	0.457±0.02^{A}	0.187±0.051^{E}
4	1.337±0.06^{DE}	0.388±0.07	0.128±0.03^{B}	0.157±0.29	0.274±0.04^{A}	0.437±0.02^{AB}	0.554±0.110^{C}
5	1.520±0.11^{CD}	0.360±0.03	0.122±0.03^{B}	0.130±0.02	0.278±0.05^{C}	0.396±0.04^{D}	0.731±0.200^{B}
6	1.434±0.24^{DE}	0.333±0.08	0.138±0.05^{B}	0.124±0.03	0.257±0.06^{C}	0.407±0.03^{CD}	0.342±0.107^{D}
7	1.360±0.18^{DE}	0.378±0.06	0.129±0.05^{B}	0.133±0.03	0.295±0.09^{BC}	0.435±0.02^{ABC}	0.178±0.052^{E}
8	1.230±0.11^{E}	0.364±0.08	0.126±0.03^{B}	0.128±0.03	0.264±0.03^{C}	0.420±0.02^{BCD}	0.310±0.055^{D}
P	0.001	NS	0.010	NS	0.003	0.001	0.001

Values were presented as mean ± SE. Means denoted with different superscripts within the same column are statistically significant, ***NS:*** *Not Significant*

Histopathological Findings

The hepatic accumulation of lipid was evident in the high fat diet group (Group 1 and 2). Macro and micro-vesicular type lipid vacuoles were clearly seen by light microscopy. Semi-thin sections also revealed lipid globules present in hepatocyte cytoplasms *(Fig. 1)*. Lipid vacuoles were more prominent in the periacinar area and it was mostly macrovesicular type. Hepatic accumulation of lipid was considerably higher in the ovariectomised and the high fat diet-fed mice than in the low fat diet controls. However, gemfibrozil supplemented high fat diet inhibited fat accumulation in the liver of mice. Gemfibrozil supplemented diet-fed mice exhibited signs of mild hydropic degeneration in the hepatocytes accompanied by dilated central veins and mild anisocytosis.

Mild hyperplasia was seen in the Langerhans islets of the pancreas in the high fat diet-fed mice. Size of Langerhans islets increased with irregular contours, in some cases islets were expanding into surrounding exocrine pancreatic tissue. Acinar cells were vacuolated in the high fat diet-fed mice (Group 1 and 5) while no acinar vacuolization was detected in the other groups. Cytoplasmic basophilia

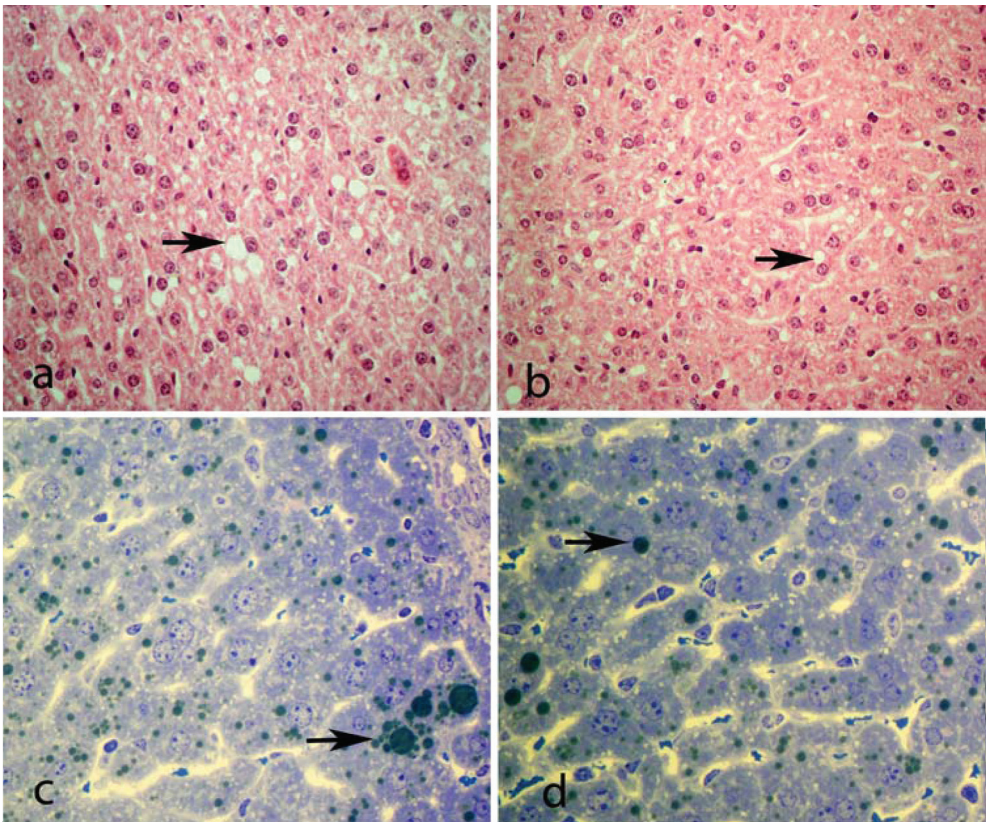

Fig 1. The histological appearance of fatty liver in the high fat diet groups. In Group 1 (a), and (b) 5 are showing sharply bounded fat vacuoles in hepatocytes in mice *(arrows)*. (H & E x 260). In (c) Group 1, and (d) 5 are semi-thin sections lipid globules *(arrows)* present in hepatocyte cytoplasms (toluidine blue x 750)

Şekil 1. Yüksek yağlı diyet uygulanan farelerde şekillenen karaciğer yağlanmasının histopatolojik görünümü. (a) Grup 1'deki ve (b) 5'teki farelerde hepatositlerdeki keskin sınırlı yağ vakuolleri *(oklar)*. (H&E x 260). (c) Grup 1'deki ve (d) 5'teki farelerde yarı ince kesitlerde hepatositlerdeki yağ globülleri *(oklar)* (Toluidin mavisi x 750)

increased in the pancreatic acinar cells of the gemfibrozil supplemented groups (Groups 2, 3, 6, and 7).

Lipocytes in ommental fat tissue, fat layer covering the outer surface of the kidneys and inguinal fat were microscopically hypertrophied in the high fat diet-fed mice. Adipose tissue was highly vascularised in the high fat diet-fed mice than in the other groups.

Immunohistochemistry

Ovariectomy, high fat diet and gemfibrozil supplementation altered tissue PPAR-α and -γ expression levels and reaction densities. However, PPAR-β signals were not affected by ovariectomy, high fat diet and gemfibrozil supplementation. PPAR-α, -β, and -γ positive cells (%) and their statistical significances were summarised in *Table 3*. PPAR-α signals were pronounced particularly in the liver, kidney and heart of the mice.

PPAR-α levels were significantly increased following high fat diet and gemfibrozil supplementation. However, ovariectomy reduced tissue PPAR-α expression levels. PPAR-α positive signals were detected in both hepatocyte cytoplasm and nuclei. PPAR-α staining intensity and distribution differed between groups.

PPAR-α positive cell numbers and staining intensity were higher in the livers of mice in Group 2 compared to that of Group 1 *(Fig. 2)*. In Groups 1, 2, 5, and 6 PPAR-α positive hepatocyte numbers and staining intensities were higher than remaining Groups of 3, 4, 7 and 8. In Group 3, PPAR-α positive signals were mostly present in the periacinar area. In contrast to this finding, high fat diet caused a partial shift of staining from periacinar area to periacinar and midzonal region and partly towards hepatocytes located in the periportal area. Hepatocytes in high fat diet plus gemfibrozil given groups revealed that almost all hepatocytesin liver parenchyma stained positively.

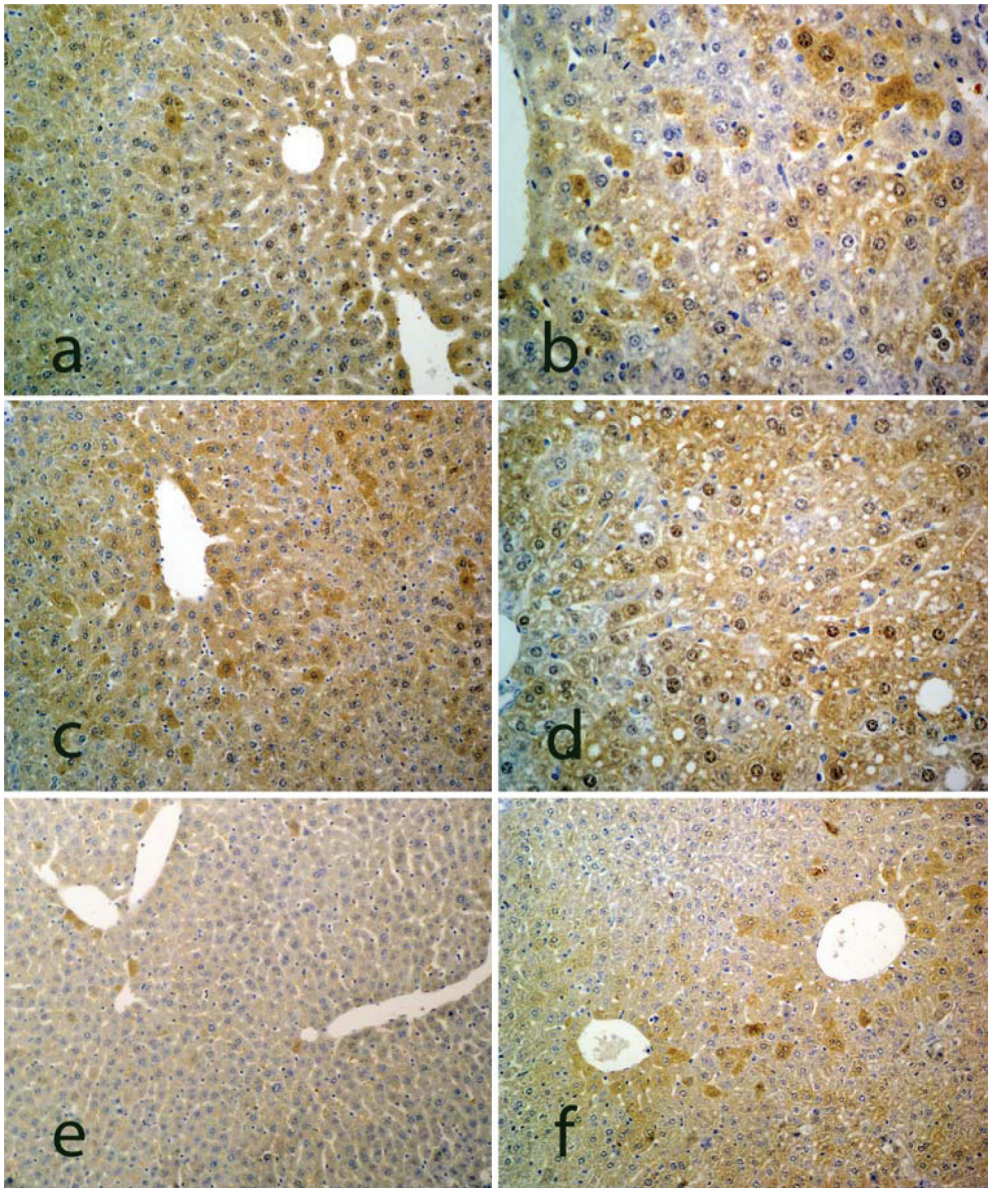

Fig 2. Figures are showing PPAR-α immunoreactivitiy in the liver of mice with the high fat diet (a, b, c and d), low fat diet (e and f), gemfibrozil (b and f), ovariectomised (a and c) and sham operated (b and d). Avidine biotine peroxidase complex (ABC). PPAR-α staining intensity and the number of positively stained cells were greater in gemfibrozil treated mice compared to the mice with no gemfibrozil treatment (b>a, d>c, e>f), and also in the high fat diet fed mice compared to the low fat diet fed mice (a, b, c, and d>e and f). Ovariectomy application in the high fat diet fed mice reduced the PPAR- α expression in liver (c>a, d>b). Magnification: a,c and f: x180; b and d: x260; e: x90)

Şekil 2. Şekillerde yüksek yağlı diyet (a, b, c ve d), düşük yağlı diyet (e ve f), gemfibrozil (b ve f), ovariektomi (a ve c) ve sham operasyonu uygulanan (b ve d d) farelerin karaciğerindeki PPAR-α immunoreaktivitesi görülüyor. Avidin biotin peroksidaz Kompleks (ABC). PPAR-α boyanma yoğunluğu ve pozitif boyanan hücre sayısı gemfibrozil uygulananlarda (b>a, d>c, e>f) gemfibrozil uygulanmayanlara göre, yüksek yağlı diyet ile beslenenlerde (a, b, c, d>e,f) düşük yağlı diyetle beslenenlere göre daha fazla. Yüksek yağlı diyetle beslenen farelere (a,b,c,d) ovariektomi uygulaması karaciğerde PPAR-α ekspresyonunu azalttı (c>a, d>b). (Büyütme: a,c ve f: 180; b ve d: x260; e: x 90)

In kidneys, PPAR-α positive signals were detected mainly in the proximal tubular epithelial cells. In all groups, almost all proximal tubular epithelial cells were stained for the PPAR-α antibody. Gemfibrozil supplemented high fat diet extended PPAR-α staining to distal tubular epithelial cells.

PPAR-α positive signals were also detected in the stomach and intestinal epithelial cells, adreno-cortical cells, heart muscle and smooth muscle cells, adipocytes and macrophages.

No significant difference was detected for PPAR-β expression amon the groups. PPAR-β immunoreactivity was seen in almost all tissue samples. Both cytoplasmic and nuclear, granular type PPAR-β signals were mostly

Table 3. *PPAR-α, -β, and -γ positive cells (%) in the liver and their statistical significances*
Tablo 3. *Karaciğerde PPAR-α, PPAR-β ve PPAR-γ primer antikorlarıyla pozitif boyanan hücre yüzdesi ve gruplar arası istatistiksel farklılıklar*

Immunoreactivity (positivity %)	Groups								P
	1	2	3	4	5	6	7	8	
PPAR-α	28.4 ± 3.1^{F}	55.5 ± 2.2^{B}	37.2 ± 5.2^{E}	6.1 ± 1.6^{H}	48.7 ± 4.5^{C}	66.8 ± 5.3^{A}	44.4 ± 5.1^{D}	11.5 ± 2.3^{G}	0.001
PPAR-β	29.7±8.4	28.0±13.1	27.9±6.2	28.6±9.1	30.5±8.1	29.0±12.4	27.8±10.7	29.7±9.7	NS
PPAR-γ	8.2 ± 0.9^{BC}	8.8 ± 0.7^{B}	5.8 ± 0.7^{D}	5.2 ± 1.0^{D}	10.9 ± 1.5^{A}	11.0 ± 1.8^{A}	8.1 ± 1.3^{BC}	7.3 ± 1.0^{C}	0.001

Values were presented as mean ± SE. Means denoted with different superscripts within the same line are statistically significant, ***NS:*** *Not Significant*

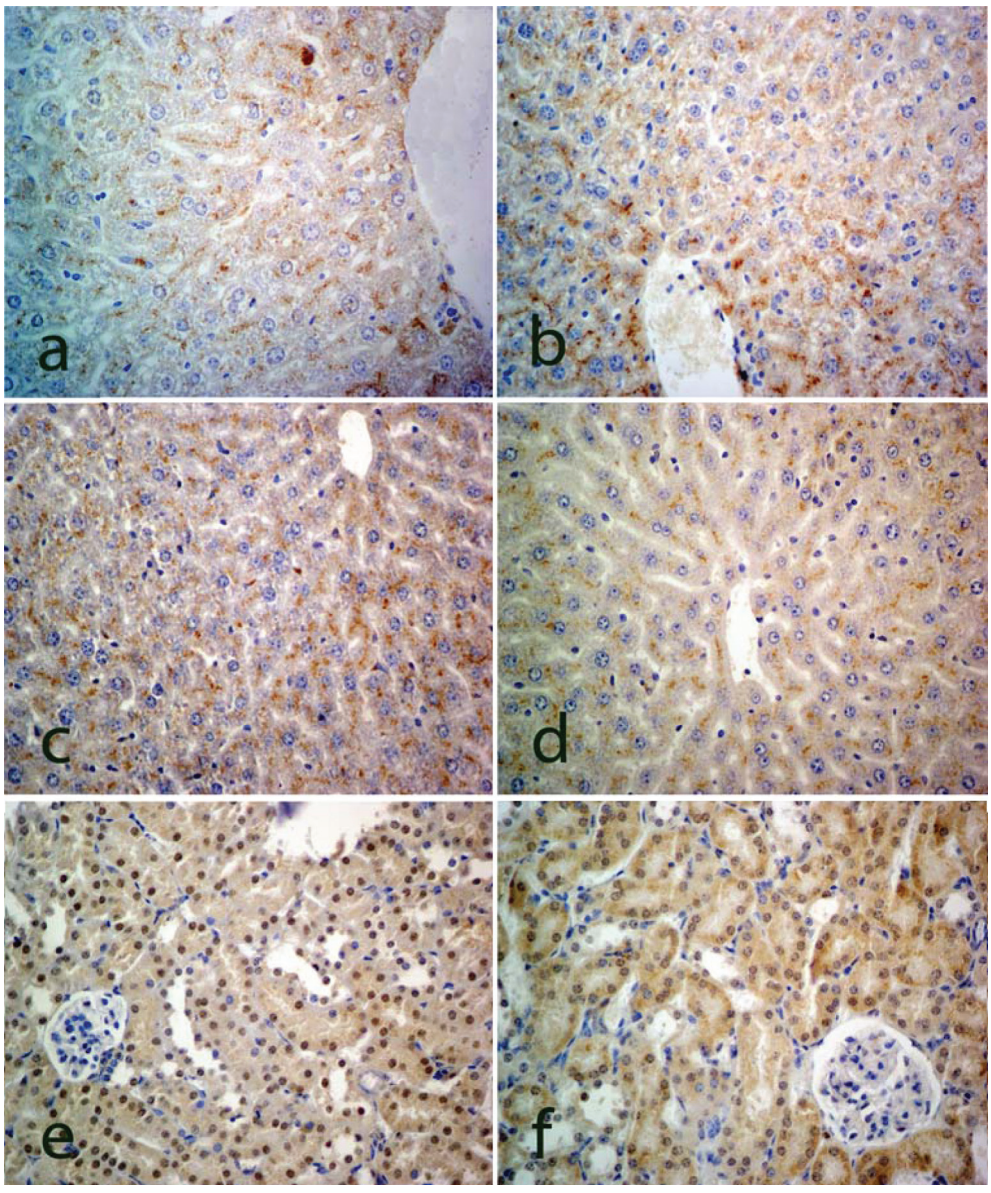

Fig 3. PPAR-β expression in the liver and kidneys. Avidin-biotin peroxidase complex (ABC).Following ovariectomy (a, c, d), sham (b, d, f), high fat diet (a, b, d, e, f) and gemfibrozil (b and d) administration (a, b, c, d) hepatocytes mostly revealed granular cytoplasmic and positive immunoreaction while in kidneys the staining was (e, and f both cytoplasmic and nuclear. There is no difference between groups in terms of staining intensity and the number of positively stained cells (Magnification: a, b, c, d and f: x 260)

Şekil 3. Karaciğer ve böbreklerdeki PPAR-β ekspresyonu. Avidin Biotin Peroksidaz Kompleks (ABC). Ovariektomi (a, c, d), sham (b, d, f), yüksek yağlı diyet (a, b, d, e, f) ve gemfibrozil (b ve d) uygulamaları sonunda karaciğerde (a, b, c, d) çoğunlukla sitoplazmik ve granüler tarzda pozitif reaksiyon görülürken böbreklerde (e ve f) hem sitoplazmik hem nükleer pozitif reaksiyon görülmekte. Pozitif boyanan hücre sayısı ve boyanma yoğunluğu açısından ise gruplar arası farklılık yok (Büyütme: a, b, c, d ve f: x 260)

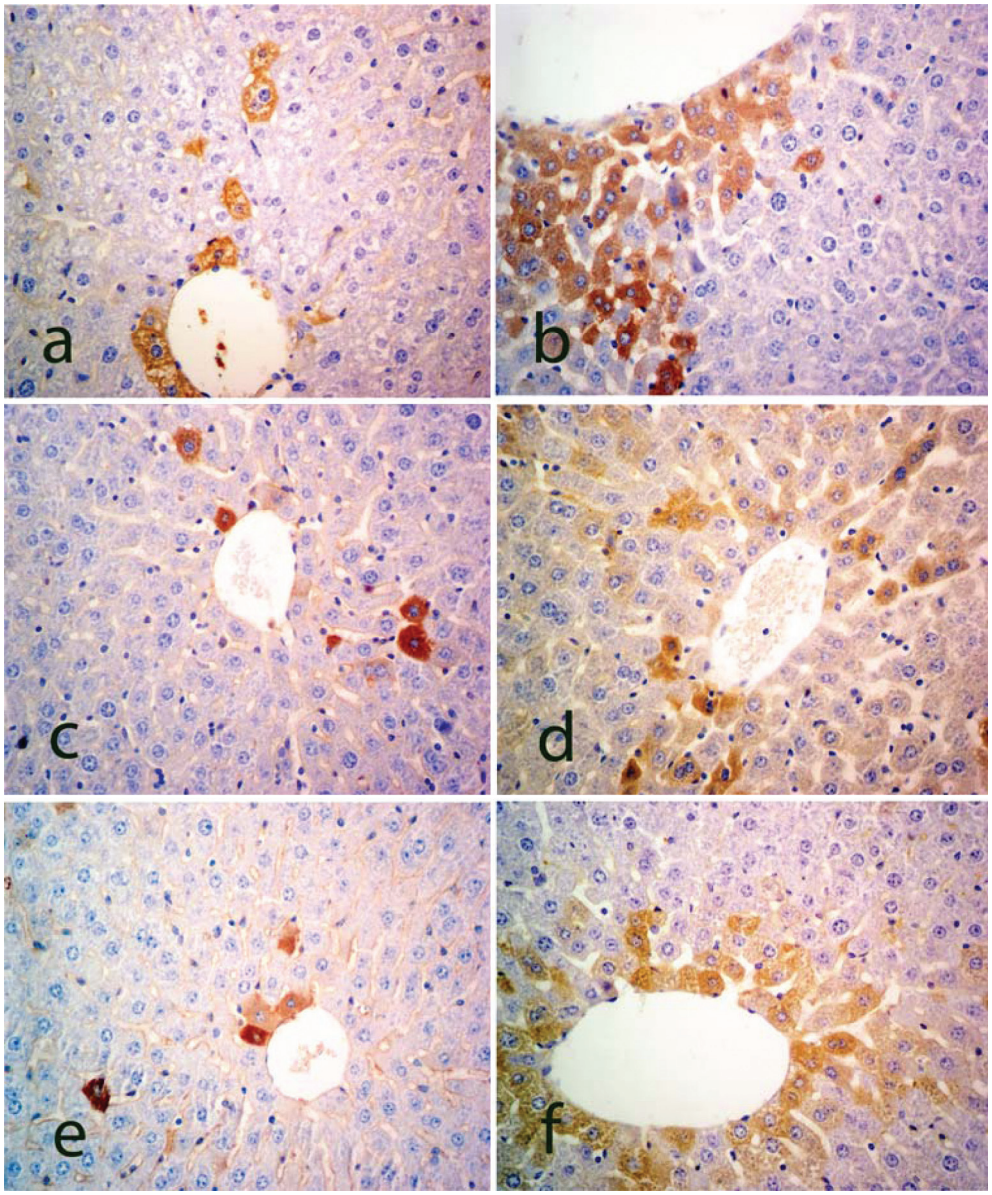

Fig 4. PPAR-γ expression in the liver. Avidin-biotin peroxidase complex (ABC). While there is no difference for the positively stained cells and staining intensity of PPAR-γ in the ovarioectomised (a, c, e), sham (b, d, f), high fat diet (a and b) and high fat diet plus gemfibrozil (c and d) applied mice groups staining intensity is elevated in the low fat diet and gemfibrozil (e and f) administered groups (Magnification: a, b, c, d, e and f: 260)

Şekil 4. Karaciğerde PPAR-γ ekspresyonu. Avidin Biotin Peroksidaz Kompleks (ABC). Ovarioektomi (a, c, e), sham (b,d,f), yüksek yağlı diyet (a ve b) ile yüksek yağlı diyet ve gemfibrozil (c ve d) uygulanan farelerde pozitif boyanan hücre sayısı ve boyanma yoğunluğu açısından fark görülmezken düşük yağlı diyet ve gemfibrozil (e ve f) uygulanan farelere göre PPAR-γ ekspresyonu daha fazla (Büyütme: a, b, c, d, e ve f: 260)

Table 4. *Biochemical alterations in the serum of treatment groups and their statistical significance*
Tablo 4. *Çalışma da kullanılan farelerin serum örneklerindeki biyokimyasal değişiklikler ve gruplar arası istatistiksel farklılıklar*

Gorups	AST (U/L)	ALT (U/L)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	VLDL (mg/dl)
1	280.62±10.05^{A}	86.21±6.09^{A}	131.81±8.71^{A}	178.17±20.67^{A}	30.84±5.81^{A}
2	120.80±20.07^{C}	55.70±11.38^{CD}	98.60±15.03^{BC}	138.40±20.33^{B}	25.90±4.86^{B}
3	86.79±15.80^{D}	50.01±10.57^{DE}	80.00±13.06^{EF}	69.38±14.06^{D}	16.45±1.88^{C}
4	147.00±21.28^{B}	61.43±15.05^{C}	86.00±8.93^{DE}	150.14±15.02^{B}	26.29±4.64^{B}
5	275.60±22.16^{A}	70.80±7.73^{B}	107.50±11.91^{B}	176.70±13.12^{A}	30.20±4.16^{A}
6	85.54±15.82^{D}	42.63±11.26^{EF}	71.58±8.82^{F}	69.30±18.76^{D}	15.79±3.65^{C}
7	87.46±16.89^{D}	48.31±12.96^{DEF}	94.08±12.74^{CD}	94.46±17.71^{C}	18.77±5.73^{C}
8	132.30±17.28^{BC}	38.80±6.91^{F}	74.00±12.62^{F}	143.40±15.74^{B}	25.00±2.54^{B}
P	0.001	0.001	0.001	0.001	0.001

Values presented as mean ± SE. Means denoted with different superscripts are within the same column are statistically significant, ***AST:*** *aspartate amino transferase,* ***ALT:*** *alanine aminotransferase,* ***VLDL:*** *very low density lipoprotein*

localised in the periacinar hepatocytes *(Fig. 3)*.

Diffuse and granular type PPAR-β staining was observed in the cardiac myocytes and adrenal cortical cells. Both cytoplasmic and nuclear PPAR-β immunopositivity were also recorded in the stomach and intestinal epithelial cells and adipocytes.

β cells of islets of Langerhans revealed weak cytoplasmic immunoreactivity againt PPAR-β antibody while α cells located at the periphery of the islets exhibited rather dense cytoplasmic and nuclear reaction. Nuclear PPAR-β staining was detected in the acinar cells from the exocrine component of the pancreas.

PPAR-β reaction was detected in the central nervous system particularly in the dentate gyrus, hippocampus, thalamic nuclei, cerebellum and telencephalic cortical neurons.

PPAR-γ staining was detected in the capillary endothelial cells, macrophages, adipocytes, Kupffer cells and hepatocytes. PPAR-γ staining was mostly observed in the periacinar hepatocytes and to less extend in the cytoplasm of hepatocytes present in the midzonal and periportal areas, Kupffer cells and vascular endothelial cells *(Fig. 4)*.While gemfibrozil supplementation did not alter PPAR-γ immunoreactivity ovariectomy and high fat diet ameliorated PPAR-γ expression levels. The number of PPAR-γ positive hepatocytes and staining intensities were higher in the high fat gemfibrozil supplemented diet group (Group 2) and only high fat diet group (Group 1) compared to only high fat diet applied group (Group 1) and low fat diet group (Group 3), respectively. PPAR-γ positive hepatocytes were generally localised in the periacinar region. However, individual hepatocytes present in the midzonal and periportal areas were reacted positively for the PPAR-γ antibody.

PPAR-γ immunoreactivity was detected in other organs including submucosally located macrophages present in the stomach and intestines, alveolar macrophages, glomerular parietal and visceral epithelial cells, adipocytes and vascular endothelial cells of all the organs subjected to this study.

Serum Assay Results

Serum AST, ALT, total cholesterol, triglyceride, and VLDL levels were higher in the high fat diet fed groups compared to low fat diet fed groups. However, gemfibrozil supplementation to high fat diet significantly reduced serum AST, ALT, total cholesterol, triglyceride, and VLDL concentrations, compared to only high fat diet fed mice. Ovariectomy and sham operated mice serum AST and ALT levels were not significantly altered. However, serum cholesterol, triglyceride and VLDL levels were higher than that of the sham operated mice. Serum assay results and their statistical significance were summarised in *Table 4*.

DISCUSSION

It has long been known that estrogen has an effect on adiposity in humans, rodents and other species. Decrease of estrogen levels upon senescence or ovariectomy in humans and rodents alter metabolic processes and cause an increase in adiposity [20]. In the present study, body weights of ovariectomised mice from Groups 1 and 4 increased 30.4% and 6.5%, respectively. Increased body weight following ovariectomy could be explained by direct effects of the estrogen on lipid metabolism and energy homeostasis [16,20,21]. Elevated food consumption of ovariectomised mice versus sham operated mice of the present study supports this hypothesis. PPAR-α like estrogen, also plays significant role in the β-oxidation of fatty acids [8,22]. Long chain fatty acid catabolism capacities of PPAR-α null mice are rather low and consequently dyslipidemia occur [23,24] and in the long term body fat content elevates [25]. In the present study, gemfibrozil supplementation prevented mice from weight gain despite high fat diet continued for 15 weeks. Gemfibrozil effectively prevented weight gain due to estrogen deficiency which occurred following ovariectomy. Moreover, gemfibrozil supplemented low fat diet reduced body weights in Group 3 (12.7%) and Group 7 (14.4%). It was suggested that PPAR-α agonists reduce food intake and thus contribute to weight loss [26]. However, in our study gemfibrozil supplementation, instead of reducing feed consumption, increased feed intake in the gemfibrozil supplemented high fat diet group (Group 6) compared to only high fat diet applied group (Group 5). However, there was no difference between gemfibrozil supplemented low fat diet group (Group 3) and only low fat diet group (Group 4) in terms of feed consumption. Gemfibrozil supplementation reduced abdominal fat content regardless ovariectomy operation. Present study showed that abdominal adiposity which could occur as a result of hypoestrogenism could be prevented by gemfibrozil, a well-known exogenous ligand for PPAR-α.

Estrogen inhibits lipogenesis and adiposity by reducing lipoprotein lipase enzyme activity which regulates lipid storage in the adipocytes. However, ovariectomy induces lipoprotein lipase enzyme activity and consequently increases lipid storage in the adipocytes and adiposity [27]. Hormone replacement therapy in the post-menopausal women reduces the incidence of cardiovascular disease [28,29]. Moreover, in women hormone replacement therapy reduces circulating LDL levels while increasing HDL and as a result of this cardiovascular disease possibility decreases significantly [28]. In our study, ovariectomy partly increased plasma triglycerides, total cholesterol and VLDL levels. This increase was most prevalent in the high fat diet fed group. However, gemfibrozil regulated plasma lipid profile.

Implementation of the high fat diet for the 15 weeks led to mild to moderate liver steatosis in these animals. Hepatic

steatosis was more prominent in the ovariectomised mice. This was associated with increased PPAR-α and γ expression levels while PPAR-β expression level did not alter. High fat diet associated with elevated PPAR-α expression levels is probably related to the increase in the density of fatty acids in hepatocytes [24]. PPAR-α target genes are related to the mitochondrial and peroxisomal oxidation of fatty acids and intracellular other lipid metabolisms [4,9,24]. In this context, it could be suggested that free fatty acids could induce PPAR-α expression and consequently increase oxidation of fatty acids in the liver [4,8]. Unsaturated fatty acids are natural ligands of the PPAR-γ as in PPAR-α [30]. PPAR-γ target genes are in charge of adipocyte differentiation, lipid storage and glucose metabolism [30,31]. PPAR-γ is very important for adipocyte differentiation both *in vitro* and *in vivo* [30-32]. In the present study, high fat diet increased the weight of abdominal fat tissue was possibly associated with increased expression of PPAR-γ. In the study, in contrast to PPAR-α and PPAR-γ PPAR-β expression level did not change following high fat diet although it has significant role in the oxidation of fatty acids. These findings possibly indicate that PPAR-β is not activated by fatty acids.

Interestingly, ovariectomy reduced PPAR-α and-γ expression levels. Various mechanisms could be put forward to explain this finding. Because estrogen alone, is not a ligand of PPAR-α and –γ [33] also they do not form in heterodimers of PPAR and estrogen *in vivo* [34]. Therefore, the relationship between estrogen and PPARs could be explained by indirect mechanisms. For example, PPAR-γ is activated by prostaglandins, and especially the J2 series of prostaglandins [35]. Estrogen regulates the synthesis of prostaglandins in the target tissues such as uterus [36]. Estradiol increases arachidonate which is one of precursor of the prostaglandins [37]. PPAR-γ expression decreased with ovariectomy could be related to reduced effects of estrogen on the prostaglandin synthesis in target tissues. On the other hand, changes in the PPAR-γ and -α expression levels could also be related to estrogen receptors.

In this study gemfibrozil which is an exogenous ligand for PPAR-α, induced PPAR-α expression more potently than that of high fat diet. Gemfibrozil supplemented high fat diet increased PPAR-α expression more significantly in comparison with gemfibrozil alone. This finding was supported by our previous study in which Wistar-Albino rats were fed high fat diet plus clofibrate and we reported that clofibrate alone increased PPAR-α expression more effectively in comparison with high fat diet alone [8]. However, in the previous study [8], there was no significant difference in between clofibrate plus high fat diet and control diet supplemented with clofibrate which is differed from our present findings. This difference could originate either from species specific difference of ligand-mediated activation of PPAR-α or gemfibrozil application and implementation of high fat diet could create a synergism to activate PPAR-α more potently.

In the present study, gemfibrozil administration ameliorated hepatic steatosis in ovariectomised and high fat diet fed mice as well as sham operated and high fat diet fed mice. Although, hepatic steatosis is not yet fully elucidated, the beneficial effects of PPAR-α ligands were attributed to enhanced oxidation of fatty acids by inducing enzymatic activities of rate-limiting peroxisomal (AOX) and mitochondrial (ACD) β-oxidation in the hepatocyte mitochondria and peroxisomes [8,45].

In conclusion, the high fat diet and ovariectomy induced fatty liver model of mice showed that gemfibrozil supplementation was effectively ameliorated hepatic steatosis and abdominal adiposity. Our ovariectomised mice model suggests that gemfibrozil potently activates PPAR-α whereas it does not effect the expression of PPAR-β and -γ. Furthermore, high fat diet induced the expression and activation of PPAR-α and -γ whereas it was ineffective for the PPAR-β. On contrary, ovariectomy reduced the expression of PPAR-α and -γ. And also gemfibrozil administration effectively prevented liver steatosis in mice.

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RESEARCH ARTICLE

Kadmiyum, Bakır ve Kurşunun *İn Vitro* İnek Uterus Kasılmaları Üzerine Etkileri [1]

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Özet

Bu çalışma, *in vitro* koşullarda kadmiyum, bakır ve kurşunun folliküler ve luteal evredeki izole inek uterus kasılmaları üzerine olan etkilerinin belirlenmesi amacı ile yapıldı. Çalışmada; Kırıkkale mezbahasında kesilen sağlıklı Holstein ırkı ineklerin myometrial şeritleri kullanıldı. Bu şeritler, izole organ banyosuna 2 g ön gerimle asıldı. Spontan, 2.5 mIU/ml oksitosin ve 10^{-6} M prostaglandin F_2 alfa (PGF_2 alfa) ile oluşturulan kasılmalar üzerine 10^{-5} M kadmiyum, 10^{-4} M bakır ve 10^{-4} M kurşunun etkileri değerlendirildi. Kadmiyum, folliküler evrede oksitosin ve PGF_2 alfa'nın, luteal evrede ise spontan, oksitosin ve PGF_2 alfa' nın oluşturduğu kasılmaların tüm parametrelerini (maksimum ve ortalama amplitüd, frekans değerleri) anlamlı olarak azalttı. Bakır; folliküler evrede spontan kasılmaların ortalama ve maksimum amplitüd değerlerini, oksitosin kasılmalarının tüm parametrelerini, PGF_2 alfa kasılmalarının frekans ve maksimum amplitüd değerlerini düşürdü. Luteal evrede bakır, spontan kasılmaların maksimum amplitüdünü, oksitosin kasılmalarının frekans ve maksimum amplitüdünü, PGF_2 alfa kasılmalarının ortalama ve maksimumum amplitüd değerlerini düşürdü. Kurşun, folliküler ve luteal evrede PGF_2 alfa ve oksitosin kasılmalarının frekans değerini azaltırken, luteal evrede spontan kasılmaların ortalama amplitüd değerini artırdı. Sonuç olarak; kadmiyum, bakır ve kurşunun inek uterusunun kasılma yanıtlarını değiştirdiği ve buna bağlı olarak fertiliteyi etkileyebileceği kanısına varıldı.

Anahtar sözcükler: *İnek uterusu, İn vitro, Kadmiyum, Bakır, Kurşun*

The Effects of Cadmium, Copper and Lead on *In Vitro* Bovine Uterine Contractility

Summary

The aim of this study was to investigate *in vitro* effects of cadmium, copper and lead on isolated bovine uterus contractions at follicular and luteal stage. In this study myometrial strips obtained from healthy Holstein cows slaughtered in Kırıkkale slaughterhouse were used. The strips were mounted to isolated organ bath under a basal tension of 2 g. The effects of 10^{-5} M cadmium, 10^{-4} M copper and 10^{-4} M lead on the spontaneous, 2.5 mIU/ml oxytocin and 10^{-6} M PGF_2 alpha induced contractions were examined. At follicular stage, all contractility parameters (maximum and mean amplitudes and frequency) of uterine induced by oxytocin and prostaglandin F_2 alpha (PGF_2 alpha), and at the luteal stage all contractility parameters of spontaneous, oxytocin and PGF_2 alpha induced contractions were decreased significantly by cadmium. At follicular stage the maximum and mean amplitude of spontaneous contractions, all contractility parameters induced by oxytocin, the frequency and maximum amplitude caused by PGF_2 alpha and, at the luteal stage the maximum amplitude of spontaneous contractions, the frequency and maximum amplitude of uterine contractions induced by oxytocin, and maximum-mean amplitude of contractions induced by PGF_2 alpha were decreased by copper. While lead decreased the frequency of uterine contractions induced by PGF_2 alpha and oxytocin at folicular and luteal stage, at luteal stage it increased the mean amplitude of spontaneous contractions. It was suggested that cadmium, copper and lead changed the contractility of bovine uterine; therefore this may be effect the fertility in cattles.

Keywords: *Bovine uterus, In vitro, Cadmium, Copper, Lead*

GİRİŞ

Çiftleşme sırasında uyarılan serviks, uterus ve ovidukt kasılmaları; spermatozoaların fertilizasyon bölgesine ulaşmasına yardım eder [1]. Ayrıca, uterus kasılmalarının doğum ve östrus siklusu [2] ile puerperal dönemde uterusun involüsyonunda [3] ve erken postpartum dönemde uterus lumenindeki fazla sıvı ve kalıntının atılmasında rol oynadığı [4]

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bildirilmiştir. Östrusta ve östrusun hemen ardından kasılmaların frekansının en yüksek seviyeye ulaştığı, luteal dönemde ise azaldığı kaydedilmiştir [5]. Folliküler ve luteal dönemler arasında hormonel farklar bulunmaktadır. Folliküler dönemde progesteron bazal seviyenin altına inerken östrojen seviyesi artar. Luteal dönemde ise progesteron seviyesi yüksektir [6].

Çiftlik hayvanlarında uterus kasılmaları üzerine yapılan çalışmaların, (1) östrus siklusu ile uterotonik etkinlik arasındaki ilişki, (2) doğum sırasındaki uterotonik etkinlik, (3) uterusu uyaran maddelerin etkisi üzerine odaklandığı belirtilmiştir [2]. Uterus kasılmaları, herhangi bir ilaç ya da yabancı maddeden etkilenebilmektedir [7]. Çevresel kirletici olarak bilinen kadmiyum, bakır ve kurşunun uterus kasılmaları üzerine etkisinin araştırılması bu açıdan önemlidir.

Kadmiyum, bakır ve kurşuna maruziyet birçok yolla olmaktadır. Bu yollar; hatalı olarak yeme katılmaları, bu metalleri fazla miktarda ihtiva eden bitkilerin alınması, meraların ya da hayvan yemi olarak kullanılacak bitki ve yemlerin herbisid, insektisid ve funguside maruz kalması şeklinde sıralanabilir. Endüstriyel atıkların kazara çevreye bulaşması da sebepler arasında yer alır [8].

Kadmiyum, bakır ve kurşunun farklı dokular üzerine etkilerini gösteren çalışmalar bulunmaktadır [9-17]. Kadmiyum, insan myometriumunda kalsiyum kanallarını inhibe etmektedir [9]. Sıçan, kedi ve gebe sığır uterusunda kasılmaları tamamen engellendiği saptanmıştır [10]. İnsan ve değişik hayvan türlerinde bakırın uterus üzerine etkisi çalışılmış [11-14] ve bakırın *in vivo* ve *in vitro* olarak uterusun etkinliğini değiştirebileceği ortaya konmuştur. Schild [11], depolarize rat uterusunda, S-S polipeptidlerin kasılma potensini bakır (II) sülfat için sıfır olarak bildirmiştir. Bakır klorür tavşan myometriumunun kasılmalarını derişime bağlı bir şekilde artırmıştır [12]. Kurşun asetatın ise derişime bağlı olarak sıçan trakea halkalarında [15] ve tavşan aortasında [16] kasılma oluşturduğu gözlemlenmiştir. Santos ve ark.[17] kurşun asetat verilen sıçanların izole gastrik fundusunda, kurşunun adrenerjik ve kolinerjik olmayan gevşemeleri önlediğini ortaya koymuşlardır. Ancak; yapılan literatür taramalarında, hem folliküler hem de luteal evrede bu metallerin izole inek uterusu üzerine etkilerini araştıran bir çalışmaya rastlanmamıştır.

Bu çalışmanın amacı; hayvanlarda doğrudan veya çevre ve besin kirliliği şeklinde akut, subakut, kronik zehirlenmelere yol açabilen kadmiyum, bakır ve kurşunun, folliküler ve luteal dönemde inek uterus kasılmaları üzerine olan etkilerini araştırmaktır.

MATERYAL ve METOT

Hayvan Materyali

Çalışma materyali olarak folliküler ve luteal evredeki ineklerden alınan uterusa ait myometrial şeritler kullanıldı. Bu şeritler, Kırıkkale Belediyesi'ne ait mezbahada veteriner hekim kontrolünde kesilen sağlıklı Holstein ırkı ineklerden elde edildi. Hasta ve postpartum dönemdeki inekler çalışmada kullanılmadı. Her bir metal ve dönem için farklı uterus myometriumu kullanıldı.

Cihazlar

Çalışmada elde edilen veriler force displacement transducer (FDT 05 MAY, Commat, Türkiye) ile ölçülüp, Biopac System (MP35, ABD) ile kayıt altına alındı.

Kimyasal Madde ve Çözeltiler

Tirod çözeltisi her deneyde taze olarak, çözelti içeriğinde; mM cinsinden 136.9 NaCl, 2.68 KCl, 1.05 $MgCl_2$, 0.42 NaH_2PO_4, 5.5 glukoz, 1.8 $CaCl_2$, 11.9 $NaHCO_3$ ve pH: 7.4 olacak şekilde distile suda çözdürülerek hazırlandı. Kadmiyum klorür hemipenta hidrat, ACS reagent (Kodu: 239208) ve kurşun II klorür %98'lik (Kodu: 268690) Aldrich'den, bakır (II) klorür hidrat (Kodu: C3279), oksitosin liyofilize toz (Kodu: O3251) ve prostaglandin F_2 alfa tris tuzu %99 saflıkta (Kodu: P0424) Sigma'dan sağlandı. Tüm kimyasal maddeler distile suda çözdürülerek -20°C'de muhafaza edildi ve deneyler sırasında kullanıldı.

Deney Protokolü

Sağlıklı ineklerin öncelikle hangi evrede olduğu tespit edildi. Bu amaçla her iki ovaryumun makroskobik morfolojisi değerlendirildi. Regrese korpus luteum olsun veya olmasın 10 mm'den büyük follikülü olan hayvanların folliküler, aktif korpus luteumu olan hayvanların ise luteal dönemde oldukları kabul edildi. Aktif yapının bulunduğu taraftaki kornu uterinin üst 1/3'lük kısmı kesilerek ayrıldı [18] ve ovaryum tarafının karıştırılmaması için cerrahi ipek iplikle dikiş atılarak işaret konuldu. Alınan bu kısmın çevre yapıları temizlendikten sonra, soğuk zincirde (+4°C), %95 oksijen %5 karbondioksit ile önceden gazlanmış Tirod çözeltisi içerisinde, yaklaşık 15-20 dk.'da laboratuvara getirildi. Uterus dokusundan yaklaşık 1.5-2 cm boyunda, 0.5 cm eninde şerit izole edildi ve cerrahi ipek iplikle ovaryuma bakan kısmı trandusere gelecek şekilde izole organ banyosuna asıldı. Deneyler boyunca dokular 10 ml Tirod çözeltisi içinde devamlı olarak %95 oksijen %5 karbon dioksit ile gazlandı. Sıcaklık 37°C'de sabitlendi.

Her bir metal için uygulanan deney protokolü: Myometrial şeritlere 2 g ön gerim verildi. Kas bu gerime uyum sağlayana kadar en az 1 saat beklendi. Bu esnada dokular 15 dk aralıklarla spontan kasılmalar başlayana kadar taze Tirod çözeltisi ile en az 4 kez yıkandı. Bekleme süresi sonunda dokuların canlılığına oksitosinle bakılarak, tekrar 5 dk. ara ile en az 3 kez yıkama yapıldı. Ardından kadmiyum (10^{-5} M), bakır (10^{-4} M) ve kurşun (10^{-4} M)'un hayvanların hem folliküler hem de luteal evrede spontan, oksitosin ve PGF_2 alfa'nın oluşturduğu uterus kasılmaları üzerine etkilerine bakıldı. Kadmiyum derişimi Kara ve ark.'nın [10], bakırın derişimi Laudanski ve ark.'nın [13], kurşunun derişimi

ise Sopi ve ark.'nın [19] çalışmalarından seçildi. Her bir metal için ayrı uterus dokusu kullanıldı. Kontrol olarak 10 dk. boyunca spontan kasılmalar alındı, ardından yıkama yapılmadan metal uygulanarak, 10 dk. boyunca beklendi. Doku 5 dk. aralıklarla Tirod çözeltisi ile en az 3 kez, normale dönene kadar yıkandı ve kontrol olarak 2.5 mIU/ml oksitosin uygulandı. On dk. bekleme periyodunun ardından metal uygulandı, aynı protokol 10^{-6} M PGF_2 alfa için de tekrarlandı. Oksitosin ve PGF_2 alfa'nın inek myometriumunda kasılma oluşturan derişimleri seçildi [20]. Deneyler sırasında uterusun spontan, oksitosin ve PGF_2 alfa ile oluşturulan kasılmalarının frekansları, ortalama amplitüdleri ve maksimum amplitüdleri değerlendirildi. Bütün uygulamalarda 10 dk.'lık zaman periyodu içinde şekillenen değişimler dikkate alındı. Bazal çizgiye göre oluşan kasılmaların amplitüdleri ölçülerek ortalamaları ve ulaşılan en yüksek kasılma maksimum amplitüd değerleri mg cinsinden belirlendi. Ayrıca bu süre içinde oluşan kasılmaların tepe noktaları sayılarak frekans değerleri adet cinsinden tespit edildi.

İstatistiksel Analizler

İstatistiksel hesaplamalarda SPSS 15 for Windows paket programı kullanıldı. Normal dağılım gösteren parametrelerde eşli gruplar t testi, normal dağılmayanlarda Wilcoxon Signed Ranks non parametrik testi kullanıldı. P<0.05 değeri istatistiksel olarak anlamlı kabul edildi. Veriler ortalama ± standart hata şeklinde verildi.

BULGULAR

Kadmiyumun inek uterusuna etkileri *Tablo 1* ve *2*'de verilmiştir. Folliküler evrede kadmiyum uygulaması ile spontan kasılmaların frekansının uygulama öncesine göre istatistiksel olarak daha düşük çıkma eğiliminde olduğu bulundu (P=0.054). Oksitosin ve prostaglandinin oluşturduğu kasılmaların ise tüm parametrelerini (ortalama amplitüd (P<0.05), frekans, maksimum amplitüd (P≤0.01) anlamlı olarak düşürdü. Luteal evredeki inek uterusunda kadmiyum spontan kasılmaların ortalama amplitüd ve frekansını (P≤0.01), maksimum amplitüdünü (P≤0.001), oksitosin ve prostaglandinin oluşturduğu kasılmaların tüm parametrelerini (P≤0.001) anlamlı olarak azalttı *(Şekil 1)*.

Bakırın inek uterusuna etkileri *Tablo 3* ve *4*'te özetlenmiştir. Folliküler evredeki inek uterusunda bakır; oksitosin yanıtlarının tüm parametrelerini (ortalama amplitüd ve frekans (P≤0.01), maksimum amplitüd (P<0.05)), prostaglandin yanıtlarının frekans ve maksimum amplitüd yanıtlarını (P<0.05) azalttı. Spontan kasılmaların amplitüd yanıtları bakır tarafından düşürüldü (P≤0.01). Luteal evredeki inek uterusunda bakır oksitosin yanıtlarının ise frekans ve maksimum amplitüdünü (P≤0.01) düşürdü. Prostaglandin yanıtlarının ise ortalama (P<0.05) ve maksimumum amplitüd (P≤0.01) değerlerini azalttı *(Şekil 2)*.

Kurşunun inek uterusu üzerindeki etkileri *Tablo 5* ve *6*'da sunulmuştur. Folliküler evredeki inek uterusunda kurşun, oksitosin ve prostaglandin kasılmalarında frekansları (sırasıyla P<0.05, P≤0.01) azalttı. Luteal evredeki inek uterusunda kurşun spontan kasılmaların ortalama amplitüd değerini artırırken (P≤0.01), oksitosin ve PGF_2 alfa ile oluşturulan kasılmaların ise frekansını (sırasıyla P≤0.01, P<0.05) azalttı *(Şekil 3)*.

Tablo 1. *Folliküler evredeki inek uterusunda kadmiyumun spontan, oksitosin ve PGF_2 alfa ile oluşturulmuş kasılmalar üzerine etkisi*
Table 1. *The effect of cadmium on the spontaneous, oxytocin and PGF_2 alpha induced contractions on bovine uterus at follicular stage*

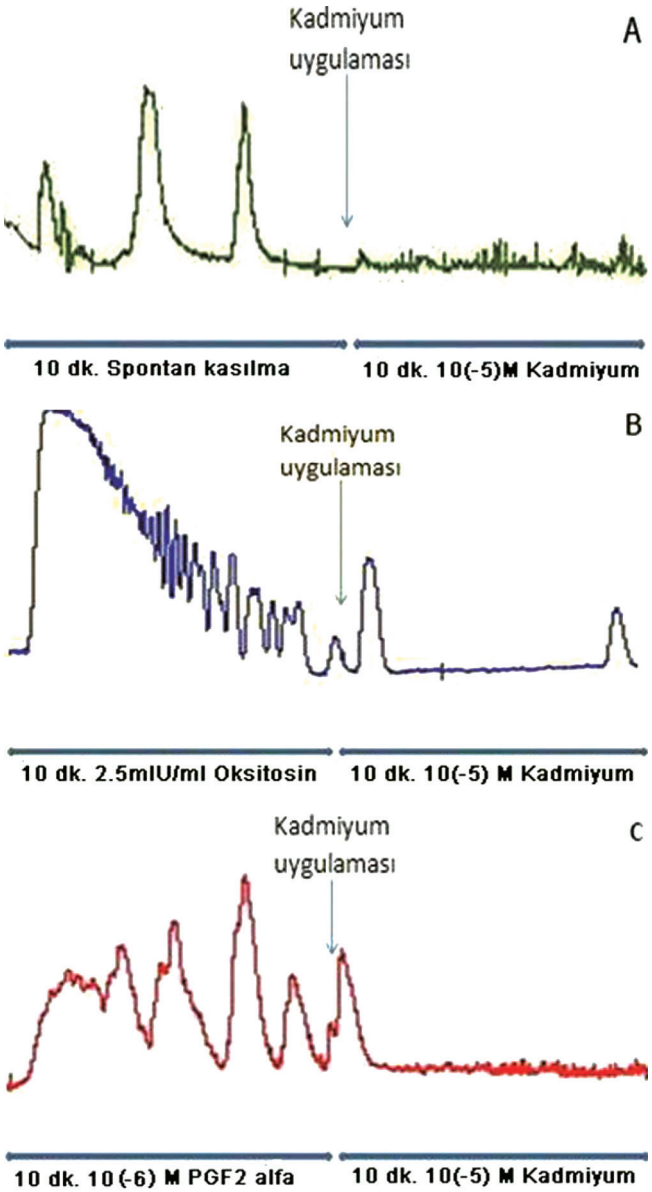
Parametreler	n	Kontrol	10^{-5}M Kadmiyum	P	2.5 mIU/ml Oksitosin	10^{-5}M Kadmiyum	P	10^{-6}M PGF2α	10^{-5}M Kadmiyum	P
Ortalama Amplitüd (mg)	11	989.00±194.41	617.18±214.44	AD	1160.73±152.92	816.91±153.52	*	1176.55±128.47	791.82±207.73	*
Frekans (adet)	11	5.55±0.94	3.55±0.94	AD	17.82±2.75	7.73±1.26	**	12.91±2.59	5.00±0.93	**
Maksimum amplitüd (mg)	11	1355.27±283.11	984.55±371.37	AD	2023.45±267.04	1266.45±246.39	**	1816.82±210.31	1097.82±248.22	**

** P<0.05, ** P≤0.01,* ***n:*** *izole inek uterusu sayısı,* ***AD:*** *Anlamlı değil*

Tablo 2. *Luteal evredeki inek uterusunda kadmiyumun spontan, oksitosin ve PGF_2 alfa ile oluşturulmuş kasılmalar üzerine etkisi*
Table 2. *The effect of cadmium on the spontaneous, oxytocin and PGF_2 alpha induced contractions on bovine uterus at luteal stage*

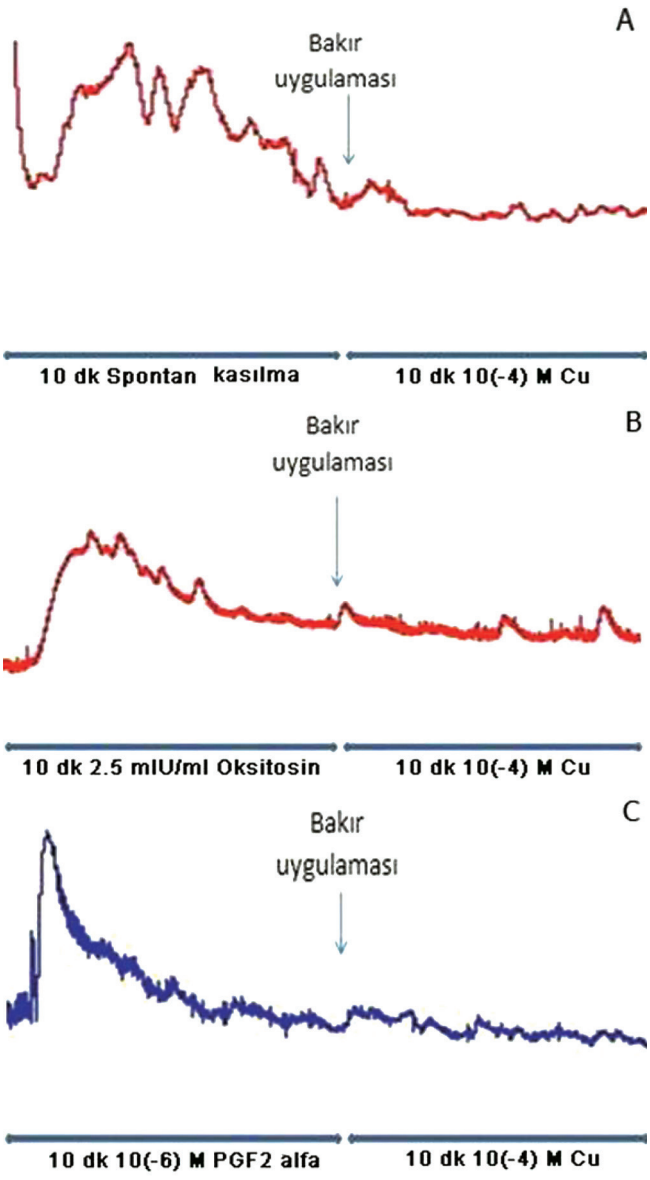
Parametreler	n	Kontrol	10^{-5}M Kadmiyum	P	2.5 mIU/ml Oksitosin	10^{-5}M Kadmiyum	P	10^{-6}M PGF2α	10^{-5}M Kadmiyum	P
Ortalama Amplitüd (mg)	21	630.81±218.24	444.14±212.55	**	881.19±123.24	561.19±106.89	***	981.57±194.51	595.76±110.24	***
Frekans (adet)	21	5.29±1.31	3.57±1.35	**	9.43±1.39	5.52±0.79	***	8.67±1.22	5.14±0.98	***
Maksimum amplitüd (mg)	21	1020.52±281.14	546.00±217.54	***	1325.86±157.14	742.19±132.04	***	1480.57±215.19	730.05±121.19	***

*** P<0.01, *** P≤0.001,* ***n:*** *izole inek uterusu sayısı*



Şekil 1. Kadmiyum klorürün izole inek uterus kasılmaları üzerine etkileri. (A) Kadmiyumun luteal evrede spontan kasılmalar üzerine etkisi, (B) Kadmiyumun folliküler evrede oksitosin ile başlatılan kasılmalar üzerine etkisi, (C) Kadmiyumun luteal evrede PGF_2 alfa ile başlatılan kasılmalar üzerine etkisi

Fig 1. The effects of cadmium chloride on isolated bovine uterus contractility (A) The effect of cadmium on spontaneous contractions at luteal stage, (B) The effect of cadmium on oxytocin induced contractions at follicular stage, (C) The effect of cadmium on PGF_2 alpha induced contractions at luteal stage



Şekil 2. Bakır klorürün izole inek uterus kasılmaları üzerine etkileri. (A) Bakırın luteal evrede spontan kasılmalar üzerine etkisi, (B) Bakırın folliküler evrede oksitosin ile başlatılan kasılmalar üzerine etkisi, (C) Bakırın folliküler evrede PGF_2 alfa ile başlatılan kasılmalar üzerine etkisi

Fig 2. The effects of copper chloride on isolated bovine uterus contractility (A) The effect of copper on spontaneous contractions at luteal stage, (B) The effect of copper on oxytocin induced contractions at follicular stage, (C) The effect of copper on PGF_2 alpha induced contractions at follicular stage

TARTIŞMA ve SONUÇ

Myometriyel kasılmalar, *in vivo* olarak hormonel değişikliklerden etkilenmektedir. Östrus siklusunun çeşitli dönemlerinde, farklı hormonlar baskın durumdadır. Folliküler dönemde östrojen, luteal dönemde progesteron hormonu yüksek seviyede salgılanmaktadır. Östrojenler, östrojen ve progesteron reseptörlerini up regüle ederek, oksitosin reseptör sayısında artışa sebep olur. Progesteron yeni östrojen reseptörlerinin oluşmasını bloke eder ve östrojen seviyesinin düşmesine neden olur. Böylece progesteron oksitosin reseptörlerini down regüle eder. Sonuç olarak, östrojenler oksitosinin myometrium üzerine etkisini artırırken, progesteron önler [21]. Çalışmada dönemsel olarak uterusun reseptör seviyelerinde farklılık olabileceği göz önüne alınarak, kadmiyum, bakır ve kurşunun etkileri folliküler ve luteal dönemde kendi içlerinde değerlendirilmiştir.

Kadmiyum, hem folliküler hem de luteal evredeki inek uterusunda oksitosin ve prostaglandinin oluşturduğu kasılmaların tüm parametrelerini (frekans, ortalama amplitüd

Tablo 3. *Folliküler evredeki inek uterusunda bakırın spontan, oksitosin ve PGF_2 alfa ile oluşturulmuş kasılmalar üzerine etkisi*
Table 3. *The effect of copper on the spontaneous, oxytocin and PGF_2 alpha induced contractions on bovine uterus at follicular stage*

Parametreler	n	Kontrol	10^{-4}M Bakır	P	2.5 mIU/ml Oksitosin	10^{-4}M Bakır	P	10^{-6}M PGF2α	10^{-4}M Bakır	P
Ortalama Amplitüd (mg)	11	1023.64±232.60	507.55±217.32	**	1117.91±398.93	796.82±281.98	**	422.72±86.36	270.64±95.08	AD
Frekans (adet)	11	7.82±1.72	5.64±1.85	AD	10.55±1.97	5.73±1.07	**	6.18±1.29	4.64±1.03	*
Maksimum amplitüd (mg)	11	1536.64±415.45	659.82±273.76	**	1639.18±527.52	1128.27±384.14	*	679.73±151.19	361.28±119.58	*

** P<0.05, ** P≤0.01,* ***n:*** *izole inek uterusu sayısı,* ***AD:*** *Anlamlı değil*

Tablo 4. *Luteal evredeki inek uterusunda bakırın spontan, oksitosin ve PGF_2 alfa ile oluşturulmuş kasılmalar üzerine etkisi*
Table 4. *The effect of copper on the spontaneous, oxytocin and PGF_2 alpha induced contractions on bovine uterus at luteal stage*

Parametreler	n	Kontrol	10^{-4}M Bakır	P	2.5 mIU/ml Oksitosin	10^{-4}M Bakır	P	n	10^{-6}M PGF2α	10^{-4}M Bakır	P
Ortalama Amplitüd (mg)	14	445.14±119.08	286.29±120.38	AD	576.57±88.67	404.79±96.74	AD	13	544.46±95.68	350.08±169.26	*
Frekans (adet)	14	4.21±0.72	3.43±0.63	AD	6.29±0.56	4.36±0.46	**	13	3.69±0.51	3.08±0.72	AD
Maksimum amplitüd (mg)	14	661.00±162.83	358.79±141.06	*	1069.00±149.51	560.43±118.20	**	13	868.69±157.28	418.85±183.85	**

** P<0.05, ** P≤0.01,* ***n:*** *izole inek uterusu sayısı,* ***AD:*** *Anlamlı değil*

Tablo 5. *Folliküler evredeki inek uterusunda kurşunun spontan, oksitosin ve PGF_2 alfa ile oluşturulmuş kasılmalar üzerine etkisi*
Table 5. *The effect of lead on the spontaneous, oxytocin and PGF_2 alpha induced contractions on bovine uterus at follicular stage*

Parametreler	n	Kontrol	10^{-4}M Kurşun	P	2.5 mIU/ml Oksitosin	10^{-4}M Kurşun	P	10^{-6}M PGF2α	10^{-4}M Kurşun	P
Ortalama Amplitüd (mg)	9	1901.11±484.20	1933±602.11	AD	2437.56±584.58	2092.89±507.74	AD	2354.67±521.28	1664.11±441.73	AD
Frekans (adet)	9	7.67±2.27	6.56±1.94	AD	11.89±2.21	7.67±1.19	*	8±1.35	6.33±1.29	**
Maksimum amplitüd (mg)	9	2877.00±750.39	2773.00±877.05	AD	3249.78±781.29	3297.11±798.41	AD	3385.00±781.46	2480.67±686.42	AD

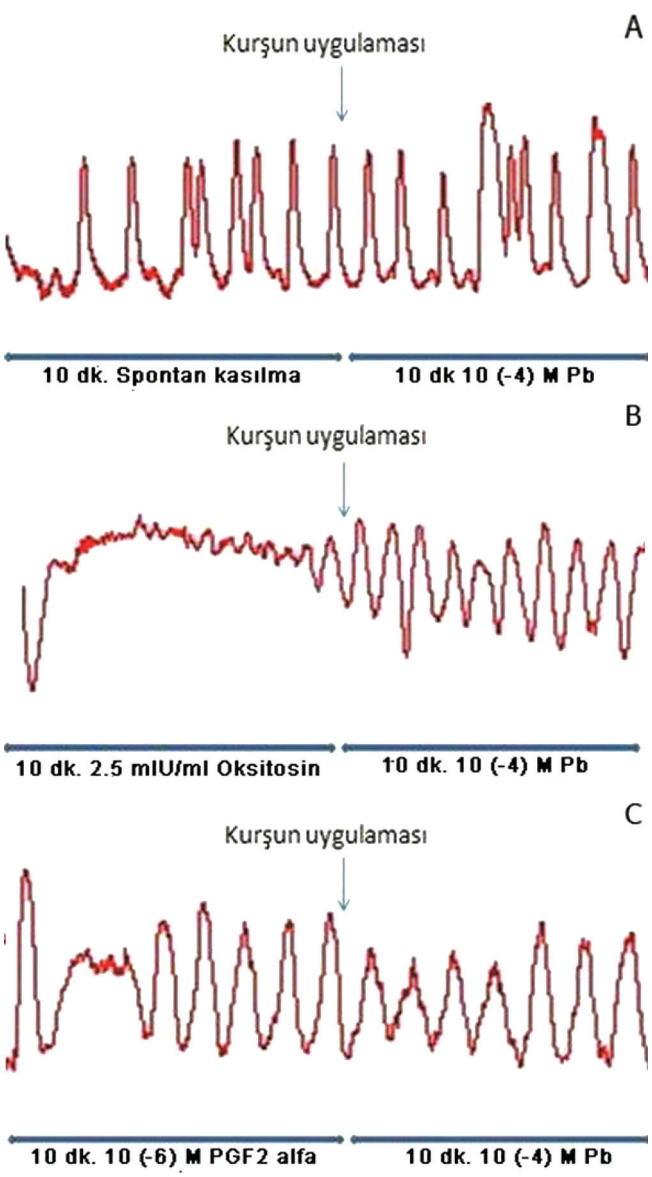
** P<0.05, ** P≤0.01,* ***n:*** *izole inek uterusu sayısı,* ***AD:*** *Anlamlı değil*

Tablo 6. *Luteal evredeki inek uterusunda kurşunun spontan, oksitosin ve PGF_2 alfa ile oluşturulmuş kasılmalar üzerine etkisi*
Table 6. *The effect of lead on the spontaneous, oxytocin and PGF_2 alpha induced contractions on bovine uterus at luteal stage*

Parametreler	n	Kontrol	10^{-4}M Kurşun	P	2.5 mIU/ml Oksitosin	10^{-4}M Kurşun	P	10^{-6}M PGF2α	10^{-4}M Kurşun	P
Ortalama Amplitüd (mg)	12	1393.92±400.23	1566.17±404.75	**	2337.92±298.84	2362.50±290.40	AD	2026.83±332.61	1919.33±342.31	AD
Frekans (adet)	12	10.75±1.70	10.17±1.54	AD	13.75±1.29	10.50±0.66	**	12.42±1.03	10.17±0.99	*
Maksimum amplitüd (mg)	12	2162.67±371.10	2056.08±389.03	AD	2984.33±337.22	3009.42±331.67	AD	3123.58±412.43	2863.75±357.33	AD

** P<0.05, ** P≤0.01,* ***n:*** *izole inek uterusu sayısı,* ***AD:*** *Anlamlı değil*

ve maksimum amplitüd) azaltmıştır. Benzer şekilde Kara ve ark.[10] da yaptıkları araştırmada, 0.01 mM kadmiyumun gebe sığır, gebe olmayan sıçan ve kedi uterusunda 0.01 mM oksitosin ile oluşturulan kasılmaların hem maksimum amplitüd hem de frekans yanıtlarının azaldığını göstermişlerdir. Spowicz ve ark.[9], gebe insan myometriumunda kadmiyumun 10^{-9}-10^{-3} M derişimlerde spontan kasılmaları bloke ettiğini, düşük derişimlerde (10^{-9} ve 10^{-8} M) ise kalsiyum ve oksitosin yanıtlarını artırırken, daha yüksek derişimlerde azalttığını belirlemişlerdir. Başka bir çalışmada kadmiyumun *in vitro* sıçan ekstraoküler kas kasılmasında da azalmalara sebep olduğu belirtilmiştir [22]. İçme suyuna 1 ve 2 ay boyunca günlük 15 ppm kadmiyum verilen sıçanlardan izole edilen duodenum şeritlerinde, asetilkolinin kasılma yanıtlarının azaldığı tespit edilmiştir [23]. Beyazıt ve ark.[24], 3 ay boyunca kadmiyum verilen sıçanlarda, idrar kesesinin detrusor kasında hem nörojenik hem de myojenik kasılmaların bozulduğunu göstermişlerdir. Kadmiyumun bu etkisi, dokularda kalsiyum kanallarını bloke etmesi ve hücre içi uyarılma-kasılma mekanizmalarıyla ilişkili olmasından



Şekil 3. Kurşun klorürün izole inek uterus kasılmaları üzerine etkileri. (A) Kurşunun folliküler evrede spontan kasılmalar üzerine etkisi, (B) Kurşunun luteal evrede oksitosin ile başlatılan kasılmalar üzerine etkisi, (C) Kurşunun luteal evrede PGF_2 alfa ile başlatılan kasılmalar üzerine etkisi

Fig 3. The effect of lead chloride on isolated bovine uterus contractility (A) The effect of lead on spontaneous contractions at follicular stage, (B) The effect of lead on oxytocin induced contractions at luteal stage, (C) The effect of lead on PGF_2 alpha induced contractions at luteal stage

kaynaklanmaktadır. Kadmiyum doğrudan ya da dolaylı yoldan hücresel kalsiyum girişini azaltır [9,23].

