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Research Article

The Effect of Microwave Heating on the Some Quality Properties and Shelf Life of Yoghurt

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Abstract

This study was carried out to determine the properties of set type yoghurts made using microwave heating after yoghurt fermentation during storage period of 28 d at 4±1°C. In this way, it will be possible to increase the yoghurt shelf-life, by preventing post-acidification. Yoghurts samples were subjected to three different microwave heating (10, 20 and 30 sec) at 720 Watt power level. Viability of yoghurt bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) was assessed during 28 d of storage. Samples were subjected also physicochemical analysis (pH and titratable acidity). The results showed that microwave applications significantly affected viable counts of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* ($P<0.05$). The counts of *L. bulgaricus* and *S. thermophilus* increased in heated yoghurts but a delay was observed during of storage period. Also the results indicated that the microwave heating had significant effect on the titratable acidity values ($P<0.05$). The titratable acidity and pH showed similar patterns of increase or decrease during storage period. During refrigerated storage, decline in pH was greater in control group yoghurts than heated yoghurts. The titratable acidity increased in all yoghurt samples. However, the rate of increase was affected by the microwave heating time. The results of the study showed that the microwave heating had significant effect on the acceptability of yoghurt during shelf-life.

Keywords: *Yoghurt, Microwave heating, Shelf-Life, L. Lactobacillus delbrueckii ssp. bulgaricus, Streptococcus thermophilus*

Mikrodalga Isıtma Uygulamasının Yoğurdun Raf Ömrü ve Bazı Kalite Özellikleri Üzerine Etkisi

Özet

Bu çalışma yoğurt fermantasyonu sonrası mikrodalga ısıtma kullanılarak üretilen ve 4±1°C'de 28 gün depolanan set tipi yoğurtların bazı özellikleri ve mikrodalga ısıtmanın yoğurt bakterilerinin canlılıkları üzerindeki etkisini belirlemek amacıyla yapılmıştır. Bu sayede depolama sırasında asitlik artışı geciktirilerek yoğurdun raf ömrünün uzatılması mümkün olabilecektir. Yoğurt örnekleri fermantasyondan sonra 720 Watt mikrodalga güç seviyesinde üç farklı süreyle (10, 20 ve 30 sn.) mikrodalga ısıtmaya tabi tutulmuştur. Yoğurt bakterilerinin (*Lactobacillus delbrueckii* ssp. *bulgaricus* ve *S. thermophilus*) depolama boyunca sayıları ve yoğurt örneklerinin bazı fiziko-kimyasal özellikleri (pH ve titre edilebilir asitlik) belirlenmiştir. Bulunan sonuçlar mikrodalga uygulamasının, canlı *L. bulgaricus* ve *S. thermophilus* sayıları üzerinde etkili olduğunu göstermiştir ($P<0.05$). Sonuçlar aynı zamanda mikrodalga ısıtmanın titre edilebilir asitlik değerleri üzerinde de önemli etkiye ($P<0.05$) sahip olduğunu göstermiştir. Çalışma, mikrodalga ısıtma uygulmasının raf ömrü süresince yoğurdun kabul edilebilirliği üzerinde önemli bir etkisi olduğunu göstermiştir.

Anahtar sözcükler: *Yoğurt, Mikrodalga ısıtma, Raf ömrü, Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophilus*

INTRODUCTION

Fermented dairy products have been considered to have health- promoting properties on consumers' health for a long time. The consumption of fermented milk products is reported to have many benefits to human health around the world [1-6]. Yoghurt is the most known fermented dairy product with high consumption worldwide mainly because of the many health-promoting effects [7-9].

The shelf life of a product is described as the time in which the food product will remain safe, be certain to retain desired sensory, chemical, physical and microbiological characteristics, and will comply with any label declarations of nutritional data when stored under the recommended conditions [10]. The possibility to offer a higher shelf life than its competitors constitutes a primary competitive advantage for fresh food producers. As all fresh products, yoghurt has a relatively short shelf life. The shelf life of yoghurt produced under normal conditions is about

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8-10 d at <10°C. Furthermore, consumers tend to buy the product with the longest possible shelf- life [11].

Microwaves are basically high frequency electromagnetic energy in the frequency range of 300-3000 MHz generated by a magnetron. Electromagnetic energy at 915 and 2450 MHz can be absorbed by water containing materials and converted to heat [12]. Microwave energy contributes to the heat transfer by electromagnetic radiation and subsequent volumetric heating [13]. The purpose of thermal processing was to extend the shelf life of food products without compromising food safety [14]. The application of microwave heating in food processing is a partly recent development. Although microwave ovens are widely used as a means of food preparation, insufficient information is available on the consequences for the composition and nutritional quality of food [13].

The storage life of yoghurt depends on the degree of sanitation during processing and packaging. Several methods are used to increase the shelf life of the product such as different packaging [15], addition of antibiotics [16-18], addition of gas [19], application of preservatives, heat treatment after incubation [18] and carbonation process [20].

The aim of this study is to increase the yoghurt shelf-life, by preventing post-acidification, the effects of microwave heating on pH, titratable acidity and viable *S. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* population, important in view of existing legal regulations for yoghurts, of yoghurt produced from cow's milk. The other aim of this study is to investigate the possibility of using microwave heating in the production of yoghurt and to make a contribution to the novel food manufacture techniques.

MATERIAL and METHODS

Raw Milk, Bacterial Cultures and Propagation

Raw cow's milk used in this study was obtained from the Atatürk University, Pilot Dairy Factory of Food Engineering, Erzurum, Turkey. The some physicochemical properties of the cow's milk were as follows: pH 6.48, titratable acidity 0.15%, dry matter 8.76% (Solid Not- Fat, SNF) and fat 3.4%.

Yoghurt starter cultures *(L.* delbrueckii ssp. *bulgaricus* and *S. thermophilus*) were obtained from the Food Engineering Department at Atatürk University. The yogurt strains were isolated from our previous one and then identified and stored. Strains were stored frozen (-80°C) as stock cultures in with 20% (v/v) glycerol (Merck, Darmstadt, Germany). Frozen cultures were prepared in the following way. The cells were routinely propagated on three successive days in MRS Agar (Merck) and M17 Agar (Merck) and incubated at 37°C anaerobically for *L. bulgaricus (*Anaerocult C system; Merck), and aerobically for *S. thermophilus* for 24-48 h respectively. Finally, the strains were inoculated in reconstituted (10%, w/w) skimmed milk and incubated at 43±1°C. Milk for the production of yoghurt was inoculated with this culture at a ratio of 2.5%. The identification of these strains was confirmed API 50 CH (bioMerieux, France) kits as previously described by Turgut et al.[21].

In this study, 230 V- 50 Hz, 23 L. 1400 Watt programmable microwave oven (Vestel MD GD23, Manisa, Turkey) were used. Microwave oven's dimensions are 340 × 220 × 320 mm and there is the round glass drying tray in the bottom. This study was carried out at 2450 MHz and 720 Watt microwave power level.

Experimental Design

The study designed completely randomized design in a factorial arrangement. There were four treatment groups with seven storage periods of 1, 5, 7, 10, 15, 20 and 28 d. The control yoghurt group was prepared without microwave treatment. Yoghurt samples were prepared by heating with microwave oven for 10, 20 and 30 sec at 2450 MHz. Every group consisted of 8 cups. One batch was taken as a control group (control) and not microwave heated. Other batches were transferred in microwave oven respectively. Other batches (H1, H2, and H3) heated at 720 W power level for 10, 20 and 30 sec respectively. Two replicates were accomplished for a total of 56 yoghurt samples. All these procedures were conducted twice and all analysis was carried out in parallel order.

Manufacture of Yoghurts

Yogurt samples were manufactured from cow's milk. The dry matter of homogenized milk was increased to 14% (SNF) by evaporation under vacuum pressure (60°C 450 mm Hg). Then evaporated milk heated for 10 min at 90°C and cooled to 43±1°C for incubation. The yoghurt culture was added to milk and the mixture was divided in to 150 mL plastic cups. The cups incubated at 42±1°C until the pH decreased to 4.7. After incubation, H1, H2 and H3 yoghurts were subjected to the microwave heating but control groups were not. All yogurt samples stored at +4°C for 28 d.

Enumeration of Yoghurt Bacteria

The viable *L. delbrueckii* ssp. *bulgaricus and S. thermophilus* counts were determined 1, 5, 7, 10, 15, 20 and 28 d of storage periods at +4°C. Samples of yoghurt (10 g) were serially diluted (w⁄v) with ¼ Ringer's solution and up to 10^{-7}, then spread-plated (0.1 mL) in duplicate onto plates of MRS agar for the enumeration of *L. delbrueckii* ssp. *bulgaricus* and M17 Agar for *S. thermophilus.* Inoculated plates were incubated anaerobically (Anaerocult C; Merck) at 43°C for 48-72 h for *L. bulgaricus* and aerobically for *S. thermophilus* at 35-37°C for 48 h. The counts of *L. bulgaricus* were enumerated according to the method of Dave and Shah [22]. The counts of *S. thermophilus* were enumerated according to the method of Harrigan [23]. Plates containing 25 to 250 colonies were enumerated and the colony forming units per gram (CFU/g) of the product was calculated.

Yoghurt Analysis

All the analyses were carried out during storage on 1, 5, 7, 10, 15, 20 and 28th d. The pH values of the yoghurt samples were determined by direct measurement with a digital microprocessor pH meter (Hanna Instruments 211, Romania) after calibration with pH 4.0 and 7.0 standard buffer. Titratable acidity (lactic acid, %) was determined by the titrating with 0.1 N NaOH using phenolphthalein indicator previously described by Kurt et al.[24]. All analyses were conducted twice.

Statistical Analysis

A factorial arrangement was set up to study the influence of microwave heating time (4) and storage periods (7) using 2 replicates. A total of 56 samples were investigated for microbiological, acidity and pH analyses on days 1, 5, 7, 10, 15, 20 and 28. All analyses were conducted twice. Data obtained from analysis of the samples were evaluated by variance analysis and the differences among means were detected by Duncan's multiple range tests (IBM SPSS ver 20).

RESULTS

Changes in pH and Titratable Acidity

The results of mean pH and acidity values are presented in *Table 1*. The pH value of the yoghurts was significantly affected by heat treatment after incubation. The initial mean pH values of the control sample and H1, H2 and H3 yoghurts were 4.22, 4.08, 4.20 and 4.41 respectively. At the end of 28 d, the values were 4.04, 3.99, 4.16 and 4.21. The pH values of the yoghurt samples during refrigerated storage period are shown *(Fig. 1)*. The initial pH values of the different yoghurt types were ranged from 4.08 to 4.41 and decreased slowly during storage. The declining trend in the pH was comparatively similar for all the yoghurt samples throughout storage. The yoghurt samples have significant difference ($P<0.05$) in pH measurements. The heated H2 and H3 yoghurt samples had higher pH values than the control and H1 yoghurt samples. Eventually, pH decreased for all samples and the decrease in pH values continued for up to 28 d. There were statistically differences between pH values and microwave heating time ($P<0.05$). The pH values of the H3 yoghurt samples were significantly different from each other ($P<0.05$) *(Table 1)*.

Microwave treatment also significantly affected the acidity of the yoghurts. During the storage period, titratable acidity of the yoghurts samples increased and differences between the values were found significant ($P<0.05$). The effect of microwave treatment after incubation on titratable acidity values of yoghurts during storage are shown *(Fig. 2)*. In the control and H1 yoghurts, a rapid increase continued for up to 28 d of storage, while the acidity of the H2 and H3 yoghurt increased less the during storage period. Acidity values also showed insignificant changes which supports the results obtained for pH.

There were statistically differences between titratable acidity values and microwave heating time ($P<0.05$). The titratable acidity of the H3 yoghurt samples was significantly different from each other ($P<0.05$). The titratable acidity of the H3 and H2 yoghurt samples had less than the control and H1 yoghurt. Though not close to the titratable acidity values of the H1 and H2 yoghurt, the differences were not found statistically significant ($P>0.05$).

Table 1. *Viable counts of L. bulgaricus and S. thermophilus of yoghurt samples*
Tablo 1. *Yogurt örneklerindeki L. bulgaricus ve S. thermophilus sayıları*

Yoghurt samples	pH (X±Sx)		Titratable Acidity (%) (X±Sx)		*L. delbrueckii* ssp. *bulgaricus* (log cfu/g) (X±Sx)		*Streptococcus thermophilus* (log cfu/g) (X±Sx)	
Storage time (days)	**		**		**		**	
1	4.23	0.04^{a}	0.84	0.02^{a}	7.844	0.09^{a}	8.400	0.08^{ab}
5	4.21	0.04^{ab}	0.89	0.02^{ab}	7.937	0.09^{a}	8.367	0.08^{ab}
7	4.14	0.04^{ab}	0.91	0.02^{bc}	7.854	0.09^{a}	8.261	0.08^{a}
10	4.12	0.04^{b}	0.95	0.02^{cd}	7.884	0.09^{a}	8.497	0.08^{abc}
15	4.10	0.04^{b}	0.99	0.02^{de}	8.091	0.09^{ab}	8.618	0.08^{bc}
20	4.10	0.04^{b}	1.03	0.02^{ef}	8.246	0.09^{b}	8.710	0.08^{c}
28	4.10	0.04^{b}	1.07	0.02^{f}	8.285	0.09^{b}	8.707	0.08^{c}
Heating time	*		**		**		**	
Control	4.07	0.03^{a}	1.04	0.01^{a}	8.588	0.07^{a}	8.744	0.06^{a}
H1	4.03	0.03^{a}	1.04	0.01^{a}	8.378	0.07^{b}	8.597	0.06^{ab}
H2	4.19	0.03^{b}	0.92	0.01^{b}	8.099	0.07^{c}	8.474	0.06^{b}
H3	4.29	0.03^{c}	0.82	0.01^{c}	7.015	0.07^{d}	8.219	0.06^{c}

*Means followed with the different superscript alphabet within each column are significantly different; *, ** are significant at 0.05 and 0.01 probability levels respectively*

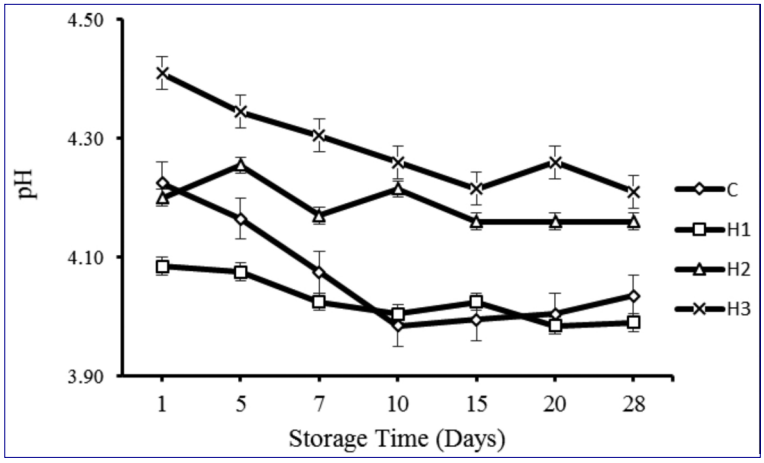
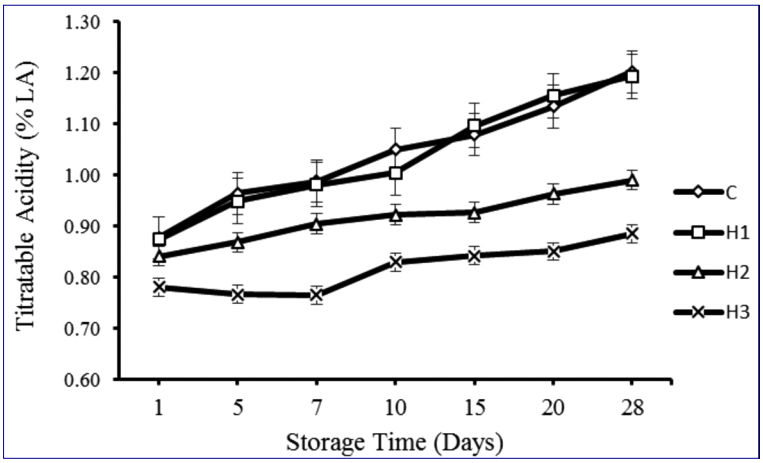


Fig 1. pH values of the yoghurt samples during refrigerated storage period

Şekil 1. Yoğurt örneklerinin depolama sırasındaki pH değerleri

Fig 2. Titratable acidity values of the yoghurt samples during refrigerated storage period

Şekil 2. Yoğurt örneklerinin depolama sırasındaki titre edilebilir asitlik değerleri



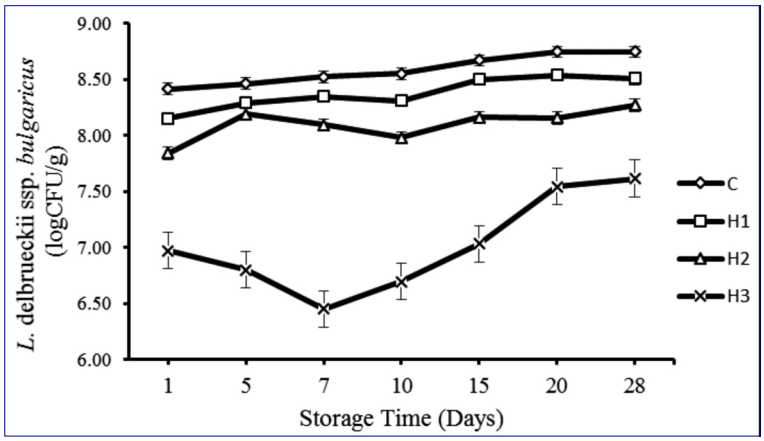


Fig 3. Viable counts of *L.* delbrueckii ssp. *bulgaricus* in the yoghurt samples during refrigerated storage period

Şekil 3. Depolama sırasında yoğurt örneklerindeki canlı *L.* delbrueckii ssp. *bulgaricus* sayıları

Changes in the Viable Counts

Microwave application significantly affected viable counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *S. thermophilus*. Changes in the counts of yoghurt bacteria in during refrigerated storage are presented in *Table 1*. *L. bulgaricus* showed a more marked increase than *S. thermophilus* during the storage period. The counts of *L. bulgaricus* increased in each type of yoghurt samples during the storage time. The effects of microwave heating on viable counts of *L. bulgaricus* in yoghurts are shown *(Fig. 3)*. The effect of storage period on viable counts of *L. bulgaricus* was found statistically significant ($P<0.05$). There were statistically differences between the counts of *L. bulgaricus* and microwave heating time. The values for the counts of *L. bulgaricus* in H3 yoghurt samples were significantly different from each other ($P<0.05$). In H3 yoghurt, *L. bulgaricus* counts were lower by at least 1 log order than those for H1 and control yoghurts. Similarly, the values for the counts of *L. bulgaricus* in H1, H2 and control type yoghurt were not statistically significantly different for one another ($P>0.05$).

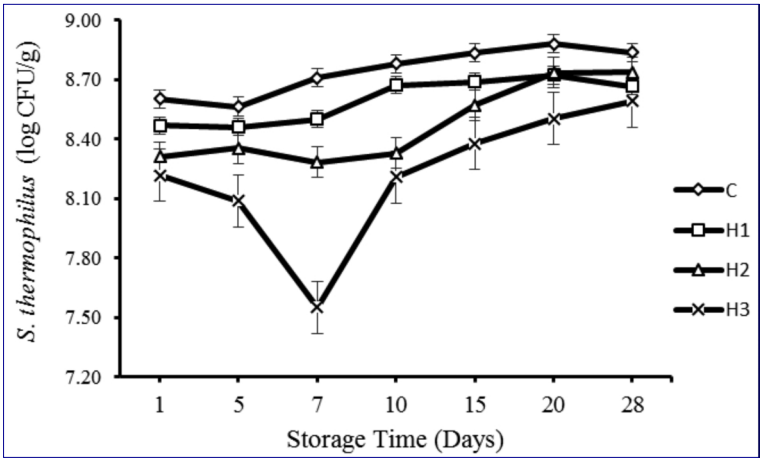


Fig 4. Average viable counts of *S. thermophilus* in the yoghurt samples during refrigerated storage period

Şekil 4. Depolama sırasında yoğurt örneklerindeki canlı *S. thermophilus* sayıları

The effects of microwave heating on viable counts of *S. thermophilus* in yoghurts during storage are shown *(Fig. 4)*. There were small differences between the yoghurt samples and the number of bacteria increased slightly during the storage in terms of the counts of *S. thermophilus*. After 7 days of storage, the counts of *S. thermophilus* showed a decline but then the counts of *S. thermophilus* increased. At the end of storage, the number of bacteria increased almost 0.4 log unit in the samples and these increases were not found statistically significant from initial counts ($P>0.05$). There were statistically differences between the counts of *S. thermophilus* and microwave heating treatment. The viable counts of *S. thermophilus* in the H3 yoghurt samples were significantly different from control group ($P<0.05$). Similarly, the values for the counts of *S. thermophilus* in H1 and H2 yoghurt were not statistically significantly different from each other ($P>0.05$).

DISCUSSION

Microwave heating application significantly affected the pH and acidity of yoghurt samples. The heated H2 and H3 yoghurt samples had higher pH values than the control and H1 yoghurt samples. There were significant differences in titratable acidity values between the groups of yoghurt samples. This difference in pH values are likely caused by exposure microwave heating time. The temperature of the yoghurt samples was rising a bit during the microwave heating. The results of the study showed that microwave heated yoghurt samples had lower acidity than no heated yoghurt Titratable acidity of the all yoghurts samples tended to increase; however, the rate of increase was affected by the microwave heating time. There were statistically significant differences in titratable acidity values between the yoghurt samples. Whereas there was no statistical difference between pH values ($P>0.05$). L. bulgaricus and S. thermophilus are responsible for the post acidification of yoghurt during cold storage. This study has proved that microwave heating can serve as an excellent method for long storage of yoghurt without adding any preservative agents. It is possible to keep the acidity low through microwave heating for 30 sec. The microwave heating also did not change flavour or compositional characteristics of yoghurt.

Microwave application significantly affected viable counts of yoghurt bacteria also. Heat treatment at first resulted in a decline in counts of *S. thermophilus* and *L. bulgaricus*. Thus it will be possible to maintain yoghurt longer time without any additives. Karagül-Yüceer et al.[20] studied on the protective effect of carbon dioxide in fruit-flavored yoghurt and reported that carbon dioxide did not affect the growth of yoghurt bacteria but carbonated yoghurt samples had lower acidity than noncarbonated yoghurt. In our study, H3 yoghurt samples had lowest titratable acidity values and lowest *S. thermophilus* and *L. bulgaricus* numbers. According for these results of microwave heating treatment it can be said to be more beneficial than CO_2 application. An increase in the viable bacterial numbers is most likely to be due to the long storage of the yoghurts, resulting in the growth of yoghurt bacteria. Contrary to our results, Akalın et al.[25] concluded that viable counts of *L. bulgaricus* decreased in yoghurt containing prebiotic and probiotic culture during the storage. Hamann and Marth [26] reported that the count of *L. bulgaricus* decreased during refrigerated storage of plain yoghurts.

The highest counts of *S. thermophilus* were found in control yoghurt and lowest numbers were found in the H3 yoghurt. Our results in terms of the viable number of *S. thermophilus* are compatible with those of Kim et al.[27] and Akalın et al.[25]. Karagül-Yüceer et al.[20] stated that counts of *L. bulgaricus* and *S. thermophilus* decreased in yoghurt during 90 d of storage period. Dave and Shah [28] found similar viable counts of *S. thermophilus* in yoghurts containing whey powder and cystein. Çakmakcı et al.[29] also found similar results in probiotic yoghurt with banana marmalade. This study has proved that microwave heating can serve as an excellent method for long storage of yoghurt without adding any preservative agents.

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Research Article

Effect of Rapamycin on Maternal Aggression in Rats [1] [2]

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Abstract

Rapamycin which is an inhibitor of mammalian target of rapamycin (mTOR), has effects as antineoplastic, retarding aging, anti-inflammatory and neuroprotective. Aim of this study is to investigate the effects of rapamycin on maternal aggression in rats. In this study 63 Wistar female rats were used. The animals were divided into 3 groups: the solvent (DMSO) group, the 5 mg/kg rapamycin group, and the 10 mg/kg rapamycin group. For behavioral testing the resident-intruder paradigm was used. The groups were compared in terms of the latency to the first aggressive behavior, the number of attacks, the total duration of aggressive behaviors and the intensity of attacks. When the groups were compared in terms of the latency to the first aggressive behavior, it was found that 5 and 10 mg/kg rapamycin groups were significantly prolonged latencies compared to the control group. When were evaluated the number of aggressive behaviors, total duration of aggressive behaviors and average severity of attacks it was found that 5 mg/kg rapamycin group's values were significantly lower than the control groups. These results show that acute administration of rapamycin, especially in 5 mg/kg dose of rapamycin prolongs the latency of maternal aggression, and decreased the number of attacks, the intensity of attacks and the total duration of aggressive behaviors in rats. Therefore rapamycin may have potential for use as a sedative drug, however it is necessary to conduct further studies.

Keywords: *Maternal aggression, Rapamycin, mTOR, Rat*

Sıçanlarda Rapamisinin Maternal Agresyon Üzerine Etkisi

Özet

Memelideki rapamisin hedefinin (mTOR) bir inhibitörü olan rapamisin antineoplastik, yaşlanmayı geciktirici, anti-inflammatuar ve nöroprotektif etkilere sahiptir. Bu çalışmadaki amacımız sıçanlarda rapamisinin maternal agresyon üzerine etkisini araştırmaktır. Çalışmada 63 adet dişi Wistar sıçan kullanıldı. Hayvanlar çözücü (DMSO) grubu, 5 mg/kg rapamisin grubu ve 10 mg/kg rapamisin grubuna ayrıldı. Davranış testi için ev sahibi-yabancı paradigması kullanıldı. Gruplar; ilk agresif davranışın başlama zamanı, toplam atak sayısı, agresif davranışın toplam süresi ve atak şiddeti açısından karşılaştırıldı. Gruplar ilk agresif davranış başlama zamanı bakımından karşılaştırıldığında 5 ve 10 mg/kg rapamisin gruplarının kontrol grubuna göre başlama zamanını anlamlı düzeyde uzattığı bulundu. Toplam agresif davranış sayısı, agresif davranışın toplam süresi ve ortalama atak şiddeti değerlendirildiğinde 5 mg/kg rapamisin grubunun değerleri kontrol grubuna göre anlamlı düzeyde düşük bulundu. Bu sonuçlar akut rapamisin uygulamasının, özellikle 5 mg/kg dozda, sıçanlarda maternal agresyonun başlama zamanını uzattığını, toplam atak sayısı, atak şiddeti ve agresyonda geçen toplam süreyi kısalttığını göstermektedir. Fakat rapamisinin sedatif bir ilaç olarak kullanılabilme potansiyeline sahip olabilmesi için daha ileri çalışmalar gerekmektedir.

Anahtar sözcükler: *Maternal agresyon, Rapamisin, mTOR, Sıçan*

INTRODUCTION

Many animals may show aggression to protect their children and defend their habitats from other animals (intruders), or do harm to other animals, for preying or outflank the mating. There are many types of aggression in animals. One of the important aggression types is maternal aggression in animals. Many of the lactating female mammals display an aggressive behavior, which is called maternal aggression, to protect their pups from intruders in their living area [1,2]. In lactating females aggression is temporarily increased, and this aggressive behavior is a remarkable feature during the first two or three weeks after delivery. After the third weeks of delivery,

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aggressive behavior in lactating females decreases and later disappears even if the lactation continues [1]. Despite alterations in many hormones and neurotransmitters (i.e. oxytocin, serotonin, dopamine, GABA…etc) levels within the brain during lactating period, underlying mechanisms of maternal aggression still remains unclear [3,4].

In modern medicine rapamycin, which is a macrolide antibiotic is used as an immunosuppressive serine-threonine kinase inhibitor. Rapamycin at low doses has immunosuppressive effects, and at higher doses it shows antifibroblast, antiproliferative and antineovascularization effects. Rapamycin (Sirolimus) binds to intracellular receptor FKBP12 to form immunophilin complex in the cell, and this immunophilin complex binds directly to the FKBP12-Rapamycin Binding (FRB) domain of mTOR for inhibiting mTOR's activity [5].

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase (PI3K), and it is a member of the phosphatidylinositol 3-kinase (PI3K) related kinase family. mTOR consists two multi-protein complexes defined as distinct protein binding partners with Rapamycin (Sirolimus). The first complex is mTORC1, which is known mTOR's rapamycin sensitive complex, and the other one is mTORC2, which is largely insensitive to rapamycin [6]. In studies about the mTOR's effect on the nervous system by using the mTOR inhibitors like rapamycin, it has been shown that mTOR is very important for the development of the central nervous system's cell survival, differentiation, axonal development, synaptogenesis; in adult synaptic plasticity, such as long-term potentiation which plays an important role in the process of learning and memory in hippocampus [7-10]. mTOR which is inhibited by rapamycin, is known to be effective in various cellular and molecular processes like neurotransmitter, receptor and ion channel expression, neuronal death, apoptosis and neuronal excitability in the CNS.

Changes in maternal behavior have been observed in mice, rats and other laboratory animals [2,11]. Differences in maternal aggression have been observed between the species, strains and even subtype [11]. The effects of various substances on maternal aggression in different animal species or different species or strains of laboratory animals has not been studied yet. Experimental evidence indicates that most mother rats, mice or hamsters that have recently given birth do not display maternal aggression [12,13]. In a study by Gammie and Nelson [13], it was found that 43% of the lactating animals did not show aggressive behavior. Similarly in another study 47% of the animals did not show any aggressive behavior [14]. It has been suggested in some of the studies that, maternal aggressive behavior occurs in response by gender of the intruding animal [2,12]. For this reason we used female virgin rats as intruders in our studies. In this study, all experimental animals were randomly divided into different groups regardless of whether they had exhibited maternal aggression to female intruders naturally or not. Animals were compared with each other with regard to selected drug effects. In these groups, it was included mother rats which had not displayed aggressive behavior under all test conditions and in natural settings.

In the present study, our aim was to investigate the acute effects of 5 mg/kg and 10 mg/kg of rapamycin on maternal aggression in rats.

MATERIAL and METHODS

Animals and Housing

All the experiments were performed in accordance with the Declaration of Helsinki and the local Bioethical Standards of Animal Experiments. The experimental protocol was approved by the Animal Ethics Committee at Abant Izzet Baysal University (2013-37). The animals which were used in the study were provided by Abant Izzet Baysal University, Experimental Animals Research Center. Sixty-three Wistar female rats (90 days old, body weight 225±25), which were used for the experiment, were kept in the laboratory conditions; 23°C room temperature, 60±5% humidity and 12:12 light-dark cycle, in optimal values, and with access to food and water ad libitum. Animals were housed in standard polycarbonate cages; the floor of the cages was covered with wood shavings, which were used as bedding and nesting material. Each female rat (70 days old) was housed with a stud male rat in the same cage until the female rats get pregnant. Vaginal smear tests were performed in order to confirm that the animal was pregnant. After the beginning of pregnancy; male rats were removed from the cages.

Drugs and Doses

In this study, the effects of rapamycin on maternal aggression, in the doses of 5 mg/kg and 10 mg/kg were investigated. Rapamycin was purchased from LC Labs (Woburn, MA, USA), and 5 mg/kg and 10 mg/kg rapamycin which were solved in 99% dimetylsulfoxide (DMSO; LobaChemie, India) used as subtances and 1 mL/kg saline was used as control.

The animals were randomly divided into three groups: 5 mg/kg rapamycin (n=16), 10 mg/kg rapamycin (n=16) and DMSO (n=16). As treatment control, 1 mL/kg intraperitoneal (i.p) saline was given to lactating rats. Basal aggression levels were measured in three groups on Day 2 and Day 3. A dose of 5 mg/kg or 10 mg/kg rapamycin, which can be considered as effective dose in pharmacological studies were applied to animals on Day 2 and Day 3 [15].

Rapamycin Treatment

Before administration, rapamycin was first dissolved in DMSO. DMSO defined as vehicle for carrying rapamycin. To determine effects of rapamycin on maternal aggression we used lactating rats both as control and test groups.

We chose the crossover experimental design as optimal method for the study. In the crossover experimental design, each group receives a series of treatments over time. Treatment in a predetermined sequence is given to the each experimental group. The time points at which substance was administered are usually called periods. In a crossover design, each experimental group serves as its own control. Thus, a crossover design should give smaller standard errors for comparisons between treatment groups than a design where treatment groups are assigned to different subjects [16]. Moreover crossover experimental design is used in studies to eliminate the effect of sequence of substance administration.

According to experimental evidence appropriate aggressive behaviors may vary from species to species and even individual to individual [14]. Therefore in our study, it has been used each substance group as its own control instead of using different control group. 5 mg/kg rapamycin, 10 mg/kg rapamycin, DMSO or saline i.p. were given to the lactating rats (each group n=16) for two consecutive days (Day 2 and Day 3), before done the series of behavioral tests with using cross-over experimental design.

In the experiments each group (n=16) was divided into two subgroups (n=8), and then saline, substance and DMSO were administered as following;

For 5 mg/kg rapamycin group (n=16); substance was administered to lactating rats on day 2, and on the same subgroup saline was administered on Day 3 (n=8). For the other subgroup, (n=8) saline was administered on day 2 and substance was administered on Day 3.

For 10 mg/kg rapamycin group (n=16); substance was administered to lactating rats on day 2, and for the same subgroup, saline was administered on Day 3 (n=8). In the other subgroup (n=8) saline was administered on day 2 and substance was administered on Day 3.

For the DMSO group (n=16); DMSO was administered to lactating rats on day 2, and in the same subgroup saline was administered on Day 3 (n=8). In the case of the other subgroup (n=8) saline was administered on day 2 and substance was administered on Day 3.

During the behavioral tests, each lactating rat was recorded with camera for 20 min, and these recordings were watched and evaluated by two trained observer.

The Behavioral Test

There are many paradigms for testing animals' aggressive behavior. One of these paradigms used in the maternal aggression studies is resident-intruder paradigm. The resident-intruder paradigm is a well-validated model of maternal aggression. The date of birth was considered as postpartum Day 0. Animals were tested on postpartum Day 2 and Day 3 after birth, because mother rats display high aggressive behavior towards female intruders mainly in the first lactation week. Aggressive behavior has usually decreased by the second lactation week [17].

On Day 2 and Day 3, the substances were given to the resident rats intraperitoneally 30 min before the experiment. The pups were removed from the home cage 5 min before the aggression test to prevent any harmful situation [14,18,19]. Thirty min after - administration of subtances to the resident rat, an intruder female rat was placed into the home cage and then the aggressive behaviors of the resident rat were observed and recorded with video camera for 20 min. To prevent intruder's odorant becoming permeated into the cages or resident animals becoming familiar with the intruders, the cages were cleaned after the each test and a different female rat was used in each test as an intruder. All intruders were young female virgins and their body sizes were not bigger than lactating mothers.

Aggressive behaviors displayed by lactating rats towards the intruder rats were observed and recorded according to the following parameters [12,14]:

a) The latency to the first aggressive behavior: The time of first aggressive attack that directed towards the intruder. If there was not any aggressive behavior, total test time (1200 s) was used as data of the latency for the first aggressive behavior.

b) The number of attacks: The number of attacks which were exhibited towards the intruder rat.

c) The total duration of aggressive behaviors: The total time of aggressive behaviors which were exhibited towards the intruder rat.

d) The intensity of attacks: The intensity of attack exhibited by the resident rat to the intruder which was scored as follows;

(0); no aggressive manifestations;

(1); scattered mild aggressive posture or attack towards the intruder, no vocalizations

(2); scattered upright aggressive posture, violent attack or boxing with the intruder, low vocalizations, but no biting or continuous fighting

(3); continuous fighting or attempts to bite the intruder rat by resident, loud vocalizations.

The lactating rats which did not exhibit any aggressive behavior, were accepted as non-aggressive rats, and they were removed from the study.

Statistical Analysis

In this study, data was collected on the following variables; the latency to first aggressive behavior (sec), the number of attacks, total duration of aggressive behaviors (sec) and the intensity of attacks. Descriptive values of these

variables were computed as mean±SD. Values obtained from the study have not shown normal distribution according to Kolmogorov-Smirnov test. After logarithmic transformation was applied, values have shown normal distribution. Levene test was used for homogeneity of variable of parameters. A crossover experimental design which is taking into account effects of sequence was used for data analysis. During the experimental procedure, experiments were done on Day 2 and Day 3 after birth, and all lactating rats were divided to the groups randomly. Least Significant Difference method with Bonferroni correction as a Post hoc test was used for detecting significant differences. Related crossover experimental design was given at the *Table 1*. If the P value obtained from calculations was smaller than 0.05, the results would be accepted as statistically significant. NCSS (version 11) packaged software was used in calculations.

RESULTS

All of the 63 female rats were used for the experiment, 48 of the mother rats (76%) displayed maternal aggressive behavior in at least one test day, and 15 of the mother rats (24%) have did not show any maternal aggressive behavior in both test days. These non-aggressive rats were removed from further experiments and were not included in further statistical analysis. Because we thought that they did not contribute to the aims of our study which were related to the effects of substances on maternal aggression.

After logarithmic transformation was applied, values have shown normal distribution. Variances of groups were found homogeneous for latency and number of attack, but variances were found nearly homogeneous for total duration of aggressive behaviors and the intensity of attacks.

Crossover analysis of variance was used for the comparisons of the groups in terms of latency to first aggressive behavior, the number of attacks, total duration of aggressive behaviors and the intensity of attacks. Results of analysis showed that there was no significant effect of sequence of administration of the subtances, so crossover model was found as applicable in comparison of substance with saline. Differences between groups were evaluated based on these results and obtained following results.

Effects of Rapamycin on the Latency to First Aggressive Behavior

Following the administration of 5 and 10 mg/kg rapamycin, the latency to first aggressive behavior was significantly increased compared to control (P values; 0.028 and 0.024 respectively). However there was no statistically significant difference between the dose groups (5 and 10 mg/kg), DMSO and other test days' values in terms of the latency to the first aggressive behavior (P>0.05) *(Table 2, Fig. 1A)*.

Effects of Rapamycin on the Total Attack Number

It was found that rapamycin reduced the total number of aggressive attacks. At the dose of 5 mg/kg of rapamycin, the average total number of attack number was significantly low compared to control (P=0.014). However there was no statistically significant difference between the dose groups (5 and 10 mg/kg), DMSO and other test days' values in terms of the total attack number (P>0.05) *(Table 2, Fig. 1B)*.

Effects of Rapamycin on the Total Duration of Aggressive Behaviors

The effect of rapamycin of the total duration of aggressive behavior was found significantly. The total duration of aggressive behaviors for the group administered 5 mg/kg rapamycin was significantly shorter than the total duration of aggressive behavior in the control group (P=0.033). However, there was no statistically significant difference between the dose groups (5 and 10 mg/kg), DMSO and other test days' values in terms of the total attack number (P>0, 05) *(Table 2, Fig. 1C)*.

Effects of Rapamycin on the Intensity of Attack

As a result of the assessments made in terms of the effects of rapamycin on the intensity of attack, it has been found that the group given 5 mg/kg rapamycin showed lower total number of attacks than the control group (P=0.0125). However there was no statistically significant difference between the dose groups (5 and 10 mg/kg),

Table 1. *Crossover experimental design*
Tablo 1. *Çapraz tasarım deney düzeni*

Groups	Non-aggressive (N)	Aggressive (N)	Administered Substance	
			Day 2	Day 3
5 mg/kg rapamycin	4	8	5 mg/kg rapamycin	1 mL/kg saline
	3	8	1 mL/kg saline	5 mg/kg rapamycin
10 mg/kg rapamycin	2	8	10 mg/kg rapamycin	1 mL/kg saline
	1	8	1 mL/kg saline	10 mg/kg rapamycin
DMSO	2	8	1 mL/kg DMSO	1 mL/kg saline
	3	8	1 mL/kg saline	1 mL/kg DMSO

Table 2. *The effects of saline (control), DMSO, and 5 mg/kg and 10 mg/kg doses of rapamycin on latency of maternal aggression, total attack number, total duration of aggressive behaviors and intensity of attack*

Tablo 2. *Salin (kontrol), DMSO, 5 mg/kg ve 10 mg/kg rapamisin dozlarının maternal agresyonun başlama latensi, toplam atak sayısı, agresyonda geçen toplam süre ve atak şideti üzerine etkileri*

Parameters	Group	N	Mean+	Median	SD	Log-M	Log-SD	P
Latency	DMSO	8	447.75	146.00	497.357	2.310	0.610	0.036
	DMSO control	8	280.44	82.50	407.505	2.065	0.575	
	5 mg/kg rapamycin	8	595.44	359.50	514.963	2.536*	0.526	
	5 mg/kg control	8	303.38	113.00	412.361	2.146	0.563	
	10 mg/kg rapamycin	8	572.94	420.00	463.564	2.575Δ	0.456	
	10 mg/kg control	8	456.25	222.50	430.655	2.461	0.452	
Total Attack Number	DMSO	8	3.63	3.00	3.324	0.596	0.308	0.045
	DMSO control	8	6.31	4.00	6.954	0.669	0.435	
	5 mg/kg rapamycin	8	2.88	2.00	4.334	0.518*	0.349	
	5 mg/kg control	8	6.19	4.00	7.423	0.709	0.330	
	10 mg/kg rapamycin	8	3.38	2.00	3.735	0.597	0.291	
	10 mg/kg control	8	3.56	3.00	3.226	0.520	0.360	
Total Duration of Aggressive Behaviors	DMSO	8	14.75	6.00	22.335	1.040	0.481	0.048
	DMSO control	8	38.00	8.00	70.301	1.174	0.653	
	5 mg/kg rapamycin	8	13.63	3.00	24.503	0.971*	0.601	
	5 mg/kg control	8	19.69	11.00	29.129	1.147	0.391	
	10 mg/kg rapamycin	8	8.13	4.50	8.793	0.962	0.342	
	10 mg/kg control	8	8.13	7.00	7.553	0.8700	0.393	
Intensity of Attack	DMSO	8	5.75	4.00	5.825	0.952	0.390	0.043
	DMSO control	8	11.63	4.00	15.684	0.806	0.572	
	5 mg/kg rapamycin	8	5.44	2.00	10.308	0.674*	0.482	
	5 mg/kg control	8	12.00	6.50	18.889	0.921	0.398	
	10 mg/kg rapamycin	8	4.69	3.00	4.729	0.754	0.283	
	10 mg/kg control	8	5.25	3.50	5.994	0.620	0.447	

+ Original data mean, ***SD;*** *standard deviation,* ***Log-M;*** *logarithmic mean,* ***Log-SD;*** *logarithmic standard deviation, * statistically significant differences compared to 5 mg/kg control group, Δ statistically significant differences compared to 10 mg/kg control group*

DMSO and other test days' in terms of the total attack number (P>0.05) *(Table 2, Fig. 1D)*.