Bakır, folliküler evredeki inek uterusunda oksitosin yanıtlarının hepsini azaltırken, PGF_2 alfa yanıtlarında frekans ve maksimum amplitüd, spontan kasılmaların ise amplitüd yanıtlarını azaltmıştır. Luteal evredeki inek uterusunda ise spontan kasılmaların ortalama amplitüd ve frekanslarını değiştirmemiş, oksitosin yanıtlarının frekans ve maksimum amplitüdünü düşürmüştür. Ayrıca PGF_2 alfa yanıtlarının frekans değerini değiştirmezken, ortalama ve maksimumum amplitüd değerlerini azaltmıştır. Verdugo ve ark.[25], $2x10^{-5}$ M bakırın sıçan myometriumunun kasılmasında artışa, $8x10^{-5}$ M derişimin üzerinde ise spazmodik kasılmaya yol açtığını ortaya koymuşlardır. Laundanski ve ark.[12], bakırın tavşan myometriumunun kasılmasında artışa neden olduğunu göstermişlerdir. Bu sonuçlar, sunulan çalışmada elde edilen bulgulardan farklıdır. Farklılığın sebebi; çalışılan hayvanın türüne ya da kullanılan bakır derişimine (10^{-4} M) bağlanabilir. Nitekim Laudanski ve ark.[13], 10^{-4} M bakır klorürün insan myometriyal kasılmalarını inhibe ettiğini göstermişlerdir. Bu çalışmada da bakır klorür, myometriumun PGF_2 alfa'ya verdiği yanıtları azaltmıştır. Bakır iyonları myometriyel etkinliği yüksek derişimlerde azaltırken, düşük derişimlerde oluşan uyarıcı etkisini endometriyel PG sentezi ve salınımına bağlı olarak gerçekleştirmektedir [13].

Kurşunun dokular üzerine etkilerini araştırmak amacıyla yapılan birçok çalışma olmasına rağmen uterusta yapılan bir çalışmaya rastlanılamamıştır. Valencia-Hernandez ve ark.[26], sıçan ve tavşan aortasında 0.1-3.1 mM kurşun asetatın derişime bağımlı kasılma yaptığını, bu kasılmaların tavşan aortasında çok daha güçlü olduğunu saptamışlardır. Sopi ve ark.[19], kurşun asetatın trakea halkalarında kasılma yaptığını, epiteli alınmış halkalarda bu kasılmanın arttığını saptamışlardır. Gupta ve Fahim [15], 10^{-12} M-10^{-4} M kurşun asetatın derişime bağlı olarak sıçan trakea halkalarında, Tomera ve Harakal [16] ise tavşan aortasında kasılma oluşturduğunu gözlemlemişlerdir. Kurşun asetat (10^{-10} M-10^{-3} M) mezenterik tavşan arterlerinde de kasılmalara neden olmuştur [27]. Sunulan çalışmada kurşun, luteal evredeki uterusun spontan kasılmalarının ortalama amplitüd değerini artırmıştır. Santos ve ark.[17], içme suyu ile 15, 30, 120 gün %0.0008 kurşun asetat alan sıçanların izole gastrik fundusunda, kurşunun adrenerjik ve kolinerjik olmayan gevşemeleri önlediğini ortaya koymuşlardır. Zhang ve ark.[28], 24 ve 48 saat boyunca 1 ppm kurşun ile kültüre edilen sıçan aortasının serotonin yanıtlarını artırdığını saptamışlardır. Zhang ve ark.[29], 24 saat boyunca 1 ppm kurşun ile birlikte edilen sıçan aortalarında asetilkolin gevşemelerinin de azaldığını saptamışlardır. Kurşun damar düz kasları üzerine etkisini doğrudan göstermektedir, bu etki hücre içi kalsiyum düzeyinin artmasına ve protein kinaz C ile etkileşimine bağlanmaktadır [27,30]. Uterus kasılmalarında da kalsiyumun hücre dışından hücre içine girişi en önemli mekanizma olarak düşünülmektedir [31]. Çalışmada spontan kasılmaların ortalama amplitüd değerinin artması hücre içi kalsiyum düzeyinin artması ile ilişkili olabilir.

İneklerde döl veriminin fertilite parametreleri sınırlarında yer alması büyük önem arz etmektedir. Uterus kasılmaları; fertilizasyon, gebelik, doğum ve postpartum süreç için çok önemlidir ve ağır metallerden etkilenebilmektedir. Hayvanlar kadmiyum, bakır ve kurşuna hatalı olarak yeme katılmaları, bu metalleri fazla miktarda ihtiva eden bitkilerin alınması, meraların pestisidlerle ilaçlanması yoluyla maruz kalmaktadır. Sonuç olarak; kadmiyum, bakır ve kurşunun

uterusunun kasılma yanıtlarını değiştirdiği ve bu değişimin fertiliteyi etkileyebileceği kanısına varılmıştır. Bu bulguların *in vivo* çalışmaların değerlendirilmesinde de göz önüne alınmasının uygun olacağı sonucuna ulaşılmıştır.

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RESEARCH ARTICLE

Comparison of Different Methods for the Detection of *Salmonella* spp. in Minced Meat Samples [1] [2] [3]

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Summary

The presence of *Salmonella* spp. in minced meat that is consumed in nine different sites of Istanbul is evaluated by using conventional culture (ISO 6579:2002), immunomagnetic separation (IMS) and fluorescent in situ hybridization (FISH) methods. *Salmonella* spp. was isolated from five of 50 (20%) minced meat with ISO 6579 method, and three of 50 (6%) minced meat using IMS method. Bacteria isolated from both ISO and IMS methods were identified as *Salmonella choleraesuis ssp. arizonae* and *Salmonella* spp. The presence of *Salmonella* spp. was determined from 37 of 50 (74%) minced meat by using FISH method. In the current study, it has been shown that ISO 6579 method was found more to be susceptible than IMS method for determining presence of *Salmonella* spp., FISH method is the best method to determine the presence of *Salmonella* spp. Even if the quick determination of the epidemics of international importance occured as a result of the contamination by pathogens derivated from foods, the results of the use of new methods should be supported by the conventional culture method.

Keywords: *Conventional culture method (ISO 6579 reference method), Fluorescent in situ hybridization (FISH) method, Immunomagnetic separation method, Minced meat, Salmonella*

Kıyma Örneklerinde *Salmonella* spp. Tespitinde Farklı Yöntemlerin Karşılaştırılması

Özet

İstanbul'un dokuz farklı semtinde tüketime sunulan kıyma örneklerinde geleneksel kültür yöntemi (ISO), immünomanyetik ayırma (İMA) ve floresanlı yerinde hibritleme (FISH) yöntemleri kullanılarak *Salmonella* cinsi bakterilerin varlığı tespit edilmiştir. İncelenen 50 kıyma örneğinin, geleneksel kültür yöntemi ile 5' inden (20%), İMA yöntemi ile 3' ünden (6%) *Salmonella* cinsi bakteri izole edilmiştir. Gerek geleneksel kültür yöntemi gerekse de İMA yönteminde besiyeri üzerinde üreyen kolonilerden elde edilen izolatlar *Salmonella choleraesuis ssp. arizonae* ve *Salmonella* spp. olarak tanımlanmıştır. İncelenen 50 kıyma örneğinin 37'sinde (74%) ise FISH yöntemi ile *Salmonella* cinsi bakterilerin varlığı tespit edilmiştir. Çalışmamızda *Salmonella* cinsi bakterilerin varlığını belirlemede geleneksel kültür yönteminin, İMA yöntemine göre daha duyarlı bir yöntem olduğu, FISH yönteminin ise *Salmonella* cinsi bakterilerin varlığını belirlemede en iyi yöntem olduğu tespit edilmiştir. Gıda kaynaklı patojenlerle kontaminasyon sonucunda ortaya çıkan uluslararası önemdeki salgınların hızlı tespiti ne kadar önemli olsa da, uygulanan yeni yöntemlerin sonuçlarının geleneksel kültür yöntemi ile desteklenmesi gerekmektedir.

Anahtar sözcükler: *Geleneksel kültür metodu (ISO 6579 referans yöntemi), Floresanlı yerinde hibritleme yöntemi (FISH), İmmünomanyetik ayırma yöntemi, Kıyma, Salmonella*

INTRODUCTION

Foods contaminated with microorganisms like pathogenic bacteria, parasitic helmints and protozoons cause great problems concerning the public health [1]. Another food type which bears great risk regarding the

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public health is meat and meat products. Under normal conditions, meat is sterile; however, it is contaminated according to cutting methods, cleanness of the knife used in the cutting process, water used in the cleaning process and to the preserving conditions and becomes dangerous regarding the microbial growth [2,3].

Salmonella bacteria are generally spread with food, contaminated with feces and are the primary factor for the food infection known as salmonellosis [4]. It is determined that in developed and developing countries, the main portion of salmonellosis cases, which are rated first in food infection and intoxication cases, involves consumption of contaminated animal originated food. For this reason, all stages of food production and consumption must be monitored and the food must be safe. Especially, in developed countries, the presence of *Salmonella* bacteria and serotype distribution in animal products such as minced meat containing the risk group are regularly monitored. Thus, a healthy database about proliferation of bacteria has been produced, and it has shed light on the epidemiological studies [5].

Generally, in order to determine the presence of *Salmonella* bacteria in foods, conventional culture method is used. With this method, results are obtained in nearly one week [6]. In food microbiology, new methods are needed to determine the specific bacteria and to confirm the lifeness of bacteria and known metabolic activities. Conventional culture methods which are used to examine water and food are deprived of the sensibility which is needed in direct determination of food pathogens. For this reason, before verification tests are applied, enrichment cultures are used to increase the number of pathogens which are in low number and identification of bacteria is performed after enrichment process [7].

Immunomagnetic separation (IMS) method is generally used in great efficiency in acquisition of damaged cells after enrichment process or in isolation of specific pathogen bacteria from heterogeneous cell suspensions [7-11]. Although IMS method takes the place of enrichment media, when it is used in combination with pre-enrichment and enrichment media, positive results are increased [12-14]. The first applications of IMS method in food microbiology are known to be performed in Brie (a salty and soft cheese type produced in Northern France), milk powder, yoghurt, meat, and vegetables [10].

For food microbiologists, direct monitoring of microbial populations in food products is an important issue. This is especially important when compared with trad conventional microbiological methods, which give results after a long time. In food samples, pathogen bacteria which are in a very low number might be under stress or be damaged. Therefore, bacteria might not proliferate in selective media, causing false results. Molecular methods like FISH method are used frequently in order to show the presence of viable but nonculturable bacteria [15]. FISH method is a fast and specific tool in determination of complex microbial population in not only food products, but also in environment like soil and mud [15].

In this study, it was aimed that 50 minced meat specimens obtained from 27 butchers and 23 supermarkets in nine different towns in Istanbul were investigated for the presence of *Salmonella* bacteria by conventional culture method (ISO 6579 reference method), immunomagnetic separation method and fluorescent *in situ* hybridization (FISH) method.

MATERIAL and METHODS

Sampling

In the current study, a total of fifty minced meat samples were purchased from a variety of retail outlets and supermarkets. Samples were transported to the laboratory in a refrigerated container and stored at 4°C until examined.

The Detection of Salmonella spp.

Under aseptic conditions, 25 g samples were taken, homogenised in 225 mL of Buffered Peptone Water (HiMedia, India) in a Stomacher (IUL Instrument, Spain) for 1 min and incubated at 37°C for 24 h [16] for pre-enrichment. Each culture was used for further testing with the three methods.

After incubation, three different methods were used to determine *Salmonella* bacteria. These methods were conventional culture (ISO 6579), immunomagnetc separation (IMS) and fluorescent *in situ* hybridization (FISH) methods.

Conventional Culture Method

Conventional culture method procedures for isolation of *Salmonella* were performed according to the International Organization for Standardization (ISO 6579) [13,17]. An aliquot of 0.1 mL BPW pre-enriched samples was inoculated to 9.9 mL Rappaport-Vasiliadis (RV) broth (HiMedia, India) and incubated at 42°C for 24 h [18]. After 24 h incubation, enriched broth (RV broth) was diluted from 10^{-1} to 10^{-4}. 25 µL of enriched broth was streaked on to Salmonella Shigella (SS) agar (HiMedia, India) and brilliant green phenol red lactose sucrose (BPLS) agar (HiMedia, India) and incubated at 37°C for 24 h. Following 24 h incubation, plates were examined for typical colonies, picking at least one colony of each typical colonial type from each of the plates for identification. Colonies of presumptive *Salmonella* are subcultured, and are confirmed by using API 20E. Serological verification of the *Salmonella* bacteria was made by using commercially available test kits (RTA Laboratories, Gebze).

Immunomagnetic Separation Method (IMS)

In the current study, Dynabeads anti-*Salmonella*

conjugates (Dynal®, Norway) and Dynal® MX3 sample mixer were used [19]. Anti-*Salmonella* paramagnetic beads are anti-*Salmonella* antibodies that bound covalently bacteria surface. The immunomagnetic separation was accomplished starting from 1 mL the pre-enrichment broth was transferred to an Eppendorf tube containing 20 µL of the immunomagnetic microbeads coated with anti-*Salmonella* [20,21]. The tubes were shaken on a Dynal® MX3 sample mixer (Dynal®, Norway) at room temperature for 10 min. Following 10 min. incubation, they placed to a magnetic separator (Dynal®, Norway) [18], and the supernatant was removed from the tubes by Pasteur pipette. 1 mL of phosphate buffer saline (PBS) containing 0.05% of Tween 20 was added to the tube containing the microbeads, the tube was shaken on a Dynal® MX3 sample mixer (Dynal®, Norway) at room temperature for another 3 min. The tubes were placed back in a magnetic separator (Dynal®, Norway). This washing process was repeated 3 times. After washing procedure, 0.1 mL PBS was added to sample and sample was diluted from 10^{-1} to 10^{-4}. Aliquots (25 µL) were streaked on SS agar and BPLS agar. After this step, the procedure was continued as described above, the conventional culture method.

Fluorescent in situ Hybridization (FISH) Method

FISH analysis was carried out to identify *Salmonella* spp. using the VIT-*Salmonella* kit (Vermicon, Munich, Germany). FISH method was applied according to the instructions of manufacturer company as follows:

0.1 mL pre-enrichment sample was transferred to 9.9 mL Rappaport Vassiliadis (RV) broth. After incubation at 42°C for 4-6 h, 2 mL sample was centrifuged at 2.700-4.000 g for 5 min. Then supernatant fluid was removed and 4 drops of 'B2 solution' were added on the sediment. Then 5-10 µL of the prepared sample was added to each well of the slide and the slide was dried horizontally at 46°C for 15-30 min. After incubation, one drop 'Solution B2' was added to each well and it was dried again horizontally at 46°C for 15-30 min. The tank was inserted a small way into the VIT-Reactor. 25 drops of 'Solution C6' was placed around in the tank and then the slide it fully into the VIT-Reactor. 1 drop of 'negative control' (brown), 1 drop of 'VIT (Sal)' (green) and 1 drop of 'positive control' (red) were added to wells. The slide was inserted into the VIT-Reactor carefully and it was dried horizontally at 46°C for 90 min. After incubation VIT Reactor was opened and the slide was removed carefully. 'Solution D6' (washing solution) that was diluted twenty-fold with distilled water, was preheated at 46°C for 30 min. VIT-Reactor was filled with the preheated washing solution. The slide was inserted carefully into the VIT-Reactor and it was closed. The drops were let run into each other and it was incubated at 46°C for 15 min. After 15 incubation, VIT-Reactor was opened and slide and washing solution were removed. VIT-Reactor was filled with distilled water and the slide was inserted into the distilled water and then slide was removed immediately. The slide was dried 46°C for 15 min. A small drop of 'Finisher' was placed between the wells on the slide. The slides were examined under epifluorescent green under the blue light (EX-465-495), and also fluorescent red under the green light (EX-510-560) were evaluated as *Salmonella* and photographs were taken.

Determination of Sensitivities of Conventional Culture and Immunomagnetic Separation Methods

The sensitivities of conventional culture (ISO) and immunomagnetic separation (IMS) method, which are used to detect *Salmonella* bacteria, are calculated by using the formula described in Boer and Beumer [22]:

$$\text{Sensitivity} = \frac{\text{Number of positive samples (P)}}{\text{P + Number of negative samples (FN)}} \times 100$$

If *Salmonella* bacteria can be isolated from the sample, P is the number of true positive samples. If *Salmonella* can not be isolated from the sample, N is the number of true negative samples. When *Salmonella* bacteria can be isolated with at least one method, if the method can not isolate *Salmonella* bacteria, false negative is present and shown with FN.

RESULTS

In the current study, *Salmonella* bacteria in minced meat samples were detected by using conventional culture method, IMS and FISH methods. By using conventional culture method, five of fifty minced meat samples showed *Salmonella* bacteria, while IMS method showed three and FISH showed thirty-seven. 12 out of 50 (24%) minced meat samples did not show any presence of *Salmonella* bacteria with three methods used. Only one sample (sample 30) showed *Salmonella* bacteria by using three methods. *Salmonella* bacteria were detected, by FISH method, in 37 of 50 minced meat samples *(Table 1, Fig. 1)*.

As seen in *Table 1*, conventional culture and immunomagnetic separation methods helped isolation of *Salmonella* bacteria in seven samples. According to the formula described in Boer and Beumer [21], the sensivities of the methods used are found to be as 71.4% for conventional culture method, and 42.8% for IMS. Based on the data and calculations given above, conventional culture method was found to be 28.6% more sensitive than immunomagnetic separation method.

DISCUSSION

With conventional culture method, five of fifty minced meat samples showed the presence of *Salmonella* bacteria. Tekinşen et al.[23] reported that no samples out of 20 ready to use minced meat samples sold in supermarkets in Ankara had *Salmonella* bacteria. The reason why *Salmonella* bacteria did not show in these types of food products is thought to be the presence of some organisms

Table 1. *Comparison of methods used* for the isolation of *Salmonella bacteria*
Tablo 1. *Salmonella cinsi bakterilerin tespitinde kullanılan yöntemlerin karşılaştırılması*

Sample Number	Conventional Culture Method	IMS Method	FISH Method	Sample Number	Conventional Culture Method	IMS Method	FISH Method
1	-	-	+	26	-	-	+
2	-	-	+	27	-	-	+
3	-	-	+	28	+	-	+
4	-	+	+	29	+	-	+
5	-	-	+	30	+	+	+
6	-	-	-	31	-	-	+
7	-	-	+	32	-	-	+
8	-	-	+	33	-	-	+
9	-	-	+	34	-	-	+
10	-	-	+	35	-	-	+
11	-	-	+	36	-	-	+
12	-	-	-	37	-	-	-
13	-	-	+	38	-	-	+
14	-	-	+	39	-	+	+
15	-	-	+	40	-	-	-
16	-	-	+	41	-	-	-
17	-	-	+	42	-	-	+
18	+	-	-	43	-	-	-
19	-	-	+	44	-	-	+
20	-	-	+	45	-	-	-
21	+	-	+	46	-	-	-
22	-	-	+	47	-	-	-
23	-	-	-	48	-	-	+
24	-	-	+	49	-	-	-
25	-	-	+	50	-	-	-

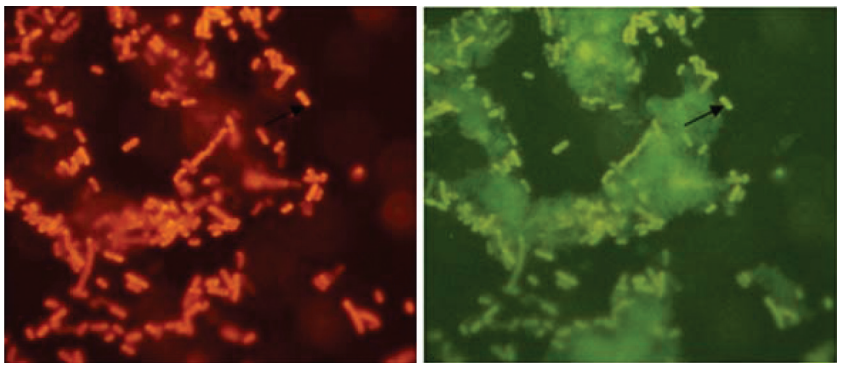

Fig 1. *Salmonella* positive sample by FISH method

Şekil 1. FISH yöntemiyle *Salmonella* pozitif örnek

producing lactic acid, which hampers the production of Gram-negative bacteria dramatically [24]. In another study by Sarıgöl [25], one of twenty minced meat samples obtained from butchers in Elazığ showed the presence of *Salmonella* bacteria, and another study by Gökalp et al.[26] yielded that one of forty-eight samples in Erzurum (obtained from butchers and Meat and Fish Institution) had *Salmonella* bacteria. Aabo et al.[27] found *Salmonella* bacteria in only one specimen out of forty-eight cattle minced meat samples, by using the conventional culture method. Erol [5] reported the isolation of *Salmonella* bacteria from only four of one-hundred-and-twenty samples (3.3%).

Detailed literature surveys showed that the presence of *Salmonella* bacteria examined by IMS method is generally found in chicken [18] and pork, cacao, powdered milk and sausage [8]; however, minced meat samples are found to be investigated in a very low number of publications. In the current study, as an alternative to the conventional culture method, IMS method showed that only three samples out of fifty samples examined had *Salmonella* bacteria. Cudjoe and Krona [13] investigated the presence of *Salmonella* bacteria by conventional culture and IMS methods, and they reported that the cultures obtained with conventional culture method had a more dense growth of competitive

flora. For this reason, it was suggested that the use of IMS method can prevent largely the competitive flora development and this will speed up the selection of suspicious *Salmonella* colonies from selective solid media [13,28]. Although some researchers [13,29] reported that IMS method gave better results than conventional culture method, our study shows that conventional culture method is more effective than IMS method. Jeníková et al.[4] published an article which investigated the use of IMS method for detecting the presence of *Salmonella* bacteria in minced meat samples, and reported that foods with high fat content were not suitable for IMS. They also reported that in a food like minced meat, which has a high fat content, magnetic beads will be lost in food matrix and therefore cannot be acquired by magnetic field, so IMS method is not successful. In such a case, because of competitive microflora (especially, bacteria of *Enterobacteriaceae* family) yield cross-reactions and other bacteria than *Salmonella* can bind to the anti-*Salmonella* Dynabeads. These factors lead to the decrease in sensitivity for IMS method, used to detect *Salmonella* bacteria [4]. Some researchers conclude this failure as both immunomagnetic particles are lost in meat with high fat content and as not using a medium with better selectivity [12,16]. In a study published by Mercanoğlu et al.[20], it was determined that anti-*Salmonella* globules could capture *Enterobacter aerogenes, Escherichia coli* and *Klebsiella pneumoniae* bacteria. It was observed that the bindings which are not specific to anti-*Salmonella* globules of these bacteria did not prevent *Salmonella* bacteria to bind to globules and after IMS application, different type of bacteria can develop with these bacteria in selective solid media [19,30]. In addition, it is thought that the cross reactions might be caused from bacterial strain and environmental conditions [31]. Our study supported the study carried out by Jenikova et al.[4] in which cross-reactions caused by competitive microflora was detected. After the use of IMS method, on the selective medium, *Salmonella* bacteria along with bacteria belonging to *Enterobacteriaceae* family such as *Proteus vulgaris, Proteus mirabilis, Citrobacter freundii, Citrobacter youngea* and *E. coli* were found. In our study, on the contrary to the study by Mercanoğlu et al.[20], as a result of cultivation on BPLS agar after IMS application, it was determined that anti-*Salmonella* globules captured *P. vulgaris* and growth of this bacteria determined on the medium. With conventional culture method and IMS method, it is thought that isolated erroneous positive strains like *C. freundii, Citrobacter brakii, C. youngeae, P. vulgaris, P. mirabilis, Morganella morganii,* and *Hafnia alvei* suppress the growth of *Salmonella* bacteria. Especially, it is considered that bacteria out of *Salmonella*, captured by magnetic particles by IMS method, cause diminisment of the sensitivity of the method.

With IMS method, it is reported that when detergents like Tween 20 or protamin are added to PBS solution, which is used as a washing solution in IMS method, there are reductions in the numbers of non-specific bindings [19,32,33]. In our study, it was found that Tween 20, added in a concentration of 0.05 mL/100 mL, to the washing solution (PBS solution) in IMS method could not prevent cross reactions and that along with *Salmonella* bacteria, other bacteria belonging to the *Enterobacteriaceae* family could grow in the medium. With IMS method, another reason for low recovery percentages of *Salmonella* bacteria might be the first washing procedure which might remove *Salmonella* bacteria with other bacteria [19,30,33]. If the number of *Salmonella* bacteria is greater than anti-*Salmonella* globules, *Salmonella* bacteria might not be captured by globules and with the first washing procedure, bacteria which did not bind to the globules might be washed out. However, even if there is only one *Salmonella* bacterium in the sample, this bacterium can be captured by anti-*Salmonella* globule. Literature survey shows that there is a few number of publications about investigation of *Salmonella* bacteria in minced meat. Erol [5] reported that *Salmonella anatum, Salmonella telaviv, Salmonella typhimurium* were isolated from minced meat samples, whereas Fratamico [34] reports that *Salmonella cerro, S. typhimurium, S. anatum* and *Salmonella infantis* were isolated from minced meat samples. In the study by Gökmen and Alişarlı [35], *Salmonella* spp. were isolated from minced meat samples in Van. In our study, conventional culture method yielded *Salmonella* spp. and *S. choleraesuis ssp. arizonae* to be isolated in five of fifty samples.

In our study, the presence of *Salmonella* bacteria was searched by conventional, IMS, and also by FISH method. Our study showed that conventional culture method yielded *Salmonella* bacteria in 5 samples out of 50 total while FISH method found this bacteria in 37 samples out of 50 total. Literature survey showed that with FISH method, *Salmonella* bacteria were searched generally in pork [17,36]. In a publication by Vieira - Pinto et al.[36], 16 out of 47 samples yielded the presence of *Salmonella*-type bacteria in pork samples. In another study it was determined that FISH method gave better results than conventional culture method [17]. The reason why FISH method gives better results than conventional culture method is considered to be due to the presence of actually dead or viable but non-culturable *Salmonella* bacteria for several stress factors [17,37]. In FISH method, these viable but non-culturable cells can show metabolic activities with their ribozomes in low number. However, FISH method is not affected from physical and chemical properties of food (temperature, salt concentration and pH), which cause *Salmonella* cells to undergo stress and cause bacteria not to be cultured. FISH method is more advantageous because a) it is not affected from inhibitory factors, b) it uses less material than conventional culture method and c) it is faster.

FISH method can detect *Salmonella* bacteria among high number of competitive microflora. However, in sample18, conventional culture method was successful in detecting

Salmonella bacteria but FISH method failed. The possible cause being the presence of a low amount of bacteria within the scanned area by microscope [40]. In addition, erroneous negative result might be due to experimental errors.

Time is a very important factor in detecting the pathogens in foods and for this reason, it is needed to use new methods which can give results in a short time. However, fast detection of epidemics due to food contaminated with pathogens is important, but sources of bacteria and obtaining bacterial isolates are more important. For this reason, the results obtained with new methods in short time must be compared with and supported by the gold standard, that is, conventional culture method.

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RESEARCH ARTICLE

Evaluation of *Smilax excelsa* L. Use in Experimentally Induced Nephrotoxicity [1]

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Summary

The protective effect of an aqueous extract of the shoots and leaves of *Smilax excelsa* L. against acute carbon tetrachloride (CCl_4)-induced toxicity and the changes in antioxidative defense activities in kidney of rats were investigated. Female Wistar rats were supplied with *S. excelsa* shoots and leaves aqueous extract once a day for 9 days (orally at a dose of 100, 200 and 400 mg/kg of body weight) prior to renal injury induction through intraperitoneal injection with a single dose of CCl_4 (1 ml/kg body wt, in a 20 % v/v olive oil solution) on the 10th day. 24 h after CCl_4 intoxication serum and tissue biochemical and hispathological analyses were undertaken after sacrification under anesthesia. Administration of the extract reversed the antioxidant parameters which were impaired in CCl_4 group, in a dose dependent manner and at a dose of 400 mg/kg of body weight the levels of almost all the parameters were almost back to normal Control group. Nevertheless, the extract did not completely improve the CCl_4-induced degenerative changes observed microscopically in kidney tissue. The results of this study suggest that *S. excelsa* could protect the kidney tissue against CCl_4-induced nephrotoxicity in rats, probably by increasing antioxidative defense activities.

Keywords: *Smilax excelsa, Renal damage, Carbon tetrachloride, Antioxidant effect, Nephrotoxicity*

Deneysel Olarak Oluşturulmuş Böbrek Hasarı Üzerine *Smilax excelsa* L. Kullanımının Değerlendirilmesi

Özet

Bu çalışmada *Smilax excelsa* L.'nın genç sürgün ve yapraklarının sulu ekstresinin sıçanlarda karbon tetraklorür (CCl_4) ile deneysel olarak oluşturulmuş böbrek hasarına olan etkisi ve antioksidan savunma sistemindeki değişiklikleri incelendi. Wistar albino dişi sıçanlara, 9 gün süre ile, *S. excelsa* extresinin 100, 200 ve 400 mg/kg dozunda oral olarak uygulanmasının ardından, 10. gününde CCl_4'ün %20'lik zeytin yağındaki solüsyonunun 1ml/kg intraperitoneal verilmesiyle böbrek harabiyeti oluşturuldu. CCl_4 uygulanmasından 24 saat sonra, sıçanlar anestezi altında öldürüldü, ardından serum ve böbrek dokusunda biyokimyasal ve histopatolojik analizler yapıldı. CCl_4 grubunda bozulan tüm değerlerin, ekstrenin verilmesiyle doza bağlı olarak düzeldiği ve 400 mg/kg dozunda aşağı yukarı normal Kontrol grubu değerlerine ulaştığı gözlemlendi. Buna karşın, ekstre CCl_4 ile oluşturulan, böbrek dokusunda mikroskobik olarak gözlenen dejeneratif değişiklikleri tam olarak iyileştirmedi. Bu çalışmanın sonucunda, *S. excelsa*'nın sıçanlarda böbrek dokusunu CCl_4 ile oluşturulan hasardan koruduğu ve bu etkisini organizmanın antioksidan savunma sistemlerini arttırarak gösterdiği ileri sürülebilir.

Anahtar sözcükler: *Smilax excelsa, Böbrek hasarı, Karbon tetraklorür, Antioksidan etki, Nefrotoksisite*

INTRODUCTION

Traditional medicines and extracts from medicinal plants have been extensively used as alternative medicine for better control and management of kidney diseases.

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Plants from the genus *Smilax* are used to treat syphilis, acute bacillary dysentery, acute and chronic nephritis, eczema, dermatitis, cystitis, and mercury and silver poisoning [1]. Rhizomes of several *Smilax* species from this genus possess a variety of bioactivities including anti-cancer [2,3], anticonvulsant [4], antiinflammatory and anti-nociceptive [5-7], hepatoprotective [8-10], antihyperuricemic and nephroprotective [11] actions. A survey of the literature showed that several Smilax species contain phenyl-propanoid glycosides [12], anthocyanins [13], flavonoid glucosides [2,14] and steroid saponins [15,16]. The antioxidant potential of *Smilax* species has predominantly been derived from *in vitro* and *in vivo* studies on the rhizomes of the plant [17-19].

Smilax excelsa L. (sarsaparilla, *Liliaceae*) is a climbing scrub up to 20 m, known as "Melocan, Melvocan, Silcan, Diken otu, Mamula (Rize), Melevcen, Sıraca (Mersin), Kırçan and Çıtırgı" in Turkey. It occurs in the deciduous forests, the scrubs and the roadsides. The shoots of the plant are consumed as vegetables [20] and it is known as a medical and economic plant, as well [21]. *Smilax excelsa* is used in folk medicine for the treatment of breast cancer, stomach pain and bloating [22]. The plant used here has no scientific proof of its use in renal disorders.

As the evidence of earlier studies shows that the leaves and shoots of *Smilax excelsa* possess flavonoids and anthocyanins, which are the major chemical constituents responsible for exhibiting antioxidant activity [23] the present study has been undertaken to evaluate the protective effects of water extract of the shoots and leaves of *Smilax excelsa* in kidney tissues in CCl_4-induced oxidative stress.

MATERIAL and METHODS

Plant Material

S. excelsa L. shoots and leaves were collected in September from Istanbul, Turkey and identified by Prof. Dr. Kerim Alpinar from the Faculty of Pharmacy, Istanbul University. The shoots and leaves were separated from the other parts, washed in running tap water and dried at room temperature. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Istanbul University (ISTE); Herbarium code number: ISTE 81928. The dried leaves were manually ground to a fine powder before extraction.

Preparation of the Extract

80 g of ground dried shoots and leaves were extracted with boiling water (2.000 ml) for 15 min while stirring. The extracts were filtered and evaporated to dryness under reduced pressure at 40°C. The yield was 25 g. Appropriate dilutions were made before each experiment.

Animals

The experimental protocol described in the present study was approved on 19.06.2007 by the Animal Assays Ethics Comittee of Istanbul University. Female Wistar rats weighing 200-240 g were supplied from Istanbul University, Institute of Experimental Medicine (DETAE). Animals were acclimatized to their environment for one week prior to experimentation. The animals were housed in a room with a 12 h light/dark cycle at about 22°C and fed on standard diet with *ad libitum* access to drinking water.

Induction of Renal Injury

The animals were divided into 5 groups each containing 6 animals. The treatment was as follows:

Group 1 (Control), which served as normal control, received water and basal diet for 10 days.

Group 2 (CCl_4) which served as toxin control, received water and basal diet for 10 days and was treated i.p. with CCl_4 (1 ml/kg body weight in 20% olive oil, v/v) on the 10th day.

Group 3, 4 and 5 ($S_{100+CCl_4}$, $S_{200+CCl_4}$ and $S_{400+CCl_4}$) were separately treated with 100, 200 and 400 mg/kg body weight of the extract of *S. excelsa* shoots and leaves once daily for 9 days 24 h prior to CCl_4 intraperitoneal administration on the 10th day.

24 h after CCl_4 intoxication, the rats were lightly anesthetized and sacrificed. Blood samples were taken from each rat through direct intracardiac intervention and centrifuged at 3.000 x g for 10 min to separate the sera. Urea, creatinine levels, paraoxonase/aryl esterase (ARE) activity and the extent of LPO measured as MDA were determined in serum. Immediately after collecting the blood samples, the kidneys were excised, rinsed in ice-cold normal saline solution followed by ice-cold 0.15 M potassium phosphate buffer, pH 7.4, blotted, dried, and weighed. Part of kidney tissues were used for histopathological examination. With another part of kidney tissues, 10% w/v homogenates were prepared in ice-cold 0.15 M potassium phosphate buffer using Art-MICCRA D-1 homogenator and centrifuged at 13.000 rpm for 5 min at 4°C (Megafuge Hereaus 1.0R). The supernatants, thus obtained were used for the estimation of antioxidant parameters like catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), myelo-peroxidase (MPO). Lipid peroxidation (LPO) reduced (GSH) and oxidized (GSSG) glutathione, GSSG/GSH, protein carbonyl content (PCC), carbonic anhydrase (CA) activity, were also estimated. All biochemical assays were done in triplicate using different homogenates. Serum and kidney samples were aliquoted and stored in a freezer (-80°C) for use in biochemical analyses.

Biochemical Assays

Shimadzu Spectrophotometer UV-(1800) was used for

all spectrophotometric measurements.

Assessment of serum biochemical parameters: Serum creatinine and urea levels were evaluated by Jaffe reaction [24], and diacetylmonooxime method [25], respectively.

Serum samples were assayed for paraoxonase/arylesterase activity using 1.0 mM phenylacetate as substrate and 0.9 mM $CaCl_2$ in 20 mM Tris/HCl, pH 8.0. The reaction was initiated by the addition of the serum sample, and the increase in the absorbance at 270 nm was recorded over a 90-s period. Enzymatic activity was calculated from the molar extinction coefficient 0.00131 $mM^{-1}cm^{-1}$. A unit of arylesterase activity was defined as 1µmol phenylacetate hydrolyzed per min under the above assay conditions [26].

Assessment of antioxidant status via antioxidant enzymes in kidney homogenates: CAT activity was measured by the ethod of Aebi [27] and expressed as mmol H_2O_2/mg protein.

SOD activity was assayed by the method described by Aruoma et al.[28]. Results were expressed as U/mg protein. One unit of SOD inhibits the rate of increase in absorbance at 560 nm by 50% under the conditions of the assay.

The activity of glutathione peroxidase (GPx) was measured using a coupled enzyme assay system linked with glutathione reductase (GR) as described by Lawrence and Burk [29].

GR activity was determined by following the oxidation of NADPH at 340 nm as described by Carlberg and Mannervik [30]. GR and GPx activities were expressed as mmol NADPH oxidized/min/mg protein using the molar extinction coefficient for NADPH at 340 nm of 6.22 mM^{-1}/ cm^{-1}.

GST activity using 1-chloro-2,4-dinitrobenzene as substrate was assayed spectrophotometrically as described by Habig and Jakoby [31]. Specific activity was expressed as mmol conjugate formed/min/mg protein using a molar extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Tissue MPO levels were measured according to Hillegass et al.[32]. Results were expressed as units of MPO per gram of protein of supernatant as determined by method of Lowry.

The formation of LPO products was assayed by the measurement of thiobarbituric acid reactive substances (TBARS) levels on the basis of MDA reaction with thiobarbituric acid at 532 nm according to Buege and Aust [33]. The values of TBARS were calculated using an extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} and expressed as nmol of MDA/g wet weight.

The levels of GSH and GSSG were measured in kidney tissue by the enzymic recycling procedure using 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB) and glutathione reductase (DTNB-GSSG reductase recycling assay) as described by Anderson [34].

The PCC was assayed by the modification of the procedure described by Reznick & Packer [35], using dinitrophenylhydrazine (DNPH) dissolved in HCl, accompanied by blanks in HCl alone. Results were expressed as nmol of protein carbonyl per mg of protein (determined on the HCl blank pellets using a BSA standard curve in 6 M guanidine-HCl and reading the absorbance at 280 nm) using a molar extinction coefficient of 22.000 $M^{-1}cm^{-1}$ for DNPH.

The *p*-nitrophenylacetate esterase activity of CA was measured by the method of Verpoorte et al.[36]. One unit of enzyme activity was expressed as mmol nitrophenol formed per minute at 0°C using a molar extinction coefficient of 5 mM^{-1}/ cm^{-1}.

Histopathological Assay

Dissected kidney tissues were taken immediately and fixed in Bouin's solution for histopathological examinations. The tissues were dehydrated and embedded in paraffin. The paraffin sections of 5 µm thickness were stained with Masson's trichrome stain (Masson) and Periodic Acid-Schiff stain (PAS). All sections were examined under Olympus-CX 41 light microscope.

Statistical Analysis

Biochemical results were evaluated using an unpaired *t*-test and ANOVA variance analysis using the NCSS statistical computer package. The values were expressed as mean ± SD. Analysis between control and experimental groups was performed using the Mann-Whitney test. $P<0.05$ was considered as significant.

RESULTS

Assessment of Serum Biochemical Parameters

Serum urea levels were significantly raised in the CCl_4 group compared to the control group ($P<0.005$). Treatment with *S. excelsa* leaf extract at 400 mg/kg dose only, restored urea levels to control values ($P_{ANOVA}=0.0001$). Thus, pretreatment of rats with aqueous extract of *S. excelsa* attenuated the CCl_4-induced rise in serum urea level confirming the protective effect of the extract on serum kidney parameters. Nevertheless no significant change in serum creatinine levels was observed ($P_{ANOVA}=0.487$; *Table 1*).

The serum TBARS (expressed as MDA) concentration in the CCl_4 treated group was significantly higher ($P<0.0001$) than that of the normal controls. Treatment with *S. excelsa* aqueous extract with 100, 200 and 400 mg/kg doses, prior to the CCl_4 administration decreased the enchanced MDA level significantly ($P<0.005$, $P<0.05$ and $P<0.0001$ respectively compared to CCl_4 group). Reduction in the levels of MDA in the group treated with 400 mg/kg aqueous extract was almost close to normal group ($P_{ANOVA}=0.0001$; *Table 1*).

CCl_4 did not produce a significant change in serum ARE levels *(Table 1)*. Whereas treatment with *S. excelsa* extracts at 200 (P<0.005 *versus* Control group) and 400 mg/kg (P<0.05 *versus* Control group) increased significantly serum ARE activity (P_{ANOVA}=0.001).

Assessment of Antioxidant Status in Kidney Homogenates

Antioxidant enzyme activities (CAT, SOD, GPx, GR, GST and MPO) in kidney of Control and tested groups are shown in *Table 2*.

No significant change in CAT activity of all the experimental groups (P>0.05) was recorded (P_{ANOVA}=0.647).

SOD activity was significantly decreased in the CCl_4 group (P<0.05 *versus* Control group), administration of the extracts at the three doses reversed this effect (P_{ANOVA}= 0.002).

Although no significant difference was seen in the CCl_4 group, the activities of GPx, GR, and GST were significantly enhanced by pre-treatment of CCl_4-intoxicated rats with *S. excelsa* extracts at 100 mg/kg, 200 mg/kg, 400 mg/kg doses compared with those of CCl_4 group (P_{ANOVA}=0.0001, 0.002, 0.008 respectively).

In the control CCl_4 treated group and the group pretreated with 100, 200 and 400 mg/kg aqueous extract of *S. excelsa* renal MPO levels were significantly (P<0.05) higher than those of the normal Control group. A significant (P<0.05) reduction was observed in the group treated with 400 mg/kg of aqueous extract of *Smilax excelsa* in comparison with those observed in the control CCl_4 treated group and the group pretreated with 100 and 200 mg/kg. However, MPO levels remained greater than control (P_{ANOVA}=0.011; *Table 2*).

Table 1. *Effect of pretreatment with Smilax excelsa L. aqueous extract after CCl_4 intoxication, on serum urea, creatinine and MDA levels and aryl esterase (ARE) activity in rats*

Tablo 1. *Sıçanlarda CCl_4 ile oluşturulmuş böbrek hasarında Smilax excelsa L. sulu ekstresinin serum üre, kreatinin ve MDA düzeyleri ile arilesteraz (ARE) aktivitesi üzerine etkisi*

Group	Urea mg/dl	Creatinine mg/dl	MDA mmol/l	ARE kU/l
Control	25.2±4.3	0.76±0.4	1.4±0.3	29.6±9.4
CCl_4	44.2±13.5[a]	1.00±0.4	3.4±0.4[d]	29.7±19.4
$S_{100+CCl_4}$	43.3±11.4[a]	0.78±0.4	2.5±0.4[d,e]	36.1±27.6
$S_{200+CCl_4}$	44,3±9.3[b]	0.81±0.2	2.7±0.6[c,e]	79.7±25.2[a,g]
$S_{400+CCl_4}$	28.6±7.9[c]	0.77±0.2	1.6±0.4[f]	64.3±21.8[c,e]
P_{ANOVA}	0.000	0.487	0.0001	0.001

[a] *P<0.005 vs. Control;* [b] *P<0.001 vs. Control;* [c] *P<0.05 vs. CCl_4;* [d] *P<0.0001 vs. Control;* [e] *P<0.05 vs. Control;* [f] *P<0.0001 vs. CCl_4;* [g] *P<0.005 vs. CCl_4*

Table 2. *Effect of pretreatment with Smilax excelsa L. aqueous extract after CCl_4 intoxication, on activies of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) and myeloperoxidase (MPO) in kidney tissue of rats*

Tablo 2. *Sıçanlarda CCl_4 ile oluşturulmuş böbrek dokusu hasarında Smilax excelsa L. sulu ekstresinin katalaz (CAT), süperoksit dismutaz (SOD), glutatyon peroksidaz (GPx), glutatyon redüktaz (GR), glutatyon peroksidaz (GST) ve miyeloperoksidaz (MPO)aktiviteleri üzerine etkisi*

Group	CAT U/mg protein	SOD U/mg protein	GPx U/mg protein	GR U/mg protein	GST U/mg protein	MPO U/g tissue
Control	81.7±14.8	2.5±0.2	523.9±71.6	90.6±14.6	92.1±23.4	0.18±0.1
CCl_4	76.9±14.1	2.1±0.3[a]	553.7±31.8	86.0±10.0	89.2±24.9	0.99±0.5[d]
$S_{100+CCl_4}$	88.3±10.6	2.6±0.3[b]	723.8±53.9[c]	106.0±27.0	124.8±9.3[a]	0.76±0.2[c]
$S_{200+CCl_4}$	85.0±13.9	3.0±0.5[b]	759.3±97.5[d]	124.0±24.3[a]	133.9±37.5[a]	1.22±0.3[c]
$S_{400+CCl_4}$	86.0±14.2	2.5±0.0[b]	759.5±23.5[c]	124.4±11.1[d]	128.7±20.5[a]	0.50±0.1[c,b]
P_{ANOVA}	0.647	0.002	0.0001	0.002	0.008	0.0001

[a] *P< 0.0 5 vs. Control;* [b] *P< 0.05 vs. CCl_4;* [c] *P< 0.001 vs Control;* [d] *P< 0.005 vs. Control*

Table 3. *Effect of pretreatment with Smilax excelsa L. aqueous extract after CCl_4 intoxication, on lipid peroxidation (LPO), reduced (GSH) and oxidized glutathione (GSSG) levels, GSSG/GSH ratio, protein carbonyl content (PCC), carbonic anhydrase (CA) activity in kidney tissue of rats.*

Tablo 3. *Sıçanlarda CCl_4 ile oluşturulmuş böbrek dokusu toksisinde Smilax excelsa L. sulu ekstresinin lipit peroksidasyonu (LPO), indirgenmiş (GSH) ve oksitlenmiş (GSSG) glutatyon düzeyleri, GSSG/GSH oranı, protein karbonil miktarı (PCC), ve karbonik anhidraz (CA) aktivitesi üzerine etkisi.*

Group	LPO nmol MDA/g tissue	GSH mmol/g tissue	GSSG mmol/g tissue	GSSG/ GSH	PCC nmol/mg protein	CA U/mg protein
Control	24.1±7.2	0.58±0.1	0.05±0.01	0.10±0.01	1.1±0.4	168.8±24.5
CCl_4	38.6±9.4[a]	0.31±0.1[b]	0.06±0.04	0.18±0.07[a]	2.9±0.3[f]	230.3±21.7[b]
$S_{100+CCl_4}$	36.0±9.5[a]	0.58±0.1[d]	0.05±0.03	0.11±0.02[c]	2.9±1.4[b]	216.7±29.1[a]
$S_{200+CCl_4}$	38.9±8.4[b]	0.53±0.1[e]	0.09±0.03[a]	0.18±0.08[a]	2.6±1.7[a]	238.3±24.6[b]
$S_{400+CCl_4}$	27.1±6.9[c]	0.67±0.2[e]	0.04±0.01	0.09±0.01[c]	1.5±0.6[g]	176.5±22.0[e]
P_{ANOVA}	0.011	0.003	0.046	0.004	0.002	0.0001

[a] *P< 0.05 vs. Control;* [b] *P< 0.005 vs. Control;* [c] *P< 0.05 vs. CCl_4;* [d] *P< 0.001 vs CCl_4;* [e] *P< 0.005 vs CCl_4;* [f] *P< 0.0001 vs. Control;* [g] *P< 0.0001 vs CCl_4*

The content of GSH was significantly decreased in the CCl_4-treated rats compared with normal control (P<0.005). In the group of rats pretreated with 100, 200 and 400 mg/kg aqueous extract of *S. excelsa*, GSH levels were comparable to those of normal Control group (P>0.05). The differences between all the groups was found significant (P_{ANOVA}=0.003). There was no significant difference between the kidney GSSG levels of all the experimental groups (P_{ANOVA}=0.046; *Table 3*). The GSSG/GSH ratio which was significantly increased in the CCl_4 group (P<0.05 versus Control group), was decreased to normal Control group levels by administration of *S. excelsa* extract at 400 mg/kg dose (P_{ANOVA}=0.004; *Table 3*).

The significant raise in the PCC content, in the kidney of CCl_4 treated group was only significantly reduced with pretreatment with *S. excelsa* leaf extract at 400 mg/kg (P<0.05 *versus* CCl_4 group), thus restoring it back to the controls range (P_{ANOVA}=0.002; *Table 3*).

The CA activity in the CCl_4 treated group and the group pretreated with 100 and 200 mg/kg aqueous extract of *S. excelsa* was also observed to be highly significant (P<0.005, P<0.05 and P<0.005 respectively) as compared to that in the normal Control group. Pretreatment with 400 mg/kg *S. excelsa* brought the activity near to that of the Control rats (P<0.05 *versus* CCl_4 group; P_{ANOVA}=0.0001; *Table 3*).

Histopathological Assessment

Under light microscope, the cortex in the kidneys of Control group animals has a large number of glomeruli that proximal and distal tubules located around them *(Fig. 1 A)*. CCl_4 caused moderate degenerative changes primarily in proximal tubules, in addition to less in distal tubules in the renal tissues of rats. The renal injury induced by CCl_4 consisted of histopathological changes such as cytoplasmic debris and desquamated nuclei in the widened lumens of proximal and distal tubules, shortening and rupturing at the brush border of proximal tubular cells, vacuolisation and hypertrophy in the proximal and distal tubular cells, hyperemia in the interstitial vascular areas. Besides these histopathological changes, necrotic areas were observed in the kidney tissues of some animals given CCl_4 *(Fig. 1 B)*. However, the degenerative changes were partially apparent in the kidneys of rats received *Smilax excelsa* extract at the 100 mg/kg *(Fig. 1 C)*, 200 mg/kg *(Fig. 1 D)* and 400 mg/kg *(Fig 1. E)* dose together with CCl_4. PAS positive reaction in brush border and basal membrane of proximal tubular cells in the kidney tissue of all groups were similar.

DISCUSSION

In recent years, the search for herbal and natural drugs with antioxidant activity has gained importance as the

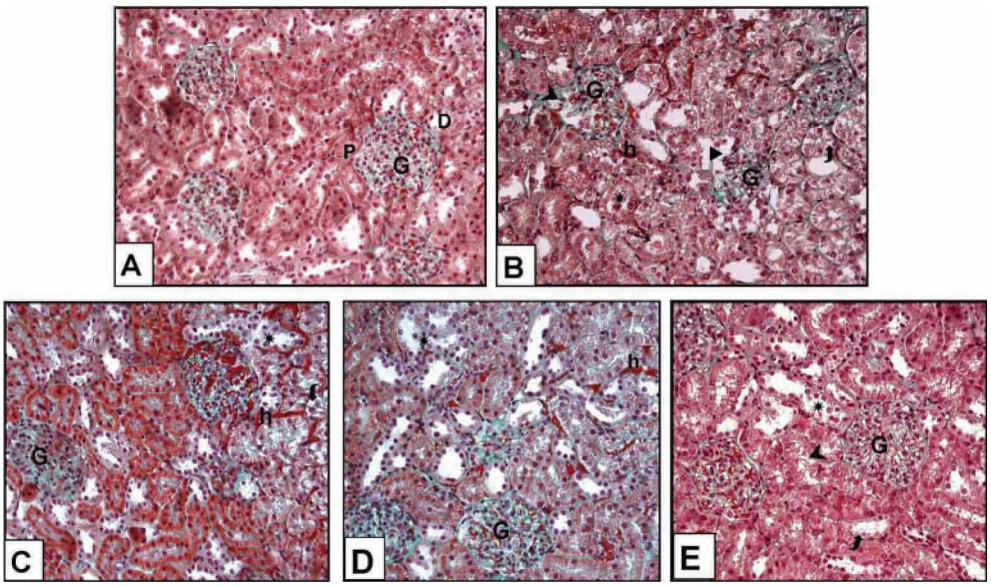

Fig 1. Light microscopic appearance of kidney tissue of a control animal (A). The kidney section of rats given CCl_4 (B), light micrographs of *S. excelsa* (100 mg/kg)-treated CCl_4 group (C), of *S. excelsa* (200 mg/kg)-treated CCl_4 group (D), and of *S. excelsa* (400 mg/kg)-treated CCl_4 group (E). Proximal tubule (P), distal tubule (D) and glomerulus (G). Cytoplasmic debris and desquamated nuclei (✶) in the widened lumens of proximal and distal tubules, shortening (→) and the rupturing (➤) at the brush border of proximal tubular cells, hypertrophy (➥) in tubular cells, hyperemia (h), necrotic area (▶). Masson. Original magnification x400

Şekil 1. Kontrol hayvanın böbrek dokusunun ışık mikroskobik görünümü (A). CCl_4 verilen sıçana ait böbrek kesiti (B), *S. excelsa* (100 mg/kg) verilen CCl_4 grubuna ait (C), *S. excelsa* (200 mg/kg) verilen CCl_4 grubuna ait (D) ve *S. excelsa* (400 mg/kg) verilen CCl_4 grubuna ait (E) ışık mikrografları. Proksimal tübül (P), distal tübül (D) ve glomerulus (G). Proksimal ve distal tübüllerin genişlemiş lümeninde sitoplazma artıkları ve dökülmüş nukleuslar (✶), proksimal tübül hücrelerinin fırça kenarlarında kısalma (→) ve kopma (➤), tübül hücrelerinde hipertrofi (➥), hiperemi (h), nekrotik alan (▶). Masson. Orijinal büyütme x400

dietary intake of antioxidants obtained from natural sources is considered to be relatively safe and involves no side effects. *Smilax excelsa* shoots and leaves constitutes a particularly interesting source of biologically active phytochemicals as it contains a variety of phenolic compounds with substantial *in vitro* antioxidant activity [1]. However, until recently, there have been few studies about the pharmacological effects of the leaves of *Smilax excelsa*. *In vitro* antioxidant properties of leaf extracts were shown in one study on *Smilax china* [37] after our research [23]. Nephroptotective activity of extracts obtained from *S. china* rhizomes in hyperuricemic animals was demonstrated in a recent study without histopathological observation [38]. Despite the many beneficial biological properties of *Smilax excelsa*, its protective effect against CCl_4 nephrotoxicity has not so far been explored. The current study was aimed at identifying biochemical and renal histopathological abnormalities that occur with the evolution of nephrotoxicity in rats and to appreciate their possible reversal after the treatment with the water extract of *Smilax excelsa* shoots and leaves.

Nephrotoxicity is one of the most common kidney problems and occurs when body is exposed to drugs or chemical reagents. Several medicinal plants are reviewed for their nephroprotective activities [39]. CCl_4 is commonly used in rat experimental models to investigate the oxidative stress induced in various organs. In addition to its hepatic toxicity, it was reported that CCl_4 also causes disorders in kidneys.

The elevation in blood urea, and the observed histopathological alterations recorded in this work indicated that CCl_4 caused moderate impairment in renal function along with significant oxidative stress in the kidneys which is consistent with other studies [40].

Because, generally, the effect of CCl_4 is observed after 24 h of its administration and withdrawal of blood and excising of kidneys were carried out after 24 h of CCl_4 intoxication, serum creatinine levels remained normal 24 h after the induction of renal toxicity in rats, which is in accordance with the observation reported by Tirkey at al.[41] who failed to observe any increase in BUN nor serum creatinine levels after CCl_4 administration.

Our histopathological findings were in agreement with the degenerative structural changes reported to occur in kidney tissues after the application of CCl_4 [42,43]. Our results showed that CCl_4 leads to moderate degenerative changes mainly necrosis, hyperemia, and vacuolar degeneration in the kidney of rats. It was observed that pretreatment with the aqueous extracts of *Smilax excelsa* leaves did not completely diminish CCl_4-induced degenerative injury in kidney tissue, morphologically. Thus, the improvement in antioxidant enzymes system seem to have been incapable of neutralizing increased CCl_4 toxicity seen in the histopathology of kidney cells.

There was a significant increase in serum MDA levels, which is an indirect measure of lipid peroxidation that suggests the posibility of enhanced production of free radicals as reported by Manna et al.[40] and Tirkey at al.[41]. In recent years paraoxonase/arylesterase activities have been used as oxidative stress markers. It has been suggested that they may play a protective role under oxidative stress. Paraoxonase was found to use efficiently not only lipoprotein-associated peroxides (including cholesteryl linoleate hydro-peroxides), but also hydrogen peroxide (H_2O_2) [44]. In this study the induction of paraoxonase/arylesterase activity may merely be a manifest of antioxidant response to the increased oxidative stress. It was reported that flavonoids can act as potent inhibitors of LDL oxidation via preserving or increasing serum paraoxonase/arylesterase activity thus promoting hydrolisis of LDL-associated lipid peroxides [45]. The presence of high quantity of flavonoids in *Smilax excelsa* leaves could be responsible for the protective effect against the oxidative stress of CCl_4 in kidneys of rat.

CAT, SOD, GR, GPx, GST and GSH were evaluated as an index of antioxidant status of kidney tissues. SOD generates hydrogen peroxide as a metabolite, which must be scavenged by catalase or GPx. In this study the small degree of changes in renal CAT and SOD activities did not attain statistical significance between CCl_4 treated control and groups pretreated with *S. excelsa*. May be CAT and SOD are easily inactivated by lipid peroxides, which are scavenged by GPx. GPx faciliate the conjugation of hydrogen peroxide to reduced glutathione (GSH) leading to generation of water and oxidized glutathione (GSSG), which is then reduced to GSH by the NADPH-dependent GR. The increase in GR and GPx activities may be due to the elevated GSH content. Some of the changes in glutathione antioxidant system may possibly reflect an inter-organ antioxidant response to a generalized increase in tissue oxidative stress associated with intoxication, possibly through the export of hepatic glutathione for the subsequent uptake by extrahepatic tissues. The absence of a change in the GSSG/GSH ratio, a potent inhibitor of tissue oxidative stress in the group pre-treated with 400 mg kg of *S. excelsa* leaves is in accordance with the suggestion that free radical damage is attenuated. The increase in GST activity which is involved in the detoxification of the lipid peroxidation products may also be an early adaptive response to oxidative stress. It may be concluded that administration of *Smilax excelsa* leaves protected the antioxidant status of the kidney as revealed by the enhanced level of GR, GPx, GST and GSH in this experiment. Earlier studies have also shown that different plant extracts comprehensively ameliorated the renal injuries induced through CCl_4 intoxication by increasing antioxidant enzyme activities [40,41].

In the present study, the administration of CCl_4 resulted in a significant elevation in renal PCC and MDA levels, indicating increased protein and lipid oxidation,

respectively, leading to tissue damage and failure of the antioxidant mechanisms to prevent the production of excessive free radicals. Interestingly, pretreatment of 400 mg/kg *S. excelsa* leaves markedly reduced the extent of protein and lipid oxidation by decreasing the PCC and MDA levels, which confirms the nephroprotective effect of *S. excelsa* leaves against the renal protein oxidation and lipid peroxidation induced by CCl_4.

Increased MPO enzyme activity is an indicator of inflammation. This enzyme is highly enriched in the azurophilic granules of polymorphonuclear leukocytes (PMNs) recruited to injured tissue to mediate the acute phase of the inflammatory response [46]. Reduced MPO levels in the group pretreated with 400 mg/kg *S. excelsa* extract indicate that the extract may cause significant supression of neutrophil infiltration. These results were in accordance with those of Tsumbu et al.[47] who reported that plant polyphenols could decrease the MPO activity released by the neutrophils.

Carbonic anhydrases (CAs) are key enzymes that regulate acid-base homeostasis in both normal and pathological conditions. The fact that CA III in humans as well as in other species is abundantly expressed in skeletal muscle and some other tissues [48] make it a physiologically significant pool of reactive sulfhydryls that function as oxyradical scavengers. The concentration of CA III in these cells could reach the same order of magnitude as that of glutathione. Thus it may provide an important physiological mechanism of protecting tissues against oxidative damage [49]. Increased CA activity in the CCl_4 treated group and the group pretreated with 100 and 200 mg/kg aqueous extract of *S. excelsa* observed in this study indicate that CA may have a direct role in cellular response to oxidative damage.

The present study confirmed and extended the results of other studies showing that oxidative stress occurs depending on the increase of reactive oxygen species production in different diseases. The administration of antioxidants could conceivably protect tissues from the effects of free radicals and lipid peroxidation and thereby retard the progress of many diseases [44,50-52].

The biochemical findings of the present study revealed that pretreatment with 400 mg/kg of body weight *S. excelsa* leaves of CCl_4-treated rats ameliorated the toxic effects of CCl_4 by restoring the markers mentioned above to normal levels.

In conclusion, our results indicated the protective role of *S. excelsa* leaves against CCl_4-induced nephrotoxicity. The mechanisms of protection include the inhibition of protein and lipid oxidation processes and the increase in antioxidant enzymes activities, which results in the recovery only of biological parameters but not contribute to the integrity of kidney histological aspects.

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RESEARCH ARTICLE

The Effects of Factors on Death Rate in the Broiler Farms

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Summary

The objective of this research is to determine the effects of factors on the death rate in the Broiler farms in Mersin province, Turkey. The data of this study is obtained in the 87 Broiler farms in 2012. The chi-square test has been applied to the acquired data. The results have shown that the fewer death rates of animals are observed while the budget for the production period increases. Besides, it is also found out that the enlargement on the field of the management, 7 days long of curtain opening procedure and selection of the chicks regarding average weight as 40 g are among the factors, reducing the death rate of the animals. Moreover, usage of the fully equipped vehicles determined by the EU standards for the transportation of animals, heating the coops and 21 days of relaxation period of the coops would have statically significant effects on the decrease of the death rates. The average death rate has been found as 9.68. With the possibility 7% reducing, the average production increase 508 kg/period for each management.

Keywords: *Broiler farms, Death rate, Chi square test, Mersin*

Broiler İşletmelerinde Ölüm Oranı Üzerine Etkili Olan Faktörler

Özet

Bu çalışma Broiler işletmelerinde, hayvanların ölüm oranını etkileyen üretici ve işletmeye ait faktörlerin belirlenmesi amacıyla yapılmıştır. Çalışmanın verileri 2012 yılında 87 Broiler işletmesinden elde edilmiştir. Elde edilen verilere Ki kare testi uygulanmıştır. Yapılan test sonucunda bir üretim dönemine ayrılan bütçe artıkça kümeslerdeki ölüm oranının azaldığı tespit edilmiştir. Bunun yanı sıra işletmenin alanının genişlemesi, perde açma süresinin yaklaşık 7 gün olması, işletmeye gelen civcivlerde ortalama 40 g ağırlığın tercih edilmesinin de ölüm oranını azalttığı belirlenmiştir. Ayrıca civcivlerin taşınmasında tam donanımlı AB mevzuatına uygun araçların kullanılması, otomatik sobalarla kümesin ısıtılması ve kümes dinlenme sürelerinin en az 21 gün olmasının ölüm oranlarını azaltıcı yönde etki yapacağı tespit edilmiştir. İşletmelerde ortalama ölüm oranı 9.68 olarak bulunmuştur. Bu oran %7'ye düşürüldüğünde işletme başına üretim ortalama 508 kg/dönem artacaktır.

Anahtar sözcükler: *Broiler işletmeleri, Ölüm oranı, Ki-kare testi, Mersin*

INTRODUCTION

All people need protein, carbohydrate, vitamin and mineral, so that they can live a healthy life. Animal originated foods are one of the main elements of the balanced nutrition. People compensate their needs for these foods through meat, milk and egg. Among these proteins, with its high level of nutrition rate and lower rates of fat and cholesterol, and comparing to the red meat its cheapness and easy digestibility, the consumption of chicken meat is increasing regularly. This makes the poultry an important sector among the other meat producing sectors. The poultry is still an important economic activity and a source of food in developing countries [1].

With 35-45 days of production period, intensive producing opportunity in unit area, high rate of transformation of animal feeds into the meat and lower need of workforce comparing to the other agricultural managements, broiler production has a special place in the animal production [2,3].

Besides the advantages mentioned above for the producer, broiler has also several disadvantages. The poorly modernized conditions for chick transportation, inappropriate set up of the coops [4] and the other circumstances of the coop such as temperature, lightning,

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ventilation and noise [5] could be the counted reasons of these disadvantages.

The poultry production in Turkey has begun in 1930 with the establishment of the Ankara Central Poultry Institution. In 1963, the imported hybrid broiler and layer type of chickens, which were kept for breeding, have been delivered to the commercial managements. After the 1980s, poultry production has become a really important sector [6-10], which meets the national demand for the chicken meat. The broiler institutions have tried to enlarge their market shares by modern farms. However, some external factors have affected the sector negatively. For instance, the bird flu also known as influenza or bird plague has quite large negative effects starting with 2005. In the city of Mersin, in which 14.74% (23 million number) of Turkey's yearly broiler poultry production (162 million number) have been supplied, because of the spreading bird influenza there has been detected 20 million of decrease on the production. Later that year, broiler consumption have started to increase slowly again and in 2011 in Turkey 3.40% (5 million number) of the production were performed in Mersin [11]. Not only in the world but also in Turkey, many studies have been carried out to increase the broiler production. The usage of pads on the effectiveness of broiler production [12], poultry place preference and its effects [13], free breeding system and its effects [14], the cost of production [15], the density of the poultry (population) [16], the death incidents during the transportation of the chickens [17,18], the economic analysis of the broiler institutions [19], factors affecting profits of broiler enterprises [20], financial effects of avian influenza [21] and the factors on the performance of the producers have been surveyed [22]. Yet, about the factors for the broiler death rate there has not been any research has been conducted. This study attempts to fill this void by analyzing the effects of factors on the death rate in the broiler farms in Mersin.

MATERIAL and METHODS

The primary findings of the study have been acquired by the questionnaire with the broiler farms in Mersin. The secondary findings have been obtained by the research on the records in the Province Municipality of Mersin.