Effects of Pup Number on the Maternal Aggression

When examined relationship between number of pups and maternal aggression, it was determined that the latency to first aggressive behavior decreased significantly when increase in number of pups (P=0.028). In addition, the number of attacks showed increases significantly when number of pups get higher (P=0.049). However, a significant difference was not observed between increasing the number of pups, the total duration of aggressive behavior and severity of attack (P values; 0.090 and 0.094 respectively).

DISCUSSION

In the present study, the effects of i.p administration of 5 mg/kg and 10 mg/kg rapamycin on maternal aggressive behavior which occurred in lactating rats were investigated. Maternal aggression was evaluated by using resident intruder paradigm in this study.

In our study, latency to first aggressive behavior was observed between 3 and 7 min on average. In terms of latency, the longest time was found in the 5 mg/kg and 10 mg/kg rapamycin dose group. In terms of the total duration of aggressive behaviors, the total attack number and intensity of attack, significant decrease was observed only in 5 mg/kg dose group. On the other hand significant changes were observed in 10 mg/kg rapamycin dose group, however it was not significant statistically. DMSO which was used as a solvent did not have any effect on maternal aggression.

In a number of studies, it has been reported that maternal aggressions often seen in lactating rats do not occur in every individual [14]. Gammie and Nelson [13] used the house mouse in their study, and they also reported that the proportion of animals exhibiting aggressive behavior is 57% (n=14). On the other hand in a study in

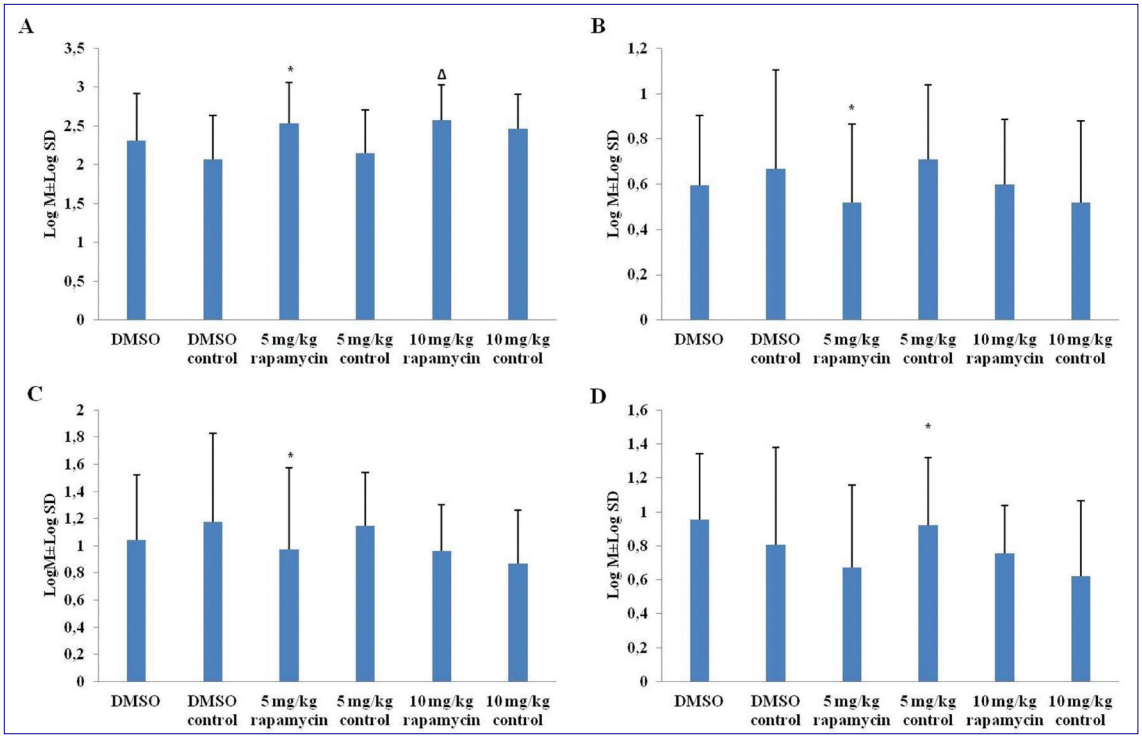

Fig 1. The effects of saline (control), DMSO, and 5 mg/kg and 10 mg/kg doses of rapamycin on latency of maternal aggression (A), total attack number (B), total duration of aggressive behaviors (C) and intensity of attack (D). * statistically significant differences compared to 5 mg/kg control group, Δ statistically significant differences compared to 10 mg/kg control group

Şekil 1. Salin (kontrol), DMSO, 5 mg/kg ve 10 mg/kg rapamisin dozlarının maternal agresyonun başlama latensi (A), toplam atak sayısı (B), agresyonda geçen toplam sure (C) ve atak şideti (D) üzerine etkileri. *5 mg/kg kontrol grubu ile karşılaştırıldığında istatistiksel olarak anlamlı, Δ 10 mg/kg kontrol grubu ile karşılaştırıldığında istatistiksel olarak anlamlı

which the wistar rats were used, it was reported that the proportion of animals exhibiting aggressive behavior was 75% [20]. Ankarali et al.[14] reported that the proportion of animals exhibiting aggressive behavior was 53%. In our study, the proportion of the study subjects displaying maternal aggression was 76% (48 of the 63 animals). In some studies, it has been reported that mothers with birth experience show more aggressive behavior [21]. In our study some of the lactating rats (n=15) did not display maternal aggressive behavior, and the reason that lactating rats did not exhibit aggressive behavior may due to laboratory environment, or mother rats inexperience on birth and puppy care. Lactating rats aggressive behaviors toward intruders depends on the intruders' age and hormonal status [22].

This study is the first study about rapamycin's effect on the time to the onset of first episode of aggressive behavior (latency), the total number of attacks, the intensity of attacks and the total duration of aggressive behaviors. There is only one previous study on effects of rapamycin on aggression seen in pilocarpine animal model for epilepsy. In the study which is done by Huang et al.[15], the sex of the animals was not specified, and resident-intruder paradigm was used. Huang et al.[15] reported that rapamycin reduced aggression in pilocarpine induced epilepsy in an animal epilepsy model. Our study findings that rapamycin administration reduced aggressiveness were compatible with the literature. However, the time to first episode of maternal aggressive behavior, the total number of attacks, the total duration of aggressive behavior and the intensity of attacks could not compared due to lack of studies in literature.

Psychiatric disorders are common in persons with epilepsy [15,23]. Aggression is one of several psychiatric disorders that have long been observed in epileptic patients including those with temporal lobe epilepsy [24], cortical dysplasia [25] and tuberous sclerosis [15,26]. The relationship between aggression and epilepsy has been shown in several animal models, including models of pilocarpine [27] and domoic acid [28]. The underlying molecular mechanisms of association between aggression and epilepsy remain unknown.

Furthermore, in a study related to aggression and epilepsy, not only epileptic seizure has occurred in animals but also aggression level of the animals has been observed increase substantially in pilocarpine induced epilepsy model. Huang et al.[15] has reported that pilocarpine induced status epilepticus is accompanied by an increase aggressive behavior in rats, and this increase in aggression was by 5 mg/kg rapamycin. These data support our results which demonstrated that 5 mg/kg dose of rapamycin reduced the maternal aggression. Rapamycin is a potential mTOR inhibitor, and has been shown to reduce aggressive-

ness. This suggests that there is a relationship between mTOR hyperactivation and mechanism of aggression.

Generally, it is assumed that maternal aggression may be triggered by changes in level of hormones on last period of pregnancy or after delivery [29]. Although different results have been obtained in many studies, changes levels of estrogen, oxytocin and prolactin are thought to be responsible for observed maternal aggression.

There are very few studies regarding the role of GABAergic activity on maternal aggression. Increased GABA release may be important for maternal aggression in rodents. Because GABA agonists administered to virgin female rats treated like lactating mother rats, however GABA antagonists administered lactating mother rats' maternal aggression have decreased [30]. GABAergic activity is necessary for maternal aggression in rats. It has been demonstrated that maternal aggression is generated in specific areas of the brain. When bicuculline, which is a receptor antagonist of $GABA_A$, is infused into hypothalamus or medial amygdala reduces aggressive attacks [31], and infusion bicuculline into the central periaquaductal grey matter inhibits maternal aggression [32]. Although Bjork et al.[33] have reported that there is positive relationship between plasma level of GABA and maternal aggression based on their family history of psychiatric patients, how a connection between plasma GABA levels and nerve tissue is not yet established.

Weston et al.[34] found that mTOR regulates growth of GABAergic neurons and synaptic transmission in their study. However they have also reported that use of rapamycin inhibits these regulations. By suppressing mTOR activity, rapamycin inhibits GABAergic activity, and consequently leads to reduction in maternal aggression. This supports the findings of our study.

Although mTOR's molecular mechanism play role in regulation of maternal aggression, these mechanisms have not been fully studied yet. It is known that mTOR pathway is involved in many processes like the regulation of axonal growth, synaptogenesis, receptor and channel expression. All of these processes may cause to increased excitability in the brain, and this increased excitability may lead to maternal aggression [35].

Results from our previous and present studies indicate that rapamycin has dual antiaggression properties [36]. Again, some antiaggressive drugs such as diazepam and buspirone also possess dual activities [37]. It will be interesting to see if other antiaggressive drugs have a similar effect in this model in the future.

Consequently, it is thought that as a result of suppression or reduction of all specified process (mTOR pathways and its process) by rapamycin regulates maternal aggressive behavior. We expect from the future studies that the role of rapamycin on the maternal aggression will be clarified. Rapamycin remains a prospective agent for treatment of maternal aggression.

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Conflict of Interest

The authors declare that there are no conflicts of interest. All authors approved the final manuscript.

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Research Article

Effect of Quercetin on Cortisol and Oxytocin Levels, Oxytocin Receptor Gene Expression and Morphometry of Uterus in Rats Exposed to Bisphenol A

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Abstract

This study was carried out to evaluate effects of quercetin and bisphenol A (BPA), alone and in combination, on plasma cortisol and oxytocin levels, oxytocin receptor gene expression and morphometry of uterus in rat. After one week of acclimatization, twenty female rats were divided into four treatments as: control, received ethanol saline as injection intraperitoneally and gavaged hydroxypropyl-methylcellulose (HPMC). The second group injected with 50 µg BPA per kg body weight dissolved in ethanol saline two times per week for 4 weeks. The third group gavaged 30 mg quercetin per kg body weight suspended in aqueous solution of HPMC two times per week for 4 weeks, while the fourth group was treated with quercetin along with BPA. Two days after final injection and gavage, rats were anesthetized and uterine and blood samples were collected. BPA alone increased plasma MDA level, decreased total antioxidant capacity (TCA) and had no effect ($P>0.05$) on oxytocin and cortisol levels in the plasma compared with control group. Quercetin along with BPA decreased MDA level, but had no effect ($P>0.05$) on TCA, oxytocin and cortisol levels compared to control. Quercetin and BPA, alone or in combination, resulted in increase ($P<0.05$) oxytocin receptor gene expression. Treatment with quercetin along with BPA increased ($P<0.05$) endometrium, but had no effect ($P>0.05$) on myometrium and epimetrium. It was indicated that quercetin may be a potential compound for reducing oxidative stress damages in uterus layer and increasing blood oxytocin level and its receptor in uterine, but cannot completely ameliorate the negative effects of BPA.

Keywords: *Bisphenol, Oxidative stress, Oxytocin, Quercetin, Rat*

Bisfenol A Verilen Ratlarda Kuersetinin Kortizol ve Oksitosin Seviyeleri İle Oksitosin Reseptör Gen Ekspresyonu ve Uterus Morfometrisi Üzerine Etkileri

Özet

Bu çalışma birlikte ve ayrı ayrı kuersetin ve bisfenol A (BPA) uygulamalarının ratlarda plazma kortizol ve oksitosin seviyelerine, oksitosin reseptör gen ekspresyonuna ve uterus morfometrisine etkilerini araştırmak amacıyla yapılmıştır. Bir haftalık alıştırma dönemi sonrasında 20 dişi rat dört gruba ayrıldı. Kontrol grubuna intraperitoneal etanol-salin enjeksiyonu ile birlikte hidroksipropil-metilselüloz (HPMC) gavaj yoluyla uygulandı. İkinci gruba 4 hafta boyunca haftada iki kere olmak üzere etanol-salinde çözdürülmüş BPA 50 µg/vücut ağırlığı dozunda enjekte edildi. Üçüncü gruba 4 hafta boyunca haftada iki kere olmak üzere HPMC içinde çözdürülmüş edilmiş kuersetin 30 mg/vücut ağırlığı dozunda gavaj yoluyla verilirken dördüncü gruba kuersetin ile birlikte BPA verildi. Son enjeksiyon ve gavaj uygulamasından 2 gün sonra ratlar anestezi uygulanarak uterus dokuları ve kan örnekleri toplandı. Tek başına BPA uygulaması plazma MDA seviyesini artırırken total antioksidan kapasiteyi düşürdü (TCA). BPA uygulaması plazma oksitosin ve kortizol seviyelerinde ise konrol grubu ile karşılaştırıldığında bir değişime neden olmadı ($P>0.05$). Kuersetin ve BPA'nın birlikte uygulanması kontrol grubuyla karşılaştırıldığında MDA seviyesini düşürürken TCA, oksitosin ve kortizol seviyelerinde ise bir değişime neden olmadı ($P>0.05$). Kuersetin ve BPA'nın tek başlarına veya birlikte uygulanması oksitosin reseptör gen ekspresyonunun artmasına neden oldu ($P<0.05$). Kuersetin ve BPA'nın birlikte uygulanması endometrium kalınlığını artırırken ($P<0.05$) myometrium ve epimetriuma etki etmedi. Kuersetinin uterusda oksidatif stres hasarını azaltmada ve kan oksitosin seviyesi ile oksitosin reseptör gen ekspresyonunu artırmada potansiyel bir madde olabileceği ancak BPA tarafından oluşturulan negatif etkileri tümüyle ortadan kaldıramadığı belirlenmiştir.

Anahtar sözcükler: *Bisfenol, Oksidatif stres, Oksitosin, Kuersetin, Rat*

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INTRODUCTION

Bisphenol A (BPA), a monomer of plastics, causes cytotoxicity and adverse effects on brain, reproductive system, and liver, because of oxidative stress [1-3]. Patisaul et al.[4] reported that adolescent rats exposed to BPA exhibited significantly higher levels of anxiety, because of oxytocin reduction by BPA exposure. They also found that adolescent rats on the soy-rich diet did not exhibit anxiety. Finally, Patisaul et al.[4] suggested that the phytoestrogens may mitigate the effects of BPA.

Quercetin, a potent phytoestrogen, acts as an antioxidant and has been identified to occur naturally in many vegetables and fruits at relatively high concentrations [5]. Both BPA and quercetin have a week estrogen-like feature and disrupt or mimic the normal action of estradiol [6,7]. According to the previously reported studies [8,9], estradiol could increase the frequency and duration of uterine contractility. It was reported that BPA could stimulate uterine contraction via oxytocin-related pathway in immature rats [6]. Wolstenholme et al.[10] showed that BPA exposure results in change of oxytocin receptor gene expression in brain.

In the literature, information concerning effect of BPA and quercetin, alone or together, on blood oxytocin level and gene expression of oxytocin receptor in uterine is scarce. It was hypothesized that estrogenic activity of these compounds involved the oxytocin secretion and its receptor gene expression in the uterine. Therefore, the present study was carried out to evaluate the effects of BPA and quercetin, alone or together, on the concentration of cortisol and oxytocin levels, oxytocin receptor gene expression and morphometry of uterus in rat.

MATERIAL and METHODS

The study was approved by the Ethics and Research Committee of Islamic Azad University, Science and Research Branch (Approval date: 15.04.2014; no: 1424).

Chemicals and Reagents

Quercetin (CAS Registry No. 117-39-5) used in this assays was provided by Merck KGaA (Darmstadt, Germany). Quercetin was suspended in 10 mL of a 0.25% aqueous solution of hydroxypropyl methylcellulose (HPMC) to a final concentration of 30 mg quercetin/mL. Bisphenol A was purchased from Sigma Chemical Company (CAS Registry No. 80-05-7). BPA was dissolved in 5% ethanol solution. The solvents of quercetin and bisphenol were used as the negative control. All other materials were of analytical grade, obtained from standard sources.

Animals and Experimental Design

Twenty female Wistar rats (age, 28 days; average body weight, 80 g) were supplied from the Razi Institute (Karaj, Iran). The animals were kept in plastic cages, fed a standard diet and access to free and fresh water. Rats were maintained at 20 ± 2°C, and exposed to a 12 h light/dark cycle. The animals were quarantined for 7 days before beginning the experiments. Rats were handled in accordance with the standard guide for the care and use of laboratory animals. After one week of acclimatization to the laboratory conditions, rats were randomly divided into four treatments with five rats in each. Treatments were as: rats of the first group served as a control group and was injected ethanol saline intraperitoneally and gavaged HPMC solution, both two times per week (Sunday morning and Wednesday evening) for 4 weeks. The second group (BPA) received bisphenol A at a dose of 50 µg/kg body weight two times per week (Sunday morning and Wednesday evening) for 4 weeks intraperitoneally and received HPMC solution via gavage. Rats in the third group (Q) received quercetin at a dose of 30 mg/kg body weight two times per week for 4 weeks (Sunday morning and Wednesday evening) via gavage and injected ethanol saline intraperitoneally. The fourth group (Q + BPA) received in combination, bisphenol and quercetin. Treatment of quercetin was started five days (two gavages at days 1 and 4 of experiment) before bisphenol injection and continued throughout the experiment (eight gavages during 28 days). Other rats received aqueous solution of HPMC via gavage two times during mentioned period. Bisphenol A was injected eight times and quercetin was gavaged 10 times during experiment period. The doses of bisphenol and quercetin were calculated according to the animal's body weight before each administration.

Blood Sampling and Measurements

Two days after final injection and gavage, rats were anesthetized with diethyl ether and blood sample was collected into heparinized tubes from heart. The blood was then centrifuged and the plasma was collected and kept at -20°C for the determination of malondialdehyde (MDA), total antioxidant, oxytocin, and cortisol levels. Oxytocin level was measured using ELISA kits that provided from Enzo Life Sciences (Farmingdale, NY, USA). Cortisol level was measured using ELISA kit that provided from Mono Bind Company (Lake Forest, CA, USA). Plasma MDA level was determined using commercial kit (Pars Azmoon, Tehran, Iran) based on the method described by Dropper et al.[11]. Total antioxidant capacity of plasma was measured according to the method of Benzie and Strain [12].

Tissue Processing

Left uterine corn was fixed in buffered 10% formalin solution. After 72 h fixation, uterine corn was cut to 5 mm sections. Sections were dehydrated by passing them through increasing concentrations of ethanol (70%, 80%, 95% and 100%) for 1 min each, and then placed in xylene for 1 min to clear. Then sections were stabilized in paraffin and sliced by a microtome (Leica M20, Leica Microsystems,

Germany) with 6 µm thickness. At least 6 slices per section were fixed on a glass slides, processed by hematoxylin-eosin and then mounted by entellan rapid mounting medium. Slides were evaluated under computer-connected microscope using image analyzer software.

Quantification of Oxytocin Receptor Gene Expression

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

Statistical Analysis

Data were subjected to analysis of variance procedures appropriate for a completely randomized design using the General Linear Model procedures of SAS software. Mean comparison was done using Tukey test at $P<0.05$.

RESULTS

The effect of different treatments on plasma MDA levels is shown in *Fig. 1*. There were significant differences (P=0.0003) among treatments for plasma MDA level. There was no significant difference for MDA level between rats received quercetin and the control group. Injection of BPA increased significantly MDA as compared with the control group. Rats received quercetin along with BPA had lower MDA as compared to those received BPA alone.

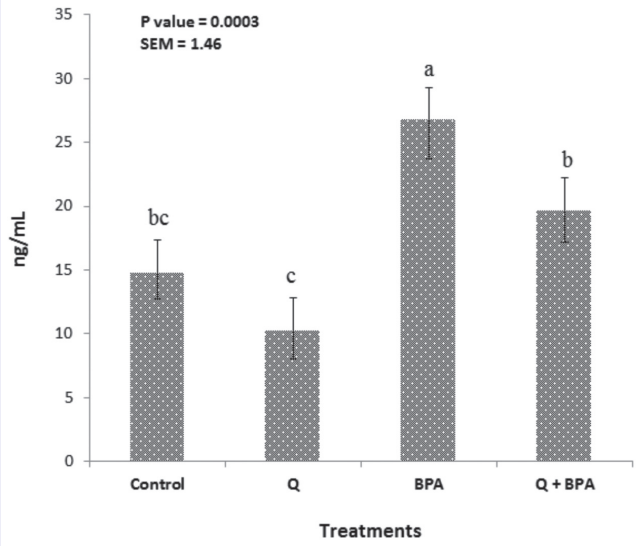

Fig 1. The effect of treatments (Q, quercetin; BPA, bisphenol) on plasma malondialdehyde level of rats

Şekil 1. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) plazma malondialdehit seviyelerine etkileri

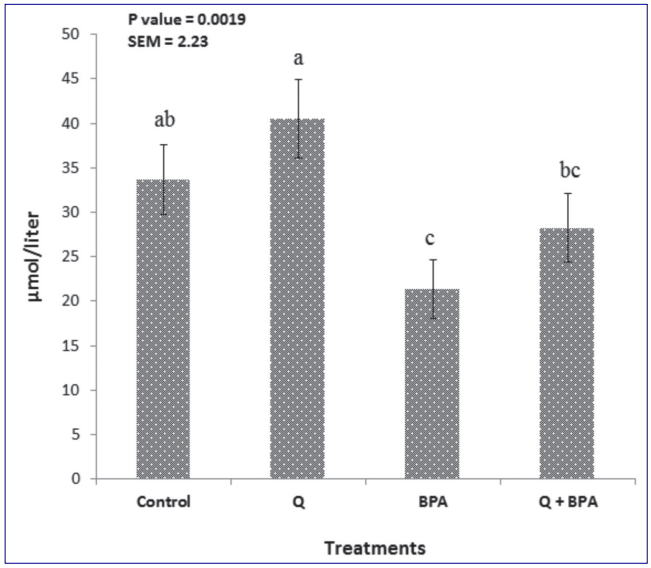

Fig 2. The effect of treatments (Q, quercetin; BPA, bisphenol) on plasma total antioxidant activity level of rats

Şekil 2. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) plazma total antioksidan aktivite seviyelerine etkileri

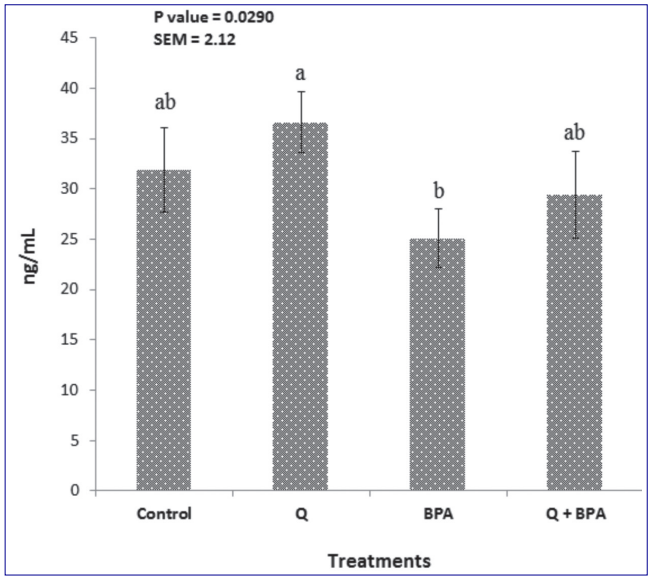

Fig 3. The effect of treatments (Q, quercetin; BPA, bisphenol) on plasma oxytocin level of rats

Şekil 3. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) plazma oksitosin seviyelerine etkileri

Total antioxidant capacity in plasma was differ (P=0.0019) among treatments *(Fig. 2)*. A significant difference found between rats received quercetin and BPA alone. Quercetin alone had no effect (P>0.05) on TAC, but injection of BPA decreased it as compared to the control group. Treatment of rats exposed to BPA with quercetin could not increase (P>0.05) the plasma antioxidant capacity as compared with those received BPA alone.

Plasma oxytocin level of rats is presented in *Fig. 3*. Injection of BPA alone reduced the oxytocin level

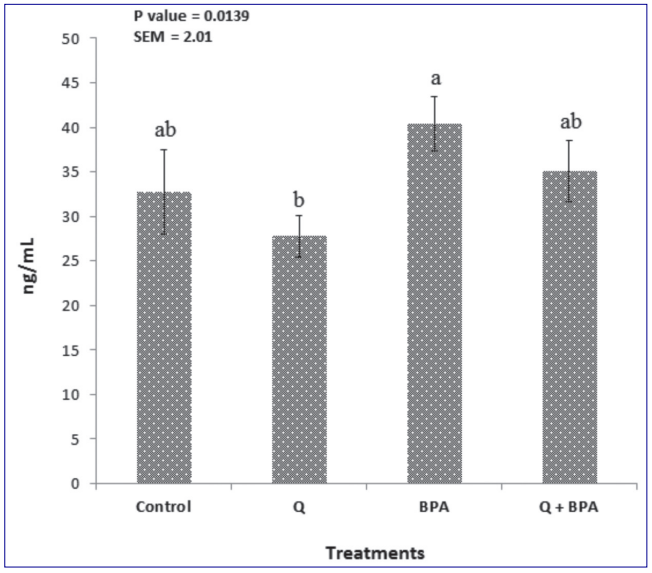

Fig 4. The effect of treatments (Q, quercetin; BPA, bisphenol) on plasma cortisol level of rats

Şekil 4. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) plazma kortizol seviyelerine etkileri

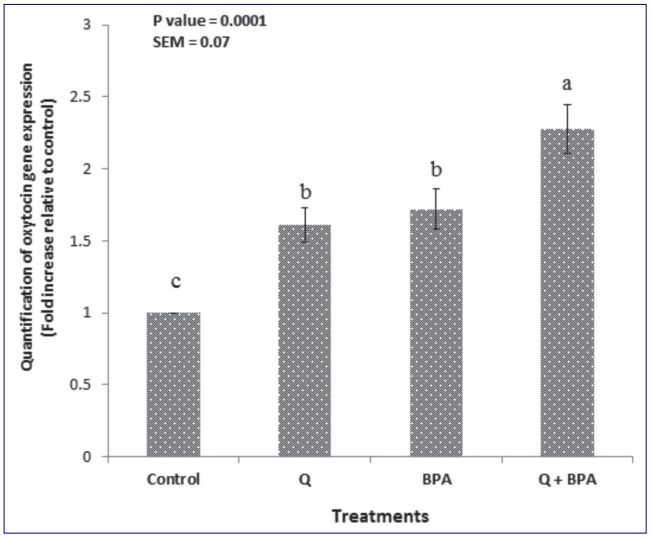

Fig 5. The effect of treatments (Q, quercetin; BPA, bisphenol) on gene expression of oxytocin receptor in uterine

Şekil 5. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) oksitosin reseptörü gen ekspresyonu *üzerine* etkileri

as compared to quercetin alone (P=0.029). Treatment with quercetin along with BPA had no significant effect (P>0.05) on oxytocin level as compared with the control group and also those received these compounds alone.

There was a significant difference (P=0.0139) for plasma cortisol level among treatments *(Fig. 4)*. BPA increased the plasma cortisol level compared to quercetin. Administration of quercetin to rats exposed to BPA could not decreased (P>0.05) cortisol level as compared to those received BPA alone.

Table 1. *The effect of different treatments (Q, quercetin; BPA, bisphenol) on diameter of various layers of uterine*

Table 1. *Uygulamaların (Q, kuersetin; BPA, bisfenol) uterus katmanlarının kalınlıklarına olan etkileri*

Treatments	Endometrium µm	Myometrium µm	Perimetrium µm
Control	255±15.7^{b}	275±10.7^{ab}	18.3±2.07^{b}
Q	355±17.5^{a}	307±13.1^{a}	23.6±2.08^{a}
BPA	193±16.2^{c}	260±12.0^{b}	11.5±1.52^{c}
Q + BPA	238±15.1^{b}	262±16.1^{b}	13.6±1.32^{bc}
SEM*	9.29	7.61	1.032
P value	0.0001	0.0089	0.0001

*^{a,b,c} Means without a common superscript letter differ within each part of a column (P<0.05); * SEM: standard error of means*

The gene expression of oxytocin receptor in uterine is shown in *Fig. 5*. The gene expression was differ (P=0.0001) among treatments. Administration of quercetin and BPA, alone or in combination, resulted in increase the expression of oxytocin receptor gene. Quercetin and BPA in combination had higher impact on gene expression.

The effect of different treatments on various layers of uterine is shown in *Table 1*. Treatment with quercetin resulted in significant increase and exposure of rats to BPA resulted in significant decrease in various layers of uterine. Treatment with quercetin along with BPA significantly increased endometrium, but had no effect on myometrium and epimetrium.

DISCUSSION

The main objective of this study was to evaluate the effect of bisphenol and quercetin administration, alone and together, on plasma cortisol and oxytocin levels, oxytocin receptor gene expression and morphometry of uterus in rat. In the literature, there was limited information about the effects of administration of bisphenol and quercetin, alone or in combination, on these parameters in animals and we conducted this research with hypothesis that quercetin could ameliorate the adverse effects of bisphenol.

The level of plasma MDA in rats exposed to BPA increased and in those received quercetin decreased. In agreement to our results, Sangai et al.[13] reported a significant increase in the serum MDA level in rats exposed to BPA compared to the control group. Exposure of animals to BPA resulted in increase of reactive oxygen species and decrease in antioxidant content and activity [14-16]. A study [17] revealed that BPA increase the free radical formation and decrease body ability to detoxify reactive oxygen species. It was reported [18] that BPA can induce cytotoxicity by impairing mitochondrial function and a consequent decrease in the cellular levels of ATP. Thus, changes in energy metabolism and glutathione redox balance could

be considered as potential mechanisms for inducing adverse effects by bisphenol A. This condition leads to lipooxidation and cell membrane damages and finally increases in plasma MDA.

In this study, BPA treatment caused a statistically significant reduction in total antioxidant capacity compared with the control group. In a study [13], BPA treatment reduced the activities of catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase, also glutathione and total ascorbic acid contents compared with control. Reduction in anti-oxidants activity and content resulted in decrease of plasma total antioxidant activity.

Co-treatment with quercetin and BPA could not alleviate the changes in plasma MDA level, as well total antioxidant activity. It decreased plasma MDA, but its effect was not significant. In contrast to our results, Sangai et al.[13] reported that quercetin could ameliorate the oxidative stress created by BPA through lowering MDA levels and increasing enzymatic and non-enzymatic anti-oxidants in mice. This discrepancy may be related to higher dose of quercetin, as they treated mice with 60 mg/kg body weight. Another study [1] showed that concurrent addition of bisphenol A and quercetin caused concentration-dependent amelioration in bisphenol A-induced cytotoxicity. Also, it was shown [13] that treatment with quercetin along with bisphenol A could significantly ameliorates bisphenol A-caused changes in the activities of ATPase in liver and kidney of mice. The restoration of ATPase activity suggests the ability of quercetin to protect the sulfydryl group from oxidative damage through inhibition of lipid peroxidation [18]. In our study, the diameter of uterus layers for rats exposed to BPA decreased, because of oxidative damage. Quercetin had positive effect on them by antioxidant activity and co-treatment results in ameliorating the negative effects of BPA.

Plasma cortisol level decreased in rats treated with quercetin as compared with the control group. A study demonstrated [19] that during times of prolonged stress, quercetin has been shown to suppress the release of cortisol. Over time the quercetin dose had the strongest suppression on cortisol levels. They then examined the rats' brains and observed that quercetin can inhibit the release of corticotropin-releasing hormone [20]. One of the reasons for decrease in cortisol level with quercetin treatment is elevation of oxytocin. Oxytocin is a hormone that helps relax and reduce blood pressure and cortisol levels.

The plasma level of cortisol increased in group received BPA. Increases in cortisol level in rats received BPA may be related to estrogenic activity. The study of Edwards and Mills [21] showed that estrogen administration lead to elevated plasma cortisol level. An interesting study [22] demonstrated that bisphenol A, similar to estrogen [23], could increase cortisol production by enhancing phosphorylation of CREB (cAMP response element-binding protein) in normal human adrenocortical cells. Another study [24] showed that BPA could induce corticotropin-releasing hormone expression in the placental cells. A study [25] demonstrated that BPA exposure to the dam throughout gestation and lactation led to elevated corticosterone concentrations in female litters. Further, BPA-treated female rats exhibited higher basal corticosterone than control [26]. Also, Kawabata et al.[27] demonstrated that quercetin have suppressive effect on acute stress-induced hypothalamic-pituitary-adrenal axis response in rats. Quercetin co-treatment with BPA could not ameliorate its effect on cortisol level. In the literature, there was no study concerning effect of BPA on blood oxytocin levels and uterus oxytocin receptor gene expression, but many studies were found about its effects on brain oxytocin level, brain oxytocin receptor gene expression and behavior.

Brains from 18.5 days post-coitus male mice exposed to BPA express less oxytocin, oxytocin receptor gene expression, and vasopressin than control males [28]. In their study, expression of oxytocin receptor gene in brain of female mice was higher than control that is consistent with our finding in uterine. The estrogenic effect of BPA may be related to increase in gene expression of oxytocin in uterine.

Quercetin treatment in this study increased plasma oxytocin level and its gene expression in uterine. Information about quercetin effects on oxytocin level and gene expression is limited. A study [29] showed that quercetin could inhibit the PGF2α-induced uterine contraction. They reported that quercetin could reduce oxytocin level that is inconsistence with our finding. Co-treatment of quercetin and BPA resulted in increase of oxytocin gene expression in uterine 2.3 folds higher that control. The additive estrogenic effects of these compounds may be resulted to increase the gene expression higher than their individual effect.

It was indicated that quercetin may be a potential compound for reducing oxidative stress damages in uterus layer and increasing blood oxytocin level and its receptor in uterine, but cannot completely ameliorate the negative effects of BPA.

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Research Article

A New Flap Model in the Treatment of Non-Healing Elbow Wounds in Dogs: The Island Arterial Composite Flap Comprising the Skin Originating from the Thoracodorsal Artery, the Cutaneous Trunci Muscle and Fascia of the Latissimus Dorsi Muscle

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Abstract

In the treatment of wounds with tissue loss in the elbow region, despite the most frequently used method being the tube flap of thoracodorsal artery origin, this method has the disadvantages of necrosis, opening of sutures and tension. The aim of this study is to present the results of the island arterial composite flap, containing the skin of the thoracodorsal area, the cutaneous trunci muscle and fascia of the latissimus dorsi muscle, for the reconstruction of chronic wounds in the elbow region. The material of the study consisted of 14 dogs with either non-healing wounds or wounds with tissue loss in the elbow region. Firstly, debridement of the elbow defect was carried out. Next, an elliptical incision depending on the size of the defect was made in the thoracodorsal skin. Once the thoracodorsal artery and its pedicle were exposed, this was passed through the tunnel formed in the axillary region and placed over the debrided defect in the elbow region. It was sutured using non-absorbable monofilament suture material and simple interrupted sutures and the results were evaluated. In conclusion, the skin originating from the thoracodorsal artery, muscle and fascia island artery flap may be an effective method in the reconstruction of wounds in the elbow region.

Keywords: *Island flap, Cutaneous trunci muscle, Non-healing elbow wounds, Dog, Composite flap, Latissimus dorsi muscle fascia*

Köpeklerde İyileşmeyen Dirsek Yaralarının Sağaltımında Yeni Bir Flep Modeli: Torakodorsal Arter Kökenli Deri, Cutaneöz Trunci Kası ve Latissimus Dorsi Kası Fasyasını İçeren Ada Arteriyel Kompozit Flebi

Özet

Dirsek bölgesindeki doku kayıplı yaraların sağaltımında en çok kullanılan yöntem torakodorsal arter kökenli tüp flebi olmasına karşın, nekroz, dikişlerde açılma, gerginlik gibi dezavantajları bulunmaktadır. Bu nedenle çalışmamızda, torakodorsal bölgedeki deri, kutaneöz trunci kası ve latissumus dorsi kasının üzerindeki fasya dokusunun, kompozit ada arteriyel flebi olarak, dirsek bölgesindeki kronik yaraların rekonstrüksyonu için kullanılmasının sonuçlarının ortaya konması amaçlandı Dirsek bölgesinde doku kayıplı veya iyileşmeyen yarası bulunan 14 köpek çalışmanın materyalini oluşturdu. Öncelikle dirsek bölgesindeki kronik yara yenileştirildi. Daha sonra torakodorsal bölgedeki deri üzerinde, mevcut yaranın boyutuna göre değişen elips şeklinde bir ensizyon yapıldı. Torakodorsal arter pedikülü ortaya çıkarıldıktan sonra koltuk altında oluşturulan tünelden geçirilerek dirsek bölgesindeki yenileştirilmiş yara üzerine taşındı ve monoflament emilmeyen dikiş materyali ile basit ayrı teknikle dikilerek sonuçları değerlendirildi. Sonuç olarak torakodorsal arter kökenli deri, kas ve fasya ada arteriyel kompozit flebinin, dirsek bölgesindeki iyileşmeyen yaraların rekonstrüksiyonunda etkili bir yöntem olabileceği düşünüldü.

Anahtar sözcükler: *Ada flep, Kutaneöz trunci kası, İyileşmeyen dirsek yarası, Köpek, Kompozit flep, Latissimus dorsi kası fasyası*

INTRODUCTION

Wounds of the elbow region in dogs may be caused secondary to external trauma, as a result of ischemia or pressure sores due to long time compression of the skin (decubital ulcers), following revision of damaged skin wound or as a complication of an abscess, infection or bursa extirpations. The treatment is long and arduous. Options

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for the treatment of elbow wounds in dogs include: second intention, simple appositional closure of the wound edges by suturing, applying axial model flaps, island artery flaps, myocutaneous flaps and skin grafts [1].

Axial model flaps are skin flaps with their own direct cutaneous artery and vein, the same length as the flap [2]. Clinically the easiest axial model flaps to produce are thoracodorsal and caudal epigastric axial model flaps [3]. Therefore, in the treatment of elbow region wounds in veterinary practice, apart from second intention and opposition and suturing of wound edges, the most selected method is the thoracodorsal axial model flap technique. However, similar to other axial model flaps, complications such as opening of the sutures, edema, infection, seroma formation and distal flap necrosis may be observed [4].

In dogs, the cutaneous trunci muscle originates from the profund pectoral muscle and covers the dorsal, lateral and ventral abdominal wall in a thin layer [5]. Also this muscle, enables the abdomen to twitch when exposed to external irritants [6]. Cutaneous trunci muscle circulation is provided by the direct cutaneous arteries feeding the skin above and also small muscular branches. The combined separation of the skin and cutaneous trunci muscle from the underlying tissues protects the vascular relationship of these two structures [7].

A flap containing two or more tissue elements including skin, muscle, bone or cartilage is termed a composite flap. For the closure of foreleg defects in veterinary practice, the cutaneous trunci muscle and its overlying skin has been described and used as a myocutaneous style composite flap [8]. However, composite flaps do not have widespread usage in veterinary practice [1].

Island artery flaps are created using direct cutaneous arteries described for axial model flaps. However, unlike axial model flaps, these are transferred onto the recipient site after total detachment of the skin connection [9,10]. The island arterial flaps can be rotated up to 180 degrees, so this provides a significant advantage in cases where the defect location is close to the base of a flap. The ventral deep circumflex iliac and cranial superficial epigastric axial pattern flap regions have been reported to be the most suitable areas for island artery flap rotation [10].

The fascial tissue has been used in the reconstruction of various tissues due to its properties such as; its easy vascularization [11], the fact that it is easily obtainable, does not cause any loss of function afterwards and has a high tolerance to ischemia, forms a slippery surface and its resistance to stretching [12,13]. For this purpose, the most commonly used fascial tissues in veterinary practice are the thoracolumbar fascia and fascia lata [14,15].

In the authors' opinion, the fact that this composite flap prepared from the thoracodorsal region was created in the island artery style, passed through a tunnel and incorporated the fascial tissue on the bottom layer, may decrease complications previously reported to be encountered in axial model flaps.

MATERIAL and METHODS

In this study, 15 defects in the elbow region of 14 dogs of different breed, age and gender, were treated using the island arterial composite flap consisting of thoracodorsal artery-originating skin, the cutaneous trunci muscle and the fascia overlying the latissimus dorsi muscle. Patients were induced with 4-6 mg/kg IV propofol (Pofol, Sandoz®, 10 mg/ml ampule) and intubated. Anesthesia was maintained with 2.5% isoflurane (Isoflurane®, Adeka) and 100% oxygen. The patient was positioned in lateral recumbency with the extremity with the defect uppermost. The affected extremity was shaved with razor blade and disinfected up to the lateral thoracal wall and distal antebrachium. Firstly, the lesion in the elbow region was freshen up by cut out of the small pieces of the skin from the wound edges circumferentially and an elliptical recipient site surrounded with healthy skin tissue was established *(Fig. 1a)*. Dimensions of the defect were then measured in two planes by metric fashion. The cranial side of the flap was formed by an incision along the length of the spina scapula. The caudal border was made parallel to the first incision and twice the distance from the first incision to the caudal shoulder depression (caudal border of scapula). The caudal and cranial incisions were connected and the flap borders were established *(Fig. 1b)*. Next, the skin was dissected together with the underlying cutaneous trunci muscle and latissimus dorsi muscle fascia, starting from the caudo-dorsal location of the area near the transversal processes of the thoracal vertebrae and was continued in a cranio-ventral direction towards the caudal shoulder depression. During dissection, any bleeding in both the latissimus dorsi fascia forming the bottom layer of the flap and the donor site was cauterized. Also, care was taken not to damage the thoracodorsal artery pedicle located at the dorsal level of the acromion in the caudal shoulder depression [10]. When the caudal shoulder depression was reached, for ease of flap transfer, the soft tissues surrounding the thoracodorsal artery and vein were carefully separated. Thus, a vascular pedicle of approximately 9-10 cm was obtained *(Fig. 1c)*. The composite flap with its free vascular pedicle was passed through a tunnel, under the skin in the axillary region, made in the direction of the defect *(Fig. 1f)*. It was observed that the anatomical course of the thoracodorsal artery pedicle was almost parallel to the sternum and that the prepared flap was bent approximately 90 degrees during transfer to the elbow region. The flap positioned over the defect was sutured with simple interrupted sutures using either Number 0 or Number 1 non-absorbable monofilament suture material (Prolene®, Ethicon) depending on the thickness of the skin *(Fig. 1g)*. The same procedure was repeated for the donor site on the thorax. In all cases, two drains were placed in

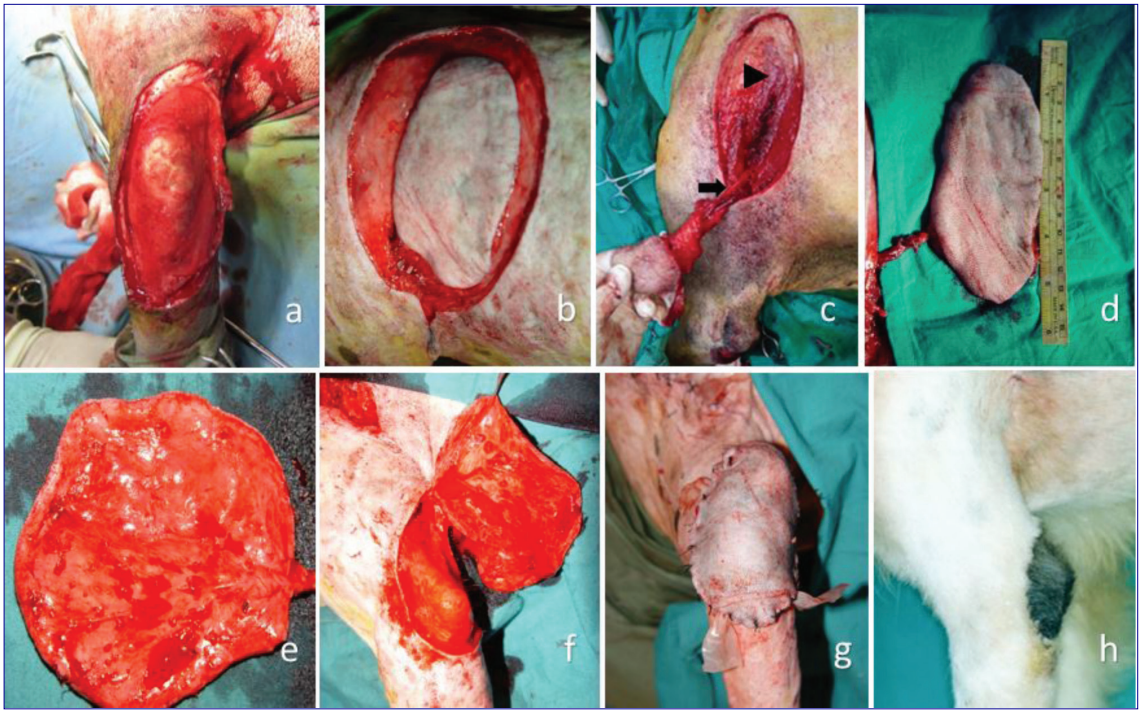

Fig 1. Preparation and application stages of the island arterial composite flap comprising thoracodorsal artery-originating skin, the cutaneous trunci muscle and fascia of the latissimus dorsi muscle. Debridement of the defect in the elbow region and formation of a circular recipient site surrounded by healthy skin tissue on to which the flap can be sutured, **a;** elliptical donor site created by connecting cranial and caudal incisions, **b;** freeing the island arterial flap without damaging the blood vessel pedicle, *(black arrow)* thoracodorsal artery pedicle *(black arrow head),* latissimus dorsi muscle without its fascia, **c;** elliptical flap and thoracodorsal artery pedicle, **d;** appearance of the internal side of the flap, **e;** advancing the flap through the tunnel made towards the defect, **f;** suturing flap on to defect and placement of drains, **g;** appearance of area on post-operative day 20, **h**

Şekil 1. Torakodorsal arter kökenli deri, cutaneous trunci kası ve latissimus dorsi kası fasyasını içeren ada arteriyel kompozit flebinin oluşturma ve uygulama aşamaları. Dirsek bölgesindeki defektin yenileştirilmesi ve flebin üzerine dikilebileceği, sağlıklı deri dokusuyla çevrili daire şeklinde bir alıcı bölge oluşturulması, **a;** kranial ve kaudal ensizyonların birleştirilmesiyle oluşturulan elips şeklindeki donör bölge, **b;** ada arteriyel kompozit flebinin damar pedikülüne zarar vermeden serbestleştirilmesi, *siyah ok* thorakodorsal arter pedikülü, *siyah okbaşı,* üzerinden fasyası sıyrılmış latissimus dorsi kası, **c;** elips şeklindeki flep ve torakodorsal arter pedikülü, **d;** flebin iç yüzünün görünümü, **e;** flebin, deri altından, defekte doğru oluşturulmuş tünelden ilerletilmesi, **f;** flebin defekt üzerine dikilmesi ve drenlerin yerleştirilmesi, **g;** bölgenin postoperatif 20. gün görüntüsü, **h**

the recipient site and 20 mg/kg IM ceftriaxone disodium trisesquihydrate (Unacefin®, Fako) was administered for 5 days. Elizabethan collar was placed to all dogs after awaking in reanimation. The patients were hospitalized for 2 days after the operation and discharged following drain removal on day 2. Patient owners were advised to keep their dogs on soft flooring and that there was no elevation on which the dogs could rise up on their hind legs and rest their elbows. Post-operative bandage application was not performed to avoid iatrogenic obstruction of flap blood supply and partial or full flap necrosis. The patients were called back for a check-up on days 10 and 20 and healing was evaluated. Assessment of flap viability was performed by visual observation.

RESULTS

Of the 14 dogs included in the study, 10 (71.5%) were male and 4 (28.5%) were female with ages ranging 1-11 (mean 4.6, SE 1.03) years and mean body weight was 31.9 (SE 2.47) kilogramme. Breed distribution of the dogs was: 6 mixed breed, 5 Turkish Kangal, 1 Rottweiler, 1 Saint Bernard and 1 German Shepherd. Reasons for chronic wounds forming in the elbow region were: bursitis olecrani (elbow hygroma) extirpation in 7 (50%) dogs and suture failure following tumor extirpation in 5 (35.7%) dogs, pressure sores in 1 (7.15%) and unknown cause in 1 (7.15%) dog *(Table 1)*. Post-operative histopathological investigation had not been performed in 4 of 5 cases following previous tumor extirpation and in one case (case no.11) the histopathology report result had showed extensive granulation tissue. In one case (case no.13), it was reported that the dog had been reluctant to stand for approximately 1 month due to neurological reasons and preferred to sit with its elbows on the floor. Physical examination revealed an ischaemia-based pressure wound with bilateral tissue loss observed in the olecranon on the caudal aspect of the elbow joint. Following improvement of neurological signs with medical treatment and the patient starting to walk again, both defects were closed with the skin, muscle, fascia composite flap described in this study. In case no.12, the reason for the elbow wound was unknown.

The patients presenting with elbow hygroma compli-

Table 1. *Patients information*
Tablo 1. *Olgulara ait veriler*

No	Breed	Cause of the Elbow Region Wounds	Side	Sex	Age (Year)	Body Weight (kg)	Number of Prior Surgeries	Flap Dimensions (cm)
1	Rotweiller	Elbow hygroma	Left	M	1,5	34	1	13×8
2	Turkish Kangal	Elbow hygroma	Right	M	2	41	2	12×7
3	Mix Breed	Tumor Extirpation	Right	CM	6	23	1	12×6
4	Mix Breed	Elbow hygroma	Left	SF	1	21	1	8×5
5	Mix Breed	Tumor Extirpation	Right	F	11	26	2	9×5
6	Mix Breed	Tumor Extirpation	Left	M	8	25	2	10×5
7	Turkish Kangal	Elbow hygroma	Left	F	1	37	1	10×6
8	Mix Breed	Elbow hygroma	Left	SF	1.5	32	1	10×6
9	Turkish Kangal	Elbow hygroma	Right	M	2	39	1	10×6
10	Saint Bernard	Elbow hygroma	Left	M	1	52	4	12×7
11	Mix Breed	Tumor Extirpation	Right	CM	11	19	2	9×5
12	Turkish Kangal	Unknown	Left	M	8	38	Unknown	11×6
13	German Shepherd	Pressure Sores	Bilateral	CM	3	25	0	8×5
14	Turkish Kangal	Tumor Extirpation	Right	M	8	34	2	10×5

M: *intact male;* ***F:*** *Intact female;* ***SF:*** *spayed female;* ***CM:*** *castrated male*

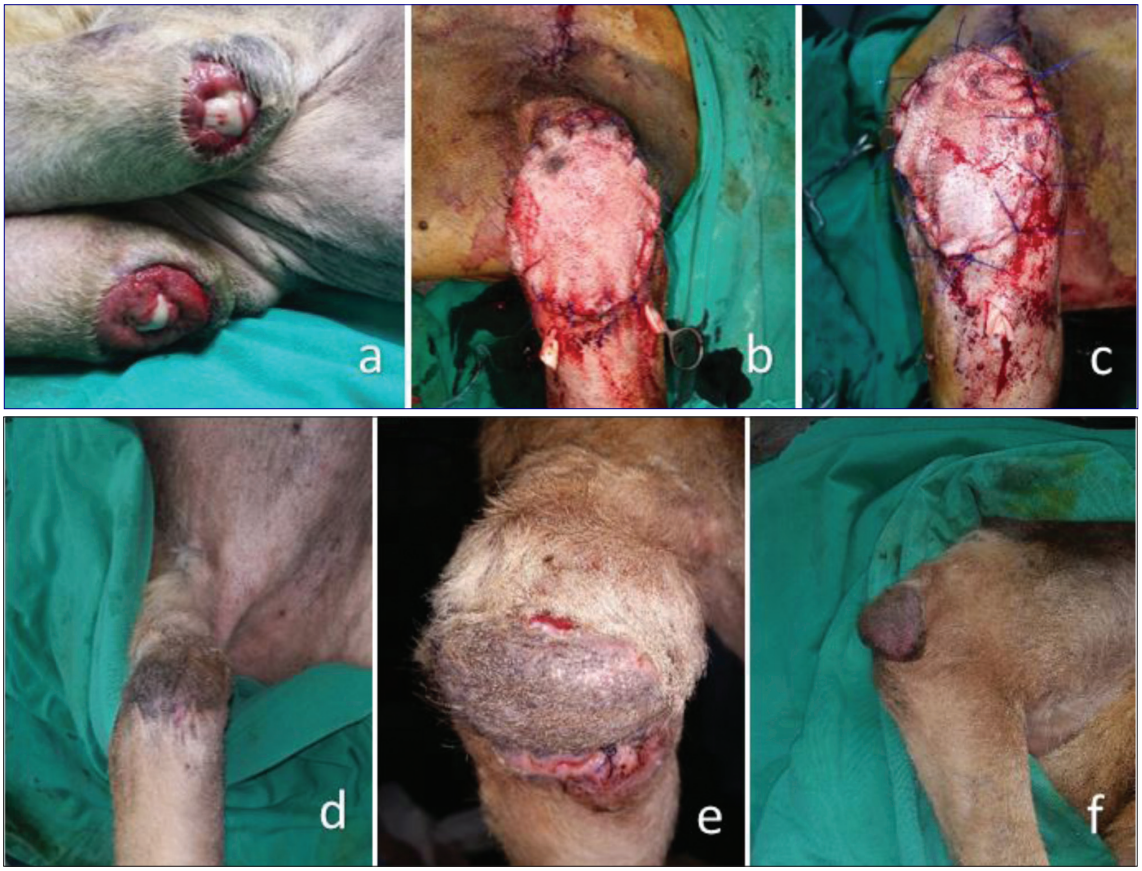

Fig 2. Pre-operative appearance of defect in both caudal elbow regions in case no.13, **a;** immediate post-operative appearance of right elbow region, **b;** immediate post-operative appearance of left elbow region, **c;** post-operative day 10 appearance of left elbow region, **d;** post-operative day 10 appearance of right elbow region. Sutures in the distal part of the flap have opened and flap ends curled medially, **e;** post-operative day 20 appearance of right elbow region, **f**

Şekil 2. Onüç numaralı olgunun her iki kaudal dirsek bölgesindeki defektin preoperatif görüntüsü, **a;** sağ dirsek bölgesinin akut postoperatif görüntüsü, **b;** sol dirsek bölgesinin akut postoperatif görüntüsü, **c;** sol dirsek bölgesinin postoperatif 10. gün görüntüsü, **d;** sağ dirsek bölgesinin postoperatif 10. gün görüntüsü. Flebin distal kısmındaki dikişler açılmış ve flebin uçları mediale doğru kıvrılmış, **e;** sağ dirsek bölgesinin 20. gün postoperatif görüntüsü, **f**

cation had a mean age of 1.4 (SE 0.17) years and mean bodyweight of 36.5 (SE 3.56) kg, while the patients presenting with mass extirpation complications had a mean age of 8.8 (SE 0.97) years and a mean bodyweight of 25.4 kg (SE 2.45). In 13 dogs, the chronic wound in the elbow region was unilateral and in one case it was bilateral. Among the unilateral cases, the lesion was on the left side in 7 (53.8%) and on the right side in 6 (46.2%) cases. In 12 of the cases, at least one surgical intervention had been previously performed for the treatment of the chronic wound in the elbow region.

No complications such as; seroma, edema, distal or total flap necrosis or paresthesia was encountered in any of the cases. During suture removal in the follow-up examination of one case (case no.4) on day 10, two sutures were seen to be absent in the distal flap area. However, since subcutaneous tissues were not exposed, no additional treatment (bandage, ointment administration etc.) was carried out. In the examination of this case on day 20, healing was seen to have completed without any problem. In the day 10 examination of case no.13, sutures in the distal flap region were seen to have opened and rotated internally on the wound edge of the flap side *(Fig. 2e)*. The wound edges were revised and sutured using the simple interrupted suture technique and Number 0 non-absorbable monofilament suture material (Prolene®, Ethicon). On day 20, healing without complication was observed and the sutures were removed.

DISCUSSION

Surgical resection, severe trauma, infection, radiation necrosis and some congenital abnormalities may cause hard-to-close large defects in the body. The aim in closing these defects is to provide functional unity without producing tension. In the reconstruction of full thickness tissue losses of the body wall, it is important to select a method to close the defect with no dead spaces and protect the tissues beneath the defect [16].

In dogs, wounds of the elbow region may occur due to external traumas, ischemia in the skin resulting from exposure to long-term pressure, tumor, abscess, infection and as a complication of bursa extirpations [1,4].

Hygromas are described as chronic tissue swellings containing serous fluid and are caused by repetitive trauma on soft tissue overlying bony prominences. They are seen mostly in giant breeds (Great Dane, Irish Wolfhounds, Newfoundlands) and dogs younger than 2 years of age [17]. The 7 dogs included in this study due to their elbow hygroma complications had a mean age of 1.4 years and this was consistent with literature. The reason for the difference regarding breed distribution can be explained by the fact that dog breeds such as the Great Dane, Irish Wolfhound and Newfoundlands are not common breds in the geographical location in which the study was carried out.

Serosanguinous fluid accumulating in the dead space, formed as a result of loss of connection between the skin and subcutaneous tissue during reconstruction, is called seroma formation. This situation may cause secondary complications such as opening of sutures, and may be caused by failure to place a drain in the area following surgery [4,18]. In this study, two drains were applied to the recipient site during surgery and seroma formation was prevented. Also preparation of an island-style flap and transferring it to the recipient site through a tunnel greatly prevented formation of dead space.

Despite not being objectively measured, during suturing of the flap to the recipient site, the excessive tension may cause opening of the sutures, tissue ischemia and necrosis [18,19]. Particularly in axial pattern flaps, the diameters of blood vessels decrease towards the tip of the flap. In addition, the tension created during suturing may cause distal flap necrosis. Also, bending of the lifted flap during its transfer to the recipient site directly slows down cutaneous artery circulation, constituting another cause for distal flap necrosis [18]. In this study, it was observed that the anatomical course of the thoracodorsal artery pedicle was almost parallel to the sternum and that the prepared flap was bent approximately 90 degrees during transfer to the elbow region. Considering island artery flaps can be rotated up to 180 degrees [10], the fact that no distal or total flap necrosis was encountered in any of the cases in this study may be related to the preservation of flap circulation with this technique.

On the other hand, it has been reported that, as long as the cutaneous artery and vein are not damaged directly, there is no difference with regard to flap vitality between traditional axial pattern flaps and island artery flaps [20]. This also suggests that, observation of no complications, such as distal or total flap necrosis, cannot be solely attributed to the fact that the flap was prepared as an island artery flap. At this point, the mode of transfer of axial model flaps to the recipient site becomes important.

While the middle section of axial model flaps may be prepared in the shape of a tube for closure of distant wounds, a bridge incision may also be made to connect donor and recipient sites. Sometimes they may also be prepared in an "L" shape to close large and irregular defects. Peninsular flaps are larger than "L" shaped, "90 degrees" or "hockey stick" shaped flaps. However, it has also been expressed that, the more the flap is rotated, the more functional distance will decrease and the more the skin will fold [21]. Consequently, to the author's knowledge, since axial model flaps and island artery flaps transferred differently to the recipient site have not been compared separately, it may be wrong to assume that there is no difference regarding flap vitality between axial model flaps and island artery flaps [20].

Similar to the overlying cutaneous trunci muscle and skin, the latissimus muscle also feeds from branches of the thoracodorsal artery [5]. However, in this study, the latissimus dorsi muscle was not included in the flap. One reason for this is the fact that the latissimus dorsi muscle ends in the caudal shoulder depression and is not suitable for use as an island artery flap. Also, it has been observed that defects in the elbow region are not deep enough to require closure with another muscle layer. The fact that the bottom layer of the flap prepared in this study was formed by fascial tissue was a particular preference of the group of researchers.

The fascial tissue is an easily obtainable, easily vascularized tissue resistant to ischemia. In veterinary practice, the thoracolumbar fascia and fascia lata have been used for the reconstruction of various tissues like oral cavity mucosa, esophagus and urethra [11-15,22]. In the traditional cutaneous trunci myocutaneous composite flap, it has been reported that, when the flap is lifted, the latissimus dorsi and subcutaneous tissue between should not be included in the flap [8]. This situation prevents benefitting from the previously mentioned properties of the fascial tissue. Also, the fact that the fascia of the latissimus dorsi muscle covered the bottom section of the flap in this study, was seen as an important factor in lessening the tension on the sutures by forming a slippery surface in the recipient site. As a complication, opening of the sutures was only encountered in case numbers 4 and 13. The reason for suture opening and inward folding of the distal flap region in case no.13 was attributed to the fact that despite receiving medical treatment in the pre-operative period, the patient had laid on the floor for long periods during the post-operative period. The opening in the sutures in case no. 4 was too small to warrant revision. The reason for this opening could not be identified since there were no complications such as presence of edema or seroma formation.

Despite place a bandage over the flap area has some advantages like decrease dead space and reduce swelling, that is also known that bandage application may cause hypoxia, tissue damage and flap necrosis [21]. On the other hand, bandage application may not be performed after island arterial flap procedure [23]. In our study, postoperative bandage application was not performed to avoid to fail circulation in the thoracodorsal artery pedicle and to ensure adequate blood supply of the flap. Eventually, any common complication due to avoid bandage application was not observed.

The longer duration of surgery compared to other techniques is the only disadvantage of this method.

In the authors' opinion, the fact that the flap was prepared as an island arterial flap in this study was transferred to the recipient site via a subdermal tunnel and that the surface in contact with the defect was covered with fascial tissue, were reasons for the low rate of complications. Also in this flap model, it was seen that the defect in the elbow region could be closed without causing tension and with minimal dead space. In conclusion, the island arterial composite flap consisting of the skin, cutaneous trunci muscle and fascia of the latissimus dorsi muscle may be an effective method in the reconstruction of chronic wounds in the elbow region.

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Research Article

Effects of Pyometra on Some Oxidative Stress Parameters, Inflammatory Mediators and Neutrophil Segmentation in Bitches [1]

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Abstract

Pyometra is characterized by uterine bacterial infection with the accumulation of pus in the uterus often initiates systemic inflammation in the body and may lead to multiple organ dysfunction syndrome (MODS). The main objectives of this study is to evaluate C-reactive protein (CRP), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), superoxide dismutase (SOD), catalase (CAT), and thiobarbituric acid-reactive substances (TBARS) in bitches with pyometra and control bitches to explore the possible variety between the groups to detect the morbidity of the disease and contribution of these parameters in the condition of MODS. All bitches underwent ovariohysterectomy (OVH) and blood sampling was performed just before and after 15 days of the surgery. TNF-α, CRP, and IL-6 levels were significantly higher in pyometra group before the OVH ($P<0.05$, $P<0.001$, $P<0.001$, respectively), and CRP and IL-6 levels were significantly high after 15 days of OVH ($P<0.001$, $P<0.05$, respectively). Two-segmented neutrophils were significantly lower in pyometra group before ($P<0.05$) and after OVH ($P<0.01$). It is revealed that with this study, CRP and IL-6 could be used for inflammation detection when leucocyte number was normal in operated bitches with pyometra. Oxidative stress was not apparent in bitches with pyometra. Also, platelet (PLT) level and neutrophil segmentation index (NSI) might be good indicators to identify the chronic phase and the severity of the disease and risk of disseminated intravascular coagulation (DIC) in bitches with pyometra.

Keywords: *Pyometra, C-reactive protein, Disseminated intravascular coagulation, Platelet, Neutrophil segmentation index, Dog*

Köpeklerde Pyometranın Bazı Oksidatif Stres Parametreleri, İnflamatorik Mediyatörler ve Nötrofil Segmentasyonu Üzerine Etkileri

Özet

Uterusun bakteriyel invazyonu sonucu irin toplanmasıyla karakterize olan pyometra sıklıkla sistemik bir enflamasyonu başlatabilir ve bu da hastayı çoklu organ yetmezliğine sürükleyebilir (MODS). Çalışmanın amaçlarından biri, pyometralı ve sağlıklı köpeklerde kanda C-reaktif protein (CRP), tümör nekrozis faktör-α (TNF-α), interlökin-6 (IL-6), süperoksit dizmutaz (SOD), katalaz (CAT) ve tiyobarbitürik asit reaktif ürünleri (TBARS) gibi parametrelere bakılarak gruplar arasındaki varyasyon tespitiyle morbidite tahmininin yapılması ve bu parametrelerle MODS arasındaki ilişkinin ortaya çıkarılmasıdır. Çalışmaya dahil edilen köpeklerden kan alınarak ovariohisterektomi operasyonu uygulandı, on beş gün sonra ikinci kez kan alınarak aynı parametreler tekrar değerlendirildi ve bu süreçte grup içi ve gruplar arasındaki değişime bakıldı. TNF-α, CRP ve IL-6 düzeyleri operasyon öncesi pyometralı grupta önemli oranda yüksek bulundu (sırasıyla, $P<0.05$, $P<0.001$, $P<0.001$), Ovariohisterektomi sonrasında 15. gün yapılan kan değerlendirmelerinde ise CRP ve IL-6'nın pyometralı grupta hala yüksek olduğu görüldü (sırasıyla, $P<0.001$, $P<0.05$). Çift segmentli nötrofillerin sayısı pyometralı köpeklerde operasyon öncesi ve sonrası gruplara ve zamana bağlı olarak önemli düzeyde düşük olduğu tespit edildi ($P<0.01$). Bu çalışmayla CRP ve IL-6 değerlerinin pyometralı köpeklerde ovariohisterektomi sonrası enflamasyonun devam edip etmediğinin bir göstergesi olarak kullanılabileceği sonucuna varıldı. Yine trombosit (PLT) düzeyi ve nötrofil segmentasyon indekslerinin (NSI) pyometranın kronik yapısının ve şiddetinin belirlenmesinde iyi birer gösterge olduğu kanaatine varılmıştır. Aynı zamanda, pyometralı köpeklerin kanamayı arttıran dissemine intravasküler koagülasyon (DIC) riski altında olabileceğinin ortaya konulduğu düşünülmektedir.

Anahtar sözcükler: *Pyometra, C-reaktif protein, Dissemine intravaskuler koagülasyon, Trombosit, Nötrofil segmentasyon indeksi, Köpek*

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INTRODUCTION

Pyometra is a frequent endometrial problem in bitches, prolonged effects of oestrogen and following progesterone dominance in the uterus lead to hormonally mediated differences in the endometrium [1]. Accumulated endometrial secretions, lack of uterine contractility, reduced leucocyte response and bacterial invasion from ascendant genitourinary system may lead to uterine infection and consequent pyometra in bitches [2,3]. High percentage of occurrence makes pyometra one of the most important diseases in intact bitches [4].

Systemic inflammatory response syndrome (SIRS) is a response to stimulus severe enough to cause the release of inflammatory mediators into the blood stream. Pyometra sometimes might turn into SIRS when it is not diagnosed properly or treated adequately. In under some conditions SIRS might lead to multiple organ dysfunction syndrome (MODS) and the development of MODS can increase the risk of mortality [5]. An imbalanced immune response to proinflammatory cytokines may cause SIRS turn into MODS. Proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumour necrosis factor-α (TNF-α) are considered to be associated with MODS. Some of the criteria in animals with SIRS are fever or hypothermia, tachycardia, hypocapnea or tachypnea, decreased or increased leucocyte number and increased percentage of band neutrophils. Unfortunately, SIRS cannot be only detected with these criteria and only 64% of sepsis condition can be diagnosed in dogs. In humans, TNF-α level increases in the presence of SIRS and MODS, IL-6 and C-reactive protein (CRP) levels increase in septic conditions [5].

Increased levels of reactive oxygen species (ROS)-superoxide radical ($\cdot O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$)- in a cell may be a cause for DNA damage and it leads to inhibition of some proteins. In addition to this process decreased antioxidant levels called literally oxidative stress may be a cause of many diseases [6]. ROS are toxic substances and they are susceptible to causing damage as a starter of lipid peroxidation on cell structure and cell membrane [7]. Detoxification of ROS materials depends on the antioxidant defense system that includes some enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathion peroxidase (GPX) [8]. Malondialdehyde (MDA) is by-product of lipid peroxidation in the body and its assessment shows lipid peroxidation especially to determine the oxidative damage on the cellular membranes. MDA is measured as Thiobarbituric acid-reactive substances (TBARS) [8-10]. The published data for reference values of oxidative stress parameters in dogs and cats are very limited with many contradictory results.

Haematological abnormalities occur in bitches with pyometra. Because of the infection increased white blood cell (WBC) counts commonly with a regenerative left shift and decreased red blood cell (RBC) counts also present in bitches with pyometra. Causes of anaemia are might be different and one of them is thought to be neutrophil oxidative metabolism that destructs the cell membranes of erythrocytes leading to pyometra-associated anaemia [10].

The aim of this study are 1) to measure CBCs, serum SOD, CAT, TBARS and plasma concentrations of CRP, TNF-α and IL-6 in healthy old intact dioestrus bitches, 2) to determine whether these parameters are associated with severity of the pyometra and used as a diagnostic marker in pyometra bitches 3) to determine whether these oxidative stress parameters, inflammatory mediators and neutrophilic segmentation/or other haematological parameters are differentiated during the healing period of the OVH in healthy old and pyometra bitches.

MATERIAL and METHODS

Animals

The study was performed on 27 bitches with pyometra (pyometra group; PG) and 8 clinically healthy bitches in dioestrus (control group; CG). Mean body weight of PG was 20.18±2.39 kg and CG was 15.15±5.20 kg. All of the bitches were geriatric exception of one in each group. Bitches submitted to study were treated against internal and external parasites and vaccinated against common diseases by their owners. Health status of bitches was checked by laboratory and physical examinations that also include vaginoscopy and transabdominal ultrasonography (Easote Piemedical MyLab Five Vet, Netherlands). OVH was performed in both groups on the same day of the admission to hospital/diagnosis of pyometra.

Anamnesis Data

Written informed consent was obtained from all dog owners after being explained the purpose of the study. A detailed case history of the animals (polyuria, polydipsia, appetite loss, vomiting, antiparasitic drug status, vaccination schedule, and any other diseases etc.) was taken and recorded with thorough clinical examination. All procedures were approved by the Istanbul University Ethic Committee (Proposal #1702011).

Haemotology and Biochemistry Analyses

Blood samples were collected from the cephalic or jugular vein under aseptic precautions when the bitches come to hospital (before the anaesthesia of OVH) and transferred into two EDTA and one plain blood collection tubes (three tubes for each blood sample). The plain tubes were centrifuged after immediately blood collection at 4°C and 3.000 rpm for 15 min and sera-separated before analysis of biochemistry parameters. One of the EDTA tubes were then subjected to centrifugation at 4°C and 3.000 rpm for 10 min to segregate plasma and erythrocytes. Complete blood cell count (CBC) that includes measurement of RBC,

WBC, PLT, haemoglobin (HGB), haematocrit (HCT), medium cell volume (MCV), medium cell haemoglobin (MCH), medium cell haemoglobin concentration (MCHC) and some biochemical parameters (glucose, creatinine, urea, alanine and aspartate aminotransferase) were analysed at Istanbul University Veterinary Faculty's Teaching Hospital according to our routine laboratory methods. Remaining sera and plasma were transferred into eppendorf tubes and stored at -86°C for the analysis of TBARS, SOD, CAT, TNF-α, IL-6 and CRP.

Blood sampling were performed two times just before and two weeks after the OVH. Administration of mix of vitamins was not applied after surgeries not to cause any possibility of interaction with the antioxidant levels.

OVH, Tissue Collection and Histopathologic Examination

OVHs were performed routinely under general isoflorane (Forane Liquid; Aesica Queenborough, UK) anaesthesia. Three transverse sections from different parts of each uterine horn were cut for the histopathological examination, and all uterine tissues were fixed in 10% histologic grade buffered formalin (Histologic Grade formaldehyde; Sigma-Aldrich) embedded in paraffin, and standard sectioning and staining procedures were used. Sections were cut at 5 to 7 µm, mounted on slides, and stained with haematoxylin and eosin. Slides were evaluated at magnification × 20.

Antioxidants and Inflammatory Markers

The concentrations of all parameters were measured with commercially available canine sandwich enzyme-linked immunosorbent assay (ELISA) kits. TNF-α was measured using the canine TNF-α ELISA (CK-E90814, Hangzhou Eastbiopharm Co. Ltd., China), IL-6 was measured using the canine IL-6 ELISA (CK-E90948, Hangzhou Eastbiopharm Co. Ltd., China), CRP was measured using the HS-CRP canine ELISA (CK-E91348, Hangzhou Eastbiopharm Co. Ltd., China), TBARS was measured using the canine TBARS ELISA (CK-E91350, Hangzhou Eastbiopharm Co. Ltd., China), SOD was measured using the canine SOD ELISA (CK-E91351, Hangzhou Eastbiopharm Co. Ltd., China), and CAT was measured using the canine CAT ELISA (CK-E91349, Hangzhou Eastbiopharm Co. Ltd., China). Assay procedures were performed according to the manufacturer's instructions. Finally, microplates were read using a microtiter reader (µQuant, BioTek Instruments, USA). The detection range of CRP was 0.05-30 mg/L, IL-6 was 5-2.000 ng/L, TNF-α was 5-1.000 ng/L, SOD was 0.5-200 ng/mL, CAT was 1-300 ng/mL and TBARS was 0.5-100 nmol/mL. Intra- and inter-assay coefficient of variations (CV) for all parameters were <10% to <12%, respectively.

Blood Smear Preparation and Staining

Slides were prepared for each blood sample (both pyometra and control) and a single smear were made per slide. For the smear preparation well mixture of whole blood sample anticaogulated with 2.0 mg EDTA per millilitre was used and smeared uniformly with the method by Chung et al.[11]. The slides were air-dried for 5 min before staining and May-Grünwald's-Giemsa's azur eosin methylene blue solution (contains methanol) stain (Merck KGaA, Darmstadt, Germany) was used for staining. Stained slides were rinsed with deionized water, air-dried and evaluated.

Counting of WBCs and Neutrophil Segmentation Index (NSI) in the Blood Smears

The slides of 35-blood smear were analysed and WBCs enumerated with a conventional binocular Olympus light microscope system with 100× immersion objective (UPLSAPO-100XOPH, Olympus). A drop of immersion oil was placed directly at the feathered edge of the smears for observing and a total of hundred leukocytes were differentiated manually for each blood smear sample. Neutrophils were additionally classified in accordance to the number of lobes and NSI was calculated (number of neutrophil with 5 lobes or more × 100/number of neutrophil with 4 lobes). According to this formula values greater than 16.9 were considered abnormal and considered as a sensitive indicator for right shift [12].

Body Condition Score (BCS) and Type of Diet

"Five-point" BCS was accepted for the study. Type of food fed to the bitch during the past year was asked to the owner and diet categories were determined with the method by Lund et al.[13].

Statistics

Descriptive statistics (mean and standard error) and repeated ANOVA test was applied for haematological and biochemical analyses. Group (pyometra and control) was added to statistical model, as Between-Subject Effects while sampling time and Sampling Time×Group interaction were included in the model as within subject effects. Moreover, independent sample *t*-test was applied to compare for each sampling time. Additionally paired *t*-test was used to compare the values obtained at 0^{th} day and 15^{th} day. A value of $P<0.05$ was used to indicate the statistical significance.

RESULTS

None of the bitches in PG had ruptured uterus noticed during surgery and none of them died during or within 15 days of the surgeries (mortality rate 0%). PG consists of six Terriers, four Cocker Spaniels, four Mongrels, four Golden Retrievers, three German Shepherds, two Pekignese, two Rottweillers, a Siberian Husky, and a Samoyed, whereas CG consists of four Terriers, a German Shepherds cross, a King Charles Spaniel, a Rottweiller, and a Pekignese. Both group of bitches were fed with mostly popular dry + homemade food as follows by popular dry food.

Table 1. *Haematology and serum biochemistry variables measures in CG and PG before and 15 days after OVH*
Tablo 1. *Kontrol ve pyometra grubu köpeklerde ovariohisterektomi öncesi ve 15 gün sonrası hematoloji ve serum biyokimyası değerleri*

Variable	Control (n=8) Mean (±SE)	Pyometra (n=27) Mean (±SE)	T-test Significance	Group	T	G×T
RBC 0th day (×10⁶ µL)	6.74±1.35	5.82±0.20	*	*	NS	NS
RBC 15th day (×10⁶ µL)	6.63±0.54	5.59±0.20	*			
Significance	NS	NS				
WBC 0th day (×10³ µL)	14.03±1.70	38.30±5.25	*	*	NS	NS
WBC 15th day (×10³ µL)	14.11±1.51	19.50±2.34	NS			
Significance	NS	**				
HCT 0th day (%)	39.07±1.28	47.50±2.68	**	**	NS	NS
HCT 15th day (%)	37.93±1.42	46.88±8.94	**			
Significance	NS	NS				
HGB 0th day (g/dL)	15.54±0.10	12.79±0.40	**	**	NS	NS
HGB 15th day (g/dL)	15.20±1.10	12.23±0.50	**			
Significance	NS	NS				
PLT 0th day (×10³ µL)	496.75±79.60	314.30±35.00	*	NS	NS	NS
PLT 15th day (×10³ µL)	463.88±77.52	470.40±53.26	NS			
Significance	NS	**				
MCV 0th day fl	72.00±1.48	67.30±0.54	***	**	NS	NS
MCV 15th day fl	72.00±1.74	67.37±1.29	NS			
Significance	NS	NS				
MCH 0th day (pg)	23.50±0.33	21.90±0.21	***	***	NS	NS
MCH 15th day (pg)	23.25±0.31	21.48±0.23	***			
Significance	NS	*				
MCHC 0th day (%)	32.13±0.44	32.63±0.24	NS	NS	***	NS
MCHC 15th day (%)	31.50±0.42	31.37±0.26	NS			
Significance	*	***				
Glucose 0th day (mg/dL)	109.8±5.37	103.60±7.90	NS	NS	NS	NS
Glucose 15th day (mg/dL)	104.13±5.88	100.44±2.92	NS			
Significance	NS	NS				
Urea 0th day (mg/dL)	30.8±3.4	42.22±8.8	NS	NS	NS	NS
Urea 15th day (mg/dL)	36.25±9.6	44.74±8.0	NS			
Significance	NS	NS				
Creatinine 0th day (mg/dL)	0.9±0.05	1.10±0.13	NS	NS	NS	NS
Creatinine 15th day (mg/dL)	0.9±0.06	1.09±0.14	NS			
Significance	NS	NS				
AST 0th day (IU/L)	26.13±1.73	47.80±7.61	NS	NS	NS	NS
AST 15th day (IU/L)	27.3±2.2	31.10±3.70	NS			
Significance	NS	*				
ALT 0th day (IU/L)	40.5±8.94	28.89±4.30	NS	NS	NS	NS
ALT 15th day (IU/L)	38.3±7.73	33.52±6.32	NS			
Significance	NS	NS				

Abbreviations: ***CG****, control group;* ***PG,*** *pyometra group;* ***OVH,*** *ovariohysterectomie;* ***RBC,*** *red blood cell;* ***HCT,*** *hematocrit;* ***HGB,*** *hemoglobin;* ***PLT,*** *platelet;* ***MCV,*** *medium cell volume;* ***MCH,*** *medium cell hemoglobin;* ***MCHC,*** *medium cell hemoglobin concentration;* ***AST,*** *aspartate aminotransferase;* ***ALT,*** *alanine aminotransferase;* ***T****, Time;* ***G×T,*** *Group × Sampling Time interaction; Significance, comparison within the same group for each sampling time. *P<0.05, **P<0.01 ***P<0.001,* ***NS:*** *P>0.05*

Table 2. *Antioxidant status and inflammatory mediators of bitches in CG and PG before and 15 days after OVH*
Tablo 2. *Kontrol ve pyometra grubu köpeklerde ovariohisterektomi öncesi ve 15 gün sonrasında antioksidan ve inflamatorik mediyatörlerin durumu*

Variable	Control (n=8) (Mean±SE)	Pyometra (n=27) (Mean±SE)	T-test Significance	Group	T	G×T
SOD 0th day (ng/mL)	1.71±0.35	1.26±0.19	NS	*	NS	NS
SOD 15th day (ng/mL)	1.91±0.30	1.07±0.17	*			
Significance	NS	NS				
CAT 0th day (ng/mL)	1.05±0.20	0.88±0.11	NS	NS	NS	NS
CAT 15th day (ng/mL)	1.56±0.27	1.03±0.15	NS			
Significance	NS	NS				
TBARS 0th day (nmol/mL)	1.94±0.30	1.07±0.16	*	*	*	NS
TBARS 15th day (nmol/mL)	1.14±0.23	0.92±0.12	NS			
Significance	NS	NS				
CRP 0th day (mg/L)	2.06±8.03	58.35±4.37	***	***	*	*
CRP 15th day (mg/L)	1.69±5.47	31.49±2.98	***			
Significance	NS	***				
IL-6 0th day (ng/L)	10.19±9.20	63.73±5.01	***	***	***	***
IL-6 15th day (ng/L)	10.10±3.78	20.62±2.06	*			
Significance	NS	***				
TNF-α 0th day (ng/L)	4.89±3.84	14.03±2.09	*	*	NS	NS
TNF-α 15th day (ng/L)	4.44±1.80	6.13±0.98	NS			
Significance	NS	**				

Abbreviations: : CG, *control group;* ***PG,*** *pyometra group;* ***OVH,*** *ovariohysterectomie;* ***SOD,*** *super oxide dismutase;* ***CAT,*** *catalase;* ***TBARS,*** *thiobarbituric acid reactive substances;* ***C-RP, C*** *reactive protein;* ***IL-6,*** *interleukin 6;* ***TNF-α,*** *tumour necrosis factor alpha.* ***T,*** *Time;* ***G×T,*** *Group × Sampling Time interaction; Significance, comparison within the same group for each sampling time. *P<0.05, **P<0.01, ***P<0.001,* ***NS:*** *P>0.05*

Clinical Signs, Haematology and Biochemical Parameters

The most common clinical signs in PG were vaginal discharge (open-cervix; 81.48%), inappetance (51.85%), polyuria/polydipsia (48.15%), lethargy (33.33%), weight loss (25.92%), and vomiting (14.81%). Mean BCS of PG was 3.15±0.72 and CG was 3.25±0.46. Mean body temperature of PG was 38.58±0.14°C, and CG was 38.16±0.08°C. Heart rate was 100±18 beats min^{-1} in PG, and 90±16 beats min^{-1} was in CG. Respiratory rate was 30±12 breaths min^{-1} in PG, and 20±6 breaths min^{-1} was in CG.

The mean and the standard error values of CBC and biochemical parameters are presented in *Table 1*. At 0th day in PG, WBC count ranged from 9.0-105.0 ($\times 10^3$ µL), and in CG 9.4-16.6 ($\times 10^3$ µL). WBC counts decreased after OVH, but were still elevated in 16 bitches in PG. The mean and the standard error values of SOD, CAT, TBARS, TNF-α, CRP, and IL-6 are presented in *Table 2*.

Leukocyte Profile and NSI

Leukocyte profile and NSIs of all bitches are presented in *Table 3* and *Table 4*, respectively. Although WBC counts were in normal range, NSI were high in two bitches with pyometra (#p22, #p23) before, and 15 days after OVH in #p22 *(Table 4, Table 5)*. PLT counts were high in accordance with NSIs in #p22 and #p23 bitches before, and 15 days after OVH in #p22. Both bitches had open cervix pyometra. The graphs in *Fig. 1* provide a good example of how three bitches with pyometra responded to uterine infection differently.

Histopathology

Endometrial epitheliums were enlarged, columnar, and vacuolated in both groups. Papillary proliferations and hyperplasia were seen on the surface of the epithelium. In the CG, endometrial proliferation and glandular elongation were prominent. The stroma of the uterine tissues was oedematous and secretions were frequently present in the lumens of the glands.

In the PG, endometrium had moderate to severe cellular infiltration composed of many viable and degenerative neutrophils, fewer epitheloid macrophages, plasma cells and lymphocytes. Inflammatory cells, fibrin and karyorrhectic debris diffusely filled the lumen infiltrating the ulcerated endometrium. The surface epithelium was composed of columnar cells with foamy cytoplasm partially detached from the lamina propria. Varying sized cystic glands and papillary changes in the glandular epithelium were also found. Endometrial lymphatics were ectatic and blood vessels were congested.