The number of the questionnaires has been decided with the Proportional Sampling Method. The formula for the finite population there is an example below on the known or estimated rate. On the occasions, on which P is unfamiliar, it would be better to work with the maximum sample volume by approving the P=0.5, so that we can reduce the possible mistake [23,24].

$$n = \frac{N * p * (1-p)}{(N-1) * \sigma_p^2 + p * (1-p)}$$

According to the formula; n: Sample Size, N: Operating number in the population, σ^2_p: Variance of the rate, r: Deviation from the average (10%), p: The rate of the operations to the population.

$$\sigma_p^2 = \frac{0.1}{1.96} = 0.051$$

$$n = \frac{896 * 0.5 * 0.5}{895 * (0.051)^2 + 0.5 * 0.5} = 86.89 \cong 87$$

In this formula the sample consumer for the survey has been determined as 87 with the 95% of confidence parameters (z=1.96) and 10% of average deviation.

Chi Square Test

Chi square test is a nonparametric statistical analyzing method often used in experimental work where the data consist in frequencies or counts as distinct from quantitative data optained from measurement of continuous variables such as age, income, budget, and so on. The most common use of the test is to assess the probability of association or independence of facts. Managements have been divided into 2 by the death rates, which have been decided by the companies as 7% regarding whether they are below or above the death rate. In this study, for the determination of the effects belonging to the producer in broiler farms, the Chi-square test has been applied to the work. Chi-square test is being used for the exact determination between the relations of intermittent variables and also the systematic relations among the frequency based variables, which are pictured on a cross-table. The chi-square test is based mainly on the resolving of the difference between the estimated and observed frequencies regarding if they are expressive or not [25].

RESULTS

The 92% of the broiler producers participating in the questionnaire are male. The age of the participants is varied from 22 to 70 years and the average has been calculated as 45.39. The education years of the participants are varying from 5 to 15 years and the average is 6.16 years. This shows that approximately 72% of the broiler producers have been graduated from only primary school. Averagely, there are 4.03 individuals living in the producer's household and the helping individuals are detected as 1.53. The duration of stay or in other words, aging until the poultry time for the chicks can differ 35 to 45 days. Average cutting age of the chicken is calculated as 42.69 days. The producers have produced from 4 to 7 times a year and the average is 5.53 times. The average income of the producers is conducted as 6979,31 Turkish liras (TL) and their average budgets are 1922,41 TL *(Table 1)*.

In over populated coops, the best capacity for

Table 1. *Descriptive statistics*
Tablo 1. *Tanımlayıcı istatistikler*

Variables	Min.	Max.	$X \pm S_x$
Gender (female:0, male:1)	0	1	0.92±0.27
Age (year)	22	70	45.39±9.79
Education (year)	5	15	6.16±2.22
The number of household members	1	6	4.03±1.07
Household members helping the broiler production	1	3	1.53±0.52
Cutting Age (days)	35	45	42.69±1.08
The number of yearly periods	4	7	5.53±0.59
Income (TL/period)	1.000	20.000	6979.31±4891.74
Budget (TL/period)	250	8.000	1922.41±1704.18
Coop area (m^2)	180	1.600	768.10±377.94
Capacity of the coop (number)	2.000	25.000	11318.39±6154.85
The number of chickens for each m^2	11	17	14.47±1.55
The distance of chick transportation (km)	20	950	349.63±331.09
Species of the chick (Hubbard: 0, Ross: 1)	0	1	0.86±0.35
Curtain opening time (day)	4	11	6.72±2.26
Heating system (Classical stove: 0, Automatic stove:1)	0	1	0.22±0.42
The weight of the chick in the broiler man. (g)	35	43	38.75±1.85
Live chicken weight for cutting (g)	2.000	2.700	2391.72±131.15
Provender transformation rate (FCR)	1.06	2.86	1.67±0.23
Coop relaxation time (day)	10	36	23.57±4.29
Death rate of the management (%)	3	20	9.68±3.60
Preferred death rate by the comp. (%)	5	7	6.76±0.61

preventing the deceleration and increment of the death rates is decided as 14-18 chicken per m^2 in the coop [26]. The average production area of the participants is 768.10 m^2 and the 11318.39 chickens for each farm. The average chicken number for each m^2 is 14.47. The chicks cover 349.63 km distances until they have been brought to the management. In the 86% of the managements is breeding the Ross type and 14% of them is breeding the Hubbard kind of chicks. In order to make the heating easier, the coops have been divided into by the curtains. These curtains have been opened up, when the chicks have grown adequately. The average curtain opening time is calculated as 6.72 days. For the providing the heating in the coops, classical and automatic stoves have been used. The 22% of these managements have chosen the automatic stoves. The average weight of the chicks is 38.75 g and averagely after 43 days of growing up they weigh 2391.72 g and ready to be cut and to be eaten. The coops relaxation period has been described as the time between the cleaning of the units and the interval of the period for each unit. The coops relaxation time could vary between 10 to 36 days and the average calculated is 23.57 days *(Table 1)*. By splitting the average weight of chick until they are ready to be cut and the amount of the provender, the Feeding Change Ratio hereafter FCR is calculated [27,28]. FCR could vary from 1.60 to 1.75 and the average is 1.67. The death rate in the managements is changing from 3% to 20% and the average is 9.68%, on the other hand the wished or desired death rate is 6.36%, also among the other companies around the death rate is limited max 7% *(Table 1)*. With the possibility 7% reducing, the average production increase 508 kg/period for each management.

The socioeconomic and demographic factors of the producers on production have quite a big role for the decreasing of the death rate. The chi-square test results have been shown in the *Table 2*. The budget for one producing period ($P<0.01$), transportation of the chicks to the facility ($P<0.01$), the coop area ($P<0.05$), coop relaxation time ($P<0.05$), curtain opening time ($P<0.05$), the weight of the chicks($P<0.10$), the age of the producer ($P<0.10$), the number of the family members helping the production process ($P<0.10$) and the heating system ($P<0.10$) have been found statically important.

There has been a positive oriented relation between the age of the producer and the death rate. Usually the producer, who are more than 50 years old, do not pay mostly any attention for modernizing the coops and produce through the old classical methods. Just as in the producers under the age of 50 years there has been lower death rate observed. There has been also a negative oriented relation between the helping family members and the

Table 2. *The results of Chi Square Test*
Tablo 2. *Ki kare test sonuçları*

Variables		Death Rate		Chi-square	P-value
		Less than %7	%7 or more		
Age	<50	26.15	73.85	2.803	0.094
	50+	9.09	90.91		
Education	Primary	25.40	74.60	1.710	0.425
	Jun. High	13.33	86.67		
	High school	11.11	88.89		
Household members helping the broiler prod.	No	14.29	85.71	2.714	0.099
	Yes	28.89	71.11		
Income (TL/term)	<5000	14.71	85.29	1.906	0.386
	5000-9500	24.24	75.76		
	10000+	30.00	70.00		
Budget (TL/term)	<1000	5.00	95.00	10.845	0.004
	1000-2500	18.75	81.25		
	3000+	47.37	52.63		
Coop area (m^2)	<550	12.12	87.88	6.340	0.042
	550-1000	21.05	78.95		
	1050+	43.75	56.25		
Heating system	Stove	17.65	82.35	3.206	0.073
	Automatic stove	36.84	63.16		
Coop relaxation period (day)	<21 days	8.82	91.18	5.539	0.019
	21+	30.19	69.81		
Curtain opening time	<6	36.67	63.33	5.928	0.052
	6-9	14.63	85.37		
	10+	12.50	87.50		
The kind of the chicks	Haverd	25.00	75.00	0.081	0.775
	Ross	21.33	78.67		
The average weight of the chicks (g)	<40	15.09	84.91	3.614	0.057
	40+	32.35	67.65		
The transportation distance of the chicks (km)	<70	11.11	88.89	6.285	0.012
	70+	33.33	66.67		

death rate. The more the family members help with the producing process; the lower becomes the death rate in broiler farms. There has been also a negative oriented compound between the death rate and the producer's budget. The more budget means the more quality and amount of chicken baits and also a better maintenance. It covers producing labor, disinfection, pad, lime, chicken feed or bait, medication, heating and lightning. Another negative relation has been observed between the coop area and the death rate. The larger the coop becomes, the less chicken on every m^2 locate, which decreases the death rate. Just as the relation between the heating system of the coop and the death rate.

The death rate in the coops with automatic heating system has been less than the ones with the classical stove system. The automatic systems are sensible to the heating changes and work precisely, that is why they protect the coop against the sudden temperature alterations. The coop relaxation period covers the cleaning-disinfection period and the time for the relaxation and lasts 3 weeks averagely. In this time (21 days) the fertilizer is removed from the coop and the curbs and mangers are washed and disinfected. After the coop is washed and dried off, it is disinfected and conducted the liming process [29]. There is a negative oriented relation between the relaxation period and the death rate as well. In the coops with the relaxation period of 21 days or more, the death rate has been detected less than the other coops. Relaxation period enables the coop and its equipment to be cleaned and disinfected for a longer time. The curtain opening time has affected the death rate also negatively. The longer the curtain opening

time has been, the chicks have grown and their range of motion has been narrowed down and this results with the more death rates. Another negative oriented relation has occurred between the weight of the chicks and the death rate. If the chicks weigh are 40 g or more, it is not likely to encounter the death incidents. The chicks weighing 40 g or more, could accommodate themselves more easily to a new living circumstances than the others and their survival rate has increased *(Table 2)*.

According to the EU regulations or legislations, it is required that all the containers and their equipment must protect the animals from suffering and wounding and provide their security against the harsh weather conditions such as extreme cold or hot and other climate conditions. They must be also easy to be cleaned and disinfected [27]. The transportation distance has also effects on the death rate of the chicks. If the transportation distance is more than 70 km, the death rate decreases since such transportation has been provided with the high equipped vehicles, which enables the chick comfort. For lower distances most of the time old or downgraded vehicles have been used for the transportations *(Table 2)*.

DISCUSSION

The poultry, as a sub-branch of livestock, has an increasing importance on the national economy regarding the procurement for the protein deficiency, due to the lack of production in the red meat sector, which has minor share in the agriculture production of Turkey. The objective of this study is to determine the factors which have effects on the death rate in the broiler farms. According to the results of the survey, there has been an average 5.53 production periods in a year. This results resemble the findings in İstanbul (5-6 periods/year) [30] and in South Georgia, USA (6 periods/year) [31]. However, the average production periods have been found high in Czechoslovakia (3.6-4.7 periods/year) [32].

The number of chickens in the coops for a square meter has a vital importance in terms of productivity. In crowded coops the development decreases and the death rate increases. The most suitable amount is decided as the 14-18 chickens in a square meter during the cutting ages [26]. In this research the average number of chicken has been decided as 14.47 chicken/m^2. There is a parallel relation between the density and the death rate in the coops. In the farms, where the density is around 13, 15 and 17 chicken/m^2, the bait consumption and utilization has been conveyed more useful and the death rate has been detected as less than 1% [33]. The similar results have occurred in the surveys in Saudi Arabia [34] and Turkey [35] as well.

In broiler farms the death is a really vital factor, which affects the success and the income of the manufacturer directly. In these researched farms, the death rate has been found as 9.68% but the desired rate by the firms is 6.76%. The results of the high death rate in the farms could be lined up basically as the weight of the chick, the transportation and the heating of the coops. In a management the death rate is 8.86% [36], in another between 6.2% to 8.2% [37]. According to a research made in Bangladesh, the death has the third place among the important problems [38].

According to the results of the Chi-square test; the age of the producer, the number of the family members helping the producing, the budget for a producing term, the area of the management, heating system, coop relaxation time, curtain opening time, weight of the chicks in the broiler and the transportation distance have been found statistically significant.

The broilers need a certain budget provide for their expenditures such as heating, lightening and work-labor. In the research area, it has been observed that the more the budget increases, the less the death rates become. According to the research in Bangladesh, the improvement regarding the yearly income of the families in broiler farms, have affected the performance of the manufacturers positively [38].

In the previous researches, the sudden altering on the temperatures has raised the death rates obviously [39-41]. According to the analysis results of this research, in the broiler farms with the automatic heating system the death rate is relatively less in comparison to the other farms. The automatic heaters are sensible to the temperature in the coop and as they function sensitively they prevent and obstruct the temperature changes. This is the reason of the necessity for building modern coops with automatic closed heating systems in Turkey.

The research shows us that if the transportation distance of the chicks is less than 70 km away, the death rate decreases in the coops. The research in the Czech Republic claims that the transportation distance of over 50 km increases the death rate among the chicks [42]. The reason of that are the deficiencies in the transportation trucks, which should protect the chicks against the harsh climate conditions, be eligible to be cleaned and disinfected. The regulations should be implemented in regards to the European Union for the transportation of the animals in general.

As a result, the decreasing of the death rates and increasing of the productivity provide for the manufacturers with the higher incomes, better life standards and contribution on the national economy.

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RESEARCH ARTICLE

The Effect of L-Arginine on Growth Performance, Some Serum Biochemical Parameters and Duodenal Motility in Broilers [1]

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Summary

The aim of this study was to evaluate the effect of diet supplemented with L-arginine (L-Arg)on growth performance, some serum biochemical parameters and duodenal motility of broilers during three time periods: 0 to 10, 11 to 28 and 29 to 42 days old. A total of 500, mixed sex, one-day-old Ross-308 broiler chicks were divided into five groups as follows: Arg deficient group and four experimental groups. Each group was then divided into five subgroups of 20 chicks each. Arg deficient group for all time periods was fed by basal diet which contained 10% less L-Arg than optimum Arg requirement recommended by the breeder. Experimental groups were fed by basal diet supplemented with L-Arg which was progressively 10% increased in groups. The highest body weight gain (BWG) was observed on days 11-28 and 0-42 in experimental group fed by basal diet supplemented with 110% L-Arg, whereas the lowest feed conversion ratio (FCR) was determined on days 29-42 and 0-42 in the same experimental group. Feed intake did not change in all three periods, while serum urea nitrogen level in the experimental group in which diet supplemented with 10% L-Arg, was lower than other groups on day 0-10. On contractility studies, it was observed that L-Arg inhibited the amplitude of contractions in duodenum in a dose-dependent manner *in vitro*. These results suggest that the basal diet formulated with 90-130% Arg is not effective on growth performance of chicks on days 0-10, whereas the diet supplemented with 10% L-Arg more than optimum Arg requirement is adequate during the days 11-28 and 29-42. Moreover, although the L-Arg decreased the duodenal contractility *in vitro,* it is suggested that the diet supplemented with 10% L-Arg more than optimum Arg requirement may be negatively affected the FCR in broilers.

Keywords: *Broiler, L-arginine, Growth performance, Duodenal contractility*

L-Arjininin Broylerlerde Büyüme Performansı, Bazı Biyokimyasal Parametreler ve Duodenal Motilite Üzerine Etkisi

Özet

Bu araştırma 0-10., 11-28. ve 29-42. günler arasında broylerlerde L-arjinin (L-Arj)nin rasyonlara katılmasının büyüme performansı, bazı biyokimyasal parametreler ve duodenal motilite üzerine etkisini belirlemek amacıyla yapılmıştır. Araştırmada toplam 500 adet, bir günlük yaşta, karışık cinsiyette Ross 308 broyler civciv: Arj yetersiz grup ve dört deneme grubu olmak üzere beş gruba ayrılmıştır. Her bir grup da 20'şer civcivden oluşan 5 alt gruba ayrılmıştır. Arjininçe yetersiz grup tüm deneme boyunca üretici tarafından önerilen optimum Arj gereksiniminin %10'undan düşük L-Arj içeren temel rasyon ile beslenmiştir. Deneme grupları ise, temel rasyona %10 progresif artan düzeylerde L-Arj ilave edilen rasyonlarla beslenmiştir. Araştırma sonunda ihtiyacın %110 düzeyinde Arj bulunan diyetle beslenen grupta 11-28 ve 0-42. ($P<0.01$) günlerde en yüksek canlı ağırlık artışı (CAA), buna karşın aynı deneme grubunda 29-42. ve 0-42. günlerde en düşük yemden yararlanma oranı (YYO) bulunmuştur. Her üç dönemde YT gruplarda değişmezken serum üre azot düzeyi optimum Arj gereksiniminin %10 Arj ilaveli rasyondan oluşan deneme grubunda 10. günde diğer gruplara göre düşmüştür. *İn vitro* ortamda ise L-arjininin doza bağlı olarak duodenumda kasılımları baskıladığı görülmüştür. Sonuç olarak, broylerlerde arjinin gereksiniminin %90-130'unu karşılayacak şekilde hazırlanan rasyonların 0-10. günler arasında büyüme performansı üzerinde etkili olmadığı, ancak 11-28. ve 29-42. günler arası dönemlerde optimum Arj gereksiniminden %10 daha fazla L-Arj içeren rasyonun yeterli olduğu belirlenmiştir. Bununla birlikte, L-arjininin *in vitro* duodenum kasılımları azalttığı, bu bağlamda optimum L-Arj gereksinimin %10'nun daha fazla L-arjininli rasyonla beslenen broylerlerde YYO'nun olumsuz etkileneceği öngörülmektedir.

Anahtar sözcükler: *Broyler, L-arjinin, Büyüme performansı, Duodenal kasılım*

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INTRODUCTION

It is important to add a balanced amount of Arg to poultry diets for growth development [1-3], nitrogen balance [3] and protein metabolism [4]. However, a decrease in body weight (BW) and BW gain (BWG), as well as in feed intake (FI) and feed conversion ratio (FCR), due to supplementation of Arg more than optimum Arg requirement of diet of broilers, has been reported [5]. Nitric oxide (NO) is synthesized from L-Arg in a two-step enzyme reaction by nitric oxide synthase (NOS) [6]. Nitric oxide is involved in the intestinal water transport by acting directly on the epithelium and blood flow or indirectly by stimulating neuronal reflexes. Noncholinergic nonadrenergic neural mechanisms involving nerves containing NO have been shown to modulate smooth muscle in the gastrointestinal tract and therefore NO may be important in the regulation of cyclical small intestinal motility [7]. Moreover, the nicotinamide adenine dinucleotide phosphate diaphorase activity in nerve fibres of jejunum in chickens has been reported [8]. It has been also well documented that the presence of neuronal NOS enzyme in nerve fibres of proventriculus in chickens [9].

Previous studies in broiler chickens have demonstrated the effect of adequate or higher Arg levels on growth performance [5,10,11]. However, to the authors' knowledge, there have been no reports performed to investigate the effect of diet formulated with decreased and progressively increased Arg on growth performance during three time periods, as well as on intestinal motility and NO metabolism. Therefore, the present study was designed to evaluate the effect of different levels of Arg on growth performance, serum biochemical parameters and duodenal motility in 0-10, 11-28 and 29-42 days old broilers.

MATERIAL and METHODS

Animals, Housing and Experimental Desing

This study was carried out at the Animal Research Center of Afyon Kocatepe University, after approval by the local ethical committee (B.30.2.AKU.09.Z.010). Five hundred, one-day old, mixed sex chicks (Ross-308) were obtained from a commercial hatchery, weighed and randomly separated into five groups. Each group was then divided into five subgroups that consisted of 20 birds each. Experiments were carried out for 42 days. Feed and water were provided *ad libitum* and the daily lighting regimen was 23 h of light and 1 hour of dark throughout the study.

The chicks were reared on the floor of pens in a curtain-sided broiler house. Pine wood shavings were used as litter material. The pens were 2 m^2 in size and the stocking density was 12 chicks per square meter. The temperature was 34±1°C during the first week of the study and was gradually reduced to 26±1°C by the third week. Thereafter, the study was maintained at a room temperature of 24°C.

Dietary responses to L-Arg (Sigma A5131 powder) were evaluated from 0 to 10, 11 to 28 and 29 to 42 day old. The diet of Arg deficient group for all time periods was consisted of basal diet which was formulated 10% less L-Arg than optimum Arg requirement recommended by the breeder. The Arg-deficient diet contained 1.35, 1.14 and 0.99% Arg for all time periods, respectively. All nutrients met or exceeded the nutrient requirements for broiler chickens [12]. The levels of crude protein (CP) and Arg of the basal diet, including corn, corn gluten, soybean meal, full-fat soybean and meat and bone meal, were analysed before being used in formulation *(Table 1)*. Experimental groups were fed basal diet which included progressive increments of 10% L-Arg thereby; Arg requirement was achieved with the rate of 100%, 110%, 120% and 130% in groups.

The levels of CP and amino acids of the feed ingredients, including corn, corn gluten, soybean meal, full-fat soybean and meat and bone meal, were calculated before being used in the design of the diet formulations. The CP levels in the diets and raw feed materials was analysed using methods of AOAC [13]. L-Arg levels in the diet were determined by LC-MS-MS (Applied Biosystems API-3200) in a laboratory (ANT Technical Devices Lab.) *(Table 1)*.

Growth Performance

The mortality rate of broilers was recorded daily. Body weights were recorded by pen on days 0, 10, 28, and 42. Feed intake and BWG per pen was recorded 0-10, 11-28 and 29-42 days. Feed conversion rations were calculated by dividing the cumulative feed intake per pen by the live body mass per pen at the end of the measurement periods.

Biochemical Parameters

Blood samples were collected into non-heparinized tubes from 10 birds from each group (2 birds/replicate) on days 10, 28 and 42 during sacrifying and serum was collected by centrifugation. Serum was harvested and stored (−20°C) before analysis. Sera were analysed for concentrations of alkaline phosphatase (ALP), alanine aminotranspherase (ALT), aspartate aminotranspherase (AST), creatinine, urea nitrogen (BUN)in an autoanalyzer (Tokyo Boeki Prestige 24i, Japan).

The Preparation of Isolated Smooth Muscle Strips of Duodenum for Contractility Experiments

Duodenum was collected about 15 min after exsanguinations and transported on ice to the laboratory within 30 min. Then, samples were put into a dissecting Petri dish containing Krebs' solution (KS: NaCl 118 mmol/l KCl 4.7 mmol/l, $CaCl_2$ 2.5 mmol/l, $MgSO_4$ 1 mmol/l, KH_2PO_4 1 mmol/l, glucose 11 mmol/l, $NaHCO_3$ 25 mmol/l), which were continuously ventilated with a gas mixture (95% O_2 and 5% CO_2). Five mm-long ring strips of samples were

Table 1. C*omposition of the Arg deficient diets in different periods (%)*
Tablo 1. *Farklı dönemlerde Arj yetersizliği oluşturulan diyetlerin bileşimi (%)*

Ingredients	Phase (days)		
	0 to 10 d	11 to 28 d	29 to 42 d
Corn	53.53	55.38	57.22
Corn gluten meal	6.60	9.00	9.00
Boncalite	-	-	5.00
Soybean meal	14.75	3.20	0.43
Full fat soybean	17.14	24.00	19.63
Meat bone meal	4.00	4.00	4.50
Vegetable oil	1.50	2.00	2.50
Calcium carbonate	0.64	0.60	0.28
Dicalcium phosphate	0.62	0.55	0.25
Salt	0.19	0.20	0.20
DL-Methionine	0.22	0.20	0.07
L-Lysine HCl	0.31	0.37	0.27
Sodium bicarbonate	0.20	0.20	0.20
Vitamin premix*	0.20	0.20	0.30
Mineral premix**	0.10	0.10	0.15
Calculated composition, %			
ME, kcal/kg	3023	3159	3173
Crude protein	22.20	20.80	19.00
Calcium	0.97	0.92	0.80
Available phosphorus	0.46	0.44	0.42
Methionine + Cystine	0.99	0.96	0.80
Lysine	1.31	1.24	1.04
Arginine	1.38	1.21	1.01
Analyzed composition, %			
Crude protein	22.14	21.57	19.65
Arginine	1.35	1.14	0.99

** Vitamin premix provides per 2.5 kilogram of diets: 12.000.000 IU vitamin A, 2.500.000 IU vitamin D_3, 40.000 mg vitamin E, 5.000 mg vitamin K_3, 3.000 mg vitamin B_1, 6.000 mg vitamin B_2, 5.000 mg vitamin B_6, 20 mg vitamin B_{12}, 25.000 mg niacin, 12.000 mg pentatonic acid, 1.000 mg folic acid, 50 mg biotin, 10.000 mg BHT, ** Mineral premix provides per 2.5 kilogram of diets: 100.000 mg calcium, 100.000 mg magnesium, 70.000 mg manganese, 150 mg cobalt, 400 mg iota, 150 mg selenium, 25.000 mg ferric, 5.000 mg cupper, 60.000 mg ZnO*

dissected from the middle point of related tissue and incised longitudinally. Thereafter, longitudinal smooth muscle strips were carefully isolated and one edge of each tissue preparation was fixed to platinum ring electrodes. The opposite edge of the tissue was connected to a force-displacement transducer (model 10-A; MAY, Commat, Ankara, Turkey). Isolated strips were placed in a four chambers organ baths (IOBS 99 Isolated Tissue Bath Stand Set, Commat) filled with 20 ml KS (pH 7.4), which were continuously oxygenated (95% O_2 and 5% CO_2) at 37°C. The isometric smooth muscle activity of duodenum were monitored and recorded by computer via the force transducer and an acquisition system (model MP30 WSW with Biopac Student Lab, PRO Software, Biopac Systems, Commat).

Recording of Isometric Duodenum Contractility

Duodenum in organ baths were kept in KS for at least 1 h before the recordings to enable the tissues to adapt to the environment and the solution was refreshed at 15 min intervals. The appropriate resting tension for the strips was determined in initial experiments. The strips were placed under progressive increments of tension. Optimal tension relationships were achieved with resting tensions of 1 g for the strips. After the completion of the 30 min baseline period, contractions of longitudinal strips of duodenum for each animal were visualized and recorded to determine normal spontaneous contractions. Thereafter, the strips were treated with Arg (Sigma, Cat # A8094) at increasing concentration (10^{-5} – 10^{-3} M) to determine endogenous NO

activity. The mean tension of spontaneous contractions for each strip calculated for a 10-min period before administration of examined substances was set as 100% (control period). Thereafter, changes in contractions caused by the examined substances were recorded and compared to the control period [14,15].

Statistical Analysis: Data from treatment means were analyzed as a completely randomized design using the General Linear Models procedure of the SPSS for windows. When differences (P<0.05) among means were found, means were separated using Tukey's Studentized range test. Linear and Quadratic Arg dose response curves were plotted using the GLM procedure of SPSS.

RESULTS

Broilers fed graded levels of Arg had showed quadratic responses for BWG in 11 to 28 and 0 to 42 d of age, FCR in the 29 to 42 and 0 to 42 d of age.

It was observed that BWG on days 11-28 (P<0.05) and 0-42 (P<0.01) in experimental group which chicks were fed with diet contained 110% Arg increased when compared to experimental diet groups contained 90 and 130% Arg. Similarly, FCR was more adequate in the same groups on days 29-42 and 0-42 (P<0.001). Feed intake did not show any significant difference between groups in all time periods *(Table 2)*.

It was determined that the levels of serum ALP, ALT, AST and CRE did not differ between groups, while BUN in the experimental group in which diet supplemented with 10% L-Arg, was lower than other groups on day 0-10 *(Table 3)*.

The amplitude of spontaneous contractility of duodenum in groups did not show any significant difference during the days 0-10, 11-28 and 29-42 *(Table 4)*. However, increasing concentrations of Arg from 10^{-5} to 10^{-3} M decreased the amplitude of duodenal contractility in all time periods (P<0.001). Moreover, the most effective level of Arg in decreasing the amplitude of duodenal contractility was 10^{-3} M *(Fig. 1A, B, C)*. The percentage of inhibition of contractility analysis showed no significant differences in all groups in the days 0-10, 11-28 and 29-42 *(Table 4)*.

DISCUSSION

The present study was performed in three time periods. Arg requirement of broilers was provided by the diet which was 10% less than optimum Arg level recommended by the breeder and 10% progressive increments of Arg for each experimental group. Consequently, Arg requirement was accomplished with the rate of 90-130% on days 0-10, 11-28 and 29-42 in broilers. It has been reported that Arg in diet is required for optimum BWG [2,4,16,17], however the level of Arg below requirement [16] or 25% higher than requirement in diet [5,11] decreases BWG. Burton and Waldrop [18], Cuca and Jensen [1] and NRC [19] (1994) have suggested that the sufficient Arg level in diet is 1.25-1.40% until the day 28, 1.24 to 1.28% in first three weeks and 1.25% between days 0-21, 1.1% between days 22-42, respectively. In the present study, we observed that BWG increased in experimental group which the Arg requirement corresponded to 110% Arg on days 11-28 and 0-42 when compared to other experimental groups (P<0.01).

In the current study, FI did not differ in groups in each time periods. This result was consistent with Kidd et al.[2] and Corzo et al.[3] who suggested that various levels of L-Arg

Table 2. *The growth performance of broilers fed by the diet which consisted of 90, 100, 110, 120 and 130% of Arg requirement*
Tablo 2. *Arj ihtiyacının %90, 100, 110, 120 ve 130'unun karşılandığı diyet ile beslenen broylerlerin büyüme performansı*

Growth Performance	Days	Treatment					SEM	P	
		90	100	110	120	130		Linear	Quadretic
Body weight gain, g	0 to 10	225.3	227.6	230.7	225.4	220.2	1.56	0.263	0.316
	11 to 28	983.3b	1029ab	1060a	1015ab	998.1b	8.49	0.763	0.014*
	29 to 42	965.0	1004	1032	1008	949.8	11.49	0.723	0.078
	0 to 42	2173b	2261ab	2322a	2248ab	2168b	17.03	0.803	0.004**
Feed intake, g	0 to 10	297.7	294.8	291.5	296.7	300.3	3.17	0.829	0.856
	11 to 28	1662	1781	1731	1747	1739	15.14	0.250	0.140
	29 to 42	2938	2708	2646	2697	2745	30.86	0.107	0.133
	0 to 42	4896	4785	4669	4741	4785	38.76	0.329	0.453
Feed conversion ratio, g/g	0 to 10	1.32	1.29	1.26	1.31	1.36	0.012	0.237	0.102
	11 to 28	1.69	1.73	1.63	1.72	1.74	0.013	0.254	0.062
	29 to 42	3.05a	2.69c	2.56c	2.67c	2.89b	0.04	0.061	0.000***
	0 to 42	2.25a	2.11bc	2.01c	2.10bc	2.20ab	0.02	0.340	0.000***

*Letters (a, b, c) indicate significant differences between them in each column, * P<0.05, ** P<0.01, *** P<0.001*

Table 3. *Effects of Arg on levels of serum alkaline phosphatase (ALP), alanin aminotranspherase (ALT), aspartat aminotranspherase (AST), creatinin, urea nitrogen (BUN) on days 10th, 28th and 42nd*

Tablo 3*. Serum alkalen fosfataz (ALP), alanin aminotransferaz (ALT), aspartat aminotransferaz (AST), kreatinin ve üre azot (BUN) düzeyi üzerine 10., 28. ve 42. günlerde Arj'in etkisi*

Parameters	Day	Treatment					SEM	P	
		90	100	110	120	130		Linear	Quadretic
ALP, U/L	10	21.21	18.50	10.51	11.12	13.21	6.33	0.458	0.433
	28	631.3	765.5	443.0	634.2	773.6	121.3	0.919	0.128
	42	976.4	1253	1388	1360	1335	87.33	0.574	0.314
ALT, U/L	10	4.87	2.75	2.50	3.50	3.50	0.87	0.157	0.98
	28	3.00	1.75	2.50	2.37	2.14	0.12	0.357	0.210
	42	2.80	3.00	2.28	2.12	2.71	0.22	0.709	0.460
AST, U/L	10	159.8	182.0	154.1	170.4	148.5	15.43	0.293	0.356
	28	194.3	152.2	168.0	151.5	153.3	7.22	0.062	0.376
	42	210.6	249.3	238.9	283.2	308.8	12.74	0.128	0.783
Creatinin, mg/dl	10	0.10	0.12	0.07	0.11	0.12	0.01	0.742	0.567
	28	0.061	0.03	0.07	0.03	0.04	0.01	0.156	0.098
	42	0.14	0.12	0.12	0.12	0.13	0.04	0.316	0.060
BUN, mg/dl	10	5.73a	6.37a	4.16b	6.60a	5.71a	0.28	0.019*	0.021*
	28	3.50	2.50	3.71	3.12	2.71	0.44	0.238	0.567
	42	4.50	3.75	4.42	4.00	3.71	0.33	0.068	0.256

*Letters (a, b) indicate significant differences between them in each column, * P<0.05*

Table 4. *The amplitude of spontaneous contractility of duodenum on days 10th, 28th and 42nd*

Tablo 4. *Spontan duodenum kasılımlarının 10., 28. ve 42. günlerde amplitüdü (g)*

The amplitude of Spontaneous Contractility (g)		Treatment					SEM	P
		90	100	110	120	130		
Day	10	0.50	0.52	0.48	0.53	0.50	0.022	0.975
	28	0.62	0.68	0.69	0.61	0.56	0.028	0.589
	42	0.70	0.76	0.71	0.63	0.64	0.026	0.685

supplementation did not show any effect on FI in broilers. In contrast to our finding, it has been reported that Arg-deficient diet [16] or high level of Arg in diet [3,5] decreased the FI in broilers. However, Skalan and Plavnik [20] reported that the effect of Arg on FI and BWG was related to protein level in diet. Therefore, it is suggested that discrepancy in FI and BWG in above-mentioned reports may be different CP levels in diet.

We clearly demonstrated that the level of Arg corresponded to 90-130% of Arg requirement did not alter FCR on days 11-28 and 0-10 however, FCR was more adequate in the experimental group which the diet contained 110% Arg on days 29-42 and 0-42. This finding supports the previous observations that either Arg deficiency [2,16] or high level of Arg in diet [5,21] decreases the FCR level. Moreover, it has been emphasised that the Arg:lysine ratio in diet should be balanced between days 21 and 42 and FCR may be negatively affected by the changes of Arg:lysine ratio [22]. In the present study, the discrepancies of FCR in Arg deficient group and the diet group which contained 130% Arg during the 29 to 42 day period may be explained by the occurrence of alteration in the Arg:Lysine ratio in diet.

In the present study, serum ALP, ALT and AST levels did not show any significant difference between groups. Besides the serum concentrations of ALP, ALT and AST are valuable indicators to detect any abnormality of liver [22], it is suggested that the presence of Arg in diet corresponded to 90-130% of Arg requirement is ineffective on liver enzymes.

Creatinine is mainly released from liver and pancreas and excreted from the body via glomerular filtration and tubular secretion. Therefore, creatinine is a gold standard for kidney damage, when compared to BUN [23]. There was statistically significant difference in BUN concentration in the current study (if it is not 2 fold higher than normal value) [22]. Arginine did not produce important difference in terms of kidney function test *(Table 3)*. In the current study, a significant difference was observed in the concentrations of BUN levels between groups on day 0-10,

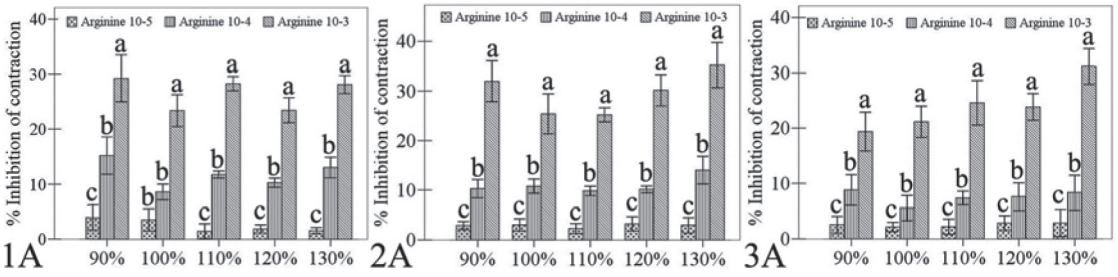

Fig 1. Percentage inhibition of spontaneous contractions in duodenum on 10th, 28th and 42nd days by application of Arg at doses of 10^{-5}, 10^{-4} and 10^{-3} M

Letters (a,b,c) indicate significant differences between them in each column. *P*-value was <0.001 in groups determined by ANOVA. 1A) 0 to 10 d of age, 1B) 11 to 28 d of age 1C) 29 to 42 d of age

Şekil 1. Duodenumda 10., 28. ve 42. günlerde Arj'in 10^{-5}, 10^{-4} and 10^{-3} M dozlarının spontan kasılımlarda oluşturduğu yüzde inhibisyon

Her bir kolonda farklı harfler (a,b,c) istatistiksel önemi gösterir. *P*-değeri ANOVA tarafından gruplarda < 0.001 belirlendi. 1A) 0-10 günlük yaş, 1B) 11-28 günlük yaş,1C) 29-42 günlük yaş

Table 5. *Percentage inhibition spontaneous contractions in groups in the presence of Arg (10^{-5}, 10^{-4}, 10^{-3} M) on isolated strips of duodenum on day 10th, 28st and 42nd*

Tablo 5. *Gruplarda 10., 28. ve 42. günlerde izole duodenum örneklerinde Arj (10^{-5}, 10^{-4}, 10^{-3} M) spontan kasılımlarda oluşturduğu yüzde inhibisyon*

Inhibition Spontaneous Contractions (%)	Treatment					SEM	P
	90	100	110	120	130		
10th day							
Arg10^{-5} M	2.40	3.49	2.02	1.84	1.55	0.48	0.784
Arg10^{-4} M	11.30	8.55	11.57	10.24	11.24	0.62	0.570
Arg10^{-3} M	30.86	23.38	28.28	23.44	28.12	1.13	0.140
28th day							
Arg10^{-5} M	2.87	3.00	2.28	3.21	2.94	0,49	0.987
Arg10^{-4} M	2.86	10.28	10.78	9.83	10.12	0.76	0.440
Arg10^{-3} M	31.97	25.41	25.20	30.17	35.20	1.69	0.280
42nd day							
Arg10^{-5} M	1.90	2.14	2.17	2.86	2.86	0.61	0.967
Arg10^{-4} M	7.17	5.56	7.33	7.60	8.32	0.98	0.946
Arg10^{-3} M	21.56	21.14	24.59	23.83	31.17	1.51	0.220

whereas creatinine did not show any significant difference between groups. It has reported that if these enzymes does not increase two fold higher as compared to their normal values, no kidney damage would be expected [23]. Therefore, it is suggested that no changes in kidney function occurs due to Arg in diet.

Nitric oxide was reported to be an important regulator of gastrointestinal motility [24-26]. It has been reported that NO is an inhibitor neurotransmitter in canine isolated ileocolonic junction and longitudinal smooth muscle of duodenum [25] and rat gastric fundus [26]. It has been also reported that oral administered Arg have a regulatory role in esophageal and gall bladder motility [27,28]. Moreover, it has been demonstrated that intravenous or intragastric administration of large amounts of Arg delays gastric emptying in humans [29,30] and dogs [31]. Therefore, the present study also focused on the effect of Arg supplemented to diet on motility of duodenum of chickens throughout three time periods. In the present study, spontaneous contractions of duodenum did not change on days 0-10, 11-28 and 29-42 *(Table 4)*. It is suggested that this may be attributed to the metabolisation of Arg before reaching the duodenum or to the absence of NO in these tissues due to short half-life of NO itself.

Arginine shows its relaxing effect on smooth muscles via self-conversion to NO by using NO synthase in these tissues [27]. It has been observed that L-Arg completely inhibits smooth muscle contractility at doses of 0.01–1 mM, *in vitro* [32]. Bulbul et al.[14] demonstrated that the most effective dose of L-Arg was 10^{-5} M in rat intestinum. In the present study, Arg inhibited the spontaneous contractions of duodenum and this finding is consistent with above-mentioned reports. However, it was observed the inhibition rate did not show any significant difference between

groups. The tissue or serum NO concentrations are changed by the alteration of NO synthase expression [33]. Similarly, it has already been reported that SNP, a exogenous NO donor and L-NAME, a selective NOS inhibitor changes the nNOS expression in jejunum but not in duodenum and ileum in broilers [34]. In this study, no difference was observed in the inhibition of duodenum contractility between groups, *in vitro*. Therefore, it is suggested that oral administration of Arg may not be affected by intestinal NOS enzymes.

In conclusion, in agreement with the contractility data that dietary Arg affected the motility in the duodenum by decreasing the amplitude of contraction, it is indicated that the diet supplemented with 110% Arg is found to be satisfactory between days 11-42 in broiler nutrition.

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RESEARCH ARTICLE

The Role of Red and Infrared Low Level Laser Therapy on Unmeshed Full-Thickness Free Skin Autograft in Rabbits: As An Animal Model

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Summary

The effect of laser on wound healing has been approved but the role of Low Red (LR) and Infrared Low Level Laser (ILLL) on unmeshed full-thickness free skin autograft (UFFSA) is not clear yet. The aim of the present experimental study is to determine the effectiveness of LR and ILLL in UFFSA in rabbits. The study was conducted on 15 New-Zealand white rabbits were divided into three groups, Control (C), Low Red (LR), Low Infrared (LIR). A 5×5 cm UFFSA was obtained then rotated 90 degrees and repositioned on its own bed and sutured. The rabbits in LR and LIR received LR and ILLL for 6 days post-operatively. On days 3, 5, 7, 14, and 30 skin biopsies were obtained. Graft size was measured. There were significant differences in epitheliazition, polymorphonuclears, fibroblast, and collagen among groups on days 3 and 5 and new vessels on day 3. LR and LIR had similar role in new vessels till day 5 and collagen synthesis on day 3 for group C. There was no significant difference in epithelialization in groups on days 7, 14, and 30. LIR showed significant differences of length, area, peripheral, and diameter during study and of width on day 7. We concluded LIR played the more effective role on early phase (on day 5) of healing and more acceptable appearance to LR and C groups on quantitative measures.

Keywords: *Unmeshed full-thickness free skin autograft, Low red laser, Infrared low level laser, Rabbit*

Düşük Yoğunluklu Kırmızı ve Kızılötesi Lazer Işın Tedavisinin Tavşanlarda Birbirine Geçmeyen Tam Kalınlıklı Serbest Deri Otogrefti Üzerine Etkisi: Bir Hayvan Modeli

Özet

Lazer ışınlarının yara iyileşmesi üzerine etkisi kanıtlanmış olmasına rağmen Düşük Yoğunluklu Kırmızı (LR) ve Kızılötesi Düşük Yoğunluklu Lazer (ILLL)'in birbirine geçmeyen tam kalınlıklı serbest deri otogrefti (UFFSA) üzerine etkisi açıkca bilinmememktedir. Bu deneysel çalışmanın amacı tavşanlardaki UFFSA üzerine LR ve ILLL'nin etkililiğini belirlemektir. Çalışma Kontrol (C), Düşük Kırmızı (LR), Düşük Kızılötesi (LIR) olma üzere üç gruba ayrılan 15 Yeni Zelanda beyaz tavşanı üzerinde gerçekleştirildi. Beş×beş cm'lik UFFSA elde edilip daha sonra 90 derece döndürüldü ve kendi yatağında yeniden konumlandırılıp ve dikildi. LR ve LIR içindeki tavşanlar operasyon sonrası 6 gün boyunca LR ve ILLL uygulaması aldı. Üçüncü, 5., 7., 14. ve 30. Günlerde deri biyopsileri elde edildi. Greft boyutları ölçüldü. Gruplararası önemli farklılıklar 3. ve 5. günlerde epitelizasyon, polimorfnükleer hücreler, fibroblast ve kollajen üzerine gözlemlenirken 3. gün yeni damarlar üzerinde belirlendi. Grup C için, LR ve LIR'nin 5. güne kadar yeni damarlar üzerine ve 3. gün kollajen sentezi üzerine benzer rol oynadığı belirlendi. Gün 7, 14, ve 30'da gruplar arasınad epitelizasyon açısından anlamlı bir fark bulunmadı. LIR uzunluk, alan, çevre ve çap üzerine çalışma süresince, genişlik üzerine ise 7. günde önemli farklılık gösterdi. LIR'ın erken dönem (5. gün) iyileşme aşamasında daha etkin rol oynadığı ve LR ve C gruplarınnın kantitatif ölçümler üzerine daha kabul edilebilir görünümde olduğu sonucuna varıldı.

Anahtar sözcükler: *Birbirine geçmeyen tam kalınlıklı serbest deri otogrefti, Kırmızı düşük yoğunluklu lazer, Kızılötesi düşük yoğunluklu lazer, Tavşan*

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INTRODUCTION

Skin grafts are indicated when there is major loss of skin from trauma particularly on limbs, tumor removal, and resurfacing full thickness burns after major thermal injuries [1-3]. A skin graft (unmeshed full-thickness free skin autograft) is one choice for complicated wound therapy [2]. The main advantages of UFFSA include as pliable, movable, resistant to trauma, normal skin appearance, minimum contraction, increase the size and adequate protection and with no immune response [1,2]. Presence of seroma formation between donor and recipient sites prevents graft adherence [3]. So fluid accumulation within or under the graft and movement of the graft prevent good vascular connection from developing between the graft and the bed [2,3]. Therefore this type of graft used in spite of admitted post-surgical appearance especially in burns, contaminated bed and improper granulation bed [3]. Moreover survival time of this type of graft is not survive as well as meshed full-thickness autograft [3]. Regarding of the problem, UFFSA is not sometimes taken to the wound bed that it is not seen in meshed full-thickness autograft [1,3]. It is worthy that the latter technique does not meet cosmetic criteria, so for better appearance; UFFSA is suggested [3]. According to the struggle, unmeshed graft is not used for the reason of probable consequences. During the three last decades, many reports showed that monochromatic light sources and photomedicine were used for acceleration treatment of skin wound healing [4]. Many researchers also have indicated that the low-power laser light has therapeutic role to promote the repair processes of connective tissues as skin, ligaments, tendons, nerve, pulp of tooth, bone and cartilage in variety of animal models and as well as ulcers of a wide range of etiologies in humans [5-9]. Few studies supported the effect of low level laser and other monochromatic light sources in UFFSA and increasing its success. Therefore beneficial role of laser on "taken" and appearance of this type of graft is underquestion. As there was lack of information and few investigations deal with effect of low red and low infrared lasers and dose of them on UFFSA and on its survival time and success, so the purpose of this study was to clarify the effect of red and infrared of the low level laser therapy on UFFSA of rabbit skin.

MATERIAL and METHODS

The study was approved by the Animal Ethics Committee of the Iranian laboratory animal ethic frameworks under the reference code IAEC 1-12.

For this study, 15 mature New Zealand white rabbits of both genders were exposed to in the same light, temperature, humidity and diet for two weeks. All rabbits were then randomly allocated into three equal groups, Control (C), Low Red (LR) and Low Infrared (LIR).

First stage

On the day of the operation, left side of all rabbits was prepared for operation and clipping and aseptic surgical techniques were employed. A combination of Xylazine HCl (5 mg/kg, IM) and Ketamine HCl (40 mg/kg, IM) was given for anesthesia. All rabbits of three groups were then placed in the right lateral position and the left thoracoabdominal region was scrubbed with 1% povidone-iodine solution. After preparation of rabbits and surgical team, a 5×5 cm full-thickness skin graft was obtained and separated from the region, and then the graft was rotated 90 degrees repositioned and sutured to the surrounding skin with simple interrupted suture by 4-0 monofilamet polyamide *(Fig. 1)*. For this purpose, firstly, sutures were placed of the four corners of grafted skin and secured in bed and continued to oppose the two other borders of skin in each groups *(Fig. 2)*. Post-operatively, animals were given penicillin procaine (60.000 IU/kg, deeply SC, three days) and Gentamicin (2 mg/kg, SC, three days). Analgesic agent was not prescribed post-operatively. Every rabbit kept in solitary cage for 30 days and all of them took the same diet during study.

Second stage

The irradiation protocol established in rabbits of group LR and LIR using 1 J/cm^2 using red light (wavelength 635

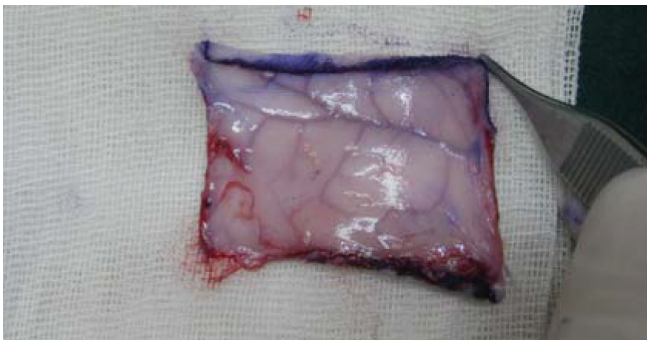

Fig 1. Skin removed, inner side, the vessels of skin are visible
Şekil 1. Serbestleştirilen derinin iç yüzü ve damarları görülmektedir

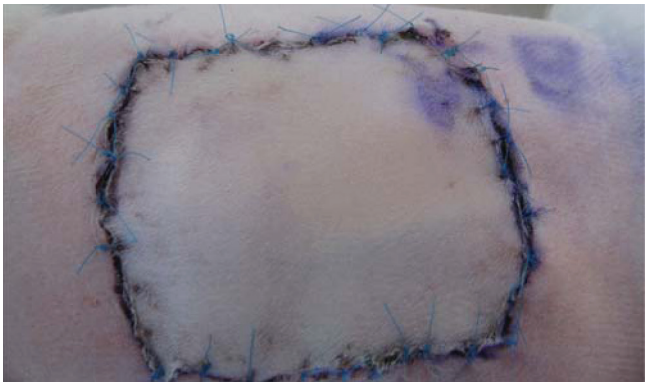

Fig 2. The skin after suturing
Şekil 2. Dikiş sonrası derinin görünümü

nm) and infrared light (wave length 850 nm) by laser (Mustang 2000, Russia) equipment. Time of irradiation was 60 sec centripetally once a daily for 6 days, with the first application immediately after surgical procedures. Rabbits in group C did not receive any laser irradiation. On days 3, 5, 7, 14, and 30 skin biopsies were obtained using scalpel blade No. 11 from the cranial end of the ventral side on day 3, from the middle of caudal side on day 5, from the middle of dorsal side on day 7, from the middle of cranial side on day 14 and from the caudal end of ventral side on day 30 of the junction of square shaped graft and skin. The sample was fixed in formalin and embedded in paraffin for sectioning. The samples were sent to laboratory and 6 micrometers section was done and stained by haematoxylin and Eosin dye for microscopic study. The evaluation of qualitative histopathologic data was scaled based of *Table 1*. Length, width, area, perimeter and diameter of graft sides were measured in each rabbit on the same days and record.

Statistical Analysis

The histologic evaluation of grafts was done qualitatively and quantitatively. Statistical data were analyzed by SPSS software version 16.0. One-way ANOVA, Tukey Post-Hoc test and repeated measures analysis with 95% confidence interval was done by General Linear Model (GLM). This procedure was used of continuous variables with normal distribution. The Kruskal-Wallis and Friedman tests were used to evaluate the ordinal variables. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Immediately after excision of graft because of the tension lines, the area of the graft expanded and after transplanting, the shrinking process of the graft is seen simultaneously. The wound dehiscence wasn't occurred in animals of all groups during study. All rabbits tolerated surgery and survive during study. Qualitative data were as follows:

Epithelialization

Based on microscopic study, there were significant differences in epithelialization among groups (C, LR, and LIR) on days 3 and 5 and LR and LIR were similar and showed significant differences to group C. There was no difference in epithelialization in all groups days 7, 14, and 30 *(Table 2, Fig. 3-14)*.

Polymorphonuclears (PMNs)

The number of PMNs showed significant differences on days 3, 5, and 14. There were no significant differences on days 7 and 30 among groups *(Table 2, Fig. 3-14)*.

Table 1. *Explanation of used scale in the semi-quantitative evaluation of histological sections*
Tablo 1. *Histolojik kesitlerin yarı kantitatif değerlendirmesinde kullanılan ölçekler*

Scale	Epithelization	PMNL	Fibroblasts	New Vessels	Collagen
0	thickness of cut edges	absent	absent	absent	absent
1	migration of cells (<50%)	mild ST	mild-ST	mild-SCT	minimal-GT
2	migration of cells (≥50%)	mild DL/GT	mild-GT	mild-GT	mild-GT
3	Bridging the excision	moderate DL/GT	moderate_GT	moderate_GT	moderate_GT
4	keratinization	marked DL/GT	marked-GT	marked-GT	marked-GT

*(**ST** – surrounding tissue, i.e. tissue out of GT; **DL** – demarcation line; **SCT** – subcutaneous tissue; **GT** – granulation tissue)*

Table 2. *Mean ± SE of quantitative meaures (length, width, area, perimeter and diameter) in different days ±*
Tablo 2. *Farklı günlerde ortalamaSH'nın kantitatif değerleri (Uzunluk, genişlik, alan, çevre ve çap)*

Day	0			3			5			7			14			30		
Group	C	LR	LIR	C	LR	LIR	C	LR	LIR	C	LR	LIR	C	LR	LIR	C	LR	LIR
L	5± 0.00	5± 0.00	5± 0.00	4.45± 0.07	4.22± 0.0	3.54± 0.13	4.10± 0.10	3.62± 0.19	3.05± 0.09	3.50± 0.10	3.24± 0.17	2.55± 0.09	3.56± 0.12	3.05± 0.12	2.74± 0.05	3.04± 0.05	1.92± 0.10	2.25± 0.14
W	5± 0.00	5± 0.00	5± 0.00	4.5± 0.04	4.70± 0.0	4.65± 0.06	4.45± 0.04	4.45± 0.09	4.40± 0.14	4.16± 0.05	4.24± 0.10	3.82± 0.07	3.90± 0.04	3.90± 0.10	3.64± 0.05	3.14± 0.12	2.56± 0.22	3.14± 0.05
A	25± 0.00	25± 0.00	25± 0.00	21.51± 047	19.54± 0.47	16.57± 0.69	18.37± 0.52	16.27± 1.15	13.47± 0.64	15.77± 0.63	13.79± 1.06	11.01± 0.45	13.55± 0.50	11.99± 0.68	9.97± 0.29	9.56± 0.50	4.96± 0.58	7.17± 0.49
PM	20± 0.00	20± 0.00	20± 0.00	15.52± 0.021	17.54± 0.2	16.44± 0.31	17.16± 0.23	16.40± 0.68	14.55± 0.36	15.92± 0.29	15.12± 0.52	13.40± 0.26	14.92± 0.26	13.92± 0.35	12.76± 0.19	12.36± 0.33	13.92± 0.38	10.55± 0.35
D	7.07± 0.00	7.07± 0.00	7.07± 0.00	6.56± 0.07	6.32± 0.07	5.87± 0.10	6.07± 0.08	5.76± 0.18	5.3± 0.13	5.63± 0.10	5.34± 0.18	4.79± 0.89	5.25± 0.08	4.96± 0.13	4.56± 0.07	4.37± 0.12	3.21± 0.21	3.55± 0.10

*In each raw, the heterogeneous letters show the statistical differences among groups in each day (P<0.05), **L**=Length, **W**=Width, **A**=Area, **PM**=Perimeter, **D**=Diameter*

Fibroblast

Similar to epithelialization, there were significant differences among groups (C, LR, LIR) on days 3 and 5. There was no significant difference among groups on days 7, 14, and 30 *(Table 2, Fig 3-14)*.

New *vessels* (Neovascularization)

Formation of vessels was significant on day 3, 14, and 30, but there were no significantly differences on days 5 and 7 *(Table 2, Fig. 3-14)*.

Collagen synthesis

Regeneration of new collagen showed significant differences among groups (C, LR, LIR) on days 3 and 5. There was no significant difference among groups on days 7, 14, and 30. Another interesting result was the separation of epidermal from dermal layer in all groups in the third day. Histopathologic image showed that in group LIR, re-epithelialization is formed faster (on day 5) than another groups *(Table 2, Fig. 3-14)*.

So, finally all of results indicated successful "taken" of graft with acceptable appearance in group LR and particularly group LIR and efficient adhesion between bed and donor with no gap and fluid accumulation and seroma formation in all groups *(Fig. 15-17)*.

Quantitative data

The analysis of quantitative data of diameter, perimeter, length and area showed the differences among groups on day 3, 5, 7 and 14 ($P<0.05$, *Table 2*). In group LR, differences were seen in diameter, width and area on day 30 ($P<0.05$, *Table 2*). The appearance of graft of LIR and LR were more acceptable to control group and healing rate was more rapidly in former groups, so was significant in LIR particularly on day 3 and 5 *(Fig. 6-8)*.

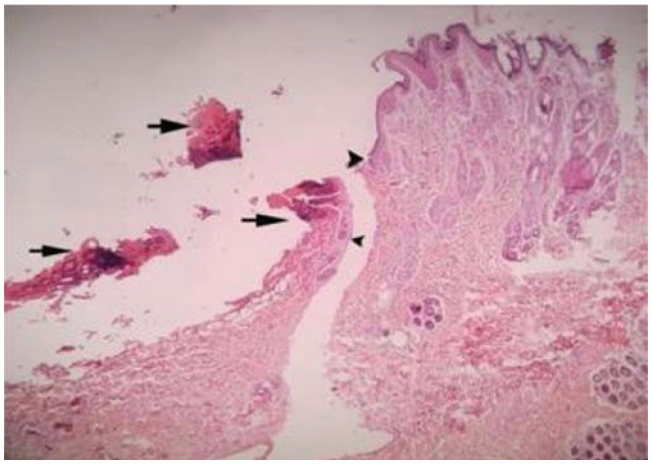

Fig 3. Re-epithelialization *(arrow head)* and necrotic epidermis *(arrow)* in C, H&E × 64

Şekil 3. C'de re-epitelizasyon *(ok başı)* ve nekrotik epidermis *(ok)*, H&E × 64

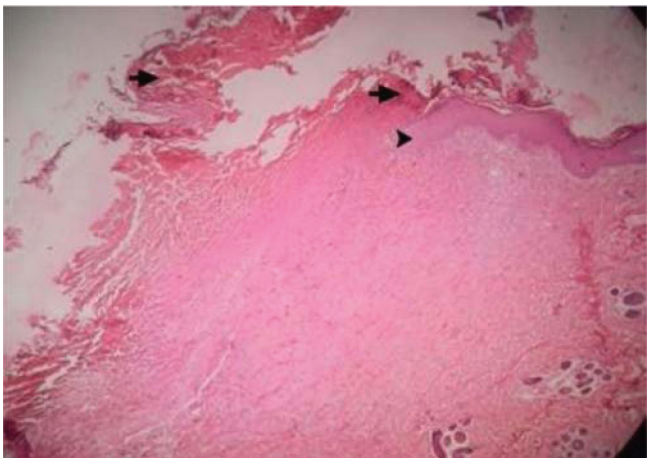

Fig 5. Re-epithelialization *(arrow head)* and necrotic epidermis *(arrow)* in LR, H&E × 64

Şekil 5. LR'de re-epitelizasyon *(ok başı)* ve nekrotik epidermis *(ok)*, H&E × 64

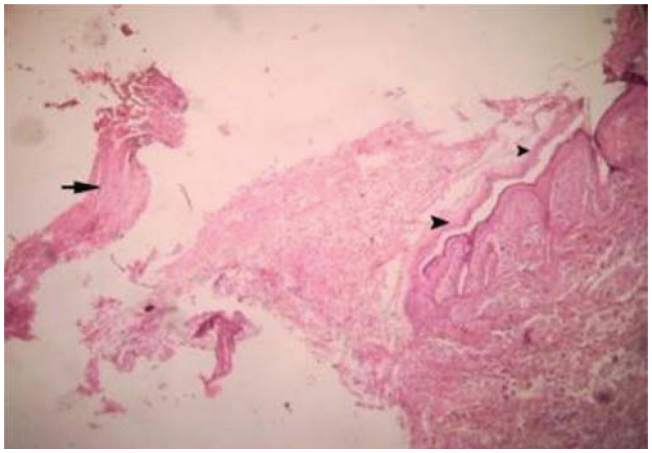

Fig 4. Re-epithelialization (arrow head) and necrotic epidermis *(arrow)* in LIR, H&E × 64

Şekil 4. LIR'de re-epitelizasyon *(ok başı)* ve nekrotik epidermis *(ok)*, H&E × 64

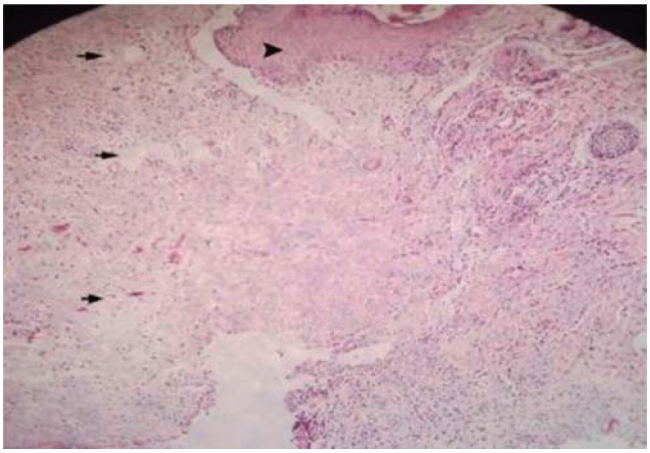

Fig 6. Re-epithelialization *(arrow head)*, collagen fiber *(arrow)* and necrotic epidermis (N) in C, H&E × 64

Şekil 6. C'de re-epitelizasyon *(ok başı)*, kollajen lif *(ok)* ve nekrotik epidermis (N) H&E × 64

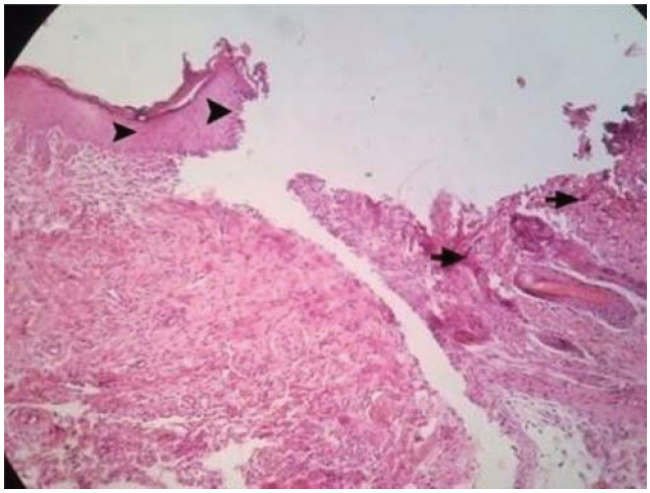

Fig 7. Re-epithelialization *(arrow head)* and necrotic epidermis (N) in LIR, H&E × 64

Şekil 7. LIR'de re-epitelizasyon *(ok başı)* ve nekrotik epidermis (N) H&E × 64

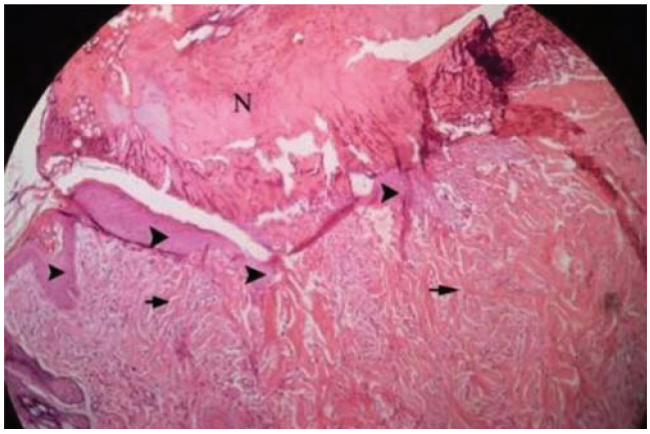

Fig 8. Re-epithelialization *(arrow head),* collagen fiber (arrow) and necrotic epidermis (N) in LR, H&E × 64

Şekil 8. LR'de re-epitelizasyon *(ok başı),* kollajen lif *(ok)* ve nekrotik epidermis (N) H&E × 64

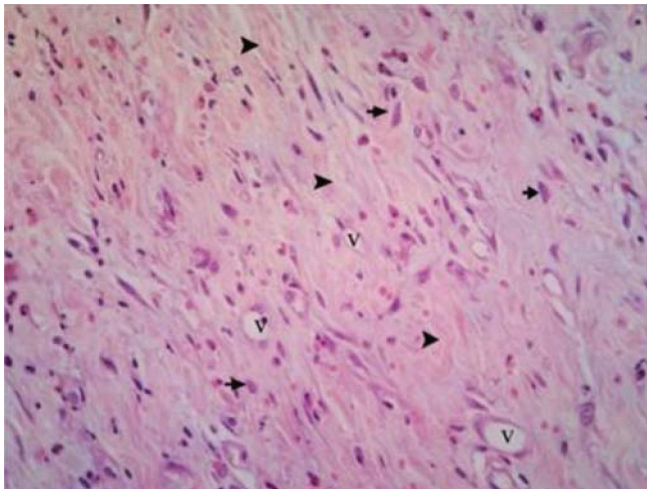

Fig 9. New vessels (V), collagen fiber *(arrow head)* and fibroblast *(arrow)* in C, H&E × 64

Şekil 9. C'de yeni damarlar (V), kollajen lif *(ok başı)* ve fibroblast *(ok)* H&E × 64

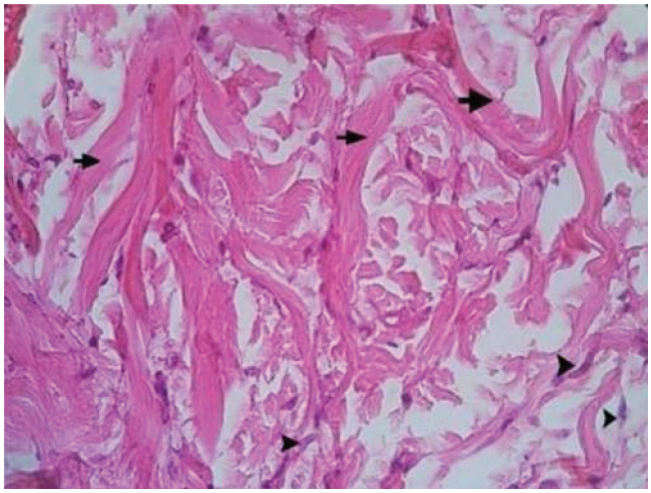

Fig 10. Collagen fibers *(arrow)* and fibroblasts *(arrow head)* in LIR, H&E × 640

Şekil 10. LIR'de kollajen lif *(ok)* ve fibroblast *(ok başı)* H&E × 64

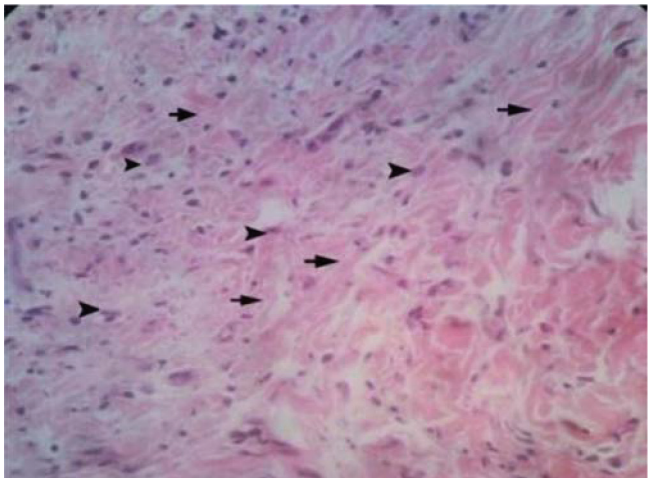

Fig 11. Collagen fibers *(arrow)* and fibroblasts *(arrow head)* in LR, H&E × 640

Şekil 11. LR'de kollajen lif *(ok)* ve fibroblast *(ok başı)* H&E × 64

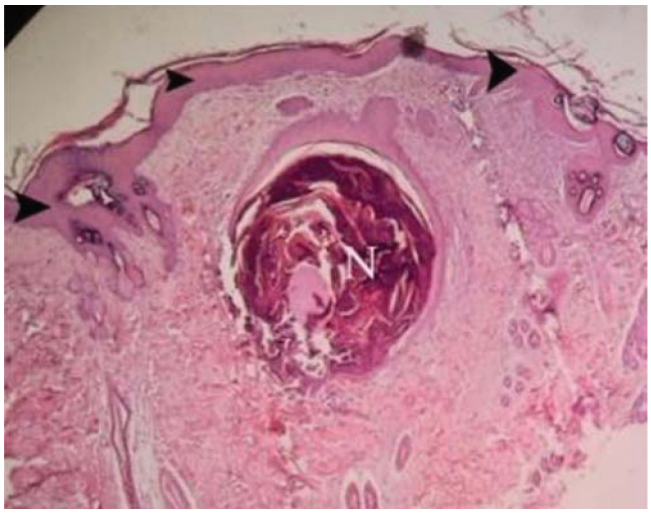

Fig 12. New epidermis *(arrow head)* and necrotic tissue (N) in C, H&E × 64

Şekil 12. C'de yeni epidermis *(ok başı)* ve nekrotik doku (N) H&E × 64

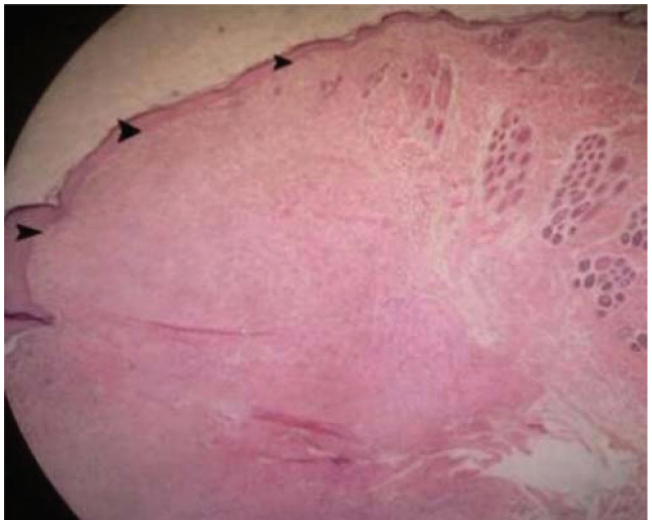

Fig 13. New epidermis *(arrow head)* and cornified in LIR, H&E × 64
Şekil 13. LIR'de yeni epidermis *(ok başı)* ve boynuzsu hali, H&E × 64

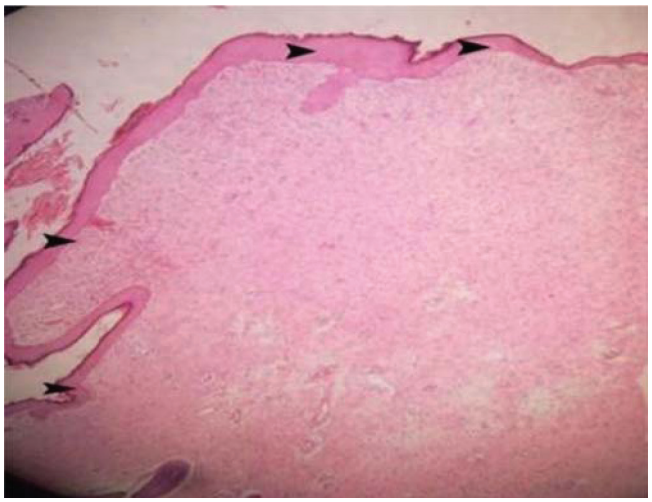

Fig 14. New epidermis *(arrow head)* and cornified in LR, H&E × 64
Şekil 14. LR'de yeni epidermis *(ok başı)* ve boynuzsu hali, H & E×64

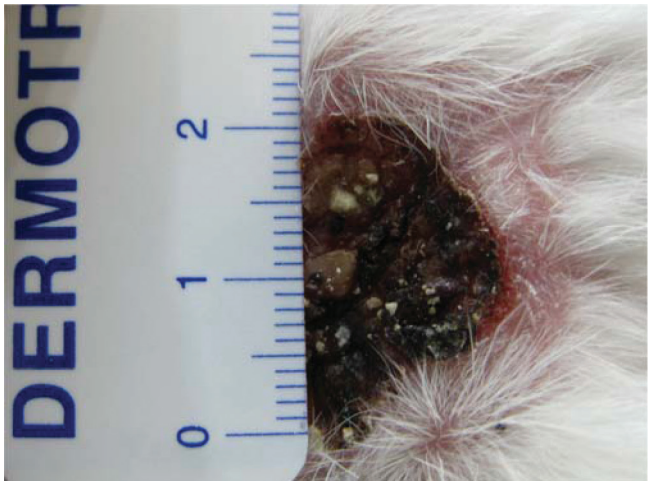

Fig 15. Appearance of a rabbit skin in control group at day 30
Şekil 15. Otuzuncu günde kontrol grubunda bir tavşan derisinin görünümü

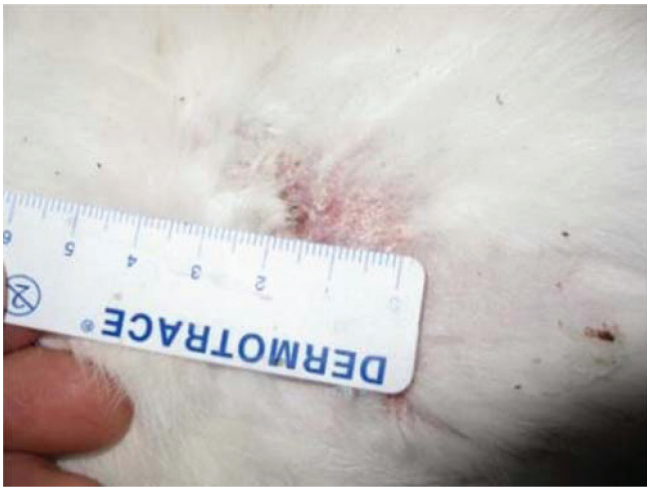

Fig 16. Appearance of a rabbit skin in LIR group at day 30
Şekil 16. Otuzuncu günde LIR grubunda bir tavşan derisinin görünümü

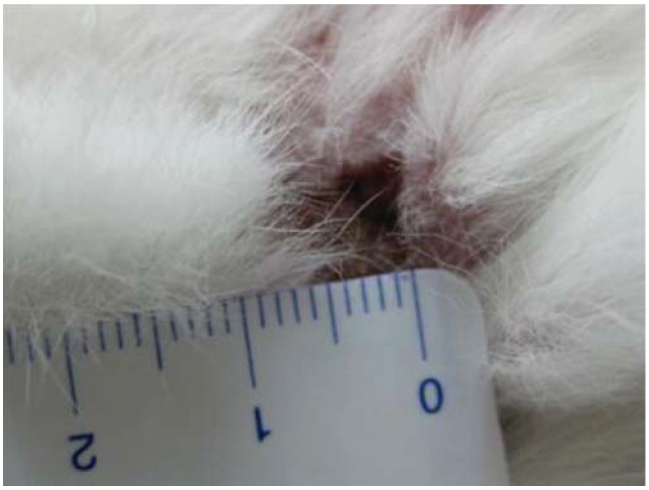

Fig 17. Appearance of a rabbit skin in LR group at day 30
Şekil 17. Otuzuncu günde LR grubunda bir tavşan derisinin görünümü

DISCUSSION

The closure and repair of large defect of skin is one of the main concerns of patients, clinicians and investigators for years [10-12]. The unmeshed full-thickness free skin autograft is one of the choices for this purpose with maximum acceptable appearance, but the success rate of graft taken depends on establishment of arterial and venous connections with less fluid accumulation and seroma formation between them [1-3,13]. Hence some plastic surgeons bypass the latter consequence with meshed pattern for diminishing seroma formation and resolving postoperative complication related to UFFSA, but it meets less cosmetic appearance [1-3]. Many investigations were done on the laser role on wound healing process but there was lack of information deal with effect of low level red and low level infrared and dose of them on unmeshed full-thickness skin autograft and "taking" graft. So the role of low level laser on unmeshed full-thickness free skin auto-

graft is unknown. Although, using low level laser therapy (LLLT) in health care has been documented in the literature for more than three decades [7]. Many researchers have demonstrated that LLLT reduces pain and has effective role in wound healing (initial, second, and final phases; pro-inflammatory, proliferation, and remodeling, respectively), and can evoke vaso-dailation, early epithelialization increased fibroblastic reaction (gingival fibroblast *in vitro*), lukocytic infiltration, encouraging the formation of type I and type III procollagen specific pools of mRNA, enhancement of ATP synthesis within the mitochondria, activating lymphocytes, and increasing their ability to bind pathogens, reduction of nicotine side effects on graft, and neovascularization [14-19]. Moreover, it was revealed that using LLLT for months or years accelerates formation of bone [20]. Regarding to above effects the time required for complete graft closure will reduced [21]. The work of Karu also showed bio-stimulation effects of the rate DNA synthesis of blue (404 and 454), red (620 nm), and near infrared (760 and 830 nm) wavelengths in vitro [15]. Moore and his colleagues demonstrated that maximum cell proliferation occurred with 665 and 675 nm light, whereas 810 nm light was inhibitory to fibroblast [17]. Rodrigo's study showed that local and systemic effects using combination red and infrared laser on the repair of back wound of rats in control and other experimental groups earlier on the 7th postoperative day [22]. Current study was showed that significant effect of low red and low infrared laser on epithelialization, polymorphonuclears (PMNs), fibroblast, new vessels (angiogenesis), and collagen synthesis in UFFSA of LIR group. Significant changes on day 3 and 5 on are related to shortening the inflammatory phase of repair and enhancing release of factors stimulating the proliferative stage of repair and increasing vaso-dailation and lukocytic infiltration in inflammatory phase by Low Infrared Laser [5,8]. So these findings indicate LIR (850 nm) had significant effect to the other groups in early phase of graft taking particularly on day 3 and less effective on day 5 and the remaining days of study. As it was mentioned, former finding does not agree with Moors's study that demonstrated inhibitory effect of the wavelength and agrees with some investigators [17,23]. Walsh and et al showed that infrared (830 nm) enhances vaso-active effects by its actions on mast cells and facilitates entry of leukocyte [5,16]. Therefore, vaso-dilation, with increased local blood flow and shortening of pro- and inflammatory phase caused by laser in LIR and even LR groups [5,16]. Fluid accumulation mechanically separates the grafts from their bed, impairing nutrition and revascularization [3]. We also did not find any fluid accumulation and seroma formation between recipient and donor. The absence of seroma accumulation in the experimental and even control groups can be because of the healthy, free of debris, irregularities and non-infectious wound bed.