Table 3. *Leucocyte profile and segmented neutrophils' of bitches in CG and PG before and 15 days after OVH*
Tablo 3*. Kontrol ve pyometra grubu köpeklerde ovariohisterektomi öncesi ve 15 gün sonrasında lökosit profili ve nötrofil segmentasyonu*

Variable	Control (n=8) Mean (±SE)	Pyometra (n=27) Mean (±SE)	T-Test Significance	Group	T	G×T
Band neutrophil 0th day	23.88±2.87	35.63±3.74	NS			
Band neutrophil 15th day	21.88±2.42	18.56±1.75	NS	NS	*	NS
Significance	NS	NS				
Two segmented neutrophil 0th day	20.00±1.30	13.30±1.43	*			
Two segmented neutrophil 15th day	25.63±2.01	16.22±1.50	**	**	*	NS
Significance	NS	NS				
Three segmented neutrophil 0th day	20.00±1.74	12.90±1.40	*			
Three segmented neutrophil 15th day	18.25±1.86	20.11±1.77	NS	NS	NS	*
Significance	NS	NS				
Four segmented neutrophil 0th day	6.63±1.92	8.30±1.33	NS			
Four segmented neutrophil 15th day	7.00±0.80	10.63±1.15	NS	NS	NS	NS
Significance	NS	NS				
Five segmented neutrophil 0th day	1.88±0.90	3.33±0.84	NS			
Five segmented neutrophil 15th day	1.25±0.62	3.96±1.33	NS	NS	NS	NS
Significance	NS	*				
Six segmented neutrophil Day 0	0.13±0.13	0.96±0.47	NS			
Six segmented neutrophil 15th day	0	1.00±0.30	NS	NS	NS	NS
Significance	NS	**				
Seven segmented neutrophil 0th day	0	0.19±0.9	NS			
Seven segmented neutrophil 15th day	0	0.44±0.21	NS	NS	NS	NS
Significance	NS	*				
Eight segmented neutrophil 0th day	0	0.04±0.04	NS			
Eight segmented neutrophil15th day	0	0.11±0.06	NS	NS	NS	NS
Significance	NS	NS				
Nine segmented neutrophil 0th day	0	0	NS			
Nine segmented neutrophil 15th day	0	0	NS	NS	NS	NS
Significance	NS	NS				
Lymphocyte 0th day	17.25±2.99	11.37±1.46	NS			
Lymphocyte 15th day	17.13±1.46	16.00±1.47	NS	NS	NS	NS
Significance	*	NS				
Monocyte 0th day	7.88±1.01	9.67±1.12	NS			
Monocyte 15th day	3.38±0.94	5.41±0.60	NS	NS	**	NS
Significance	*	NS				
Eosinophil 0th day	2.13±1.63	0.44±0.19	NS			
Eosinophil 15th day	5.50±1.13	3.89±0.85	NS	NS	**	NS
Significance	NS	NS				
Basophil 0th day	0	0.07±0.05	NS			
Basophil 15th day	0	0.33±0.13	NS	NS	NS	NS
Significance	NS	NS				

Abbreviations: ***CG,*** *control group;* ***PG,*** *pyometra group;* ***OVH,*** *ovariohysterectomie;* ***T,*** *Time;* ***G×T,*** *Group × Sampling Time interaction; Significance, comparison within the same group for each sampling time.*P<0.05, **P<0.01, ***P<0.001,* ***NS:*** *P>0.05*

Table 4. *0th day and 15th day of NSIs' in both groups of bitches*
Tablo 4. *Her iki gruptaki köpeklerde 0. ve 15. gün nötrofil segmentasyon indeksleri*

Bitch no.	0th Day NSI	15th Day NSI	Bitch no.	0th Day NSI	15th Day NSI
P1	1.27	0	P19	0	3.61
P2	4.84	5.66	P20	4.41	3.61
P3	0	4.94	P21	1.27	0
P4	0	2.63	P22	**70.27**	**102.86**
P5	0	10.53	P23	**44.23**	3.33
P6	0	0	P24	2.53	3.33
P7	2.38	13.43	P25	0	7.23
P8	7.14	4.94	P26	3.57	10.53
P9	0	2.63	P27	4.05	0
P10	16	3.61	C1	9.33	1.39
P11	0	3.92	C2	1.82	1.56
P12	5.88	3.92	C3	2.53	0
P13	1.30	0	C4	5.17	5.71
P14	12.68	13.43	C5	0	0
P15	3.23	0	C6	2.86	5.71
P16	2.66	3.61	C7	0	0
P17	14.47	2.63	C8	1.39	0
P18	10.66	7.23			

Abbreviations; P, *pyometra bitches;* ***C,*** *control bitches. Values bold indicate high segmentation index with significance*

Table 5. *Haematology variables measures in #p22 and #p23 bitches before and 15 days after OVH*
Tablo 5. *p22 ve p23 numaralı köpeklerde ovariohisterektomiden hemen önce ve 15 gün sonrasına ait hemogram değerleri*

Variable	#p22	#p23	Reference Intervals
RBC 0th day (×10⁶ µL)	6.28	**5.24**	5.6-8
RBC 15th day (×10⁶ µL)	7.50	5.60	
HGB 0th day (g/dL)	**13.70**	**11.30**	14-19
HGB 15th day (g/dL)	**15.90**	**12**	
HCT 0th day (%)	43	**37**	40-55
HCT 0th day (%)	52	**35**	
WBC 0th day (×10³ µL)	15.90	9	6-16.6
WBC 15th day (×10³ µL)	12.40	7	
PLT 0th day (×10³ µL)	**571**	**490**	150-400
PLT 15th day (×10³ µL)	**516**	400	
MCV 0th day fl	69	70	65-75
MCV 15th day fl	70	70	
MCH 0th day (pg)	22	22	22-26
MCH 15th day (pg)	**21**	23	
MCHC 0th day (%)	**32**	**31**	33-36
MCHC 15th day (%)	**30**	**32**	

Abbreviation: OVH, *ovariohysterectomie. Bold values indicate abnormality*

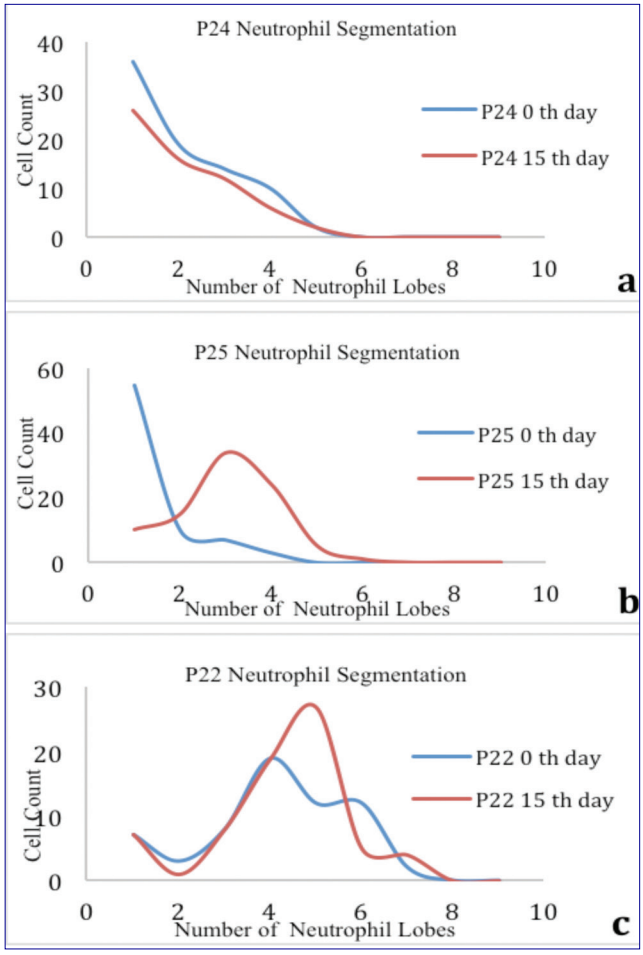

Fig 1. Neutrophilic segmentation profiles in #p24, #p25, #p22 bitches with pyometra. Blue line shows the first blood sampling (0th day), red line shows the second blood sampling (15th day). X-axis shows the segmentation of the neutrophils (0-9 lobes), and Y-axis shows the number of neutrophils. a. #p24 shows left shift at 0th and 15th days, b. #p25 shows left shift at 0th day, and moderate right shift at 15th day, c. #p22 shows intense right shift at both sampling days

Şekil 1. p24, p25 ve p22 no'lu pyometralı köpeklerde nötrofil segmentasyon profili. Mavi çizgi ilk kan örneklemesini (0. gün), kırmızı çizgi ikinci kan örneklemesini (15. gün) göstermektedir. X ekseni nötrofil segmentasyonunu (0-9 lob), Y ekseni nötrofil sayısını göstermektedir. a. #p24 0. ve 15. günlerde sola kayma, b. #p25 0. günde sola kayma ve 15. günde orta düzeyde sağa kayma, c. #p22 ise her iki günde de sağa kayma göstermektedir

DISCUSSION

One of the main aims of our study was to determine a clinically more specific marker in veterinary medicine for the prognosis of pyometra by measuring biochemical parameters. The ideal parameter should estimate the morbidity and mortality of critically ill patients at high risk of development SIRS and consequently MODS. In the present study, comparison was made between the evaluation of CBCs, NSI, IL-6, TNF-α, CRP, SOD, CAT, and TBARS in bitches with or without pyometra before and after 15 days of OVH.

In our study mortality rate in PG was 0% within the 15

days of OVH. In a previous study, the mortality rate in PG was 4% [5]. In humans with pyometra accompanied by SIRS, the mortality rate was found 6-7% [14] that is much higher compared to dogs. Finding of 0% mortality in our study indicates that the risk of development of MODS through SIRS in PG is very low. Because the majority of bitches in our study had open-cervix pyometra this might lowered the endotoxemia and SIRS and consequently impacts on the mortality rate. Oxidative stress was not obviously occurred in PG in this study and also this might reflected on the mortality rate. Respiratory and heart rates and body temperatures were similar with the previous studies [5,15]. Breed distribution in PG was similar with the breeds with high risk, but three German shepherd bitches in PG that was determined to have a low risk of developing pyometra was in contrast by Egenvall et al.[4].

Although BW of bitches in PG presented higher than CG in our study, BCSs were higher in CG. Our result with higher BW in PG was in accordance with the previous study [14], but our opinion here BCSs is more predictive value about the adipose tissue in the body and this high BW of the bitches in PG only shows more medium and large breed of dogs involved in the PG. Inflammatory parameters such as CRP, IL-6 and TNF-α are known to systematically rise in obesity [16,17], but higher levels of those in PG were associated with the inflammation of the uterus, not with the obesity because of this group had normal BCS.

Of the variables, RBC, WBC, PLT, HCT, HGB, MCH and MCV showed significantly different between the PG and CG at 0th day in this study. Lower levels of RBC, MCH, and MCV in PG show microcytic anaemia and also high HCT levels shows dehydration of the bitches which points the chronic phase of the disease. Microcytic anaemia in PG may be occurred because of the blood loss into the uterus, iron deficiency, and toxic effects of the pyometra on the bone marrow or lipid peroxidation damage on erythrocytes membranes. In contrast with the other studies [5,18] none of the biochemical variables were found significantly different between the groups, before and 15 days after OVH. PLT was significantly lower in PG before the OVH, but increased after 15 days. This result of decreased PLT in pyometra bitches was in accordance with the previous studies [5,19] and it was noted that bitches with endometritis-pyometra were under risk of disseminated intravascular coagulation (DIC), which decreases the PLT, and other coagulation factors and increase the risk of haemorrhage [19]. In another study [20], it was revealed plasma coagulation factors were decreased in dogs with sepsis but PLT levels were not differed. Haemorrhagic shock after the day of the pyometra surgery was seen previously in a bitch [21] and diagnosed as DIC. Also some studies [22,23] have shown that *Escherichia coli* endotoxemia is responsible for DIC in bitches with pyometra. In contrast, in our study, PLT levels were higher in some bitches with open cervix-pyometra for example in two bitches (#p22, #p23) with high NSI that shows the chronic phase of the pyometra disease. PLTs are known to be having an important role in inflammatory processes and in a study [24] high PLT and plateletcrit levels were found to be significantly associated with advanced endometriosis that is a chronic inflammatory disease in women. In our opinion, in chronic phase of pyometra especially in open-cervix pyometra which is not performed for a long time because of the unconcern of the owner, PLT level and NSI get higher because of the chronic inflammation of the uterus and aging of neutrophils in circulation, but in closed-cervix pyometra endotoxemia occurs more rapidly causing DIC with thrombocytopenia and consequent haemorrhage.

Cytokines acts as mediators in immune system may give valuable information about the mechanism, the severity and the prognosis of the disease [25]. In our study we focused on IL-6 and TNF-α cytokines that are considered to induce synthesis of acute phase proteins such as CRP. Previously, IL-6 has been shown to have major importance in acute phase response and hepatic regeneration [25]. In our study, IL-6 values were significantly different before and 15 days after OVH in PG and higher than expected (3.24-16.6 ng/L) in CG. Neuman et al.[25] reported that IL-6 concentration in healthy bitches should be <1 pg/mL, this value is similar with the human studies [26,27] and with that study it was found that IL-6 concentrations were much higher in dogs with acute disease. Because pyometra is an acute disease, measurement of IL-6 concentrations in pyometra cases may give an opinion about the course of the disease. In that study, IL-6 level in bitches with pyometra similar with dogs with acute hepatitis and varied between 1-202 pg/mL. In a newer study [28] revealed that, IL-6 concentrations were ranged from 45-4656 pg/mL in the non-surviving dogs while the surviving dogs had a range of 0-405 pg/mL in an intensive care unit. IL-6 found to be a good prognostic factor in critically ill dogs (P<0.001) but its concentrations in bitches with pyometra (a part of the survivors) were not given to the reader [28]. Our results of IL-6 concentrations within the accordance with this study, and none of the bitches died during the study. In another study [5], IL-6 concentrations were not significantly differed between pyometra and control groups, although Dabrowski et al.[29] found out that assessment of serum IL-6 and IL-10 were good prognostic factors in the healing period after OVH in bitches with pyometra.

The TNF-α concentrations were significantly higher before and 15 days after OVH in PG. This result is in accordance with the Fransson et al.[5]. It is known that TNF-α has a very short half-life and difficult to detect after starting of an infection within 24 h [5]. In our opinion, high levels of TNF-α concentrations after OVH might due to continuing of systemic infection in some bitches with pyometra in our study. The CRP concentrations were significantly higher before and 15 days after OVH in PG. This result is in accordance with the other studies and it was

able to distinguish SIRS status with CRP concentration [5,18,30]. In our opinion, CRP can serve clinically as an important marker in pyometra for the determination of the severity and continuity of the inflammation in case of no other inflammation occurs in other parts of the body.

It is reported that TBARS and CAT levels increase but SOD levels decrease by age [8,31-34]. In our study, SOD and CAT values were higher in CG although not significantly difference was found between the groups before OVH. According to our results none of the groups did show oxidative stress before and after OVH and additionally CAT and TBARS values were elevated after 15 days of OVH exception of SOD value. In another study [35] in healthy bitches, OVH was related with oxidative stress after 30 days of the surgeries. Our results have shown accordance with the earlier results of that study. Also similar results of oxidative stress parameters in PG and CG may be explained with the majority of the bitches in our study had open-cervix pyometra. The food type were fed to the bitches was not constant in our study but very much similar between the both groups and the results of any parameter in this study was not affected by the diet.

A response characterized by leukocytosis with a left shift and neutrophilia was mostly apparent in PG before the surgery. Each bitch was responded in different ways to inflammation (pyometra) and stress (OVH) as observed by neutrophil segmentation profiles *(Fig. 1a-c)*. Some of the bitches in PG still had an infection 15 days after OVH as shown by a left shift *(Fig. 1a)*, ceasing of the infection *(Fig. 1b)*, and had leukocytosis or normal leucocyte number with a right shift (high NSI) before and after OVH *(Fig. 1c)*. This bitch *(Fig. 1c)* is believed to have a uterine infection for longer duration before seeking a veterinary care. Right shift-hypersegmentation of neutrophils is a nonspecific indicator of aging of these cells in circulation [36] and refers to chronicle of the disease like in these two bitches with pyometra (#p22, #p23). Band neutrophils were found higher but not significant before OVH in PG while in other studies significantly higher in pyometra bitches [5,19]. In our study, two segmented neutrophils were found significantly lower in PG before and after OVH. Lymphopenia, eosinophilia, and monocytosis were seen in PG before OVH and this result within the accordance with a similar study [37].

Different breeds of dogs that have different cytokines, hormones and humoral factors, and many different breeds were included in our study and in our opinion this heterogeneity might affected some of our results.

As a conclusion, CRP and IL-6 could be used for inflammation detection after OVH in pyometra. Oxidative stress was not apparent with the parameters of TBARS, CAT, and SOD in PG. Also, surgery itself did not generate oxidative stress in bitches of both groups as detected after 15 days of the OVHs. Because thrombocytosis is a marker of chronic inflammation [24], in future studies PLT and other factors relevant with PLT should be determined for the chronic phase of the pyometra and sepsis in bitches. Additionally, NSI can give very valuable information about the severity of the pyometra and blood smear should be prepared from especially critically ill cases to see how immune system respond to an infection by an individual.

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Research Article

Detection of Diacylglycerol Acyltransferase 1 (DGAT1) Gene Polymorphism in Imroz and Chios Sheep Breeds in Turkey Using PCR-RFLP Method [1]

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Abstract

DGAT1 which catalyzes the last step of triacylglycerol synthesis has two alleles of the in animals ($DGAT1^A$ and $DGAT1^K$), the allele carrying the amino acid Lysine (K) is associated with milk with high fat content and low milk yield, whereas the allele carrying the amino acid Alanine (A) is the contrary. These alleles are known as candidate marker genes in choosing the animals for breeding. The aim of this study was to examine the genetic structures to the DGAT1 gene by PCR-RFLP method in Imroz (n=60) and Chios (n=52) sheep breeds. In this study, it was found that DGAT1 gene's allelic frequencies varied significantly between the two sheep breeds. The AA genotypic frequency was found the highest in Chios sheep breed (11.538%); the KK genotypic frequency was found the highest in Imroz sheep breed (68.333%) and the KA genotypic frequency was found the highest likewise in Imroz sheep breed (36.538%) in DGAT1 gene. In this study, the A allele frequency (0.702) was found higher than the K allele frequency in Imroz sheep breed. But, the K allele frequency (0.817) was found higher than the A allele in Chios sheep breed. It is believed that by increasing the DGAT1-like loci in order to ameliorate the milk yield capacity of native sheep breeds and merging the results obtained from these loci with the data and pedigree records of animals will prove especially useful to make better deductions and discoveries.

Keywords: *Sheep, Imroz, Chios, DGAT1*

Türkiye'deki Gökçeada ve Sakız Koyun Irklarında Diacylglycerol Acyltransferase 1 (DGAT1) Gen Polimorfizminin PCR-RFLP Yöntemi İle Belirlenmesi

Özet

Triasilgliserol sentezinin son basamağının katalizini yapan DGAT1'in hayvanlarda iki alleli ($DGAT1^A$ve $DGAT1^K$)var olmakla birlikte, bunlardan Lysin aminoasiti (K) taşıyan varyant süt içinde yüksek yağ oranına ve düşük süt verimine sahip iken, Alanin (A) taşıyan ise tam tersidir. Bu alleller, marker yoluyla seçilim için elverişli olarak bilinmektedirler. Bu çalışmada, Gökçeada (n=60) ve Sakız (n=52) ırkı koyunlarda DGAT1 gen yapısının PCR-RFLP yöntemiyle incelenmesi amaçlanmıştır. Yapılan çalışmada incelenen iki koyun ırkında DGAT1 geninin allel frekanslarının önemli derecede farklı olduğu belirlenmiştir. DGAT1 geninin; AA genotip frekansının en yüksek (%11.538) Sakız koyun ırkında, KK genotip frekansının en yüksek (%68.333) Gökçeada koyun ırkında ve KA genotip frekansının ise benzer şekilde en yüksek (%36.538) Gökçeada koyun ırkında görüldüğü belirlenmiştir. Bu çalışmada incelenen koyun ırklarından Gökçeada koyunlarında A allel frekansı (0.702), K allel frekansından yüksek bulunmuştur. Sakız koyunda ise K allel frekansı (0.817), A allel frekansından yüksek bulunmuştur. Yerli koyun ırklarında süt verim kabiliyetinin arttırılması için DGAT1 benzeri lokus sayısının da arttırılması ile bu lokuslardan elde edilecek sonuçların hayvanlara ait verim ve pedigri kayıtları ile birleştirilmesi daha verimli çıkarımlar sağlayacağına inanılmaktadır.

Anahtar sözcükler: *Koyun, Gökçeada koyunu, Sakız koyunu, DGAT1*

INTRODUCTION

Humans, for their well-being, should consume a sufficient quantity of vegetal and animal based food products. However, even though all around such food products are produced in sufficient quantities, their consumption shows an uneven distribution. In the future, with the increase of the world's population along with

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global warming, this imbalance is going to grow worse and worse as time goes by.

It is a known fact that between developed countries and countries in development there is a significant difference in consumption of animal based food products per person. While in the future, no increase in animal based food demand is expected within developed countries, it is being reported that by 2020s, the demand for meat and milk is going to be doubled within developing countries, population of which is rapidly increasing [1]. This means that milk production which is limited to bovines is going to be less and less sufficient therefore requiring alternative ways for milk production. This, as a result, shows us that agriculture and animal husbandry is a sector which needs to be ameliorated at a national scale [2].

It is a known fact that there are lots of similarities between cow, goat and sheep milk and that sheep milk is preferred for the production of milk with alternative proteins because of its nutritional contents. Since sheep milk is more concentrated and has twice the fat percentage and proteins, it is preferred over cow and goat milk. It has been reported that sheep milk contains 40% more proteins than cow and goat milk [3]. As a result, relationships between milk protein/fat polymorphism and milk yield have become more and more important in time.

Diacylglycerol acyltransferase (DGAT) uses Diacylglycerol and Acyl-CoA as substrates and therefore catalyzes the last step of triacylglycerol synthesis [4]. This enzyme also has an important role in adipose tissue and intestinal fat absorption [5]. In animals, there are two variants of DGAT1 gene (them being DGAT1^{A} and DGAT1^{K}). Of these variants, the one carrying the Lysine amino acid is associated with milk with high triglyceride content and low milk yield, whereas the variant carrying the Alanine amino acid is associated with high milk yield and low triglyceride content. Both of these variants code for integral membrane proteins between which there is no sequence homology [6]. DGAT1 is the first gene that is known to code for a protein that has a DGAT activity [7]. Mice without DGAT1, *i.e.* carrying the mutant allele, are viable, fertile and resistant to diet-induced obesity [8]. Absence of DGAT1 on one hand, causes an increase in saturated fat levels while decreasing unsaturated fat levels thereby modifying the fatty acid compositions in adipose tissues and skeletal muscles on the other hand alters the triglyceride metabolism in tissues such as mammalian gland tissues and as a result causes an absence of milk production [9]. Among European cattle breeds, Lysine to Alanine substitution at the loci 10433 and 10434 on exon 8 (DGAT1 K232A) is reported to possess several polymorphic characteristics [10]. Mutation of the allele "K" can increase the milk fat content [11,12]. The medical literature also reports that the allele K is responsible for the saturated fat levels in milk [13,14]. Individual characteristics in the aforementioned loci in cattle breeds can affect the DGAT1 K232A polymorphism and thus have different effects on milk fat [10]. Allele frequencies can even vary among the aforementioned cattle breeds [15]. As a result, studies on K232A polymorphism in cattle is highly important for the milk industry and for the consumer since it improves the milk quality.

DGAT1 which is located under the centromeric region of the 14th Cattle Chromosome, is also defined as one of the Quantitative Trait Loci (QTL) which influence the milk production properties [16,17]. Within animals possessing the known QTL genotypes, the DGAT1 gene was sequenced and a non-conserved K>A mutation was detected which in certain dairy cattle breeds had a significant effect on milk yield and content [12,16,17]. In cows DGAT1 has become a strong candidate gene for milk yield, milk fat and intra-muscular fat content. In dairy sheep breeds, the importance of role of DGAT1 in fat metabolism has made it an intriguing candidate gene in explaining the variations of the influence of DGAT on the milk content. Recently, Scata et al.[18] have found that in sheep breeds with high milk fat had an SNP which was present at 5´UTR of DGAT1 whereas in breeds containing low milk fat (Sarda sheep breed) this SNP was negatively correlated with milk fat content. With the advancement of methods in molecular genetics and biotechnology, these two areas found widespread use in livestock husbandry and as a result, several links were found between structural gene loci and milk yield, fat and protein levels in cattle. Thus these genes were reported as being possible marker genes in choosing the animals for breeding [11,19]. Geneticists performed selection studies on these genes in order to increase the frequency of alleles which have a positive effect on a chosen trait [20]. The variations which are economically important among these genes are used for markers to be used in marker based selections [21]. That's why, the data obtained in this study on the relationship between milk yield and milk fat percentage is going to be important when it comes to choosing the candidate animals to breed and therefore reveal the genetic value of them. The main purpose in selection is to guess the genetic value of the animal as best as possible and thus increase the genetic gain.

In livestock husbandry, in studies in which the relationships between polymorphisms in molecular markers and yield are investigated, the possibility of there being a relationship between milk proteins and milk fat content has been becoming more and more important [22,23]. In the light of studies in recent years, a relationship between certain genes and their allelic composition and milk yield was found and these genes were reported to have the potential to be used as a genetic marker [19,24].

The aim of this study was to determine the polymorphism of the DGAT1 gene in Imroz and Chios sheep breeds using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

MATERIAL and METHODS

Genomic DNA Extraction

Peripheral blood samples collected from Imroz sheep (n=60) and Chios sheep (n=52) breeds which were raised in various farms within the Marmara Region. The blood samples were kept within blood collection vials containing anticoagulants. Genomic DNA was extracted using Genox Genome Extraction Kit. The quantity of acquired DNA as well as its purity was determined using a spectrophotometer with a ratio of $O.D._{260/280}$.

Detection of the DGAT1 Gene

Following the DNA isolation, the alleles Lysine (K) and Alanine (A) of the DGAT1 gene were identified using PCR amplification and *AluI* (EURx) endonuclease digestion. Simple PCR amplification was made with a total volume of 26 µL. For each primer, 12.5 µL of 2X PCR master mix, 0.5 µM of (forward primer: 5′-GCATGTTCCGCCCTCTGG-3′, reverse primer: 5′-GGAGTCCAACACCCCTGA-3′) [11] and 50 ng of DNA sample were used. For the PCR optimization, 5% DMSO was added to the solution. These two alleles were added in order to ameliorate the amplification and thus get better results. Next, the following the PCR amplification cycle was executed:

The PCR condition consisted of initial denaturing at 95°C for 15 min followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and after the last cycle the samples were kept at 72°C for 3 min. About 10 µL of the PCR product was digested with 5U of *AluI* restriction endonuclease enzymes. After the cutting process of the prepared mixture kept for 4.5 h at 37°C the tubes for the enzyme inactivation was performed by keeping 65°C for 20 min. The DNA bands were dyed with ethidium bromide and detected in 2% agarose gel. A 2.5 µL 100bp standard DNA ladder (Analitika Lab.) containing all the locus specific alleles was used.

Statistical Analyses

The allelic and genotypic frequencies were calculated according to Cerit et al.[25]. Standard χ^2 test was performed in order to calculate the statistical significance between the observed and expected frequencies. For each genotype, expected and observed heterozygosity were also calculated.

Genotypic distribution for the Hardy-Weinberg equality was tested via the statistical application GENEPOP (Version 1.2) [26], PIC (Polymorphic Information Content) value for each genotype and effective allele count were also calculated [27,28].

Ethics Approval

Following the application to the Local Ethics Committee for Animal Experimentations of University of Istanbul, it has been concluded that this study did not require any ethics committee approval.

RESULTS

The purity of the obtained DNA was calculated with the ratio OD_{260}/OD_{280} for the DNA obtained from 112 samples and was found to be 1.9273 on average which is 19.28 ng/µL. Wild type and mutant bands were detected under 2% agarose gel electrophoresis. *AluI* enzyme was identified and it cut the mutant variant A into two strands of DNA; 272 and 37 bp. Because *AluI* enzyme was unable to recognize the allele K, wild type allele K was seen as a 309 bp single band. KA genotype appeared as three bands at 309, 272 and 37 bp. KK homozygote, AA homozygote and KA heterozygote individuals must show single, double and triple bands, respectively *(Fig. 1, Fig. 2)*.

Genotypic and allelic frequencies, expected and observed heterozygosity and Hardy-Weinberg Equilibrium (HWE) values were shown on *Table 1* and *2*. PCR analysis has shown us that DGAT1 gene was present in both sheep breeds. In Imroz sheep breeds, the allelic frequencies of the alleles K and A were0.817 and 0.183 whereas in Chios sheep breeds, the alleles K and A presented allelic frequencies of 0.298 and 0.702 respectively *(Table 1)*.

Effective allele number shows us how much of the alleles within a population contributes to the genetic diversity, which is the expected heterozygosity *(Table 2)*. It has been found that this number is 1.72 and 1.43 in Chios and Imroz breeds respectively. This means that since the expected heterozygosity in Chios breeds was higher than Imroz breeds, the former breeds therefore have higher

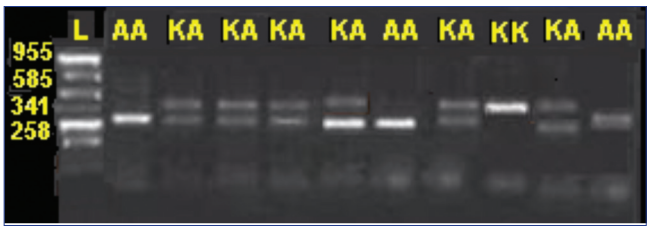

Fig 1. DNA band images obtained from Chios sheep breeds
Şekil 1. Sakız koyun ırkından elde edilen DNA bant görüntüleri

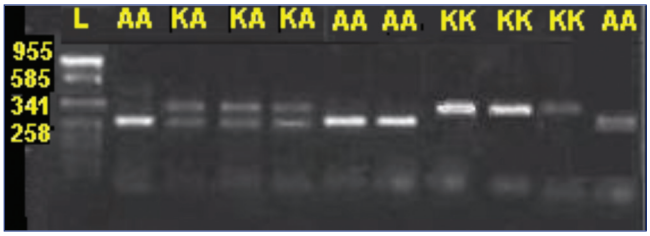

Fig 2. DNA band imagesobtained from Imroz sheep breeds
Şekil 2. Gökçeada koyun ırkından elde edilen DNA bant görüntüleri

Table 1. *Genotypic and allelic frequencies in Imroz and Chios sheep breeds*
Tablo 1. *Gökçeada ve Sakız koyun ırklarında genotipik ve allelik frekanslar*

Breeds	n	Genotypic Frequency (%)			Allelic Frequency (%)	
		KK	KA	AA	K*	A
Imroz	60	68.333 (n= 41)	26.667 (n= 16)	5.000 (n= 3)	0.817	0.183
Chios	52	51.923 (n= 27)	36.538 (n= 19)	11.538 (n= 6)	0.298	0.702

** (P<0.01)*

Table 2. *Genetic polymorphism parameters of the DGAT1 gene in Imroz and Chios sheep breeds*
Tablo 2. *DGAT1 geninin Gökçeada ve Sakız koyun ırkları arasındaki genetik polimorfizmi parametreleri*

Breeds	n	Heterozigosity		HWE[a]	PIC[b]	Effective Allele Number
		Observed	Expected			
Imroz	60	0.267	0.299	0.397	0.255	1.427
Chios	52	0.365	0.418	0.360	0.331	1.720

[a] Hardy-Weinberg Equilibrium, [b] Polymorphic Information Content

allelic contribution. In Imroz and Chios breeds, PIC is 0.255 and 0.331, respectively which indicates low genetic variation.

DISCUSSION

In cattle husbandry, for a long time, milk yield and milk fat content has been of great importance. In this study, it was found that DGAT1 gene's allelic frequencies varied significantly between the two sheep breeds, Chios and Imroz. Between these two breeds, the allele K which was observed the most in Imroz breed (0.817) was dominant whereas in Chios sheep breed, it was the complete opposite, with the allele A being more prominent and possessing the dominant characteristic (0.298). However, since it was observed that the allele A was responsible for the significant increase of the average milk yield in both of the breeds, it can be deduced from the findings in this study that when it comes to the production of milk with high yield and low fat ratio, genetic selection highly favors the Chios sheep breed. Since for the production of dairy products which require milk with high fat content, such as butter, buttermilk and cheese, allele K is the most favorable choice, Imroz sheep breed would be the best choice for such products since it has the highest allelic frequency when compared to the Chios breed. When it comes to the low-fat dairy products, choosing the allele A which promotes the production of low-fat milk and therefore choosing the Chios sheep breed where this allele is the most frequent.

The allele K variant of DGAT1 gene is involved in the significant decrease of protein and milk yield as well as the significant increase of fat content while, on the contrary, the allele A is associated with the significant increase of milk and protein yield and the significant decrease of fat content [29-31]. Kaupe et al.[32], in their study where they investigated the K232A polymorphism on DGAT1 locus among 1748 belonging to 38 different cattle breeds have found that beef cattle possessed a higher allele A frequency while it was the opposite in dairy cattle where the frequency was lower. DGAT1 is identified as being the most important gene when it comes to milk fat content and milk yield and it is known to increase the milk fat content by around 55% by encoding the Lysine amino acid. Schennink et al.[33], in one of their studies, have reported that in Holstein-Friesian cattle, DGAT1 gene had affected the milk fat content by 50%. According to the findings in this study, sheep with high allele K frequency (Imroz breeds) had significantly higher milk fat content than sheep with low allele K frequency (Chios breeds). Sun et al.[34] reported that theallele K of the DGAT1 gene increased the fat concentration in milk while decreasing the milk yield, which is in accord with the findings.

Scata et al.[18] in their studies have found that in sheep breeds indigenous to Italy (Sarda, Altamurana and Gentile di Puglia) the mutation g.5553C-T in the DGAT1 gene caused a decrease in milk fat percentage which corresponds to the findings in this study related to the allele A. However, contrary to their findings, genotypic frequencies of the alleles A and K found in this study are significantly different between the two breeds. Again, in a study on the same mutation done by Nanekarani et al.[35], the allelic frequencies of the studied alleles C and T were 0.562 and 0.438 respectively. They concluded that the allelic frequencies were almost the same, which is in contrast to the findings of this study. Which means that in Chios and Imroz sheep breeds, there exists a specific preference for the alleles A and K respectively which as a result influences the milk content of these breeds.

Hardy-Weinberg Equilibrium for Chios and Imroz sheep breeds was calculated to be 0.360 and 0.397 respectively which means that both of the breeds are in Hardy-Weinberg

Equilibrium (P<0.05). This would mean that the DGAT1 locus of these breeds is not undergoing any kinds of mutations, selections or migrations. In the aforementioned study done by Nanekarani et al.[35], they found that Lori sheep breed was not in HWE, in contrast to the findings in this study.

Bal and Akyuz [19], in their studies, have investigated the allelic frequencies of A and K in DGAT1 among three different cattle breeds (Holstein, Eastern Anatolian Red and Native Black). In Holstein and Eastern Anatolian Red breeds, allele A was found to be of higher frequency (0.75 and 0.62 respectively) while in Native Black breed, allele K was slightly more frequent (0.51). In the molecular marker study done by Ozdemir and Dogru [36] it was stated that this variant could be used as a genetic marker in selection studies. Also, the results obtained in this study is in accord with the findings coming from another study of Ozdemir [37] where the allelic frequency of the DGAT1 variants A and K in Native Black breeds was in the range of 0.35-0.60 (N=25). The values of HWE for the both sheep breeds indicate stability of the variants A and K. In a study done by Nanekarani et al.[35] where the exon 16-17 of DGAT1 gene in Lori sheep breed was studied. The alleles C and T of this gene had an allelic frequency of 0.562 and 0.438 respectively (n=118).

PIC value denotes the relationship between genotypic variation and alleles. If PIC is zero, then there's no genetic variation, if it's 1, then it means that each variation corresponds to an allele. In this study, the PIC value was found to be low in both sheep breeds, which indicates low allelic variation. According to the findings by Yang et al.[11] in their study done on four different sheep breeds (Tan, Oula, Ganjia and Qiaoke), the PIC value was also low, indicating low allelic variation, which is in accord with this study.

In conclusion, we believe that DNA typing is an effective method in identifying the alleles K and A of DGAT1 gene. Also, according to the findings in this study, there is a statistically significant difference in relation to the milk yield and fat content parameters between the sheep breeds with high allele K frequency (Imroz breed) and low allele K frequency (Chios breed). In selection studies which will be done according to the milk yield parameter, the use of Chios sheep breed which carry the allele K232A of DGAT1 gene which in turn corresponds to a high milk yield is believed to be favorable. It has been reported that DGAT1 and its allele K appeared in European sheep breeds long ago, having been inherited to sheep breeds from their ancestral species [29]. However, it has been reported that the allelic frequencies of the native breeds are lower than those of pure blood European breeds [38]. Correct usage of crossbreeding and artificial insemination techniques on native breeds in order to increase the allelic frequencies will be beneficial on the amelioration of milk quality parameters which in turn make it possible to provide the consumer with higher quality milk [39]. For example, cattle carrying the allele A of the gene ABCG2 (which codes the Breast Cancer Resistant Protein, Bcrp) have been reported to be selected since they are economically favourable [40]. In sheep husbandry, using DGAT1 gene's polymorphism will be useful for selection studies although more studies similar to this one need to be done in order to find the exact relationships between milk quality parameters and DGAT1's genetic variance. Polymorphism data acquired in this study can be used in certain areas related to animal husbandry in order to develop correct selection methods. It is believed that by increasing the DGAT1-like loci in order to ameliorate the milk yield capacity of native sheep breeds and merging the results obtained from these loci with the data and pedigree records of animals will prove especially useful to make proper deductions and discoveries. Finally, further studies are needed in order to elucidate the mechanisms of action of genetic polymorphisms of DGAT1 genes which have an impact on economically important production traits in sheep such as milk yield and milk fat content.

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Research Article

The Effectiveness of Patellar Anti-rotational Suture Techniques on Treatment of Lateral Patellar Luxation in Calves [1]

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Abstract

In this study, it was aimed to investigate that clinical efficacy of patellar anti-rotational suture technique of luxation patellae in calves. A total of 17 Holstein calves aged between 5 days to 40 days and weighed 35 to 60 kg were subjected. All of patellar luxation cases were congenital and lateral direction. After parapatellar skin incision, subcutaneous tissues were dissected as a blunt and the joint capsules were opened in all cases. A screw (3.5 x 32 mm) was inserted where the lateral condyles and a non-absorbable suture material (size 3 or 4 silk) was passed behind the screw and around the patella and then it was tied tightly to stabilize the patella and the joint capsule was slightly narrowed. Skin and subcutaneous tissues were closed routinely. Calves started to walk without limping end of 3rd week.

Keywords: *Lateral patellar luxation, Anti-rotational suture technique, Calf*

Buzağılarda Lateral Patellar Lukzasyon Tedavisinde Patellar Antirotasyonel Dikiş Tekniğinin Etkinliği

Özet

Bu çalışmada, buzağılarda patellar lukzasyonun tedavisinde patellar antirotasyonel dikiş tekniğinin klinik etkinliğinin araştırılması amaçlanmıştır. Bu amaçla, 5-40 günlük yaş ve 35-60 kg canlı ağırlık aralığında değişen toplam 17 adet buzağı ele alınmıştır. Buzağılardaki patellar lukzasyonların tamamının konjenital ve lateral doğrultuda olduğu belirlenmiştir. Parapatellar deri ensizyonunu takiben, derialtı dokular küt olarak diseke edilmiş ve tüm vakalarda eklem kapsülü açılmıştır. Lateral kondüle dik olarak bir vida (3.5 x 32 mm) yerleştirilmiş, vidanın arkasından ve patellanın etrafından dolanan emilemeyen bir dikiş materyali (ipek iplik 3-4 no) sekiz şeklinde sıkıca bağlanmış daha sonra eklem kapsülü biraz daraltılmıştır. Deri ve derialtı dokular rutin şekilde kapatılmıştır. Buzağılar, üçüncü haftanın sonunda topallamadan yürümeye başlamıştır.

Anahtar sözcükler: *Lateral patellar lukzasyon, Antirotasyonel dikiş tekniği, Buzağı*

INTRODUCTION

Patellar luxation is a common problem in small animals and it is usually occurs in the medial direction in both small and large breed dogs [1-3]. In calves, although there are little number of publications on this problem and its treatment, it has been reported that it is rare in the lateral direction [4,5] or conversely, congenital patellar luxations are almost always formed in the lateral direction [6-9].

Luxation of the patella may be congenital or acquired. Congenital causes include; coxa-varum, coxa-valgum, genu-valgum, lateral torsion which occurring in one third of the distal femur, medial rotation of the tibia, shallow femoral trochlear groove, incorrect development of the femoral trochlear condyle [3]. However, a femoral nerve deficit associated with difficult parturition or a nerve damage that affects the quadriceps muscle group may trigger patellar luxation because of normal lateral pulling force of gluteo biceps muscle [7]. Acquired causes are indirect or direct traumas [3,7].

The extensor (or quadriceps) mechanism of the stifle joint consists of the quadriceps, patellar tendon, patella, patellar ligament, and tibial tubercle. In healthy animals, this mechanism reaches to the middle of the knee from

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the proximal femur in a straight line. On the other hand, if patellar luxation develops in animals, the underlying cause is commonly malalignment of the extensor mechanism [3].

When prevents movement of the patella on sulcus trochlea, lameness occurs. Development of the erosion and/or osteophyte formations on the edge of the sulcus trochlea and articular face of the patella and thickening of the joint capsule exacerbates the lameness [10,11].

Displacement of the patella can be detected by clinical examination. While stifle joint is in extension position, patella is forced to luxation. Position of the patella can be determined by antero-posterior radiographic examination [4,12,13]. Also, the trochlear groove and its shape can be assessed by a tangential view of the flexed stifle (skyline imaging technique) [7]. Differential diagnosis of the patellar luxation includes avascular necrosis of the femoral head, coxofemoral luxation, and joint distortion. Prognosis is favorable for constant luxations but it is guarded for habitual luxations [3].

Patellar luxations are classified from grade I to grade IV in small animals [11]. For farm animals it is used with slight modification [7].

Species and age of the animal, luxation grade, accompanying complications (trochlear deformation, depth of the trochlear groove, concomitant cruciate ligament injury etc.) should be considered in determining treatment method [14]. There are many methods defined for treatment of the patellar luxation in small animals. These include; soft tissue reconstruction procedures such as overlap of lateral or medial retinaculum, fascia lata overlap, patellar and tibial anti-rotational suture ligaments, desmotomy and partial capsulectomy, quadriceps release and bone reconstruction procedures such as trochleoplasties, tibial tuberosity transposition, patellectomy, corrective osteotomies [2,3,13].

In farm animals and horses, small number of studies has been performed about patellar luxation and there are few methods described for its treatment. These methods are lateral release and medial or lateral imbrications [7], trochleoplasty depending on trochlear condition [7,8], medial patellar desmotomy [15].

In this study, it was intended that usage of the patellar anti-rotational suture ligaments used in small animals by adapting to calves and observation of healing period after surgery.

MATERIAL and METHODS

Study materials were consisted by a total of 17 Holstein calves, 11 female and 6 male, which were presented to Veterinary Faculty Animal Hospital with clinical history of severe lameness or inability to stand up on hind limb since birth, an ability to fully extend the stifle. Calves were 5 d to 40 d of age and weighed 35 to 60 kg.

According to anamnesis, difficulty in standing up and gait deficit has been started in all of calves just after delivery *(Table 1)*. In all calves, physical, hematological and radiographical examinations were performed. In clinical and radiological examination, unilateral patellar luxation was detected in all of the calves *(Fig. 1/A)*. All luxations were in lateral direction and the affected stifle joint was right in 9 calves and left in 8 calves. Also, obvious gait deficit with the calf in a unilateral crouch position was observed in all cases. Luxations were classified according to grading system for farm animals [7].

Nine calves (case number 1,4,5,6,8,9,10,12,16) had occasional patellar luxation. In these cases, the patella easily luxated manually at full extension and did not readily return to normal position when released. These cases were classified grade 2. Eight calves (case number 2,3,7,11,13,14,15,17) had permanently patellar luxation. After reposition of the patellas, they did not stay in place when the joint flexed. The depth of the trochlear groove may be different than normal. These cases were classified grade 3 *(Table 1)*.

Calves underwent surgery following 12 h starving period. Anesthesia was performed with combination im xylazine HCl 0.2 mg/kg body weight (Alfazyne®, Egevet, Turkey) and ketamine HCl 1.1 mg/kg body weight (Alfamine®, Egevet, Turkey). After disinfection of the region, a 10 cm medial parapatellar skin incision was made from distal one of fourth of femur to tibial tuberosity, than the subcutaneous tissues were dissected as a blunt. The joint capsules were opened in all cases, but in 6 cases (Case No. 2, 3, 6, 7, 12, 15) although a shallow trochlear groove was seen, no intervention was made to the joint surfaces and lateral femoro-patellar ligament was not transected. A screw (3.5 x 32 mm) was inserted where the lateral fabella settled in dogs and a non-absorbable suture material (size 3 or 4 silk, Orhan Boz®, Turkey) was passed behind the screw and around the patella, then it was tied just tight enough to stabilize the patella *(Fig. 1/B, C, D, E, F)*. Position of the patella was checked and the joint capsule was slightly narrowed. Skin and sub-cutaneous tissues were closed routinely. Post operative antibiotic (im, 1 mg/kg, ceftiofur HCl, Cefcloren®, Provet, Turkey) and nonsteroidal antiinflammatory drug (im, 2.2 mg kg, flunixine meglumine, Finadyne®, Ceva-DİF, Turkey) were prescribed to the all of cases for 5 day.

RESULTS

Age, breed, gender, weight, and clinical signs of the calves which had been operated with patellar luxation diagnosis are presented in *Table 1*.

The pulse, respiration, temperature and hematological

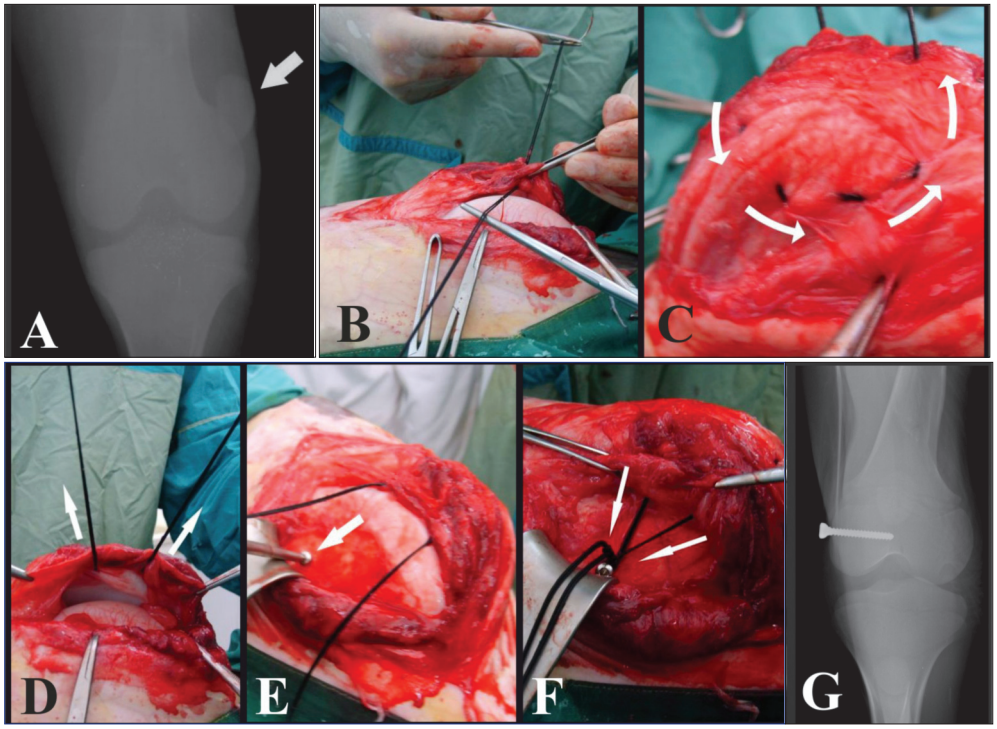

Fig 1. A, Preoperative antero-posterior radiograph of the affected leg (luxated patella are showed by arrow); **B,** Start of the circular suture; **C,** Placement of the suture material around the patella as shown by arrows; **D,** The view of suture material from articular side and stretching of the suture material in the direction of the arrows; **E,** Placement of the screw as shown by arrow; **F,** Tying of the suture material to the screw, arrows shows tightening direction; **G,** Postoperative antero-posterior radiograph of the affected leg

Şekil 1. A, Etkilenen bacağın antero-posterior preoperatif radyografisi (lukze olmuş patella ok ile gösterilmektedir); **B,** Sirküler dikişin başlangıcı; **C,** Oklar ile gösterildiği şekilde dikişin patella etrafına yerleştirilmesi; **D,** Dikiş materyalinin artiküler yüzeyden görünüşü ve dikiş materyalinin oklar yönünde gerdirilmesi; **E,** Ok ile gösterildiği şekilde vidanın yerleştirilmesi; **F,** Dikiş materyalinin vidaya bağlanması, gerdirme yönü okla gösterilmektedir; **G,** Etkilenen bacağın antero-posterior postoperatif radyografisi

parameters were normal. Both stifles were flexed, calves were unable to stand and when assisted adopted a crouching position. During palpation, the patella was found displaced laterally and there were atrophy muscles. The patellas were permanently luxated along with tibial torsion and misalignment of the distal tuberosity with the patellar groove and the patella could not manually reduced in grade III. The patellas were temporary luxatedand the patella could manually reduce in grade II.

When was entered to the joint space, medial femoropatellar ligament was found to be flexible. Femoral condyle was nearly normal in all calves, but the trochanteric ridge was low and trochelar groove was shallow. Therefore, the patellas were not have a good, deep, secure groove to ride in and easily pulled out. There was no increase in joint fluid with exception of Case 13. The same calf had thickening of joint capsule and surrounding tissue depending on the patellar position.

In case 1, from postoperative day 7, the calf began to touch the ground with toes of the affected foot and tried to bear weight on it. It began to bear weight on the affected foot from the postoperative day 14 and was able to walk almost normal from the postoperative day 21.

Postoperatively, the calves started to touch the ground with toes of the affected foot and tried to bear weight on it within 7 day. They began to bear weight on the affected foot from the postoperative day 14 and were able to walk almost normal from the postoperative day 21.

Lameness scoring was performed at the day 15 examination. For this purpose, each of the calves was observed by investigator while an assistant made the calf walk at least 10 m, and lameness level was scored as; not exist, mild, moderate and severe *(Table 1)*. According to scoring which performed at day 15, lameness level was mild in 12 calves, mild to moderate in 4 calves and moderate in 1 calf (Case no. 13). Subsequently, it was learned that 16 calves started to walk without limping at week 3 and 1 calf (Case no. 13) at week 4 *(Table 1)*. Patients were followed up until the end of 3rd month with the phone call and at the end of this period the owners said that the calves have completely normal gait.

DISCUSSION

Patellar luxations in the cattle mostly occur in dorsal direction [4,5,7,12]. According to literature, patellar luxations

Table 1. *Information about the calves included the study*
Tablo 1. *Çalışmaya dahil edilen hayvanlara ilişkin bilgiler*

[1]CN	[2]B	[3]A	[4]G	[5]W	[6]SLAP	[7]LS	[8]AL	[9]DL	[10]LG	[11]PO-SUAL	[12]LS-15	[13]PO-WWL
1	H	5	M	45	Day 1	C	R	L	2	Day 3	ML	Week 3
2	H	5	F	45	Day 1	C	L	L	3	Day 7	MM	Week 3
3	H	5	F	40	Day 1	C	R	L	3	Day 3	MM	Week 3
4	H	6	F	35	Day 1	C	R	L	2	Day 3	ML	Week 3
5	H	9	M	40	Day 1	C	L	L	2	Day 2	ML	Week 3
6	H	10	M	45	Day 1	C	R	L	2	Day 3	ML	Week 3
7	H	11	F	35	Day 1	C	R	L	3	Day 3	ML	Week 3
8	H	8	F	40	Day 1	C	R	L	2	Day 3	ML	Week 3
9	H	5	F	40	Day 1	C	L	L	2	Day 2	ML	Week 3
10	H	6	F	45	Day 1	C	L	L	2	Day 3	ML	Week 3
11	H	9	M	50	Day 1	C	L	L	3	Day 2	MM	Week 3
12	H	10	F	35	Day 1	C	R	L	2	Day 2	ML	Week 3
13	H	40	F	60	Day 1	C	R	L	3	Day 10	MD	Week 4
14	H	15	M	40	Day 1	C	L	L	3	Day 7	MM	Week 3
15	H	10	F	35	Day 1	C	R	L	3	Day 2	ML	Week 3
16	H	12	F	40	Day 1	C	L	L	2	Day 3	ML	Week 3
17	H	7	M	45	Day 1	C	L	L	3	Day 3	ML	Week 3

*[1]**CN:** Case Number; [2]**B:** Breed (H, Holstein) [3]**A:** Age (day); [4]**G:** Gender (M, Male; F, Female); [5]**W:** Weight; [6]**SLAP:** Start the Lameness After Parturition; [7]**LS:** Lameness Status (C, Constant; I; Intermittent); [8]**AL:** Affected Leg (R, Right; L, Left); [9]**DL:** Direction of the luxation (L, Lateral); [10]**LG:** Luxation Grade; [11]**PO-SUAL:** Start to use the affected leg postoperatively; [12]**LS-15:** Lameness Scoring at Day 15 (ML, Mild; MD, Moderate; MM, Mild to Moderate); [13]**PO-WWL:** Post-operative walking without limping*

are rare in calves [8,16], and nearly always in lateral direction if they are congenital [6-9]. In all of 17 cases which represented our clinics with complain of lameness and diagnosed as patellar luxation, all of the luxations were determined in lateral direction.

Consistent with the literature [7,8], extremely flexed knee joint with instability and abducted hindlimbs were determined in clinical examination. Nondurable structure of the medial femora-patellar ligaments were noticed during surgery. It was learned that there were an uneventful pregnancy periods and normal delivery from histories of all calves. Based on these information, the cases were considered as congenital because of any trauma had been never happen.

The radiographs of the stifle joint revealed lateral displacement of the patella of related hind limb. The radiographs also showed a flattened and shallow trochlear groove in cases. The findings were consistent with grade II and III patellar luxation [11] and also with the previous reports on congenital lateral patellar luxation in calves [7,11].

Surgical correction of the patellar luxation required realignment of the extension mechanism and stabilization of the patella in the femoral trochlea. Narrowing of the joint capsule (capsuloraphy), sulcoplasty in young animals (trochleoplasty), fixation from the fabella to the patellar ligament (patelloplasty), wedge osteotomy in adult small animals (wedge resection), transposition of the tibial tuberosity areused individually or in combination for treatment of patellar luxations [7,8,15].

While a little number of publication was encountered on treatment of patellar luxation in calves, compared to dogs much smaller number treatment methods were found for calves in classical literature [6-9]. Lateral release and medial or lateral imbrications can be applied separately or in combination and also these methods can be combined with one of the trochleoplasty methods when trochlear groove is shallow. According to the literature about both small and large animal, trochlear groove is not very distinct after just after delivery and it becomes deeper during growth of the animal when the patella is in it [2,3,7]. Therefore young animals with lateral patella luxation will not have a ready-made trochlear groove. Patellar luxation causes tightening of the capsular tissues on the side of luxation. In a study with 2 calves, which one is 3 months old and other one is 1 months old, trochleoplasty was performed for patellar luxation treatment and the method was successful for both cases [8]. However, ossification of the trochlea in calves is complemented by 3 months of age [7].

Kilic et al.[9] treated congenital bilateral patellar luxations in 16 calves by using transposition of partial patellar tendon and m. vastus lateralis [9]. They declared that an organic material usage may be ideal option when

considering growth periods of the calves. In this study, there was no complication during long-term follow-up period of patellar anti-rotational suture ligaments and capsulorraphy. Also, because of the eldest calf was 40 d old, ossification of the trochlea was not yet completed. Therefore, even is the trochlear groove was shallow, it was thought that the presence of the patella in its place after reduction would provide deepening of the groove. Hence, any intraarticular intervention was not performed, only the joint capsule was slightly narrowed by opening for to support the reduction.

An additional medial support to the retinaculum was constructed by placing a non-absorbable suture around the screw and anchoring it through the patellar ligament to encircle the patella on allsides. This suture reinforced the patella-fabellar ligament and should maintain tension on the patella throughout the normal range of movement.

The suture was not placed through the tendon of origin of the gastrocnemius. Care was taken to ensure that the suture material does not come directly into contact with the articular cartilage. Medial retinacular overlap was performed at the same time.

Also there are no reported or estimated risks of complication of the patellar anti-rotational suture ligaments technique, while lateral or medial trochlea become more susceptible to breakage in trochleoplasty procedure if too much abaxial bone is removed out [7].

Medial patellar desmotomy technique which is applied for treatment horses with persistent or intermittent upward patellar fixation may predispose horses to distal fragmentation of the patella due to increased stress on the middle patellar ligament [13,15]. Patellar anti-rotational suture ligaments technique can be used in these cases by using lateral condylar screw placement without patellar fragmentation risk. Also, especially in smaller weight animals with a grade IV luxation, tibial crest repositioning for to increase the line of tension of the quadriceps axial to the femoropatellar joint can also be performed. Avoidance of tibial crest transposition can be reasonable for heavier animals because of the complications rate of this procedure (i.e. implant failure and/or nonunion) [7].

For the patellar anti-rotational suture ligaments technique, which is mainly applied to small animals, it has been declared that fibrous tissue formation around the suture and realignment of soft tissues will maintain the position of the patella even if suture material has been ruptured or loosened [2]. When planning procedure applied, it was thought that fibrous tissue formation can keep the patella in its position regardless of the animal's weight. Despite the increase in weight there was no problem in the animals which were followed up to the third month after the operation.

As a result, it was concluded that the patellar luxation is not rare problem in calves. This study suggests that grade II and III lateral patellar luxation in calves can be satisfactorily treated with patellar anti-rotational suture technique in combination with capsuloplasty along medial retinacular reinforcement of the joint capsule without sulcoplasty. If technique is performed early in the course of the disease, and the affected animal may regain the normal functions of limb in a short time.

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Research Article

Effects of Different Fixative Solutions on Kidney Volume [1] [2]

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Abstract

With this study, it is aimed to determine the volumetric changes at kidneys of 40 sheep (left and right kidneys, in total 80 kidneys) before and after treated with different fixation solutions by using a stereological method. In the study, 80 sheep kidneys provided from our faculty slaughterhouse were used. Kidneys were divided into four groups of 20 kidneys in each group (10 left and 10 right kidneys). During slaughtering of the sheeps, kidneys were rapidly taken out from abdominal cavity of the animal and fresh kidneys volumes which sectioned to 0.5 cm thickness were determined by Cavalieri Principle. It was noticed that this process did not exceed 30 min in order to prevent autolysis. Afterwards, groups of kidneys each including 20 were put into Formalin, Ethyl alcohol, Zenker and Bouin solutions and fixed upon determinated fixing durations. Volume redeterminations by using Cavalier Method were also performed on fixed kidney cross sections. Number of intersected points and other parameters were entered to the macro program (Microsoft Excel XP) and volumes were determined. Volumetric changes occured on kidneys before and after fixation were evaluated, and statistical calculations were performed. It was observed that all of the fixing solutions used in this study have different rate of diminish effect on kidney volume. However this effect were not statistically significant. The evaluation of interaction on volume changes; solution, direction and solution x direction were also not significant (P>0.05). We think that data gathered at anatomical and histological research using fixed materials may be used as references.

Keywords: *Fixative solutions, Kidney, Stereology, Volume*

Farklı Tespit Solüsyonlarının Böbrek Hacmi Üzerine Etkileri

Özet

Bu çalışma ile tespit öncesi ve farklı tespit solüsyonları kullanılarak fikse edilmiş 40 adet koyunun (sağ böbrek ve sol böbrek olmak üzere toplam 80 böbrek) böbreğindeki hacimsel değişikliklerin stereolojik bir yöntem kullanılarak ortaya konması amaç edinilmiştir. Çalışmada fakültemiz mezbahasından temin edilen 80 adet koyun böbreği kullanılmıştır. Böbrekler 20'şer (10 sağ ve 10 sol böbrek) olmak üzere 4 gruba ayrılmıştır. Koyun kesimi sırasında böbrekler hayvanın karın boşluğundan hızlı bir şekilde çıkartılmış ve 0.5 cm kalınlığında kesitlere ayrılan fresh böbreklerin Cavalieri prensibi ile hacimleri hesaplanmıştır. Otolizden korumak için bu işlem süresinin 30 dakikayı geçmemesine dikkat edilmiştir. Ardından 20'şerli gruplar halinde böbrekler Formalin, Etil alkol, Zenker ve Bouin solüsyonlarının içerisine konarak belirlenmiş sürelerde tespit edilmesi sağlanmıştır. Tespit olmuş böbrek kesitlerinin üzerinde Cavalieri prensibine göre tekrar hacim hesaplamaları yapılmıştır. Kesişen nokta sayıları ve diğer parametreler programa girilmiş ve hacim değerleri ortaya çıkarılmıştır. Tespit öncesi ve tespit sonrası böbrek hacimlerinde meydana gelen değişiklikler değerlendirilmiş ve istatistiki hesaplamalar yapılmıştır. Çalışmada kullanılan tespit solüsyonlarının böbrek hacmi üzerinde belirli oranlarda küçülme yarattığı gözlenmekle birlikte bu solüsyonların küçülme üzerine etkisinin istatistiki olarak önem taşımadığı belirlenmiştir. Hacim değişimi üzerine interaksiyonlar değerlendirildiğinde ise solüsyon, yön ve solüsyon x yön'ün böbrek hacmi üzerinde etkisinin de önemsiz olduğu gözlenmiştir (P>0.05). Yapılacak olan anatomik ve histolojik çalışmalarda fikse edilmiş materyaller kullanıldığında bu çalışmadan çıkarılacak olan verilerin referans olarak kullanılabileceğini düşünmekteyiz.

Anahtar sözcükler: *Fiksatif solüsyonlar, Böbrek, Stereoloji, Hacim*

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INTRODUCTION

Preserving the cells as they are in the live body is called fixation. The tissues that were taken out of the body will start degrading and spoiling in a short period of time unless specials measures are taken. Therefore, the tissues or organ parts taken should be fixed before any degradation occurs in the cells.

The requirements for fixation include selection and accurate preparation of an appropriate fixative solution, use of the solution 10 times higher in volume than the tissue volume [1,2], strictly following the fixation schedule, and selecting an appropriate temperature [3].

The agents used for the fixation are chemical substances. These are called as fixatives. A decent fixative should have lethal, penentrant, and hardening effects in the tissue. However, undesired characteristics are dissolution of the tissue components, shrink of the tissue, changes in the protein structure, and destruction of the nucleic acids [4].

The most frequently used fixative solutions are aldehydes, mercurials, alcohols, and picric acids. In the aldehyde group, there are Formaldehyde and Glutheraldehyde. Fixation is achieved by establishing cross bridges with the proteins of the tissue. 10% Formaldehyde has been used for fixing animal cadavers in the anatomy laboratories and fixing the tissues in histology and pathology laboratories. Mercurials contain mercury chloride. It is one of the most functional salts used for tissue fixation. It is seldom used alone becuase it cause shrinkage reaction. Its solutions are known as B5 and Zenker. Alcohols show their effects by causing denaturation of the proteins by dehyrating the tissue. They fix the tissue slowly but they harden and shrink the tissue. Ethyl and Methyl alcohols are in this group. Picric acid presicpitates all proteins and forms pitrat molecules that are water-soluble. It fixes rapidly. It cause shrink of the cell that can be easily seen [3,4]. The most important picric acid solution is Bouin solution.

Volume, concentration, temperature and time interval are among the factors affecting the fixation. Fixing procedure should start within maximum 30 min after the fresh tissue sample was taken because autolysis start immediately after death and specialized organs such as brain, kidney are more severely and faster affected than those rich in elastic fibrils and collagen. To be fixed sufficiently within 12-18 h, most tissues require a 10-15 times higher volume of freshly prepared fixative solution [4].

Cavalieri principle, which is a stereological method has been used frequently in a number of studies to calculate the volume of different tissues and organs [5-10]. Stereological methods have been widely used in recent years for evaluating the organs as a whole in experimental medical studies [9] as well as for evalutions at cell level [6,11].

Volumetric changes of the entire kidney or in its a certain part can be helpful for evaluation of kidney development, pathology and anomalies. Therefore, many studies on kidney volume focused on this issue. Unlike from previous studies, fresh kidney volumes and volumes of kidneys treated with different fixatives were calculated in our study. The tissues will be fixed by each of four groups of fixatives classified based on their mode of actions. The tissue fixatives usually work by shrinking the tissue which is an undesired effect. In this study, it was aimed to study volumetric changes in left and right kidneys of 40 sheep (total of 80) as fresh or after fixing in different solutions by a steorological method.

MATERIAL and METHODS

In this study, left and right kidneys of 40 sheep, total of 80, that were obtained from the teaching slaughterhouse of our faculty were used. The kidneys were divided into 4 groups of 20 in each (10 left and 10 right). During the dressing of sheep carcasses in the slaughterhouse, the kidneys were removed from the abdominal cavity and weighted using a digital scale (±0.01 mg of precision). As the first step of Cavalieri principle, the kidneys were cross-sectioned [12]. An electrical pastrami slicer with adjustable thickness feature was used for cross-sectioning. Depending on the size of the kidneys, 9 to 12 slices were obtained from each kidney. Then, 0.5 cm thick slices were arranged as the surfaces of the same direction will be upward. For the whole kidney volume, a point grid with 0.5 cm interval (distance between the encircled points) was used. Point grid is a transparent paper with points spaced equally on it *(Fig. 1)*. The point grids were randomly tossed onto the crossections of the kidneys and the numbers of encircled points on the tissue were used for calculating the kidney volume *(Fig. 2)*.

The points on the cross sections were counted and inserted in the appropriate places in below equation. The equation was computerized with a macro program prepared using Microsoft Excel (XP) *(Fig. 3)*.

$$V = t \times a/p \times \sum P$$

V = Volume; t = Section thickness; a/p = Area represented by each point on point grid; ∑P = Total point count on section surface area

This procedure was repeated for each cross section and finally the volume of the whole kidney was calculated. The macro program also automatically calculated the volume and error (CE).

Each of the fresh kidney section was then placed into the fixative solution in a locked jar by keeping the same direction of the surfaces used for measurements. The jars were groupped as to contain 10 left and 10 right kidneys for each fixative solution. The fixative solutions included

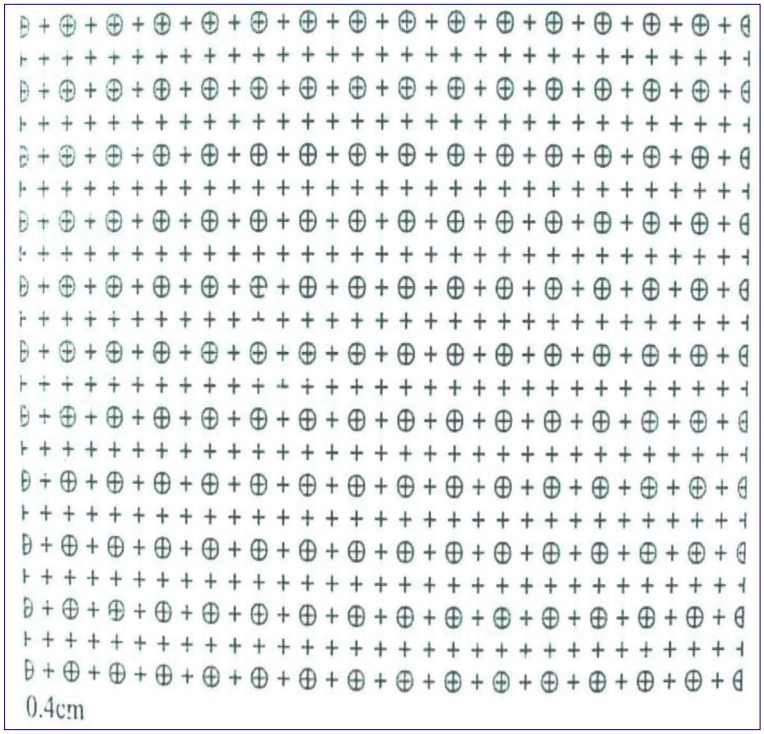
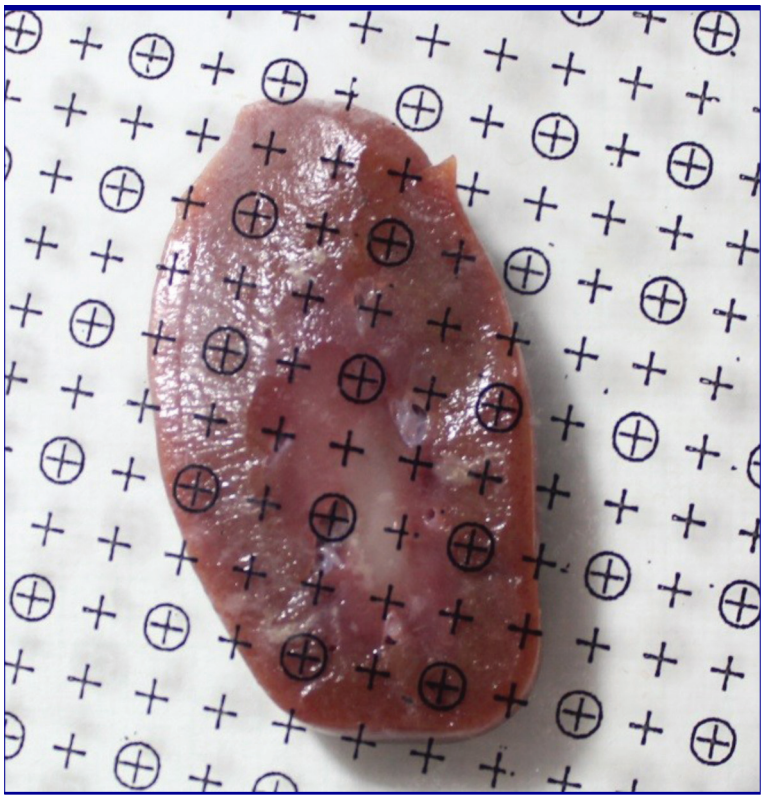


Fig 1. Point grid

Şekil 1. Nokta sayım cetveli

Fig 2. Superimposition of the point grid onto the crossections of the kidneys

Şekil 2. Böbrek kesitleri üzerine rastgele atılan bileşik noktalı alan ölçüm cetveli



Formalin, Ethyl alcohol, Zenker, and Bouin. The commercial 37% Formaldehyde was diluted with distilled water to 10% Formalin. Ethyl alcohol was prepared by mixing absolute alcohol and formalin. Zenker's fixative solution was prepared by using $K_2Cr_2O_7$, $HgCl_2$, glacial asetic acid and distilled water as described in the literature. Bouin solution was made from picric acid, formalin, and glacial acetic acid [2,3].

Fixation times of the numbered sections of the 10 left and 10 right kidneys were 24 h for Formalin, 24 h for Ethyl alcohol, 12 h for Zenker solution, and 18 h for Bouin solution. The amount of the fixative solutions in the jars were at least 10 fold of the volumes of the tissues. At the end of the fixation procedure, the tissues were weighted (±0.01 mg of precision) and all step that were used for the

Fresh:	Whole kidney
Solution:	
Direction	Counted
Section number	Point
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	
15	
16	
17	
18	
19	
20	
Total number of points hitting the sectior	0
Number of section=	
(t) Section thickness=	0,5
(SL) Scala lenght (measured by ruler)=	1
(SU) Scala unit=	1
(d) Distance between the points of the grid	0,5
b/a	10
	0,50
A	0,25
Nug	0,00
Variance of total area (Var srs)	0,00
Total variance	0,00
%nug	#SAYI/0!
CE (Coefficient error)	#SAYI/0!
Kidney Volume	0,00
Weight	

Fig 3. A macro program prepared using Microsoft Excel (XP)

Şekil 3. Microsoft Excel (XP sürümü) programı kullanılarak hazırlanmış olan makro program

volume calculations of the fresh sections were repeated on the fixed sections. The numbers of the intersecting points on the surfaces and other parameters were inserted to the program and volumes were calculated.

The changes in the volumes and weights of the left and right kidneys before and after the fixing were evaluated and statistical analysis were carried out. GLM procedures of the SPSS 13.0 software were used to determine the effects of the type of solution and the direction on the ratio of changes in weight and volume. The fixed effects of solution, direction, and solution x direction interaction were included in the GLM model. The photographs were taken by using Canon 550d brand camera.

RESULTS

The effects of fixative solution, direction and their interaction were not significant (P>0.05). The reduction in the volumes after fixing were found as 5.21% in Bouin solution, 6.4% in Ethly alcohol, 4.83% in Formalin, and 5.44% in Zenker's solution.

Effect of the fixative type on the weight change were significant (P<0.001). There was 26.91% decrease in Ethyl alcohol solution. The difference between Ethyl alcohol and other fixatives were significant. A 2.7% increase in Zenker solution was determined but this increase was lower compared to those found in Bouin and Formalin solutions. The difference between Bouin solution and Formalin was not significant *(Table 1)*.

DISCUSSION

The swelling or shrink of the cells occurs as an adverse effect of the fixation depending on the type of solution. As long as the effects of the chemical agents called as fixative is known, there will be less chance for error. Ethyl alcohol diffuse to the tissue slowly causing hardening and shrinking. Mercury chlorid and picric acid always cause shrinkage while glacial acetic acid results in the swelling of the cells, thus it is not used alone [2,3]. According to Fox *et al.*[13], the most important effect of Formaldehyde and other fixatives is to cause shrinkage. Many researchers attempt to explain how such shrinkage occur. However, these may result in many discrepancies because such shrinkage phenomenon was not determined by observation but by taking measurements before and after the fixing procedure [14-16].

In a study on rabbit kidney by Bolat *et al.*[14], the researchers observed that fixing the kidneys with Formalin

Table 1. *The effect of solution, direction and solution-direction on volume and weight change*

Tablo 1. *Hacim ve ağırlık değişimi üzerine solüsyonun, yönün ve solüsyon-yön interaksiyonunun etkisi*

Property	Solution (S)				Direction (D)		SEM	Significance		
	Bouin	Ethyl alcohol	Formalin	Zenker	Right	Left		S	D	SxD
Volume change, %	-5.21	-6.40	-4.83	-5.44	-5.78	-5.16	0.311	NS	NS	NS
Weight change, %	10.19a	-26.91c	9.62a	2.70b	-1.17	-1.03	0.298	***	NS	NS

**** P<0.001;* ***NS:*** *P> 0.05;* $^{a,b,c:}$ *Mean values carried different letters in the same line significantly different (P<0.05)*

caused an increase in the volume of the kidneys. Similarly, Warui and King [16] reported an 18% increase in volume of chicken kidney after fixing with glutaraldehyde via perfusion method. In contrary, Malas *et al*.[15] reported that Formaldehyde resulted in a 48.16% shrinkage in rat kidney volume. In our study, the volume of sheep kidney reduced at 4.83% when it was fixed in Formalin. Volumes of fresh organs was determined using the fluid replacement principle (Archimedes principle) in all previous studies, in our study, we used Cavalieri method to carry out a reliable comparison of fixed and fresh kidney volumes.

CE prediction is essential in stereological studies for deciding whether the frequency of the points on the grid and the numbers of the sections were sufficient. In general, a CE value of less than 10% is acceptable [17]. Şahin *et al*.[10] reported, the upper limit for CE as 5% in their study. In our study, the average CE value calculated for each section using Microsoft Excel program was found as 5%. It is thought that this value would be even lower if the numbers of the sections increase. However, we should note that slicing the fresh kidney by the slicer was somewhat difficult because the tissue was too soft, thus increasing the numbers of sections was not possible.

The experimental studies revealed that the Formalin had cancerogenic effect as well as harmful effects on many other systems including nervous system [18] and reproductive system [19]. Serious physical symptoms, irritation of eyes, lacrimation, irritation of airways and dermatitis are among the adverse effects that students and academicians experience [20]. Despite all these harmful effects, the reasons why this fixatives is still commonly used in making cadaver includes the low cost, ease of use [21], and high toxicity on the microorganisms [22]. When effects of the fixatives on the kidney volume was evaluated, which was also the objective of the current study, the least shrinkage of 4.83% was observed in Formaldehyde-fixed kidneys. In the literature, to our knowledge, there is no published study on the effects of Zenker's or Bouin's fixative solutions on the volume of organs. The reasons why Zenker and Bouin solutions are preferred for fixing biopsy materials which are small in size are thought to be difficulty of preparation of the solutions, higher cost of the chemical substances used in the formula of the solutions. In addition, coloring tissues to yellow and rendering the tissues fragile are considered as disadvantages of Bouin solution.

It is concluded that the difference in weight after fixing the tissues results from different mechanisms of the fixatives. Bolat *et al*.[14] fixed kidney with Formaline and found the weight increase was 7.33% in the left and 7.56% in the right kidney. In our study, the weight increase after fixing with Formaldehyde was 9.62% on average. Increase in weight was also observed after fixing with Zenker and Bouin solutions while Ethyl alcohol resulted in loss of weight after fixing. It has been thought that this loss might be due to the dehydrating effect of ethyl alcohol or breaking down the lipids.

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Research Article

The Effects of Vitamin E on Antioxidant Enzyme Activity in HepG2 Cells [1]

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[1] *This study was presented as oral presentation in the 7th National Veterinary Biochemistry and Clinical Biochemistry Congress (28-30 May 2015, Samsun)*

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Abstract

It is aimed to investigate the effect of vitamin E, powerful antioxidant (alpha-tocopherol succinate) on antioxidant enzyme activities in hepatocellular carcinoma (HepG2) cells. The hepatocellular carcinoma cell line HepG2 was used and the cells were cultured in the absence (control) or presence of different dose of vitamin E (50 mM, 50 µM and 10 µM vitamin E) for 24 h. The effect of vitamin E (alpha-tocopherol succinate) on catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activities in hepatocarcinoma cells were measured by spectrophotometry. A significant decrease in GPx activity was detected in 50 mM vitamin E treated HepG2 cells. However a significant decrease occurred in 10 µM and 50µM vitamin E applied HepG2 cells. SOD activity in study groups were lower than in control cells. In addition to this, the decrease in SOD activity in 50 mM vitamin E applied cells was significant. CAT enzyme activity in 50 µm vitamin E applied HepG2 cells was higher and, in 10 µM and 50 mM vitamin E applied HepG2 cells were lower than in control group. It was determined that vitamin E has a dose-dependent effect on antioxidant enzyme activity in HepG2 cells.

Keywords: *Catalase, Glutathione Peroxidase, HepG2, Superoxide dismutase, Vitamin E*

Vitamin E'nin HepG2 Hücrelerinde Antioksidan Enzim Aktivitesi Üzerine Etkileri

Özet

Güçlü bir antioksidan olan vitamin E'nin (alfa-tokoferolsüksinat) HepG2 hücrelerinde antioksidan enzim aktiviteleri üzerine etkisinin araştırılması amaçlanmıştır. Çalışma materyali olarak HepG2 hücre hattı kullanılmıştır. Vitamin E uygulanan hücreler çalışma grubunu vitamin E uygulaması yapılmayan hücreler kontrol grubunu oluşturmuştur. Çalışma grubu hücrelerine 10 µM, 50 µM, 50 mM dozlarda vitamin E uygulaması yapılarak, 24 saat sonunda HepG2 hücrelerinde antioksidan enzimlerden katalaz (CAT), süperoksitdismutaz (SOD) ve glutasyonperoksidaz (GPx) aktiviteleri spektrofotometrik olarak ölçülmüştür. 50 mM vitamin E uygulanan HepG2 hücrelerinde GPx enzim aktivitesinde anlamlı bir artış saptanmıştır. Ancak, 10 µM ve 50 µM vitamin E uygulanan HepG2 hücrelerinde ise anlamlı bir azalma meydana gelmiştir. Vitamin E uygulaması yapılan hücrelerdeki SOD aktivitesi vitamin E uygulaması yapılmayan kontrol grubuna göre daha düşük ölçülürken, 50 µM vitamin E uygulanan HepG2 hücrelerinde SOD aktivitesinde anlamlı azalma tespit edilmiştir. 50 µM vitamin E uygulanan HepG2 hücrelerindeki CAT enzim aktivitesi kontrol grubuna göre daha yüksek bulunurken, 10 µM ve 50 mM vitamin E uygulanan hücrelerdeki CAT enzim aktivitesi kontrol grubuna göre daha düşük tespit edilmiştir. Vitamin E'nin HepG2 hücrelerinde antioksidan enzim aktiviteleri üzerinde doz-bağımlı etkisinin olduğu belirlenmiştir.

Anahtar sözcükler: *Glutatyon peroksidaz, HepG2, Katalaz, Süperoksit dismutaz, Vitamin E*

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INTRODUCTION

Hepatocellular carcinoma (HCC) is common malignant disease associated with high mortality rate [1]. It has been known that Vitamin E (Vit. E), which is a fat-soluble vitamin, is the most effective antioxidant in biological systems. Vit. E converts superoxide and hydroxyl radical, singlet oxygen and lipid peroxides to lesser active form and hence, it protect against lipid peroxidation and oxidative tissue damage [2]. According to the analyses results, it has been found that Vit. E has an anti-oxidant activity and it exists in 8 different forms including four tocopherol and four tocotrienol [3]. Alpha-tocopherol is most common and active form of Vit. E in nature [4].

In humans, the free radicals that are formed as a result of a reaction between polyunsaturated fatty acids in membranes lipids and oxygen may have a role in tumor mechanism [5]. Vit. E may prevent certain tumor formation as a powerful anti-oxidant by protecting cells and DNA from the damage caused by free radicals [5,6]. In the laboratory studies, it has been shown that nutritional antioxidants including Vit. E prevent the growth of cancer cells [7]. The anti-oxidant effect of Vit. E refers researchers to investigate the protective effect of Vit. E in chronic diseases such as cardiovascular diseases, atherosclerosis and cancer. Many epidemiological studies have shown a relationship between high-dose Vit. E intake and cardiovascular diseases [8,9].

Opposite to its antioxidant activity, pro-oxidative effects of vitamin E are also observed *in vitro*. It has to be considered that vitamin E, like every redox-active compound, may exert anti- and pro-oxidative effects [10]. In a study performed by Heisler et al.[11], it was stated that Vit. E does not inhibit the growth of pancreatic cell lines. In another study performed on animals, it has been indicated that the mechanism of reduction of liver metastasis in pancreas cancer may be affected in accordance with increased GPx and SOD activities and decreased levels of thiobarbituric acid-reactive substances (TBARS).According to result of study, it was concluded that antioxidant vitamins prevent oxidative stress in hepatocytes [12]. It has also been reported that Vit. E intake has a protective effect on the progression of certain cancer types [13,14]. Vit. E has been shown to protect liver damage induced by oxidative stress in animal experiments [15,16], but effects of Vit. E on cancer cells it has not been well studied. Thus, in the present study, it is aimed to investigate the effect of Vit. E on antioxidant enzyme activity in hepatocellular carcinoma (HepG2) cells.

MATERIAL and METHODS

The HepG2 cell line was used in the present study (ATCC Cat No. HB- 8065). Cells were grown in RPMI 1640 Medium containing 10% Fetal Bovine Serum (FBS), 50 mg/L Gentamicin sulfate and 300 mg/L L-glutamine in a cell culture incubator at 37°C in the presence of 5% CO_2.

According to cell viability test, the optimum proliferation conditions for HepG2 cell were determined as 100.000 cell/well for 24 h. The HepG2 cells were incubated with Medium containing different doses of Vit. E (50 mM, 50 µM and 10 µM) for 24 h; whereas Vit. E-free medium was added in cells of control group. The enzyme activities of SOD, CAT and GPx in cell lysates were measured at the end of 24 h.

Determination of Superoxide Dismutase (SOD) Activity

SOD activity was measured by the method developed by Sun et al.[17]. This assay involves inhibition of nitroblue tetrazolium reduction, with xanthine-xanthine oxidase. In the assay, xanthine-xanthine oxidase used as a superoxide generator.

Determination of Catalase (CAT) Activity

CAT activity was measured by the method stated by Aebi [18]. According to the method, decrease in absorbance at 240 nm of a reaction mixture consist of H_2O_2, in phosphate buffer, and sample is determined. The decrease in absorbance is proportional to enzymes activity in sample.

Determination of Glutathione Peroxidase (GPx) Activity

GPx activity was measured by the method stated by Paglia and Valentine [19]. Glutathione peroxidase (GPx) reduces the CumeneHydroperoxide while oxidizing glutathione (GSH) to oxidized glutathione (GSSG). The generated GSSG is reduced to GSH with consumption of NADPH by glutathione reductase (GR). The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP+ is indicative of GPx activity and the decrease of NADPH is proportional to GPx activity.

Bradford method was used for protein quantitation in cell lysates [20].

The Bradford assay is a protein determination method that involves the binding of CoomassieBrilliant Blue G-250 dye to proteins. This blue protein-dye form is detected at 595 nm by a spectrophotometer or microplate reader.

Statistical Analysis

Statistical comparison between treated and control groups were performed using one-way ANOVA with post hoc Duncan test. P values <0.05 were considered statistically significant.