The wave-length, power, power density, energy density, treatment duration, treatment intervention time post-injury, and method of application are the causative factors of response of tissue to laser [18,21]. Woodruff's study revealed that energy density was the only treatment parameter with predictable dose dependent treatment effects in outcome [24]. They revealed that energy densities ranging from 19 to 24 J/cm^2 had the largest average effect size [24]. Walsh and et al and the other related researches have demonstrated a range of bio-stimulation effects at dose from 2 to 10 J/cm^2 in red and infrared wavelengths [5]. Although deciding about the right dosage to obtain best result for the healing has not been the main concern of this study, we managed to achieve positive results by applying dose 1 J/cm^2 with 850 nm wavelength. These results (positive bio-stimulation effects) were in accordance with of results of Walsh's study with different dosage from 2 to 10 J/cm^2. Therefore it can be drawn that in our study using dosage 1 J/cm^2 brings about positive results and agree with Walsh's study.

The other finding was contraction of graft on its bed that started on day 3 and continued till day 30. As was mentioned, the shrinkage of graft size in group LIR was more significant. The previous studies had revealed contraction in thin and split-thickness grafts by the time. Few reports indicated contraction of unmeshed full-thickness free skin grafts. The main reason of shrinkage in LIR could be the presence of numerous myofibroblasts especially in LIR group on day 3 to the two groups and maturity of collagen between graft and its bed. So our results agreed with results of other investigations.

Finally, the significant results of our study could represent the beginning of a paradigm of laser therapy (red and especially infrared) and its role on graft (UFFSA) and diminished well known post-operative complications.

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RESEARCH ARTICLE

The Role of Nitric Oxide in the Effects of Ovarian Steroids in the Duodenum [1]

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Summary

The purpose of this study is to determine the distribution and expression of nNOS, eNOS and iNOS and to evaluate the role of ovarian steroids in NOS expressions in the duodenum tissues of rats. Rats in the control group (Ov group) were injected intramuscularly with sesame oil for 10 days, whereas rats in the first, second, and third experimental groups received intramuscular administration of progesterone (P group), 17β-estradiol (E2group) and 17beta-estradiol and progesterone (E2 + P group), respectively. According to the results of an immunohistochemical evaluation, a severe nNOS expression was observed in both the crypts and nerve fibers of the duodenum in the Ov, P and E2 groups but decreased in the E2 + P group. It was determined that iNOS expression increased in the surface epithelial cells and crypts of P group while it only decreased in crypts of E2 group. There was severe eNOS expression in crypts in all treatment groups, while it only increased in the surface epithelial cells in the E2 group. The results of the current experiment suggest that the effect of ovarian steroids on duodenum absorbtion and microvascular protection can be mediated by crypt and epithelial nNOS, iNOS and eNOS expressions in rats. Besides the estradiol and progesterone administration together inhibit nNOS activitiy in the duodenum.

Keywords: *Ovarion steroids, nNOS, iNOS, eNOS, Duodenum, Immunohistochemistry*

Duodenumda Ovaryum Steroidlerinin Etkisinde Nitrik Oksidin Rolü

Özet

Bu çalışmanın amacı rat duodenum dokularında NOS ekspresyonları üzerinde ovaryum steroidlerinin rolünü değerlendirmek ve nNOS, eNOS ve iNOS ekspresyon ve dağılımlarını belirlemektir. Kontrol grubu ratlara (Ov grup) intramüsküler olarak 10 gün susam yağı enjekte edildi. Bunun yanında birinci, ikinci ve üçüncü deney gruplarına sırasıyla progesteron (P grup), 17β-estradiol (E2group) ve 17beta-estradiol ve progesteron (E2 + P group) uygulandı. İmmunohistokimyasal değerlendirme sonuçlarına göre, Ov, P ve E2 gruplarında duodenumun hem kriptlerinde ve hem de sinir fibrillerinde şiddetli nNOS ekspresyonu gözlendi, E2 + P grubunda ise reaksiyon şiddeti azaldı. P grubunda kriptlerde ve yüzey epitel hücrelerinde iNOS ekspresyonu artarken, E2 grubunda sadece kriptlerde reaksiyonun azaldığı belirlendi. Tüm gruplarda kriptlerde şiddetli eNOS ekspresyonu bulunurken, E2 grubunda sadece yüzey epitel hücrelerinde reaksiyon şiddetinin arttığı belirlendi. Yapılan çalışmanın sonucu, ovaryum steroidlerinin etkisinde duodenumda absorbsiyon ve mikrovasküler korumanın, sıçanlarda kript ve epitellerde nNOS, iNOS ve eNOS ekspresyonu aracılığıyla olabildiğini, bunun yanında duodenumda östrojen ve progesteronun birlikte uygulanmasının nNOS aktivitesini inhibe ettiğini göstermektedir.

Anahtar sözcükler: *Ovaryum steroidleri, nNOS, iNOS, eNOS, Duodenum, İmmunohistokimya*

INTRODUCTION

Nitric oxide (NO) is an intercellular and endocellular signal molecule, and has an important role in the physiological process of the intestines. NO can regulate muscular contraction and blood circulation in the intestine [1-2].

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Endogenous nitric oxide is derived from L-Arginine by the enzyme called nitric oxide synthase (NOS) [3]. NOS is widely distributed in the intestine, and has several isoforms, such as constitutive nitric oxide synthase (nNOS and eNOS) and inducible NOS (iNOS) [4,5]. iNOS is the NOS isoform most broadly implicated in the processes of inflammation and carcinogenesis in the GI system [6]. Neuronal NOS (nNOS) constitutes the predominant source of NO in neurons; once nitric oxide diffuses into smooth muscle cells, it inhibits the contractibility of smooth muscles by activating guanylate cyclase [7]. Endothelial NOS (eNOS) is the predominant source in the endothelium [8,9]. eNOS expression most prominent in endothelial cells lining vascular channels [10,11] throughout the gut, liver, and pancreas [11]. eNOS also regulates a number of cellular and physiologic functions within the GI and hepatic systems including vasodilation and protection of the mucosal barrier function.

The precise mechanism by which sex steroids modulate changes in gastrointestinal motility is also not known. NO is known to mediate relaxation of gastrointestinal smooth muscle [12-14] and to be involved in the pregnancy associated decrease of gastrointestinal motility [15]. Besides, there is increased NOS expression in the gastrointestinal tissues during pregnancy [16], and NOS expression also increased by estradiol in various tissues [16,17], including the gastrointestinal tract and neuronal tissues [16].

Therefore, the aim of this study was to determine the effects of ovarian steroids on the distribution of NOS protein expression in rat duodenum.

MATERIAL and METHODS

Animals and Experimental Design

Duodenum tissues were obtained from animals used in TÜBİTAK project (Project No. VHAG-2097). In this project, female Sprague-Dawley rats were bought from the Gulhane Military Medical Academy Research Center Department of Laboratory Animals. The experimental protocols were approved by the Animal Care and Use Committee at Afyon Kocatepe University (15.09.2004; B.030.2.AKÜ.0.8Z. 00.00/115) and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

The rats in all groups were fed ad libitum with a commercial rat diet. A total of 40 rats were anesthetized by intraperitoneal ketamine (21.2 mg/kg) and xylazine (4.2 mg/kg) and ovariectomized bilaterally. Two weeks after the operation, the rats were randomly assigned to 4 groups of 10 rats each. Rats in Ov group received daily 0.2 ml intramuscular administrations of sesame oil. Rats in P and E2 groups were administrated with progesterone (2 mg/rat/day; i.m.) and 17β-estradiol (10 mg/rat/day; i.m.) respectively. Rats in E2 + P group were injected with both progesterone and 17 β -estradiol at the same dosages. The hormone treatments were continued for 10 days. After the treatments, rats were killed by cervical dislocation.

Determination of Distribution of nNOS, iNOS and eNOS in Duodenal Tissues

The duodenal samples were removed immediately after sacrifice and fixed in 10% buffered formol-saline solution and processed routinely for embedding in paraffin. Immunohistochemistry for nNOS, iNOS and eNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed using Universal LSAB Kits (Zymed Histostain Plus Broad Spectrum, South San Francisco, CA, US) according to manufacturer's protocol. Briefly, paraffin sections (5µ) were treated with nNOS (1:1000 dilution), iNOS (1:1000 dilution) and eNOS (1: 1000 dilution) primary antibody for 30 min at 37°C. Paraffin sections were washed three times in phosphate buffered saline (PBS), and incubated with the biotinylated secondary antibody for 30 min at room temperature (RT). The samples were then washed three times in PBS and incubated in Streptavidin-HRP for 30 min at RT. Following rinsing in PBS for 3X5 min, the sections were rinsed in distilled water, and incubated in DAB (Zymed, 00-2020) for 5 min to reveal peroxidase. Counterstaining was carried out with haematoxylin. Sections were cleared in xylene and mounted with Entellan. Negative controls were processed using antibody diluent reagent solution instead of the primary antibody. Relative immunreaction of positive cells was evaluated by two independent observers in a blinded fashion and given a score as followed: 0- no reaction; 1- weak reaction; 2- moderate reaction 3- strong reaction, 4- very strong reaction [18].

Statistics

Statistical analysis of results was performed by a one-way analysis of variance (ANOVA) followed by Duncan tests. Values are presented as means ± SE. Group differences were declared significant at $P<0.05$.

RESULTS

NOS expressions in duodenal tissue sections are shown in *Fig. 1, 2* and *3*. nNOS, iNOS and eNOS immunoreactions were identified on the epithelial layers of the villi and crypts. The staining pattern was very similar along the length of the villi. Moreover, nNOS expression was also present in nerve fibers. Because nNOS and eNOS expression were faint in the Brunner glands we did not statistically evaluate this expression in the table. Besides we did not find any iNOS immunoreaction in the Brunner glands.

Immunohistochemistry Scoring Results (Semiquantitative)

Semiquantitative observations of the nNOS, eNOS and

iNOS expressions in the duodenum are presented in *Table 1*, *2* and *3*.

Neuronal NOS Expression

nNOS expression on the surface epithelium of the villi was moderate in all treatment groups, although the intensity of immunreaction on crypt epithelium was strong in the Ov, P and E2 groups and moderate in the E2 + P group. The intensity of immunostaining on the surface epithelium of the villi was weaker than that of the crypts *(Fig. 1)*. There was no significant difference between nNOS expression in the surface epithelium of the villi among the groups and in the crypts in the Ov, P and E2 groups, whereas the expression significantly decreased in the E2 + P groups (P<0.001). nNOS expression was strong in nerve fibers among the groups. But the expression (P<0.05) were significantly lower in E2 + P group compared with the Ov, P and E2 groups.

Endothelial NOS Expression

eNOS expression on the surface epithelium was moderate in the Ov, P and E2 + P groups and strong in the E2 group. The intensity of immunreaction was strong in the crypt region in all groups. The intensity of immuno-

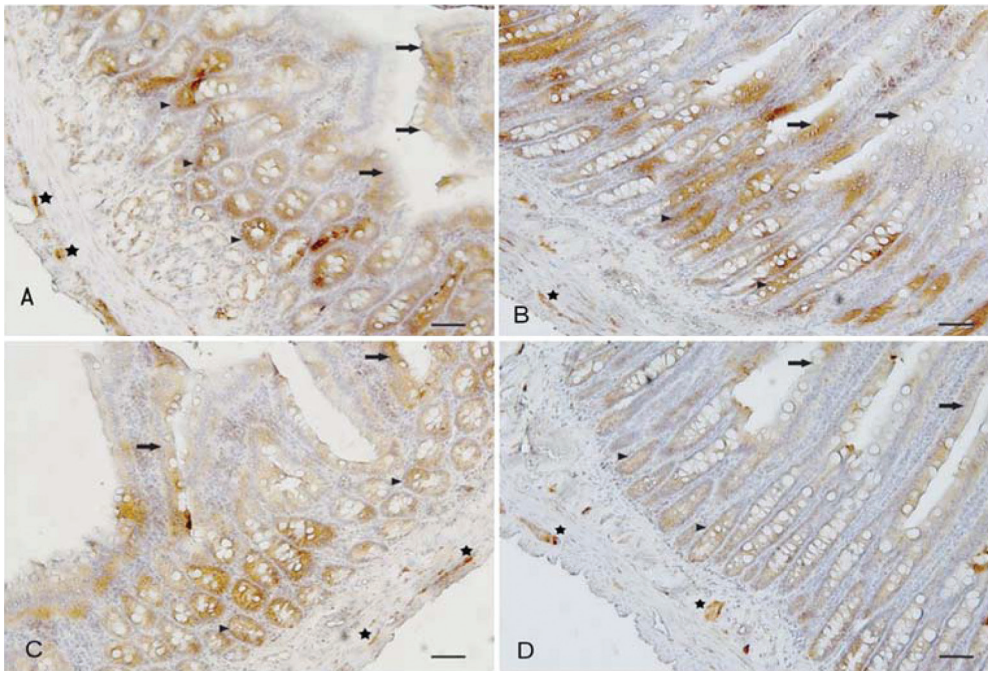

Fig 1. nNOS expression in surface epithelium of the villi *(arrows)* and crypts *(arrow head)* and nerve fibers (*). **A-** Ov group, **B-** P group, **C-** E2 group, **D-** E2+P group, Bar 50 µm

Şekil 1. Villus yüzey epiteli *(ok)*, kriptler *(ok başı)* ve sinir fibrillerinde (*) nNOS ekspresyonu. **A-** Ov grubu, **B-** P grubu, **C-** E2 grubu, **D-** E2+P grubu, Bar 50 µm

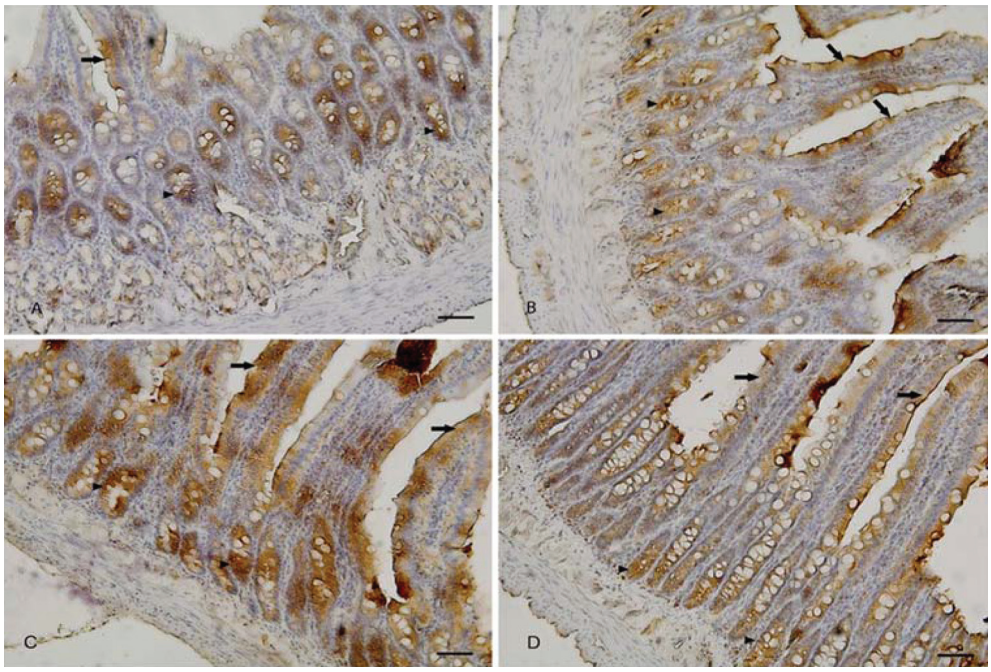

Fig 2. eNOS expression in surface epithelium of the villi *(arrows)* and crypts *(arrow head)*. **A-** Ov group, **B-** P group, **C-** E2 group, **D-** E2+P group, Bar 50 µm

Şekil 2. Villus yüzey epiteli *(ok)* ve kriptlerde *(ok başı)* eNOS ekspresyonu. **A-** Ov grubu, **B-** P grubu, **C-** E2 grubu, **D-** E2+P grubu, Bar 50 µm

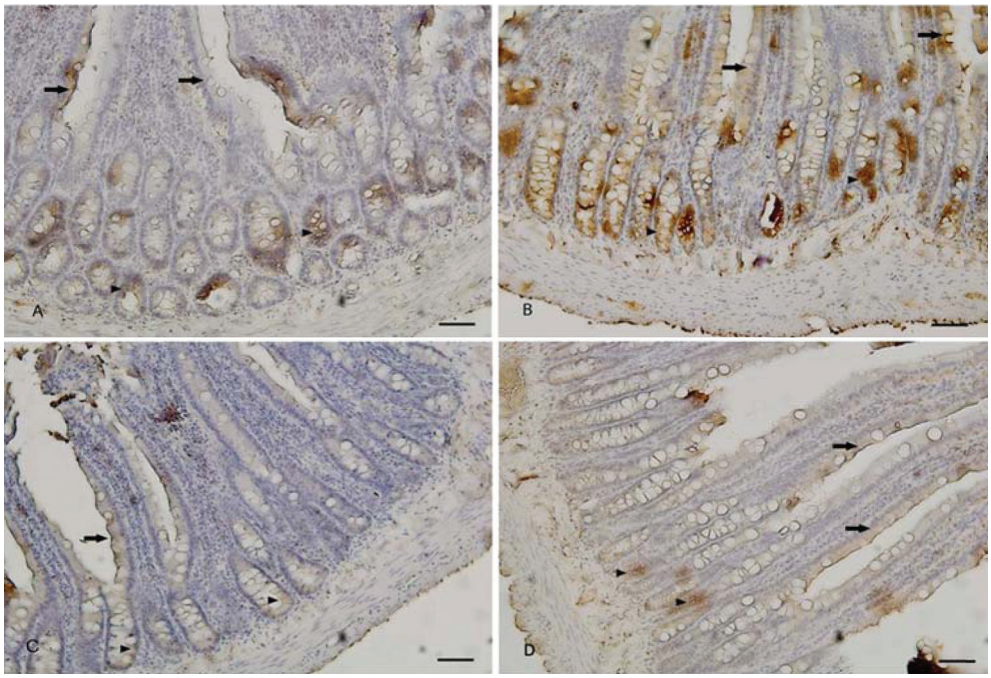

Fig 3. iNOS expression in surface epithelium of the villi *(arrows)* and cripts *(arrow head)*. **A-** Ov group, **B-** P group, **C-** E2 group, **D-** E2+P group, Bar 50 µm

Şekil 3. Villus yüzey epiteli *(ok)* ve kriptlerde *(ok başı)* iNOS ekspresyonu. **A-** Ov grubu, **B-** P grubu, **C-** E2 grubu, **D-** E2+P grubu, Bar 50 µm

Table 1. *nNOS expression scores in duodenal surface epithelium and crypts and nerve fibers, in all groups*
Tablo 1. *Gruplarda duodenum yüzey epiteli, kriptler ve sinir fibrillerinde nNOS enzim skorları*

Group / Expression	Ov	P	E_2	E_2+P	P
Surface epithelium	1.30±0.15	1.80±0.13	1.70±0.15	1.60±0.16	N.S
Crypt region	3.20±0.13a	3.30±0.15a	2.80±0.25a	2.10±0.28b	0.000***
Nerve fibers	3.00±0.21ab	3.50±0.17a	3.30±0.15a	2.70±0.15b	0.013*

*Letters (a, b, c) in the same line indicate significant differences between different letters, * P<0.05, *** P<0.001*

Table 2. *eNOS expression scores in duodenal surface epithelium and crypts in all groups*
Tablo 2. *Gruplarda duodenum yüzey epiteli ve kriptlerde eNOS enzim skorları*

Group / Expression	Ov	P	E_2	E_2+P	P
Surface epithelium	1.60±0.22b	1.70±0.21b	2.70±0.26a	1.70±0.21b	0.004**
Crypt region	3.10±0.10	3.10±0.18	3.10±0.28	2.90±0.10	NS

Letters (a, b, c) in the same line indicate significant differences between different letters, ***NS:*** *not significant, ** P<0.01*

Table 3. *iNOS expression scores in duodenal surface epithelium and crypts in all groups*
Tablo 3. *Gruplarda duodenum yüzey epiteli ve kriptlerde iNOS enzim skorları*

Group / Expression	Ov	P	E_2	E_2+P	P
Surface epithelium	0.50±0.17bc	1.10±0.10a	0.20±0.13c	0.80±0.13ab	0.000***
Crypt region	1.00±0.00b	3.00±0.21a	0.50±0.17c	0.80±0.13bc	0.000***

*Letters (a, b, c) in the same line indicate significant differences between different letters, *** P<0.001*

staining on the surface epithelium of the villi was weaker than that of the crypts *(Fig. 2)*. The intensity of eNOS expression on the surface epithelium was higher in the E2 group than in the Ov, P and E2 + P groups (P<0.01) and no differences were observed in crypts among groups.

Inducible NOS Expression

iNOS expression was weak on the surface epithelium in all groups. The intensity of immunreaction in crypts was weak in the Ov, E2 and E2 + P groups, but strong in the P group *(Fig. 3)*. The intensity of iNOS expression in the

surface epithelium did not change among the Ov and E2 and E2 + P groups. It was greater in the P group compared with the Ov and E2 groups (P<0.001). iNOS expression in the crypt region was significantly increased in the P group compared with the Ov, E2 and E2 + P groups (P<0.001).

DISCUSSION

Recent studies showed that NO was a neurotransmitter in the non-adrenergic non-cholinergic (NANC) inhibitory nerves of the gut [15,19-21]. During nerve stimulation, NO generated by nNOS in nerve terminals regulates the release of vasoactive intestinal polypeptide (VIP), which diffuses to muscle cells to participate in muscle relaxation [22,23]. Our study showed that nNOS was localized in the nerve fibers, and this expression of nNOS in the nerve fibers correlated with other researches [10,24-27].

Previous studies indicated that the release of NO by NANC nerves was an important factor controlling gut motility *in vivo* [8] and in vitro [2,3]. Some research indicated that the nNOS protein [19] and nitrergic activity [15] were increased in nerve fibers in the gastric fundus and colon but not in the ileum in late pregnancy. We observed strong nNOS protein expression in nerve fibers but it did not increase with estrogene or progesterone. Conversely, in our study, a combination of estrogene and progesterone decreased the nNOS reaction, this decrease may be associated with longer (10 day) progesterone treatment. We also observed strong nNOS expression in crypts. The expressions decreased in the E2 + P group. Furthermore, nNOS localized weakly in the Brunner's glands in all groups.

In the present study, eNOS localizations were identified on the epithelial layers of the villi and crypts in the duodenum. The crypts showed a stronger immunoreaction than that of the epithelial layer of the villi but there were no differences between groups. eNOS is the key NOS isoform responsible for NO-regulated vasodilation in the GI system and eNOS-derived NO is produced under basal conditions [28]. eNOS expression is found throughout the gut [11] and lamina propria of villi [10]. Previous research has shown that gastrointestinal smooth muscle cells are able to generate NO by a Ca21-dependent constitutive NOS [29]. And myogenic NO is responsible for the regulation of smooth muscle contractility [30]. Some research claims that NOS isoforms are not expressed by intestinal smooth muscle cells [31]. Bani at al.[27] showed that eNOS and iNOS immunoreactions occur in the circular and longitudinal smooth muscle layers of the ileum in mice, and also found eNOS protein in intestinal smooth muscle cells [32]. In the current study, we did not find any eNOS immunoreaction in duodenum smooth muscle cells. Our results are not consistent with Vannucchi et al.[32] because the technique and antibodies they used differ from ours. Research indicated that inhibition of basal NO production enhanced epithelial mucosal permeability, thus implicating constitutive NO production in the protection of the GI mucosal barrier function [33]. In this study there was also a strong expression in the surface epithelium in the E2 group. NO generated by those cells may play an important role in absorption and the protection of microvasculature. Estrogen may have an important role in the duodenal mucosal system and for epithelial function. Furthermore, we observed strong eNOS expression in the crypts and weak immunostaining in the Brunner glands in all groups. It may have a function in stimulating the high rate of macromolecular synthesis in this active secretory tissue.

The intestinal epithelial cells expressed iNOS in a large amount [34]. Previous research detected a strong iNOS reactivity in the basal and apical side of enterocytes in villi epithelium, in duodenal crypts, in connective tissue cells and in the endothelial cells of lamina propria vessels in human duodenum [35]. iNOS expression in intestinal cells indicates that the isoform of the enzyme is expressed in the small intestine without inflammation. In our study, similarly, an iNOS reaction was found in surface epthelium and duodenal crypts. And these immunoreactions were significantly greater in the surface epithelium and crypts in the P group and it decreased in the crypts in E2 group compared to the Ov group. Crypts represent regions of rapid mitosis and differentiation, providing a continual turnover of cells that differentiate into columnar and goblet cells as they migrate from the crypts towards the luminal end of the villi [36,37]. Previous studies also revealed the presence of iNOS immunoreactivity in myenteric neurons, in smooth muscle cells, and in endothelium of the rat duodenum with postembedding immunoelectronmicroscopy [26]. These findings are different from our result. This difference may be a function of the techniques used.

In conclusion, ovarian steroids may cause some alterations mediated by nNOS, iNOS and eNOS expression on the duodenal mucosal system, differentiation, mucosal barrier function, and on the absorption and protection of microvasculature.

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RESEARCH ARTICLE

Capsular Typing and Antimicrobial Susceptibility of *Pasteurella multocida* Isolated from Different Hosts

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Summary

In this study, 75 *Pasteurella multocida* isolates from different animal species of cattle, sheep, goat and rabbit isolated during 2001-2012 were identified and characterized by multiplex PCR for 5 capsular types of A, B, D, E, and F. Thirty six of isolates were isolated from cattle (19 lungs, 12 nasal swabs and 5 milk samples), 33 from sheep lungs, 3 from goat lungs and 3 from rabbit lungs. Each isolates was obtained from different herds/flocks except 9 bovine nasal swab isolates (6 and 3), which were from two herds. All *P. multocida* isolates identified by conventional methods were confirmed by multiplex PCR. Thirty three (91.6%) of 36 bovine isolates were serogroup A; 11 (33.3%) of 33 sheep isolates were serogroup D, 2 (6.0%) were serogroup A, 1 (3.0%) was serogroup F and 19 (57.5%) were untypable; 2 of 3 goat isolates were serogroup D. All of 3 rabbit isolates were untypable. Overall 26 (34.6%) isolates were untypable. No strain was detected from serogroup B and E. Antimicrobial susceptibility test results of 50 *P. multocida* strains using disk diffusion test showed that all strains were susceptible to ceftiofur, enrofloxacin, florfenicol and trimethoprim-sulfamethoxazole. Six (12.0%) strains were resistant to spectinomycin, 6 (12.0%) to tetracycline, 3 (6.0%) to tilmicosin and 3 (6.0%) to erythromycin. Multiple resistance was detected in 7 (4 tetracycline+spectinomycin, 2 tilmicosin+erythromycin, 1 tetracycline + tilmicosin + erythromycin) of 11 resistant strains isolated from 10 cattle and 1 sheep.

Keywords: *Pasteurella multocida, Capsular typing, PCR, Antimicrobial susceptibility, Cattle, Sheep*

Farklı Hayvan Türlerinden İzole Edilen *Pasteurella multocida* Suşlarının Kapsüler Tiplendirilmesi ve Antimikrobiyal Duyarlılıkları

Özet

Bu çalışmada 2001-2012 yılları arasında sığır, koyun, keçi ve tavşan gibi değişik hayvan türlerinden izole edilen *Pasteurella multocida* suşlarının multipleks PCR ile identifikasyonu ve kapsüler serogrupları (A, B, D, E ve F) araştırıldı. Toplam 75 suşun 36'sı (19 akciğer, 12 burun svabı, 5 süt) sığır, 33'ü koyun, 3'ü keçi ve 3'ü de tavşan akciğerlerinden izole edildi. İki işletmeden alınan 9 (6 ve 3'er adet) sığır burun svabı dışındaki örneklerin her biri farklı işletmelerden gelen hayvanlara aitti. Konvansiyonel yöntemlerle *P. multocida* olarak identifiye edilen suşların tümü multipleks PCR ile doğrulandı. Kapsüler tiplendirme sonucu 36 sığır suşunun 33 (%91.6)'ü serogroup A; 33 koyun suşunun 11 (%33.3)'i serogrup D, 2 (%6.0)'si A, 1(%3.0)'i F olarak belirlendi ve 19 (%57.5)'u tiplendirilemedi; 3 keçi suşunun 2'si serogrup D olarak tiplendirildi; 3 tavşan suşunun hiçbiri tiplendirilemedi. Toplam 26 (%34.6) suş tiplendirilemedi. Serogrup B ve E'ye ait suş tespit edilmedi. Disk difüzyon yöntemiyle 50 suşa yapılan antimikrobiyal duyarlılık test sonucunda suşların tümü ceftiofur, enrofloxacin, florfenicol ve trimethoprim-sulfamethoxazole'e duyarlı bulundu. Spectinomycin ve tetracycline'e 6 (%12.0)'şar suş, tilmicosin ve erythromycin'e 3 (%6.0)'er, suş dirençli bulundu. Dirençli 11 (10 sığır, 1 koyun) suşun 7'sinde çoklu direnç (4 suşta tetracycline + spectinomycin, 2 suşta tilmicosin + erythromycin, 1 suşta tetracycline + tilmicosin + erythromycin) tespit edildi.

Anahtar sözcükler: *Pasteurella multocida, Kapsüler tiplendirme, PCR, Antimikrobiyal duyarlılık, Sığır, Koyun*

INTRODUCTION

Pasteurella multocida causes important diseases as primary or secondary pathogen in many domestic or wild mammalian and avian hosts. Major diseases include haemorrhagic septicaemia in cattle and buffaloes, atrophic

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rhinitis in swine, snuffles in rabbit and pneumonias in cattle and sheep and, fowl cholera in poultry [1,2]. Occasionally, it can cause mastitis, localized infections and abortion in cattle [3]. *P. multocida* is also commonly found in the upper respiratory tract flora of many animal species [4-6]. Capsule and lipopolysaccaride (LPS) are the major components of *P. multocida* cell surface [7]. *P. multocida* are classified into 5 serogroups as A, B, D, E, and F based on capsule structures and further 16 serotypes based on LPS composition [1]. Capsule is considered as one of important virulence determinants that allow *P. multocida* to avoid innate host defense systems [4,8,9]. All *P. multocida* capsules are carbohydrate polymers, but composition of capsule is different in each serogroup. It was shown that, capsular material of serogroup A strains contains hyaluronic acid, serogroup D heparine, serogroup F chondroitin sulfate, serogroup B was composed of arabinose, mannose (or N-acetyl mannosaminuronic acid) and galactose [1,7,8,10]. Conflicting reports exist on capsular polysaccharides of *P. multocida* as protective antigen. Although pure polysaccharides are nonimmunogenic, antigens responsible for the capsule specific reactions are not completely understood [1,10]. It was suggested that immunogenicity of capsule is associated with LPS and purified capsule antigens behave as haptens in certain animals [1].

Capsular serogrouping is frequently used for typing of *P. multocida,* and capsular types generally correlate with particular disease and host [4,8]. The original and most common method for capsular serogrouping is indirect haemagglutination test. One of the limitations of the test is difficulties in preparation of high titer specific capsule antisera and their commercially unavailability [1]. In addition, inagglutinability of some capsulated fresh cultures with homolog capsular antiserum or reduced capsular material by serial subculture often renders such strains untypable in this method [1,6]. These problems of indirect haemagglutination test caused to attempts to devise non-serological tests. Non-serological tests such as hyaluronidase for serogroup A and acriflavin flocculation test for serogroup D were shown to be unreliable in a comparative study with PCR [11]. Multiplex PCR facilitates capsular serogrouping and provides fast, reliable and inexpensive typing [10].

Respiratory diseases including *P. multocida* infections in livestock are one of the most common causes of antimicrobial use for prophylaxis, metaphylaxis and treatment. However, imprudent use of antimicrobials creates a high risk of selecting resistance [12,13]. Continuous monitoring of antimicrobial susceptibilities of important animal pathogens with standard methods provides useful information on trends in resistance development [14]. In practice, in the case of respiratory problems veterinarians prefer to start antimicrobial treatment immediately, before disease does not progress. Therefore national or regional antimicrobial susceptibility data can help practitioners in their initial antibiotic choice.

Little is known about the serotype and antimicrobial susceptibility profiles of *P. multocida* isolates in Turkey. Except very few studies in cattle performed by conventional serological methods [15,16], no study is present about capsular types of *P. multocida* from animal species using molecular methods. In other countries most of studies were carried out with bovine isolates and little is known about serogroups and antimicrobial susceptibilities of *P. multocida* isolated from small ruminants. The aim of this study was to investigate capsular serogroups of *P. multocida* isolated from different animal species during 2001-2012, by multiplex PCR and to determine their antimicrobial susceptibility profiles.

MATERIAL and METHODS

Samples

A total of 75 *P. multocida* isolated from cattle, sheep, goat and rabbit submitted to diagnostic laboratory of Konya Veterinary Control Institute during 2001-2012 were tested *(Table 1)*. Among 75 isolates, 36 were from cattle (19 lung, 12 nasal swab, and 5 milk samples), 33 from sheep lungs, 3 from goat lungs and 3 from rabbit lungs. Except 6 and 3 nasal swab samples of calves taken from per herd, each isolate was belonging to animals from different herds. All lungs and nasal swab samples were from animals (aged 1-7 months) with respiratory problems as evidenced by clinical, macroscopic and microscopic findings after necropsy and, milk samples from animals with mastitis. Samples submitted mainly from Konya province in Central

***Table 1**. Distribution of P. multocida isolates according to hosts species and years*
***Tablo 1.** P. multocida suşlarının konakçı türleri ve yıllara göre dağılımı*

Years	Animal Species				Total Number of Isolates
	Cattle	Sheep	Goat	Rabbit	
2001	1	2			3
2002	2	1			3
2003	2	1		3	6
2004	3	6	1		10
2005	3		2		5
2006	5	2			7
2007	2	1			3
2008	1	4			5
2009	10	14			24
2010	2				2
2011	3	1			4
2012	2	1			3
Total	36	33	3	3	75

Anatolia, however, in recent years bovine herds are enlarging and animals from other provinces are gathered into herds, because of the widespread animal movements, origin of some animals may belong to other provinces. No reliable information was available for previous antibiotic use in animals.

Isolation and Identification

Swab and organ specimens were plated onto blood agar base No.2 (Oxoid) with 5% sheep blood. Agar plates were incubated at 37°C overnight. Presumptive identification of isolates as *P. multocida* was made on the basis of colony characteristics, no haemolysis on blood agar, no growth on MacConkey agar, Gram staining and microscopic morphology, positive catalase and oxidase tests [1,17]. Following presumptive identification, isolates were stored at -85°C in Microbank (Pro-Lab Diagnostics) tubes.

PCR for Identification and Capsular Typing

For the confirmation of isolates and determination of their capsular types, a multiplex PCR using primers specific for *P. multocida* [18] and for 5 capsular serogroups [10] based on capsule biosynthesis genes were used. To perform PCR, isolates were recovered from stocked cultures by streaking on blood agar, after overnight incubation at 37°C several colonies were suspended in PCR grade water and used as template following incubation at 100°C for 10 min.

PCR mixture contained 2 mM $MgCl_2$, 200 µM dNTPs, 3.2 pmol of each primers (*Cap*A, B, D, E and F; KMT1T7, KMT1SP6), 1.25 U Taq DNA polymerase (Bioron), 5 µl template DNA in total volume of 50 µl. Cycling parameters were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 90 sec. Products were electrophoresed on 1.5% agarose gel in TBE containing 0.5 µg/ml ethidium bromide for 1 h at 90 V, with 100 bp DNA ladder. All isolates were also tested second time without *P. multocida*-specific primers (KMT1T7, KMT1SP6) using only capsular primers to get clear amplicon bands for capsular serogroups. The vaccine strain of serogroup B provided from Pendik Veterinary Control Institute, İstanbul was used as control strain.

Antimicrobial Susceptibility Testing

A total of 50 *P. multocida* strains from 24 cattle, 20 sheep, 3 goats and 3 rabbits were tested by disc diffusion test in Mueller-Hinton agar (Oxoid) supplemented with 5% defibrinated sheep blood according to Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, CLSI: M31-A3 [19]. *S. aureus* ATCC 25923 was used as quality control strain. Following antimicrobial discs (Oxoid) were used: spectinomycin, ceftiofur, enrofloxacin, trimethoprim-sulfamethoxazole, tilmicosin, florfenicol, tetracycline, erythromycin and penicillin. To interpret results zone diameters described in CLSI: M31-A3 were used *(Table 2)*.

RESULTS

All isolates were confirmed as *P. multocida* which produced about 460 bp *P. multocida*-specific fragment in multiplex PCR *(Fig. 1)*. In capsular typing, 33 of 36 bovine isolates were determined as serogroup A. Among bovine isolates, only 2 milk and 1 nasal swab samples were untypable. Of 33 sheep isolates 11 (33.3%) were serogroup D, 2 (6.0%) serogroup A, 1 (3.0%) serogroup F and 19 (57.5%) was untypable *(Table 3)*. Two of 3 goat isolates were serogroup D. All of 3 rabbit isolates were untypable. Serogroup B and E were not determined. An amplified product of about 1044 bp, 657 bp, 851 bp and 760 bp for serogroup A, D and F strains, and for serogroup B of the control strain were obtained respectively (*Fig. 1, 2* and *3*). When samples were tested without *P. multocida* specific

Table 2. *Zone diameters and interpretation criteria of tested antimicrobial agents according to CLSI Standard M31-A3 (2008) used in the study*
Tablo 2. *Test edilen antimikrobiyal etkenlerin CLSI Standardı M31-A3 (2008)'de belirtilen zon çapları ve değerlendirme kriterleri*

Antimicrobial Agent	Disk Content	CLSI Zone Diameter (mm)		
		Susceptible	Intermediate	Resistant
Spectinomycin	100 µg	≥ 14	11-13	≤ 10
Ceftiofur	30 µg	≥ 21	18-20	≤ 17
Enrofloxacin	5 µg	≥ 21	17-20	≤ 16
Trimethoprim-sulfamethoxazole*	1.25/23.75 µg	≥ 16	11-15	≤ 10
Tilmicosin	15 µg	≥ 11	-	≤ 10
Florfenicol	30 µg	≥ 19	15-18	≤ 14
Tetracycline*	30 µg	≥ 19	15-18	≤ 14
Erythromycin*	15 µg	≥ 23	14-22	≤ 13
Penicillin*	10 units	≥ 29 ≥ 15	- -	≤28 (for Staphylococci) ≤ 14 (for Enterococci)

** Because no zone diameter was determined specifically for P. multocida in CLSI Standard, zone diameters defined for other bacteria were used*

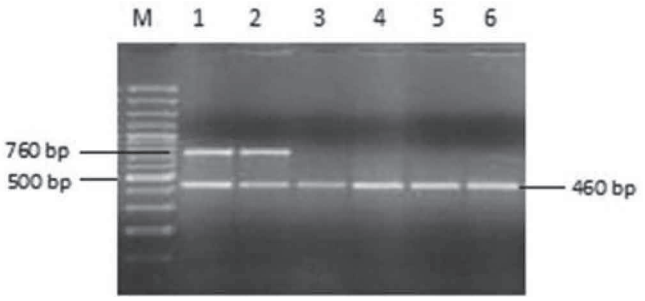

Fig 1. Multiplex PCR for *P. multocida*. M: 100 bp marker; Lane 1-2: Positive control (capsular type B), *P. multocida* specific 460 bp+760 bp bands; Lane 3-6: Untypable isolates, only *P. multocida* specific 460 bp bands

Şekil 1. *P. multocida* multipleks PCR. M: 100 bp marker; 1-2: Pozitif kontrol (kapsüler tip B), *P. multocida* spesifik 460 bp+760 bp bandlar; 3-6: Tiplendirilemeyen *P. multocida* suşları, sadece *P. multocida* spesifik 460 bp bandlar

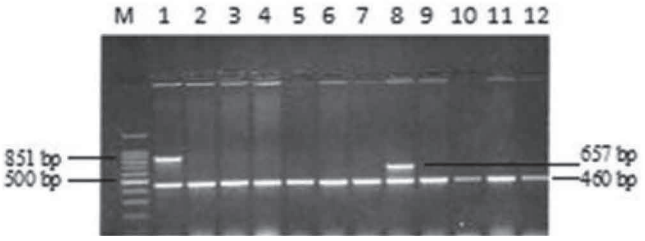

Fig 2. Multiplex PCR with *P. multocida* isolated from sheep. M: 100 bp marker; Lane 1-12: *P. multocida* specific 460 bp band; Lane 1: 460 bp+851 bp bands (capsular type F); Lane 8: 460 bp+657 bp bands (capsular type D)

Şekil 2. Koyunlardan izole edilen *P. multocida* suşları ile multipleks PCR. M: 100 bp marker; 1-12: *P. multocida* spesifik 460 bp band; 1: 460 bp+851 bp bandlar (kapsüler tip F); 8: 460 bp+657 bp bandlar (kapsüler tip D)

primers (only by capsular primers) in multiplex PCR, number of serogroup A positive samples increased from 1 to 33 *(Fig. 3)* and number of serogroup D and F positive samples did not changed.

In 9 (9/75) cases (in 5 sheep, 3 calf and 1 kid lung samples) *P. multocida* was isolated together with *Mannheimia haemolytica* and in 4 cases (in 2 sheep, 1 calf and 1 kid lung samples) it was isolated with *Arcanobacterium (Trueperella) pyogenes*.

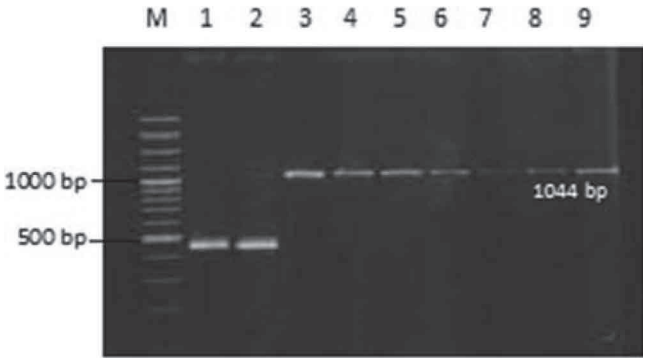

Fig 3. Multiplex and capsular PCR for *P. multocida*. M: 100 bp marker; Lane 1-2: Products of multiplex PCR including primers specific for *P. multocida* and for 5 capsular types (only 460 bp bands); Lane 3-9: Products of PCR with primers for capsular serogroups without *P. multocida* specific primers (capsular type A specific 1044 bp bands)

Şekil 3. *P. multocida* multipleks ve kapsüler PCR. M: 100 bp marker; 1-2: *P. multocida* spesifik ve 5 kapsüler primeri içeren multipleks PCR ürünleri (sadece *P. multocida* spesifik 460 bp bandlar); 3-9: Sadece 5 kapsüler serogrup primerleri ile yapılan PCR ürünleri (kapsüler tip A spesifik 1044 bp bandlar)

Antimicrobial susceptibility test results of 50 *P. multocida* strains from 24 cattle, 20 sheep, 3 goats and 3 rabbits are shown in *Table 4*. All isolates were susceptible to ceftiofur, enrofloxacin, florfenicol and trimethoprim-sulfamethoxazole. All isolates had ≥ 20 mm zone diameter for penicillin. In total, 11 strains were resistant to antimicrobial agents and 7 of them were multiresistant *(Table 5)*.

DISCUSSION

Studies indicate that *P. multocida* is one of the most common bacterial pathogens of bovine respiratory disease complex in different countries [20-23] and even in some studies its isolation from calf pneumonia appear to be increasing [24]. In a previous study carried out in Konya province [25], and in other studies from Turkey [26,27], isolation rate of *P. multocida* from pneumonic cattle and sheep lungs was found higher than that of *M. haemolytica*. In the present study, in 12% (9/75) of cases *P. multocida* was

Table 3. *Multiplex capsular PCR results of P. multocida isolates from different animal species*
Tablo 3. *Farklı hayvan türlerinden izole edilen P. multocida suşlarının multipleks kapsüler PCR sonuçları*

Animal Species	No. of Isolates	Isolated Samples	Capsular PCR Typed Isolates		Untypable Isolates	
			No	%	No	%
Cattle	36	19 Lung	19 serogroup A	100	-	8.3
		12 Nasal swab	11 serogroup A	91.6	1	
		5 Milk	3 serogroup A	60.0	2	
Sheep	33	Lung	11 serogroup D	42.4	19	57.5
			2 serogroup A			
			1 serogroup F			
Goat	3	Lung	2 serogroup D	66.6	1	33.3
Rabbit	3	Lung	-	-	3	100
Total	75		49	65.3	26	34.6

Table 4. *Antimicrobial susceptibility test results of 50 P. multocida strains isolated from cattle (24), sheep (20), goat (3) and rabbit (3) during 2001-2012*

Tablo 4. *2001-2012 yıllarında sığır (24), koyun (20), keçi (3) ve tavşan (3)'lardan izole edilen 50 P. multocida suşunun antimikrobiyal duyarlılık test sonuçları*

Antimicrobial Agent	Number of Strains		
	Susceptible	Intermediate	Resistant
Spectinomycin	44 (88%)	-	6 (12%)
Ceftiofur	50 (100%)	-	-
Enrofloxacin	50 (100%)	-	-
Trimethoprim-sulfamethoxazole	50 (100%)	-	-
Tilmicosin	38 (76%)	9 (18%)	3 (6%)
Florfenicol	50 (100%)	-	-
Tetracycline	42 (84%)	2 (4%)	6 (12%)
Erythromycin	7 (14%)	40 (80%)	3 (6%)
Penicillin	50 strains: ≥ 20 mm (22: 20-24 mm, 28: ≥ 25 mm)		

however, no such an interference was occurred with the other serogroups.

In the study, 91.6% (100% of lung isolates) of bovine isolates were capsular type A and only 3 isolates from 2 milk and 1 nasal swab samples were untypable. Similarly, studies from different countries showed that most of *P. multocida* isolates from cattle with respiratory disease complex including neonatal calf pneumonia and shipping fever were capsular type A and occasionally other capsular types have been isolated [28-31].

Contrary to previous studies [15,16] in Turkey which were carried out by conventional methods, capsular type B strains were not detected in this study. Serogroup B and E strains reported to cause haemorrhagic septicaemia generally in Asian and African countries respectively. An inactivated vaccine with Robert type 1 strain (serogroup B) was produced in Pendik Veterinary Control Institute, İstanbul until recently. As far as we know, in cattle no case

Table 5. *Properties and resistance profiles of P. multocida strains found resistant in antimicrobial susceptibility testing*

Tablo 5. *Antimikrobiyal duyarlılık testinde dirençli bulunan P. multocida suşlarının özellikleri ve direnç profilleri*

Resistant Strains	Isolation Year	Isolated Animal Species	Samples	Capsular Serogroup	Resistance Profile
1	2002	Cattle	Nasal svab	A	Tetracycline
2	2003	Cattle	Milk	A	Tetracycline + Spectinomycin
3	2003	Cattle	Lung	A	Tetracycline + Spectinomycin
4	2004	Cattle	Lung	A	Spectinomycin
6	2006	Cattle	Lung	A	Tilmicosin + Erythromycin
7	2007	Cattle	Lung	A	Tetracycline + Spectinomycin
8	2007	Cattle	Lung	A	Tetracycline + Spectinomycin
9	2008	Cattle	Milk	A	Spectinomycin
10	2011	Cattle	Lung	A	Tetracycline + Tilmicosin + Erythromycin
11	2006	Sheep	Lung	Untypable	Tilmicosin + Erythromycin

isolated together with *M. haemolytica* and in 4 cases it was isolated with *A. (Trueperella) pyogenes*. Results indicated that in majority (62/75) of samples *P. multocida* was isolated alone.

In this study, *P. multocida* isolates previously identified by phenotypic properties were confirmed by multiplex PCR. Capsular PCR method reported to be used reliably determining capsular serogroups of bovine, ovine, porcine and avian *P. multocida* isolates [6,28]. Results showed that in the multiplex PCR, *P. multocida* specific primers can be used in combination with 5 set of capsular primers except with serogroup A strains. *P. multocida* specific primers interfered with serogroup A primers and resulted in no or weak band. When all isolates were tested one more time using only capsular set of primers, serogroup A specific bands appeared in previously negative bovine isolates, related with haemorrhagic septicemia symptoms was observed for a long time in Turkey and the vaccine is not produced anymore. Capsular typing provides helpful information about distribution of isolates in different host with different disease conditions. It has been observed that haemorrhagic septicemia and pneumonic pasteurellosis are frequently confused. Use of reliable methods for capsular typing is quite important to determine real disease conditions in animal species. In this respect PCR is quite helpful.

In the study, 11 (33.3%) of 33 sheep isolates were serogroup D, 2 (6%) serogroup A, 1 (3%) serogroup F and majority of them (57.5%) were untypable. Similar to sheep isolates, 2 of 3 goat isolates were serogroup D. Little information is present worldwide about capsular serogroups of *P. multocida* from sheep and goats. In several

studies in Turkey, *P. multocida* was the most commonly isolated bacterial agent from sheep pneumonias [27,32]. Nevertheless, no study is present about capsular types of *P. multocida* from small ruminants in Turkey. Few studies examining limited number of isolates showed that in small ruminants, serogroup D [11] and A [30,33] were commonly detected capsular types. Capsular type D so far most frequently isolated from porcine atrophic rhinitis. Sheep and goat isolates of capsular type D should be further characterized for differentiation from porcine isolates.

Antimicrobial susceptibility test with commonly used antimicrobial agents for the therapy of respiratory diseases of livestock animals, such as enrofloxacin, florfenicol, ceftiofur, oxytetracycline, trimethoprim-sulfamethoxazole, tilmicosin, spectinomycin and erythromycin showed that resistance development among *P. multocida* isolates was generally low. Resistance was determined against spectinomycin, tetracycline, tilmicosin and erythromycin. Other studies [24,34,35] also pointed out that, tetracycline, tilmicosin and spectinomycin were less susceptible antimicrobial agents for *P. multocida* strains. In the study, majority of isolates (76%) were intermediately susceptible to erythromycin according to taken criteria *(Table 2)*. Similarly, in other studies [33,36], high rate of intermediate strains was reported among *P. multocida* strains. In other studies in Turkey, in sheep [27] and in cattle [36] resistance development was not high and erythromycin was less susceptible antimicrobial agent. This is not surprising, because erythromycin is an antimicrobial agent susceptible mostly for Gram positive microorganisms.

It is known that occurrence of antimicrobial resistance varies between countries and regions. Antimicrobial susceptibility data of *P. multocida* isolated from cattle over a three year period (2002-2004) in different European countries showed that resistance against *P. multocida* isolates were generally low and most common resistance observed against tetracycline which was 24% in Italy, 20% in Holland, 12% in Denmark and France in some years [14]. In a monitoring study [37] in Germany in 2002-2003, only 1.5% of 132 bovine *P. multocida* isolates were found resistant against ceftiofur and enrofloxacin. In a 4 year survey [38] in the USA including 318 *P. multocida* from cattle, similar to our study, it was reported that all strains were susceptible ceftiofur, highest resistance were detected against erythromycin (84%) followed by tilmicosin (41.1%), tetracycline (28.9%) and spectinomycin (23.6%). In the Netherland study [39], in agreement with this study, no resistance to ceftiofur and florfenicol but higher resistance to tetracycline was reported. In this study, while no resistance was detected against enrofloxacin and ceftiofur, tetracycline resistance was not as high as in other countries. High resistance rates against some antimicrobial agents may be due to intensive production systems and common use of antimicrobial in these types of productions. There is limited study on small ruminants. In a study [40] all of 28 *P. multocida* isolated from sheep and goats was reported to be susceptible to ceftiofur and florfenicol.

In the study, 11 (22%) of 50 isolates were resistant at least one antimicrobial agent. Among these resistant isolates, majority (10/11) were from cattle 24 strains and only 1 was from sheep 20 strains. This may be related to more antimicrobial use in cattle populations than in sheep populations, generally. While 10 of all bovine strains were serogroup A, one sheep strain was untypable. Resistant strains were not cumulated in any specific year.

In conclusion, results of the study provide information about capsular types of *P. multocida* isolates mainly from pneumonic cattle and sheep by using PCR method in Turkey. In cattle, majority (33/36) of isolates was capsular type A and 8.3% was untypable. In sheep and goat, capsular type D was predominant (13/36) and 55.5% was untypable. In general, antimicrobial resistance development to commonly used antimicrobial agents for respiratory diseases was not high among *P. multocida* isolates.

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RESEARCH ARTICLE

Sunset Yellow FCF' nin Tavuk Embriyosu Deri ve Barsak Mast Hücrelerinin Degranülasyonu Üzerindeki Etkileri [1]

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Özet

Bu çalışmada, hazır gıdalarda yapay renklendirici olarak kullanılan sunset yellow'un (E110) tavuk embriyosu dermal ve mukozal mast hücrelerindeki degranülasyon etkisi histolojik yönden incelenmiştir. Sunset yellow (2.5 mg/kg) inkübasyonun 15. gününde vitellusa enjekte edilmiştir. Enjeksiyonundan 6, 12 ve 24 saat sonra dermal ve mukozal mast hücrelerindeki degranülasyon etkisi incelenmiştir. Dermal mast hücrelerinde (deri) en fazla kısmi degranülasyon sunset yellow enjeksiyondan 12 saat, mukozal mast hücrelerinde (barsak) 6 saat sonra, en fazla ileri düzeyde degranülasyon ise deride enjeksiyondan 24 saat, barsakta 12 saat sonra gözlenmiştir. Barsaktaki mukozal mast hücrelerinde ileri degranülasyonun enjeksiyondan 24 saat sonra da devam ettiği belirlenmiştir. Her iki dokuda kısmi degranüle mast hücresi granüllerinin gevşek ve kaba granüller oluşturduğu, ileri degranülasyon gösteren mast hücrelerinin ise daha az granül içerdiklerinden dolayı daha aydınlık sitoplazmaya sahip oldukları görülmüştür.

Anahtar sözcükler: *Sunset yellow, Tavuk embriyosu, Mast hücresi, Deri, Barsak*

Degranulation Effects of Sunset Yellow-FCF on Dermal and Intestinal Mast Cells of Chicken Embryo

Summary

In this study, the degranulation effect of sunset yellow (E110) which is used as artificial coloring agent in ready food was histologically examined on dermal and mucosal mast cells of chicken embryo. Sunset yellow (2.5 mg/kg) was injected into vitellus of eggs on the 15th day of incubation. Degranulation of dermal and mucosal mast cells were evaluated 6, 12, 24 h after injection. The most of partial degranulated in dermal mast cells (skin) was observed after 12 h from injection of sunset yellow, and after 6 h in mucosal mast cells (intestine). High level degranulation in skin was showed after 24 h from injection, and after 12 h in intestine. Also, it was seen that high level degranulation in the mucosal mast cells of intestine were continued after 24 h from injection. In both types of tissues, it was found that partially degranulated mast cells formed loose and coarse granules while mast cells showing high level of degranulation have brighter cytoplasm since they contain less granuless.

Keywords: *Sunset yellow, Chicken embryo, Mast cell, Skin, Intestine*

GİRİŞ

Gıda boyaları, gıda katkı maddeleri içerisinde önemli bir grubu oluşturmakta ve gıdaların cazibesinin artışında önemli bir rol oynamaktadır [1]. Sunset yellow hazır gıdalarda renklendirici olarak kullanılan ve E kodu 110 ile tanınan gıda renk maddelerinden biridir. Oldukça kompleks kimyasal yapıya sahip bir azo boyasıdır. Monoazo sınıfından olan sunset yellow'un, Cl Food Yellow 3, Orange Yellow S, Cl (1975) no. 15985. INS No.110 isimleri de bulunmaktadır. Kimyasal adı ise Disodium 6-hydroxy-5-(4-sulfonato-phenylazo)-2-naphthalene-sulfonate'dır. Kimyasal formülü $C_{16}H_{10}N_2Na_2O_7S_2$, molekül ağırlığı 452.38 g/mol' dür [2]. Sunset yellowun kullanıldığı gıdalar arasında portakal suyu, jel şekerlemeler, tahıl, pasta, tatlı, çerez, dondurma ve konserve balık bulunur. Ayrıca Berocca, Polaramine ve Ventolin şurup gibi ilaçların üretiminde kullanılır [3,4]. Kabul edilebilir günlük alım miktarı: vücut ağırlığı üzerinden 2.5 mg/kg'dır [2].

Gıdalarda renk maddelerinin araştırıldığı çeşitli çalışmalarda, kullanımına izin verilen renk maddelerinin yüksek

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miktarda kullanıldığı ve ayrıca izin verilmeyen renk maddelerine de rastlanıldığı rapor edilmiştir [5-7]. Sokakta satılan tüketime hazır gıdalarda tip, boyut ve kullanılan renkler üzerine yapılan bir araştırmada incelenen 545 numunenin büyük bir kısmında izin verilen oranların aşıldığını, en çok kullanılan gıda boyasının da tartrazin ve sunset yellow olduğunu saptanmıştır [8].

Azo boyaları ve diğer katkı maddelerinin sebep olduğu rahatsızlıklar arasında ürtiker, astım, rhinitis, angioedema, purpura ve nadir olarak da anaflaktik şok, baş ağrısı ve gastrointestinal bozukluklar sayılmaktadır [9,10]. Ürtikerli veya anjioedema'lı hastaların Allura Red AC, Amaranth, Sunset Yellow FCF, Ponceau 4R ve tartrazine hassasiyetleri rapor edilmiştir [11]. Başka bir çalışmada kronik veya kronikleşmenin nüksettiği ürtikerli hastaların %4'ünde intolerans tanımlanmış ve bunun gıda katkı maddelerinden (benzoatlar, sorbik asit, tartrazine, sunset yellow) kaynaklandığı açıklanmıştır [12]. Üç yaşındaki hiperaktif çocuklar üzerinde yapılan bir araştırmada, diyetten yapay renklendiriciler (tartrazin, azorubin, sunset yellow, ponceau-4R) ve koruyucu maddelerden sodyum benzoat çıkarıldığında, hiperaktivitenin düzeldiği, bu maddeleri içeren içeceklerin verilmesiyle davranış bozukluklarının tekrar ortaya çıktığı belirlenmiştir [13].