RESULTS

The SOD activities of HepG2 cells treated with different Vit. E doses in comparison to the control group are presented in *Fig. 1*. The SOD activity in Vit. E-treated cells was found to be lower in comparison to control group. A significant decrease was determined in SOD activity of cells treated with 50 µM Vit. E.

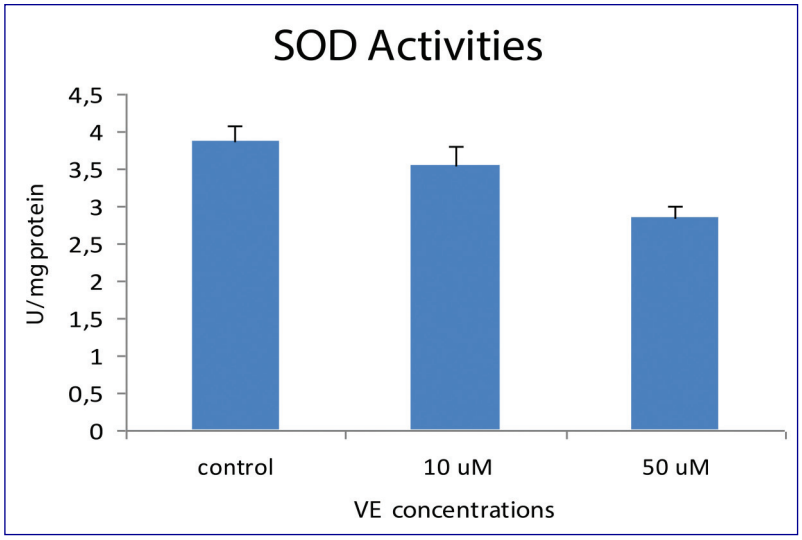
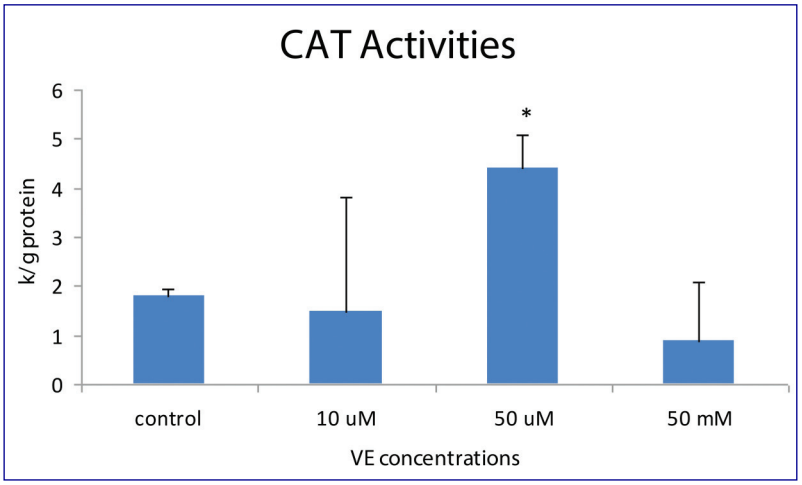


Fig 1. The SOD activities of HepG2 cells treated with different Vit. E doses

Şekil 1. Farklı dozlarda Vit. E uygulanan HepG2 hücrelerinde SOD aktiviteleri

Fig 2. The CAT activities of HepG2 cells treated with different Vit. E doses

Şekil 2. Farklı dozlarda Vit. E uygulanan HepG2 hücrelerinde CAT aktiviteleri



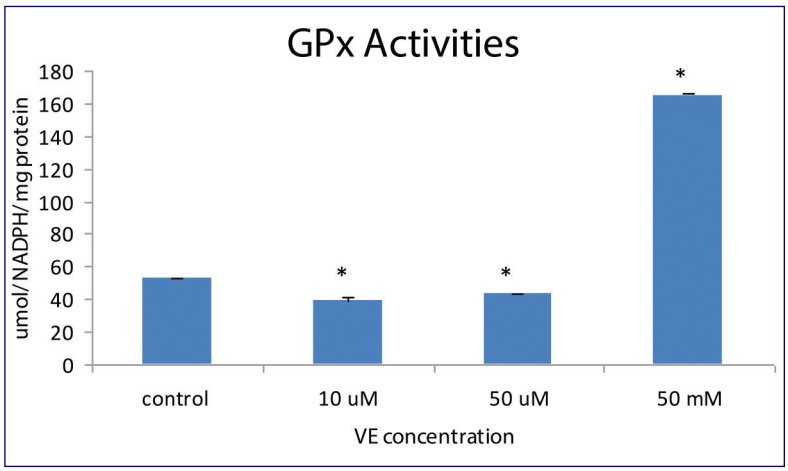


Fig 3. The GPx activities of HepG2 cells treated with different Vit. E doses

Şekil 3. Farklı dozlarda Vit. E uygulanan HepG2 hücrelerinde GPx aktiviteleri

The CAT activities of HepG2 cells treated with different Vit. E doses in comparison to the control group are presented in *Fig. 2*. A significant increase was determined in HepG2 cells treated with 50 μM Vit. E. However, a non-significant decrease was observed in HepG2 cells treated with 10 μM and 50 mM Vit. E.

The GPx activities of HepG2 cells treated with different Vit. E doses in comparison to the control group are presented in *Fig. 3*. Although, the GPx activity in HepG2 cells treated with 50 mM Vit. E was found to be higher in comparison to control group, it was lower than control group in cells treated with 10 μM and 50 μM.

DISCUSSION

It has been known that oxidative damage has a role in cancer pathogenesis. Oxidative damage and ongoing process may lead to DNA damage which is the basis of cancer development [21]. The deficiency at the level of antioxidant defense is important in the defense mechanism against free radicals. Antioxidants are the chemical components preventing the activation and formation of free radicals [22]. Enzymatic and non-enzymatic antioxidants are the first defense mechanism against toxicity generated by free radicals. The balance between prooxidants and antioxidants are important for normal cellular function. Vit. E shows its antioxidant activity by affecting free radicals on cell membranes [23].

CAT and GPx both act to scavenge SOD products, hydrogen peroxide. In the absence of adequate amounts of CAT, hydrogen peroxide might be expected to undergo conversion to highly toxic hydroxyl radicals by way of the Fenton type reaction [24]. Therefore, when the H_2O_2 product of SOD is probably more scavenged by GPx, and CAT, its activity is eventually decreased. Level of SOD activity compared with CAT activity is a key factor for efficient SOD activity, and that the combined activity of CAT and SOD could potentially lead to either positive or negative effects on the antioxidant defense potential [25].

In an *in vitro* study, the activities of intracellular antioxidant enzymes, SOD and CAT, were increased in rats receiving Vit. E diet. It has been claimed that Vit. E increases the strength of endogenous antioxidant defense by this way. Vitamin E can indirectly effects the activity of several transcription factors involved in the transcriptional regulation of Mn-SOD. Furthermore, as ROS are potent inhibitors of SOD, vitamin E, through its superoxide scavenging activity, can up-regulate SOD activity [26].

The SOD enzyme has three different isoforms; cytoplasmic Cu/Zn-SOD (SOD1), extracellular EC-SOD (SOD3) and mitochondrial Mn-SOD (SOD2). *In vivo* studies showed that the cytoplasmic SOD activity did not change with different doses of Vit. E (10, 30 and 100 mg/kg BW); however, an increase was observed in mitochondrial SOD activity. In these studies, Vit. E directly increased the transcriptional activity of SOD gene by upregulating the mRNA activity of mitochondrial SOD. The over-expression of cytoplasmic SOD gene has been observed in increased ROS production and oxidative catabolism [26]. In the present study, SOD activity was performed in cell line lysate. The absence of any variation may be due to the lack of prooxidant effect at low Vit. E levels.

In a study performed by Hajiani et al.[26], no significant variation was determined in CAT activity with low-dose Vit. E administration. On the other hand, the CAT activity was significantly increased at the Vit. E doses of 30 and 100 mg/kg. An increase in high dose Vit. E administration was observed after a long time. In the present study, no significant variation was observed in 10 uM Vit. E administration; whereas, the CAT activity was increased in 50 uM Vit. E administration.

Another issue that has been emphasized in studies performed with Vit. E is the determination of required dose for the formation of antioxidant effect. It has been thought that low-dose Vit. E is unable to show adequate antioxidant effect; however, high-dose Vit. E, which is defined as mega dose, may cause synergic toxicity by interacting with other substances [27]. This is originated from dose-dependent antioxidant effect of Vit. E. In the present study, the CAT activity in HepG2 cells treated with 50 µM Vit. E was found to be higher in comparison to control group. On the other hands, it was lower in cells treated with 10 µM and 50 mM in comparison to control group. It is thought that Vit. E increases the strength of antioxidant defense by showing higher antioxidant effect at the dose of 50 mM in HepG2 cells.

While the GPx activities of HepG2 cells treated with 10 µM and 50 µM Vit. E were found to be decreased, a significant increase was determined in HepG2 cells treated with 50 mM Vit. E in comparison to the control group. In certain types of cancers, such as prostate, breast, skin and lung cancer, it has been reported that Vit. E may block cancer progression, and *in vivo* and *in vitro* progression of prostate tumor was slowed down by Vit. E in mice treated with different doses of chemotherapeutic agents [28,29]. The radical forms of Vit. E develop at high-doses of Vit. E (alpha-tocopherol) as a result of prooxidant effect. The radicals of Vit. E require glutathione (GSH) for regeneration [30]. The increase in the activity of GPx in cells treated with 50 nm Vit. E may be the result of an increase in GPx activit due to the induction of Vit. E radicals.

Pre-data study results of molecular studies investigating the mechanism of action of Vit. E on antioxidant enzymes have showed that Vit. E shows its dose-dependent activity by effecting intracellular enzyme activity in HepG2 cells.

It is required to measure the antioxidant enzyme activities and cellular oxidant levels in extensive studies involving long-term administration of Vit. E.

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Research Article

Acute Effects of N-Acetylcysteine on Total Antioxidant Capacity, Total Oxidant Capacity, Nitric Oxide Level and Gammaglutamyl Transpeptidase Activity in Rabbits

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Abstract

N-acetylcysteine (NAC), acetylated derivative of cysteine amino acid, is widely used mucolytic agent. In this study, it was aimed to investigate the acute effects of NAC on total antioxidant capacity (TAC), total oxidant capacity (TOC), nitric oxide (NO), albumin, globulin, glucose levels and gammaglutamyl transpeptidase (GGT) activity. Eleven New Zealand rabbits were used in the study. Blood samples from the rabbits were taken before the start of the experiment to determine control values. NAC (100 mg/kg) was injected to rabbits as intramuscularly. Blood samples were collected from vena auricularis of rabbits at 3rd, 6th and 9th h. While TAC levels were found high ($P<0.05$) at 6 and 9 h, TOC levels were found low ($P<0.05$) at 3rd, 6th and 9th h after NAC injection compared to the values before the experiment. NO levels were found low ($P<0.001$) at 6th and 9th h, GGT activities were found low ($P<0.05$) at 3rd and 6th h, total protein and albumin levels were found low ($P<0.05$) at 3rd h after NAC injection compared to values before the experiment. As a result, after intramuscular injection of NAC, plasma TAC levels of rabbits increased, while TOC and NO levels decreased. According to the results, it can be concluded that NAC may show antioxidant effect in a short time. Therefore, NAC could be a good alternative when the antioxidant system needs to be strengthened due to its beneficial properties.

Keywords: *N-acetylcysteine, Total antioxidant capacity, Total oxidant capacity, Nitric oxide*

Tavşanlarda N-Asetil Sisteinin Total Antioksidan Kapasite, Total Oksidan Kapasite, Nitrik Oksit Düzeyleri ve Gamaglutamil Transpeptidaz Aktivitesi Üzerine Akut Etkileri

Özet

Sistein amino asidinin asetilenmiş türevi olan N-asetilsistein (NAS) mukolitik olarak kullanılan bir moleküldür. Yapılan çalışmada tavşanlara verilen NAS'ın total antioksidan kapasite (TAK), total oksidan kapasite (TOK), nitrik oksit (NO), albumin, globulin, glukoz düzeyleri ve gama glutamil transpeptidaz (GGT) aktivitesi üzerine akut etkilerini araştırmak amaçlanmıştır. Çalışmada 11 adet Yeni Zelanda ırkı tavşan kullanıldı. Denemeye başlamadan önce kontrol değerlerini saptamak için kan numuneleri alındı. Daha sonra tek doz kas içi NAS (100 mg/kg) enjekte edildi ve enjeksiyondan 3, 6 ve 9 saat sonra kan numuneleri alındı. Deneme öncesine göre total antioksidan seviyesi 6. ve 9. saatlerde yüksek ($P<0.05$) bulunurken, total oksidan seviyesi NAS verildikten sonra 3., 6. ve 9. saatlerde düşük ($P<0.05$) saptandı. Nitrik oksit düzeyleri deneme öncesi ile karşılaştırıldığında, denemenin 6. ve 9. saatlerinde düşük ($P<0.001$) bulundu. GGT aktivitesi deneme öncesi değerlerine göre, 3 ve 6. saatte düşük ($P<0.05$) saptandı. Benzer şekilde total protein ve albumin düzeyleri deneme öncesi değerlerine göre denemenin 3. saatinde istatistiksel olarak düşük ($P<0.05$) saptandı. Sonuç olarak, tavşanlara intramuscular NAS enjeksiyonunun plazma TAK düzeyini arttırırken TOK ve NO düzeylerini azalttığı bulundu. Bu sonuçlara göre NAS'ın kısa bir süre içinde antioksidan etki yapabileceği ve antioksidan sistemin güçlendirilmesine ihtiyaç duyulan durumlarda iyi bir seçenek olabileceği düşünülmektedir.

Anahtar sözcükler: *N-asetil sistein, Total antioksidan kapasite, Total oksidan kapasite, Nitrik oksit*

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INTRODUCTION

N-acetylcysteine (NAC) is the N-acetyl derivative of the L-cysteine amino acid. NAC is used in the treatment of respiratory tract diseases such as Chronic obstructive pulmonary disease (COPD), chronic bronchitis [1] and cardio-vascular system diseases such as myocardial infarction and coronary failure [2]. It was also reported that it played a role in detoxification of toxic chemicals in the liver [3], septic shock induced by oxidative stress [4] and reduction of harmful effects of reactive oxygen types (hydroxyl radical and singlet oxygen) [5]. N-acetylcysteine plays a role in the synthesis of an important antioxidant molecule, known as reduced glutathione (GSH), by giving the cysteine residue [6,7]. Furthermore, since it carries thiol group in its structure and it is a sulfhydryl group donor, it binds or reduces free radicals non-enzymatically, helping to clean the medium from hydroxyl radical, the same way other thiols function [5].

NAC, which is the precursor of glutathione, plays a role in the increase of intracellular glutathione storage and in keeping the cytoplasmic reserves constant [6]. GSH is found in two forms: reduced (GSH) and oxidized (GSSH). The proportion of these two forms (GSH/GSSH) was reported as a marker for the antioxidative cellular capacity [8].

Circulating concentrations of different antioxidants or oxidants can be measured separately, but the measurements are time-consuming, labor-intensive, and costly and they require complicated techniques. Because the measurement of different antioxidant/oxidant molecules, is not practical and antioxidant/oxidant effects are additive, total anti-oxidant response (TAR) or total oxidant status (TOS) of a sample is measured and this is named as total antioxidant capacity [9] or total peroxide [10].

Nitric oxide (NO) is a neurotransmitter with a toxic effect, small molecules and a short half-life, which is soluble in lipids, and moves through cellular membranes easily and has a high reaction capacity [11,12]. NAC, when administered orally or intravenously, blocks the reabsorption of NO precursors, nitrate and nitrite in the kidneys and causes their excretion, lowering the NO levels in the blood [13]. NAC also inhibits the inducible nitric oxide synthase (iNOS) activity, which is one of the enzymes that provides for NO production [14].

Serum GGT (GGT; EC 2.3.2.2) activity increases as a result of liver damage or when the immune system is activated [15]. The main function of GGT is to hydrolyze gamma glutamyl peptide bonds, transferring them to a receptor. The most important substrate of this reaction is the glutathione. Another significant function of the GGT is to protect the intracellular glutathione levels and to control the NO production from GSNO (S-Nitrosoglutathione) [16].

Based on these findings, it was aimed to investigate the effect of NAC on TAC, TOC, NO, albumin, globulin, glucose levels and GGT activity.

MATERIAL and METHODS

Eleven New Zealand Rabbits (Laboratory Animal Unit of the University of Kafkas, Kars, Turkey) of both sexes, aged between 7 and 9 months were used. Before the experimental procedure, consent for the study was taken from Kafkas University Animal Experiments Local Ethics Committee (KAÜ-HADYEK: 2012-65). The body weight was 2.200-3.000 g. They were kept in cages at room temperature (22-25ºC) with a 12:12 h light: dark cycle and fed a special pelleted rabbit diet as *ad libitum*. Before the experiment, blood samples were collected to obtain zero hours values. Then, animals were injected with single intramuscular dose of NAC, 100 mg/kg body weight (Asist®, Hüsnü Arslan İlaçları A.S, Istanbul, Turkey). Blood samples were taken from the marginal ear vein at 3rd, 6th and 9th h after injection into heparin treated tubes. Plasma was obtained by centrifugation at 3.000 rpm for 10 min and stored at -25°C until analyses.

Biochemical Analyses

All analyses were determined via spectrophotometer (PowerWave XS, BioTek, Instruments, USA)

Determination of total antioxidant and oxidant capacity: Total antioxidant capacities were determined colorimetrically using commercial kit (Rel Assay®, Gaziantep, Turkey) in plasma samples. Antioxidants in the sample reduce dark blue-green colored 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical to colorless reduced ABTS form. The change of absorbance at 660 nm is related with total antioxidant level of the sample. Plasma total oxidant capacities were determined with commercial kit (Rel Assay®, Gaziantep, Turkey). Oxidants present in the sample oxidize the ferrous ion-chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are present in reaction medium. The ferric ion makes a colored complex with chromogen in an acidic medium. The color intensity is related to the total oxidant molecules present in the sample at 530 nm. Trolox and hydrogen peroxide standards were used for total antioxidant and total oxidant capacities [9,10].

Determination of nitric oxide levels in plasma: Nitric oxide concentrations were determined with chemical method in plasma samples. Plasma samples were de-proteinized with 10% zinc sulphate. Total NO (nitrate and nitrite) concentrations were determined colorimetrically by the acidic Griess reaction [17].

Determination of other biochemical parameters in plasma: Gamma glutamyl transpeptidase (GGT) activity, total protein, albumin and glucose levels in plasma were determined commercial kits via colorimetrically

(TML®, Ankara, Turkey). The globulin concentration was calculated by subtraction of the albumin value from the total protein value.

Statistical Analysis

The data for biochemical parameters were analyzed by ANOVA followed by post hoc Tukey test using SPSS Windows 10.0. All data were presented as mean ± SE. Values were considered statistically significant if P value was less than 0.05.

RESULTS

After NAC injection, total antioxidant level was found higher at 6th and 9th h (P<0.05), total oxidant capacity level was found lower at 3rd, 6th, and 9th h (P<0.05) when compared to pre-experiment measurements. Nitric oxide levels were found statistically higher at 6th and 9th h when compared to pre-experimental measurements and the 3rd h of the experiment (P<0.001). GGT activity identified in the study was statistically lower only 3rd and 6th h after NAC injection than before experiment (P<0.05). Total protein and albumin levels found in the samples were lower on the 3rd h of the experiment compared to the pre-experiment levels (P<0.05). No statistically significant variations were observed in globulin and glucose levels during the experiment *(Table 1)*.

DISCUSSION

N-Acetylcysteine is reported as a molecule with antioxidant action due to the free thiol groups that exist in its easy cell-permeable structure and its role in reduced glutathione synthesis [18,19]. Effects of NAC on several toxic substances and disease conditions were studied [19-22]. However, there are not sufficient number of studies on acute effects of NAC on the antioxidant system and biomolecules without the existence of a disorder. Thus, it was aimed to research the effects of NAC on antioxidant system and certain biochemical parameters in healthy rabbits and the duration of these actions in the present study.

In this study, intramuscular application of 100 mg/kg NAC demonstrated its effects generally after 3 of application on most measured plasma parameters. It resulted in a statistical increase in TAC level after 3 h of the application and the increase lasted until the end of the experiment. In fact, the studies demonstrated that NAC displayed antioxidant properties and was effective. It was reported that the antioxidant action of NAC was through increasing liver blood flow, GSH levels and by removing free oxygen radicals [23,24].

In the present study, it was determined that there was a statistically significant decrease in TOC levels 3 hours after NAC application. This could be due to reactive oxygen type removal of NAC [25,26]. Bioactive aldehydes such as malondialdehyde (MDA) and hydroxy alkenal which are produced as a result of oxidation of lipid acids create cellular damage and cause an increase in oxidant molecule levels. Measurement of MDA levels is an important indicator to identify the cellular oxidant system when TOC level could not be determined. Altinoz et al.[27] investigated the effect of NAC against acrylamide toxicity in rats, it was reported that MDA levels increased as a result of acrylamide toxicity and MDA levels were reduced with NAC application [27]. Similarly, in a study studying the effect of NAC on myocardial ischemia-reperfusion damage, it was found that NAC reduced the MDA levels [28]. In another study, it was observed that lung and kidney MDA levels were lower in NAC administered group [29]. Results of the previous studies demonstrated that NAC caused reduction in MDA level, which is a product of lipid peroxidation and an indicator of oxidant capacity.

Khanna et al.[22] investigated the effect of NAC in cadmium toxicity on Leydig cells isolated from a 28 days old male mouse. In that study, it was argued that NAC

Table 1. *Plasma TAC, TOC, NO, albumin, total protein, globuline, glucose levels and GGT activity in N-acetylcysteine given rabbit*
Tablo 1. *N-asetil sistein verilen tavşanlarda plazma TAK, TOK, NO, total protein, globulin ve glukoz düzeyleri ile GGT aktivitesi*

Parameters	Before Experiment	After Experiment Hours			P
		3rd	6th	9th	
TAC (mmol Trolox Eq/L)	0.4±0.03^{b}	0.5±0.04^{ab}	0.6±0.7^{a}	0.6±0.2^{a}	0.05
TOC (µmol H_2O_2 Eq/L)	7.6±0.3^{a}	6.7±0.3^{b}	6.5±0.3^{b}	6.4±0.3^{b}	0.05
NO (mikromol/L)	29.1±2.9^{a}	25.3±2.7^{a}	13.4±1.2^{b}	18.0±2.0^{b}	0.001
Albumin (mg/dL)	3.8±0.04^{a}	3.5±0.08^{b}	3.6±0.06^{b}	3.7±0.03^{ab}	0.05
Total Proteine (mg/dL)	6.0±0.10^{a}	5.5±0.1^{b}	5.4±0.08^{b}	5.5±0.1^{b}	0.05
Globuline (mg/dL)	2.2±0.1	1.9±0.08	1.9±0.1	1.9±0.2	Ns
Albumine/Globuline	1.8±0.1	1.9±0.1	2.0±0.1	2.2±0.3	Ns
Glucose (mg/dL)	90.1±1.3	89.6±1.8	91.7±1.9	91.7±1.5	Ns
Gamma Glutamyl transpeptidase (U/L)	9.5±0.3^{a}	7.2±0.5^{b}	7.7±0.5^{b}	8.2±0.5^{ab}	0.05

^{a,b} The groups in the same line labeled different letters are statistically significant (P<0.05, P<0.001), ***Ns:*** *Non significant*

application lowered cadmium induced oxidative stress in Leydig cell, decreased the formation of oxidant molecules and could be used as a potential protective agent.

In fact, the findings of the present study demonstrated that NAC rapidly decreased the oxidant capacity. The decrease in TOC level could be explained by two mechanisms. One could be associated with the antioxidant effect of NAC due to the sulfhydryl groups in its structure, the other could be the increase in antioxidant capacity induced by the increase in reduced glutathione synthesis.

A paramagnetic free radical, NO, is formed as a result of oxidation of L-arginine citrulline by nitric oxide synthase enzyme. After synthesis and its action, NO is rapidly neutralized by haemoglobin or superoxide anion and transformed into nitrites or nitrates [30]. A statistical decrease in NO levels 6 hours after the experiment was identified in the present study. It was reported that intravenous NAC application reduced nitrate and nitrite excretion from kidneys both in healthy individuals and individuals who had heart surgery, decreasing bioavailability of NO. It was asserted that application of NAC and its metabolites (cysteine and glutathione) inhibits reabsorption of nitrite and nitrate by possibly inhibiting renal carbonic anhydrase (CA) activity or inorganic anion carriers such as bicarbonate, which provides alkalinity in urine [13]. In the present study, NAC application lowered nitric oxide levels in the plasma. One reason for that could be the inhibition of the reabsorption of NO metabolites nitrate and nitrite in urine. It was also argued that the effect of NAC on NO could be via removal of superoxide anions and inhibition of NO synthase activity [31]. In a study by Lee et al.[29] conducted to examine the effect of NAC to reduce organ injuries in Sprague-Dawley rats in a series of empirical and clinical studies, it was demonstrated that NO levels were significantly lower in the NAC administered group. This result clearly demonstrates the effect of NAC on NO metabolism. In a study that investigated the effect of NAC on cyclophosphamide-induced cardiotoxicity in rats, it was recorded that NAC application prevented oxidative and nitrosative stress and promoted the protection of antioxidant enzyme activity [32]. The changes observed in TAC, TOC, and NO levels in samples after NAC injection were generally similar with previous studies [23,24,28,31].

In the present study, a statistical decrease was found in albumin levels after 3 and 6 h after the experiment but on the 9th h albumin level reached to normal limits. Similarly, a statistical decrease was identified in total protein levels 3 h after NAC injection. Findings of the present study differed from the results shown in other studies. In a study that investigated the effects of ursedeoxycholic acid, resveratrol and NAC in nonalcoholic hepatic lipidosis in rats, it was reported that joint administration of resveratrol and NAC brought glucose, albumin, MDA, GSH, triglyceride, low density lipoprotein and leptin levels back to normal [33]. In the current study, following the NAC injection, no variations were observed in globulin and glucose levels when compared to pre-experiment measurements.

Gammaglutamyl trans peptidase is a ectopeptidase responsible for the degradation of glutathione in the gammaglutamyl cycle [34]. In the present study, it was determined that GGT activity started to decrease 3 h after the experiment and reached normal levels on the 9th hour. This shows that NAC could be a key enzyme in the synthesis of glutathione and GGT.

Glutathione biosynthesis via gammaglutamyl cycle is important for maintaining GSH homeostasis and normal redox status. GGT also initiates the metabolism of glutathione S-conjugates to mercapturic acids by transferring the gamma-glutamyl moiety to an acceptor amino acid and releasing cysteinylglycine [35]. If GGT activity is above normal, GSH is destroyed by GGT enzyme and this situation is extremely important for the antioxidant defense system. In fact, the decrease in GGT activity as a result of the injection was consistent with the information mentioned above.

As a result, after intramuscular injection of NAC, plasma TAC levels of rabbits increased, while TOC and NO levels decreased. According to the results, it can be concluded that NAC may show antioxidant effect in a short time. Therefore NAC could be a good alternative when the antioxidant system needs to be strengthened due to aforementioned properties.

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Research Article

Localisation of Estrogen Receptor Alpha and Progesterone Receptor B in Goat Ovaries During Breeding and Non-Breeding Season [1] [2]

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[2] *Summary of this study has been presented in "48th Annual Conference Physiology and Pathology of Reproduction and simultaneously 40th Joint Congress of Veterinary and Human Medicine February Conference, Zurich, Switzerland, 11th - 13th February 2015"*

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Abstract

The main objective of this study was to investigate the localisation of estrogen receptor alpha (ER-α) and progesterone receptor B (PR-B) by immunohistochemistry in goat ovaries during in- and out of breeding season. The ovaries obtained from goats slaughtered in breeding season (n=10) and non-breeding season (n=10) were used. From the same animals, blood samples were taken to determine the levels of serum steroid hormones (E_2, P_4). The ER-α and PR-B immunohistochemical distributions within the ovaries were determined by the ABC method. In breeding season, the ER-α was detected in the germinal epithelium and follicular granulosa cells. The PR-B was determined to concentrate on the corpus luteum (CL) cells. The same receptors were also found to be weak in the theca externa cells of preovulatory follicles. In non-breeding season, the ER-α germinal epithelium and smooth muscle cells of certain blood vessels showed a weak positive reaction. The PR-B was positively stained in the germinal epithelium and few stroma cells. It was suggested that the average steroid hormone profiles in breeding (E_2: 11.83±1.70 pg/mL, P_4: 10.08±1.58 ng/ml) and non-breeding season (E_2 2.33±0.85 pg/ml, P_4 0.21±0.04 ng/ml) correlated well with the localisation intensity of receptors in goat ovaries.

Keywords: *Goat, Ovary, Immunohistochemistry, Estrogen receptor α, Progesterone receptor B*

Keçi Ovaryumunda Üreme Sezonu İçinde ve Dışında Östrojen Reseptör alfa ve Progesteron Reseptör B'nin Lokalizasyonu

Özet

Sunulan bu çalışmada, üreme sezonu içinde ve dışındaki keçi ovaryumlarında, östrojen reseptör alfa (ER-α) ve progesteron reseptör B'nin (PR-B) yerleşim yerlerinin immünohistokimyasal olarak araştırılması amaçlandı. Mezbaha şartlarında kesilen dişi keçilerden, üreme sezonu içinde (n=10) ve dışında (n=10) elde edilen ovaryumlar kullanıldı. Aynı hayvanların, serum steroid hormon düzeylerini (E_2, P_4) belirlemek için kan örnekleri alındı. ER-α ve PR-B'nin ovaryumlardaki yerleşimleri immünohistokimyasal olarak ABC metodu ile belirlendi. Üreme sezonu içinde, ER-α reseptörleri germinatif epitelyum ve foliküllerin granüloza hücrelerinde saptandı. PR-B reseptörlerinin ise corpus luteum (CL) hücrelerinde yoğunlaştığı belirlendi. Aynı reseptörlerin, preovulator foliküllerin teka eksterna hücrelerinde de zayıf olarak bulundukları gözlendi. Üreme sezonu dışında, ER-α reseptörleri germinatif epitelyum ve bazı kan damarlarının düz kas hücrelerinde zayıf pozitif reaksiyon gösterdi. PR-B ise germinatif epitelyum ve bazı stroma hücrelerinde pozitif boyandı. Keçilerde, üreme sezonu içi- (E_2: 11.83±1.70 pg/mL, P_4: 10.08±1.58 ng/mL) ve dışındaki (E_2: 2.33±0.85 pg/ml, P_4: 0.21±0.04 ng/ml) ortalama steroid hormon profillerinin bu reseptörlerin ovaryumlardaki lokalizasyon yoğunlukları ile uyumlu olduğu kanısına varıldı.

Anahtar sözcükler: *Keçi, Ovaryum, Immünohistokimya, Östrojen reseptör α, Progesteron reseptör B*

INTRODUCTION

Essential steroid hormones for reproduction are secreted from the ovary; such that progesterone is secreted from the corpus luteum (CL) while estrogen is secreted from ovarian follicles in different developmental stages [1]. These hormones play a crucial role in morphologic and functional changes in the reproduction organs in females [2].

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Estrogen is a well-known regulator in steroidogenesis and folliculogenesis. It regulates follicular development by stimulating the ovarian granulosa cell proliferation. Follicle stimulation hormone (FSH) secretion is also very important in the regulation of gap junctions between the granulosa cells [3,4]. There are two specific estrogen receptors, so called as the estrogen receptor alpha (ER-α) and estrogen receptor β (ER-β), that are localised dispersedly within the ovarian tissue and responsible for the intraovarian movements [5,6]. Localisation and distribution of these two receptors given have been defined in immunohistochemical studies conducted in various animal species [7-10]. The ER-α and ER-β are localised within the germinal epithelium, interstitial tissue and follicles in different developmental levels. Localisation places of two receptors could change according to the species of the animal and hormonal periods [5].

Progesterone hormone, secreted from the CL, is also critical in regulating reproductive cycles in mammals. It has primary function in implantation; supporting uterus with uterine milk and sustaining pregnancy following the embryo enters into the uterus [11]. The progesterone (P_4) gets activated after binding to the intracellular progesterone receptors (PR) in target tissues. There exist two isoforms of progesterone receptors A - B, (PR-A, PR-B) [12,13]. Further, there is a progesterone receptor C (PR-C), but it is seen in pathologic situations such as breast cancer [14]. Progesterone receptors have been studied in women [6], cows and bitches in different stages of their estrous cycles [15,16].

Researchers have long been working on reproduction to increase the productivity in goat breeding, as in other farm animals. In the literature, no data could be found on the immunohistochemical investigation and comparison of ER-α and PR-B in goat ovaries during breeding or non-breeding seasons. Therefore, the aim this study was to determine the localisation areas of ER-α and PR-B immunohistochemically in the ovaries of goats during in- and non-breeding season.

MATERIAL and METHODS

Animals and Tissues

An official approval from the YYU Animal Experiments and Ethical Council was received (dated on 16.05.2013, with decision number 05) prior to this study. In breeding- (n=10) and non-breeding season (n=10), goat ovaries were acquired from Van City slaughterhouse. Prior to slaughtering animals, 5 ml of blood samples were collected and their sera were extracted to determine estrogen (E_2) and P_4 hormone levels. The concentrations were measured with appropriate kits with ELISA system [17,18].

The ovaries collected were fixed with 10% formaldehyde for 24 h after morphologic evaluation; tissues were blocked with paraplast after routine histologic processes [19]. Serial sections of 5 µm obtained from each tissue were stained immunohistochemically to determine the ER-α and PR-B receptors.

Hormonal Procedures

Following samples of 5 ml blood were taken and serum extracted, estrogen and progesterone hormone levels were measured by Goat Estradiol (E_2)/Progesterone (P_4) ELISA Kits (Cusabio) respectively, according to the manufacturer's recommendations.

Immunohistochemical Procedures

Tissues were stained immunohistochemically with ABC method [20]. Tissue sections (4 µm) were taken on polysine-coated slide (Thermo scientific, Menzel -Glaser, Germany). Antigen retrieval (citrate buffer 10%, pH: 6) was applied for 40 min boiling after rehydration of deparaffinised sections. Peroxidase blockage was applied in H_2O_2 for 20 min to prevent non-specific bindings after cooling to room temperature (in 3% methanol). Protein blockage was performed by 10 min incubation with 1/5 concentrated rabbit serum after washing in phosphate buffer solution (PBS). They were incubated at room temperature for 2 h with 1/100 anti-estrogen receptor α (Abcam, ab75635), 1/50 anti-progesterone receptor B (Abcam, ab2765) primary antibodies. Then, PBS washing was performed. Conjugated secondary antibody with biotin was applied for 20 min. Streptavidin peroxidase was applied for 20 min after PBS washing for 20 min. Afterwards, firstly staining with AEC for 10 min (Zymed, 3- Amino-9-ethylcarbazole) and then counterstaining with Mayer's haematoxylin, enclosed with an aqua-based glue, were performed. In the immunohistochemistry for ER-α and PR-B, the staining intensity was graded semi-quantitatively as; no immunostaining (-), weak staining (+), moderate staining (++), and strong staining (+++).

Statistical Analysis

Data from the estrogen and progesterone concentrations of goats in breeding- and non-breeding seasons were analysed by regression analysis using MINITAB. Differences of means (±SEM) between the experimental groups were considered significant when $P<0.05$ [21].

RESULTS

Hormonal Results

Average levels of E_2 and P_4 hormones in goats were 11.83±1.70 pg/ml and 10.08±1.58 ng/ml during breeding season, and 2.33±0.85 pg/ml and 0.21±0.04 ng/ml in non-breeding season, respectively *(Fig. 1, Fig. 2)*.

Immunohistochemical Results

Stained sections were evaluated according to the frequency of reaction (-), (+), (++), and (+++) in the ER-α

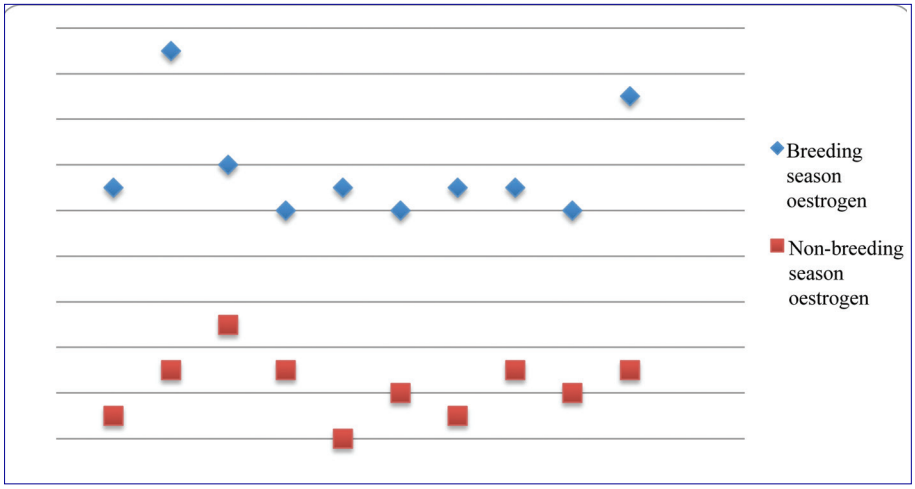


Fig 1. Seasonal distribution of serum estrogen levels (pg/ml)

Şekil 1. Sezonal serum östrojen düzeyi dağılımı (pg/ml)

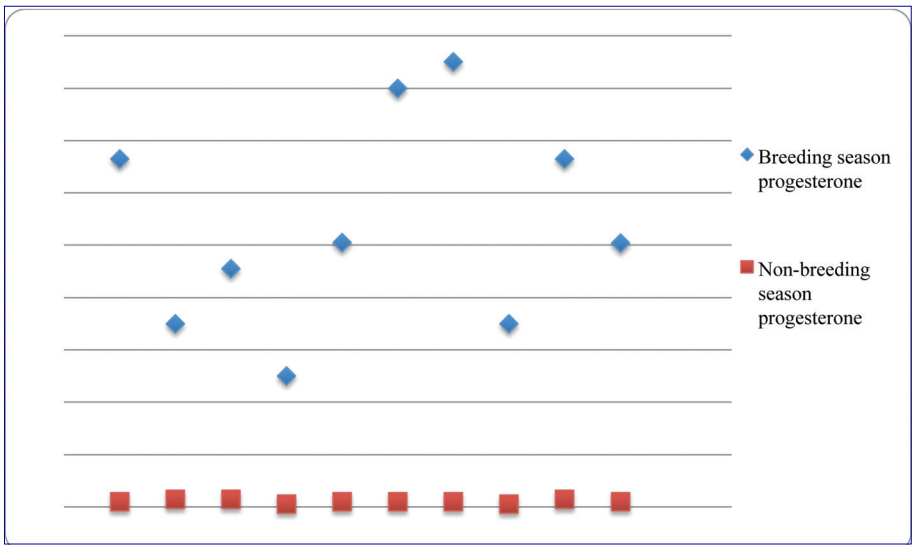


Fig 2. Seasonal distribution of serum progesterone levels (ng/ml)

Şekil 2. Sezonal serum progesteron düzeyi dağılımı (ng/ml)

Table 1. *ER-α and PR-B receptor reaction intensity*
Tablo 1. *ER-α ve PR-B reseptör reaksiyon yoğunluğu*

Receptor	Breeding	Non-Breeding
ER-α	Primary, secondary, tertiary follicle, preovulatory follicle granulosa cells (+++) Germinative epithelium (++) Wall of some vessels (+)	Germinative epithel (++) Wall of some vessels (+)
PR-B	In CL luteal cells (+++) Corpus albicans (++) epithelium, Theca int/ext (+)	Corpus albicans (++) Germinative epithelium (+) Stromal cells (+)

and PR-B receptor staining, and photos were taken (Leica ICC50) *(Table 1)*.

In breeding season, the ER-α reacted as (+) in ovarian primary, secondary, tertiary, and preovulatory follicles, as well as germinative epithelium and some smooth muscles in the walls of some blood vessels *(Fig. 3A-B)*. Reactions were observed for the PR-B as (+) in ovarian CL, corpus albicans, germinative epithelium, theca interna and externa, and stroma *(Fig. 4A-B)*.

In non-breeding season, the ER-α was observed (+) in ovarian germinative epithelium and the wall of small-shaped vessels *(Fig. 3D)*. The PR-B was observed (+) in ovarian germinative epithelium, corpus albicans cells, and stroma cells *(Fig. 4D)*.

Negative control was photographed in- and out of breeding season *(Fig. 3C, Fig. 4C)*.

DISCUSSION

There appears to be no data available on the ER-α and PR-B in goat ovaries in the literature survey, thus making the present study scientifically unique and valuable. So,

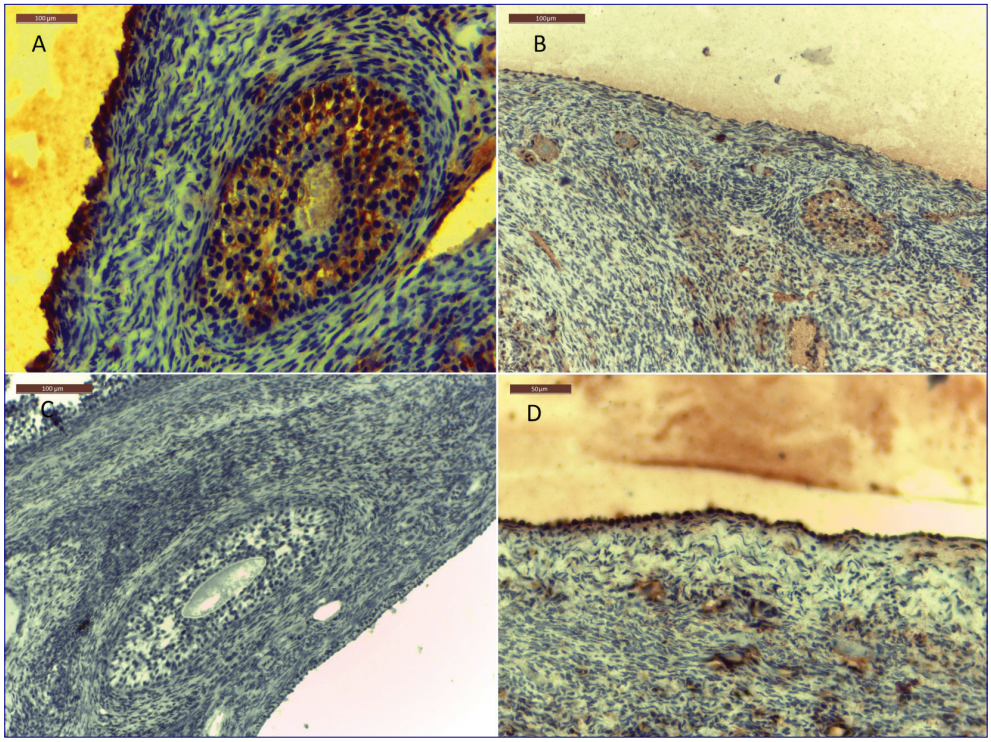
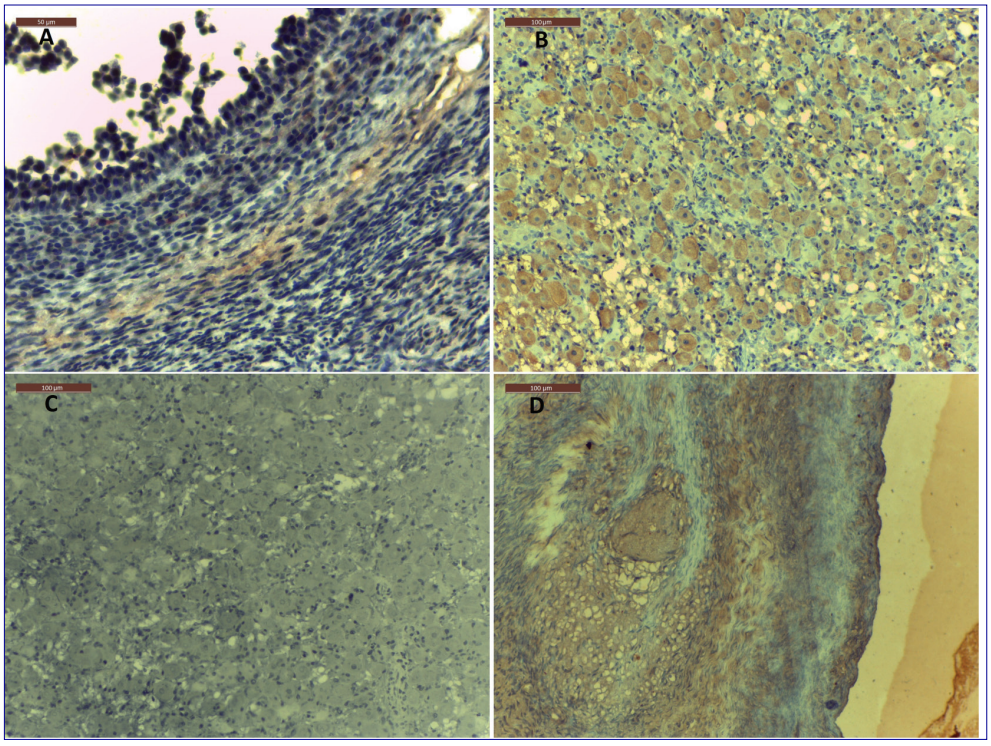


Fig 3. A- The ER-α in breeding season as (+) in ovarian, secondary follicles, germinative epithelium and some smooth muscles within the wall of some blood vessels, **B-** The ER-α in breeding season as (+) in ovarian primary and secondary follicles, germinative epithelium and some smooth muscles within the wall of some blood vessels, **C-** The ER-α as negative control in breeding season, **D-** In non-breeding season, the ER-α as (+) in ovarian germinative epithelium and wall of small-shaped vessels

Şekil 3. A- Ovaryumda üreme sezonunda, sekonder foliküller, germinatif epitelyum ve kan damarları duvarındaki düz kaslarda ER-α (+), **B-** Ovaryumda üreme sezonunda primer, sekonder foliküller, germinatif epitelyum ve kan damarları duvarındaki düz kaslarda ER-α (+), **C-** Üreme sezonunda ER-α negatif kontrol, **D-** Ovaryumda üreme sezonu dışında germinatif epitelyum ve bazı kan damarları duvarında ER-α (+)

Fig 4. A- The PR-B as (+) in ovarian theca externa, **B-** The PR-B as (+) in ovarian CL, **C-** The PR-B as negative control in breeding season, **D-** The PR-B (+) in ovarian germinative epithelium, corpus albicans cells, and stroma cells during out of breeding season

Şekil 4. A- Üreme sezonunda ovaryumda teka eksterna PR-B (+), **B-** Üreme sezonunda corpus luteumda PR-B (+), **C-** Üreme sezonunda PR-B negatif kontrol, **D-** Üreme sezonu dışında germinatif epitelyum, corpus albicans ve stroma hücrelerinde PR-B (+)



our results obtained herein will be compared with those in other animal species studied rather scarcely.

In cattle, the PR localised in secondary and tertiary follicles, granulosa cells, theca interna cells, and theca externa cells. It was concluded that progesterone production is regulated not only by granulosa cells but also by theca interna and theca externa. Also, we claimed herein that progesterone has negative impact on the PR immunohistochemical staining, as reported earlier in cattle [22]. Further, we observed that there was a positive reaction of PR-B with theca interna and theca externa cells in breeding season. Indeed, positive reactions were also observed in the cells of CL, germinal epithelium and stroma.

Further, The PR in cows was investigated and progesterone

membrane component 1 stained immunohistochemically in estrous cycle. The cells of granulosa, theca, stroma, endothelium, germinal epithelium and the CL showed (+) reactions in both follicular and luteal phases. In this study, the localisation of receptors was determined in cytoplasm and nucleus of the cells of granulosa, theca, endothelium, stroma, germinal epithelium, and the CL. We concluded that the PR showed weak reaction in follicular and luteal cells. The present study in goats had similarities with studies like immunohistochemically staining (+) of PR in germinative epithelium, theca and CL luteal cells [23].

To prove the relationship between steroid hormones and progesterone, Vermeirsch et al.[16] determined in dog ovaries that although the PR stained (+) in surface epithelium, and the cells of follicle, theca, and the CL, but PRs stained (-) in vessel wall, stroma cells and oocyte. Herein, we observed that the PR showed (+) reactions immunohistochemically with surface epithelium, theca, and luteal cells. Unlike the findings in dog ovaries, we found that there was (+) staining in ovarium stroma. In dogs, progesterone level in estrus was 3.33±2.49 ng/ml while the E_2 was 12.42±8.05 pg/ml. In this study, progesterone hormone level in anestrous period was 0.61±0.56 ng/ml, while E_2 hormone level was 4.26±3.49 pg/ml. Differences in both P_4 and E_2 levels arise mainly the stage of sexual cycle and species of the animals (estrus in dogs *vs.* anoestrus in goats).

In mouse, the relationship between the age and reproduction cycles of animals was investigated based on androgen and PR localisation and distribution in the ovary. It was concluded that the PRs are more connected with the cycle phase rather than the animal's age [24]. In the present study however, no such comparison was made since animals used herein were all mature individuals being slaughtered.

In another study conducted in cows, localisations and densities of E_2 and progesterone receptors of ovaries during the luteal and follicular phases were compared [20]. It was reported that the ER-α was immunohistochemically stained (+) in all developmental phases in the cells of granulosa, germinal epithelium, stroma, theca, the CL and corpus albicans from primary follicle to mature follicle, while the PR-B gave immunohistochemically positive results with germinal epithelium, stroma cells, corpus albicans and the CL. According to the localisation of receptors given, similar results were obtained in goats studied herein, as compared to those in cow ovaries.

Hulas-Stasiak and Gawron [5] studied the ER-α and ER-β receptors immunohistochemically in spiny mouse ovaries and found the localisation of ER-α. Granulosa cells carried α receptors in all stages of developing follicle. Nucleus contained the ER-α in the cells of prenatal follicles, theca, and pre-CL. However, the atretic follicles did not show such staining. Similar to their study, the receptors in granulosa cells of developing follicles showed staining in goat ovaries. Further, the ER-α were lacking in any phase of the CL cells herein. Also, Li et al.[25], studying distribution of ER-α and PR in mouse ovaries, uterus and oviduct, reported that ovarian ER localised in granulosa cell nuclei and interstitial cells, while the PR localised in prenatal follicle nuclei. They obtained some receptor localisations similar to the ovarian findings herein. Additionally, Sar and Welsch [26], studying about separating the ER-α and ER-β in rats, determined that beta-receptors are localised in the middle of some follicle nuclei. The ER-α was detected in uterus and oviducts of adolescent and non-adolescent rats. In our study however, the receptors were observed in nucleus.

Korte et al.[27] determined that the more distributed the receptors the lesser the hormone (P_4) concentration in rabbit ovaries. In the ovaries of canine [16], primate [7] and porcine [23], a profound negative correlation was observed between hormone levels and receptor distributions. In the present study, we found similar results (negative correlation between hormone level and receptor localisation) in goats.

It was concluded that, as expected, the localisation intensity of estrogen and progesterone receptors within the ovaries correlated well with their respective steroid hormone profiles in goats during breeding and non-breeding seasons. Determining the estrogen and progesterone profiles (including their intraovarian receptor sites), thus understanding the ovarian functions in more detail would provide crucial data for future studies on goat reproduction in different seasons. Present results might also be helpful (with receptor sites and their localisation density) for greater understanding the relationship of endocrinological and receptor cascades of reproduction in other animals showing seasonal breeding (such as ewe, mare, bitch, etc.).

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Research Article

Effect of N-Acetylcysteine (NAC) on Post-thaw Semen Quality of Tushin Rams [1]

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Abstract

The aim of the present study was to investigate the effect of N-acetylcysteine (NAC) on freezability of Tushin ram semen. Ejaculates from four Tushin rams were collected with artificial vagina and then pooled. Pooled semen was divided into four aliquots to be diluted with skim milk-based-egg yolk-glycerol (SEG) extender supplemented with various concentrations of NAC (0, 0.25, 0.5 and 0.75 mM). The semen was loaded into 0.25 mL straws, equilibrated (at 4°C for 2 h), frozen in liquid nitrogen (LN) vapour (at -120°C for 15 min) and stored in LN (-196°C). After thawing (at 37°C for 1 min), sperm motility, dead-live ratio, morphology, abnormal acrosome and membrane integrity (HOST) were evaluated. Results showed that 0.75 mM NAC has detrimental effects on motility, compared to the other three NAC doses evaluated (P<0.05). Membrane integrity was higher in 0.25 and 0.5 mM NACs. There was significant differences in semen viability among NAC doses (P<0.05). In conclusion, higher doses of NAC, especially used with SEG extender, may have some detrimental effects on freezability of ram semen. Moreover, although modest doses of NAC slightly improved freezability of Tushin ram semen.

Keywords: *Tushin ram, Semen, Cryopreservation, N-acetylcysteine, Post-thaw semen quality*

N-Asetilsistein (NAC)'in Dondurulmuş-Çözdürülmüş Tuj Koçu Spermasının Kalitesi Üzerine Etkisi

Özet

Çalışmanın amacı, Tuj koç spermasının dondurulabilirliği üzerine N-asetil sistein (NAC)'in etkisini araştırmaktı. Dört tuj koçundan suni vajen ile alınan sperma birleştirildi. Karışım sperma dört eşit hacme bölündü ve 0 mM, 0.25 mM, 0.5 mM ve 0.75 mM NAC içeren yağsız süt yumurta sarısı ve gliserollü sulandırıcı ile sulandırıldı. Sperma 0.25 mL'lik payetlere çekildi, ekilibre edidi (4°C'de 2 saat), sıvı azot buharında dondurulduu (-120°C'de 15 dak.) ve sıvı azot (-196°C) içinde saklandı. Çözüm (37°C'de 1 dak.) sonrası, sperm motilitesi, ölü-canlı oranı, morfolojisi, anormal akrozom ve membrane bütünlüğü (HOST) değerlendirildi. Sonuçlar, 0.75 Mm NAC'ın, diğer NAC dozları ile karşılaştırıldığında, motilite üzerine olumsuz etkilere sahip olduğunu gösterdi (P<0.05). Membran bütünlüğü 0.25 ve 0.5 mM NAC derişimlerinde en yüksekti (P<0.05). Ayrıca, NAC dozları arasında, sperma canlılığı açısından önemli farklar da belirlendi (P<0.05). NAC'ın yüksek dozlarının, düşük dozları dondurulabilirliği bir miktar iyileştirse de, özellikle sütlü sulandırıcılarla kullanıldığında, koç spermasının dondurulabilirliği üzerine bazı olumsuz etkileri olabileceği sonucuna varıldı.

Anahtar sözcükler: *Tuj koçu, Sperma, Dondurma, N-asetilsistein, Çözüm sonu sperma kalitesi*

INTRODUCTION

Tushin sheep breed of the mutton-wool-milk type was bred in Georgia in the 13th-14th centuries under conditions of year-long range husbandry. The breed derives its name from the Tushins, the people of Tushetia, the mountain district where they were developed. According to the breed regionalization plan, the Tushin breed is raised mainly in the Georgian and partly in Azerbaijan, in today's Armenia and in some districts of North East of Turkey (Kars province). Tushin sheep have accommodated to this region hard climate's condition. Its number is estimated as 200.000 and approximately 0.65% of total sheep presence in Turkey [1]. Therefore, this breed can be considered at risk of

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extinction. Cryopreservation of semen has been considered most important assisted reproductive technique used for protecting extinction breeds. There have been limited number studies carried on cryopreservation and freezability of Tushin ram semen. The reactive oxygen species (ROS: H_2O_2, O_2, OH, ROOH) and antioxidant defences have been shown to play an important role in fertility and infertility [2,3]. Direct and indirect evidence indicated that some steps of cryopreservation of semen involve the production of toxic ROS [4,5]. Also Griveau and Le Lannou [6] reported that ROS in the ejaculate are produced by the sperm and by leucocytes that infiltrate semen. One of the various ROS, the hydrogen peroxide (H_2O_2) decreased sperm motility in many species and it is known that thiols may prevent H_2O_2 mediated loss of spermatozoa motility in cryopreserved bull semen [7-9] and also in chilled equine semen [10]. Thiols are a large class of antioxidants that includes cysteine, N-acetylcysteine (NAC), and glutatione (GSH). NAC is precursors of intracellular GSH biosynthesis [11]. There is not any study on Tushin ram semen cryopreservation and also NAC supplemented to milk based extenders in ram semen. Therefore, the aim of the present study was to evaluate the quality of Tushin ram semen after it had been extended and cryopreserved with milk based extender containing different amount of NAC.

MATERIAL and METHODS

The experiment was carried out according to guidelines for animal research from the National Instutites of Health and all procedures on animals were approved by the Kafkas University Ethic Committee on Animal Research in current study (Approval date/Number: KAÜ-HADYEK 2009/02). All chemicals in current study were provided from Sigma-Aldrich if not stated.

Animals

Four healthy and sexually mature (2 years old) Tushin rams were used in this study. Rams were housed at Education Research and Practice Farm, Faculty of Veterinary Medicine, University of Kafkas Turkey at 40°34'33"N, 43°02'35"E at an altitude of 1751 m. Rams were trained for artificial vagina and were used routinely as semen donors twice a week before the study. They were fed 0.91 kg of concentrate daily, and good quality hay and water were supplied *ad libitum*.

Semen Collection

The four rams were trained to mount a ewe in estrus and serve the artificial vagina. Ejaculates were obtained from each ram by artificial vagina according to the technique previously described by Salamon and Maxwell [12]. Ejaculates were collected twice a week during this experiment. The volume and mass activity were recorded before the tube was placed in a water bath at 37°C. Each ejaculate with 3+ mass activities was analysed to determine its semen concentration, total number of spermatozoa per ml and semen motility, so that adequate semen quality was secured before ejaculates of the two rams were pooled. Only ejaculates with motility >75%, sperm concentration of >2 x 10^9 spermatozoa/mL and semen volume of >0.5 mL were included in this study. Sperm concentration was determined using hemocytometric method, after diluting semen with Hayem solution (Dilution rate 1/500). The percentage of motile spermatozoa was estimated by subjective microscopic examination using a phase contrast microscope supplied with heated stage at 37°C and magnification 400x after dilution with extender (Dilution rate 1:10).

Semen Cryopreservation and Experimental Groups

The skim milk based extender was all prepared within 1 week period and kept at 5°C. The composition of skim milk based solution was 10 g skim milk powder, 0.9 g glucose, formulated on the basis of 100 mL. 10% (v/v) egg yolk and 5% (v/v) glycerol were added to skim milk based solution. NAC (0 mM, 0.25 mM, 0.5 mM and 0.75 mM) were added to skim milk-egg yolk-glycerol (SEG) extender containing 500 IU of penicillin and 500 µg of streptomycin sulphate per mL. Immediately after collection and evaluation, semen was extended with SEG supplemented with 0 mM, 0.25 mM, 0.5 mM or 0.75 mM NAC. Diluted semen was loaded into 0.25 ml French straws constituting doses of 100 X 10^6 spermatozoa per straw. Plastic straws were sealed with polyvinyl alcohol powder. The straws were placed in refrigerator at 5°C. The semen was allowed to equilibrate for 2 h before freezing. After equilibration, the straws were frozen horizontally on a rack about 4 cm above liquid nitrogen (LN_2) held in an insulated container. The nitrogen vapour reduced the temperature within the straws to -120°C in approximately 15 min. Then the straws were transferred rapidly to LN_2 containers at -196°C. The straws were stored in LN_2 until evaluation time.

Post-thawing Semen Analyses

After 2 months storage, two straws from each treatment groups (0 mM, 0.25 mM, 0.5 mM, 0.75 mM NAC) were thawed in a warm bath (37°C). After 1 min, the contents of the straw were examined microscopically as described at below. Totally 6 replications were carried out to determine effect of NAC on cryopreservation of ram semen. After thawing of straws, a 3 µL aliquot of each sample was placed on a warmed (37°C) slide and covered with a cover slip before examination under a phase-contrast microscope (Nikon Eclipse E400, Nikon Corp., Japan) at 400x magnification. After observing four or five different fields, the percentage of motile sperm cells was recorded for each sample. Throughout the experiment, two technicians evaluated all the samples without knowing experimental groups and their mean values were recorded as a percentage. For evaluation of acrosomal and morphological abnormalities, one drop sample from groups was diluted in

1 mL Hancock's solution (prepared with 62.5 mL formalin, 150 mL sodium saline solution, 150 mL buffer solution, and 500 mL distilled water [13], placed under a coverslip and evaluated by phase-contrast microscopy (Nikon Eclipse E400, Nikon Corp., Japan) under immersion. The morphological alternations were classified as described by Ax et al.[14]. To evaluate membrane integrity, 50 µL of semen sample was diluted with 450 µL of 100 mM hypotonic solution (composed of 9 g fructose plus 4.9 g sodium citrate per liter of distilled water). After 1 h, smear was prepared and evaluated considering sperm tail curling (%) using bright-field microscopy (Olympus CX21, Olympus Optical Co. Ltd., Japan) (Hypo-osmotic swelling Test/HOST), spermatozoa with curved tail were considered as membrane intact [15]. Percentage of viability in NAC doses after thawing was evaluated with eosin staining (Eosin-Y 1.67 g and sodium citrate 2.9 g dissolved in 100 mL distilled water) as described by Ax et al.[14]. The sperm smears were prepared by mixing a drop of semen with two drops of stain on a warm slide and spreading the stain immediately with the aid of a second slide. The viability was assessed by counting 200 sperm cells with bright-field microscopy (400x) (Olympus CX21, Olympus Optical Co. Ltd., Japan). Sperm showing partial or complete colorization were considered non-viable or dead. Only sperm showing strict exclusion of the stain were considered to be alive [14].

Statistical Analysis

The mean post-thaw semen parameters of motility, dead spermatozoa, abnormal spermatozoa and hypo-osmotic swelling test (curling tail spermatozoa) for the 6 trials carried out during this study were subjected to analysis of variance (One way ANOVA), and differences among means were tested for significance by the Fisher's PLSD. The SPSS 10.0 software was used for all statistical analyses. Differences with values of P<0.05 were considered to be statistically significant.

RESULTS

Results were summarized in *Table 1*. All experimental doses of NAC had similar progressive motility when the results according to six replications were considered to evaluate different doses of NAC, except 0.75 mM NAC (19.1%) (P<0.05). 0.25 mM dose of NAC provided lowest percentage of abnormal acrosome (35.2%). Whilst 0.5 mM NAC had highest membrane integrity and viability percentage (43.7 and 34.7% respectively), 0.75 mM NAC had lowest membrane integrity and viability percentages (37.3 and 28.7 respectively), compared with the other doses of NAC (P<0.05). Moreover, 0.75 mM NAC had highest dead spermatozoa (lowest viability), abnormal spermatozoa and abnormal acrosome percentages, compared with other groups (P<0.05). Briefly, NAC supplementation just improved post-thaw membrane integrity compared with control (P<0.05).

DISCUSSION

In current study, high dose of NAC (0.75mM) especially suppressed the post-thawing motility, while lower doses of NAC there were not any beneficial or detrimental effect on post-thawing sperm parameters, compared with control group. The production of toxic reactive oxygen species, damaging sperm motility and viability [2,16] has been pointed out to be involved in some steps of cryopreservation [5]. Antioxidants such as superoxide dismutase, catalase, cytochrome C and glutathione peroxidase in liquid presentation solutions improved the motility and acrosome integrity of ram spermatozoa, and there was improvement in survival of spermatozoa with increasing dose of antioxidants [17].

Glutathione taking part in Thiols including NAC is one of the most effective protective agents against oxidative damage of bull spermatozoa [18] and also thiols including NAC were demonstrated to prevent hydrogen peroxidase mediated loss of sperm motility in frozen thawed bull semen [8].

The skim milk and the egg yolk contains proteins and lipoproteins shown to have antioxidant properties [19]. However, skim milk and egg yolk contains very few enzymatic antioxidants, especially α-tocopherol, carotenoids, casein and milk proteins [20]. If these non-enzymatic antioxidants are mainly oxidized and not regenerated, this could partly explain why skim milk and egg yolk is not very efficient against oxidative stress and why addition of NAC may have been beneficial to frozen-thawed sperm motility and other parameters in skim milk based egg yolk extender.

Table 1. *Post thaw parameters of Tushin ram semen extended with milk based extender supplemented with different doses of NAC (Mean ± Standard Error)*
Tablo 1. *Farklı dozlarda NAC eklenmiş süt bazlı sulandırıcı ile sulandırılmış Tuj koçu spermasının çözüm sonu parametreleri (Ortalama ± Standart Hata)*

Concentration of NAC	Motility (%)	Viability (%)	Abnormality (%)	Abnormal Acrosome (%)	Membrane Integrity (%)
0.25 mM (n=6)	26.6±2.4^{b}	34.4±3.9^{b}	39.1±1.8^{b}	35.2±2.0^{b}	42.6±3.9^{b}
0.5 mM (n=6)	28.3±4.4^{b}	34.7±4.0^{b}	43.9±2.7^{ab}	38.5±2.0^{ab}	43.7±2.6^{b}
0.75 mM (n=6)	19.2±4,6^{a}	28.7±4,5^{a}	49.7±4.8^{a}	44.7±4.5^{a}	37.3±5.1^{a}
0 mM(Control/n=6)	28.3±5.2^{b}	32.3±5.6^{b}	43.7±4.6^{b}	37.9±3.4^{ab}	37.5±4.5^{a}

^{a,b,c} *Means with different letters differ significantly within the same column (P<0.05);* ***n:*** *Number of replication for cryopreservation;* ***NAC:*** *N-acetylcystein*

NAC was added to Tris-glucose-egg yolk extender with high and low doses (0.5 or 1.5 mM) [21-23]. When different doses of NAC were supplemented to Tris egg yolk based extender [21-23] or milk based extender [10], beneficial effects on sperm motility were determined. Although Michael et al.[21,22] determined that supplementation of 1.5 mM NAC to Tris based extender improved sperm motility of frozen-thawed and chilled canine semen, we qualified that higher doses of NAC (i.e. 1 mM and higher) supplemented to skim milk based extender had some directly toxic effects on ram semen, compared low doses of NAC (0.75 mM and lower doses) in our preliminary study [24]. Moreover, in current study, 0.75 mM NAC detrimentally affected motility, viability, morphology and membrane integrity of frozen-thawed ram semen, compared lower doses of NAC groups (0.25 and 0.5 mM) and control (0 mM NAC). The reason of detrimental effect of higher NAC doses may have been originated from acidity of NAC and changing of extender pH. Acidity may cause augmentation and precipitation of milk components especially casein. Also we observed with naked eye that 1 mM and higher doses of NAC supplemented to skim milk based extender caused precipitation and directly toxic effect on ram semen extended with NAC supplemented extender in our preliminary study. Yildiz et al.[24] determined that 5 and 10 mM NAC doses may have toxic effect on ram semen during cryopreservation process. They found that higher NAC doses detrimentally affect sperm motility.

Although there has been limited study [24] about NAC supplementation to skim milk based extender in ram semen, Pagl et al.[10] supplemented 0.2 mM NAC to defined milk protein fraction extender in storage of equine semen at 5°C. They evaluated sperm motility with determined times, they determined that 0.2 mM NAC did not have any detrimental or beneficial effects on sperm motility, compared control group. However, in our current study, it was determined that moderate doses of NAC (0.25 and 0.5 mM) slightly improved sperm viability, morphology, acrosome and membrane integrity. Furthermore, moderate doses of NAC did not show any detrimental or beneficial effect on sperm motility.

Micheal et al.[23] supplemented different NAC doses (0, 0.5, 1, 2.5 or 5 mM) in canine semen extenders and determined that NAC supplementation of semen extenders is beneficial to semen motility of canine spermatozoa during chilling with the 0.5 mM concentration being the most effective, although no significant ROS inhibition was observed at 72 h. It may be said that moderately doses of NAC could improve sperm motility, viability and membrane integrity not only via antioxidant effects but also via other unknown ways.

In conclusion, moderate doses of NAC (i.e. 0.5 mM and lower) supplemented in skim milk based extenders can be used to protect ram sperm cells from oxidative stress without detrimental effect on freeze-ability of ram semen. Furthermore, higher doses of NAC (i.e. 0.75mM and higher), especially used with milk based extender, may have some detrimental effects on freeze-ability of ram semen. It warrants further evaluation in fertility trials.

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Research Article

Heavy Metal Levels in Farmed and Wild Fishes of Aegean Sea and Assessment of Potential Risks to Human Health

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Abstract

The aim of this study was to determine the levels of Ag, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn in wild seabream (*Sparus aurata),* blotched picarel *(Spicara maena)*, pandora *(Pagellus erythrinus)* fished from the Aegean Sea and the farmed seabream *(Sparus aurata),* and to assess the risks depending on their consumption by humans. A total of 40 fishes were collected and the metal levels were determined using ICP-OES. The metal levels were determined to be Ag (Nd), Al (3.20-8.87), Co (0.01-0.01), Cr (0.02-0.51), Cu (0.96-1.28), Fe (0.23-4.30), Mn (0.15-0.39), Ni (0.13-0.22), Pb (0.00-0.10) and Zn (6.18-7.30) (mg kg^{-1} w.w.) in the study. No Ag or Cd were found in the samples. The heavy metal levels were below the national and international legal limits. The target hazard quotient (THQ) and total target hazard quotient (TTHQ) method was used to determine the possible risks of the heavy metals on human health. THQ and TTHQ values were all less than 1 and it was concluded that they do not pose any risks in terms of human health.

Keywords: *Aegean Sea, Heavy metal, Pagellus erythrinus, Sparus aurata, Spicara maena, Risk assessment*

Ege Denizi Vahşi ve Çiftlik Balıklarının Ağır Metal Düzeyleri ve İnsan Sağlığı Üzerine Potansiyel Risklerinin Değerlendirilmesi

Özet

Bu çalışmada Ege Denizinden avlanan vahşi çipura (*Sparus aurata*), izmarit (*Spicara maena*), mercan *(Pagellus erythrinus)* ve çiftlik çipura *(Sparus aurata)* türlerinin Ag, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb ve Zn düzeylerinin belirlenerek tüketimlerine bağlı olarak insan sağlığı risk değerlendirmesi yapılması amaçlandı. Toplam 40 adet balık toplandı ve metal düzeyleri ICP-OES ile belirlendi. Çalışmada balık türlerindeki metal düzeyleri Ag (Nd), Al (3.20-8.87), Co (0.01-0.01), Cr (0.02-0.51), Cu (0.96-1.28), Fe (0.23-4.30), Mn (0.15-0.39), Ni (0.13-0.22), Pb (0.00-0.10) ve Zn (6.18-7.30) (mg kg^{-1} y.a.) olarak belirlendi. Örneklerde Ag ve Cd'a rastlanmadı. Belirlenen ağır metal düzeyleri ulusal ve uluslararası yasal limitlerin altında bulundu. Ağır metallerin insan sağlığı üzerine olası risklerinin değerlendirilmesinde target hazard quotient (THQ) ve total target hazard quotient (TTHQ) metodu kullanıldı. THQ ve TTHQ değerleride 1'in altında bulundu ve insan sağlığı açısından herhangi bir risk oluşturmadığı sonucuna varıldı.

Anahtar sözcükler: *Ege Denizi, Ağır metal, Pagellus erythrinus, Sparus aurata, Spicara maena, Risk değerlendirmesi*

INTRODUCTION

Water pollution due to heavy metals is an important issue today. Is not only threatens the aquatic ecosystems, but also human health [1]. The passage of heavy metals into ecological systems is due to human activities mostly [2,3]. Especially during the two centuries since the industrial revolution, thermal power plants, garbage and waste cremation facilities have been the main sources of heavy metal contamination. Heavy metals create potential hazards for humans because they remain in the environment for a long time, have high levels of toxicity, tend to accumulate in living tissues and affect the top of the food chain more [4,5].

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Fish is high quality and valuable food that contains essential amino acids, required for humans, essential fatty acids (omega-3 etc.), carbohydrates, fat soluble vitamins and important macro and micro elements such as Ca, Mg, and Se. Therefore, fish consumption is important to maintain and improve human health [6,7]. The accumulation of heavy metals in fishes is due to the absorption of ions dissolved with suspended particles and foods from the lipophilic membranes in intestines and gills [8,9]. Potentially toxic metals such as Cd, Pb, Ni may harm to human health if they are ingested for long times even in small concentrations. On the other hand, essential minerals such as Cu, Zn, Fe, Mn may become toxic at higher levels [10,11]. Cr causes allergic dermatitis and cancer, short term exposure to Cu causes gastrointestinal problems and long term exposure to it causes liver and kidney disorders and the Wilsons disease. Cd and Pb have toxic effects on kidneys, in addition to chronic toxic effects such as lowering breeding capacity, atherosclerosis, learning difficulties, hypertension, tumors, function disorders in the liver and other organs [5,12].

The southern part of the Aegean Sea is being heavily polluted by discharges from tourism, agriculture, mining and industry. However the literature concerning the levels of metal in the tissues of wild fish that are heavily fished in the fish farms and their environs are scarce [7,13]. Determination of metal levels in these fish, and assess them in terms of human health is very important with regard to public health. Metal accumulation in fish is affected by physiological (species, size, sexual maturity and feeding habits) and environmental (seasonal changes, water quality and pollution level) factors [7,9,14].

This study aimed to determine and compare the heavy metal levels in muscle tissues of farmed fish and the wild fish caught around them, and to evaluate the health risks that may arise in humans according to consumption scenarios.

MATERIAL and METHODS

Samples, Study Area and Preparation

The muscle tissues excised from the same regions of the fishes were used to determine Ag, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn levels. 10 each of *Sparus aurata* Linnaeus, 1758 *(SaW), Spicara maena* L., 1758 *(Sm)* and *Pagellus erythrinus* Linnaeus, 1758 *(Pe)* were fished around the fish farms in the region between the Göltürkbükü, Torba, Güvercinlik Bay around the Güllük Bay in the city of Muğla at the Aegean shore, and Kuşadası (N 30°30′ and E30°30′) by hook fishing. Additionally, 10 farmed *Sparus aurata Linnaeus,* 1758 *(SaF)* samples were collected from marine products sales points in the same region to reach a total of 40 fishes. Microwave acid digestion (BERGHOF, Germany) system was used to burn the organic parts to determine the amount of metals in the muscle tissues. Homogenized muscle tissue samples of 0.5 g each were weighed into vessels. Then 8 mL nitric acid (HNO_3; 65%, Merck, Germany) and 2 mL hydrogen peroxide (H_2O_2; 30%, Merck, Germany) were added before burning the samples in a 4 step process compliant with the procedure for 15 min at 85% power and 200°C. The samples were filtered through a filter paper and filled up to 25 mL with deionized water and kept in plastic tubes at +4°C prior to analysis.

Analysis

The levels of heavy metal in the filtrates containing Ag, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn were determined with the Inductively Coupled Plasma Optical Emission Spectrophotometer (ICP-OES) model Optima 3000DV sequential (Perkin-Elmer-Norwalk, USA) device according to USEPA (Method 200.7) [15]. Measurements were triplicated for each sample. Heavy metal concentrations in fish muscles were expressed as mg kg^{-1} wet weight (ww). The data quality was checked by using standard reference material, the recovery results was satisfactory and ranged from 97.6% to 105.3%. Instrument Limit of Detections (LOD) (mg kg^{-1}) were 0.001 for Cd and Co, Ag, 0.002 for Al, Cr, Cu, Fe and Zn, 0.003 for Mn and Ni, 0.007 for Pb.

Health Risk Assessment

The metal levels of muscle tissues were used to determine the potential health risks arising from *SaW, Sm, Pe* and *SaF* consumption. The target hazard quotient (THQ) method [16] with the formula given below, determined by The US Environmental Protection Agency was used in the study to assess the health risks of heavy metal accumulation in fishes.

$$\mathrm{THQ} = \frac{\mathrm{E_F} \times \mathrm{E_D} \times \mathrm{F_{IR}} \times \mathrm{C}}{\mathrm{R_{fD}} \times \mathrm{W_{AB}} \times \mathrm{T_A}} \times 10^{-3}$$

Where E_F is exposure frequency (365 days/year), E_D is exposure corresponding to average lifespan (70 years), F_{IR} is the consumption rate (g/person/day) varying according to the consumption habits which is 17 g/person/day (6.3 kg/person/year) [17] for Turkey and 52 g/person/day (19.2 kg/person/year) for the world [18], C is the metal concentration in the food (mg kg^{-1} ww), R_{fD} is the oral reference dose (mg kg^{-1}/day) [19], W_{AB} is the average body weight (bw) (70 kg) and T_A is the average non-carsinogenic exposure duration (E_FxE_D) [7,12,20,21]. THQ values less than 1 shows that the exposed population is not under risk. The Total THQ (TTHQ) with the formula given below is the total of THQ values used in recent studies [12,20,21].

$$\mathrm{TTHQ} = \mathrm{THQ_{Ag}} + \mathrm{THQ_{Al}} + \mathrm{THQ_{....}} + \mathrm{THQ_{Zn}}$$

Statistical Analysis

One-way analysis of variance (ANOVA) was performed to determine statistically significant differences. The level of $P<0.05$ was considered statistically significant.

RESULTS

The heavy metal concentrations determined through ANOVA tests are given as mg kg^{-1},wet weight ± sd in *Table 1*. The results of the comparative Tukey multiple test only show a statistically significant difference between the *SaF* for Cr accumulation and *SaW* (P<0.05). The average metal levels in fish muscle tissues were Zn>Al>Fe>Cu> Mn>Cr>Ni>Pb>Co. The metal levels in terms of fish species were ordered *Sm>SaF>Pe>SaW*.

The length (cm) of fish samples ranged from 16.3 to 29.6 for *SaW* with a mean of 22.7 cm, 14.7 to 26.8 for *SaF* with a mean of 20.6 cm, 9.6 to 20.1 for *Sm* with a mean of 15.8 cm and 9.5 to 25.2 *Pe* with a mean of 16.2 cm. The weight (g) of fish samples ranged from 136 to 284 for *SaW* with a mean of 193 g, 92 to 212 for *SaF* with a mean of 156 g, 19.8 to 56.0 for *Sm* with a mean of 33.2 g and 28.9 to 210.2 *Pe* with a mean of 68.8 g.

According to the results, the highest metal levels were (mg kg^{-1}) Al 8.87, Cr 0.51, Cu 1.28, Fe 4.30, Mn 0.39, Ni 0.22, Pb 0.10 and Zn 7.30. The average was 0.01 for Co in all samples, while Pb, Cd and Ag were not detected in *SaW* and *SaF*.

The heavy metal levels for *SaF* were found to be on average (mg kg^{-1}) Ag *(Nd),* Al (6.06±5.14), Cd (Nd), Co (0.01±0.01), Cr (0.51±0.25), Cu (0.96±0.79), Fe (3.96±4.90), Mn (0.15±0.10), Ni (0.13±0.13), Pb (*Nd*) and Zn (6.92±1.95), *for SaW* they were Ag (Nd), Al (3.20±1.92), Cd (*Nd*), Co (0.01±0.01), Cr (0.10±0.06), Cu (1.15±2.45), Fe (1.76±2.14), Mn (0.39±0.32), Ni (0.22±0.15), Pb *(Nd)* and Zn (6.18±3.36). No Ag, Cd and Pb were detected for *SaF* and *SaW*. In wild *(SaW)* and farmed *(SaF) S. aurata*, there were no statistically significant differences in heavy metal levels except Cr between the farmed and wild subtypes (P<0.05).

The average metal levels in the muscule tissues of the fish samples in the calculation of the THQ and TTHQ values are given in *Table 2*. THQ values for all fish species were ranged as follows: 0.0E+00 for Ag, 7.8E-04 and 6.6E-03 for Al, 0.0E+00 for Cd, 8.1E-03 and 2.5E-02 for Co, 1.6E-03 and 1.3E-01 for Cr, 5.8-03 and 2.4E-02 for Cu, 8.0E-05 and 4.6E-03 for Fe, 2.6E-04 and 2.1E-03 for Mn, 1.6E-03 and 8.2E-03 for Ni, 0.0E+00 and 1.9E-02 for Pb, 5.0E-03 and 1.8E-02 for Zn. The TTHQ values on the other hand varies between 3.2E-02 and 2.0E-01.

DISCUSSION

Although water products are important for the human diet, they are a source of food that needs to be constantly monitored and assessed for risks in terms of public health due to the risk of heavy metal accumulation [7,21,22]. The absorption of essential metals which are more than the physiological requirements or the absorption of toxic metals which are more than the tolerable limits may cause harmful effects [21]. The aim of this study was to determine the levels of heavy metals in wild and farmed fish in the Aegean Sea, and to compare them to assess the risks caused by metals depending on consumption. The results of the study shows that the accumulation in the muscle tissues of fishes consumed by human were all below the limits determined by national and international authorities (maximum allowed heavy metal amounts in fish muscle tissue, mg kg^{-1}) (Cu:20, Cd:0.05, Pb:0.30 and Zn:50) [23,24].

Dalman et al.[25] have found Pb: <0.02-0.4, Cd: <0.01-0.04, Cu: <0.1, Zn: <0.5-7.2 mg kg^{-1} in their study for the fish *(Dicentrarchus labrax)* obtained from fish farms in the Güllük Bay.

Table 1. *Mean metal concentrations in the muscle tissues of fish species*
Tablo 1. *Balık türleri kas dokuları ortalama metal konsantrasyonları*

Metals	S. aurata		S. maena	P. erythrinus	Total n:40
	Wild (n:10)	Farmed (n:10)	Wild (n:10)	Wild (n:10)	
Ag	*Nd*	*Nd*	*Nd*	*Nd*	*Nd*
Al	3.20±1.92	6.06±5.14	8.87±5.59	5.42±3.05	5.76±4.32
Cd	*Nd*	*Nd*	*Nd*	*Nd*	*Nd*
Co	0.01±0.01	0.01±0.1	0.01±0.01	0.01±0.01	0.01±0.01
Cr	0.10±0.06^{a}	0.51±0.25^{b}	0.24±0.28^{ab}	0.02±0.05^{a}	0.22±0.26
Cu	1.15±2.45	0.96±0.79	1.28±3.14	1.06±0.74	1.16±1.93
Fe	1.76±2.14	3.96±4.90	4.30±1.78	0.23±0.41	2.60±3.16
Mn	0.39±0.32	0.15±0.10	0.31±0.34	0.37±0.26	0.30±0.27
Ni	0.22±0.15	0.13±0.13	0.19±0.29	0.20±0.17	0.19±0.19
Pb	*Nd*	*Nd*	0.10±0.13	0.09±0.11	0.05±0.09
Zn	6.18±3.36	6.92±1.95	6.83±1.64	7.30±0.82	6.81±2.05

** Different letters in the same column indicate significant differences between fish species (P<0.05)*

Table 2. *THQ and TTHQ estimate for individual metals caused by the consumption of wild S. aurata, S. maena, P. erythrinus and farmed S. aurata for inhabitants*
Tablo 2. *Tüketiciler* için *vahşi S. aurata, S. maena, P. erythrinus ve* çiftlik *S. aurata tüketimine bağlı* çalışılan *metaller* için *hesaplanan THQ ve TTHQ*

Metals	THQs and TTHQs	*S. aurata*		*S. maena*	*P. erythrinus*
		Wild	Farmed	Wild	Wild
Ag	THQ^f	0.0E+00	0.0E+00	0.0E+00	0.0E+00
	THQ^t	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Al	THQ^f	2.4E-03	4.5E-03	6.6E-03	4.0E-03
	THQ^t	7.8E-04	1.5E-03	2.2E-03	1.3E-03
Cd	THQ^f	0.0E+00	0.0E+00	0.0E+00	0.0E+00
	THQ^t	0.0E+00	0.0E+00	0.0E+00	0.0E+00
Co	THQ^f	2.5E-02	2.5E-02	2.5E-02	2.5E-02
	THQ^t	8.1E-03	8.1E-03	8.1E-03	8.1E-03
Cr	THQ^f	2.5E-02	1.3E-01	5.9E-02	5.0E-03
	THQ^t	8.1E-03	4.1E-02	1.9E-02	1.6E-03
Cu	THQ^f	2.1E-02	1.8E-02	2.4E-02	2.0E-02
	THQ^t	7.0E-03	5.8E-03	7.8E-03	6.4E-03
Fe	THQ^f	1.9E-03	4.2E-03	4.6E-03	2.4E-04
	THQ^t	6.1E-04	1.4E-03	1.5E-03	8.0E-05
Mn	THQ^f	2.1E-03	8.0E-04	1.6E-03	2.0E-03
	THQ^t	6.8E-04	2.6E-04	5.4E-04	6.4E-04
Ni	THQ^f	8.2E-03	4.8E-03	7.1E-03	7.4E-03
	THQ^t	2.7E-03	1.6E-03	2.3E-03	2.4E-03
Pb	THQ^f	0.0E+00	0.0E+00	1.9E-02	1.7E-02
	THQ^t	0.0E+00	0.0E+00	6.1E-03	5.5E-03
Zn	THQ^f	1.5E-02	1.7E-02	1.7E-02	1.8E-02
	THQ^t	5.0E-03	5.6E-03	5.5E-03	5.9E-03
$TTHQ^f$		1.0E-01	2.0E-01	1.6E-01	9.8E-02
$TTHQ^t$		3.3E-02	6.5E-02	5.3E-02	3.2E-02

*^{f} Fish ingestion rate by FAO (52 g/person/day) [18];^{t} Fish ingestion rate by TURKSTAT (17 g/person/day) [17]; **THQ:** Target hazard quotient; **TTHQ:** Total target hazard quotient*

Uluturhan and Kucuksezgin [26] have determined the levels of Cd: 2.0±0.91, Pb: 59.3±34.7, Zn: 2810±142, Cu: 164±24.1 µg kg^{-1} in their study conducted in 2007 on fish from Didim. Tepe et al.[27] have determined heavy metal levels of Cd: 0.03±0.01, Co: 0.03±0.00, Cr:0.13±0.05, Cu: 1.56±0.43, Fe: 46.9±12.1, Mn: 0.37±0.07, Ni: 0.31±0.16, Pb: 0.39±0.18, Zn: 5.20±0.81 µg g^{-1} for the fish they studied in Izmir.