Mast hücreleri, salgı granüllerinin içerdikleri histamin ve serotonin gibi biyolojik aminler, heparin gibi proteoglikanlar, enzimler, prostaglandinler ve lökotrienler gibi araşidonik asit ürünleri ve bir çok interlökin içerikli sitokinler ile bağışıklık sisteminin düzenlenmesi ve vücut savunmasında önemli rol oynarlar [14]. Bu hücreler doğal ve kazanılmış bağışıklıkta vücudun ihtiyacına göre rol almakta, çevre şartlarına bağlı olarak kendini göreve hazırlayabilmektedir. Aralarında katkı maddelerinin de bulunduğu birçok etken, yangıda rol oynayan medyatörlerin salıverilmesini uyarabilmektedir. Katkı maddelerine karşı gelişen allerjik reaksiyonlarda birincil aracı madde, mast hücrelerinden salınan histamindir [15,16]. Histaminin yanı sıra prostaglandinler, leukotrienler, bradikininler ve lökosit inhibitör faktörün de (LIF) katkı maddelerine karşı meydana gelen reaksiyonlarda payının olabileceğini gösteren çalışmalar bulunmaktadır [17-19].

Bu çalışma; sunset yellow'un allerjik reaksiyonlarda önemli rol oynayan mast hücrelerinin degranülasyonu üzerindeki etkisinin araştırılması ve söz konusu katkı maddesini içeren gıdaların özellikle alerjik bünyeli ve gebelik gibi hassas dönemlerdeki tüketiciler tarafından daha bilinçli kullanılması hususunda ön bilgiler sağlayacağı düşüncesi ile gerçekleştirilmiştir.

MATERYAL ve METOT

Bu çalışma, Adnan Menderes Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'nun onayıyla gerçekleştirilmiştir (Onay No: 2011-009). Çalışmada kullanılan SPF (specific pathogen free) yumurtalar (Leghorn ırkı), T.C. Tarım ve Köy İşleri Bakanlığı Bornova/İZMİR Veteriner Kontrol ve Araştırma Enstitüsü Müdürlüğü'nden temin edilmiştir. İnkübasyonun 15. gününde enstitüden alınan döllü tavuk yumurtaları laboratuvara getirildikten ve ağırlıkları belirlendikten sonra kuluçka makinesine yerleştirilmiş, ortama adapte olmaları için yaklaşık iki saat beklenmiştir.

Kontrol grubu ikiye ayrılmıştır. I. grup hiç açılmayan ve 6, 12, 24 saat için 5'er adetten toplam 15 yumurtadan, II. grup ise sunset yellow için çözücü olarak kullanılan distile su enjeksiyonu yapılan ve 6, 12, 24 saat için 10ar adetten toplam 30 yumurtadan oluşturulmuştur. Distile su kontrol grubunda yumurtalar açıldıktan sonra 0.1 ml distile su G27 iğne ile enjekte edildikten sonra parafilmle kapatılmıştır. Daha sonra hiç açılmayan kontrol grubu ve distile kontrol grubu yumurtaları kuluçka makinesine (37.5°C, %60-80 nem) (Brinsea Octagon-40DX) yerleştirilmiştir.

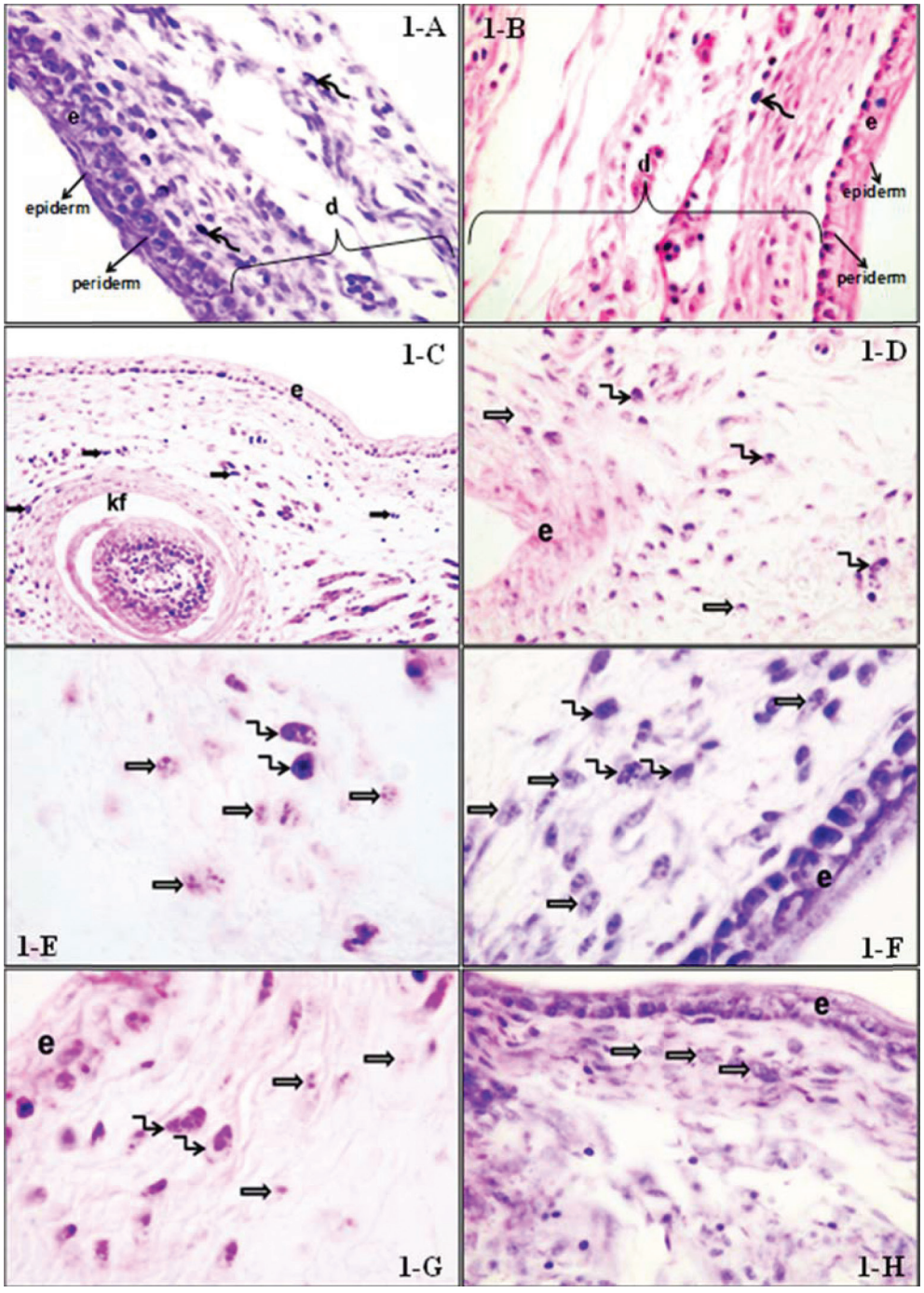
Deney gruplarını oluşturan gelişiminin 15. günündeki embriyolu tavuk yumurtalarına (6, 12, 24 saat için 15er adetten toplam 45 yumurta), sunset yellow (Aldrich, CAS No: 2783-94-0) Türk Gıda Kodeksin'de [20] önerilen doz olan 2.5 mg/kg olarak uygulanmıştır. Bu uygulama için önce yumurtalar tartılarak ortalama ağırlıkları belirlenmiştir. Daha sonra sunset yellow çözeltisi 0.1 ml de 2.5 mg/kg olacak şekilde distile su ile hazırlanmıştır. Hazırlanan bu çözeltiden 0.1 ml alınarak G27 iğne ile vitellus kesesine enjekte edilmiştir ve parafilmle kapatılmıştır. Maddenin uygulanmasından 6, 12 ve 24 saat sonra yumurtalar açılarak çıkarılan embriyolar serum fizyolojik (%0.9 $NaCl_2$) ile vitellustan arındırılmıştır.

Histolojik preparasyon için embriyolardan alınan deri ve barsak örnekleri Saint-Marie tespit çözeltisi (99 ml %95 alkol + 1 ml glasial asetik asit) ile 4°C'de 24 saat tespit edildikten sonra rutin histolojik preparasyon işlemleri (dehidrasyon, bloklama, kesit alma) yapılmıştır. Kesitler rotary mikrotomda 5 µm kalınlığında alınmış ve mast hücre proteoglikanlarını (histamin, heparin, serotonin) belirlemek için toluidin mavisi, metilen mavisi ve modifiye giemsa ile boyanmıştır [21]. Deri ve barsak doku örneklerinden hazırlanan preparatlar ışık mikroskobuyla (Olympus BX51) incelenerek farklı büyütmelerde fotoğrafları (Olympus E-330 digital kamera) çekilmiştir. Ayrıca tüm grupların doku kesitlerine ait preparatlar kör yöntemi ile incelenmiş ve mast hücrelerindeki degranülasyon (+); daha az, (++), az, (+++); orta, (++++); çok, (+++++); çok fazla şeklinde semi-kantitatif olarak analiz edilmiştir.

BULGULAR

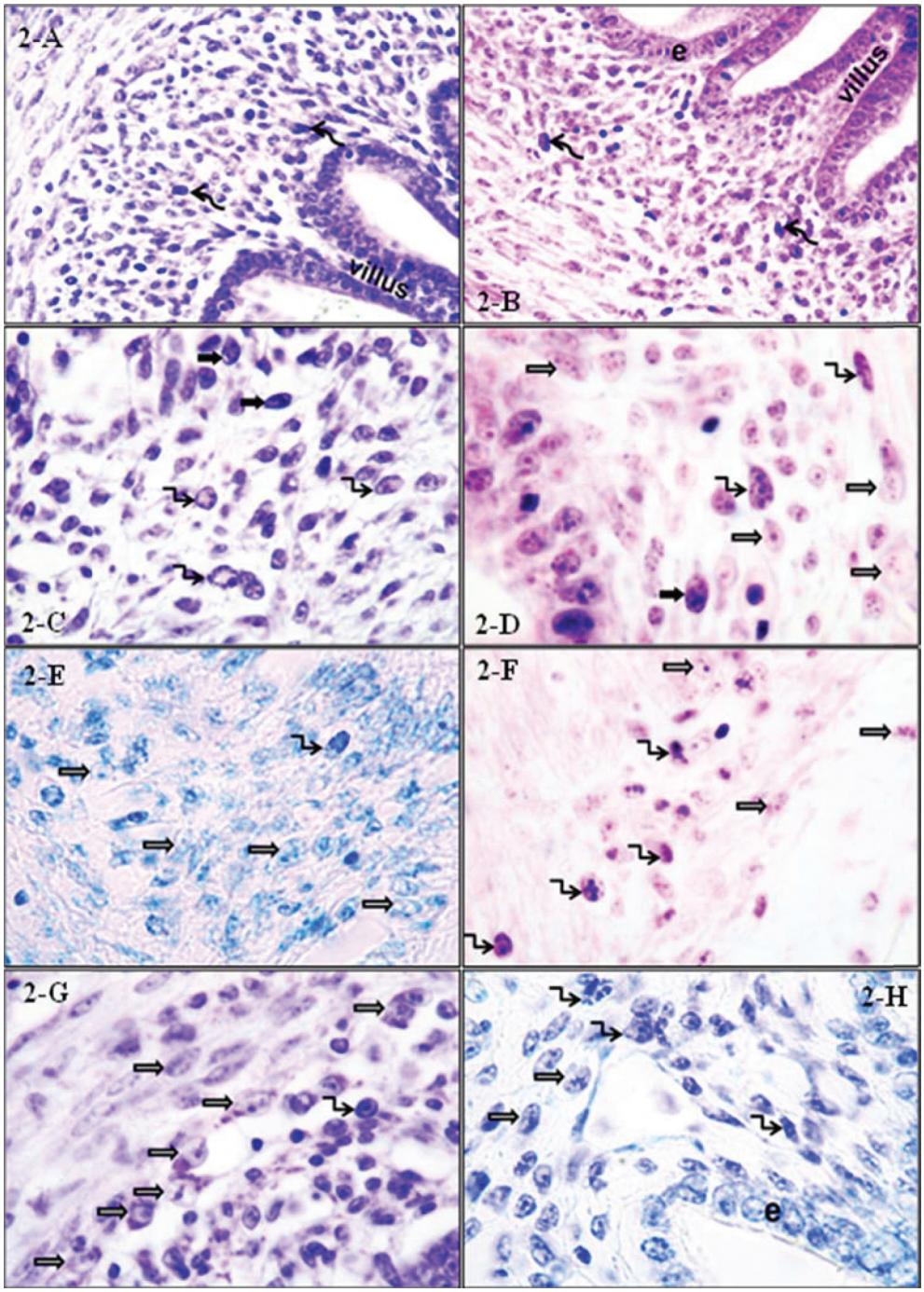
Yapılan incelemeler sonucunda, normal-kontrol ve distile su-kontrol grubu arasında histolojik açıdan farklılık bulunmamıştır. Bu nedenle kontrol grubu olarak normal-kontrol grubu temel alınmış ve bu doğrultuda histolojik

değerlendirmeler yapılmıştır. Gözlenebilen dermal mast hücrelerinin sıkı paketlenmiş granüllere sahip olduğu ve degranülasyon göstermediği tespit edilmiştir *(Şekil 1A/B)*. Kontrol grubu ile karşılaştırıldığında sunset yellow'a 6 saat maruz kalan grupta embriyonik dermal mast hücrelerinin daha fazla olduğu, pek çok mast hücresinin sıkı paketlenmiş ve yoğun boyanmış granüller içerdiği dikkati çekmiştir *(Şekil 1C, Tablo 1)*. Bu grupta ileri düzeyde degranüle mast hücrelerine orta düzeyde rastlanmıştır *(Şekil 1C/D, Tablo 1)*. Sunset yellow'a 12 saat maruz kalan gruba ait doku örneklerinde çok sayıda dermal mast hücresine rastlanmış olup, çoğu mast hücresinde kısmi ve ileri düzeyde degranülasyon gözlenmiştir *(Şekil 1E/F, Tablo 1)*. Kısmi degranüle mast hücresi granüllerinin çözülmeye başladığı, gevşek ve kaba granüller oluşturduğu saptanmıştır *(Şekil 1E/F)*. İleri derecede degranülasyon gösteren mast hücre-lerinin ise daha az granül içerdiklerinden dolayı daha soluk sitoplazmaya sahip oldukları dikkati çekmiştir. Bu grupta sıkı paketlenmiş granül içeren mast hücrelerine az rastlanmıştır *(Şekil 1E, Tablo 1)*. Sunset yellow'a 24 saat maruz kalan grupta ise sayıca çok fazla olduğu gözlenen dermal mast hücrelerinde ileri düzeyde degranülasyon tespit edilmiştir (Tablo 1). Ayrıca bu grupta sıkı paketlenmiş granüle sahip mast hücresine daha az, kısmi degranülasyona sahip mast hücrelerine ise orta düzeyde rastlanmıştır *(Şekil 1G/H, Tablo 1)*.



Şekil 1. A/B: Kontrol grubuna ait 15 günlük tavuk embriyosu derisinde sıkı paketlenmiş, yoğun granüller içeren dermal mast hücreleri *(kavisli ok)*, **C:** 6 saatlik deney grubuna ait tavuk embriyosu deri kesitinde kıl folikülü (kf) etrafında toplanmış sıkı paketlenmiş granül içeren mast hücreleri *(siyah ok)*, **D:** Enjeksiyonundan 6 saat sonra dermal mast hücrelerinde meydana gelen kısmi *(kırık ok)* ve ileri düzeyde degranülasyon *(beyaz ok)*, **E/F:** Sunset yellow' un enjeksiyonundan 12 saat sonra kısmi *(kırık ok)* ve ileri *(beyaz ok)* düzeyde degranülasyon gösteren mast hücreleri, **G/H:** SY'un enjeksiyonundan 24 saat sonra kısmi *(kırık ok)* ve ileri *(beyaz ok)* düzeyde degranüle mast hücreleri. Epidermis (e), dermis (d), Boyama; A,F,H metilen mavisi, B,C,D,E,G modifiye giemsa. Büyütme; A,B 80x; C 40x, D,H 100x; E,F,G 160x

Fig 1. A/B: Dermal mast cells with tightly packed granule of control group of 15-day chicken embryo skin *(curved arrow)*, **C:** 6 h after injection of sunset yellow, mast cells with tightly packed granule around the hair follicles (kf) in skin tissues *(black arrow)*, **D:** 6 h after injection, partial *(broken arrow)* and high level degranulation *(white arrow)* in dermal mast cells, **E/F:** 12 h after injection of sunset yellow, partial *(broken arrow)* and high level degranulation *(white arrow)*, **G/H:** partial *(broken arrow)* and high level degranulated *(white arrow)* mast cells after 24 h treatment. Epidermis (e), dermis (d), Staining; A,F,H methylene blue, B,C,D,E,G modified giemsa. Magnification; A,B 80x; C 40x, D,H 100x; E,F,G 160x



Şekil 2. A/B: Kontrol grubu tavuk embriyosu barsak dokusunda sıkı paketlenmiş granül içeren mukozal mast hücreleri *(kavisli ok)*, **C/D:** SY'un enjeksiyonundan 6 saat sonra sıkı paketlenmiş granül içeren *(siyah ok),* kısmi *(kırık ok)* ve ileri *(beyaz ok)* düzeyde degranülasyon gösteren çok sayıda mukozal mast hücresi. Modifiye giemsa, 160x, **E/F:** SY'un enjeksiyonundan 12 saat sonra kısmi *(kırık ok)* ve ileri *(beyaz ok)* düzeyde degranülasyon gösteren çok sayıda mast hücresi izlenmektedir, **G/H:** SY'un enjeksiyonundan 24 saat sonra ileri düzeyde degranülasyon gösteren çok sayıda mast hücresi *(beyaz ok)*. Boyama; A,C,G metilen mavisi; B,D,F modifiye giemsa; E,H toluidin mavisi. Büyütme; A,B 80x; C,F 100x, D,E,G,H 160x

Fig 2. A/B: Mucosal mast cells with tightly packed granule of control group of chicken embryo skin *(curved arrow),* **C/D:** 6 hours after injection, non-degranule *(black arrow),* mucosal mast cells of showing partial *(broken arrow)* and high level degranulation *(white arrow),* **E/F:** 12 h after injection, partial *(broken arrow)* and high level degranulation *(white arrow)* of mast cells, **G/H:** 24 h after injection of sunset yellow, high level degranulation *(white arrow)* of mast cells. Staining; A,C,G methylene blue, B,D,F modified giemsa, E,H toluidin blue, Magnification; A,B 80x; C,F 100x; D,E,G,H 160x

Kontrol grubunun barsak kesitlerinde gözlenebilen mukozal mast hücrelerinin sıkı paketlenmiş granüllere sahip olduğu ve degranülasyon göstermediği tespit edilmiştir *(Şekil 2A/B)*. Sunset yellow'a 6 saat maruz kalan gruba ait embriyoların barsak dokusunda mast hücrelerine kontrol grubuna göre daha sık rastlanmıştır *(Şekil 2)*. Sıkı paketlenmiş granüller içeren birçok mast hücresine rastlanmakla birlikte *(Şekil 2C, Tablo 1)* bazılarının kısmi ve ileri düzeyde degranülasyon gösterdiği tespit edilmiştir *(Şekil 2D, Tablo 1)*. Sunset yellow'a 12 saat maruz kalan grupta çoğu mukozal mast hücresinde kısmi ve ileri düzeyde degranülasyon gözlenmiştir. Bu grupta sıkı paketlenmiş granül içeren mast hücrelerine daha az rastlanmıştır *(Şekil 2E/F, Tablo 1)*. Sunset yellow'a 24 saat maruz kalan grubun mukozal mast hücrelerinde genellikle ileri düzeyde degranülasyon tespit edilmiştir. Ayrıca bu grupta ileri degranüle mast hücrelerine sıkı paketlenmiş granüle ve kısmi degranülasyona sahip mast hücrelerinden daha çok rastlanmıştır *(Şekil 2G/H, Tablo 1)*.

TARTIŞMA ve SONUÇ

Gıda maddelerinin üretimi sırasında kullanılan boyalar, istenilen kalitenin elde edilmesini sağlayarak tüketimi arttırdığı için gıda endüstrisinde önemli bir yere sahiptir.

Tablo 1. *Sunset yellow uygulaması sonrasında mukozal ve dermal mast hücrelerinin süreye bağlı degranülasyon yoğunluğu, (+: daha az, ++: az ,+++: orta,++++: çok,+++++: çok fazla)*

Table 1. *Degranulation density depending on time of mucosal and dermal mast cells after treatment of sunset yellow, (+: weaker, ++: weak ,+++: medium,++++: strong,+++++: very strong)*

Doku	Süre		
	6 saat	12 saat	24 saat
DERİ (dermal mast hücresi)			
Sıkı paketlenmiş granül içeren mast hücresi	+++++	++	+
Kısmı düzeyde degranülasyon gösteren mast hücresi	++++	+++++	+++
İleri düzeyde degranülasyon gösteren mast hücresi	+++	++++	+++++
BARSAK (mukozal mast hücresi)			
Sıkı paketlenmiş granül içeren mast hücresi	+++	+	+
Kısmı düzeyde degranülasyon gösteren mast hücresi	+++++	+++++	+++++
İleri düzeyde degranülasyon gösteren mast hücresi	+++	+++++	+++++

Gıda boyaları ve koruyucuları ile yapılan araştırmalar genellikle klinik, biyokimyasal ve fizyolojik çalışmalardır. Mast hücrelerindeki yapısal değişikliklerden ziyade organizmanın genel reaksiyonunu ele alan bu çalışmalara göre; gıdalarda ve ilaçlarda yaygın olarak kullanılan katkı maddeleri, insanlarda allerjik reaksiyonlara neden olmakta ya da bu rahatsızlıkları arttırmaktadır [22,23]. Gıda katkı maddelerinin bağışıklık mekanizmasına etki edip etmediğini belirleyebilmek için, alerjik reaksiyonlarda önemli işlevi olan mast hücreleri üzerine yapılan çalışmalar bulunmaktadır [24-26]. Bu çalışmada ise diğer yapılan çalışmaların dışında sunset yellow'un embriyonik dokulardaki mast hücrelerinde degranülasyona sebep olup olmadığı ortaya konulmuştur.

Mast hücrelerinin orijinleri, yerleşim yerleri, kullanılan tespit solüsyonuna verilen cevap, taşıdığı farklılıklar, fonksiyonel kriterler ve hücrelerin morfolojik özellikleri gibi unsurlar göz önüne alındığında mukozal (MMC) ve bağ dokusu mast hücreleri (CTMC) olarak iki temel mast hücresi tipi olduğu bilinmektedir [27]. Bu iki farklı tip mast hücresinin karşılaştırmasını yapabilmek amacıyla çalışmamızda CTMC'e örnek olarak deri, MMC'e örnek olarak barsak dokusu tercih edilmiştir.

Deride mast hücrelerinin yerleşimi türler arasında genel olarak benzerlik göstermektedir. Koyun, kedi ve köpek derisinde mast hücrelerinin dermisin yüzeysel katmanında kan damarı, kıl follikülü, yağ ve ter bezlerinin çevresinde yerleştiği bildirilmiştir [28-30]. Yaptığımız çalışmada da deri dokusundaki dermal mast hücrelerinin düzensiz sıkı bağ dokusu (dermis) içerisinde genellikle kıl follikülü ve kan damarı çevrelerine yerleşim gösterdiği gözlenmiştir. Barsaktaki mast hücreleri de mukozal gevşek bağ dokusu içerisinde kan damarlarına yakın yerleşim göstermektedir. Mast hücrelerinin deride bu bölgelerde bulunmalarının nedeni, savunma mekanizmalarının dışında, kan akımında düzenleyici rol oynadığı ve damar geçirgenliğini artırarak ya da hücreler arası maddenin sıvı durumunu ayarlayarak epidermise ait hücrelerin ve lokalize oldukları bölgelerde bulunan diğer hücrelerin beslenmesini kolaylaştırmak olabilir. Erginlerde olduğu gibi fötal deride de mast hücrelerinin perivasküler yerleşimi dolaşım düzenlenmesi ile yakın ilişkisini göstermektedir. Gelişmekte olan follikküllerin çevresinde bulunması ise gerek bağ dokunun yapılanmasında gerekse çoğalan follikül hücrelerinin beslenmesinde rolü olduğunu düşündürmektedir.

MMC'lerin heparin içermediği ve boyutlarının daha küçük olduğu; CTMC'lerin ise temel olarak heparin içerdikleri ve daha büyük oldukları söylenmektedir [31,32]. Çalışmamızda da deri ve barsakta incelenen mast hücrelerinin boyut olarak birbirinden farklı oldukları tespit edilmiştir. Ancak yukarıda sözü edilen çalışmaların aksine, bizim çalışmamızda boyut bakımından karşılaştırıldıklarında deride bulunan CTMC'lerin, barsaktaki MMC'lere nazaran daha küçük boyutta oldukları görülmüştür. Bu farklılık, incelediğimiz dokuların gelişimini henüz tamamlamamış embriyonik doku olmasından kaynaklanabileceğini düşündürmektedir. Çalışmamızda dermal mast hücrelerinin mekik şeklinde, mukozal mast hücrelerinin ise daha oval/yuvarlak şekilde oldukları dikkati çekmiştir. Bu morfolojik farklılığın derideki mast hücrelerinin genellikle kas dokuya yakın ve sıkı bağ dokuda lokalize olmaları sonucu ortaya çıkmış olabileceğini düşünmekteyiz.

Fareler ile yapılan bir çalışmada gıda ve ilaç sanayinde kullanılan tartrazinin enjeksiyonundan 1 ve 12 saat sonra mast hücrelerinde hafif derecede degranülasyon, 6 saat sonra oldukça fazla iç degranülasyon gözlenmiştir. Enjeksiyondan 24 saat sonra ise çok belirgin bir degranülasyon tespit edilememiştir [26]. Bizim çalışmamızda ise deride en fazla kısmi degranülasyon enjeksiyondan 12 saat sonra, barsakta 6 saat sonra, en fazla degranülasyon (ileri düzeyde) deride enjeksiyondan 24 saat, barsakta ise 12 saat sonra gözlenmiştir. Barsaktaki mukozal mast hücrelerinde ileri degranülasyonun enjeksiyondan 24 saat sonra da devam ettiği belirlenmiştir. Mast hücre morfolojisi ile ilgili bulgularımız, fareler ile yapılan çalışmaya [26] benzerlik göstermekle birlikte enjeksiyonda 24 saat sonra ileri degranülasyonun görülmesi bakımından farklılık bulunmaktadır. Bu farklılık kullanılan maddeden dolayı olabileceği gibi deney hayvanına ve dokuya da bağlı olabilir.

Bu çalışmada sunset yellow enjeksiyonu sonrasında embriyonik deri ve barsak dokularında mast hücrelerinin sayıca arttığı gözlenmiştir. Degranülasyon öncesi granülize olmuş bu mast hücre yoğunluğundan, mast hücrelerinin sunset yellow'u antijen olarak algıladıklarını ve aktivasyon göstererek histamin mediatörü yardımıyla immünolojik bir reaksiyon başlattığı kararına varılmıştır.

Sonuç olarak; katkı maddesi olarak kullanılan ve allerjik reaksiyonlara yol açtığı ileri sürülen sunset yellow'un, tavuk embriyosu dokularındaki mast hücrelerinde degranülasyona sebep olduğu tespit edilmiştir. Son yıllarda insanlarda artış gösteren allerjik rahatsızlıklar da gözönüne alınırsa, adı geçen maddenin gıdalarda renk maddesi olarak kullanımının kontrol altına alınması, yüksek dozlarda uzun süreli tüketilmemesi, özellikle gebelik döneminde kullanılmaması ve hatta besinlere ilave edilmesinin yasaklanması gerektiği görüşündeyiz.

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RESEARCH ARTICLE

Effect of *in ovo* Ghrelin Administration on Thyroid Hormones and Some of Serum Biochemical Parameters in Newly-hatched Chicks

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Summary

The aim of this study was to investigate the effect of *in ovo* ghrelin administration on serum thyroid hormones levels and serum total cholesterol, triglyceride, calcium (Ca), phosphate (P) concentrations and alkaline phosphatase (ALP) activity in newly-hatched chicks. Fertilized eggs were divided into 5 groups; group G1 as control (intact or without injection), group G2 (50 ng ghrelin/egg at day 5), group G3 (100 ng ghrelin/egg at day 5), group G4 (50 ng ghrelin/egg at day 10), and group G5 (100 ng ghrelin/egg at day 10). Data obtained from serum analysis showed that 100ng/egg ghrelin administration caused T_4 elevation, whereas there were no any significant changes when ghrelin was administrated in 50ng/ egg dosage. There was no any significant difference for T_3 level between experimental groups. Serum triglyceride concentration was lower in all injected groups than control group, but only the differences between control group and G2 and G4 groups were statistically significant ($P<0.01$). Serum Ca concentration was higher in G2 and G3 compared with control group. No significant differences were observed in total cholesterol, P concentrations and ALP activity among experimental groups. The present results indicate that appropriate timing of *in ovo* injection of ghrelin has considerable effects on serum Ca levels of newly-hatched chicks. Also, higher dosage of *in ovo* administrated ghrelin can elevate serum T_4 level.

Keywords: *Chicken, Ghrelin, in ovo administration, Thyroid hormones, Serum biochemical measures*

Yumurtadan Yeni Çıkmış Civcivlerin Tiroid Hormonları ile Bazı Serum Biyokimyasal Değerleri Üzerine *in ovo* Ghrelin Enjeksiyonunun Etkisi

Özet

Bu çalışmanın amacı; yumurtadan yeni çıkmış civcivlerde *in ovo* ghrelin uygulamasının serum tiroid hormonları seviyeleri ile serum total kolesterol, trigliserid, kalsiyum, fosfat konsantrasyonları ve alkalin fosfataz (ALP) aktivitesindeki etkilerinin araştırılmasıdır. Döllenmiş yumurtalar G1-G5 olmak üzere 5 gruba ayrıldılar; G1: kontrol (enjeksiyon uygulanmayan), G2: 5. günde 50 ng ghrelin/ yumurta enjeksiyonu, G3: 5. günde 100 ng ghrelin/yumurta enjeksiyonu, G4: 10. günde 50 ng ghrelin/yumurta enjeksiyonu, G5: 10. günde 100 ng ghrelin/yumurta enjeksiyonu. Serum analiz sonuçları 100 ng ghrelin/yumurta uygulamasının T_4 seviyesinde artışa neden olurken 50 ng ghrelin/yumurta uygulamasının herhangi bir değişikliğe neden olmadığını gösterdi. Deneme grupları arasında T_3 seviyesi bakımından herhangi bir değişikliğe rastlanmadı. Serum trigliserid konsantrasyonu kontrol grubu ile kıyaslandığında enjeksiyon uygulanan tüm gruplarda daha düşük olarak tespit edilmesine rağmen sadece G2 ve G4 grupları istatistiksel olarak fark göstermekteydi ($P<0.01$). Kontrol ile kıyaslandığında, G2 ve G3 gruplarında serum Ca konsantrasyonu daha yüksek olarak belirlendi. Total kolesterol, fosfat konsantrasyonu ve ALP aktivitesi yönünden deney grupları arasında bir fark gözlemlenmedi. Bu sonuçlar uygun zamanda *in ovo* ghrelin uygulamasının yumurtadan yeni çıkmış civcivlerde serum Ca miktarında önemli bir etkisinin olduğunu göstermektedir. Ayrıca, yüksek doz *in ovo* ghrelin uygulaması serum T_4 seviyesini artırabilir.

Anahtar sözcükler: *Tavuk, Ghrelin, in ovo uygulama, Tiroid hormonları, Serum biyokimyasal değerler*

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INTRODUCTION

Ghrelin is a multifunctional regulatory peptide that was discovered in the rat stomach by Kojima *et al*.[1]. Researches on avian ghrelin are less than those of mammalian ghrelin, but the findings have demonstrated a similarity on GH-releasing activity, and a difference in appetite regulation between mammals and birds, especially in chicken [2]. Chicken ghrelin includes 26 amino acids and is shorter than human or rat ghrelin with 28 amino acids [3]. *In vivo* and *in vitro* investigations showed that GH-releasing action of ghrelin has a dose-depended manner and is similar to growth hormone releasing hormone (GHRH) with a stimulatory effect on somatotrophs in chicken [4]. Regarding metabolic aspects of chicken ghrelin, anti-lipogenic effect of administrated ghrelin in neonatal broiler chicks has been reported by Buyse *et al*.[5].

Human ghrelin is expressed in fetal thyroid, but not in adult [6]. There is no available evidence for ghrelin expression in chicken thyroid. Onset of chicken thyroid activity is on second week of embryonic development. The first appearance of colloid droplets is on d 7 but it is not until about days 10-13 that the adenohypophysis thyroid interactions become established, while the growth of the embryo, as measured by body weight, skeletal size, muscle growth and growth of cartilages and bones, is greatly influenced by thyroid hormones in late embryonic development [7-9].

Khazali [10] with ventricle infusion of ghrelin in goats reported that ghrelin may increase the mean plasma concentration of T_3 and T_4. In human, the effect of ghrelin on hypothalamus-pituitary-thyroid axis and circulated T_4 was documented. In this regard, Kluge et al.[11] had stated, early fT4 increases following ghrelin infusion was possibly induced by direct action of ghrelin on the thyroid gland where ghrelin receptors have been identified. In other hand, a study conducted via intracerebroventricular administration (another injection method) of ghrelin had different result with reducing the plasma TSH and T_4 [12]. Regarding metabolic aspects of chicken ghrelin, anti-lipogenic effect of administrated ghrelin in neonatal broiler chicks has been reported by Buyse *et al*.[5].

In ovo ghrelin has been identified in albumen and yolk of fertile chicken egg [13] and its gene expression was observed during embryonic life, especially after 5-day of incubation [14]. Also, ghrelin mRNA and expression have been identified in follicles [15], pancreatic cells of chicken [16] and oviduct of quail [17].

Regardless to ghrelin's effects on plasma concentration of GH [3], Insulin [18] and prolactin [19], many effects of chicken ghrelin on serum biochemical measures such as plasma lipids, calcium (Ca) and phosphorus (P) concentrations are unclear. The aim of present study was investigate the effects of *in ovo* ghrelin administration on thyroid hormones level and some of serum indices include total cholesterol, triglyceride, Ca and P concentrations and alkaline phosphatase (ALP) activity in newly-hatched chicks.

MATERIAL and METHODS

In this study, 250 fertilized eggs were collected from commercial broiler breeder flock (Ross 308). The eggs were divided into five groups; group G1 (control or intact), group G2 (*in ovo* injected with 50 ng ghrelin/egg at day 5), group G3 (*in ovo* injected with 100 ng ghrelin/egg at day 5), group G4 (*in ovo* injected with 50 ng ghrelin/egg at day 10) and group G5 (*in ovo* injected with 100 ng ghrelin/egg at day 10). Eggs were incubated with normal hatchery (37.8°C and 60%: RH). The lyophilized Ghrelin was obtained from Sigma-Aldrich® (Rat Ghrelin - USA), dissolved in 1% acetic acid solvent and proposed concentrations of ghrelin were prepared. At day 5 and day 10 of incubation, *in ovo* injections were conducted in hygiene room at 37°C. Before injection, egg shells were marked with marker for identification of air cell position and detection of optimum injection point. At this experiment, 22G needles were used for safe *in albumin* injection. After hatching, the blood samples were collected from 75 hatched chicks (15 individual samples from 15 hatched-chicks for each group), immediately following chick decapitation. Blood samples were centrifuged and serum was obtained for determination of T_3 and T_4 level with Electrochemiluminescence immunoassay method on a Modular Analytics E170 analyzer [20], and serum biochemical analysis with Alcyon 300 auto analyzer (Abbott Park, IL., USA) and its commercial kits.

Data obtained by 15 individual samples from 15 hatched-chicks for each group (in total 75 samples) were analyzed (one-way analysis of variance) with SAS software (Ver. 9.1) by and the differences between groups were evaluated with Duncan multiple range test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Table 1 shows T_3 and T_4 concentrations in newly-hatched chicks following *in ovo* injection of exogenous ghrelin. In this study, ghrelin administration in 100 ng/egg caused T_4 elevation in G3 and G5 compared with control group, whereas there was no significant change when ghrelin was administered at 50 ng/egg dosage (G2 and G4). There was no any significant change for T_3 level between experimental groups. *Table 2* shows serum total cholesterol, triglyceride, Ca, P and ALP level in experimental groups following *in ovo* injection of exogenous ghrelin. Exogenous ghrelin administration (50 and 100 ng/egg) at embryonic day 5 or 10 has no significant effect on total cholesterol concentration. however, triglyceride level declined in G2 and G4 (57.3 and 70.6 mg/dl, respectively) compared with

Table1. *Serum T_3 and T_4 concentrations in newly-hatched chicks following in ovo injection of exogenous ghrelin*

Tablo 1. *in ovo ghrelin uygulanan yumurtadan yeni çıkmış civcivlerde serum T_3 ve Te_4 konsantrasyonları*

Experimental Groups	Injected Dosage (ng/egg)	Injection Day (Incubation Day)	T_3	T_4
G1	0	-	10.10	4.08[b]
G2	50	5	9.83	4.20[b]
G3	100	5	9.96	4.96[a]
G4	50	10	9.80	4.2[b]
G5	100	10	10.6	4.47[ab]
P value			0.0572	0.0303
SEM[1]			0.6899	0.5773

** Different letters (a, b or c) show significant difference, [1] SEM, based on pooled estimate of variance*

level of T3 in experimental groups *(Table 2)* may be indicator of high thyroid activity for releasing sufficient level of T_3 and T_4 for possible successful hatching process. Possible mechanism for increased plasma T_4 level following 100 ng ghrelin administration may be due to TRH-releasing effect of ghrelin as currently identified by Pekary and Sattin [24].

In present study, serum total cholesterol was not affected significantly by *in ovo* ghrelin administration although there were slight decline in G2, G3 and G4 groups *(Table 2)*. Triglyceride concentration was lower in G2, G3, G4 and G5 groups compared with control or solution injected groups *(Table 2)*. It has been reported that circulating human ghrelin can decrease blood lipids and it is a key factor for prevention of hyperlipidemia after dietary fat intake [25]. The high cholesterol or arthrosclerosis cause higher concentration of circulating ghrelin in comparison

Table 2. *Serum total cholesterol, triglyceride, Ca, P and ALP levels in in newly-hatched chicks following in ovo injection of exogenous ghrelin*

Tablo 2. *in ovo ghrelin uygulanan yumurtadan yeni çıkmış civcivlerde serum total kolesterol, trigiliserid, Ca, P ve ALP değerleri*

Experimental Groups	Injected Dosage (ng/egg)	Injection Day (Incubation Day)	Total Cholesterol (mg/dl)	Triglyceride (mg/dl)	Ca (mg/dl)	P (mg/dl)	ALP (U/l)
G1	0	-	577.7	[a] 120.0	10.1 [bc]	6.3	2166.7
G2	50	5	417.7	[b] 57.3	11.4 [ab]	5.7	2565.0
G3	100	5	514.1	[ab] 87.6	12.3 [a]	5.8	2328.7
G4	50	10	506.3	[b] 70.6	10.0 [bc]	4.9	2640.0
G5	100	10	618.0	[ab] 81.3	8.6 [c]	6.1	2903.3
P value			0.3597	0.0079	0.0029	0.0797	0.8732
SEM[1]			80.473	12.488	0.521	12.488	500.556

** Different letters (a, b or c) show significant difference, [1] SEM, based on pooled estimate of variance*

G1 (120 mg/dl). There were no significant differences in P concentrations and ALP activity rates among experimental groups. Ca concentration was significantly higher in G3 (100 ng ghrelin/egg injection at day 5: 12.3 mg/dl), and was slightly lower in G5 (100 ng ghrelin injection at day 1 0: 8.6 mg/dl) compared with G1 (10.1 mg/dl).

DISCUSSION

The reports about ghrelin effects on thyroid activity are different, due to experimental animal, dosages and duration. However, the effect was not investigated in chicken.

Park *et al.*[21] observed that Ghrelin enhances the proliferating effect of thyroid stimulating hormone in thyroid cells. Gjedde *et al.*[22], and Caminos *et al.*[23] reported that circulating ghrelin levels were increased in hypo-thyroid condition. Their finding that shows negative correlation of plasma ghrelin and T_4 levels are in contrast with findings of present study *(Table 1)*. In present study, ghrelin administration in high concentration (100 ng) caused subsequent elevation in T_4 level *(Table 1)* that this result is in according to Khazali [10] findings. In other hand, constant with healthy condition [26]. Buyse *et al.*[5] have reported that fatty acid synthesis decreased and there is anti-lipo-genic effect of ghrelin following ghrelin injection to chickens. Results of the present study are similar to the findings of Buyse *et al.*[5], and partially are in agreement with the report by Egecioglu *et al*.[25] on human. It is suggested that the ghrelin has a regulatory effect for lipid metabolism even in embryonic life of chickens.

The effect of ghrelin on serum Ca, P and ALP activity has not been studied in early embryonic development. In the present study *in ovo* administrated ghrelin had no significant effect on ALP activity *(Table 2)*. The role of ghrelin in ALP activity limited with some information obtained in mammalian species. Fukushima *et al*.[27] stated that ghrelin causes osteogenesis by stimulation of ALP. Maccarinelli *et al.*[28] observed high ALP activity in mice with injection of ghrelin. With attention to lack of findings in relation to the ghrelin acts in ALP activity in birds, the present data show that *in ovo* exogenous ghrelin administration had no effect on serum ALP activity in any of ghrelin-administrated groups. Our findings are different from the report of Maccarinelli *et al.*[28] on ghrelin function to ALP activity in mammalian model. G3 group (100 ng/egg ghrelin at day 5) had significantly higher Ca rate

than control group (G1) (P<0.01). However, there was no significant difference between control group and G2, G4 and G5 groups in terms of serum Ca concentrations when compared with control (T1) *(Table 2)*. Pérez-Castrillón *et al*.[29] declared that ghrelin has not any important role in Ca metabolism and serum Ca has not significant correlation with circulation ghrelin level in human. Increasing serum Ca in group G3 is opposite to ghrelin osteogenic effect and osteogenic-related Ca decreases [27,30]. *In ovo* administrated 100 ng/egg ghrelin at d 5 might stimulate uptake of *in ovo* Ca from extra-embryonic sources (yolk and eggshell), and it may divert to embryonic circulation [31] for cartilage and bone formation may occurred mainly eight day after incubation, such as tibia formation [32]. Similarly, serum P concentration had not any significant differences in any of experimental groups.

In ovo administration of 100 ng ghrelin/egg has considerable effect on serum T_4 and triglyceride levels, but minor effect on serum Ca levels, whereas it didn't affect serum total cholesterol, P and ALP of newly-hatched chickens. Further studies with different methods of *in ovo* injection and *in yolk* injection, also administration at different incubation days (pre-incubation or at third week of incubation) in further experiments can be useful to clarify an effect of maternal or *in ovo* ghrelin in avian species.

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RESEARCH ARTICLE

Determination of Phenotypic Correlations Between Internal and External Quality Traits of Guinea Fowl Eggs

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Summary

In this study, it was aimed to determine the internal and external quality traits of the Guinea fowl eggs as well as the phenotypic correlation among the these traits. Totally 100 Guinea fowl eggs were collected in three sequential days were used for this study. The birds were housed at Poultry Research Unit of the Department of Animal Science, Faculty of Agriculture, University of Akdeniz. Values of egg weight, egg length, egg width, eggshape index, eggshell weight, eggshell ratio, eggshell thickness, eggshell surface area, unit surface area and egg volume were determined as 40.14 g, 49.47 mm, 37.89 mm, 0.76%, 6.48 g, 16%, 0.54 mm, 65.69 cm^2, 0.11 g/cm^2 and 38.21 cm^3, respectively. In addition, values of yolk weight, yolk height, yolk width, yolk index, yolk ratio, albumen height, albumen width, albumen length, albumen weight, albumen index, yolk/albumen ratio and haugh unit were found as 13.58 g, 14.99 mm, 40.64 mm, 37%, 33%, 4.77 mm, 62.97 mm, 80.07 mm, 21.62 g, 6.7%, 68% and 74.97%, respectively. According to the results determined in this study, all most all external quality traits of the egg were changed at the significant levels depending on the change occurred in the egg weight. The results indicated that egg weight influences external egg quality traits of quinea fowl. The positive correlations obtained among the egg quality traits indicated that they can be improved phenotypically through selection.

Keywords: *Guinea fowl, Egg weight, Egg quality, Phenotypic correlation*

Beç Tavuğu Yumurtalarında İç ve Dış Kalite Özellikleri Arasındaki Fenotipik Korelasyonların Belirlenmesi

Özet

Bu çalışmada Beç tavuğu yumurtalarında iç ve dış kalite özellikleri ile bu özellikler arasındaki fenotipik korelasyonların belirlenmesi amaçlanmıştır. Bu amaçla toplam 100 adet Beç tavuğu yumurtası kullanılmıştır. Araştırma Akdeniz Üniversitesi Ziraat Fakültesi Zootekni Bölümü Hayvancılık Birimi'inde yürütülmüştür. Yumurta ağırlığı, yumurta uzunluğu, yumurta eni, şekil indeksi, kabuk ağırlığı, kabuk oranı, kabuk kalınlığı, kabuk yüzey alanı, birim yüzey alanı ve yumurta hacmi sırasıyla 40.14 g, 49.47 mm, 37.89 mm, %0.76, 6.48 g, %16, 0.54 mm, 65.69 cm^2, 0.11 g/cm^2 ve 38.21 cm^3 olarak bulunmuştur. Ayrıca, sarı ağırlığı, sarı yüksekliği, sarı genişliği, sarı indeksi, sarı oranı, ak yüksekliği, ak genişliği, ak uzunluğu, ak ağırlığı, ak indeksi, sarı/ak oranı ve haugh birimi de sırasıyla 13.58 g, 14.99 mm, 40.64 mm, %37, %33, 4.77 mm, 62.97 mm, 80.07 mm, 21.62 g, %6.7, %68 ve %74.97 olarak hesaplanmıştır. Yumurta ağırlığındaki değişmeye bağlı olarak yumurtanın dış kalite özellikleri önemli derecede değişmiş ve yumurta ağırlığı dış kalite özelliklerini etkilemiştir. Yumurta kalite özellikleri arasında ortaya çıkan önemli korelasyonlar bu özelliklerin fenotipik olarak seleksiyonla iyileştirilebileceğini göstermiştir.

Anahtar sözcükler: *Beç tavuğu, Yumurta ağırlığı, Yumurta kalitesi, Fenotipik korelasyon*

INTRODUCTION

Avian egg is not only a tool for reproduction but is also a valuable food source for human. Moreover, avian eggs are culturally accepted worldwide and are not submitted to any religious nor traditional interdiction. Nowadays, it is widely recognized that eggs are more than a source of nutrients, numerous studies describing biological properties potentially exploitable by pharmaceutical, food-processing and cosmetic industries [1,2]. The egg size and internal quality of eggs are important for both table and hatching eggs. The nutrient content and composition

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of an egg can thus greatly influence the development of the embryo contained within as well as its success as a hatchling. Variation in the composition of avian eggs occurs among species [3-6].

Eggs from species that are more precocial at hatching tend to have a higher proportion of yolk in the egg and a corresponding lower proportion of albumen, a composition which is perhaps necessary to ensure that eggs are provisioned with enough nutrients for the prolonged incubation and development of the embryos of precocial species [6]. The major constituent of albumen is water amounting to 88% of total weight. The most important trait of egg composition, linked to egg dry matter, is the yolk/albumen ratio [7-9]. Beside the yolk/albumen ratio, eggshell resistance to shocks is an economically primordial trait as it determines the ability of eggs to withstand transportation from producers to consumers [10]. Moreover, an intact eggshell is also necessary to impede bacterial invasions of eggs and to reduce food poisining risks [10,11]. In the future egg quality studies about egg yolk, albumen and eggshell quality will continue to increase. Parcularly, most of the studies will be investigated to reduce the cholesterol level in the egg yolk [12] and to increase the yolk/albumen ratio in the eggs [7]. Although the albumen is a major indicator of internal egg quality; air cell size, albumen and yolk quality and the presence of blood or meat spots in the eggs are the parameters, which determines the internal egg quality [13,14]. Egg yolk is still an important source for nutrients, and also used in non-food purposes like leather processing and a source of biologically active substances [15].

Chicken egg has been very well studied for its internal and external qualities as well as for its compositions, however such information are not so abundantly documented in other poultry species. So, this study are conducted to investigate egg quality traits and phenotypic correlations between internal and external quality traits of Guinea fowl eggs.

MATERIAL and METHODS

Totally 100 Guinea fowl eggs were randomly selected and evaluated in this study. The Guinea birds were housed at Research Unit of Animal Science Deparment, Faculty of Agriculture, Akdeniz University. Evaluated eggs were collected from the guinea fowl hens, reared in a floor system. The hens were at about 40 weeks of age. The birds were fed a diet containing 21% crude protein and 2.900 kcal/kg metabolic energy and provided with fresh water *ad libitum* during the laying period. In order to determine egg quality traits, eggs were stored at room temperature for 24 h before quality measurement. The eggs were numbered first and then measured with an electronic balance to the nearest 0.01 g. Subsequently, egg length and width were measured by slide calipers sensitive to 0.01 mm. After this process, the eggs were broken on a table with a glass cover in order to measure the yolk height, yolk diameter, albumen height, albumen length and albumen width by a 3-legged micrometer with an accuracy of 0.01 mm. Later on, the yolk was seperated from the albumen with the help of a spoon and weighed while the albumen weight was calculated by subtracting yolk weight and shell weight from the gross egg weight. The eggshells were washed under slightly flowing water so that the albumen remains were removed. The washed eggshells were left to dry in the open air for 24 h. Then, all eggshells were balanced together with the shell membrane. Finally eggshell samples were taken from sharp region, blunt region and equatorial parts of each egg were measured with micrometer with an accuracy of 0.01 mm, and the average eggshell thickness was obtained from the average values of these three parts [16]. The other quality traits were evaluated by methods described [17-21]. Data were subjected to analysis using SPSS 17.0 [22].

Shape Index (%)=100(Egg width/Egg height)*

*Shell Surface Area (cm^2)=3.9782*Egg weight$^{0.75056}$*

Unit Surface Shell Weight (g/cm^2)=Shell weight/Shell surface area

Shell Ratio (%)=100(Shell weight/Egg weight)*

Albumen Index (%)=100{Albumen height/[(Albumen length+Albumen width)/2]}*

Albumen Weight (g)=Egg weight-(Yolk weight+Shell weight)

Albumen Ratio (%)=100(Albumen weight/Egg weight)*

Yolk Index (%)=100(Yolk height/Yolk diameter)*

Yolk Ratio (%)=100(Yolk weight/Egg weight)*

Yolk/Albumen Ratio (%)=100(Yolk weight/Albumen weight)*

*Haugh Unit=100 log [Albumen height-(1.7*Egg weight$^{0.37}$)+7.57]*

*Egg Volume (cm^3)=[0.6057-(0.0018*Egg width)]*Egg length*(Egg width)^2*

RESULTS

Descriptive statistics of internal and external egg qualities and phenotypic correlations among the internal and external egg traits are presented in *Table 1, 2* and *3*, respectively. There was found significant correlations among the internal and external egg quality traits.

DISCUSSION

The egg weights were ranged from 34.03 g to 45.7 g and average egg weight was determined as 40.14±0.235g. The average egg weight determined in this study was similar to that found in studies carried out by Oke et al.[23,24]; Singh et al.[25]; Nahashon et al.[26,27]. But, the egg weight was found lower than those of reported by Tebesi et al.[28]; Dudusola [29];

Table 3.*The phenotypic correlations between internal and external quality traits of guinea fowl*
Tablo 3. *Beç tavuğu yumurtalarının iç ve dış kalite özellikleri arasındaki fenotipik korelasyonlar*

Traits	SW	YW	YH	YWDT	AH	AWDT	AL	EWDT	EL	ST	AI	SI	SI	SFA	SR	HU	EWL	AW	Y/A	YR	AR
EW	.658**	.569**	.292**	.329**	.243*	-.119	.082	.859**	.569**	.462**	.223*	.064	.311**	1.000**	.212*	.043	.890**	.201*	-.188	-.260*	.075
SW		.568**	.101	.360**	.078	.023	-.091	.501**	.236*	.867**	.102	-.085	.247*	.657**	.861**	-.048	.488**	.078	.274**	.049	-.498**
YW			.223*	.574**	.051	.176	.183	.469**	.236*	.430**	.005	-.092	.220*	.568**	.388**	-.064	.461**	-.064	.670**	.638**	-.669**
YH				-.255*	.677**	-.130	-.173	.219*	.098	.073	.624**	.889**	.135	.289**	-.030	.641**	.213*	.225*	-.030	-.012	.045
YW					-.087	.081	.187	.345**	.048	.274**	-.108	-.656**	.260*	.329**	.258*	-.150	.291**	-.050	.398**	.371**	-.410**
AH						-.267*	-.222*	.223*	.143	.077	.933**	.570**	.101	.240*	-.045	.976**	.233*	.269*	-.173	-.146	.161
AWDT							.510**	-.146	-.101	-.003	-.484**	-.145	-.057	-.117	.132	-.242*	-.155	-.277**	.310**	.303**	-.319**
AL								.096	-.026	-.248*	-.452**	-.247*	.117	.088	-.152	-.246*	.058	.049	.075	.131	-.023
EWDT									.404**	.331**	.202	-.007	.592**	.857**	.108	.052	.937**	.245*	-.207	-.249*	.134
EL										.143	.145	.048	-.487**	.568**	-.044	.027	.698**	.086	-.215*	-.247*	.190
ST											.115	-.066	.166	.460**	.795**	-.003	.318**	.009	.294**	.081	-.486**
AI												.543**	.077	.219*	-.008	.913**	.219*	.257*	-.193	-.181	.171
YI													-.024	.062	-.134	.573**	.015	.182	-.196	-.169	.212*
SI														.310**	.130	.043	.278**	.156	-.019	-.030	-.028
SFA															.212*	.039	.888**	.201*	-.189	-.262*	.075
SR																-.077	.071	-.092	.493**	.251*	-.704**
HU																	.055	.118	-.134	-.095	.143
EWL																		.223*	-.242*	-.288**	.175
AW																			-.778**	-.753**	.724**
Y/A																				.955**	-.955**
YR																					-.848**

*$P<0.05$, **$P<0.01$; ***EW****=Egg weight,* ***SW****=Shell weight,* ***YW****=Yolk weight,* ***YH****=Yolk height,* ***YWDT****=Yolk width,* ***AH****=Albumen height,* ***AWDT****=Albumen width,* ***AL****=Albumen length,* ***EWDT****=Egg width,* ***EL****=Egg length,* ***ST****=Shell thickness,* ***AI****=Albumen index,* ***YI****=Yolk index,* ***SI****=Shape index,* ***SFA****=Shell surface area,* ***SR****=Shell ratio,* ***HU****=Haugh unit,* ***EVL****=Egg volume;* ***AW****=Albumen weight, Y/A= Yolk/albumen,* ***YR****=Yolk ration*

Table 1. *External quality traits of Guinea fowl eggs*
Tablo 1. *Beç tavuğu yumurtalarının dış kalite özellikleri*

Traits	N	Min	Max	Mean±SE
Egg weight (g)	100	34.03	45.70	40.14±0.235
Egg length (mm)	100	46.44	52.68	49.47±0.107
Egg width (mm)	100	36.40	40.02	37.89±0.087
Shell weight (g)	97	3.01	8.97	6.48±0.080
Shell ratio (%)	97	9.00	20.00	16.10±0.162
Sharp region thickness (mm)	95	0.44	0.71	0.55±0.005
Blunt region thickness (mm)	95	0.41	0.69	0.53±0.004
Equatorial region thickness (mm)	95	0.43	0.69	0.54±0.004
Average shell thickness (mm)	95	0.43	0.70	0.54±0.004
Shell surface area (cm^2)	100	49.94	60.71	55.69±0.216
Unit surface shell weight (g/cm^2)	97	0.06	0.15	0.11±0.001
Shape index (%)	100	72.00	81.00	76.60±0.191
Egg volume (cm^3)	100	34.26	43.25	38.21±0.214

Table 2. *Internal quality traits of Guinea fowl eggs*
Tablo 2. *Beç tavuğu yumurtalarının iç kalite özellikleri*

Traits	N	Min	Max	Mean±SE
Yolk weight (g)	90	11.69	15.87	13.58±0.107
Yolk height (mm)	90	12.04	17.09	14.99±0.110
Yolk width (mm)	90	36.51	45.13	40.64±0.170
Yolk index (%)	90	28.00	44.00	37.02±0.347
Yolk ratio (%)	90	30.00	38.00	33.81±0.235
Albumen height (mm)	90	3.09	6.93	4.77±0.086
Albumen width (mm)	90	51.54	71.54	62.97±0.480
Albumen length (mm)	90	65.23	91.34	80.07±0.563
Albumen weight (g)	100	16.67	41.89	21.62±0.491
Albumen index (%)	90	4.00	10.00	6.79±0.143
Albumen ratio (%)	90	44.00	57.00	50.03±0.315
Yolk/Albumen ratio (%)	90	54.00	85.00	68.10±0.858
Haugh unit	90	60.57	87.80	74.97±0.651

Nowaczewski et al.[30]; Song et al.[31] whereas was found higher than those reported by Obike et al.[32]. The difference between the egg weights reported in the various studies, it might be due to variations in strain, stocking density, seasonal factors, feeding system and age of birds Nagarajan et al.[33]; Tanabe and Ogawa [34]. Generally eggs of birds have oval shape with small differences among the species. Despite its small differences, egg shape is considered as an important factor in characterizing bird species. In this study egg shape index ranged from 0.72 to 0.81% and average egg shape index was calculated as 0.76%, indicating that the eggs had normal shape. The average egg shape in in this study was similar to that reported in studies carried out by Tebesi et al.[28]; Nowaczewski et al.[30], whereas was found lower than those reported by Dudusola [29]; Oke et al.[24] and Singh et al.[25]. The results of investigations concerning the relationship of egg weight with egg shape index are ambiguous. However, in many studies carried out on chickens researchers reported a negative, although not always significant, correlation between the egg shape index and its weight Rozycka and Wezyk [35]; Kul and Seker [36]; Tebesi et al.[28]; Nowaczewski et al.[30]; Begli et al.[37] which would mean that heavier eggs are more elongated. In contrast, in their experiments on quinea fowls Bernacki and Heller [38] found that heavier eggs were characterized by greater shape index, these eggs were more ball-shaped. These results are further corroborated by research result obtained by Kuzniacka et al.[39] in Guinea fowls, who found a significant positive correlation between the shape of eggs and their weight (0.317).

In this study average egg length and width was found as 49.47 and 37.89 mm, respectively. The egg length and width values in this study were similar to that found in studies carried out by Tebesi et al.[28]; Singh et al.[25] and Song et al.[31]. The egg weight showed significant and positive correlation with egg length and egg width, and the values of correlations was determines as 0.569 and 0.859 for egg length and egg width, respectively. The significant and positive correlation indicates that the longer length of the egg, the higher the egg weight. Egg length had also been reported to significantly affect egg weight Momira et al.[40]. However, the association between egg weight and egg width was significant. This may be attributed to the fact that the yolk of the egg occupies the width area, thereby translating to heavier weight for eggs. This result corroborated the report of Abanikannda et al.[41]. These authors reported a phenotypic correlation of 0.78 and 0.84 between egg weight with egg length and egg width, respectively. Based on the correlations, they concluded that egg length and egg width were beter predictors of egg weight when compared to egg shape index. The findings determined in this study are also in agrement with the reports of Nwagu et al.[42]; Obike and Azu [32]; Tebesi et al.[28] they found highly significant correlations between the egg weight with egg length and egg width. Also, Apuno et al.[43] reported that significant correlations between the egg weight with egg length and egg width.

The eggshell weights were ranged from 3.01 to 8.97 g, and and average eggshell weight was calculated as 6.48 g. The averege eggshell weight of quinea fowls in this study was similar to that reported by Bernacki and Heller [38]; Kuzniacka et al.[39]; Dudusola [29]; Nowaczewski et al.[30]; Oke et al.[24]. The greater eggshell weight of the quinea fowls could have been affected not only by their greater surface area resulting from the egg size but also its thickness. The authors found a significant positive correlation between the egg weight and the thickness of its eggshell Nowaczewski et al.[30]; Tebesi et al.[28]. In this research, also found a significant positive correlation between the egg weight and eggshell weight (0,658). According to Nordstrom and Ousterhout [44], eggshell weight was significantly influenced by egg weight. These workers found that 47% of variation in eggshell weight was due egg weight. On the other hand, no such correlations were reported by Kuzniacka et al.[39]; Oke et al.[24] and Nahashon et al.[26,27] in quinea fowls.

The eggshell thickness was ranged from 0.43 to 0.70 mm, and average eggshell thickness was determined as 0.54mm. Also, average eggshell thickness was found higher at sharp region (0.55mm) and lower at the blunt region (0.53mm) implying that mineralizion was higher at sharp region. The finding on eggshell thickness was in disagrement with Song et al.[31], Dudusola [29]; Tebesi et al.[28] and Nowaczewski et al.[30] and the average eggshell value in this study was higher than the reported by these authors. The egg weight has an indirect relation with the shell quality of the egg. Thus, it has been stated by most of the researchers that the eggshell thickness has direct relation with the egg weight Choi et al.[45]; Stadelman [46]. Some researchers have mentioned a positive correlations between the egg weight and the eggshell thickness Stadelman [46]; Nowaczewski et al.[30]; Kul and Seker [36]. Also, Moreki et al.[47] who found a positive correlation between egg weight and eggshell thickness of Ross broiler breeder eggs. This implies that eggshell thickness increases with increased egg weight of broiler chicken. In this study, there was found significant correlation between the egg weight and eggshell thickness (0.462). In this study, eggshell surface area was ranged from 49.94 to 60.71 cm^2 and average eggshell surface area was calculated as 65,69 cm^2. The average eggshell surface area was lower than those of reported by Dudusola [29] and Nowaczewski et al.[30]. Also, eggshell ratio was ranged from 0.09 to 0.20% and average eggshell ratio was determined as 16%. The average eggshell ratio determined in this study was similar to that found in studies carried out by Nowaczewski et al.[30] but, was found higher than those of reported by Tebesi et al.[28] and Song et al.[31].

There was found a moderate phenotypic correlation between egg weight and albumen weight (0.201) and a highly significant corelation between egg weight and yolk weight (0.569) in this study. The findings determined in this study are in agrement with the reports of Obike and Azu [32]; Tebesi et al.[28]; Kul and Seker [36] they found highly significant correlations between the egg weight with albumen weight and yolk weight. These results suggest that the heavier weight of the albumen and the yolk, the larger egg weight which in turn leads to increase in egg weight. So, selecting for egg weight will invariably select eggs with larger albumen and yolk weight, which is needed for embryo development.

Yolk weight had a significant correlation with yolk width (0.574). The yolk width possibly constitutes the yolk portion which may have influenced the yolk weight positively. Non-significant and negative correlation was found between yolk weight and yolk index. This result is in consonance with the report of Obike and Azu [32] and Nwagu et al.[42]. There was found a negative but significant relationship (-0.656) between yolk index and yolk diameter. This result is an expected situation, because yolk diameter is the very important factor in the determination of yolk index. Inversely, the association between yolk index and yolk height was highly significant (0.889). Also, yolk index was found positively and significant correlated with albumen height (0.570). This means that improvement of yolk diameter, yolk height and albumen height will result to a beter yolk index. Depending on the this result, egg freshness will be improved since yolk index determines egg freshness.

Highly significant and positive correlation was determined between albumen index with albumen height (0.933), albumen weight (0.257) and albumen ratio (0.724), but there was found significant and negatively correlation between albumen index and albumen width (-0.484) in this study. According to Ozcelik [48], albumen index, albumen height, albumen weight and albumen ratio gives indication of the dense albumen quality anda are used in the estimation of haugh unit, which is an important factor the internal quality of the egg.

The correlation between albumen height and yolk height was found positive and significant (0.677). This observation implies that as albumen height increased, yolk height increased and albumen quality becomes beter. Similar result was determined by Obike and Azu [32]; Nwagu et al.[42]. Similarly, there was found positive and significant relationship between albumen index and yolk index (0.543). This result is in corformity with the research findings of Obike and Azu [32]; Ozcelik [48]; Kul and Seker [36]. In this study, haugh unit was not significant, but negatively correlated with eggshell thickness (-0.003), eggshell weight (-0.048) and eggshell ratio (-0.077). However, also non-significant and positive correlation was found between haugh unit with egg weight (0.043), egg width (0.052), egg length (0.027) and egg shape index (0.043). Similar result reported by Zhang et al.[49].

As a result, the study revealed that egg weight of quinea fowl is an important factor that influences external egg quality characteristics. Thus, it was possible to use egg weight in determining the eggshell weight, eggshell thickness and eggshell ratio instead of using these traits that are the determinants of the eggshell quality of the quinea fowl. It should therefore be considered in any breeding and management programme aimed at improving these traits. In additon, egg length and egg width were stronly and positively correlated with egg weight as 0.569 and 0.859, respectively. Hence, selection for egg length and egg width will invariably select eggs with heavier phenotypic weight. Based on this result, the traits should be employed as selection criteria to improve egg weight. Also, the correlations determined among the internal egg quality traits indicate that the parameters can be improved through selection. Poultry researchers will study on egg quality traits as well as the activities of the breeders who deal with the Guinea fowl eggs breeding and improvement.

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RESEARCH ARTICLE

Determination of Enterotoxigenic Gene Profiles of *Bacillus cereus* Strains Isolated from Dairy Desserts by Multiplex PCR

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Summary

The aim of this study was to investigate the presence of *Bacillus cereus* and to detect enterotoxigenic genes in dairy dessert samples utilising a multiplex PCR technique. A total of 100 samples, 25 keskul, 12 tavuk gogsu, 12 kazandibi, 30 supangle, 4 profiteroles and 17 sutlac were analysed. *B. cereus* contamination was found in 7 of 100 (7.0%) samples including 3 (25%) tavuk gogsu, 2 (6.6%) supangle, 1 (4%) keskul and 1 (5.8%) sutlac. The average number of *B. cereus* was between 2.0×10^1 - 5.0×10^2 cfu/g in these dairy dessert samples. A total of 20 isolates were collected from the 7 positive samples. The results indicated that 30% (6/20) of *B. cereus* isolates contain three enterotoxic HBL complex encoding genes *hblA, hblC* and *hblD,* whereas 70% (14/20) had no *hbl* genes. In addition, all three enterotoxic NHE complex encoding genes, *nheA, nheB* and *nheC,* were detected in 40% (8/20), two *nhe* genes (*nheA* and *nheB*) were found in 45% (9/20) and one *nhe* gene (two *nheA* and one *nheB*) was found in 15% (3/20) of the isolates. The *ctyK1* gene was not detected in any sample. The presence of *B. cereus* and their enterotoxigenic genes in dairy desserts may be a potential risk for public health.

Keywords: *B.cereus, Dairy dessert, Enterotoxin genes*

Sütlü Tatlılardan İzole Edilen *Bacillus cereus* Suşlarının Enterotoksijenik Gen Profillerinin Multipleks PCR ile Belirlenmesi

Özet

Bu çalışmanın amacı sütlü tatlılarda *Bacillus cereus* varlığını araştırmak ve enterotoksijenik genleri multipleks PCR tekniğiyle belirlemektir. Bu amaçla 25 keşkül, 12 tavuk göğsü, 12 kazandibi, 30 supangle, 4 profiterol ve 17 sütlaç olmak üzere toplamda 100 örnek analiz edilmiştir. 3'ü tavuk göğsü (%25), 2'si supangle (%6.6), 1'i keşkül (%4) ve 1'i sütlaç (%5.8) olmak üzere toplamda 7 örnek (%7.0) *B. cereus* varlığı açısından pozitif bulunmuştur. Analiz edilen sütlü tatlı örneklerinde ortalama *B. cereus* sayısı 2.0×10^1 - 5.0×10^2 kob/g olarak tespit edilmiştir. 7 pozitif örnekten toplamda 20 izolat elde edilmiştir. Sonuçta, *B. cereus* izolatlarının %70'inin (14/20) *hbl* geni taşımadığı, %30'unun (6/20) üç enterotoksik HBL kompleksi kodlayıcı geni (*hblA, hblC* ve *hblD*) bulundurduğu saptanmıştır. Ayrıca yapılan incelemede, izolatların %40'ının (8/20) üç NHE kompleksi kodlayıcı geni (*nheA, nheB* ve *nheC*), %45'inin (9/20) iki *nhe* geni (*nheA* ve *nheB*) ve %15'inin (3/20) bir adet *nhe* geni (ikisi *nheA* ve biri *nheB*) içerdiği saptanmıştır. *ctyK1* geni hiçbir örnekte belirlenememiştir. Sütlü tatlılarda *B. cereus* varlığının gıda zehirlenmelerine yol açarak halk sağlığı açısından potansiyel bir tehlike olabileceği düşünülmektedir.

Anahtar sözcükler: *B. cereus, Sütlü tatlı, Enterotoksin genleri*

INTRODUCTION

The *B. cereus* group consists of six different species: *B. cereus*, *B. mycoides*, *B. thuringiensis*, *B. anthracis, B. weihenstephanensis* and *B. pseudomycoides* [1,2]. *B. cereus* is a Gram-positive bacteria inhabiting numerous environments, including soil, plant materials and many types of foods, especially those of plant origin; however, it is also frequently isolated from meat, eggs and dairy products [3]. An ubiquitous, spore-forming bacterium, *B. cereus* causes food spoilage and can produce two distinct types of toxins that differ in the main symptoms induced in humans. *B. cereus* is the etiologic agent of two types of food-borne disease, resulting in an infection causing vomiting and

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diarrhoea as the major clinical symptoms.

Both *B. cereus*-derived diseases are caused by toxins: the diarrhoeal type occurs due to protein toxins formed in the intestinal tract by growing organisms (enterotoxins) and the emetic toxin type results from a peptide that is preformed in the food (emetic toxin or cereulide) [4,5]. Within 12 h of food consumption, the enterotoxin disease type is characterised by diarrhoea. The emetic disease type is characterised by vomiting and nausea within two hours after consumption of the suspected food. For both types of the disease, clinical signs last approximately 24 h [3].

B. cereus produces at least three different proteins, referred to as enterotoxins, that may cause foodborne illness. The haemolysin BL (Hbl), nonhaemolytic enterotoxin (NHE) and cytotoxin K (CytK) proteins are considered the primary virulence factors in *B. cereus*. The HBL complex is composed of three proteins, B, L1 and L2 [6,7], transcribed from the genes *hblC* (encoding L2), *hblD* (encoding L1), and *hblA* (encoding B). The NHE complex is composed of three proteins, NheA, NheB and NheC encoded by the three genes *nheA*, *nheB* and *nheC*, respectively [8].

B. cereus is a common contaminant of milk [9]. Some investigations have examined the occurrence of toxin genes and toxin producing strains in milk and dairy products [10,11]. Exclusion of *Bacillus* spp. from the food chain is not possible, and the only recognised control method involves maintaining a cold temperature throughout the processing, manufacture and storage stages. It had been reported that toxogenic *B. cereus* strains could affect the constituents in dairy desserts during toxin formation [12]. *B. cereus* causes problems by degrading dairy products, creating off-flavours, sweet curdling and bitter cream because of the production of proteinase, lipase and phospholipases, and it endangers public health through food contamination [13,14].

In dairy products, the presence of *Bacillus* spp. is inevitable, and the spore-formation ability of this organism allows it to survive pasteurisation easily [15]. In milk and dairy products, the presence of *B. cereus* decomposes casein peptides into amino acids and free fatty acids into milk fat, thus degrading the quality of milk products and shortening shelf life [16]. The occurrence of *B. cereus* was previously reported as a contaminant of cheese [17,18].

The objective of this study was to investigate the presence of *B. cereus* and its associated toxins in dairy dessert samples utilising an mPCR technique for special emphasis on their enterotoxigenic potential.

MATERIAL and METHODS

Materials

From June through August 2010, 100 dairy dessert samples were analysed, consisting of 25 keskul (a milk pudding containing coconut), 12 tavuk gogsu (a milk pudding containing chicken breast meat), 12 kazandibi (a milk pudding slightly burned on the bottom), 30 supangle (a milk pudding containing chocolate), 4 profiteroles and 17 sutlac (milk puddings containing rice). The samples were collected from 16 different small patisseries in Samsun. Dairy dessert samples were immediately transported to the laboratory in a refrigerated box and examined within 1-2 h of sampling for the presence of *B. cereus*.

Method

- Isolation and Identification of B. cereus

B. cereus count was determined according to ISO 7932/2004 by the surface plating method with mannitol egg yolk polymyxin (MYP) agar (Oxoid CM0929), and the plates were incubated at 30°C for 24 h. Rough and bright pink colonies with a zone of egg yolk precipitation were then transferred to nutrient agar slants, and identification was confirmed by microscopic and biochemical characterisation that included Gram stain, anaerobic utilisation of glucose, reduction of nitrate, Voges-Proskauer test, motility, oxidase production, catalase production, endospore formation and haemolysis, as suggested by other authors [19-21].

- Detection of B. cereus Enterotoxins Production and Toxin Type

The multiplex PCR technique was used to detect the presence of the *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC* and *ctyK1* genes of *B. cereus* according to the procedure provided by Wehrle *et al*.[22]. For our study, the analysis was a combination of two multiplex PCRs and a general PCR (PCR 1-3). PCR 1 and 2 each included three primer pairs for *hblA*, *hblC*, *hblD* and *nheA*, *nheB* and *nheC*. PCR 3 included a primer pair for *ctyK1* alone. All primer pairs used in this study and corresponding multiplex PCR systems (PCR 1–3) are listed in *Table 1*.

The strains NTCC 11145 *(Hbl, Nhe)*, and NVH 391/98 (*ctyK1*) were used as reference strains.

The template DNA was initially obtained using the boiling method. Two colonies were chosen and inoculated into a microcentrifuge tube containing 500 µl sterile distilled water, suspended in a water bath (Memert, Germany), and boiled for 10 min at 95°C. The tubes were centrifuged at 9500 × *g* for 10 min at 4°C (Hettich-Universal-320R, Germany), after which the supernatant containing DNA was transferred into Dnase/Rnase-free microcentrifuge tubes and stored at -20°C for use as the template DNA.

The PCR reaction was performed in a reaction mixture of 50 µl final volume containing 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.5 µM of forward and reverse primers, 1.5 U Taq polymerase in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5

Table 1. *Characteristics of PCR primers used for multiplex detection of B. cereus toxin genes*

Tablo 1. *B. cereus toksin genlerinin tespiti için kullanılan PCR primerlerinin özellikleri*

Primer	Sequence (5'-3')	Product Size (bp)	Reference
hblC F hblC R	CGAAAATTAGGTGCGCAATC TAATATGCCTTGCGCAGTTG	411	[39]
hblD F hblD R	AGGTCAACAGGCAACGATTC CGAGAGTCCACCAACAACAG	205	[39]
hblA F hblA R	ATTAATACAGGGGATGGAGAAACTT TGATCCTAATACTTCTTCTAGACGCTT	237	[40]
nheA F nheA R	GAGGGGCAAACAGAAGTGAA TGCGAACTTTTGATGATTCG	186	[39]
nheB F nheB R	CCGCTTCTGCAAAATCAAAT TGCGCAGTTGTAACTTGTCC	281	[39]
nheC F nheC R	ACATCCTTTTGCAGCAGAAC CCACCAGCAATGACCATATC	618	[41]
cytK1 F cytK1 R	AACAGATATCGGTCAAAATGC CGTGCATCTGTTTCATGAGG	623	[42]

µL 10× reaction buffer and 2 µL template DNA for PCR 1 and 2. The final reaction mixture for PCR 3 contained 0.1 mM of each dNTP, 1 mM $MgCl_2$, 0.3 µM of forward and reverse primers, 1 U Taq polymerase in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 µL 10× polymerase buffer and 2 µL of template DNA. A negative control without template DNA was included in each experiment. The mixture was then processed in a thermocycler as explained below (Bio Rad-MJ Mini-PTC-1148, Singapore).

The PCR samples were subjected to amplification according to the following program: initial denaturation at 95°C for 5 min, followed by 30 cycles comprising denaturation at 94°C for 1 min, annealing at 55°C for 1 min (PCR 1) and 49°C for 1 min (PCR 2 and 3) and an extension at 72°C for 1 min. A final extension was carried out at 72°C for 10 min.

- Gel Electrophoresis

The PCR products were detected from a 20 µl volume of the amplification mixture (supplemented with 4 µl loading dye), loaded onto a 2.0% agarose gel containing ethidium bromide (Gene Choice), and electrophoresis (BioRad, Power Pac-Basic, Singapore, BioRAD, electrophoresis tank, WideMini, Singapore) was carried out at 90 V for 1.5 h. A 1000-100 bp DNA ladder molecular weight marker was used to identify the amplified products. The PCR products were visualised under UV illumination (Wise-UV-Wuv-L50, Korea).

RESULTS

The numbers of *B. cereus*, enterotoxigenic *B. cereus*, and enterotoxin types isolated from 100 dairy dessert samples are presented in *Table 2*.

B. cereus were detected in 7 (7.0%) of the total 100 dairy dessert samples. Three of tavuk gogsu (25%), 2 of supangle (6.6%), 1 of keskul (4%) and 1 of sutlac (5.8%) samples were positive. The average number of *B. cereus* isolated from dairy dessert samples was $5.0x10^2$ and $2.0x10^1$cfu/g.

A total of 20 *B. cereus* isolates from 7 positive dairy desserts samples were analyzed for the presence of seven enterotoxic gens by PCR. The results showed that 30% (6/20) of *B. cereus* isolates contained three enterotoxic HBL complex encoding genes *hblA*, *hblC* and *hblD*, whereas 70% (14/20) had no *hbl* genes. All three enterotoxic NHE complex encoding genes *nheA*, *nheB* and *nheC* were detected in 40% of isolates (8/20), whereas two nhe genes (*nheA* and *nheB*) were found in 45% (9/20) of the isolates

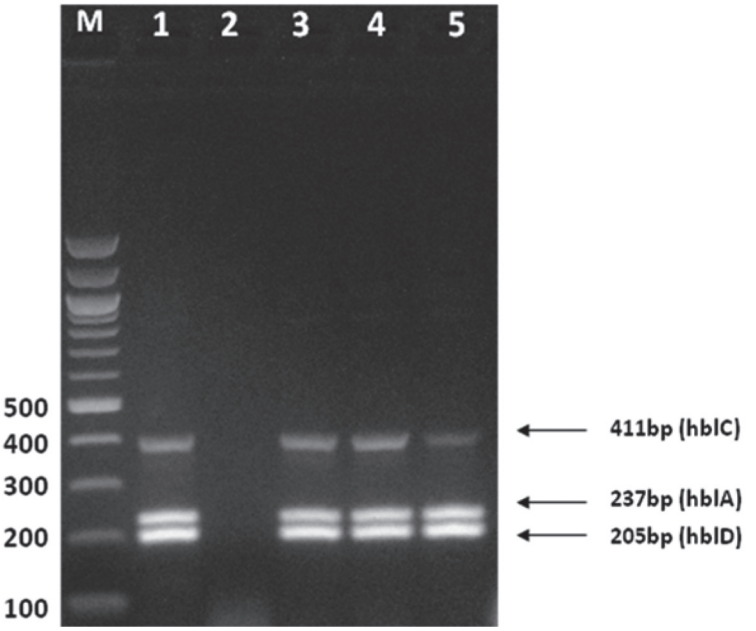

Fig 1. Demonstration of *hblA, hblC,* and *hblD* genes of *B. cereus* strains by multiplex PCR technique. M: Marker; 1: positive control; 2: negative control; 3–5: *B. cereus* isolates

Şekil 1. *B. cereus* izolatlarında *hblA, hblC* ve *hblD* genlerinin multipleks PCR tekniği ile gösterilmesi. M: Marker; 1: pozitif control; 2: negative control; 3-5: *B. cereus* izolatları

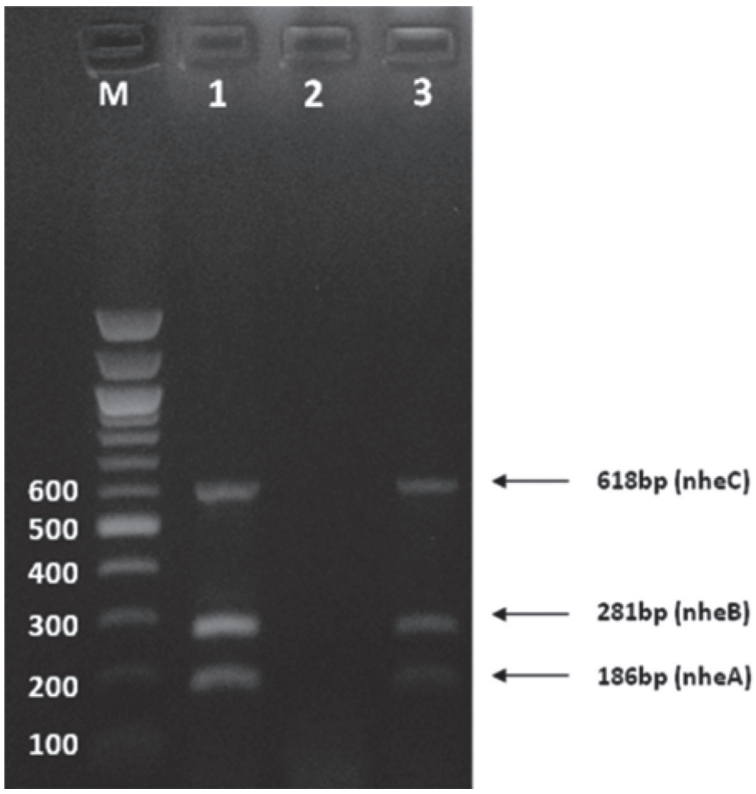

Fig 2. Demonstration of *nheA, nheB,* and *nheC* genes of *B. cereus* strains by multiplex PCR technique. M: Marker; 1: positive control; 2: negative control; 3: *B. cereus* isolate

Şekil 2. *B. cereus* izolatlarında *nheA, nheB,* and *nheC* genlerinin multipleks PCR tekniği ile gösterilmesi. M: Marker; 1: pozitif control; 2: negative control; 3: *B. cereus* izolatı

Table 2. *Prevalence of enterotoxin genes of B. cereus isolates*
Tablo 2. *B. cereus izolatlarının enterotoksin gen dağılımı*

Samples Positive/Total (%)	No. of Isolates	Haemolytic BL Complex			Non-Haemolytic Enterotoxin Complex			Cytotoxin K
		hbl A	*hbl D*	*hbl C*	*nhe A*	*nhe B*	*nhe C*	*cyt K*
Keskul 1/25 (4%)	2	-	-	-	2 (100%)	2 (100%)	-	-
Tavuk gogsu3/12 (25%)	9	6 (66.6%)	6 (66.6%)	6 (66.6%)	9 (100%)	7 (77.7%)	-	-
Kazandibi 0/12	-	-	-	-	-	-	-	-
Supangle 2/30 (6.6%)	4	-	-	-	3 (75%)	4 (100%)	3 (75%)	-
Profiterole 0/4	-	-	-	-	-	-	-	-
Sutlac 1/17 (5.8%)	5	-	-	-	5 (100%)	5 (100%)	5 (100%)	-
Total7/100 (7%)	20	6 (30%)	6 (30%)	6 (30%)	19 (95%)	18 (90%)	8 (40%)	-

and one nhe gene (two *nheA* and one *nheB*) was found in 15% (3/20) of the isolates. The *ctyK1* gene was not detected in any sample (*Fig. 1, Fig. 2* and *Table 2*).

DISCUSSION

High numbers of *B. cereus* result in food poisoning due to the presence of toxins and their subsequent production of toxins, presenting a potential risk to the consumer [23]. In addition to causing food-borne illness, *B. cereus* is responsible for the spoilage of various food products. Borge *et al.*[24], reported that psychrotolerant micro-organisms such as *B. cereus*, contaminate refrigerated foods and continue to be a safety problem. Under normal circumstances, *B. cereus* concentrations found in food are $<10^3$/g and, for the most part, $<10^2$/g. The minimum level required to cause disease from food consumption is estimated at $>10^5$/g [25]; unfortunately, a small dose ($<10^3$/g) may cause disease in susceptible individuals [26].

In this study, *B. cereus* was detected in 7.0% of dairy dessert samples which is in agreement with that of Ahmed *et al.*[27], who examined the presence of *B. cereus* in 400 milk and dairy products and isolated *B. cereus* strains from 9%, 14%, 35%, and 48% of raw milk, cheese, pasteurised milk and ice cream samples, respectively. Te Giffel *et al.*[28], analyzed 334 pasteurised milk samples from household refrigerators in the Netherlands and isolated *B. cereus* from 133 (40%) of the samples. Larsen and Jørgensen [29], examined 458 pasteurised milk samples in Denmark, and the occurrence of *B. cereus* in Danish pasteurised milk was 56%. This rate of contamination is higher than pasteurised milk from Iran and the Netherlands. In Turkey,

B. cereus contamination has been determined previously by Ozdemir [30], in pasteurised milk samples and was isolated from 56 (46.6%) of the samples. Molva *et al.*[31], also detected *B. cereus* from 6% of cheese samples.

B. cereus produces four enterotoxins, called HBL, NHE, cytK and enterotoxin-T [3,8,9]. HBL is considered one of the most important virulence factor of *B. cereus* [6,7]. NHE is a three component (nheA, nheB and nheC), enterotoxin responsible for the diarrheal food poisoning syndrome [9,24]. Cytotoxin K is responsible of diarrheal syndrome with necrotic, hemolytic and cytotoxic effects on the intestinal ephithelium [10].

The presence of diarrhoeal enterotoxin genes in the isolated strains was screened by mPCR as described by Gaviria *et al.*[32], Hansen and Hendriksen [33] and Zahner *et al.*[34]. In the detection of enterotoxin genes, 30% of *B. cereus* strains were found to carry the three *hbl* genes. Molva *et al.*[31], found that all detected strains contained *hblD* gene. Hansen and Hendriksen [33], reported that 64% of *B. cereus* strains contained this gene. Mäntynen and Lindström [35], detected the *hblA* gene in 52% of their *B. cereus* strains. Conversely, Hansen and Hendriksen [33], reported the high prevalence of the *hblA* gene in 13/22 (59%) of the *B. cereus* strains. They also reported that 16 *B. cereus* (73%) strains contained the *hblC* gene. In our study, it was determined that nhe enterotoxins contained a gene distribution of 19 (95%) *nheA*, 18 (90%) *nheB* and 8 (40%) *nheC*. Nduhiu *et al.*[36], detected 3.9% *nheA*, 19.6% *nheB* and 3.9% *nheC* genes from milk and 3.9% *nheA*, 11.8% *nheB* and 3.9% *nheC* genes from cheese from their *B. cereus* strains. However, several authors have also reported that almost all *B. cereus* strains contain *nhe* genes [31,33,37,38].

In conclusion, *B. cereus* strains isolated from dairy dessert samples should be regarded as potential enterotoxin producers according to the PCR results. Therefore, improving product quality and safety should be achieved by applying good manufacturing practice and implementing the hazard analysis and critical control point system. To minimise risk, better hygiene practices are required in the production of the foods.

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RESEARCH ARTICLE

Detection of *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi* by Cultural and Molecular Methods in Dogs in Western Turkey [1]

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Summary

The aim of this study is to exhibit the presence of *Capnocytophaga* species in Turkey and to determine the regional risks of these bacteria for the people in dogs. Totally 200 oral swab samples were taken from owned dogs which have had dental plaque problems. The diagnosis of *Capnocytophaga* infections were done by PCR using *CaL2, AS1, CaR* and *CyR* gene sequence primers. The first PCR was carried out to samples using by *CaL2* and *AS1* primers and results were estimated as *Capnocytophaga* species. The second PCR was applied to samples using *CaR* and *CyR* reverse primers and results were also interpreted as being *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi*. At the end of the first PCR, *Capnocytophaga* spp. was detected from 11 (5.5%) out of 200 samples. *CaR* and *CyR* gene were investigated in samples which were detected as *Capnocytophaga* spp. It was determined that 2 (18.2%) of the samples were positive for *CaR* gene which were identified as *C. canimorsus* and 9 (81.8%) of the samples were positive for both *CaR* and *CyR* gene which were identified as *C. canimorsus* and *C. cynodegmi*. *C. canimorsus* and *C. cynodegmi* species are concluded for generating risk for human health and due to the lacking of information about the disease it was also difficult to diagnosis these agents.

Keywords: *Capnocytophaga canimorsus, Capnocytophaga cynodegmi, Dog, Identification, PCR*

Batı Türkiye'de Köpeklerde *Capnocytophaga canimorsus* ve *Capnocytophaga cynodegmi* Türlerinin Kültürel ve Moleküler Yöntemlerle Araştırılması

Özet

Bu çalışma ile *Capnocytophaga* türlerinin Türkiye'deki varlığının ortaya çıkarılması ve köpeklerde bulunan bu bakterilerin insanlar için bölgesel risklerinin belirlenmesi amaçlanmıştır. Çalışmada dıştaşı olan sahipli köpeklerden 200 adet oral svap örneği toplandı. *Capnocytophaga* infeksiyonlarının tanısı için *CaL2, AS1, CaR* ve *CyR* gen sekans primerleri kullanılarak PCR uygulandı. İlk PCR işlemi *CaL2* ve *AS1* primerleri kullanılarak yapıldı ve sonuçta *Capnocytophaga* spp. identifikasyonları gerçekleştirildi. İkinci PCR işleminde *CaR* ve *CyR* reverse primerleri kullanıldı ve sonucunda *Capnocytophaga canimorsus* ve *Capnocytophaga cynodegmi* identifikasyonları gerçekleştirildi. İlk PCR işleminden sonra, 200 örneğin 11 (%5.5)'inden *Capnocytophaga* spp. identifiye edildi. *Capnocytophaga* spp. olarak identifiye edilen örneklerde *CaR* ve *CyR* gen bölgeleri araştırıldı. Bu örneklerin 2 (%18.2)'sinde *CaR* geni pozitif bulundu ve örnekler *C. canimorsus* olarak identifiye edildi. Örneklerin 9 (%81.8)'unda *CaR* ve *CyR* genleri pozitif bulundu ve bu örnekler *C. canimorsus* ve *C. cynodegmi* olarak identifiye edildi. *C. canimorsus* ve *C. cynodegmi* türleri insan sağlığı için risk oluşturmaktadır. Bu infeksiyonlar hakkındaki yetersiz bilgi, infeksiyonların tanısını ve bu bakterilerin identifikasyonlarını zorlaştırmaktadır.

Anahtar sözcükler: *Capnocytophaga canimorsus, Capnocytophaga cynodegmi, Köpek, İdentifikasyon, PCR*

INTRODUCTION

Millions of people are somehow bitten by animals every year in the world. Bite wounds cause complications ranging from mild injuries to serious infections. These infections may be related to the mouth flora of the animal

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and/or pathogens available on the skin flora of bitten person. The most frequently seen bacterial zoonotic agents, transmitted to the humans by animal bite, are *Pasteurella multocida, Bartonella henselae, Spirillum minus, Streptobacillus moniliformis, Francisella tularensis* and *Capnocytophaga canimorsus (C. canimorsus)* [1,2].

C. canimorsus, formerly designated Dysgonic fermenter 2 (DF-2) was first described in 1976; it is a commensal bacterium of dogs and cats saliva, which can be transmitted to man by bite (54% of cases), scratch (8.5%), or mere exposure to animals (27%) [3]. The infection distribution with dogs and cats are 24% and 10%, respectively [4]. *Capnocytophaga gingivalis, Capnocytophaga ochracea* and *Capnocytophaga sputigena* species caused localized juvenile periodontitis on the immunocompetent hosts and could be isolated from other regions as part of polymicrobial infections [3].

C. canimorsus and *C. cynodegmi* have regularly been isolated from the oral cavities of dogs. These two species are very similar in morphology and in small subunit ribosomal RNA gene (16S rRNA) sequence with a 97% similarity [5].

C. canimorsus infections were found to occur worldwide and have been reported from the United States, Canada, Europe, Australia and South Africa [6]. Australia has a canine population of about 3.75-4 million. It is estimated that around 63% of Australian households own some type of pet, with 53% owning a cat or a dog. It has been projected that each year more than 100.000 Australians are attacked by dogs, causing injuries of varying degrees of severity. The Emergency Department of Australia's public hospitals treat between 12.000 and 14.000 people for dog bite injuries and almost 1400 of those have injuries that are serious enough to warrant hospitalization. Between 4% and 25% of dog bite wounds become infected. Bacteria also caused to cellulitis, fatal sepsis, organ failure, meningitis and endocarditis [7].

Dilegge et al.[8] showed that 49.2% of canine samples (59 positive out of the total 120) carried a species of *Capnocytophaga* spp. Of the total number of canines sampled, 21.7% of which (26 positive) carried *C. canimorsus*, and 11.7% (14 positive) carried *C. cynodegmi*. Four canines carried *C. ochracea*, one canine carried *C. haemolytica*, and one carried an isolate that was either *C. gingivalis* or *C. granulose, C. canimorsus* was characterized by very mild symptoms or lead to fatal infections such as sepsis with showing severe symptoms. *C. cynodegmi* has a low possibility of creating systemic infection. Therefore most of the *Capnocytophaga* infections that have been reported were contiguous with the oropharynx, including periodontal diseases, ophthalmic lesions, respiratory tract infections, traumatic pericarditis, mediastinal or cervical abscesses and local wound infections [9,10].

Genetic and biochemical similarities between *C. canimorsus* and *C. cynodegmi* make it rather difficult to identify these species and thus there is a need for specific and enhanced molecular methods for both species identification. Reports about the presence of *C. canimorsus* strains showed that this bacteri is hardly distinguished from *C. cynodegmi* by the comparison of the 16S rRNA sequences. Therefore, to develop more convenient and specific PCR systems to identify the *Capnocytophaga* spp. is required [11].

The aim of this study is to exhibit the presence of *Capnocytophaga* species in Western of Turkey and to point out a risk by these bacteria for the human population in the region.

MATERIAL and METHODS

Isolation of Capnocytophaga spp.

A number of 200 oral swabs were taken by the convenient technique from domestic dogs having tartar in Muğla provinces in Western of Turkey the dates between January and April 2012. Samples were delivered to Adnan Menderes University Veterinary Faculty of Microbiology Department in cold chain. Of the 200 swabs samples, 77 (38.5%) were from male dogs and 123 (61.5%) were from female dogs. This study was conducted according to the Ethical Committee regulations of Adnan Menderes University (document ID 2009/54). The distribution of the samples with year and sex were shown on the *Table 1*.

It was aimed to obtain pure culture of *Capnocytophaga* spp. from oral swab samples arrived to the laboratory. For the purpose preventing the other flora bacteria growth, oral swab samples were inoculated onto sheep blood agar including gentamicin. The cultivated agar plates were incubated in 5% CO_2 atmosphere for 5 days. After the incubation Gram staining method were applied to suspected colonies. Biochemical tests were applied to the strains for the identification of *Capnocytophaga* spp.[12].

Extraction of DNA from Oral Swabs

Samples were collected using sterile swabs from 200 dogs gingival tissues (BD BBL culture swab plus) and these samples were suspended with Brain-Heart Infusion Broth and were incubated at 5% CO_2 microaerophilic atmosphere

Table 1. *The distribution of swab samples taken from dogs by year and sex*

Tablo 1. *Köpeklerden alınan svap örneklerinin yaş ve cinsiyete göre dağılımları*

Dogs Groups by Years	Number of Male Dogs	Number of Female Dogs	Total Number of Dogs
0-5	63	107	170
6-9	13	15	28
10 and over	1	1	2
Total	77	123	200

for 24 h at 35°C. Bacterial cells were harvested from broth culture and passed to DNA extraction application. DNAs were extracted from swab samples with Ultraclean Microbial DNA Isolation Kit® (MO BIO Laboratories, Inc.) as recommended by the manufacturer. DNA extracts were stored in cryo-tubes at -20°C until PCR studies [13].

Primers and PCR

CaL2, AS1, CaR, and *CyR* primers were designed for the determination for *Capnocytophaga* spp which informed as Suzuki et al.[12].

In order to obtain positive control DNAs for the PCR assays, two strains of *C. canimorsus* and *C. cynodegmi* were kindly provided by Michio SUZUKI (1-23-1 Toyama Shinjuku-ku Tokyo, Japan).

In the first PCR protocol we used, total volume of 50 µl for a sample in PCR amplification were adjusted as follows for identification of *Capnocytophaga* spp.; ViBuffer A (Vivantis®) 10x enzyme buffer solution 1x, magnesium chloride ($MgCl_2$) 50 mM, 10x dNTP, primer (for *CaL2-AS1* primer pair) 10 pmol, Pfu DNA polymerase 5U (Vivantis®) [12].

The second PCR amplifications were performed to in the same conditions using by *CaL2-CaR* and *CaL2-CyR* primers to identify *C. canimorsus* and *C. cynodegmi* [12].

PCR conditions were as follows; an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min [12]. PCR was performed in an Eppendorf Master Cycler with a capacity of 25 samples thermal cycling.

Detection of the Amplified Products

The 10 µl amplified products were detected by staining with 0.5 µg/ml ethidium bromide after electrophoresis at 80 V for 40 min in 2% agarose gels. PCR products of 124 bp for *Capnocytophaga* spp. and 427 bp for *C. canimorsus* and *C. cynodegmi* were considered evidence for identification.

RESULTS

Isolation of Capnocytophaga spp.

The *Capnocytophaga* spp. was not identified from all oral samples by conventional and biochemical tests.

PCR

By this work as a part of a postgraduate thesis, the diagnosis of *Capnocytophaga* infections which could not be made as routinely, were shown to be done by using *CaL2*, *AS1, CaR* and *CyR* gene sequence primers. In this study, 200 oral swab samples were taken from owned dogs which have dental plaque and at the end of first PCR, *CaL2* and *AS1* gene were detected from 11 (5.5%) out of 200 samples. So, these samples were evaluated as *Capnocytophaga* spp. After the DNAs of the agent identified as *Capnocytophaga* spp. they were evaluated for being positive for *C. canimorsus* or *C. cynodegmi* using *CaR* and *CyR* gene nucleotide sequences at second PCR. It was also determined that 2 (18.2%) of the samples were positive for *CaR* gene which were identified as *C. canimorsus* and 9 (81.8%) of the samples were positive for both *CaR* and *CyR* gene which were identified as *C. canimorsus* and *C. cynodegmi*. Apmlified PCR products were shown at *Fig. 1* and *2* which were positive for *Capnocytophaga* spp. and *C. canimorsus* and *C. cynodegmi*, respectively.

As the result of this study, 2 (22.2%) positive samples were detected as *C. canimorsus* and *C. cynodegmi* from male dogs and 7 (78.2%) from females. Only 2 positive samples were detected as *C. canimorsus* (100.0%) from female dogs totally. Rates for *C. canimorsus* and *C. cynodegmi* positive according to year were shown on the *Table 2*.

The distribution of positivity by year is examined and the results are as follows; out of the 11 dogs were detected as *Capnocytophaga* spp. one (9.1%) was from 6-9 year group and . ten (90.9%) were from 0-5 year group dogs. Out of nine that found as *C. canimorsus* and *C. cynodegmi* eight were (88.8%) from 0-5 year group and1 (11.2%) was

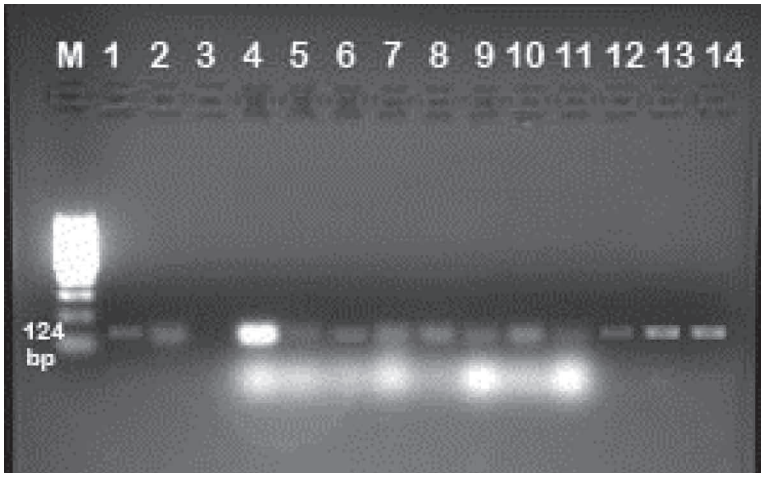

Fig 1. *Capnocytophaga* spp. PCR results (using *CaL2-AS1* primers), **M:** 100bp DNA ladder, **1:** *Capnocytophaga canimorsus* Positive Control, **2:** *Capnocytophaga cynodegmi* Positive Control **3:** Negative Control, **4-14:** *Capnocytophaga* spp. PCR Positive Samples

Şekil 1. *Capnocytophaga* spp. *CaL2-AS1* primerleri kullanılarak elde edilen PCR sonuçları, **M:** 100bp DNA işaretleyicisi, **1:** *Capnocytophaga canimorsus* Pozitif Kontrol, **2:** *Capnocytophaga cynodegmi* Pozitif Kontrol **3:** Negatif Kontrol, **4-14:** *Capnocytophaga* spp. PCR Pozitif Örnekler

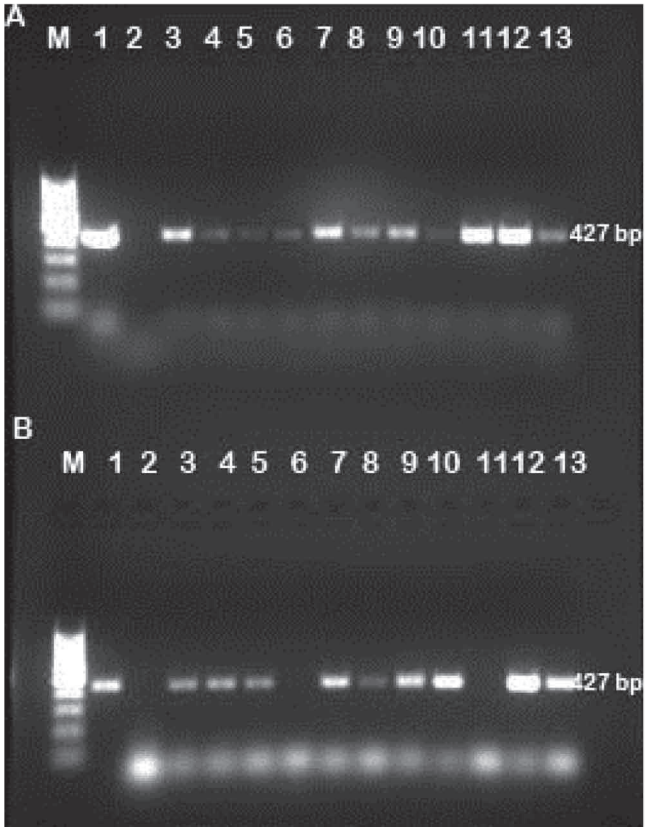

Fig 2. *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi* PCR results

A) *Capnocytophaga canimorsus* PCR results using *CaL2-CaR* primers, **M:** 100bp DNA ladder, **1:** *Capnocytophaga canimorsus* positive control, **2:** Negative Control **3-13:** *Capnocytophaga canimorsus* PCR positive samples **B)** *Capnocytophaga cynodegmi* PCR results using *CaL2-CyR* primers, **M:** 100bp DNA ladder, **1:** *Capnocytophaga cynodegmi* positive control, **2:** Negative Control **3-5:** *Capnocytophaga cynodegmi* PCR positive samples **6:** *Capnocytophaga cynodegmi* PCR negative samples **7-10:** *Capnocytophaga cynodegmi* PCR positive samples **11:** *Capnocytophaga cynodegmi* PCR negative sample **12-13:** *Capnocytophaga cynodegmi* PCR positive samples

Şekil 2. *Capnocytophaga canimorsus* ve *Capnocytophaga cynodegmi* PCR sonuçları

A) *Capnocytophaga canimorsus CaL2-CaR* primerleri kullanılarak elde edilen PCR sonuçları, **M:** 100bp DNA işaretleyicisi, **1:** *Capnocytophaga canimorsus* Pozitif Kontrol, **2:** Negatif Kontrol **3-13:** *Capnocytophaga canimorsus* PCR pozitif örnekler **B)** *Capnocytophaga cynodegmi CaL2-CyR* primerleri kullanılarak elde edilen PCR sonuçları, **M:** 100bp DNA işaretleyicisi, **1:** *Capnocytophaga cynodegmi* Pozitif Kontrol, **2:** Negatif Kontrol **3-5:** *Capnocytophaga cynodegmi* PCR pozitif örnekler **6:** *Capnocytophaga cynodegmi* PCR negative örnek **7-10:** *Capnocytophaga cynodegmi* PCR pozitif örnekler **11:** *Capnocytophaga cynodegmi* PCR negative örnek **12-13:** *Capnocytophaga cynodegmi* PCR pozitif örnekler

Table 2. *C. canimorsus and C. cynodegmi positive rates by year*
Tablo 2. *Yaşlara göre C. canimorsus ve C. cynodegmi pozitiflik oranl/arı*

PCR Positive Samples	0-5 Years	6-9 Years	10 Year and over	Total Number of Positive Samples
C. canimorsus	2	-	-	2
C. cynodegmi	-	-	-	0
C. canimorsus and *C. cynodegmi*	8	1	-	9

from a 6 years old dog. Two samples were detected as *C. canimorsus* from the 0-5 year group dogs.

DISCUSSION

In 1976, *C. canimorsus* was reported as a commensal bacterium in the oral flora of dogs and isolated from the blood and spinal fluid of a patient caused by an unidentified Gram negative bacillus, after a recent dog bite was first described [14]. Five *Capnocytophaga* species *(C. gingivalis, C. ochracea, C. sputigena, C. granulosa* and *C. haemolytica)* have been reported to be found in the human oral cavity and been associated with peridontitis. It is now clear that *C. cynodegmi* and *C. canimorsus* are part of the commensal oral microbiota of canines and more rarely of cats [6,15,16].

In the first study for *C. canimorsus* [17], the researchers examined 50 dogs samples and found *C. canimorsus* prevalence at 8%. In the latter reports *C. canimorsus* infections are associated with dog bites or close animal contact such as licking of human wounds. In a study done with the conventional culture method *C. canimorsus and C. cynodegmi* were detected in 74% and in 86% of dogs, respectively [6].

In another report including the culture and PCR results of *C. canimorsus* detection, 26% of the dogs tested were positive by both culture and PCR [18].

Currently, PCR based detection system is described which discriminates between *C. canimorsus* and *C. cynodegmi* and using this method the prevalence of both bacteria was determined in the dogs and cats [12].

The same study reported that on the basis of purely molecular screening of oral swabs using PCR amplification of a fragment of the small subunit ribosomal RNA gene (16S rRNA), that 74% of canines sampled (out of 325 canines) harbored *C. canimorsus*. The researcher determined the specificity and sensitivity of the PCR performed with different combinations of primers for discriminatory amplification of the 16S rRNA gene of *C. canimorsus* and *C. cynodegmi*. The *CaL2–AS1* primer pair could amplify the target sequences from the DNA derived from both *C. canimorsus* and *C. cynodegmi*. Specific amplification of *C. canimorsus* DNA but not *C. cynodegmi* DNA was achieved by the *CaL2–CaR*, whereas the DNA fragment of *C. cynodegmi* alone was amplified by the PCR using the *CaL2-CyR* primer pair [12].

The samples of the dogs and cats were, therefore, examined by PCR with these primers for the presence of specific sequence of *C. canimorsus* and *C. cynodegmi*. 240 of 325 (74%) dogs and 66 of 115 (57%) cats were tested positive for *C. canimorsus*, while *C. cynodegmi* was detected in 279 of 325 (86%) dogs and 97 of 115 (84%) cats. Both of these species were detected in 219 (67%) of 325 dogs and 64 (56%) of 115 cats [12].

Isolation of *C. canimorsus* (DF-2) from sheep and cattle (25-30% of the animals tested) but not from pigs was reported [19].

In this study, 200 oral swap samples which were taken from owned dogs which have had dental plaque, were examined and at the end of first PCR, *CaL2* and *AS1* gene were detected from 11 (5.5%) out of samples. Therefore, these samples were evaluated as *Capnocytophaga* spp. positive. After identifying the DNAs of the agent as *Capnocytophaga* spp. the strains were verified for being positive of *C. canimorsus* and *C. cynodegmi* using *CaR* and *CyR* gene nucleotide sequences by a second PCR. After the second PCR using the specific *CaR* and *CyR* primers, *CaR* and *CyR* gene regions were investigated in samples already detected as *Capnocytophaga* spp. positive. It was determined that 2 (18.2%) of the samples were positive for *CaR* gene which were identified as *C. canimorsus* and 9 (81.8%) of the samples were positive for both *CaR* and *CyR* gene which were identified as *C. canimorsus* and *C. cynodegmi*. As a result of this study, there were no positive samples detected for only *CyR* gene sequence.

Lavy et al.[20] reported that, *Capnocytophaga* species were found in 3 out of 17 dogs which were under 6 months of year. This year range is of a particular concern since puppies begin to lose their first teeth around 4 months of year and the teeth are completely replaced by their adult canines by 6 months.

In another study, the researcher reported that *Capnocytophaga* species were carried 72.7% in toy dog breeds. These results must be interpreted with caution that transmitted from dogs to human [8].

In this study when the result of positivity range is analyzed by year, 10 (90.9%) of the samples were detected in 0-5 years animals, and 1 (9.1%) of the sample were detected in 6 years animal out of 11 *Capnocytophaga* spp. positive samples. Eight (88.8%) of the samples were detected in 0-5 years animals and 1 (11.2%) of the sample was detected in 6 years animal out of 9 *C. canimorsus* and *C. cynodegmi* positive samples. Two (100%) samples which were only *C. canimorsus* positive, were detected in 0-5 years animals. In males, 2 (22.2%) *C. canimorsus*/*C. cynodegmi* positivity were detected while in females the corresponding figure was 7 (78.2%). Only 2 (100%) samples were detected as *C. canimorsus* positive in female animals. According to our data, female dogs were sensitive *Capnocytophaga* spp. infections.

C. canimorsus and *C. cynodegmi* species are considered for generating risk for human health and these agents hard to determine. The difficulty in diagnosis also causes the ignorance of these infections. These strains isolated from oral flora of dogs, the most common pet animal of our country, can be transmitted from dogs to human. Therefore, dogs can take an important part of *Capnocytophaga spp.* infections dissemination to human via oral excrections, such as saliva, and direct contact with mouth and tongue. Beside *Capnocytophaga* infections', these bacteria are associated with serious diseases such as meningitis, acute organ failures, intravascular coagulopathy, etc. in humans especially in immunodeficient patients. *Capnocytophaga* infections can likely be transmitted from dogs to humans by means of especially oral ways and even by kissing.

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RESEARCH ARTICLE

Effects of Heat Stress on Egg Yield and Mortality Rates of Caged Poultry Houses

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Summary

In present study, effects of heat stress due to variations in indoor temperature and relative humidity on egg yield and mortality rates of hens in caged poultry houses were investigated between the months April and August (5 months). The experimental poultry house has automated feeder and waterer and operates at 90% capacity. Each cage has 5 hens and there were a total of 9900 Isa Brown hens in the poultry house before the experiments. Hens were 27th weeks old in the beginnings of the experiment. During the experiments, indoor and outdoor climate parameters such as temperature and relative humidity, daily egg production and mortalities were continuously recorded. Structural characteristics of the poultry house were also determined. Heat and moisture gains/losses, temperature humidity index (THI), egg production rates (EPR) and mortality rates (MR) were calculated. Results revealed significantly increasing and strong relationships between indoor temperature and THI – MR and significantly decreasing relationships between indoor temperature and EPR ($P<0.01$). Indoor temperature increased from 20.7°C in April to 29.4°C in August, THI values increased from 66.1 to 77.0 during the same period. Therefore, mortality rates increased from 0.36% in April to 1.59% in August. While EPR was 88.7% in April, the value decreased to 79.4% in August. Without sufficient wall and roof insulation, it was found to be impossible to provide an indoor temperature of neither 18°C to keep EPR at high levels nor 21°C to keep MR≤‰0.1.

Keywords: *Poultry house, Temperature, Humidity, Heat stress, Egg production rate, Mortality rate*

Kafesli Kümeste Sıcaklık Stresinin Yumurta Verimi ve Mortalite Üzerine Etkileri

Özet

Bu araştırmada Nisan-Ağustos döneminde (5 ay) kafesli tip yumurta tavuğu kümesinde kümes içi sıcaklık ve bağıl nem değişimine bağlı olarak ısı stresinin tavuklarda yumurta verimi ve mortalite üzerine etkisi incelenmiştir. Otomatik yemleme ve sulama sistemiyle donatılan, %90 kapasite kullanım oranına sahip olan kümeste her kafese 5 tavuk yerleştirilmiş olup, deneme başlangıcında kümeste 27 haftalık yaşta 9900 adet Isa Brown ırkı tavuk bulunmaktadır. Araştırma boyunca kümes içi ve dış ortam sıcaklık ve bağıl nemi, kümeste günlük yumurta üretimi, günlük ölen tavuk sayısı sürekli kaydedilmiştir. Ayrıca, kümese ait yapısal özellikler ölçülmüştür. Kümese ait ısı ve nem dengeleri, Sıcaklık Nem İndeksi (THI), Yumurta Verimi (EPR) ve Ölüm Oranı (MR) hesaplanmıştır. Araştırma sonuçlarına göre; kümes iç sıcaklığı ile THI ve MR arasında artan, kümes iç sıcaklığı ile EPR arasında ise azalan istatistik yönden çok önemli ($P<0.01$) ve kuvvetli ilişkiler tespit edilmiştir. Bu ilişkilere göre Nisan ayından Ağustos ayına doğru kümes sıcaklığının 20.7°C'den 29.4°C'ye ve THI'nin 66.1'den 77.0'a artışı sonucu aylık MR Nisanda %0.36 iken aylara göre artarak Ağustosta %1.59'a kadar çıkmıştır. EPR ise aynı dönemde aylık olarak Nisan'da %88.7 iken Ağustos ayında %79.4'e kadar gerilemiştir. Bu nedenle çatı ve duvarda yeterli izolasyon olmadan ve serinletme yapmadan kümes sıcaklığını ne yüksek EPR için gerekli olan optimum 18°C sıcaklıklarda, ne de günlük MR≤‰0.1 olduğu 21°C kümes içi sıcaklık bandında tutmanın mümkün olmadığı görülmüştür.

Anahtar sözcükler: *Kümes, Sıcaklık, Bağıl nem, Sıcaklık stresi, Yumurta verim randımanı, Ölüm oranı*

INTRODUCTION

In poultry facilities, beside sufficient feeding and proper genotypes, indoor environmental conditions should also be kept at optimum levels to provide "animal welfare" and consequently optimize the operation and thus maximize

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the income. It is not possible to reach the desired yield levels only by selecting high-yield genotypes and implementing the best feeding programs. Improper indoor environmental conditions definitely hinder the expected outcomes from the facility.

Environmental conditions play a significant role in laying hen facilities to provide desired productivity levels. Especially indoor temperature and relative humidity have direct impacts on physiological activities of hens. Hens are able to keep body temperatures and some mechanisms only within certain temperature intervals and they can not adapt to high temperatures. Their higher production performance and feed conversion efficiency make today's chickens more susceptible to heat stress than ever before [1].

High temperatures create some health effects on hens such as vaso-dilatation, decrease in blood flow rates toward glands forming the shell, increase in respiration rates, respiratory alkalosis, decrease in blood ionic Ca level, decrease in carbonic anhydrate enzyme activities in kidney and egg-shell glands, decrease in Ca mobilization from bone-deposits. All these health-effects decrease egg yields [2-5].

Researches to determine upper (18-32.2°C) and lower (7.2-19°C) limits of proper indoor temperatures and to determine optimum growth temperatures vary based on the region where the poultry house is located, type of housing, animal species and growing periods [6-17]. In case of exceeding lower and upper heats, different effects and following physical changes can be observed on chickens: i) decrease in egg weight with heat stress [6]; ii)heat stress increases with increasing temperature and relative humidity, egg yield and feed consumption decrease [7,8]; iii) decrease Egg Yield Rate and increase Mortality Rate with increasing inside temperature and/or with increasing Temperature Humidity Index [9-14]; iv) distinctive negative effects of heat stress on yield and mortality rates [15]; v) strains in metabolism, ultimate changes in sensible heat and latent moisture production [16]; vi) a 44% decrease in egg yield at 21°C poultry house [17]; and vii) decrease trend in egg production [18].

Hens perform better at constant temperatures (21-22°C) than varying temperatures (17-35°C) and health problems are less in constant temperatures [18]. Each 1°C increase in temperatures between 25-30°C results in 1.5% decrease in egg yield [19].

Relative humidity generally does not have significant impacts on hens at temperatures between 15.6-26.6°C, but relative humidity above 50% at temperatures between 26.6-37.7°C endangers the life of hens. High temperatures together with 70-75% relative humidity speed up the growth of microorganism populations. Therefore, relative humidity of poultry houses should always be kept below 80% [20]. Optimum relative humidity ranges for laying hen poultry houses are recommended as between 50-75% [21], 50-80% [22] and 60-80% [15].

Combined effect of temperature and relative humidity on poultry houses is explained by Temperature-Humidity Index (THI). A growing atmospheres with a THI value ≤70 is defined as "comfort zone", a value between 75-78 is defined as "stress zone" a value ≥78 is classified as "extreme stress zone" [23].

There are some laboratory studies about the negative impacts of temperature and relative humidity on animals. However, in-situ researches are not preferred due to population sizes, difficulties in control of animals, higher labor needs and similar reasons. Therefore, evaluations about the effects of heat stress on animal performance and yield are mostly depend on limited data. In present study, indoor and outdoor climate factors (temperature and relative humidity) of a caged poultry house were regularly measured and effects of heat stress on EPR and MR were investigated under actual conditions. Furthermore effects of aging on EPR and MR are also included to the regression models and compared to ISA Brown commercial layer production recording chart.

MATERIAL and METHODS

Caged poultry house, selected for experimental purposes, is located in Tokat Province (39°51' N and 40°55' E) of Middle Black Sea Region of Turkey. It has a capacity of 11.000 hens with automated feeder and waterer and operated at 90% capacity. Each cage has 5 hens and there were a total of 9900 Isa Brown hens aged 189 days aged (27 weeks) in the poultry house before the initiation of experiments. Although EPR and MR values are usually calculated via "age in weeks"; in this study, statistic analyses were done using "age in days". Thus

Long term average temperatures are 12.5, 16.4, 19.8, 22.4 and 22.3°C, and relative humidities are 60.0, 61.0, 59.0, 57.0 and 58.0 for April-August period (1961-2011) in the research region according to the Turkish State Meteorological Service [24,25].

Experimental poultry house is oriented along east-west direction and it is 40 m long, 11 m wide and 2.75 m high. The house is operated with natural ventilation system with 6 air outlets and 22 windows along the long axes.

Walls were constructed with 19 x 19 x 13.5 cm hallow tiles, 2 cm inner and 3 cm outer lime-cement plaster were applied over the walls. The roof was insulated by 3 cm Styrofoam over wood siding and covered by corrugated asbestos-cement roofing.

During the experimental period, daily feed consumptions, lighting and ventilation levels were kept constant. EPR and MR values were recorded daily. Indoor and outdoor temperature and relative humidity values of

April - August period were periodically measured with a "Datalogger" (HOBO RH/Temp, Type: HO8-003-02, USA) as to have 1 data/h and variations in temperature and relative humidity were monitored. Heat - moisture balance calculations were performed by using hourly and daily averages of measured data.

Criteria specified by NIH (National Institute of Health Guide for the Care and Use of Laboratory Animals) were obeyed during the experiments carried on animals.

Heat transfer coefficients of constructional members, heat - moisture balance and ventilation capacities heat and moisture production of hens were all determined by using relevant calculation procedures [9,13,15,22,24,26-28].

According to pentant principle, minimum design outdoor temperature of Tokat Province is -15°C [29] and placed into the 2nd Climate Zone. Calculated heat transfer coefficients by using these assumptions benchmarked with the recommended values for roofs and walls based on climate zone and type of housing [30,31].

Sensible heat production (SHP) and moisture production rate (MPR) of the poultry house were calculated as follows [32,33].

$$SHP = \rho \times V \times C_p \times (T_e - T_{out}) + U\,(T_{in} - T_{out}) - Q_{Sup} - Q_{Equip} \quad (1)$$

Where;

SHP : Sensible heat production rate, W

ρ : Density of inlet air, kg/m^3

V : Ventilation rate, m^3/s

Cp : Specific heat of inlet air, J/ (kg.K)

T_e, T_{out}, T_{in} : Exhaust, outdoor and indoor air temperature, respectively, °C

U : Building heat transfer coefficient, W/K

Q_{Sup}, Q_{Equip} : Heat from supplementary heaters and other internal equipment, respectively, W

$$MP = \rho \times V \times (W_e - W_o) \quad (2)$$

Where;

MPR : Moisture production rate, kg/s

W_e, W_o : Humidity ratio of exhaust and outdoor, respectively, kg/kg

Temperature-Humidity Index (THI) was calculated by [23];

$$THI = 1.8 \times T_{in} - (1 - j_{in}) \times (T_{in} - 14.3) + 32 \quad (3)$$

Where;

THI : Temperature-Humidity Index

T_{in} : Poultry house indoor temperature, °C

j_{in} : Poultry house indoor relative humidity

Descriptive statistics, correlation analysis, principle components and factorial analysis, single and multiple regression analyses were performed between treatments by using SPSS 18 statistical analysis package [34]. Furthermore effects of aging on EPR and MR also included to the model regression analyses.

RESULTS

Unit heat gains/losses were calculated by using surface areas of constructional members and their heat transfer coefficients. Results revealed the roof and walls as the largest winter/summer heat gain/loss sources *(Table 1)*.

Indoor and outdoor hourly temperature and relative humidity values for the experimental period are provided in *Table 2*.

By taking heat losses and heat production of hens into consideration, irradiative heat-up (Q_{rad}), maximum natural

Table 1. *Area, heat transfer coefficient and unit heat loses for constructional members*
Tablo 1*. Kümes yapı elemanlarına ilişkin alan, ısı geçirme katsayısı ve birim ısı kayıpları*

Construction Member	Wall	Windows	Doors	Roof
Area (m^2)	260.0	16.2	4.2	495.0
Coefficient of Heat Transfer (W/m^{2}K)	1.60	5.88	6.04	0.99
Building heat transfer coefficient (W/K)	416.0	95.3	25.4	490.1

Table 2. *Variation of hourly outdoor and indoor temperature and relative humidity values between April and August period*
Tablo 2*. Nisan-Ağustos döneminde iç ve dış sıcaklık ve nispi nem değişimi*

Month	Outdoor Temperature (°C)			Outdoor RH (%)			Indoor Temperature (°C)			Indoor RH (%)		
	Min	Max	Average	Min	Max	Average	Min	Max	Average	Min	Max	Average
April	-2.9	30.8	13.3	22	96	55	12.3	26.5	20.7	23	81	50
May	11.2	31.0	16.6	24	97	64	17.6	27.6	22.8	32	72	52
June	12.8	32.4	19.9	29	99	67	17.9	30.4	23.9	21	76	52
July	16.8	34.8	23.8	22	93	56	18.3	33.7	26.0	23	79	49
August	21.5	38.8	25.1	33	97	61	21.8	38.5	29.4	18	78	48
Apr-Aug	-2.9	38.8	19.8	22	99	60.8	12.3	38.5	24.6	18	81	49.9

Table 3. *Irradiative heat-up, natural ventilation capacity, ventilation capacity provided per hen and THI, EPR and MR as monthly average values*
Tablo 3. *Aylara göre kümeste radyasyonla ısı artışı, sağlanan doğal havalandırma kapasitesi, tavuk başına sağlanan havalandırma kapasitesi, THI, EPR ve MR ortalama değerleri*

Month	Q_{rad} (W)	V_{max} (m^3/h)	V_{max}/hen (m^3/h.hen)	THI	EPR (%)	MR (‰)
Apr	8393	52361	5.29	67.3	88.7	3.6
May	9711	58959	5.96	68.8	85.7	7.5
Jun	10185	64310	6.50	70.6	83.9	8.9
Jul	11289	91353	9.23	72.5	81.8	13.1
Aug	12948	98500	9.95	76.0	79.5	15.9
Average				71.1	83.9	9.7
Total						49.0

Table 4. *Pearson correlation coefficients between daily average values of parameters and significance levels (Probability: P-Value)*
Tablo 4*. Araştırma parametrelerine ilişkin günlük ortalama değerlerin "Pearson Correlation" katsayıları ve istatistik önem düzeyleri (Olasılık: P-değeri)*

Parameters	T_{in} (°C)	j_{in} (%)	T_{out} (°C)	j_{out} (%)	MR (‰)	EPR (%)
j_{in}	-0.189 (P<0.05)			0.116 (NS)		
T_{out}	**0.786** (P<0.01)	-0.119 (NS)				
j_{out}	0.118 (NS)	0.031 (NS)	-0.134 (NS)			
MR	**0.793** (P<0.01)	-0.160 (P<0.05)	**0.658** (P<0.01)			
EPR	-0.688 (P<0.01)	0.065 (NS)	-0.594 (P<0.01)	-0.127 (NS)	-0.613 (P<0.01)	
THI	**0.987** (P<0.01)	-0.034 (NS)	**0.781** (P<0.01)	0.128 (NS)	**0.779** (P<0.01)	-0.687 (P<0.01)

NS: *Non-Significant*

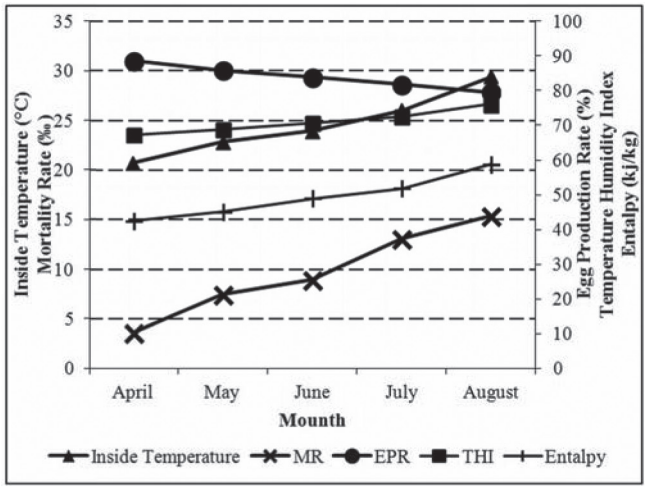

Fig 1. Variation of indoor air temperature, enthalpy, THI, EPR and MR for April-August period

Şekil 1. Araştırma kümesinde Nisan-Ağustos dönemi kümes içi sıcaklık, entalpi, THI, EPR ve MR değişimi

ventilation capacity (V_{max}), and maximum ventilation capacity per hen (V_{max}/hen) were calculated. THI was found to be as 71.1 (67.3-76.0), EPR as 83.9 (88.7-79.5) and MR as 9.5 (3.6-15.9) in monthly average values (*Table 3* and *Fig 1*).

Indoor temperature during the experimental period was ≥20°C at 91.1% of total time, ≥25°C at 40.9%, ≥27°C at 25.0%, ≥30°C at 10.6% and ≥33°C at 3% of the total time. Average indoor relative humidity was ≥50% at 51.4% and ≥%60 at 16.4% of the total time.

About 56% of THI values during the experimental period were above the threshold value of ≥70. Monthly evaluations revealed that 11.0, 40.3, 61.3, 85.4 and 99.9% of THI values respectively of the months April, May, June, July and August were above 70.

Daily values were used to see the variations in indoor and outdoor air temperatures, RH, THI, EPR and MR values. Average daily indoor air temperature was 24.60°C, indoor RH was 49.97%, THI was 71.10, EPR was 83.87% and MR was ‰0.32.

Pearson correlation coefficients between investigated parameters were calculated and significance levels were determined. The highest correlation (0.987) was observed between indoor temperature and THI *(Table 4)*.

Principal component analysis and factor analysis were performed and corresponding factor loadings were determined. Results revealed that the first 3 factors were able to explain 86.5% of the total variation. Factor 1 explained 57.1%, factor 2 explained 15.5% and factor 3 explained 13.9% of the total variation. Single evaluation of factor 1 revealed the loadings as 0.965 for daily average indoor temperature, 0.955 for THI, 0.867 for MR, 0.853 for daily average outdoor temperature and -0.796 for EPR. In factor 2, daily average outdoor relative humidity was the parameter with a loading (-0.882) value over 0.5. In factor 3, only daily average indoor relative humidity had a loading value (-0.861) of over 0.5 *(Table 5)*.

Table 5. *Unrotated factor loadings and communalities on principal component factor analysis of the correlation matrix*
Tablo 5. *Korelasyon matris faktör analizi yapılan değişken parametreler ve yükleri*

Source of Variation	Factor 1	Factor 2	Factor 3
Daily Average Indoor Air Temperature (T_{in})	0.965	-0.005	0.034
Daily Average Indoor RH (j_{in})	-0.168	-0.476	-0.861
Daily Average Outdoor Air Temperature (T_{out})	0.853	0.247	-0.168
Daily Average Outdoor RH (j_{out})	0.106	-0.882	0.431
MR (‰)	0.867	-0.025	0.060
EPR (%)	-0.796	0.115	0.045
THI	0.955	-0.083	-0.101
Variance	3.9949	1.0866	0.9727
% Variance	0.571	0.155	0.139
Total Variance (%)	0.865 (86.5%)		

equations were calculated and probability significance levels were determined *(Table 6)*. Results revealed the highest hit as R^2 97.4% between THI and inside temperature (T_{in}) (P<0.01) (*Table 6* and *Fig. 2*).

Multiple regression analysis performed to estimate EPR revealed a hit rating of 50.3% when the entire parameters are included into the model. The hit rating was observed as 47.3% when the daily average indoor temperature was included into the model and as 47.2 when THI included into the model *(Table 6; Fig. 3, Fig. 4)*.

Standard characteristic values of EPR vs Age was reported as 96% for 189 days (27 weeks) aged layers and 91% for 329 days aged layers (47 weeks) in standard performance characteristic chart from breeder company [35]. Thus it can be observed that at optimum conditions, aging of layers between 27th to 47th weeks affects EPR at 5% decreasing level. However EPR at 27th week and 47th week was recorded as 91% and 76% respectively. Thus regression analyses were done to determine which factors are related to this catastrophic decrease on EPR. Analyses results revealed that; when regression model includes T_{in}, j_{in}, T_{out}, j_{out}, MR, THI and Age hit rating was 51.4 while Age factor excluded from model hit rating decreased to 50.3% *(Table 6)*. Similarly, when model includes T_{in} and Age factors, hit rating value was determined as 48.5 whereas the model includes only T_{in}, R^2 value was calculated as 47.3%.

Multiple regression analysis performed to estimate MR revealed a hit rating of 64.3% when the entire parameters are included into the model. The hit rating was observed as 62.8% when the daily average indoor temperature was included into the model and as 60.6% when THI included into the model *(Table 6, Fig. 3, Fig. 4)*.

Table 6. *The single and multiple regression equations hit ratings (R^2) and probabilities (P)*
Tablo 6.*İkili ve çoklu regresyon eşitliklerinin isabet oranları (R^2) ve olasılıkları (P)*

Regression Equation	R^2	P
$THI = 40.1 + 1.26\ T_{in}$	97.4 %	<0.01
$EPR = 73.8 - 1.45\ T_{in} - 0.163 j_{in} - 0.105\ T_{out} - 0.0365\ j_{out} - 3.12\ MR + 1.00 THI - 0.0439 Age$	51.4 %	<0.01
$EPR = 67.7 - 2.38\ T_{in} - j_{in} - 0.148\ T_{out} - 0.0386\ j_{out} - 41.4\ MR + 1.34\ THI$	50.3 %	<0.01
$EPR = 116 - 0.256\ THI - 0.0528 Age$	49.8 %	<0.01
$EPR = 105 - 0.254\ T_{in} - 0.0574\ Age$	48.5 %	<0.01
$EPR = 109 - 1.03\ T_{in}$	47.3 %	<0.01
$EPR = 141 - 0.804\ THI$	47.2 %	<0.01
$MR = 0.124 + 0.00933\ T_{in} + 0.000418\ j_{in} + 0.000346\ T_{out} + 0.000082\ j_{out} - 0.000428\ EPR - 0.00448\ THI$	64.3 %	<0.01
$MR‰ = -0.784 + 0.0449\ T_{in}$	62.8 %	<0.01
$MR‰ = -2.14 + 0.0346\ THI$	60.6 %	<0.01

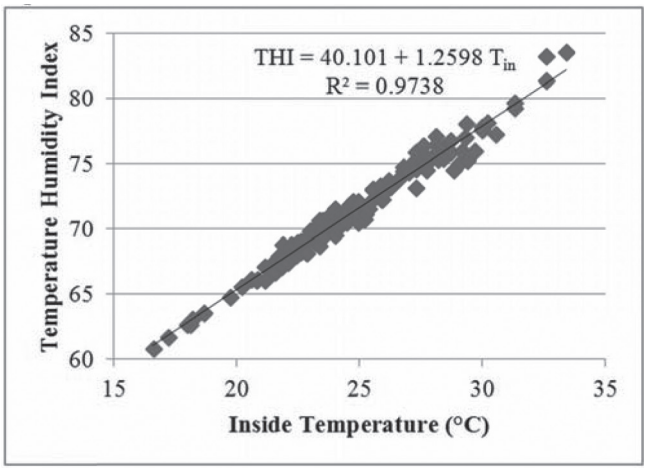

Fig 2. Relationship between THI and indoor temperature (T_{in})
Şekil 2. THI ile kümes içi sıcaklık (T_{in}) ilişkisi ve regresyon denklemi

Single and multiple regression analyses were performed between the parameters and regression equations were determined. Hit ratings of estimations made by regression

DISCUSSION

Heat balance calculations of the poultry house were performed by taking the climate zone of Tokat Province

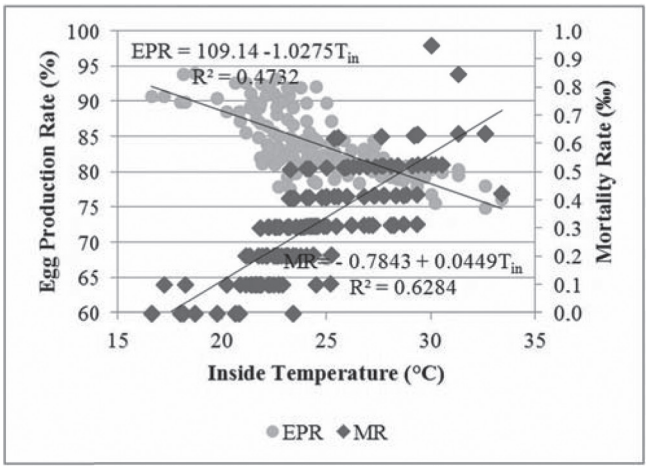

Fig 3. Relationship between EPR and MR based on variations in daily average indoor temperatures

Şekil 3. Ortalama günlük kümes içi sıcaklık değişimine göre EPR ve MR ilişkisi

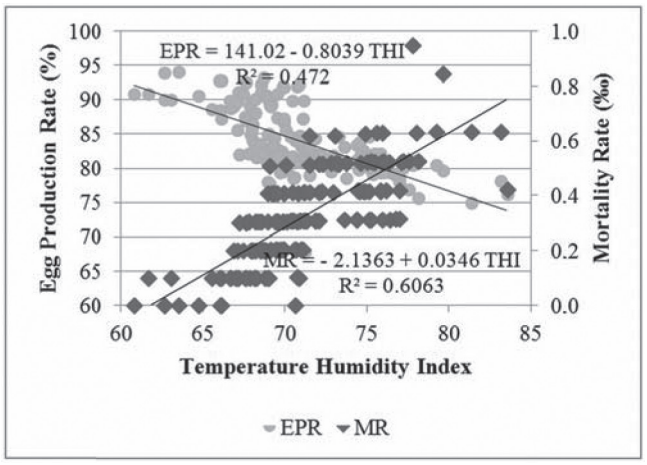

Fig 4. Relationships between THI and EPR-MR

Şekil 4. THI ile yumurta verim randımanı ve ölüm oranı arasındaki ilişki

into consideration and heat transfer coefficients of walls and roof were respectively determined as 1.60 W/m²K and 0.99 W/m²K. The ideal values should be 0.91 W/m²K and 0.33 W/m²K. Therefore, walls were found to be 78% insufficient and roof was found to be 300% insufficient compared to ideal values. Such deficiencies result in excessive heat loss during the winter months and heat gain in summer months. Supplementary insulation over the outer wall surfaces and additional roof insulation may bring the wall heat transfer coefficients to ideal values. Proper insulation and consequent heat gain/loss balance may prevent excessive THI values and fluctuations during summer/winter months. Such a case may also prevent undesired decreases in EPR and increases in MR.

Each 1°C increase in indoor temperatures between 25-30°C results in 1.5% decrease in egg yield [19]. While daily EPR of the present study was over 90% when the indoor temperatures were ≤ 20°C, the value decreased to 82.5% at 25°C and to 75% at 30°C with a 1.5% decrease corresponding to each unit increase in temperature.

A growing atmosphere for poultry houses with a THI value of ≤70 is defined as "comfort zone", a value between 75-78 is defined as "stress zone" and a value ≥78 is classified as "extreme stress zone" [23]. While EPR was >90% when the THI was ≤70, the value decreased to 80% when the THI increased to 75. Similarly, EPR rapidly decreased to 73% when the THI increased to 83. A unit increase in THI or poultry house indoor temperature may result in 1-1.5% decrease in EPR. Since the hens subjected to heat stress are not able to consume sufficient feed to present optimum performance, a decrease in egg yield is evident. Hence, EPR values over 90% in "comfort zone" (THI 60-65) decreased to 70% by moving away from comfort zone (THI 80-85), corresponding about 20% decrease in egg yield.

While daily MR was around 0.2‰ under THI values of 65-70, the value reached to 0.6-0.8‰ levels under THI value of 85 over the threshold value. If such a high THI value is persistent in poultry house, daily MR may reach to 1‰ level.

The positive high correlation (0.793) between indoor temperature and MR of present study was found to be significant (P<0.01) and indicated increased mortalities parallel to increasing temperatures.

The correlation between T_{out} and T_{in} (0.786) was also found to be significant (P<0.01). This correlation indicates insufficient acclimatization and increase or decrease of indoor temperatures with increasing or decreasing outdoor temperatures.

Positive correlations between T_{out} and THI (0.781) (P<0.01), between THI and MR (0.779) (P<0.01) and negative correlation between T_{in} and EPR (-0.688) (P<0.01) indicated that increasing THI values moved the growing atmosphere away from the "comfort zone" and decreased EPR accordingly.

The negative correlation between THI and EPR (-0.687) was found to be significant (P<0.01). Similar relationship between THI and EPR and between T_{in} and EPR (-0.688) (P<0.01) was an expected case and considered as the result of psychrometric relation between indoor temperature and THI. A 65.8% correlation (P<0.01) was observed between outdoor temperature and MR. Indoor temperature was mostly depend on outdoor temperature because of insufficient acclimatization and ventilation. Increasing outdoor temperatures rapidly increase indoor temperatures and move the growing atmosphere away from the "comfort zone" and consequently increase the mortality rates. A decreasing relationship was observed between egg yield and outdoor temperatures (59.4%) (P<0.01). Such a relationship again indicates the negative impacts of outdoor temperature on animal comfort and consequent egg yields for poultry houses without sufficient climate control.

Furthermore, it is well known fact that EPR performance losses down to 75% is an economic break-even point. According to genetic company performance charts [35], EPR values at week 27 and 47 are 96% and 91% respectively. In addition 75% EPR value occurs at week 80 for ISA Brown layers. However the results of this study showed that exceeding optimum conditions can cause more adverse effects than aging resulting an EPR value of 91% at week 27 and 76% at week 47.

Heat stress is the most significant factor to be considered in laying hen poultry houses. Beside the construction and equipments, climate-related environmental factors play a critical role in performance and yields of hens. Therefore, heat transfer coefficients of constructional members, especially of walls and roof, should be kept as low as possible to prevent excessive cooling in winter months and heat-up in summer months. Measures should be taken not only against cold stress but also against heat stress.

Indoor temperatures should be prevented not only against seasonal changes in temperatures but also against daily sudden changes in temperatures. Since it is impossible to totally eliminate heat stress-related economic losses due to physiological and metabolic changes, some kind of measures may be taken for constructional members, indoor production techniques and/or feeding practices to minimize such losses.

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RESEARCH ARTICLE

Antioxidant Activity of Cinnamon Bark Oil (*Cinnamomum zeylanicum* L.) in Japanese Quails Under Thermo Neutral and Heat Stressed Conditions

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Summary

The essential oil from bark of *Cinnamomum zeylanicum Lauraceae* was analyzed by GC and GC-MS systems in this research. The essential oil was obtained by hydrodistillation, in 0.7 (v/w) oil yields. Twelve constituents representing 99.2% of the cinnamon oil were identified. The major compounds in the oil were *cinnamaldehyde* (88.2%), *benzyl alcohol* (8.0%) and *eugenol* (1.0%). To determine antioxidant activity of the cinnamon oil, a total of 180 quails, fifteen-days-old, were allocated into 6 groups consisting of 10 birds of 3 replicates according to balanced gender and inital live weight. The birds were kept in wire cages in a temperature controlled room at 22°C for 24 h/d in thermo-neutral (TN) groups. For heat-stress (HS) groups, the birds were exposed to 34°C for 8 h/d (from 9:00 to 17:00), and later 22°C 16 h/d was performed. Relative humidity was approximately 60-65%. Basal diet was given to control groups in both TN and HS. The birds were fed with the basal diet supplemented 250 or 500 ppm cinnamon oil in the other experimental groups. Heat stress increased the malondialdehyde (MDA) levels of liver ($P<0.001$), heart ($P<0.01$) and kidney ($P<0.05$). It also induced superoxide dismutase (SOD) production of liver ($P<0.001$) and kidney ($P<0.05$). Glutathione peroxidase (GSH-Px) activity and glutathione (GSH) level of liver ($P<0.001$, $P<0.05$) and heart ($P<0.001$, $P<0.05$) were found lower under HS condition. Cinnamon oil supplementation to diet significantly increased antioxidant enzyme activity and GSH level of the tissues in both enviromental conditions ($P<0.01$). Dose of 500 ppm cinnamon oil had strong effect on antioxidant activity of the internal organs ($P<0.01$). In conclusion, cinnamon oil supplementation to diet reduced the adverse effects of heat stress and resulted the protective effect on the internal organs by activating antioxidant mechanism.

Keywords: *GC-MS, Cinnamon oil, Lipid peroxidation, Antioxidant activity, Quail, Heat stress*

Termo-Nötral ve Sıcaklık Stresi Koşullarındaki Japon Bıldırcınlarında Tarçın Kabuğu Yağının (*Cinnamomum zeylanicum* L.) Antioksidan Aktivitesi

Özet

Bu araştırmada, tarçın (*Cinnamomum zeylanicum Lauraceae*) kabuğundan elde edilen uçucu yağ GC and GC-MS sistemleri ile analiz edilmiştir. Uçucu yağın elde edilmesinde hidrodistilayon 0.7 (v/w) metodu kullanılmıştır. Elde edilen yağın %99. 2 sini oluşturan oniki bileşen tanımlanmıştır. Tarçın yağının önemli bileşenlerini *sinamaldehid* (%88.2), *benzil alkol* (%8.0) ve *öjenol* (%1.0) oluşturmaktadır. Tarçın yağının antioksidan aktivitesini belirlemek için toplam 180 adet on beş günlük bıldırcın, altı alt gruba ayrılmıştır. Her grup on bıldırcın içeren üç tekerrürden oluşturulmuş, gruplar başlangıç canlı ağırlığı ve cinsiyet bakımından dengelenmiştir. Bıldırcınlar sıcaklık kontürollü odalarda tel kafeslerde, termo-nötral (TN) grupta 22°C'de 24 saat/gün şeklinde barındırılmışlardır. Sıcaklık stresi (HS) grupları günde 8 saat süresince (9:00'dan 17:00'ye/tüm araştırma) 34°C'ye ve daha sonra 22°C'ye 16 saat/gün sıcaklığa maruz bırakılmışlardır. Bağıl nem yaklaşık %60-65 dir. TN ve HS şartlarında kontrol grubundaki bıldırcınlara temel yem verilmiştir. Diğer deneme grupları temel yeme 250 ve 500 ppm tarçın yağı ilave edilen yemle beslenmişlerdir. Sıcaklık stresi karaciğer ($P<0.001$), kalp ($P<0.01$) ve böbrek ($P<0.05$) dokularında malondialdehit (MDA) düzeyini yükseltmiştir. Stres karaciğer ($P<0.001$) ve böbrek ($P<0.05$) dokularında süperoksit dismutaz (SOD) aktivitesini artırırken, karaciğer ($P<0.001$, $P<0.05$) ve kalp dokularında ($P<0.001$, $P<0.05$) sırasıyla glutatyon peroksidaz (GSH-Px) aktivitesi ile glutatyon (GSH) seviyesini azaltmıştır. Yeme ilave edilen tarçın yağı her iki çevre şartında dokuların antioksidan enzim aktiviteleri ile GSH düzeyini önemli ölçüde yükseltmiştir ($P<0.01$). Tarçın yağının 500 ppm düzeyindeki dozu güçlü antioksidan özellik göstermiştir ($P<0.01$). Sonuç olarak, yeme katılan tarçın yağı sıcaklık stresinin olumsuz etkilerini azaltmış ve antioksidan metabolizmayı aktive ederek iç organlar üzerinde koruyucu etki göstermiştir.

Anahtar sözcükler: *GC-MS, Tarçın yağı, Oksidatif stres, Antioksidan aktivite, Bıldırcın, Sıcaklık stresi*

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INTRODUCTION

Heat stress (HS) is a very serious situation for poultry and can be described as difficulty achieving a balance between body heat production and heat loss. Air temperature, humidity, radiant heat and air speed are important factors causing acute or chronic HS in poultry. Acute HS refers to short and sudden periods of the extremely conditons, whereas chronic HS refers to extended periods of elevated the conditions [1,2]. Under the long term stress condition or repeated stress, birds start to fatigue and weak [2,3]. Long-term regulation of body in chronic stress conditions are characterized by adrenal cortical hypertrophy and increased synthesis and release of adrenal glucocorticoids, known as corticosterone (CORT) in bird [4,5]. Administration of CORT may initially induce the formation of reactive oxygen species (ROS) as indirectly reflected by an increase in lipid peroxidation (LP) [6,7]. The ROS including hydrogen peroxide (H_2O_2), hypochlorous acid (HClO) and free radicals such as the hydroxyl radical (·OH) and the superoxide anion (O_2^-) are produced in cells. ROS is a natural by-product of the routine metabolism of oxygen, however under the environmental stress (e.g., heat stress), levels of ROS can increase dramatically. This may resulted with significant damage in the cell structure. Cumulatively, this is known as oxidative stress [6]. Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in ROS causes overproduction of MDA, it is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress for the cells [8]. Superoxide dismutase (SOD) is a group of metalloenzymes whose function appears to be protection of cells from the toxic effects of the endogenously generated superoxide radicals [9]. Glutathione (GSH) is an antioxidant that play a major role in a redox potential regulation, detoxication of ROS, and also serves as a store and transport form of cysteine as well [10]. Glutathione peroxidase enzyme (GSH-Px) provides a mechanism for detoxification of peroxides (e.g. H_2O_2) and a wide variety of organic peroxides (R-OOH) to alcohols (R-OH) and water using cellular glutathione in living cells [9]. External antioxidants can directly react with free oxygen radicals, genotoxic susbtances or carcinogenics, by chelating complexes with transition metals, act as reducing agents, induce the production of antioxidative enzymes [11,12].

Cinnamon *(Cinnamomum zeylanicum* L.) is native to tropical Asia, especially Sri Lanka and India. Cinnamon bark is used as spices and for the production of essential oil. It has lots of medical properties [13] and antioxidant activity [14]. Plant tissue of cinnamon has a wide variety of phenolic compounds such as flavonoids, isoflavones, flavones, catechin and other phenolics. The phenolic compounds are dominant antioxidants that exhibit scavenging efficiency on ROS. The plant tissue has also strong antioxidant capacity because of richness from some antioxidant vitamins and minerals [15,16].

The objective of the present study is to determine antioxidant property of *Cinnamomum zeylanicum* bark oil in Japanese quails reared under thermo neutral and heat stressed condition.

MATERIAL and METHODS

Experimental Design

A total of 180 Japanese (*Coturnix coturnix Japonica*) quails, fifteen-days-old, obtained from a commercial company were used for the experiment. This study was undertaken after ethical approval of Firat University (Official form date and number: 20.01.2011 and 2011/15). The experiment was conducted at the Poultry Unit of Firat University. The birds were assigned to experimental groups at the beginning of the study with a balanced gender and inital live weight. The experimental design were performed according to 2 (thermo neutral-TN, high ambient temperature-HS) × 3 (cinnamon oil levels: 0, 250, 500 ppm) factorial design, 6 treatment groups consisting of 10 birds with 3 replicates. The birds were kept in wire cages in a temperature controlled room at 22°C for 24 h/d in TN groups. For HS groups, the birds were exposed to 34°C for 8 h/d (from 9:00 to 17:00), and later 22°C 16 h/d was performed. Relative humidity was approximately 60-65%. Basal diet was given to control groups of both TN and HS. The birds were fed with the basal diet supplemented 250 or 500 ppm cinnamon oil in the other experimental groups. The cinnamon oil was mixed in a carrier (zeolite), then added to the basal diet at a level of 1 kg/ton. The concentration of the volatile components in cinnamon oil was shown at *Figure 1* (shown as percentage peak areas of GC-MS). Diets and fresh water were given *ad libitum*. Light was provided continuously (24 h) throughout the experiment. Ingredients and chemical composition of the basal diet were shown at *Table 1*.

At the end of the study (43rd day) six male and six female quails from each experimental group whose body weight near the group average were slaughtered. After slaughtering process, liver, heart and kidney tissues were obtained from the carcasses and wrapped with aluminum foil and stored at -20°C until analysis.

Chemical Analysis

Chemical composition of feed ingredients (dry matter, crude protein, ash and ether extract) were analyzed according to the AOAC [17] procedures and crude fibre was determined by the method of CRAMPTON and MAYNARD [18].

Gas Chromatographic (GC) Analysis

The essential oil was analyzed using HP 6890 GC equipped with and FID detector and an HP- 5 MS (30 m x 0.25 mm i.d., film tickness 0.25 µm) capillary column was used. The column and analysis conditions were the same as in GC-MS. The percentage composition of the essential oil

Table 1. *Ingredients and chemical composition of basal mix diet*
Tablo 1. *Bazal karma yemin bileşimi ve kimyasal kompozisyonu*

Feed Ingredients	%	Nutritional Composition	%
Maize	29.03	Dry matter	88.25
Wheat	25.00	Crude protein	23.87
Soybean meal (48 CP)	34.29	Crude fibre	2.55
Corn Gluten	4.10	Ether extract	4.75
Vegetable oil	2.92	Ash	5.45
Dicalcium phosphate	2.02	Calcium ****	1.00
Ground limestone	0.87	Total phosphorus****	0.79
$NaHCO_3$	0.12	ME, kcal/kg****	2897
Salt	0.28		
DL-Metiyonin	0.02		
Vitamin mix *	0.25		
Mineral mix**	0.10		
Additive***	1.00		

** Vitamin premix supplied per 2.5 kg; Vitamin A 12.000.000 IU; vitamin D_3 2.000.000 IU; vitamin E 35.000 mg; vitamin K_3 4.000 mg; vitamin B_1 3.000 mg; vitamin B_2 7.000 mg; Niacine 20.000 mg; Calcium D-pantotenat 10.000 mg; vitamin B_6 5.000 mg; vitamin B_{12} 15 mg; Folik Asit 1.000 mg; D-Biotin 45 mg; vitamin C 50.000 mg; Choline chloride 125.000 mg; Canthaxanthin 2.500 mg; Apo Karotenoik Acid Ester 500 mg , ** Mineral premix supplied per kg; Mn 80.000 mg; Fe 60.000 mg; Zn 60.000 mg; Cu 5.000 mg; Co 200 mg; I 1.000 mg; Se 150 mg, *** Group Cinnamon 0 (1000 g zeolit); Group Cinnamon 250 (25 g cinnamon oil+975 g zeolit); Group Cinnamon 500 (50 g cinnamon oil + 950 g zeolit), **** Calculated*

was computed from GC - FID peak areas without correction factors.

Gas Chromatography/Mass Spectrometry (GC-MS) Analysis

The oils were analyzed by GC-MS, using a Hewlett Packard system. HP- Agilent 5973 N GC-MS system with 6890 GC in Plant Products and Biotechnology Res. Lab. in Firat University. HP-5 MS column (30 m x 0.25 mm i.d., film tickness 0.25 µm) was used with helium as the carrier gas. Injector temperature was 250°C, split flow was 1 ml/min. The GC oven temperature was kept at 70°C for 2 min and programmed to 150°C at a rate of 10°C/min and then kept constant at 150°C for 15 min to 240°C at a rate of 5°C/min. Alkanes were used as reference points in the calculation of relative retention indices (RRI). MS were taken at 70 eV and a mass range of 35-425. Component identification was carried out using spectrometric electronic libraries (Wiley, Nist).

Lipid Peroxidation

The malondialdehyde (MDA) level was measured in serum using the thiobarbituric acid reaction described by PLACER et al.[19]. The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption to the standard curve of MDA equivalents generated by acid catalyzed hydrolysis of 1,1,3,3 tetra-methoxypropane. Each sample was assayed in duplicate, and the assay coefficients of variation for MDA were less than 3%.

Superoxide Dismutase (SOD)

The plasma SOD activity was measured using xanthine and xanthine oxidases to generate superoxide radicals which react with nitroblue tetrazolium (NBT) [20]. Briefly, each sample was diluted 1:10 with phosphate buffer (50 mM, pH 7.5). The assay solution containing sodium-carbonate buffer (50 mM, pH 10), 0.1 mM xanthine, 0.025 mM NBT, 0.1 mM EDTA, xanthine oxidase (0.1 U/mL in ammonium sulfate 2 M) and sample were mixed in a cuvette. One unit of SOD activity was defined as the amount of enzyme required to cause inhibition of NBT. SOD activity was then measured at 560 nm by the degree of inhibition of this reaction on a spectrophotometer and expressed as U/mL.

Glutathione Peroxidase (GSH-Px)

The GSH-Px activity was determined according to LAWRENCE and BURK [21]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM ethylene diamine tetra acetic acid (EDTA), 1 mM sodium azide (NaN3), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 IU/ml oxidized glutathione (GSSG)-reductase, 1 mM GSH, and 0.25 mM hydrogen peroxide (H_2O_2). Enzyme source (0.1 ml) was added to 0.8 ml of the above mixture and incubated at 25°C for 5 min before initiation of the reaction with the addition of 0.1 ml of peroxide solution. The absorbance at 340 nm was recorded for 5 min on a spectrophotometer. The activity was calculated from the slope of the lines as micromoles of NADPH oxidized per minute. The blank value (the enzyme was replaced with distilled water) was subtracted from each value.

Reduced Glutathione (GSH)

The GSH content of the serum was measured at 412 nm by the method of SEDLAK and LINDSAY [22]. The samples were precipitated with 50% trichloroacetic acid and then centrifuged at 1000 × g for 5 min. The reaction mixture contained 0.5 ml of supernatant, 2.0 ml of Tris-EDTA buffer (0.2 M; pH 8.9) and 0.1 ml of 0.01 M 5,5'-dithio-bis-2-nitrobenzoic acid. The solution was kept at room temperature for 5 min, and then read at 412 nm on the spectrophotometer.

Statistical Analysis

Data were evaluated by using GLM (General Linear Model) procedure (2x3 factorial design), significant differences were further subjected to Duncan's multiple range test (SPSS) [23]. The results were considered significant when P values were lower than 0.05.

RESULTS

Twelve compounds were identified by GS-MS in C. *zeylanicum* bark oil representing 99.2% of the total oil (*Fig. 1*

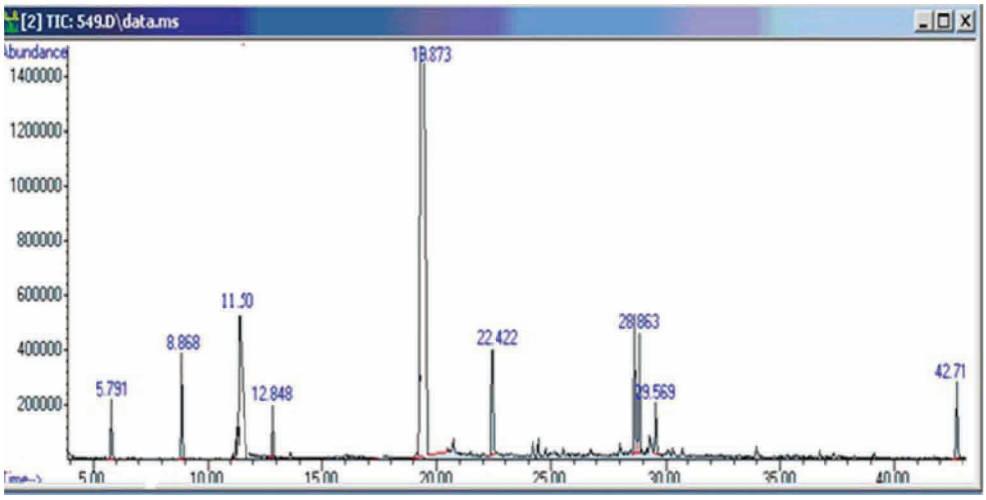

Fig 1. Total ion chromatogram of Cinnamon (*Cinnamomum zeylanicum L.)* bark volatile oil.

Şekil 1. Tarçın (*Cinnamomum zeylanicum L.*) kabuğu uçucu yağının toplam iyon kromotogramı

Table 2. *Chemical composition of cinnamon (Cinnamomum zeylanicum L.) bark volatile oil*

Tablo 2. *Tarçın (Cinnamomum zeylanicum L.) kabuğu uçucu yağının kimyasal kompozisyonu*

Peak No	RT (min)	RRI	Compounds	Peak Area (%)	Identification Method
1	5.79	976	p-Xylene	0.2	GC, GC-MS
2	8.86	1044	Benzaldehyde	0.3	
3	11.50	1102	Benzyl Alcohol	8.1	
4	12.84	1131	Formic acid	0.2	
5	13.62	1148	α-Terpinolene	0.1	
6	15.63	1192	Benzenepropanol	0.1	
7	16.69	1216	α-Terpineol	0.1	
8	19.87	1286	Cinnamaldehyde	88.2	
9	22.41	1341	Eugenol	1.0	
10	28.86	1483	Cinnamaldehyde propylene glycol acetal	0.5	
11	29.56	1493	1H-Cycloprop[e] azulene	0.1	
12	42.71	1788	Benzyl cinnamate	0.3	
Total				99.2	

RT: *Retention Time,* ***RRI:*** *Relative Retention Indices,* ***RRI=*** *((RT*100)+3865)/4.55*

and *Table 2*). Three of them are major compounds including *cinnamaldehyde* (88.2%), *benzyl alcohol* (8.0%) and *eugenol* (1.0%). Other nine compounds are minor containing in amounts less than 1%, including *cinnamaldehyde propylene glycol acetal* (0.5%), *benzaldehyde* (0.3%), *benzyl cinnamate* (0.3%), *p-xylene* (0.2%) *formic acid* (0.2%), *α-terpinolene* (0.1%), *benzenepropanol* (0.1%), *α-terpineol* (0.1%), *1H-cycloprop[e]azulene* (0.1%).

Heat stress increased the MDA levels of liver (P<0.001), heart (P<0.01) and kidney (P<0.05) tissues *(Table 3)*. SOD production of liver (P<0.001) and kidney (P<0.05) were found higher, however GSH-Px activity and GSH production of liver (P<0.001, P<0.05) and heart (P<0.001, P<0.05) were found lower under the HS condition. Cinnamon oil addition to diet decreased MDA levels of liver (P<0.05), heart (P<0.001) and kidney (P<0.05) tissues under HS condition, the current dose of cinnamon oil did not have significant effect in that condition. MDA level of heart was decreased (P<0.001) with the dose of cinnamon oil in TN condition even 500 ppm level had better effect. SOD activity of liver (P<0.001) and heart (P<0.05) increased in cinnamon oil groups in both enviromental conditions. GSH-Px activity of heart (P<0.05) in thermo-neutral condition and GSH level of heart (P<0.01) and kidney (P<0.01) were found to be higher in cinnamon groups in the both conditions.

DISCUSSION

The results of the current study show that twelve compounds representing 99.2% of the total essential oil of C. *zeylanicum* bark were identified. The major compounds in the essential oil were *cinnamaldehyde* (88.2%), *benzyl alcohol* (8.0%) and *eugenol* (1.0%). Other components analyzed in the oil were less than 1%. The composition of the essential oil of *C. zeylanicum* is quite variable, depending on the locality of growth and different part of the plant. However, *eugenol* is main component of the oil from leaf and *cinnamaldehyde* for the oil from bark [24,25]. WANG et al.[26] reported that twenty one volatile compounds identified from the essential oil of *C. zeylanicum* leaf, including aldehydes, alcohols, alkanes, ketones, ethers and sulfides. *Eugenol* (79.75%) was the major volatile component instead of *trans-cinnamaldehyde* (16.25%). FICHI et al.[27] stressed that *eugenol* (76.1%), *caryophyllene* (6.7%) and *linalool* (3.7%) were the major components in the chemical composition of the essential oil of *C. zeylanicum* leaves. Similar to the current study, UNLU et al.[28] analyzed the essential oil from the bark of *C. zeylanicum* by using GC-MS. Nine constituents representing 99.24% of the total oil were identified. The major compounds in the oil were *cinnamaldehyde* (68.95%), *benzaldehyde* (9.94%) and *cinnamyl acetate* (7.44%). YANG et al.[29] showed that *C. zeylanicum* bark essential oil was composed of three major [*cinnamaldehyde* (58.1%), *benzaldehyde* (12.2%) and

Table 3. *Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and glutathione (GSH) levels of inner organs in experimental groups*

Tablo 3. *Deneme gruplarında iç organların malondialdehit (MDA), süperoksit dismutaz (SOD), glutatyon peroksidaz (GSH-Px) ve glutatyon (GSH) düzeyleri*

Traits	HS-Heat Stress			TN- Thermo Neutral			SEM	P- Statistical Significance		
	Cinnamon Oil, ppm			Cinnamon Oil, ppm				Main Effects		CO*EC
	0	250	500	0	250	500		Environmental Conditions (EC)	Dose of Cinnamon Oil (CO)	
MDA (nmol/mL)										
Liver	12.29^{a}	8.21^{b}	8.25^{b}	6.13^{A}	5.89^{A}	5.74^{A}	0.46	***	*	NS
Heart	191.33^{a}	129.53^{b}	129.93^{b}	131.49^{A}	123.32^{B}	97.96^{C}	5.39	**	***	NS
Kidney	62.82^{a}	51.95^{b}	47.92^{b}	51.43^{A}	45.50^{A}	46.26^{A}	1.59	*	*	NS
SOD (U/Hb/mL)										
Liver	1.24^{b}	2.06^{ab}	2.37^{a}	1.28^{B}	1.35^{AB}	1.58^{A}	0.08	***	***	**
Heart	6.60^{b}	8.26^{a}	7.63^{ab}	6.73^{B}	8.20^{A}	8.15^{A}	0.21	NS	**	NS
Kidney	2.92	3.17	3.34	2.77	2.95	2.64	0.08	*	NS	NS
GSH-Px (U/g Hb)										
Liver	0.05	0.06	0.06	0.07	0.08	0.06	0.00	***	NS	NS
Heart	0.29^{a}	0.32^{a}	0.29^{a}	0.35^{B}	0.33^{B}	0.44^{A}	0.01	***	*	NS
Kidney	0.10	0.11	0.09	0.10	0.11	0.10	0.00	NS	NS	NS
GSH (nmol/mL)										
Liver	0.011	0.010	0.010	0.012	0.012	0.015	0.00	*	NS	NS
Heart	0.030^{b}	0.040^{a}	0.029^{b}	0.025^{B}	0.034^{A}	0.030^{AB}	0.00	*	**	NS
Kidney	0.018^{b}	0.013^{b}	0.024^{a}	0.020^{B}	0.016^{B}	0.036^{A}	0.00	NS	**	NS

CO*EC: *Interaction between main effects, SEM: Standart Error of Mean,* ***NS:*** *P>0.05, * P<0.05, **P<0.01, ***P<0.001,* $^{a,b,c \text{ and } A,B,C}$***:*** *Meas with different superscripts in a sub group significantly differ*

eugenol (5.1%)] and six minor constituents by GC-MS. The essential oil of cinnamon bark is known to be a unique aromatic monoterpene-rich natural source, with *trans-cinnamaldehyde* (45.62%) as the major constituents. It contains relatively high amounts of phenolic compounds (18.2% of the oil), their phenolic group plays an important role in antioxidant activity, which act as hydrogen donor [30,31]. VARALAKSHMI et al.[32] reported that bark of *C. zeylanicum* was a potential source of natural antioxidants, and could be used in any preparations for combating free radical mediated damage to the body. MATHEW and ABRAHAM [33] showed that cinnamon exracts contain a number of anti-oxidant compounds which could effectively scavenge reactive oxygen species including superoxide anions and hydroxyl radicals as well as other free radicals. CIFTCI et al.[34] suggested that cinnamon oil might play an important role as an endogenous antioxidant metabolism and could also be applicable as a protective agent against tissue damage. Increasing MDA levels of liver, heart and kidney in the current study might be due to the heat stress condition. The liver tissue was quite affected from HS force compared to heart and kidney. HS induced SOD production in liver and kidney. It decreased the GSH-Px and GSH production of liver and heart. Similarly, YANG et al.[35] mentioned about heat stress induced a significant production of ROS, function of the mitochondrial respiratory chain, anti-oxidative enzymes such as SOD, CAT, GSH-Px activity and formation of MDA. Supplementation of cinnamon oil of both doses to diet reduced MDA production in liver, heart and kidney, especially under HS condition. SCHMIDT et al.[36] mentioned about among all oxygen radicals the hydroxyl radical (OH•) was the most reactive and damages diverse biomolecules, and cinnamon oil showed high hydroxyl radical-scavenging activity because of its phenolic structure. FAIX et al.[37] observed that 0.1% level *C. zeylanicum* essential oil significantly decreased the concentration of MDA in plasma and duodenal mucosa, however it had no significant effect on the concentration of MDA in the liver and kidney tissues. The dose of cinnamon oil induced the SOD production in liver and heart tissues. Moreover, there was significant interaction between enviromental condition and dose of cinnamon oil in the liver tissue. GSH levels of heart and kidney and GSH-Px activity of heart were also found higher in cinnamon supplemented groups under different enviromental condition. These findings indicated that cinnamon oil had protective effects on liver, heart and kidney by activating antioxidant mechanism in the cells. In agree with these results, MOSELHY and JUNBI [38] reported that cinnamon contained high level of phenolic groups had a potent hepatoprotective activity by inhibiting the chain reaction of lipid peroxidation resulting decrease in MDA level and elevate in SOD activities. ULLAH et al.[39] showed that *C. zeylanicum* had strong nephroprotective effect, especially against aminoglycosides induced nephrotoxicity due to its strong antioxidant property. NOORI et al.[40] reported that

cinnamon markedly showed antioxidant activity in liver, kidney and heart tissues. In addition, cinnamon is rich from lots of vitamins and minerals [11,16]. These contents could be effective on antioxidant mechanism of cinnamon and cause well-being of the birds.

The results obtained from this study have given some clues on the chemotaxonomy of this plants. *Cinnamaldehyde* is predominant compound in essential oil of *Cinnamomum zeylanicum* bark. Chronic heat stress increased oxidative stress in hepatic, heart and renal tissues, which is characterized by reduction of the antioxidant enzyme activities and glutathione levels, and it resulted with the elevation of MDA level. Both doses of *C. zeylanicum* bark oil exhibited significant antioxidant activity in growing quails through the activation of antioxidant enzymes, especially under heat stress condition.

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RESEARCH ARTICLE

Sığırlarda *Babesia bovis* ve *Babesia bigemina*'nın Reverse Line Blotting, Nested PCR ve Real Time PCR Teknikleri İle Karşılaştırmalı Tanısı [1] [2]

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Özet

Bu çalışma, sığırlarda *Babesia bovis* ve *Babesia bigemina*'nın moleküler teşhisinde Reverse Line Blotting (RLB), Nested PCR ve Real Time PCR tekniklerinin kıyaslanması amacıyla planlanmıştır. Türkiye'nin farklı illerindeki sığırlardan daha önce farklı projelerde kullanılmak üzere toplanmış ve laboratuarda muhafaza edilen 400 adet kan örneğinden genomik DNA ekstraksiyonu yapılmıştır. Elde edilen genomik DNA'ların konsantrasyonları NanoDrop spektrofotometrede ölçülmüş ve uygun konsantrasyonları hazırlandıktan sonra RLB, Nested PCR ve Real Time PCR teknikleri ile analiz edilmiştir. RLB sonuçlarına göre incelenen örneklerin toplam 18'inin (%4.50) *B. bovis*, 59'unun (%14.75) *B. bigemina*, 16'sının (%4.00) *Babesia* spp. ve 2'sinin (%0.50) *B. bovis* + *B. bigemina*; Nested PCR ile 23'ünün (%5.75) *B. bovis*, 71'inin (%17.75) *B. bigemina* ve 7'sinin (%1.75) *B. bovis* + *B. bigemina*; Real Time PCR ile 23'ünün (%5.75) *B. bovis*, 75'inin (%18.75) *B. bigemina* ve 9'unun (%2.00) ise *B. bovis* + *B. bigemina* ile miks enfekte olduğu belirlenmiştir. Real Time PCR tekniği ile kıyaslanması sonucu Nested PCR tekniğinin %94.4 sensitivite ve %100.0 spesifite gösterdiği; RLB tekniğinin ise %88.8 sensitivite ve %100.0 spesifiteye sahip olduğu belirlenmiştir. RLB testinde *Babesia* spp. belirlenen 16 örneğin hem Real Time PCR hem de Nested PCR'da 5'inin *B. bigemina*, 9'unun *B. bovis*, 2'sinin ise *B. bovis* + *B. bigemina* ile miks enfekte olduğu saptanmıştır. Sonuç olarak bu çalışma ile sığırlarda *B. bovis* ve *B. bigemina*'nın araştırılmasında Real Time PCR yönteminin Nested PCR ve RLB tekniklerine oranla daha duyarlı olduğu belirlenmiştir.

Anahtar sözcükler: *Babesia bovis, Babesia bigemina, Sığır, Nested PCR, Revers Line Blotting, Real Time PCR*

Comparative Diagnosis of *Babesia bovis* and *Babesia bigemina* in Cattle by Reverse Line Blotting, Nested PCR and Real Time PCR Techniques

Summary

This study was carried out to compare Reverse Line Blotting (RLB), Nested PCR and Real Time PCR techniques in the molecular diagnosis of *Babesia bovis* and *Babesia bigemina* in cattle. Genomic DNA extractions were performed on 400 blood samples which were previously collected from cattle in various provinces of Turkey and stored in the laboratory with respect to use in different project studies. The concentrations of the DNAs were measured in NanoDrop spectrophotometer and analyzed by RLB, Nested PCR and Real Time PCR techniques after preparing the suitable concentrations. Totally 18 (4.50%), 59 (14.75%), 16 (4.00%) and 2 (0.50%) of examined samples were found to be infected with *B. bovis*, *B. bigemina*, *Babesia* spp. and *B. bovis* + *B. bigemina* mix, respectively by RLB. 23 (5.75%), 71 (17.75%), 7 (1.75%) and 23 (5.75%), 75 (18.75%), 9 (2.00%) of the examined samples were found to be infected with *B. bovis*, *B. bigemina* and *B. bovis* + *B. bigemina* mix by Nested PCR and Real Time PCR, respectively. When comparing the Nested PCR and RLB results with Real Time PCR assay, 94.4% and 88.8% sensitivity and both 100.0% specificity were determined, respectively. 5, 9 and 2 out of the total 16 *Babesia* spp. positivity's in RLB test were determined as *B. bigemina*, *B. bovis*, *B. bovis* + *B. bigemina* mix, respectively by both Real Time and Nested PCR. In conclusion, Real Time PCR was found to be more sensitive than Nested PCR and RLB in the investigation of *B. bovis* and *B. bigemina* in cattle with this study.

Keywords: *Babesia bovis, Babesia bigemina, Cattle, Nested PCR, Revers Line Blotting, Real Time PCR*

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GİRİŞ

Babesiosis; Apicomplexa anaç altındaki *Babesia* türlerinin meydana getirdiği, tropik ve subtropik bölgelerdeki evcil ve yabani hayvanlar ile insanlarda da görülen, zoonotik karekterli protozoer bir hastalıktır. Bu hastalığa neden olan *Babesia* türleri, Ixodidae ailesine bağlı vektör kene türleri tarafından transovarial ve transstadial olarak nakledilmektedir [1]. *Babesia bigemina* ve *B. bovis* Afrika Asya, Avustralya, Güney Avrupa, Orta ve Güney Amerika ile Türkiye'de de yaygınlık gösteren sığır babesiosis etkenlerinin başında gelmektedir [1,2].

Sığırlarda babesiosis'in Türkiye'deki durumu hakkında yapılan çalışmaların büyük bir çoğunluğunu mikroskobik ve serolojik çalışmaların oluşturduğu görülmekte olup moleküler çalışmaların sayısının oldukça sınırlı olduğu, Real Time PCR ile ilgili çalışmaların ise bu güne kadar bulunmadığı dikkati çekmektedir [3]. Türkiye'nin çeşitli bölgelerinde sığırlarda mikroskobik teşhis tabanlı çalışmalara göre *B. bovis*'in %0.5-%34.8, *B. bigemina*'nın %0.5-%32.2 [3-6], serolojik çalışmalara göre *B. bovis*'in %1.3-%51.4, *B. bigemina*'nın %0.9-%100.0 [3,4,6,7], sınırlı sayıdaki moleküler tabanlı çalışmalara göre ise *B. bovis*'in %1.8-12.7, *B. bigemina*'nın %0.77-%14.0 [2,8-11] arasında prevalans gösterdiği belirlenmiştir.

Bu çalışma, Türkiye'nin çeşitli yörelerindeki sığırlardan daha önceki çeşitli proje çalışmalarında kullanılmak üzere toplanmış ve Parazitoloji Anabilim Dalı laboratuarında muhafaza edilen EDTA'lı kanlarda sığır babesiosis'inin en yaygın ve önemli iki türü olan *B. bovis* ve *B. bigemina*'nın moleküler teşhisinde Nested PCR, Reverse Line Blotting (RLB) ve Real Time PCR yöntemlerinin kıyaslanmaları amacıyla planlanmıştır.

MATERYAL ve METOT

Hayvan Materyali ve Kan Örnekleri

Bu araştırma için gerekli etik kurul onayı Erciyes Üniversitesi Deney Hayvanları Etik Kurul Başkanlığı'ndan alınmıştır (11.11.2009 tarih ve 09/65 sayılı onay belgesi). Çalışmanın materyalini, 2007-2009 yılları arasında, TSA-09-943 kodlu ve "Kayseri Yöresinde Ruminantlarda *Theileria* Türlerinin Filogenetik Analizleri" başlıklı; TSD-08-346 kodlu ve "Karadeniz Bölgesindeki Sığırlardan Elde Edilen *Babesia bovis* Suşlarının Moleküler Karakterizasyonu" başlıklı; TSD-09-700 kodlu ve "Marmara ve Ege Bölgesindeki Sığırlardan Elde Edilen *Babesia bovis* Suşlarının Moleküler Karakterizasyonu" başlıklı; 02-50-1 kodlu ve "Kayseri Yöresinde Sığırlarda Bazı *Babesia* Türlerinin RLB ve IFA Testi ile Karşılaştırmalı Tanısı Üzerine Araştırmalar" başlıklı proje çalışmalarında kullanılmak üzere Türkiye'nin farklı illerindeki sığırlardan toplanmış ve Erciyes Üniversitesi Veteriner Fakültesi Parazitoloji Anabilim Dalı cryobankında muhafaza edilen 400 adet EDTA'lı kan örneği oluşturmuştur.

Genomik DNA Ekstraksiyonu

EDTA'lı kan örneklerinden genomik DNA ekstraksiyonu, tam otomatik DNA/RNA ekstraksiyon cihazı (Bioneer ExiprepTM 16) kullanılarak yapılmıştır. Final elüsyon 50µl olacak şekilde ayarlanmış ve elde edilen DNA miktarları Nanodrop spektrofotometre (ACT Gene ASP-3700) kullanılarak ölçülmüştür. Genomik DNA ekstraktları kullanılana dek -20°C'de muhafaza edilmiştir.

Reverse Line Blotting (RLB)

Ön aşamada gerekli olan PCR reaksiyonunda, *Theileria* ve *Babesia* soylarındaki parazitlerin 18S rRNA geninin değişken V4 bölgesinden, büyüklüğü yaklaşık 390 ile 430 bp arasında değişen bir bölgeyi amplifiye eden genel primerler (RLB-F2 ve RLB-R2) kullanılmıştır. PCR protokolü ilgili referansa göre ayarlanmıştır [12]. PCR sonucu elde edilen amplikonlar jel dökümentasyon sistemi (Gen Genuis) ile agaroz jel üzerinde görüntülendikten sonra her bir örnekten 40 µl amplikon alınarak RLB hibridizasyonunda kullanılmıştır. RLB için piroplasm türleri [Catch All (*Theileria/ Babesia*)], *Babesia* spp., *B. bovis, B. bigemina, B. divergens* ve *B. major* spesifik problarla uygun membran hazırlandıktan sonra hibridizasyon basamağına geçilmiştir. RLB sonuçlarının değerlendirilmesinde hiperfilmler üzerinde prob ve PCR ürünlerinin döküldüğü sıraların kesiştiği yerlerde meydana gelen siyah lekeler pozitif olarak kabul edilmiştir [12].

Nested-PCR

Sığır kanlarından elde edilen DNA ekstraktları ilk PCR reaksiyonunda *B. bovis* ve *B. bigemina*'nın 18S rRNA gen bölgesinden yaklaşık 582 bp'lik bölgeyi amplifiye eden KB-16 ve KB-17 primerleri ile analize tabii tutulmuştur. İlk PCR'dan sonra elde edilen amplikonlardan 0.5 µl alınarak *B. bigemina* için 262 bp'lık bölgeyi amplifiye eden KB-18 ve KB-19, *B. bovis* için ise 217 bp'lık bölgeyi amplifiye eden KB-24 ve KB-25 tür spesifik primerleri ile Nested PCR analizleri gerçekleştirilmiştir. PCR protokolleri ilgili referansa göre belirlenmiştir [13]. Amplifikasyon sonunda elde edilen PCR ürünleri (10 µl) %1.5'lik agaroz jelde elektoforeze tabi tutularak, CLP Jel Dökümantasyon Sistemi ve Gene Snap from Syngene analiz programı (UVP INC Uplant, CA) ile görüntülenip analiz edilmiştir.

Real Time-PCR

Kan örneklerinde *B. bovis* ve *B. bigemina* türlerinin Real Time PCR ile araştırılmasında sırası ile Sybergreen ve TaqMan prob bazlı qPCR kullanılmıştır. Sybergreen tabanlı qPCR'da FastStart Universal SYBR Green Master (Rox) Mix (Roche Diagnostics, Germany) kullanılarak *B. bovis*'in Msa2c gen bölgesinden 97-bp'lik bölgeyi amplifiye eden Msa2c 2F, Msa2c 2R primerleri ile örneklerin Real Time PCR analizleri gerçekleştirilmiştir [14]. Analizlerde pozitif kontrol olarak anabilim dalındaki referans *B. bovis* izolatları, negatif kontrol olarak ise steril deiyonize su kullanılmıştır. PCR

master mix üreticinin açıklamalarına göre toplam 25 µl hacimde hazırlanmış ve termal profil ilgili referansa göre belirlenmiştir [14].

TaqMan prob bazlı qPCR'da Brilliant II QPCR Master Mix (Stratagene, Agilent Technologies, USA) kullanılarak *B. bigemina*'nın RAP-1 gen bölgesinden dizayn ettiğimiz 95-bp'lik bölgeyi amplifiye eden Rap1 F (5'-TCAGCGACTAC GTCCATTTG-3') ve Rap1 R (5'- AATCAACT TGGCAGGGT CAG-3') orijinal primerleri ve aynı gen bölgesinden Rap1 P orijinal probu (5'-HEX-CCGCGTACAAGAGGTGGTACAGGAA- BHQ1-3') ile örneklerin Real Time PCR analizleri gerçekleştirilmiştir. Dizayn edilen primerler ve probun spesifiteleri blastn ve Primer Blast (http://www.ncbi.nlm.nih.gov/pubmed) analizleri ve çeşitli genetik yazılımlarla kontrol edilmiş, ayrıca anabilim dalındaki *B. bigemina*, *B. bovis*, *T. orientalis* ve *T. annulata* referans izolatları kullanılarak etkinlik ve özgünlük açısından değerlendirilmiştir. Örneklerin işlenmesinde pozitif kontrol olarak anabilim dalındaki referans *B. bigemina* izolatı, negatif kontrol olarak ise steril deiyonize su kullanılmıştır. PCR master mix üreticinin açıklamalarına göre toplam 25 µl hacimde; master mix 12.5 µl, Rap1 F (10 pmol) 1.0 µl, Rap1 R (10 pmol) 1.0 µl, Rap1 P (10 pmol) 1.0 µl, genomik DNA 50 ng µl ve steril deiyonize su 8.5 µl olacak şekilde ayarlanmıştır. Real Time PCR'da termal protokol 50°C'de 2 dk; 95°C'de 10 dk; 45 siklus, denaturation: 95°C'de 20 sn, annealing: 55°C'de 1 dk, olacak şekilde programlanmıştır.

İstatistiksel Analiz

Sığırda *B. bovis* ve *B. bigemina* enfeksiyonlarının teşhisinde kullanılan yöntemlerin istatistiksel olarak sensitivite ve spesifiteleri ilgili referansa göre belirlenmiştir [15]. Testler arasındaki uyumun belirlenmesinde Kappa testi kullanılmıştır.

BULGULAR

RLB Sonuçları

RLB analizi sonucu pozitif belirlenen bazı örneklerin hiperfilm üzerindeki görüntüsü *Şekil 1*'de verilmiştir. RLB sonuçlarına göre 400 sığırdan toplam 18'inin (%4.50) *B. bovis*, 59'unun (%14.75) *B. bigemina*, 16'sının (%4.00) *Babesia* spp. ve 2'sinin (%0.50) *B. bovis* ve *B. bigemina* miks enfekte olduğu saptanmıştır. *Babesia* soy ve tür spesifik problarla pozitiflik veren tüm örneklerin Catch-all probla da pozitif reaksiyon verdiği görülmüştür. Ayrıca incelenen örneklerde Catch-all *(Theileria/Babesia)* proba sinyal verip *Babesia* soy ve tür spesifik problara sinyal alınamayan toplam 48 örnek (*Theileria* soyunda) belirlenmiştir.

Nested PCR Sonuçları

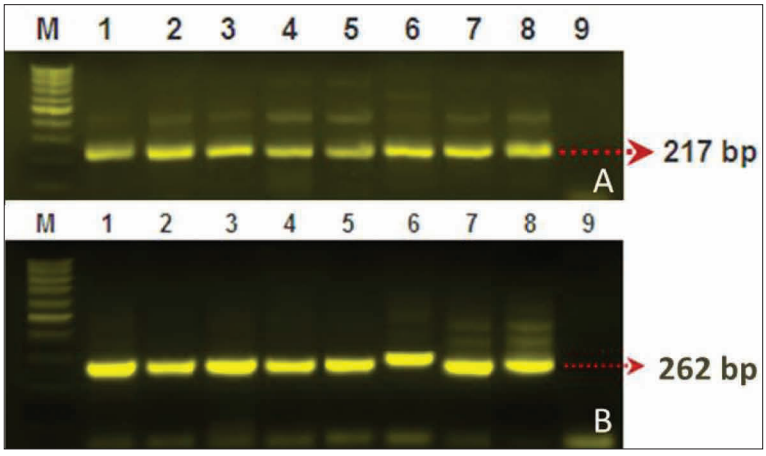
Nested PCR'ın 2. PCR basamağında *B. bigemina* için 18S rRNA gen bölgesinden 262 bp'lık bölgeyi amplifiye eden KB-18 ve KB-19 primerleriyle ve *B. bovis* için ise 18S rRNA gen bölgesinden 217 bp'lık bölgeyi amplifiye eden KB-24 ve KB-25 primerler ile analiz sonucu pozitif belirlenen bazı izolatların jel agarozdaki görünümleri *Şekil 2*'de verilmiştir. Nested PCR sonuçlarına göre 400 sığırdan toplam 23'ünde (%5.75) *B. bovis,* 71'inde (%17.75) *B. bigemina* ve 7'sinde (%1.75) ise *B. bovis* + *B. bigemina* miks enfeksiyon saptanmıştır.

Real Time PCR Sonuçları

Sybergreen tabanlı qPCR'da, *B. bovis*'in Msa2c gen bölgesinden 97-bp'lik bölgeyi amplifiye eden *Msa2c* 2F, *Msa2c* 2R primerleri ile örneklerin analizi sonucu pozitif belirlenen bazı örneklerin amplifikasyon ve melting eğrileri *Şekil 3*'te gösterilmiştir. Pozitif örneklerde ortalama çözünme sıcaklığı (Tm) 78.1°C (±0,2°C) olarak saptanmıştır. Pozitif

Şekil 1. Bazı pozitif örneklerde RLB sonuçlarının görüntüsü

Fig 1. The image of RLB results in some positive samples



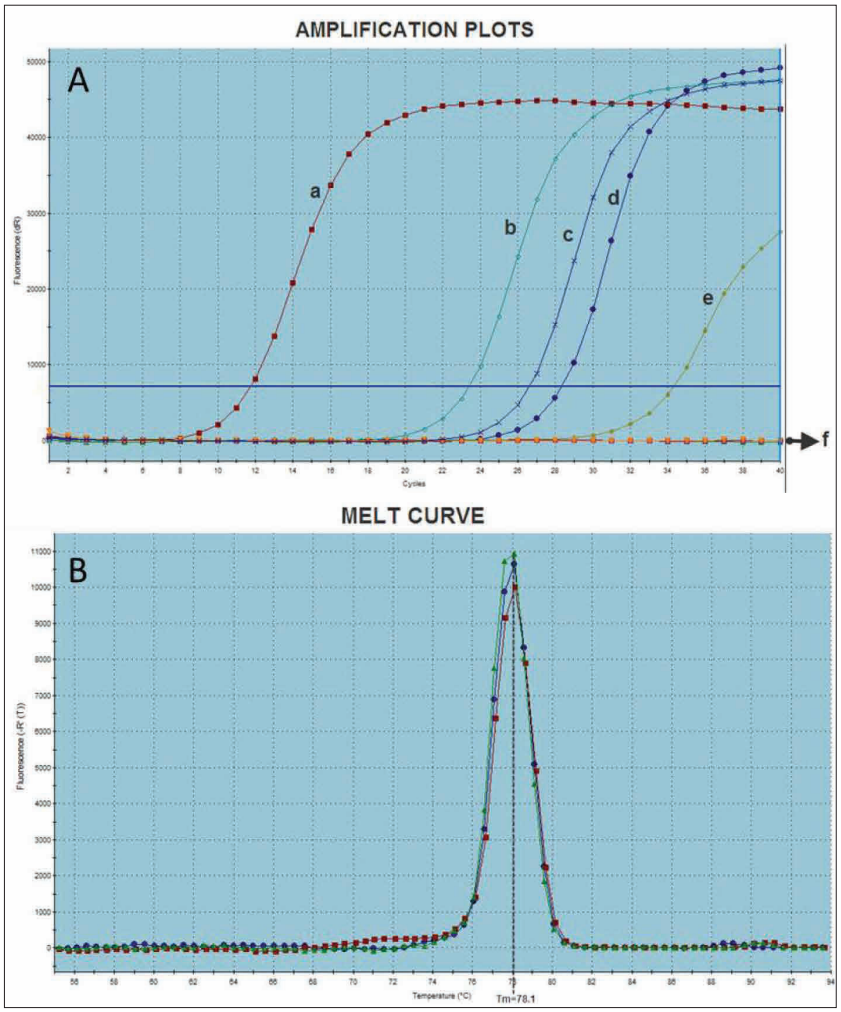
Şekil 2. Nested PCR analizinin 2. PCR basamağında *B. bovis* ve *B. bigemina* pozitif belirlenen bazı örneklerin agaroz jel üzerindeki görünümü. M: Marker (100bp), 1-7: Pozitif örnekler, 8: Pozitif kontrol, 9: No DNA

Fig 2. Some *B. bovis* and *B. bigemina* positive samples on agarose gel at the 2nd step of the Nested PCR analyses. M: Marker (100bp), 1-7: Positive samples, 8: Positive control, 9: No DNA

örneklerde parazitemi açısından belirlenen Ct (dR) (Eşik değer siklusu) değerleri ortalama 33.2 (22.3-41.8) olarak belirlenmiştir.

TaqMan prob tabanlı qPCR'da *B. bigemina*'nın RAP-1 gen bölgesinden 95-bp'lik bölgeyi amplifiye eden *Rap1* F ve *Rap1* R primerleri ve aynı gen bölgesinden dizayn edilen *Rap1* P probunun kullanılmasıyla belirlenen bazı pozitif örneklerin amplifikasyon eğrileri *Şekil 4*'te verilmiştir. Pozitif örneklerde parazitemi açısından belirlenen Ct (dR) değerleri ortalama 36.3 (15.7-44.0) olarak saptanmıştır.

Babesia bigemina'nın Rap1 gen bölgesinden dizayn ettiğimiz primer ve probların GenBank'a kayıtlı tüm *B. bigemina* Rap1 izolatları ile alignmentları *Şekil 5*'te gösterilmiştir. Rap1F primerinin M85186 izolatı ile %95, diğer tüm izolatlar ile %100; Rap1R primerinin ise AF017284 ve AF017294 izolatları ile %95, diğer tüm izolatlar ile

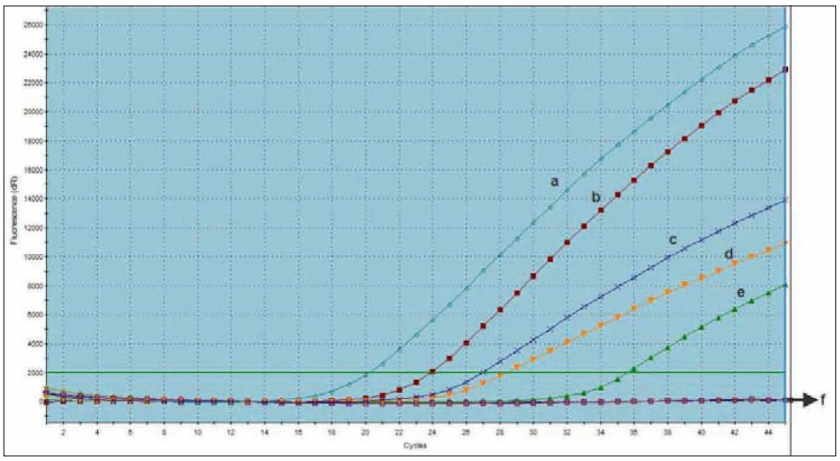


Şekil 3. Sybergreen Real Time PCR analizleri sonucu *B. bovis* pozitif saptanan bazı örneklerin amplifikasyon grafikleri (A) ve erime eğrileri (melting curve); Tm=78.1 (B). a: *B. bovis* pozitif kontrol DNA; b,c,d,e: *B. bovis* pozitif örnekler; f: No DNA, *B. bigemina, T. annulata, T. orientalis*

Fig 3. Amplification plots (A) and melting curves Tm=78.1 (B) of some *B. bovis* positive samples in Sybergreen Real Time PCR assay. a: *B. bovis* positive control DNA; b,c,d,e: *B. bovis* positive samples; f: No DNA, *B. bigemina, T. annulata, T. orientalis*

Şekil 4. TaqMan Prob bazlı Real Time PCR analizleri sonucu *B. bigemina* pozitif saptanan bazı örneklerin amplifikasyon eğrileri. a: *B. bigemina* pozitif kontrol DNA; b,c,d,e: *B. bigemina* pozitif örnekler; f: No DNA, *B. bovis, T. annulata, T. orientalis*

Fig 4. Amplification curves of some *B. bigemina* positive samples in TaqMan Prob based Real Time PCR assay. a: *B. bigemina* positive control DNA; b,c,d,e: *B. bigemina* positive samples; f: No DNA, *B. bovis, T. annulata, T. orientalis*



%100 identik olduğu belirlenmiştir. Rap1P probu ise tüm izolatlarla %100 identiklik göstermiştir. Yapılan Blastn ve Primer Blast (http://www.ncbi.nlm.nih.gov/pubmed) analizleri sonucu dizayn edilen primerler ve probun *B. bigemina* spesifik olduğu teyit edilmiştir. Bunun yanında laboratuarda mevcut *B. bovis, T. orientalis* ve *T. annulata* referans izolatlarıyla yapılan analizlerde de söz konusu izolatlar ile herhangi bir amplifikasyon olmadığı görülmüştür *(Şekil 4)*.

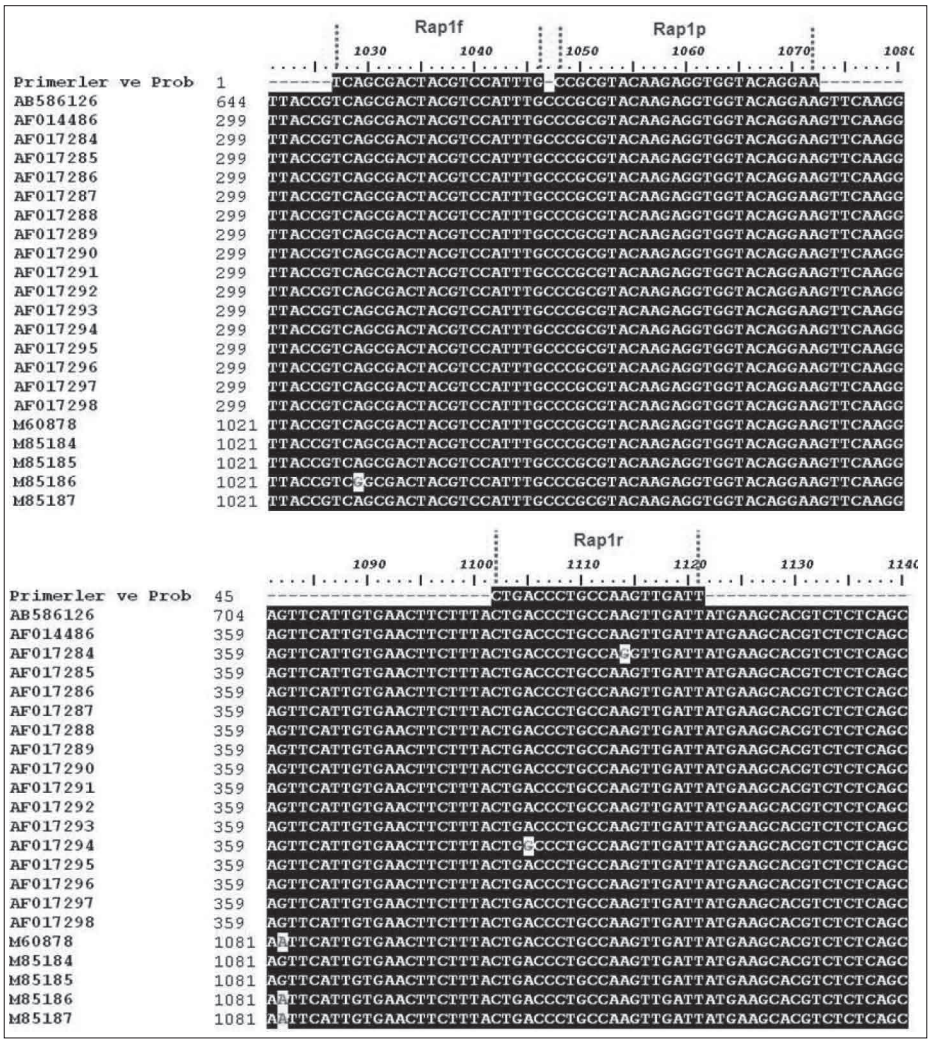
Real Time PCR sonuçlarına göre 400 sığırdan toplam 23'ünde (%5.75) *B. bovis,* 75'inde (%18.75) *B. bigemina* ve 9'unda ise (%2.00) *B. bovis* + *B. bigemina* miks enfeksiyon saptanmıştır.

RLB, Nested PCR ve Real Time PCR Sonuçlarının İstatistiksel Analizi

Sığırlarda *Babesia* enfeksiyonlarının teşhisinde RLB, Nested PCR ve Real Time PCR tekniklerinin etkinlikleri açısından yapılan istatistiksel analiz *Tablo 1*'de verilmiştir. Bu değerlendirmede gold test Real Time PCR olarak alınmıştır. Nested PCR tekniğinin Real Time PCR tekniği ile kıyaslanması sonucu %94.4 sensitivite (%88.3-97.4; %95 Güven aralığı) ve %100.0 spesifite (%98.7-100.0; %95 Güven aralığı) gösterdiği belirlenmiştir. RLB tekniğinin Real Time PCR tekniği ile kıyaslanması sonucu ise %88.8 sensitivite (%81.4-93.5; %95 Güven aralığı) ve %100.0 spesifite (%98.7-100.0; %95 Güven aralığı) gösterdiği saptanmıştır. Kappa testi ile Real Time PCR ve Nested PCR arasında %96.1 (P<0.05), Real Time PCR ve RLB arasında %92.1 (P<0.05) ve Nested PCR ile RLB arasında ise %95.9 (P<0.05) oranında uyum olduğu belirlenmiştir. RLB testinde *Babesia* spp. olarak belirlenen 16 örneğin hem Real Time hem de Nested PCR'da 5'inin *B. bigemina*, 9'unun *B. bovis*, 2'sinin ise *B. bovis* + *B.bigemina* miks olduğu ortaya konmuştur. Nested PCR'da negatif belirlenen 6 örneğin Real Time PCR'da Ct (dR) değerlerinin 40'tan büyük olduğu görülmüştür.

TARTIŞMA ve SONUÇ

Moleküler tabanlı teşhis yöntemleri son yıllarda birçok paraziter enfeksiyonun yanında sığır *Babesia* türlerinin spesifik teşhisinde ve genotiplendirilmesinde de yaygın olarak kullanılmaktadır. Bunlar arasında RLB yöntemi, PCR ürünlerinin bir membranda ayrı sıralara bağlanmış özgün problara hibridizasyonu esasıyla çalışmaktadır. Teknik,



Şekil 5. *B. bigemina*'nın Rap1 gen bölgesinden dizayn edilen primerler ve probun, GenBank'a kayıtlı diğer *B. bigemina* sekanslarıyla multiple alignmentları

Fig 5. Multiple alignments of the primers and probe designed from Rap1 gene region of *B. bigemina* with the other *B. bigemina* sequences from GenBank

Tablo 1. *Babesisosis'in teşhisinde kullanılan tekniklerin Gold Teste göre sensitivite ve spesifiteleri*
Table 1. *Sensitivity and specificities of the techniques used in the diagnosis of babesiosis according to the Gold Test*

Yöntem		Real Time PCR (Gold Test)			Sensitivite (%95 Güven Aralığı)	Spesifite (%95 Güven Aralığı)
		+	-	Toplam		
RLB	+	95	0	95	0.888 (0.814-0.935)	1.000 (0.987-1.000)
	-	12	293	305		
	Toplam	107	293	400		
Nested PCR	+	101	0	101	0.944 (0.883-0.974)	1.000 (0.987-1.000)
	-	6	293	299		
	Toplam	107	293			

birçok etkeninin eş zamanlı teşhisine imkan sağladığından oldukça pratik ve kullanışlıdır. Bu yöntemin kan protozoonlarının teşhisinde kullanılması, ilk kez Gubbels ve ark.[16] tarafından gerçekleştirilmiş olup, çalışmada 18S rRNA geninin V4 değişken bölgesini amplifiye eden primerler ile PCR'da çoğaltılan amplikonlar RLB tekniğinde kullanarak sığırlarda görülen *Theileria annulata, T. parva, T. mutans, T. taurotragi, T. velifera, B. bovis, B. bigemina* ve *B. divergens*'in eş zamanlı özgül teşhislerinin yapılabileceği belirlenmiştir. Bu çalışmada, sahip olduğu avantajlarla son yıllarda dünyada ve Türkiye'de sığır babesiosis'inin teşhisinde yaygın olarak kullanılan RLB tekniğiyle, Real Time PCR ve Nested PCR yöntemlerinin *B. bovis* ve *B. bigemina*'nın teşhisindeki etkinlikleri karşılaştırmalı olarak değerlendirilmiştir. Real Time PCR ile pozitif belirlenen 12 örnek RLB testi ile negatif belirlenmiş, 16 örneğin ise *Babesia* sp. düzeyinde pozitiflik verdiği görülürken, tür düzeyinde reaksiyon vermediği saptanmıştır. RLB testinde *Babesia* sp. probla sinyal verip *Babesia* tür spesifik problara sinyal alınamaması, kullanılan probların sensitivitesinin düşük olabileceğini gösterebileceği gibi, membranın yıkanması ve tekrar kullanılması esnasında probların membrandan uzaklaştırılmış olması ile de ilişkili olabilir. Nitekim RLB tekniğinin, spesifik olmayan PCR ve akabinde hibridizasyon prosedürü olmak üzere iki basamaklı bir prosese sahip olması sebebiyle PCR reaksiyonlarında substrat için kompetisyon ve RLB testi süresince amplifiye DNA için farklı hibridizasyon ısıları gibi çeşitli değişkenlerin her basamakta sensitiviteyi düşürebileceği kaydedilmiştir [17]. Bu sonuçlarla RLB testinin, Real Time PCR testine göre sensitivitesi %88.8 olarak belirlenmiştir. Saptanan bu sensitivite değeri Nested PCR ve Real Time PCR yöntemlerine oranla düşük olmasına karşın genel çerçevede yüksek gözükmektedir. Bunun yanında RLB testinin spesifitesi ise %100 olarak belirlenmiştir. Bu sonuçlar söz konusu testin sığırlarda *B. bovis* ve *B. bigemina* enfeksiyonlarının teşhisinde güvenilir bir şekilde kullanılabileceğini göstermektedir. Georges ve ark.[17], evcil kedilerde haemoplasmaların Real Time PCR ve RLB teknikleri ile karşılaştırmalı tanısı üzerine yaptıkları çalışmada benzer sonuçlar bildirmişlerdir. Araştırıcılar [17], RLB ve Real Time PCR ile incelenen toplam 152 kedi kanından ekstrakte edilmiş genomik DNA örneklerinde *Candidatus Mycoplasma haemominutum*'u sırasıyla 40 ve 48; *Mycoplasma haemofelis*'i 11 ve 47; *Candidatus Mycoplasma turicensis*'i ise yalnızca Real Time PCR'da 3 örnekte pozitif belirlemişler, ayrıca miks haemoplasma enfeksiyonlarını RLB ile 5, Real Time PCR ile 19 örnekte saptamışlardır. Çalışmada [17] Real Time PCR tekniği gold standart alınarak yapılan istatistiksel hesaplamalarda RLB tekniğinin %64.7 sensitivite ve %100 spesifite gösterdiği de kaydedilmiştir.

Nested PCR yöntemi, standart PCR'a farklı primerle ikinci bir amplifikasyon uygulanması esasına dayanan moleküler bir tekniktir. İlk amplifikasyonda elde edilen ürün ikinci PCR aşamasında kalıp olarak kullanılır. Kullanılan ikinci primer takımı hedef diziye özgüdür. Kompleks mikrobiyal popülasyonlara ait hedef dizilerin spesifik bir şekilde amplifikasyonu klasik PCR yöntemleriyle bazen mümkün olmadığı görülmekte ve oluşan non-spesifik fragmentlerin amplifikasyonu sonucu yanlış pozitiflikler ortaya çıkabilmektedir. Nested PCR ile söz konusu bu olumsuzluk giderilmekte, aynı zamanda düşük DNA'ya sahip örneklerde ikinci kez PCR uygulaması ile teşhisteki sensitivite artırılabilmektedir [13,18]. Son yıllarda ruminant ve vektör kenelerde babesiosis'in spesifik teşhisinde nested PCR yönteminin sıklıkla uygulandığı görülmektedir [13,19,20]. Kene ve sığırlarda *Babesia* türlerinin Nested-PCR ile araştırılması amacıyla 18S rRNA gen bölgesini hedef alan çeşitli primer dizileri dizayn edilmiştir [13,21-23]. Ancak bu primer çiftlerinin çoğunun [21-23] blastn analizi sonucu *B. bigemina* ve *B. bovis* teşhisi için GenBank'ta mevcut bazı izolatlara ait dizilimlerle uyumsuzluk gösterdiği ve bu durumun da teşhis zorluklarına ve yanlış negatifliklere yol açabileceği belirtilmiştir [13]. Bu çalışmada kullanılan ve Guerrero ve ark.[13] tarafından dizayn edilen Nested primerlerinin, araştırıcıların belirttiği gibi blastn analizi sonucu yüksek spesifite gösterdiği ve dizilimlerin GenBank'a kayıtlı tüm *B. bovis* ve *B. bigemina* 18S rRNA dizilimleri ile uyumluluk gösterdiği görülmüştür. Nested PCR yönteminin sığır babesiosis'inin teşhisindeki sensitivitesi Real Time PCR'a göre %94.4, spesifitesi ise %100 belirlenmiştir. RLB testinde negatif belirlenen 6 örnek Nested PCR ile pozitif belirlenmiş, ayrıca soy düzeyinde RLB ile pozitif belirlenen 16 örneğin bu test ile tür bazlı pozitiflik verdiği saptanmıştır. Nested PCR yönteminin göstermiş olduğu sensitivite ve spesifite

ile sığırlarda *B. bovis* ve *B. bigemina* enfeksiyonlarının teşhisinde güvenilir bir şekilde kullanılabileceği görülmektedir.

Real-time PCR tekniği, hızı, sensitivite ve spesifitesi, seçiciliği, hedef patojen veya gen bölgesinin kantitatif olarak belirlenebilmesi ve otomasyona uygun olması gibi nedenlerle son yıllarda öne çıkan tekniklerin başında gelmektedir. Ayrıca söz konusu teknik ile mutasyon analizleri ve gen ekspresyonları da hassas bir şekilde belirlenebilmektedir. Son yıllarda sığır babesiosis'inin spesifik teşhisinde de kullanılmaya başlayan 18S rRNA, mitokondrial cytochrome b ve msa2c gibi farklı gen bölgeleri hedef alınarak dizayn edilen spesifik primer ve problar ile Real Time PCR araştırmalarının yapıldığı görülmektedir [14,24,25]. Criado-Fornelio ve ark.[25], iki farklı qPCR yönteminin sığır babesiosis'inin teşhisindeki etkinliğini araştırmışlar, hem TaqMan prob hem de FRET prob bazlı qPCR denemelerinde yüksek sensitivite ve spesifite belirlemişlerdir. Her iki teknik için de saptama limiti (linear kalibrasyon eğrileri) 0.1 fg/µl-0.01 ng/µl olarak bildirilmiştir. Saha örnekleri üzerinde iki tekniğin uygulanması sonucu, spesifitelerinin %100 olmasına karşın sensitivitenin TaqMan prob bazlı qPCR yönteminde daha yüksek belirlediklerini kaydetmişlerdir [25]. Ramos ve ark.[14], *B. bovis*'in msa2c gen bölgesini hedef alarak dizayn ettikleri sybergreen tabanlı qPCR tekniğinin teşhisteki etkinliğini ortaya koyma amacı ile yaptıkları çalışmada, blastn analizleri ve *B. bigemina*, *A. marginale* ve *Bos taurus* referans DNA'larını kontrol olarak kullanarak, dizayn ettikleri primerlerin tür spesifik olduğunu kaydetmişlerdir. Çalışmada [14] qPCR tekniğinin enfeksiyonu saptama limiti, msa2c kopya sayısı ve eşik değer siklusu arasındaki linear korelasyona göre ml kanda 1000 kopya olarak saptamış, ortalama çözünme sıcaklığı (Tm) da 77.41±0.25°C belirlenmiştir. Araştırıcılar [14] qPCR'ın sensitivitesini konvansiyonel PCR'a göre yüksek belirlemişler ve iki teknik arasındaki uyumu %88.8 (Kappa indeks=0.75) bulmuşlardır. Bu çalışmada ise Real Time PCR teknikleri ile RLB testine göre 12, Nested PCR testine göre ise 6 örnek pozitif belirlenmiş olup RLB testinde soy düzeyinde pozitiflik veren örneklerin hepsinin tür identifikasyonları yapılmıştır. Ayrıca Nested PCR'da negatif belirlenen 6 örneğin Real Time PCR'da Ct (dR) (Eşik değer siklusu) değerlerinin yüksek olduğu (>40) belirlenmiş ve neticede bu örneklerde paraziteminin oldukça düşük olduğu sonucuna varılmıştır. Çalışmada elde edilen verilere ve diğer araştırmaların [14,25] sonuçlarına göre sensitivite ve spesifite hesaplamalarında Real Time PCR tekniği standart gold test olarak alınmıştır. Kappa testi ile Real Time PCR ve Nested PCR arasında %96.1 (P<0.05), Real Time PCR ve RLB arasında %92.1 (P<0.05) ve Nested PCR ile RLB arasında ise %95.9 (P<0.05) oranında uyum saptanmıştır. Elde edilen sonuçların araştırıcıların [14,24,25] bulguları ile uyum gösterdiği belirlenmiştir. Bununla birlikte Ramos ve ark.[14] tarafından dizayn edilen syber green tabanlı qPCR primerlerinin spesifiteleri blastn analizleri ve Anabilim Dalı cryobankında mevcut olan *B. bigemina, T. annulata* ve *T. orientalis* referans DNA'ları kullanılarak konfirme edilmiştir. Çalışmada *B. bovis* için bulunan 78.1±0.2°C Tm değeri Ramos ve ark.[14] tarafından bildirilen Tm değeri ile uyum göstermiştir. Apicomplexan protozoonların apikal organellerinden salgılanan proteinler, parazitin konak hücresi içerisine invaze olmasında ve hücre içinde kalmasında çok önemli rol oynamaktadır [26]. Bu proteinler arasında rhoptry-associated protein-1 (Rap-1) gen familyasının moleküler karakteri, *B. bovis* ve *B. bigemina* türlerinde tam olarak ortaya konmuş olmakla birlikte, bu genin diğer tüm *Babesia* türlerinde de bulunduğu kaydedilmiştir [27-29]. Rap-1 gen familyası proteinleri, sahip olduğu çeşitli immünolojik epitoplarla spesifik antikorların oluşumuna yol açmaktadır. Oluşan antikorların da merazoitlerin sağlam eritrositlere invaze olmasını engellediği gösterilmiştir [30,31]. Bu yüzden söz konusu proteine ait Rap-1 gen bölgesi *B. bovis* ve *B. bigemina* türlerinin moleküler tiplendirilmesinde ve çeşitli aşı çalışmalarında temeli teşkil etmiştir. Bu çalışmada *B. bigemina*'nın Rap-1 gen bölgesi hedef alınarak primer ve prob dizaynı yapılmış ve dizilimlerin GenBank'a kayıtlı tüm *B. bigemina* Rap-1 izolatları ile alignmentları gerçekleştirilmiştir. Blastn ve Primer Blast analizleri sonucu dizayn edilen primer ve probların *B. bigemina* spesifik olduğu tespit edilmiştir. Ayrıca laboratuarda mevcut *B. bovis*, *T. orientalis* ve *T. annulata* referans izolatları ile yapılan Real Time PCR analizinde de söz konusu izolatlar ile herhangi bir amplifikasyon olmadığı saptanmıştır. Elde edilen bu sonuçlar, dizayn edilen primerler ve probun *B. bigemina* enfeksiyonlarının Real Time PCR ile kantitatif spesifik teşhisinde güvenle kullanılabileceğini göstermiştir.

Sonuç olarak bu çalışma ile sığırlarda *B. bovis* ve *B. bigemina*'nın araştırılmasında Real Time PCR yönteminin Nested PCR ve RLB tekniklerine oranla daha duyarlı olduğu, özellikle düşük parazitemili rezervuar hayvanların belirlenmesi, kantitasyona olanak sağlaması ve dolayısıyla tedavi etkinliğinin tür bazlı takibi gibi çalışmalarda sahip olduğu avantajlarla öne çıktığı görülmüştür. Bunun yanında her üç tekniğin de bu enfeksiyonların teşhisinde yüksek spesifiteye sahip olduğu ve sığırlarda *B. bovis* ve *B. bigemina*'nın teşhisinde ve moleküler epidemiyolojik çalışmalarda güvenli bir şekilde kullanılabilecekleri belirlenmiştir.

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RESEARCH ARTICLE

Effects of Dietary *Saccharomyces cerevisiae* and Butyric Acid Glycerides on Performance and Serum Lipid Level of Broiler Chickens

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Summary

This experiment was conducted to evaluate the effects of dietary supplementation of live yeast *Saccharomyces cerevisiae* (SC) and butyric acid glycerides (BAG) on broiler performance and serum lipid composition. One-day-old ROSS 308 female chicks (n=378) were randomly distributed in a 3×3 factorial arrangement with three replicates for each increasing levels of (0, 0.002, and 0.004 g/g) BAG and (0, 0.003, and 0.006 g/g) SC respectively. The experiment lasted 42 d, consisting of starter (1-21 d) and grower (22-42 d) periods. Body weight (BW), feed intake (FI), and feed conversion ratio (FCR) were determined by the period for each treatment. On d 42, serum concentrations of triglycerides, cholesterol, and HDL were determined. *Saccharomyces cerevisiae* had no effect on performance in the starter period. However, chicks fed 0, 0.002, and 0.004 g/g BAG had higher BW and better FCR than control diet (P<0.05). In the grower period BW of chicks fed 0.006 g/g SC was higher than other treatments (P<0.05). For BAG both levels improved (P<0.05) BW and FCR. In serum composition, both BAG and SC decreased cholesterol concentrations (P<0.05), but the HDL levels were higher (P<0.05) only in 0.006g/g SC fed chicks. There were no significant effects in triglyceride levels among treatments. There were no SC and BAG interaction effect on response variables. In conclusion, dietary BAG improves growth performance in starter and grower periods but SC was only effective in grower period. Moreover, both BAG and SC had positive effect on serum lipid composition.

Keywords: *Saccharomyces cerevisiae, Butyric acid glycerides, Performance, Lipid composition, Broiler*

Broiler Tavuklarda *Saccharomyces cerevisiae* ve Butrik Asit Gliseridlerinin Performans ve Serum Lipid Değerleri Üzerine Etkileri

Özet

Bu çalışma; diyete canlı maya *Saccharomyces cerevisiae* (SC) ve butrik asit gliseridleri (BAG) ilavesinin broilerlerde performans ve serum lipid değerleri üzerindeki etkisini değerlendirmek amacıyla yapılmıştır. Bir günlük ROSS 308 dişi hayvanlar (n=378) her bir grup 3 kez tekrarlanacak şekilde rastgele dağıtıldıktan sonra faktöriyel olarak 0, 0.002, 0.004 g/g BAG ve 0, 0.003, 0.006 g/g SC verildi. Deneme süreci srater (1-21 gün) ve büyüme/22-42 gün) olmak üzere toplam 42 gün sürdü. Vücut ağırlığı (VA), yem tüketimi (YT) ve yem konversiyon oranı (YKO) her bir deneme için belirlendi. 42. günde trigliserit, kolesterol ve HDL konsantrasyonları tespit edildi. *Saccharomyces cerevisiae* starter döneminde performans üzerine herhangi bir etki göstermedi. Ancak, 0, 0.002 ve 0.004 g/g BAG ile beslenen tavuklar kontrol grubundakilere oranla daha yüksek VA ve daha iyi YKO değerlerine sahipti (P<0.05). Büyüme döneminde 0.006 g/g SC ile beslenen tavukların VA değerleri diğer gruplarınkinden daha yüksek idi (P<0.05). BAG verilen hayvanlarda hem VA hem de YKO değerleri gelişme gösterdi (P<0.05). Hem BAG hem de SC verilen hayvanlarda serum kolesterol konsantrasyonları düşme gösterirken (P<0.05) HDL seviyeleri sadece 0.006g/g seviyesinde SC verilenlerde daha yüksek idi (P<0.05). Gruplar arasında triglisert değerleri yönünden herhangi bir fark tespit edilmedi. Sonuç olarak; diyette BAG verilmesi starter ve büyüme dönemlerinde büyüme performansı üzerinde olumlu etki gösterirken SC verilmesi sadece büyüme devresinde olumlu etki göstermektedir. Hem BAG hem de SC serum lipid değerlerinde pozitif etkiye neden olmaktadır.

Anahtar sözcükler: *Saccharomyces cerevisiae, Butrik asit gliseridleri, Performans, Lipid Değerleri, Broiler*

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INTRODUCTION

Antibiotics benefit animal growth, performance and health. However it has been increasing pressure to reduce or even eliminate antibiotic usage in poultry due to the development of antibiotic resistance in consumers [1]. Thus, there is an increasing interest in finding other antibiotic replacements such as prebiotics, probiotics, aromatic oils and organic acids in poultry production [2]. A probiotic was defined as a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance [3]. Effects of yeast products on production and their mode of action in monogastrics have been reported in poultry [4,5]. *Saccharomyces cerevisiae* yeast has biologically valuable proteins, vitamin B-complex, important trace minerals and other plus factors [6]. Some studies have confirmed the effects of yeast products in increasing concentration of commensal microbes or suppressing pathogenic bacteria [5]. However, these effects were not reported by Van Heugten *et al.*[7].

Reduction in circulating levels of cholesterol and LDL with supplemental yeast was reported by other researchers [8,9] who stated that probiotics could contribute to the regulation of serum cholesterol concentrations by deconjugating bile salts.

Organic acids, including butyric acid are also considered potential alternations to antibiotics growth promoter [10,11]. Butyric acid has been widely reported as the major development promoter of the gut wall tissues and an important growth modulator of symbiotic intestinal microflora [10,11]. Also, butyric acid is known the main energy source for enterocytes. Kwon and Ricke [12] showed butyrate and valerate to have the greatest efficacy. Moreover, Lesson *et al.*[10] and Antongiovanni *et al.*[13] reported positive beneficial effects of butyric acid on performance traits of broilers. It has been also reported that dietary inclusion by BAG had significant reducing effect on serum compositions of total cholesterol and LDL levels [14]. Effects of SC and BAG on the performance and lipid serum composition of broiler chickens are similar. Therefore, the objective of this study was to evaluate the SC by BAG interaction effects on performance and lipid composition in broilers.

MATERIAL and METHODS

Experimental Animals and Management

Three hundred and seventy eight day-old female chicks (ROSS 308) were randomly assigned into 9 treatments, each composed of 42 birds. Birds in each treatment were placed in 3 pens, each containing 14 birds. All birds were raised on floored pen (1.2×1.8 m) and had access to feed and water *ad libitum*.

Experimental Design and Diets

Birds were distributed in a completely randomized design with 3×3 factorial arrangement with pen as the experimental unit. The composition and nutrient analysis of basal diet are shown in *Table 1* and *Table 2*. The basal diet was a typical corn-soybean meal diet as mash form that increasing amount of BAG (0, 0.002 and 0.0044 g/g) and SC (0, 0.0033 and 0.0066 g/g) were added to basal diet.

The chicks were fed the starter diet until d 21 and grower diet from d 22 until 42 based on NRC [15] nutrient requirements of chicken. The used BAG (product of BABY-C4. Silo Company, Italy) contained 25-35% monoglycerides in the 1 or 3 positions, 50-55% diglycerides in the 1 or 3 positions and 15-25% triglycerides. Unlike butyric acid,

Table 1. *The chemical composition of Saccharomyces cerevisiae*
Tablo 1. *Saccharomyces cerevisiae'nin kimyasal kompozisyonu*

Composition	***Saccharomyces cerevisiae***
Dry matter, %	93
ME, kcal/kg	1990
Crude protein, %	44.4
Crude fat, %	1
Crude fiber, %	2.7
Ca, %	0.12
AP, %	1.4

Table 2. *Composition of the basal diet fed in broilers (%)*
Table 2. *Broilerlere verilen bazal diyetin kompozisyonu (%)*

Ingredients	**Starter**	**Grower**
Corn	54.5	61
Soybean meal (44% CP)	38	32.5
Soy oil	3.5	3
Dicalcum phosphate	1.8	1.15
Oyster shell	1.3	1.45
Salt (NaCl)	0.2	0.3
DL-Methionine	0.2	0.1
Vitamin Permix[1]	0.25	0.25
Mineral Permix[2]	0.25	0.25
Calculated Composition		
ME, Kcal/kg	2990	3032
CP, %	21.45	19.48
Ca, %	1	0.9
Available P, %	0.48	0.35
Lys, %	1.1	1
Met, %	0.5	0.4

[1] Vitamin premix contained the following per kilogram of diet: vitamin A, 1100 IU; vitamin D_3, 240 IU; vitamin E, 6 IU; vitamin B_{12} 0.004 µg; biotin, 0.15 mg; folic acid, 0.2 mg; nicotinic acid, 50 mg; D-pantothenic acid, 5 mg; pyridoxine hydrocholesteroloride, 1.2 mg; riboflavin, 2.2 mg; thiamine mononitrate, 1.6 mg. [2] Mineral premix contained the following per kilogram of diet: Fe, 80 g; Cu, 8 mg; Mn, 60 mg; Zn, 40 mg; I, 0.4 mg; Se, 0.2 mg

the butyrate glycerides used in this study had only a mild buttery type odor and not the rancid odor often associated with butyric acid. The live yeast SC (containing 1×10^9 CFU/g) was provided from Klar Maya (powdery form, Iran) and chemical composition of SC is presented in *Table 1*. No antibiotics and any coccidiostate were included in the experimental diets.

Growth Performance Traits

Body weights (BW) were recorded for each replicate on days 1, 21, and 42 of age and feed intake (FI) was measured in order to calculate feed conversion ratio (FCR) for each feeding periods. Mortality ratio was recorded daily and FCR was corrected for mortality by adding body weights to the total pen weight at the end of each period.

Blood Collection and Analysis

Blood was collected at 42 day old from wing vein of 9 birds per treatment (3 birds/ replicate) and serum was separated at 5000×g for 10 min. The serum concentrations of total triglycerides, cholesterol and high-density lipoprotein (HDL) were analyzed by an automatic biochemical analyzer (Technicon RA-1000, Spain), following the instructions of the corresponding reagent kit (Pars Azmon Co., Iran).

Statistical Analysis

All data were analyzed by ANOVA using the SAS [16], GLM program. Treatment means portioned by LSMEAN analysis. The mode is:

$$X_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \Sigma_{ij}$$

Where X is the observed response. μ is the overall mean, α_i is the effect of BAG, β_j is the effect of CS, $\alpha\beta_{ij}$ is an interaction between BAG and SC and εij is the error. The level of statistical significance was present at $P\leq0.05$.

RESULTS

Growth Performance

Performance data are detailed in *Table 3*. In starter period, performance of birds were not affected by increasing levels of SC ($P>0.05$). However chicks fed 0.002 or 0.004 g/g BAG had higher BW and better FCR than control diet ($P<0.05$). Also there were no differences ($P>0.05$) between 0.002 and 0.004 g/g BAG fed chicks on broiler performance in starter period. In grower period BW of chicks fed 0.006 g/g SC was than control and 0.003 g/g SC fed chicks higher ($P<0.05$). However, increasing levels of SC had no effect on FI and FCR. For BAG both levels of 0.002 or 0.004 g/g increased BW ($P<0.05$) and decreased FCR ($P<0.05$) compared with control diet. There was no BAG by SC interaction on FI, BWG, and FCR in both starter and grower periods. There was no significant difference in mortality of all treatments.

Serum Lipids

The effects of dietary BAG and SC supplementation on serum lipid composition are shown in *Table 4*. Chicks

Table 3. *Performance of broiler chickens fed diets containing butyric acid glyceride (BAG) and Saccharomyces cerevisiae (SC)*
Table 3. *Butrik asit gliserid (BAG) ve Saccharomyces cerevisiae (SC) içeren diyetlerle beslenen broiler tavukların performans değerleri*

Groups	Starter (1-21 d)			Grower (22-42 d)		
	FI (g/bird)	BWG (g/bird)	FCR	FI (g/bird)	BWG (g/bird)	FCR
BAG, g/g						
0	628.89	477.56[b]	1.31[b]	2575.78	1120.22[b]	2.30[b]
0.002	648.33	537.89[a]	1.20[a]	2628.78	1211.67[a]	2.16[a]
0.004	663.11	553.44[a]	1.20[a]	2626.00	1211.00[a]	2.17[a]
SEM	21.75	20.51	0.02	47.72	28.45	0.03
P-Value	0.563	0.030	0.022	0.639	0.037	0.011
SC, g/g						
0	677.67	555.00	1.23	2581.78	1147.22[b]	2.24
0.003	633.89	509.78	1.24	2596.22	1163.22[ab]	2.24
0.006	628.78	504.11	1.23	2652.56	1232.44[a]	2.15
SEM	20.84	22.08	0.03	48.37	29.09	0.03
P-Value	0.260	0.157	0.093	0.500	0.043	0.093
BAG×SC						
P-Value	0.624	0.181	0.449	0.070	0.424	0.865

[a,b] Within the same row, means with different superscripts are significantly different (P<0.05)

Table 4. *Effects of butyric acid glyceride (BAG) and Saccharomyces cerevisiae (SC) on blood serum lipids of broiler chicken at 42 d age (mg/dl)*

Tablo 4. *Butrik asit gliserid (BAG) ve Saccharomyces cerevisiae (SC)'nin 42. günde broiler tavukların serum lipid değerleri (mg/dl) üzerine etkisi*

Groups	Cholesterol	Triglyceride	HDL
BAG g/g			
0	127.44[b]	87.50	90.42
0.002	120.38[a]	88.88	87.84
0.004	117.22[a]	86.33	90.26
SEM	2.70	5.26	1.94
P-Value	0.016	0.943	0.555
SC, g/g			
0	127.22[b]	94.33	88.52[b]
0.003	121.55[ab]	85.38	86.13[b]
0.006	116.27[a]	83.00	93.87[a]
SEM	2.61	4.91	1.83
P-Value	0.011	0.292	0.016
BAG×SC			
P-value	0.202	0.571	0.437

[a,b] Within the same row, means with different superscripts are significantly different (P<0.05)

fed 0.002 or 0.004 g/g BAG had the lower cholesterol concentrations compared with control diet ($P<0.05$). However there were no differences between 0.002 and 0.004 g/g BAG fed diets on cholesterol concentrations. The cholesterol concentration was lower only 0.006 g/g SC fed chicks ($P<0.05$). There were no differences between 0.003 g/g SC and control diet on cholesterol concentrations. HDL level was higher ($P<0.05$) only 0.006 g/g SC fed chicks. There were no differences between 0.003 g/g SC and control diet on serum HDL levels. Also BAG had no significant effect on serum HDL concentrations in any levels. There were no significant effects in triglyceride concentrations among treatments. There was no interaction effect of SC and BAG on cholesterol, triglyceride and LDL concentrations.

DISCUSSION

The primary role of a diet is not only to provide enough nutrients to fulfill metabolic requirements of the body but also to modulate various functions of the body. Probiotics, prebiotics, and organic acids are either beneficial microorganisms or substrates that facilitate the growth of beneficial microorganisms, which can be suitably harnessed by the food manufacturers and hold considerable promise for health care industry. Results of the present study showed that the inclusion of 0.006 g/g yeast *Saccharomyces cerevisiae* had positive effect on broiler BW gain only in grower period. Although dietary SC had no significant effects on broiler performance in starter period. These results are in agreement with Gao *et al*.[17], who reported positive effects of SC on broiler average daily gain and FCR during grower period. It seems that a period of adaptation is needed before the effects of SC inclusion can be significant because the changes in intestinal morphology and immune responses take time [17]. *Saccharomyces cerevisiae* contains live yeast as well as metabolites such as peptides, oligosaccharides, amino acids, flavor and aroma substances, and possibly some unidentified growth factors which have been proposed to produce beneficial performance responses in animal production by maintenance of beneficial microbial population [3], improving FI and digestion [18] and altering bacterial metabolism [19]. Other studies, however reported that SC had no effect on performance in poultry [4]. Differences in animal response may be related to differences in products formulations; yeast products are interchangeably classified as active dried yeast, live yeast or fermented yeast, making comparisons difficult among studies. Growth performance of birds was positively affected by dietary supplementation of BAG both in starter and grower periods. However, there were no significant differences in BWG, FI and FCR between 0.002 and 0.004 g/g fed chicks in both periods. Also FI in groups fed BAG were not significantly differences from control diet. These results are agreement with those [20-22].

Organic acids like butyric acid maintained a better microbial environment in digestive tract of birds by reducing the number of pathogenic microbes. These enhanced digestion, absorption and efficiency of utilization of feed [2,23]. Bolton and Dewar [24] indicate that free butyric acid is absorbed very quickly in the upper digestive tract, and will likely be of limited effective. By inference, butyrate needs to be stabilized, and hence the testing of butyric acid glycerides used in this study.

The present results showed that broiler chicks fed diet containing BAG or SC had significantly the lower plasma cholesterol concentrations. Similar cholesterol depressing effect due to probiotic and organic acids supplementation in broiler chicken was observed by [14,25]. The findings of our study and previous studies indicated that feeding of probiotics like SC and organic acids such as butyric acid has a cholesterol depressing effect in broiler chicken. Besides, it is reported that some of the microorganisms present in the probiotic preparation could utilize the cholesterol present in the gastro intestinal tract for their own metabolism, thus reduce to absorption the amount cholesterol [26]. Lactobacillus which better survive in low pH environment of intestinal tract specially when organic acids used to decrease pH of intestine has a high bile salt hydrolylic activity, is responsible for deconjugation of the bile salts [27]. Deconjucated bile acids are less soluble at low pH and less absorb in the intestine and are more likely to be excreted in feces [28]. Since the excretion of deconjucated bile acids is enhanced and cholesterol is its precursor, more molecules are spent for recovery of bile acids [8]. As a result of increased synthesis of this acids, it is expected the level

of serum cholesterol to be reduced. In addition, probiotic microorganisms inhibit hydroxymthyl-glutaryl-coenzyme A, an enzyme involved in the cholesterol synthesis [29].

In practical term, dietary addition of 2 g BAG per kg diet improved BWG and FCR of broiler chicks in both starter and grower periods. Supplemental SC did not affect performance during the starter period. In the grower period only 6 g SC per kg increased BW. Both agents decreased plasma cholesterol concentration. Their effects were not additive. Each of these agents might be promising alternatives for antibiotic growth promoters. The butyric acid glycerid offers a good alternative to improve poultry production.

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SHORT COMMUNICATION

Plasmodium berghei'nin *In Vitro* Kültürü: Klorokin ve Artesunat İlaç Direnç Testlerinin Uygulanması [1]

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Özet

Çalışmamızın amacı *Plasmodium berghei*'nin kısa dönem kültüründe uygulanacak ilaç direnç testlerinin laboratuarımızda yerleştirilmesi olmuştur. İlk aşamada *P. berghei*'nin genç trofozoitlerini barındıran enfekte fare kanlarının *in vitro* olarak, 24 saatlik eritrosit içi evrim döngüsünün tamamlanması gerçekleştirilmiştir. İkinci aşamada klorokin ve artesunat ilaç konsantrasyonlarının parazitin eritrosit içi gelişimini inhibe etme oranları tespit edilmiştir. Sonuçlarımıza göre, kısa dönem *in vitro* kültür testlerinde *P. berghei* parazitleri eritrositer şizogoni evrelerini tamamlamış, enfekte eritrositlerde merozoit oluşumları gözlenmiştir. Klorokin ve artesunat ile yapılan ilaç direnç testlerinde ise artesunat ilacının antipasmodiyal etkisinin daha fazla olduğu saptanmıştır.

Anahtar sözcükler: *Plasmodium berghei, in vitro, Antiplasmodiyal ilaç direnç testleri*

In Vitro Cultivation of *Plasmodium berghei*: Application of Drug Resistance Tests with Chloroquine and Artesunate

Summary

The aim of this study is to assess the drug resistance testing of *Plasmodium berghei* after short-term culture in vitro. First stage, the life cycle of the malaria parasites was completed in an average of 24 h inside the red blood cells *in vitro*. Second stage, the inhibition rates of Chloroquine and Artesunate on the infected erythrocytes were determined. The results showed that *P. berghei* parasites were completed their erythrocytic schizogony in short term *in vitro* cultivation. In the drug resistance tests with Chloroquine and Artesunate, antiplasmodial resistance did not occur with both drugs and the effect of Artesunate is higher than Chloroquine.

Keywords: *Plasmodium berghei, in vitro, Antiplasmodial drug resistance tests*

GİRİŞ

Sıtma etkeni *Plasmodium*'ların oluşturduğu ilaç direnci, *in vivo* ve *in vitro* ortamlarda bu parazitler üzerine yeni antimalarial/antiplasmodiyal maddelerin etkisini araştırmaya yönelik çalışmaların artmasına neden olmuştur. İnsanı enfekte etmeyen kemirgen sıtma parazitleriyle *in vitro* ortamda yapılan çalışmalar antiplasmodiyal etki gösteren maddelerin belirlenmesi açısından önem taşımaktadır. Yeni ilaç çalışmalarında sentez edilen veya doğal kaynaklardan izole edilen kimyasal maddelerin, klinik denemelere tabi tutulabilmeleri için öncelikle *in vitro* tarama ve toksisite deneyleri yapılmaktadır [1]. Sıtma ilaçlarının etki mekanizmaları arasında farklılıklar olduğu gibi aynı türün suşları arasında da farklılıklar bulunmaktadır [2]. Dünyanın bir çok bölgesinde, başta *P. falciparum* olmak üzere sıtma türlerinin ilaçlara karşı direnç gösterdiği bilinmektedir [3-5]. Direnç, genellikle ilaç ya da ilaç gruplarının sensitivitelerinin azalmasına neden olan spontan mutasyonların tek nokta ya da çoklu nokta mutasyonları sonucu olarak gelişmektedir [6,7]. *Artemisia annua* bitkisinden elde edilen Artemisinin ve türevlerinin

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özellikle *P. falciparum* tedavisinde uzun süre başarıyla kullanılmasının ardından, bu ilaca karşı da oluşan direnç bulgularından söz edilmeğe başlanmıştır [8,9]. Bu çalışmanın amacı, insan sıtma etkenlerinden en ağır klinik tabloya neden olan *P. falciparum*'a yakın yaşam döngü özellikleri olduğu bilinen kemirgen sıtma etkeni *P. berghei*'nin *in vitro* yaşatılıp çoğaltılmasını sağlamak ve klorokin ve artesunat ilaç direnç testlerini *in vitro* olarak uygulamaktır. Çalışmamız *in vitro* ortamda *P. berghei* üzerine uygulanan ilaç direnç testleri konusunda ülkemizdeki ilk çalışmalardan biridir.

MATERYAL ve METOT

Çalışmamızda MR4-ATCC (American Type Culture Collection) Wırginia, USA firmasından temin edilen *P. berghei* MRA-311-Anka suşu kullanılmıştır. İlk aşamada, -80ºC'de muhafaza edilen *P. berghei* genç trofozoitli eritrositleri taşıyan fare kan solüsyonu çözdürülüp, santrifüjlenerek elde edilen eritrosit çökeltisi RPMI-1640 besiyeri (%10 FCS eklenmiş) ile %10 oranında sulandırılmıştır. Kültür kapları içindeki eritrosit süspansiyonu mikrobiyolojik jar içine koyulmuş, %5 CO_2, %5 O_2, %90 N_2 karışımından gaz verilerek çalkalamalı etüve (37ºC) yerleştirilmiştir. Cam kavanozun içine yanar mumlar yerleştirilerek, CO_2 oranının artması O_2 seviyesinin düşmesi sağlanmıştır. 24 saat boyunca kültivasyon takip edilmiştir. Kültürün 22-24 saatleri arasında olgun şizont ve merozoitleri taşıyan eritrositler tespit edilmiştir. İkinci aşamada, kültür plaklarına 0.5 ml enfekte eritrosit solüsyonu ile 0.5'er ml klorokin (Sigma-C6628) ve artesunat (Sigma-A3731) ilaçlarının RPMI-1640 besiyeri ile hazırlanmış sulandırımlarından, son dilüsyonlar; 0.1, 0.4, 0.8, 1.6, 6.4 ve 12.8 µgr/ml olacak şekilde eklenmiştir. Kontrol için ayrılan çukurlardaki enfekte eritrosit süspansiyonu üzerine RPMI-1640 besiyeri koyulmuştur. Kültür plakları mikrobiyolojik jar içinde çalkalamalı etüve yerleştirilmiş ve kültivasyon başlatılmıştır [10]. İşlemler farklı zamanlarda 3 kez tekrarlanmıştır. Sonuçlar; Calcusyn version 2.1 programı ile değerlendirilmiştir.

BULGULAR

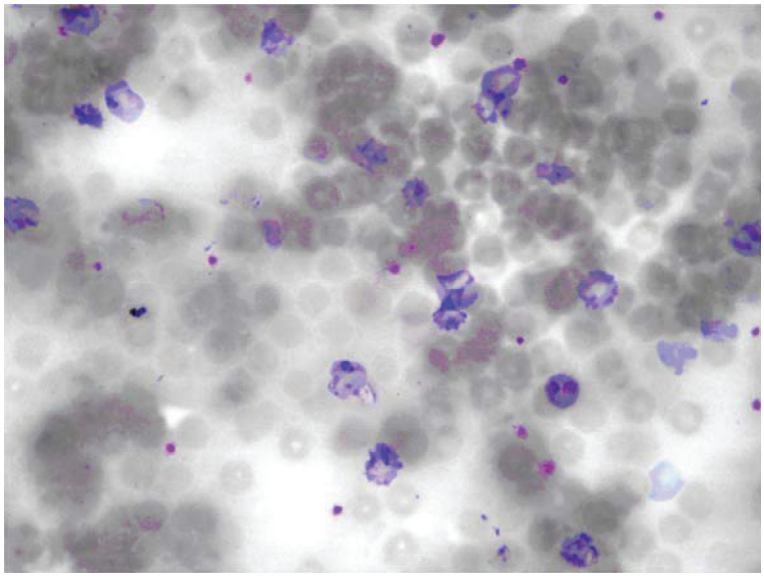
Plasmodium berghei'nin kısa dönem *in vitro* kültüründe; inkübasyon süresinin başında enfekte eritrositlerin tamamında trofozoitler bulunurken, 20-22. saatte şizontlar, 24. saatte ise merozoitler tespit edilmiştir *(Şekil 1, 2)*.

İlaç direnç testlerinde, klorokin ve artesunat ilaçlarının her ikisinde de 0.8 µg/ml dozda enfekte eritrositlerin %100'ünde trofozoit evresi bulunmuş, şizont evresine geçiş engellenmiştir. En düşük ilaç dozları olan 0.1 ve 0.4 µg/ml dozlarında, artesunat ile sırasıyla %81 ve %70 oranlarında şizont evresi tespit edilmiş, aynı dozlarda klorokin ile sırasıyla %81 ve %80 oranlarında şizont evresine geçiş olmuştur.

Klorokin ve artesunat ilaç direnç test sonuçları değerlendirildiğinde, her iki ilaç ile doz ve etki ilişkisi anlamlı bulunmuş ($P<0.05$), ED_{50} (%50'sini etkileyen doz) değerleri klorokin için 0.68431, artesunat için 0.45582 olarak hesaplanmıştır. Klorokin ve artesunat ilaç direnç testlerindeki doz etki eğrileri Calcusyn version 2.1 programı ile değerlendirilmiştir *(Şekil 3)*.

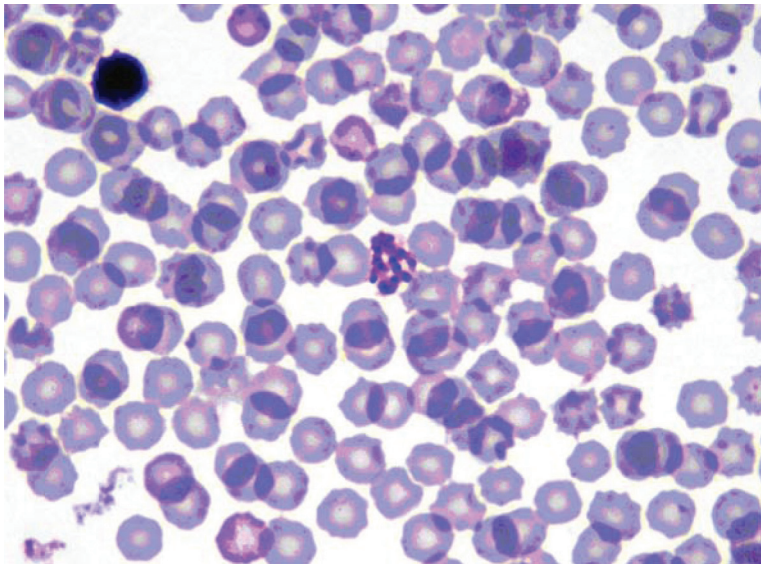
TARTIŞMA ve SONUÇ

Parazitin eritrositer dönemlerinin *in vitro* kültürleri Trager ve Jensen'in çalışmalarıyla başlamış ve sıtmanın çok yönlü araştırmalarındaki önemi sıklıkla vurgulanmıştır [11,12]. Kısa dönem *in vitro Plasmodium* kültürleri, ilaç direnci geliştirmiş suşların ayırt edilmesi amacıyla klinik izolatlara uygulanabilme kolaylığı sağlamaktadır. *In vitro* yöntemler ile yapılan *Plasmodium* çalışmaları; farklı bölgelerden izole edilen suşların aynı ilaçlara farklı direnç seviyeleri



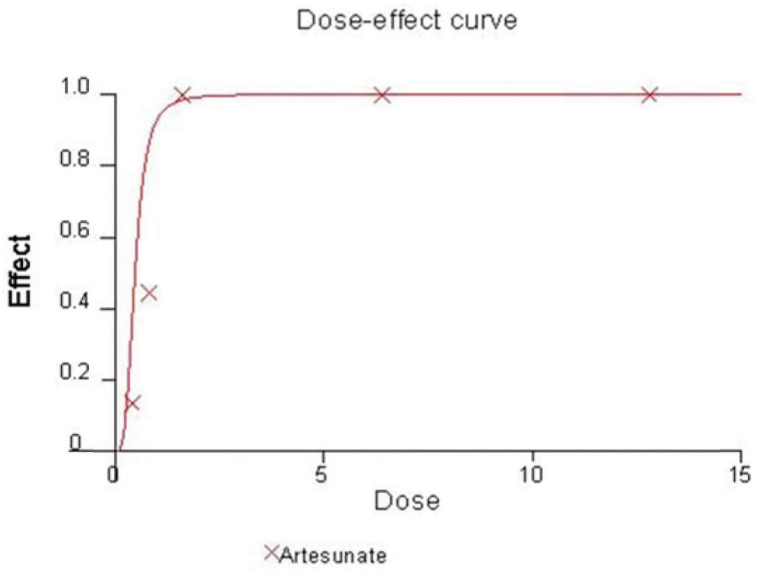
Şekil 1. Kısa dönem *in vitro* kültürün başlangıcında (0. saat) *P. berghei* ile enfekte eritrositlerde trofozoit evrelerinin görünümü

Fig 1. Appearance of trophozoite stages in erythrocytes infected with *P. berghei* at the beginning of the short-term cultivation (0. h)



Şekil 2. Kısa dönem *in vitro* kültürün 24. saatinde *P. berghei* ile enfekte eritrositlerde merozoit evresinin görünümü

Fig 2. Appearance of merozoite stages in erythrocytes infected with *P. berghei* at 24. h of the short-term cultivation



Şekil 3. Artesunat ilaç direnç testinde doz etki eğrisi (Calcusyn version 2.1)

Fig 3. The dose-effect curve of Artesunate drug resistance testing (Calcusyn version 2.1)

gösterebildiğini ve bölgesel tedavi protokollerinin belirlenmesinde bu yöntemle uygulanan ilaç direnç testlerinin önemli rol oynadığını göstermektedir [13,14]. Çalışmamızda *P. berghei* parazitleri *in vitro* kısa dönem kültürde eritrositer şizogoni geliştirerek 24 saatlik inkübasyon dönemi sonunda enfekte eritrositlerde %80 oranında şizont evresi oluşturmuştur. Klorokin ve artesunat ilaç direnç testleri sonucunda ise elimizdeki *P. berghei* suşunun her iki ilaca karşı direncinin olmadığı tespit edilmiştir. Elde ettiğimiz ED_{50} değerleri artesunat ile 0.45582, klorokin ile 0.68431'dir. Bu sonuçlar kullandığımız *P. berghei* suşu üzerinde artesunat'ın *in vitro* ortamda antiplasmodiyal etkisinin daha yüksek olduğunu göstermektedir. Bu çalışma *in vitro* üretilen *Plasmodium* parazitlerinde uygulanan ilaç direnç testleri konusunda ülkemizde yapılan ilk çalışmalardan biridir.

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SHORT COMMUNICATION

Kars Yöresi Sığırlarında Subklinik Paratuberkülozun Seroprevalansı [1] [2]

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Özet

Hayvancılık işletmelerinde ciddi ekonomik kayıplarla seyreden ve önemli bir zoonoz olan paratüberkülozis'in Türkiye'de ve Kars yöresinde prevelansına yönelik çalışmalar sınırlı sayıdadır. Sunulan çalışmada Kars yöresindeki sığırlarda subklinik paratüberkülozis'in prevalansının belirlenmesi amaçlandı. *Mycobacterium avium* subsp. *paratuberculosis* serum örneklerinde ELISA yöntemi kullanılarak tespit edildi. Bu amaçla rastgele seçilen 13 odak ve bu odaklardaki 24 işletmeden 2 yaş ve üzeri toplam 400 sığır kullanıldı. Kars yöresinde subklinik paratüberküloz'un seroprevalansı %3.5 (14/400), çiftlik prevelansı ise %41.6 (10/24) olarak belirlendi. Yaşları ≥2-<5, ≥5-<7 ve ≥7 olarak gruplandırılan sığırlarda paratüberkülozun seroprevalansı sırasıyla %1.7, %3.8 ve %5.1 olarak belirlendi. Hayvanların yaşı arttıkça pTB'a yakalanma riskinin arttığı belirlendi. Sonuç olarak, paratüberkülozun her iki odaktan birinde tespit edilmesi, enfeksiyonunun yayılma hızı, başka bölgelere bulaşma riski ve ekonomik kaybı düşünüldüğünde dikkate alınması gerekir.

Anahtar sözcükler: *Johne's Hastalığı, Mycobacterium Paratuberculosis, ELISA, Seroprevalans*

Seroprevalence of Subclinical Paratuberculosis in Cattle in Kars Region

Summary

Studies on prevalence of Paratuberculosis, an important zoonosis and cause of major economic losses to farms, are limited in Kars and Turkey as a whole. This study was therefore aimed at determining the prevalence of subclinical paratubercolos in Kars district. *Mycobacterium avium* subsp. *paratuberculosis* was determined using ELISA. For this purpose, 400 cattle above 2 years old from 24 farms located in 13 different localities were blood sampled. The seroprevalence of subclinical paratubercolos was 3.5% (14/400) and farm prevalence was 41.6% (10/24) in Kars district. Age distribution of seroprevalence of paratubercolos was 1.7% in cattle aged ≥2-<5 years old, 3.8% in ≥5-<7 years old and 5.1% in ≥7 old. The rate increased age of cattle get older. In conclusion, paratubercolos was determined in half of the localities and its incidence may pose risk of transmission to other parts of the country and of great economical losses and therefore the disease should be taken into consideration.

Keywords: *Johne's disease, Mycobacterium paratuberculosis, ELISA, Seroprevalence*

GİRİŞ

Johne's Disease ve Hohnesche Krankheit olarak da bilinen Sığır paratüberkülozu *Mycobacterium avium* subsp. *paratuberculosis* (MAP) etkeninin neden olduğu, bağırsak cidarının kalınlaşması ve tedaviye cevap vermeyen kronik bir enteritisle karakterize bulaşıcı bir enfeksiyondur. Enfeksiyon Crohn hastalığının etiyolojisinde rolü nedeniyle önemli bir zoonoz olarak da bilinmektedir [1-3]. Hayvanlar neonatal dönem dahil çok erken yaşlarda enfekte olabilmekle birlikte hastalığa ait klinik belirtiler özellikle 2-6 yaş arasındaki hayvanlarda gözlenmektedir [1]. Paratüberküloz (pTB) hayvanlarda ishal ve kilo kaybı sonucu kaşeksiye kadar zayıflama, süt veriminde düşme, kısırlık, mastitis, ölümlere neden olması ve teşhis, tedavi ve kontrol programı giderlerinden dolayı ciddi ekonomik kayıplara yol açmaktadır [1-7]. Örneğin Amerika'da enfeksiyon yıllık 200-250 milyon dolar ekonomik kayba yol açmaktadır [8]. Etkili bir tedavi ve aşısı bulunmayan enfeksiyonda etkenin süt, dışkı ve kolostrumla buzağılara, işletmedeki hayvanlara ve diğer çiftliklere ve en önemlisi insanlara bulaşmasının önlenmesinde eradikasyon gibi tedbirlerin alınması gerek-

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mektedir [3,5,7]. Bu nedenle enfeksiyonun özellikle subklinik dönemde teşhisi önemlidir [1,3]. Hastalığın seroprevalansı ve teşhisinde kültür, polimeraz zincir reaksiyonu (PZR) tekniği ve immunolojik olarak agar jel immünodiffüzyon test (AGID), komplement fikzasyon (KF) ve enzim linked immunosorbent assay (ELISA) yöntemleri kullanılmaktadır [4-6,9-11].

Kars yöresi, ülkenin farklı bölgelerine yoğun hayvan nakillerinin yapılmasından dolayı paratüberküloz gibi bulaşıcı enfeksiyöz hastalıkların tespiti, hem yayılmasını engellenmesi hem de ekonomik kayıpların önlenmesi, açısından önemlidir. Türkiye'de pTB ile ilgili çalışmaların sınırlı olduğu ve Kars yöresinde ise konuyla ilgili herhangi bir verinin bulunmadığı bilinmektedir. Dolayısıyla bu hastalığın bölgemiz ve ülkemiz hayvancılığı açısından oluşturduğu riskin boyutları tam olarak bilinmemektedir. Sunulan araştırmada Kars ve çevresindeki sığır popülasyonunda subklinik paratuberkülozun seroprevalansı ve çiftlik prevalansının belirlenmesi yanında yaş ve ırka göre dağılımının tespiti amacıyla gerçekleştirildi.

MATERYAL ve METOT

Kars merkez ve ilçelerinden rastgele seçilen 13 odak ve bu odaklardaki 24 işletmeden alınan toplam 400 sığır çalışmanın materyalini oluşturdu. Her işletmeden 2 yaşından büyük, paratuberküloz yönünden aşılanmamış hayvanların yaklaşık olarak %15-25'i kadarından rastgele serum örneği alındı. Çalışılan hayvan ırkları simental melezleri (n=156), montofon melezleri (n=190) ve yerli ırk melezlerinden (n=54) oluştu. Hayvanların yaşları ≥2 - <5 (n=116), ≥5 - <7 (n=206), ≥7 (n=78) olarak gruplandırıldı. Irk ve yaşın paratüberküloz üzerine etkisinin belirlenmesinde X^2 for trend testi kullanıldı (EPI INFO 6). MAP etkenine karşı şekillenen antikorların belirlemesinde ticari ELISA kiti kullanıldı (IDEXX Paratuberculosis Screening Ab Test).

BULGULAR

Kars yöresindeki sığırlarda subklinik paratüberkülozun seroprevalansı %3.5 (14/400) ve çiftlik prevalansı ise %41.6 (10/24) olarak belirlendi. Paratüberküloz pozitif bulunan vakaların odak ve çiftliklere göre dağılımı *Tablo 1*'de sunulmuştur. İşletmelerden 7'sinde yalnız bir sığırda, 3'ünde ise 2 veya daha fazla sığırda paratüberküloz pozitif olarak belirlendi. Paratüberkülozun prevalans oranı Simental veya melezlerinde %3.20 (5/156), Montafon veya melezlerinde %3.68 (7/190), Yerli ırk veya melezlerinde %3.70 (2/54) olarak tespit edildi *(Tablo 2)*. Pozitif 14 vakanın yaş gruplarına göre; 2'si <5, 8'i ≥5 - <7 ve 4'ü >7 yaşında olarak dağıldı. Aynı yaş gruplarında paratüberküloz prevalans oranları sırasıyla %1.72 (2/116), %3.88 (8/206) ve %5.12 (4/78) olarak belirlendi *(Tablo 2)*. Sığırların yaşı arttıkça pTB prevalans oranlarının arttığı görülmekle birlikte bu istatistiksel olarak anlamlı bulunmadı.

Tablo 1. *Subklinik Paratüberküloz pozitif vakaların odak ve çiftliklerde göre dağılımı*

Table 1. *Distribution of subclinical paratuberculose positive cattle according to locality and farms*

Odak	Çiftlik No	N1	N2
Kars Merkez	1	29	0
Sarıkamış	2	15	2
Selim	3	12	0
	4	16	1
	5	14	0
	6	17	3
Bulanık	7	20	0
	8	20	0
Cavlak	9	10	0
	10	11	0
Yeşiltepe	11	12	1
	12	16	1
Kırkpınar	13	15	0
	14	13	1
	15	13	1
Yalmaçlı	16	7	0
Halefoğlu	17	31	0
	18	34	1
Soylu	19	15	2
Arpaçay	20	16	1
Mezra	21	13	0
	22	21	0
	23	14	0
Digor	24	16	0
Toplam	24	400	14

N1: *Örneklenen Hayvan Sayısı,* ***N2:*** *Paratüberküloz pozitif Hayvan Sayısı*

Tablo 2. *Irk ve Yaşa göre subklinik paratüberküloz prevalansı*

Tablo 2. *Breed and age distribution of subclinical paratuberculose positive cattle*

Parametre		Örnek Sayısı	Pozitif Hayvan Sayısı	Oran (%)	OR
Irk	Simental veya Melezi	156	5	3.20	1.00
	Montafon veya Melezi	190	7	3.68	1.16
	Yerli Irk veya Melezi	54	2	3.70	1.16
	İstatistiks				X^2=0.05, P=0.82
Yaş	≥2 - <5	116	2	1.72	1.00
	≥5 - <7	206	8	3.88	2.30
	≥7	78	4	5.12	3.08
	İstatistik				X^2=1.7, P=0.2

TARTIŞMA ve SONUÇ

Paratüberkülozisin seroprevalansının subklinik süreçte belirlenmesi çiftlikteki hayvanlar arasında ve çiftlikten çiftliğe, enfeksiyonun yayılmasının önlenmesinde kritik bir öneme sahiptir [4,5,9]. Buzağılara doğumdan hemen sonra veya neonatal dönemde bulaşık meme, ekipman ve

kolostrum veya sütle etkeni alabildikleri ve bu dönemin enfeksiyona en duyarlı zaman olduğunun bilinmesi, etkenin ısı ve pastörizasyona dirençli olması nedeniyle süt ve süt ürünleri ile insanlara bulaşma ihtimalinin bulunması, subklinik dönemde teşhisin önemini artırmaktadır [3,6,9]. Türkiye'de varlığı uzun süredir bilinmesine rağmen pTB'la ilgili çalışmaların sınırlı olduğu görülmektedir [4-6,9,12-14]. Orta Anadolu Bölgesinde farklı yıllarda yapılan çalışmalarda mikro ve tüp komplement fikzasyon yöntemleri ile pTB' un sığırlardaki seroprevalansı sırasıyla %2.3 ve %2.7 [12] ve ELISA yöntemiyle %4.6 [5,13] olarak tespit edilmiştir. Elazığ yöresinde [6] süt örneklerinde pTB'un prevalansını PZR ve bakteriyel kültür yöntemleri ile sırasıyla %5 ve 3.4 olarak belirlenmiştir. Uşak yöresinde [9] dışkı örneklerinde pTB prevelansı Ziehl-Neelsen (ZN) boyama, Outer PZR, Nested PZR ve bakteriyolojik kültür yöntemlerine göre sırasıyla %17, %9.5, %20 ve %4 ve süt örneklerinde ise yine aynı yöntemlerle sırasıyla %15.5, %5.5, %17.5 ve %2.5 olarak tespit edilmiştir. ELISA testi ile pTB seroprevelansı Burdur yöresinde %6.2 [4] olarak tespit edilmiştir. Trakya Bölgesinde ise PZR yöntemi ile dışkı örneklerinde pTB etkeni saptamadıklarını bildirmişlerdir [14]. Çalışmamızda ELISA yöntemiyle pTB'un seroprevelansı %3.5 olarak tespit edildi. Çalışmamızda dahil genel olarak pTB Türkiye'deki prevalansını %0 ile %20 arasında değişmesi Avrupa'daki prevalans aralığı (%0-%24) ile paraleldir [2,5,15,16]. Türkiye'de pTB'la ilgili çalışmalarda çiftlik prevalansının dikkate alınmadığı görülmektedir. Fakat paratüberküluz bir işletmede tek bir sığırda bile tespit edilmesi buluşma riskinin yüksek ve kolay olması nedeniyle önem arz etmektedir. Ülkemizde sadece Burdur ili ve çevresinde yapılan çalışmada [4] pTB'un çiftlik prevalansı değerlendirilmiş ve %58 (14/24) olarak tespit edilmiştir. Çalışmamızda da çiftlik prevalans oranı %46.1 olarak diğer çalışmaya yakın bulundu. Bu iki çalışma verileri yaklaşık olarak her iki işletmeden birinde pTB'un bulunduğunu göstermektedir. Avrupa ülkelerinde pTB'un sürü prevalansının %0 ile %75 arasında değiştiği bildirilmiştir [2,5,15,16]. Çiftlikteki hayvan sayısı arttıkça ve özellikle >100 olanlarda pTB'un prevalansının da arttığı belirtilmiştir [4,16,17]. Kars ve çevresinde çiftlikler genellikle hayvan sayısı <50 olan küçük aile işletmeleri şeklindedir [18]. Bu yörede buzağılar doğumdan sonra anneleri ile birlikte barındırılması ve ilk 3 ay anne sütü ile beslendirilmesi enfeksiyonun vertikal bulaşmasına neden olabilir [3,4,6].

Prevalans oranları arasındaki farklılıklar, çalışmalara göre değişen iklim ve coğrafik şartlar, beslenme ve barındırma koşulları ve teşhis için kullanılan materyal ve yöntemlerden kaynaklanabilir [4,5,6]. Nitekim ELISA yöntemi ve serum örneklerinin kullanılmadığı bir araştırmada [9] pTB'un prevalans oranının kullanılan örnek (dışkı ve süt) ve yöntemlere göre (farklı iki PZR, kültür ve ZN boyama) göre oldukça değiştiği (%4-%20) görülmektedir. Ayrıca hastalığın dönemine göre de prevalans oranları değişebilmektedir. Örneğin İngiltere'de klinik pTB'un prevalansı %1, mezbahalarda ve klinik bulgu göstermeyen hayvanlarda ise %3.5 olarak belirlenmiştir [6]. Ayrıca, KFT yalnızca klinik pTB'un teşhisinde etkili olduğu için yalnızca bu yöntemin kullanıldığı çalışmalarda subklinik veya asamptomatik taşıyıcıları doğru belirlenemeyeceği için prevalans oranları aynı bölgelerde düşük çıkabilmektedir [6]. pTB'un subklinik dönemde teşhis önemli bir problemdir. Enfeksiyonun subklinik teşhisinde serolojik olarak spesifik antikorların belirlenmesi ve nekropsi esnasında doku veya dışkıdan MAP'ın kültürü en etkili yöntemlerdir [15]. Kültür yöntemi yüksek spesifiteye sahip olmakla beraber uzun inkübasyon süresi kullanımını sınırlandırmaktadır [9]. Ayrıca selektif vasat gereksinimi, kontaminasyon problemi ve pahalı olması bu yöntemin diğer dezavantajlarıdır. Son yıllarda moleküler biyolojide yapılan ilerlemeler sonucu geliştirilen PZR tekniği, kültür yöntemine göre daha yüksek sensitivite ile etkeni belirleyebilmektedir [9,10]. PZR yöntemi kültür yöntemine göre daha basit, hızlı, güvenilir ve yüksek spesifite ile sonuçlar verebilmektedir. Fakat özellikle ELISA gibi immunolojik teknikler kültür ve PCR yöntemlerine göre daha ekonomik ve hızlı olmasının yanında çok sayıda analizin birlikte yapılması ve kolay uygulanabilir olması gibi avantajlar sunmaktadır [4,5,19,11]. Ayrıca yapılan son güncel bir çalışmada [19] ELISA testinin sürüde enfekte hayvanları da subkutan reaksiyon testi (Avian PPD kullanılarak), dışkıda kültür (ZN boyama), PZR yöntemlerine yakın doğrulukla belirleyebildiği bildirilmiştir. Yine, pTB'la ilgili herhangi bir çalışmanın yapılmadığı bölgelerde öncelikle yüksek spesifitesinden dolayı ELISA'nın tercih edilmesi gerektiği rapor edilmiştir [11]. Son yıllarda yüksek sensitivite ve spesifite ile özellikle seroprevalans çalışmalarında ELISA hemen hemen kullanılan tek yöntem gibi görünmektedir [4-6,11,19]. Yapılan çalışmalarda ELISA'nın sensitivite değeri %30.2-%51.0, spesifite değeri ise %95 ile %99 arasında değiştiği belirtilmiştir [4,5,17,19,20]. Fakat son yıllarda geliştirilen ticari kitlerde sensitivitenin %74.1'e kadar çıktığı ve spesifite oranının ise %99'un üstünde olduğu bildirilmiştir [4,17,19,20]. Prospektüse göre, araştırmamızda kullanılan ELISA kitinin sensitivite ve spesifite oranları sırasıyla %64.7 ve %99.2 olarak belirtilmiştir. Bu nedenle pTB'un prevalansının belirlenmesinde çoğunlukla ELISA yönteminin kullanılması, kültür ve PCR yöntemlerinin ise doğrulayıcı yöntemler olarak kullanılması daha doğru bir yaklaşım olarak görülmektedir.

Çetinkaya ve ark.[7], Jersey ve Guernsey ırklarında Johne's hastalığı'nın Friesian veya diğer türlere göre daha yaygın olduğunu bildirmişlerdir. Yapılan başka bir araştırmada ise pTB'un *Bos indicus* veya melez tür sığırlarda, *Bos taurus* türlerine göre 3.5 ile 17 kat daha yüksek olabileceği bildirlmiştir. Türlere göre sonuçların değişkenlik göstermesi türe özgü duyarlık ve kros reaksiyonla ilişkilendirilmekle birlikte nedeni tam olarak bilinmemektedir [1,21]. Burdur yöresinde pTB'nin prevalansı Holştayn ırkı sığırlarda %6.2 olarak belirlenmiştir [4]. Pozitif vakaların çoğunlukla montofon veya melezlerinde ortaya çıktığı belirlenmekle birlikte çalışmamızda ırklara arasında pTB seroprevalansının önemli değişiklik göstermediği belirlendi.

Uzun süre çevrede canlı kalabilen MAP güçlü bir hücre içi patojendir. Alınan MAP etkenleri savunma sisteminde

makrofaj hücreleri içerisinde uzun süre canlı kalabilmektedir. Başlangıçta etkenin proliferasyonu bu şekilde immun sistem tarafından kontrol edilmesi inkübasyonun uzun sürmesine (1.5-2 yıl) neden olmaktadır. Kandaki antikor seviyesi direkt enfeksiyonun gelişmesiyle verilen cevaba bağlı olarak artar ve buda uzun zaman alır. Bu durum genellikle iki yaşın altında sığırlarda pTB'un teşhisini zorlaştırmaktadır ve özellikle serumun kullanıldığı prevalans çalışmalarında iki yaşından küçük hayvanlar tercih edilmemektedir. Nitekim yaşı <2 olan sığırlarda pTB'un seroprevalansını düşük çıkması da bu verileri desteklemektedir [2-6]. Ayrıca yaşı <2 sığırlarda bu nedenlerden dolayı ELISA testinin sensitivite ve spesifitesinin düşük olduğu bildirilmiştir [4,5,10,16]. Bu nedenle çalışmamızda örnekleme yapılırken inkübasyon süresi dikkate alınarak hedef popülasyon olan iki veya daha büyük yaştaki sığırlar seçildi. Çalışmamızda paratüberküloz belirlenen 14 vakanın çoğunlukla 5 ve 6 yaşında olduğu (n=8) belirlenmekle birlikte pTB'un seroprevalansı yaşı ≥7 olarak gruplandırılan sığırlarda (%5.1), yaşı ≥2 - <5 (%1.7) ve ≥5 - <7(%3.8) olarak gruplandırılan sığırlara göre daha yüksek olduğu tespit edildi. Çalışmamızda elde edilen veriler hayvanların yaşı arttıkça pTB'un seroprevalansının da arttığını bildiren çalışmaları doğrulamaktadır [2,4,16]. Çalışmamızda yaşı 2, 3 ve 4 olan sığır grubunda pTB'un prevalansının en düşük düzeyde belirlenmesi 2 yaşındaki hayvanlarda negatif oranının yüksek çıkmasından kaynaklanabilir [4]. Öztürk ve ark.[4], çalışmalarında yaşı ≥7 olan sığırlarda belirledikleri paratüberkülozun yaygınlık oranı (%6.6) çalışmamıza belirlenen orana (%5.1) yakın bulundu. Fakat Öztürk ve ark.[4], en yüksek pTB seroprevalans oranını 3 yaşındaki sığırlarda (%19.7) belirlemişlerdir.

Sonuç olarak yapılan çalışmada, hastalıkla ilgili ekonomik kayıpların tahmin edilmesi ve Türkiye'deki prevalansının belirlenmesine katkıda bulunması amacıyla Kars yöresindeki subklinik paratüberkülozun prevalansı ilk kez belirlendi. Kars yöresi kendi içinde ve diğer yörelere sık hayvan geçişi olması nedeniyle enfeksiyonu ait yüksek çiftlik prevalansı (%46.1) hastalığın yayılma potansiyeli açısından önemli risk oluşturabilir. Elde edilen veriler Kars ili ve çevresinde önemli ekonomik kayıplara yol açabilecek bir enfeksiyon olarak paratüberkülozun göz önünde tutulması gerektiğini göstermektedir. MAP etkeninin sütle ciddi oranda yayılması veya taşınması, insan sağlığı açısın -dan da önemli risk oluşturması uygun teşhis edilmesinin önemini arttırmaktadır. Türkiye'de bu alanda yapılan çalışmalar genel olarak değerlendirildiğinde paratüberkülozun ruminantlarda yaygınlığı, ülke ekonomisine zararı ve insan sağlığını için taşıdığı riskin belirlenebilmesi için ulusal düzeyde bir çalışma yapılması gerekmektedir. Ancak bu düzeyde bir çalışma hastalığa karşı etkili kontrol stratejilerinin geliştirilmesine ışık tutacaktır.

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YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup, derleme ve çeviri türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.

2- Dergide yayımlanması istenen yazılar Times New Roman yazı tipi ve 12 punto ile A4 formatında, 1.5 satır aralıklı ve sayfa kenar boşlukları 2.5 cm olacak şekilde hazırlanmalı ve resim, tablo, grafik gibi şekillerin metin içindeki yerlerine Türkçe ve yabancı dilde adları ve gerekli açıklamaları mutlaka yazılmalıdır.

Dergiye gönderilecek makale ve ekleri (şekil vs) *http://vetdergi.kafkas.edu.tr* adresindeki online makale gönderme sistemi kullanılarak yapılmalıdır.

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.

3- Yazarlar yayınlamak istedikleri makale ile ilgili olarak gerekli olan etik kurulu onayı aldıkları kurumu ve onay numarasını Materyal ve Metot bölümünde belirtmelidirler. Yayın kurulu gerekli gördüğünde etik kurul onay belgesini ayrıca isteyebilir.

4- ***Makale Türleri***

Orijinal Araştırma Makaleleri, yeterli bilimsel inceleme, gözlem ve deneylere dayanarak bir sonuca ulaşan orijinal ve özgün çalışmalardır.

Türkçe yazılmış makaleler Türkçe başlık, Türkçe özet ve anahtar sözcükler, yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, giriş, materyal ve metot, bulgular, tartışma ve sonuç ile kaynaklar bölümlerinden oluşmalı ve metin, tablo, şekil vs dahil) 10 sayfayı aşmamalıdır. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler ile birlikte 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 6 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.

Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri giriş, metin ve kaynaklardan oluşmalı ve 10 sayfayı geçmemelidir.

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.

6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.

Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.

Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.

Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.

Örnek: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed. 339-447, Lea and Febiger, Philadelphia, 1988.

Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi kaynak bilgilerinin sonuna eklenmelidir.

Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.

Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.

7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.

8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir yayın danışmanı ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.

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12- Yazarlara 50 adet ayrı baskı ücretsiz olarak yollanır.

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1- Kafkas Üniversitesi Veteriner Fakültesi Dergisi (Journal of the Faculty of Veterinary Medicine, Kafkas University) (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, review and translation on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure, table and graphic must clearly be written in both Turkish and foreign language and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

During the submission, the authors should upload the figures of the manuscript (the dimensions must not to exceed 13 X 18 cm) to the online manuscript submission system. If the manuscript is accepted for publication, the copyright transfer agreement form signed by all the authors should be send to the editorial office.

3- Authors should indicate the name of institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, editorial board may also request the official document of the ethical commission report.

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Manuscripts written in Turkish consist of the title in Turkish, summary and keywords in Turkish, introduction, material and methods, results, discussion and references and it should not exceed 10 pages including text, tables and illustrations. Manuscripts written in a foreign language should follow the title in foreign language, summary and keywords in foreign language, title in Turkish, summary and keywords in Turkish and the remaining sections described above for the manuscripts written in Turkish.

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Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but each of the summaries should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages. Additionally, they should not contain more than 4 figures or tables.

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