Comparison of the study performed by Alsaver et al.[28] on the wild and farmed fish from the Aegean Sea in Greece with the present study, the Cr and Al levels were higher while other metal levels were lower. Comparison of the study performed by Yıldız [13] except the Cu, Fe and Zn levels the Co, Cr, Mn, Ni, Pb levels were determines as higher than our study. According to data from Yıldız, Mn, Cd, Cr, Co, Pb and Ni levels were lower, Cu and Zn levels were higher and Fe level was similar [13,28].

The levels detected in different fishes in the present study are parallel with the levels in the study by Uluturhan and Kucuksezgin [26]. In the studies by Dalman et al.[25] and Tepe et al.[27] (mg kg^{-1}) the Zn (0.5-7.2), Mn (0.37), Cr (0.13) levels were close to, and the Cd (0.03), Fe (46.9), Ni (0.31), Pb (0.39), Co (0.03) and Cu (1.56) levels were higher than the levels in the present study.

The study of Yabanlı and Alparslan [12] on fish caught on the Muğla shore, reported the heavy metal levels to be (mg kg^{-1}) Cr: 0.27-0.39, Cu: 0.12-0.22 and Pb: 0.10-0.12. Compared to the data from this study, the Cr level is similar, the Cu level is higher and the Pb level is lower than the levels in our study.

In the study performed by Kalantzi et al.[7] on *SaF* species bred on the Greek shore of the Aegean, they determined the average heavy metal levels to be (mg kg^{-1}) Al: Nd-1.92, Cd: Nd, Co: Nd, Cr: 0.07-0.15, Cu: Nd, Fe: Nd-2.77, Ni: Nd, Pb: Nd and Zn: 4.29-4.99. Compared to the data from this study, the Al, Cr, Fe, Ni and Zn levels are lower, and the Cd,

Co, Cu and Pb levels are similar to ours. Moreover, they analyzed the metal levels of feeds and suggested that the cause of accumulation and the difference of metal levels in fish species may be due to the feed. The analysis results determined the metal levels in the feeds to be (mg kg^{-1}) Al: 29.11, Cd: 0.35, Co: 0.12, Cr: 0.49, Cu: 10.65, Fe: 160.13, Ni: 0.60, Pb: 0.09 and Zn: 149.89. Therefore, in this respect, it is very important to analyze the feeds used in fish farms in terms of the heavy metal levels they possess. However in study that performed by Cheng et al.[29] heavy metal concentrations in all analyzed fish species feed with commercial pellets were below the international legal levels. Basaran et al.[30] who aimed to assess the effects of fish farms on water column and sediment by feed mainly were pointed out that metal levels were tolerable for the marine ecosystem.

According to data from Tepe [31], all studied metal levels were higher on Pe caught on İskenderun Bay, Mersin Bay, Antalya Bay Northern Aegean sea except Cu on Mersin Bay, Antalya Bay Northern Aegean sea and Zn on İskenderun Bay, Mersin Bay, Antalya Bay.

Risk Assessment

R_{fD} is the oral reference dose depending on the fish consumption levels in the world and in Turkey and on the daily exposure levels. The average metal (Ag, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn) levels in the muscule tissues of the fish samples in the calculation of the THQ values are given in *Table 2*. As for TTHQ, it represents the risk of all metal THQs reveal for all fish species.

All the THQ and TTHQ values in the study were less than 1. According to these results, the consumption of the farm *(SaF)* and wild *(SaW) Sa, Sm* and *Pe* species in the study field does not represent a risk for health in terms of Ag, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn. When an assessment is made in terms of metals, while Cr (1.3E-01) represents the highest risk, Cd and Ag (0.0E+0.0) represents the lowest. This situation is most probably about the density of the metals in the marine environment and about the bioaccumulation factors. The highest THQ value was Cr in *SaF* with 1.3-E-01. According to the TTHQ values, the species are ranked as *SaF>Sm>SaW>Pe*. This situation on the other hand may be about the feeding habits (omnivorous, carnivorous, formulated food etc.), living areas *(SaW, SaF* and *Pm* demersal, *Sm* pelagic), physiologies and environmental conditions which are directly related to metal accumulation [7]. A risk assessment on the consumption of the *SaF* which is farmed and *Saw* and *Sm* which are caught in the coasts of Aegean Sea was not conducted before.

Kalantzi at al.[7] conducted study on the Al, Cr, Co, Cu, Cd, Fe, Ni, Pb and Zn levels in *SaF* fish collected from the fish farms on the Greek coasts of Aegean Sea, and have found THQ values were less than 1, similar to our study.

Yabanlı and Alparslan [12], in their study on the Cr, Cu, Cd and Pb levels on *Pe* and some other fish caught in Aegean Sea and have found THQ and TTHQ levels values were less than 1, similar to our study.

Tepe [31] conducted study on the Cd, Cr, Co, Cu, Fe, Mn, Ni, Pb and Zn levels in *Pe* fish caught on Mersin Bay, Antalya Bay Northern Aegean sea. And have found compsumption higher than 1.32 kg/week of *Pe* would be reach tolerable weekly intake (TWI) of Cd.

The levels of metals detected in the fish muscle samples analyzed in this study were below the legal and daily consumption limits according to national and international standards and these results show that there is no risk for the public health. Not detecting Ag, Cd and Pb, which are toxic heavy metals, in the wild and farm *S. aurata* fish muscle tissues and not detecting Ag and Cd in the other wild fish species caught and detecting Pb in very low levels are assessed as a positive result in terms of public health and food safety in the fisheries. Because these results which are found in the fish in this area which contribute to economy to a large extent by their export mainly to EU countries can be accepted as a promising data in terms of food safety and prospective export potential for our country. As waters are exposed to aquatic pollutants and they are important and easily accessed animal protein resources for humans, the heavy metals in the water products should be periodically monitored.

Conclusively, the Ag, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and Zn levels in the fish muscle tissues of wild *Sparus aurata* Linnaeus, 1758 *(SaW), Spicara maena* Linnaeus, 1758 *(Sm)* and *Pagellus erythrinus* Linnaeus, 1758 *(Pe)* caught in Aegean Sea and in farmed *Sparus aurata* Linnaeus, 1758 *(SaF)* were below legal limits, and THQ and TTHQ levels found depending on the feeding habits of consumers were less than 1. Therefore, the consumption of these fish does not represent any risk to human health in terms of the metals detected in the study. It is found that the consumption of *SaF* (260 g/day) being more than 5 times of the world average (52 g/day) may be risky. Therefore, it is important to monitor metal levels of the fish in the area constantly and to make periodic risk assessment on them.

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Research Article

Alternatif Kaba Yem Kaynağı Olarak Şarapçılık Endüstrisi Üzüm Atıklarının *İn Vitro* Gerçek Sindirilebilirlikleri ve Nispi Yem Değerlerinin Belirlenmesi [1]

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Özet

Bu çalışma, şarapçılık endüstrisi üzüm atıklarının yem değerlerinin belirlenmesi amacıyla yürütülmüştür. Çalışmada yem kaynağı olarak; şarap üretimi sonrası kalan kurutulmuş üzüm cibresi, üzüm çekirdekleri ve yakıt amaçlı üretilen üzüm cibresi peletleri kullanılmıştır. Atıkların, besin madde içerikleri, nispi yem değerleri (NYD), kondanse tanen (KT) içerikleri ve *in vitro* gerçek sindirilebilirlikleri (IVGS) belirlenmiştir. *İn vitro* sindirilebilirliklerin belirlenmesinde Daisy inkübatör kullanılmış, elde edilen veriler tek yönlü varyans analizi kullanılarak analiz edilmiştir. Ham protein içerikleri bakımından en düşük değer üzüm çekirdekleri (%11.05 KM), en yüksek değer ise cibre peletlerinde (%13.78 KM) belirlenmiştir. Üzüm çekirdekleri diğer formlardan daha yüksek HY; daha düşük NÖM ve daha yüksek hücre duvarı yapı elemanları içeriğine sahip olmuştur. En düşük *in vitro* gerçek sindirilebilirlik değerini en yüksek kondanse tanen içeriğine sahip olan cibre peletleri göstermiş, en iyi sindirilebilirlik değeri ise kurutulmuş cibrelerde belirlenmiştir (P<0.001). Kuru madde sindirilebilirliği, kuru madde tüketimi ve NYD dikkate alınarak yapılan sınıflandırmaya göre kuru üzüm cibresinin, diğerlerine kıyasla hayvan besleme açısından daha uygun olduğu söylenebilir. Bununla beraber daha gerçekçi sonuçların ortaya konulabilmesi açısından *in vivo* hayvan besleme denemelerinin yapılmasına ihtiyaç duyulmaktadır.

Anahtar sözcükler: *Nispi yem değeri, Pelet, Sindirilebilirlik, Şarapçılık atıkları, Üzüm cibresi*

Determination of *In Vitro* True Digestibilities and Relative Feed Values of Wine Industry Grape Residues as Alternative Feed Source

Abstract

This study was carried out to determine the feed values of grape wastes of wine industry. In this study, wastes of wine industry; dried grape pomace, grape seeds and grape pomace pellets produced for fuel were used as feed sources. The nutrient contents, relative feed values (RFV), condensed tannin (CT) contents and *in vitro* true digestibilities (IVTD) of the wastes were determined. Daisy incubator was used to determine the *in vitro* digestibilities. The obtained data were analysed by one-way analysis of variance (ANOVA). While the lowest crude protein content (11.05% DM) was determined for grape seeds, the highest content (13.78% DM) was determined for pellets. Although grape seeds with a higher ether extract (EE) content than the others, it has a lower nitrogen free extracts (NFE) and higher cell-wall structural components (CWSC). Grape seeds had higher EE and CWSC and lower NFE compared to other forms. While the lowest IVTD value was determined for grape pomace pellets which had highest CT content, the highest digestibility value was determined for dried grape pomace (P<0.001). The dried grape pomace was found superior compared to other forms in terms of dry matter digestibility, dry matter consumption and RFV. However, *in vivo* animal feeding experiments are needed to attain more reliable results.

Keywords: *Digestibility, Grape pomace, Pellet, Relative feed value, Wine waste*

GİRİŞ

Dünya'nın pek çok yerinde ruminant hayvanların beslenmesinde kaliteli kaba yem problemi yaşanmakta olup, alternatif yem kaynakları üzerinde durulmaktadır. Bu bağlamda alternatif kaba yem kaynakları ve bunların yem değerlerinin araştırılarak ortaya konması, kaba yem sıkıntısı çeken hayvancılık işletmeleri için büyük önem taşımaktadır. Şarapçılık endüstrisi atıkları da alternatif kaba yem kaynağı olma potansiyeli bakımından dikkat çekmektedir.

Dünya'da toplam üzüm üretimi 77.181.122 ton, şarap üretimi 27.421.931 ton, üzüm cibresi üretimi 3.441.122 tondur [1]. Şarap fabrikalarında üzümün suyu alındıktan sonra geriye kalan %15-25'lik kısmı üzüm cibresi olup,

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posanın %50 kadarı kabuk, %25'i çekirdek ve geri kalan %25'i de üzüm çöplerinden oluşmaktadır [2,3]. Türkiye'de yılda 3.650.000 ton yaş üzüm üretilmekte olup, bunun 3.226.473 tonu sofralık ve kurutmalık üzüm, 423.527 tonu şaraplık üzümdür [4] ve şaraplık üzümün %25'i kadar yani yaklaşık yılda 105.882 ton üzüm cibresi elde edilmektedir. Yaş cibrelerle genellikle silaj yapılmakta ya da kurutularak hayvanlara verilmektedir. Aksi halde, yaş cibreler çevreye pis koku yaymakta ve çevre kirliliğine de neden olabilmektedir. Ayrıca, bu atıkların gübre olarak tarlalara geri dönüşümü, yakıt amaçlı peletler yapılması, fide üretimi ve topraksız tarım uygulamalarında (torf, perlit ya da kaya yününe alternatif olarak) kullanılabilirliği sayesinde ekonomiye kazandırılmalarına yönelik faaliyetler bulunmaktadır [5]. Bununla beraber, üzüm posalarının içerdikleri bazı fenolik bileşiklerin (sitrik asit, tartarat, malat vb.), fonksiyonel gıdaların muhafazasında, kanser gibi bazı sağlık problemlerinin önlenmesinde önemli ölçüde faydaları bulunmaktadır [6]. Bu bağlamda hayvan yemi olarak kullanılması hayvansal ürünler üzerinde olumlu etkiler sağlayabilecektir.

Cibrelerin, yüksek lignin içeriği ve potasyum tartarat içermesi dolayısıyla karma yemlere katılması tebliğle (2005/24; Ek 1/19) yasaklanmıştır. Bununla beraber cibrelerin kuru madde bazında, %33.21 ham selüloz, %20.30 ham kül, %11.54 ham protein, %3.99 ham yağ ve %30.94 nitrojensiz öz maddeler içeriğine sahip olduğu bilinmektedir [7]. Ayrıca kullanılan çözücüye göre değişmekle birlikte üzüm cibresi çekirdeklerinde toplam fenolik maddelerin 667.87-627.98 mg GAE/g; üzüm cibrelerinde ise 29.55-45.44 mg GAE/g arasında olduğu bildirilmektedir [8]. Bu atıkların hayvan beslemede kaba yem kaynağı olarak değerlendirilmesi mümkün görülmektedir. Cibrelerin besin madde içerikleri; üzüm çeşidine (kırmızı-beyaz) ve uygulanan işlemlere bağlı olarak farklılık gösterebilmektedir [5].

Atıkların, besin madde içerikleri ve kaba yem kalitelerinin belirlenmesi yanında; sindirilebilirliklerinin belirlenmesi de oldukça önem taşımaktadır. *İn vitro* Daisy inkübatör kullanılarak sindirilebilirliklerin belirlenmesinde rumen ortamı taklit edilmekte ve sonuçlar ruminantların beslenmesinde genel kabul görmektedir.

Bu çalışmada, şarapçılık endüstrisi atıklarından; kurutulmuş cibre, üzüm çekirdekleri ve cibre peletlerinin besin madde içeriklerinin, nispi yem değerlerinin, kondanse tanen içeriklerinin ve *in vitro* gerçek sindirilebilirliklerinin belirlenmesi amaçlanmıştır. Şarapçılık atıklarının kaba yem kaynağı olarak değerlendirilebileceği, şarapçılık endüstrisinde uygulanan işlemlere göre besleme değerlerinin değişebileceği ve kondanse tanen içeriği düşük olan atıkların kaba yem değerinin yüksek olacağı hipoteziyle bu çalışma planlanmıştır.

MATERYAL ve METOT

Yem materyali olarak; özel bir firmadan temin edilen, şarap üretimi sonrası arta kalan kurutulmuş üzüm cibresi, üzüm çekirdekleri ve yakıt amaçlı olarak üretilen üzüm cibresi peletleri kullanılmış ve her bir atık 3 tekerrürlü olarak incelenmiştir. Üzüm cibreleri üzümler ezilip, sıkıldıktan sonra kalan yaş posalar olup; %10 KM içerecek şekilde açık havada kurutulduktan sonra çalışmada kullanılmıştır. Yemlerin *in vitro* gerçek sindirilebilirliklerin belirlenmesinde; birbirinden bağımsız dört adet üç litrelik kavanozdan ibaret olan ve rumen ortamını taklit eden Ankom Daisy^{II}- D220 inkübatör kullanılmıştır.

Yemlerin Besin Madde İçeriklerinin Belirlenmesi

Çalışmada kullanılan atıklarda; kuru madde (KM), ham protein (HP) ve ham kül (HK) analizleri AOAC'nin [9] bildirdiği gibi, ham yağ (HY) analizi Ankom^{XT15} ekstraksiyon sistem cihazı kullanılarak AOCS Am 5-04'e göre [10] yapılmıştır. Asit çözücülerde çözünmeyen lifli maddeler (ADF), nötr çözücülerde çözünmeyen lifli maddeler (NDF) ve ham selüloz (HS) analizleri ANKOM^{2000} Fiber Analyzer (Ankom Technology, Macedon NY) cihazı ile asit çözücülerde çözünmeyen lignin (ADL) Van Soest ve ark.'nın [11] bildirdiği gibi belirlenmiştir. Organik maddeler (OM = KM-HK), Hemiselüloz (HSel = NDF-ADF), Selüloz (Sel = ADF-ADL) ve nitrojensiz öz maddeler (NÖM = KM - (HP + HK + HY + HS)) içerikleri hesaplama yoluyla; kondanse tanen içerikleri ise Makkar ve ark.'nın [12] bildirdiği gibi belirlenmiştir.

İn Vitro Sindirilebilirliğin Belirlenmesi

İn vitro sindirilebilirlik çalışmasında kullanılan rumen sıvısı; rumen gelişimini tamamlamış, 2 yaşlarında, ortalama 450 kg canlı ağırlıkta 3 baş Holstein x Yerlikara melezi tosunun rumeninden hayvanlar kesilir kesilmez (mezbahanede), iki kat steril tülbentten süzülerek karbondioksit tüpü eşliğinde alınmış, içerisine 2 avuç rumen katı içeriği ilave edilmiş ve 39°C'deki termoslarla 15 dakika içinde laboratuvara taşınmıştır. Daisy inkübatörde 50 mm x 55 mm ebatlarında, polyester/polietilen karışımından yapılmış ve 25 µm'den büyük partiküllerin geçemeyeceği porlardan oluşmuş azot içermeyen Ankom F57 torbalar kullanılmış ve yemler 3 paralelli olarak test edilmiştir. Daisy inkübatörde sindirilebilirlik çalışmasında her bir kavanoza 2 lt'lik inkübasyon sıvısı (1600 mL tampon solüsyonu + 400 mL rumen sıvısı) CO_2 tüpü eşliğinde ilave edilmiştir. Torbalar 48 saatlik inkübasyondan sonra, kavanozlardan çıkartılıp, yıkanmış ve daha sonra 105°C'deki etüvde 3 saat kurutulmuş ve tartıldıktan sonra NDF analizi yapılmıştır. Denemede yemlerin kuru madde bazında *in vitro* gerçek besin madde (NDF) sindirilebilirlikleri (IVGS: *in vitro* gerçek sindirilebilirlik) süzgeç torba tekniği [11] kullanılarak Ankom Daisy İnkübatör'de [13] yapılmıştır. Yemlerin *in vitro* gerçek NDF sindirilebilirliği aşağıdaki formül uygulanarak hesaplanmıştır:

$$\%IVGS = 100 - ((W3 - (W1XC1)) \times 100)/ W2$$

Burada; W1: Torbaların darası, W2: Kuru örnek veya kuru örnekteki besin madde miktarı (NDF), W3: İnkübasyon sonunda torbada kalan besin madde miktarı, C1: Kör ağırlık

(inkübasyon sonrası etüvde kurutulan boş torba ağırlığı/ orijinal torba ağırlığı).

Rumen Sıvısı pH, Uçucu Yağ Asitleri (UYA) ve Amonyak Azotu (NH_3-N) Analizi

Rumen sıvısında pH ölçümleri zaman kaybetmeden dijital PH metre (Hanna Instruments 1332 model) ile; rumen sıvısı toplam uçucu yağ asitleri (TUYA) ve amonyak azotu (NH_3-N) içerikleri ise Markham [14] steam distilasyonuna göre üç tekerrürlü olarak yapılmıştır.

Yemlerin Nispi Yem Değerleri ve Kaba Yem Kalitelerinin Belirlenmesi

Yemlerin kaba yem kalitesinin belirlenmesinde nispi yem değeri indeksi (NYD) kullanılmıştır. Nispi yem değeri (NYD) indeksi, kaba yem değerlendirme ve pazarlamada kullanılan önemli bir araç olup; aşağıdaki gibi hesaplanmıştır [15].

Kuru madde sindirilebilirliği (KMS, %) = 88.9 – (0.779 x %ADF)

Kuru madde tüketimi (KMT, %CA) = 120/(%NDF)

Nispi yem değeri (NYD) = (KMS x KMT)/1.29

Kaba yem kalitesinin belirlenmesinde "The Hay Marketing Task Force of the American Forage and Grassland Council" tarafından yapılan sınıflandırmaya göre NYD bakımından yemlerde "5" (<75) reddedilecek düzeyde kötü kaliteyi; (75-86) arası 4. kaliteyi; (87-102) arası 3. kaliteyi; (103-124) arası 2. kaliteyi; (125-151) arası iyi kaliteyi ifade ederken, "prime" (>151) ise en iyi kaliteyi ifade etmektedir.

İstatistiksel Analizler

Araştırma sonunda elde edilen verilerin normallik varsayımını kontrol için Kolmogorov Smirnov testi uygulanmış ve verilerin normal dağılış gösterdiği belirlenmiştir. (Ayrıca varyansların homojenliği Levene testi ile incelenmiş olup varyansların homojen olduğu gözlenmiştir). Bu nedenle bu veri tipine en uygun analiz şekli olan tek yönlü varyans analizi ile sonuçlar %5 önem seviyesine göre incelenmiş, ortalamaların karşılaştırılması için Duncan çoklu karşılaştırma testi kullanılmıştır. Analizler SPSS 20.0 paket programında Ondokuz Mayıs Üniversitesi lisansı ile yapılmıştır.

BULGULAR

Çalışmada elde edilen bulgulara göre kurutulmuş üzüm cibresi, üzüm çekirdekleri ve üzüm cibresi peletlerine ait besin madde içerikleri *Tablo 1*'de (KM'de) verilmiştir. Buna göre; söz konusu atıklar içerisinde en yüksek HK ve NÖM içeriklerini kurutulmuş üzüm cibreleri gösterirken, en yüksek HY içeriğini üzüm çekirdekleri göstermiştir. Çalışmada NDF, ADF ve ADL içerikleri bakımından en düşük değerler kurutulmuş üzüm cibrelerinde belirlenmiştir. Şarapçılık endüstrisi atıklarının, HP içerikleri bakımından (%11.1-13.8) hayvan beslemede kullanılan pek çok yem hammaddesine yakın değerler taşıdığı görülmektedir.

İn vitro sindirilebilirlik çalışmasında kullanılan rumen sıvısına ait pH değeri 6.60 (6.55-6.69); TUYA içeriği 127 mmol/L (83-159 mmol/L) ve NH_3-N miktarı da 416 mg/L (334-525 mg/L) olarak bulunmuştur. Buna göre; kullanılan rumen sıvısının standart rumen sıvısı özelliğinde olduğu görülmektedir [16-18].

Üzüm atıklarına ait kondanse tanen (KT) içerikleri, *in vitro* gerçek sindirilebilirlikleri ile NYD, KMS ve KMT değerleri ve kaba yem değerleri *Tablo 2*'de verilmiştir. Buna göre kurutulmuş cibrenin, en yüksek NYD, KMT, KMS ve IVGS içeriklerine sahip olması ve en iyi kalitede yer almasından dolayı tercih edilebileceği söylenebilir.

Cibre peletleri kondanse tanen içeriklerinin, üzüm çekirdekleri ve kurutulmuş cibreden daha fazla olduğu belirlenmiştir (P<0.001). Bununla birlikte, kurutulmuş üzüm cibresi ve üzüm çekirdekleri KT içerikleri bakımından birbirine benzer bulunmuştur (P>0.05). Buna rağmen, üzüm çekirdeklerine ait *İVGS*'nin ve KMS değerinin kurutulmuş cibre ile benzer olması beklenilmekte iken; en yüksek değerleri kurutulmuş cibreler göstermiş, bunu üzüm çekirdekleri izlemiştir.

TARTIŞMA ve SONUÇ

Çalışmada elde edilen bulgulara göre; kurutulmuş cibre, HS ve hücre duvarı yapı elemanları bakımından üzüm çekirdekleri ve peletlenmis formdan daha düsük değerler

Tablo 1. *Şarapçılık endüstrisi üzüm atıklarının besin madde içerikleri (KM'de, %)*
Table 1. *Nutrient contents of wine industry grape residues (Dry Matter, %)*

Yemler	KM	HP	HY	HK	HS	OM	NÖM	NDF	ADF	ADL	Hsel	Sel
Kuru Cibre	88.2±0.09^{c}	12.5±0.38^{b}	4.90±0.12^{b}	8.2±0.18^{a}	19.8±2.24^{b}	91.8±0.18^{b}	54.6±2.19^{a}	49.6±0.19^{c}	38.3±0.32^{c}	32.4±0.71^{c}	11.4±0.26^{a}	5.8±0.88
Üzüm Çekirdeği	89.5±0.03^{b}	11.1±0.19^{c}	13.6±0.13^{a}	3.8±0.24^{b}	51.5±0.44^{a}	96.2±0.24^{a}	20.1±0.64^{b}	64.8±1.01^{b}	56.2±0.92^{b}	52.0±0.3^{b}	8.6±1.29^{ab}	4.2±0.84
Cibre Peletleri	90.1±0.04^{a}	13.8±0.17^{a}	5.00±0.13^{b}	3.5±0.04^{b}	55.7±0.17^{a}	96.5±0.04^{a}	22.1±0.24^{b}	69.1±0.25^{a}	62.6±0.58^{a}	57.9±0.14^{a}	6.4±0.76^{b}	4.8±0.44
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.020	0.351

^{a,b,c} *Aynı sütunda aynı harflerle gösterilen uygulamalar arasında fark yoktur.* ***KM:*** *kuru madde,* ***HP:*** *ham protein,* ***HY:*** *ham yağ,* ***HK:*** *ham kül,* ***HS:*** *ham selüloz,* ***OM:*** *organik maddeler,* ***NÖM:*** *nitrojensiz öz maddeler,* ***NDF:*** *nötr çözücülerde çözünmeyen lifli bileşikler,* ***ADF:*** *asit çözücülerde çözünmeyen lifli bileşikler,* ***ADL:*** *asit çözücülerde çözünmeyen lignin,* ***Hsel:*** *hemiselüloz,* ***Sel:*** *selüloz*

Tablo 2. *Üzüm atıklarına ait kondanse tanen, İVGS ve kaba yem değerleri*
Table 2. *Condensed tannin, IVTD and forage values of grape residues*

Atıklar	Kondanse Tanen, %	İVGS (% KM)	KMS (%)	KMT (% CA)	NYD	Kalite Sınıfı ve Tanımı
Kuru Cibre	12.64±0.11^{b}	73.80±1.17a	62.61±0.22^{a}	2.74±0.01^{a}	133.06±0.95^{a}	1 İyi
Çekirdek	12.47±0.52^{b}	51.35±0.18b	49.73±0.65^{b}	2.07 ±0.03^{b}	79.81±1.71^{b}	4 Kötü
Cibre Peletleri	16.31±0.03^{a}	47.11±0.32c	44.93±0.43^{c}	1.93 ±0.01^{c}	67.18±0.56^{c}	5. Reddedilecek düzeyde kötü
P	<0.001	<0.001	<0.001	<0.001	<0.001	

^{a,b,c} *Aynı sütunda aynı harflerle gösterilen uygulamalar arasında fark yoktur;* ***İVGS:*** *in vitro gerçek sindirilebilirlik,* ***KMS:*** *kuru madde sindirilebilirliği,* ***KMT:*** *kuru madde tüketimi,* ***NYD:*** *nispi yem değeri*

göstermiş olup, daha yüksek sindirilebilirliğe sahip olmuştur. Ancak, peletlenen cibrelerde besin madde kaybının fazla olması, peletleme işlemindeki uygulamadan (sıcaklık, toprak, yabancı maddelerle bulaşma vb.) kaynaklanmış olabilir. Üzüm çekirdekleri diğer formlardan önemli düzeyde yüksek HY içeriğine sahip olması dolayısıyla, enerjice zengindir. Ancak düşük NÖM içeriği ve yüksek hücre duvarı yapı elemanları içeriğine sahip olması dezavantajıdır.

Ensminger ve ark.'nın [19] üzüm cibresi için belirledikleri HS (%30.9), HY (%8.4), HP (%13.4), NÖM (%39.0), OM (%91.7), HK (%8.3), NDF (%53.2), ADF (%54.4) ve ADL (%35.2) içerikleri; bu çalışmada belirlenen HP, HK, ADL ve OM içerikleri ile benzer; ADF, NDF, HY ve HS içeriklerinden yüksek, NÖM içeriklerinden daha düşük saptanmıştır. Winkler ve ark.[20], beyaz ve kırmızı üzüm çeşitlerine ait cibreler için HP, HY, HS, HK ve NDF içeriklerini sırasıyla %10.9-11.2, %5.8-5.0, %24.1-21.4, %6.8-3.3 ve %42.1-34.1 olarak bildirmişlerdir. Çalışmada kullanılan cibrelere ait HS içerikleri bu bildirişe benzer sonuçlar gösterirken, NDF içerikleri daha yüksek bulunmuştur. Sousa ve ark.'nın [21] üzüm cibresinde belirledikleri HP (%8.49), HK (%4.65) ve HY (%8.16) içerikleri, bu çalışmada belirlenen HP ve HK içeriklerinden daha düşük, HY içeriklerinden daha yüksek olmuştur. Basalan ve ark.[22] beyaz ve kırmızı çeşitlerden elde edilen 28 farklı üzüm cibresine ait HK, HP, HY, NDF, ADF ve NÖM içeriklerini sırasıyla %5.0-6.3, 8.3-10.8, 4.8-4.6, 37.4-42.5, 29.4-36.1 ve 44.3-35.7 KM olarak belirlemişlerdir. Buna göre; HY içerikleri hariç diğer besin madde içerikleri bakımından kırmızı üzüm çeşitlerinin daha yüksek, NÖM bakımından ise daha düşük değerler gösterdiği ve üzüm çekirdeklerinin besin madde içeriklerinin, üzüm saplarından daha iyi olduğu belirlenmiştir. Buna göre; cibrelerin besin madde içeriklerini çeşit farklılıkları ile karışımdaki sap ve çekirdek oranları etkilemektedir.

Üzüm çekirdeklerinde HY içeriklerinin %11.6-19.6 [23,24], HP içeriklerinin %11-13 arasında değiştiği bildirilmektedir [23,25]. Çalışmada üzüm çekirdeklerinde belirlenen HY ve HP içerikleri literatür bildirişleriyle uyumludur. Alipour ve Rouzbehan [3], yaş üzüm cibresinde HP, HK, NDF, ADF ve ADL içeriklerini sırasıyla %12.6, 10.7, 50.2, 31.2 ve 19.4 KM olarak bildirmişlerdir. Çalışmada belirlenen HP ve ADF içerikleri bu bildirişe benzer bulunmuş, diğerleri ise farklılık göstermiştir. Sarıçiçek ve Kılıç'ın [24] üzüm cibresi için bildirdikleri HK (%20.30) ve HS (%33.21) içerikleri çalışmada elde edilenden daha yüksek, OM (%79.69) ve NÖM (%30.94) içerikleri daha düşük, HY (%3.99) ve HP (%11.54) içerikleri ise benzer bulunmuştur. Bu farklılıklar çalışmada kullanılan çeşit farklılığı, atıklara yapılan farklı uygulamalar, sap ve çekirdek oranı farklılıkları ile istenmeyen yabancı madde (toz, toprak vb.) içeriklerindeki farklılıklardan kaynaklanmış olabilir.

Canbolat ve ark.[26] yonca silajına ilave edilen üzüm cibresinde KM (%27.32), OM (%95.74), HK (%4.26), SÇK (% 5.67), ADF (%30.76), HP (%14.21), HY (%9.37) ve toplam tanen miktarlarını (%7.84) olarak saptamıştır. Bu değerler çalışmada belirlenen HY içeriğinden düşük, HK içeriğinden yüksek olup, OM, HP, NDF ve ADF içerikleri benzer bulunmuştur. Üzüm cibresine ait KM (%92.40-94.57), HP (%11.34-12.59), HY (%7.25-8.14), HS (%33.06-34.28), HK (%10.18-11.43) ve ADF (%52.26-55.78) değerlerinin belirlendiği çalışmalarla [27], bu çalışmada kurutulmuş üzüm cibresinde saptanan HP içeriğinin uyumlu olduğu; HS, HY, ADF ve HK içerikleri arasında ise oldukça önemli farklılıklar bulunduğu gözlenmiştir. Bu durum Varış ve ark.'nın [5] bildirdiği çeşit farklılığı ile farklı işleme ve elde ediliş yöntemine bağlanabilir. Nitekim, işleme esnasında toprak karışması HK içeriklerinin daha yüksek olmasına neden olabilmektedir. Bununla birlikte bu farklılıklar; yem bitkisi türü, gübreleme ve sulama, toprak yapısı, iklim, hasat zamanı, kurutma ve depolama şartlarındaki farklılıklar, biçim zamanı ve vejetasyon dönemi gibi bazı faktörlerden de kaynaklanmış olabilir.

Farklı üzüm cibrelerine ait kuru madde sindirilebilirliklerinin sığır, koyun ve keçilerde %26-39 arasında değiştiği belirtilmekte olup [28], kuru üzüm cibresine ait sindirilebilirliklerin yonca otu (%69) ve sudan otu (%65) sindirilebilirliklerinden oldukça düşük olduğu bildirilmektedir [28]. Bu durum farklı üzüm çeşitleri kullanımında da görülebilmekte olup, Spanghero ve ark.[29], üzüm çekirdekleri ve cibrelerin; fenolik maddeler ve saponin içeriklerinin beyaz üzüm çeşitlerinde kırmızı çeşitlerden daha yüksek olduğunu; farklı bölgelerde yetiştirilen kırmızı üzümlerin çekirdek ve posaların farklı besin madde içeriğine sahip olduklarını bildirmişlerdir.

Mirzaei-Aghsaghali ve ark.[30] tarafından, beyaz üzüm cibresine ait KM (%95.3), HP (%17.27), HS (%22.8), HY (%3.7), HK (%5.7), NDF (%59.5) ve ADF (%52.5) içerikleri

belirlenmiş olup, bu sonuçlar çalışmada belirlenen HS ve HY içerikleriyle benzer, diğerlerinden ise daha yüksek bulunmuştur. Araştırıcılar beyaz üzüm cibresinin besin madde içeriklerine göre (kısmen yüksek HP; düşük HK ve HS içerikleri ile) ruminant beslemede kaba yem kaynağı olarak kullanılabileceğini bildirmişlerdir. Kırmızı ve beyaz üzüm çekirdeklerinin HP (%12-14), HY (%7-12) ve HK (%6-9) içeriklerinin belirlendiği çalışmada [31] elde edilen değerler, çalışmada belirlenen HP ve HK değerleri ile benzer, HY içerikleri ise bu çalışmada daha düşük bulunmuştur. Kırmızı ve beyaz üzüm cibrelerinin enerji değeri ve sindirilebilirliklerin belirlendiği bir başka çalışmada ise beyaz üzüm cibresinin kırmızıya göre daha fazla şeker içeriğine sahip olduğu ve daha düşük lifli bileşenler içerdiği belirlenmiştir. Ayrıca kuru madde bazında; OM, HP, HY, HS, NDF, ADF ve ADL içerikleri beyaz üzüm cibrelerinde sırasıyla %93.3, 9.3, 4.8, 19.9, 30.6, 25.7 ve 20.2, kırmızı üzüm cibrelerinde ise %94.3, 15.5, 7.0, 31.2, 50.7, 36.5 ve 26.7 olarak bildirilmiştir [32].

Bazal diyete üzüm cibresi ilave edildiğinde yemlerin sindirilebilirlikleri ve enerji içerikleri önemli düzeyde azalmış, kırmızı üzüm cibresinde daha fazla azalma olmuştur. Ayrıca, üzüm cibresi; protein sindirilebilirliğini önemli oranda düşürmüştür [32].

Tavşan diyetlerinde yoncaya farklı oranlarda (0, 100, 200 ve 300 g/kg) üzüm cibresi ilave edilen bir çalışmada [33], üzüm cibresi ilavesinin ham protein sindirilebilirliğini ve enerji içeriğini önemli düzeyde düşürdüğü bildirilmiştir. Çalışmada elde edilen bulgular genel olarak araştırıcıların bildirdiği sınırlar içerisinde kalmıştır. Görülen farklılıkların en önemli kaynağının çeşit farklılığı, ADL içeriğinin fazlalığı, işleme metodu ve toprak yapısındaki farklılıklardan kaynaklandığı söylenebilir. Ayrıca, protein sindirilebilirliğinin önemli oranda düşmesinin sebebi ise atıkların içerdiği tanen miktarıyla açıklanabilir, nitekim tananlerin proteinleri bağladıkları (üzüm çekirdeklerinde daha kompleks bağlanma vardır) ve sindirim enzimlerini engellemesi dolayısıyla proteinleri rumende parçalamaktan koruduğu bildirilmektedir [34].

Benzer tanen içeriğine sahip kurutulmuş cibrelerle üzüm çekirdeklerinin farklı *İVGS* ve KMS değeri göstermesi; üzüm cibrelerinin sindirilebilirliklerinin sadece tanen içeriğine bağlı olmadığını, başka faktörlerin de etkisinin olduğunu göstermiştir. Abarghuei ve ark.[35] üzüm cibrelerine ait kondanse tanen içeriklerini %7.9 olarak bildirmişlerdir. Bu değer çalışmada elde edilen değerden düşük olup, çeşit farkından, toprak yapısına; üzümlerin fabrikada işlenme yönteminden, atıklardaki karışım oranına kadar pek çok faktörün bu farklılıklara neden olduğu düşünülmektedir.

Taze cibrenin %15-20 oranında kolay çözünebilir ve fermente olabilir karbonhidrat içermesi silolanabilirliği açısından önemlidir [36]. Ayrıca yüksek düzeyde (186-236 g/kg KM) tanen içermesi [37] nedeniyle silolardaki yemlerin yapısında bulunan proteinleri bağlamak suretiyle amonyak azotu (NH_3-N) şeklinde azot kaybını önlediği ve bu yolla silajlarda protein kaybını azalttığı bildirilmektedir [3,37]. Kuru üzüm cibresi de yüksek tanen içerikli bir yan ürün olarak, silaj fermentasyonunda proteinleri parçalanmaktan korumak için kullanılabilir [34]. Proteinlerin parçalanmasını önlemek için 1 kg bitki kuru maddesinde en az 5 g kondense tanen olması gereklidir [38]. Buna göre çalışmada kullanılan atıkların bu miktardan daha fazla tanen içeriğine sahip olduğu, dolayısıyla da proteinlerin parçalanmasını önleyeceği anlaşılmaktadır.

Bu çalışmada en düşük *İVGS* değerini cibre peletleri göstermiş, en iyi *İVGS* değeri ise kurutulmuş cibrelerde saptanmıştır (P<0.001). Üzüm çekirdeklerdeki *İVGS* değeri ise bu ikisi arasında kalmıştır. Yemlerin sindirilebilirliklerindeki farklılıklar içerdikleri tananlerden kaynaklanabilir. Cibre peletlerinin yapım aşamasında gördüğü ısıl işlemler dolayısıyla da sindirilebilirliklerin daha düşük olacağı düşünülmektedir. Ayrıca, üzüm çekirdeklerinde fenolik maddelerin fazla olduğu bilinmektedir. Bununla birlikte, tananler yemlerin besleme değerini ligninden daha fazla azaltabilir ve hayvan sağlığını olumsuz yönde etkileyebilir [39,40]. Üzüm cibrelerinin yüksek lignin ve tanen içeriklerinden dolayı hayvan yemi olarak kullanılabilirliği sınırlı olup, tanen bağlayıcı maddelerle birlikte kullanılması önerilmektedir [3,6]. Tananlerin, yemlerin besleme değeri üzerine olumsuz etkisini azaltmak için; yemlerin ıslatılması, üre, CaOH, NaOH ve kül solüsyonları ile muamele edilmesinde kondense tanen miktarları önemli düzeyde azalmaktadır. Bunlardan; ıslatılma sonucunda yemde bulunan kondense tanen suyla birlikte uzaklaşmaktadır [41]. Buna göre, çalışmada kullanılan üzüm atıklarının ıslatılma işlemiyle kondanse tanen içeriklerinin azaltılması ve hayvan beslemede yaygın kaba yem kaynağı olarak kullanılması mümkün olabilecektir.

Yemlerde %5-10 tanen bulunmasının tiksinme meydana getirdiği, yem tüketimi ve canlı ağırlık artışını azalttığı, besin maddelerinin sindirilme derecesi ve emilimini düşürdüğü, performansı olumsuz etkilediği ve toksik etkilerin ortaya çıkmasına neden olduğu bildirilmektedir [40]. Bu çalışmada kullanılan atıkların %5'ten fazla kondanse tanen içermesi nedeniyle hayvanlara tek başlarına kaba yem kaynağı olarak verilmesi durumunda istekle tüketiminin düşük olacağı düşünülmektedir. Ruminant hayvanlar kondense tanene karşı farklı tolerans göstermekte olup, keçilerin koyunlara göre tolerans seviyesi oldukça yüksektir. Dolayısıyla keçilerin, rasyonlarda %8-10 düzeyinde tanen bulunmasını tolere edebildiği ve üzüm atıklarının kullanımında en uygun hayvanların keçiler olduğu düşünülmektedir [39].

Çalışmada, HP içeriği bakımından en yüksek değerler cibre peletlerinde görülmesine rağmen HS, NDF ve ADF ve CT bakımından zengin, NÖM bakımından da fakir olması besleme değerini olumsuz yönde etkilemiştir. Yapılan değerlendirmede cibre peletleri 5. sınıf kaba yem (reddedilecek düzeyde kötü) yemler sınıfına girmektedir. Cibre peletleri hazırlanırken, besleme değerini artırıcı

melas vb. katkı maddeleri ilavesinin hayvan beslemede kullanılabilirliğini kısmen de olsa artıracağı ve bu sayede hayvansal üretime katkı sağlayacağı düşünülmektedir.

Sonuç olarak üzüm sanayi atıklarının kondanse tanen içerikleri bakımından %5'in üzerinde KT içeriğine sahip olmasının hayvanlarda yem tüketimini engelleyeceği, bunun için atıkların ıslatılarak tanen içeriğinin azaltılması tavsiye edilmektedir. Besin madde içerikleri dikkate alındığında üzüm atıklarının hiç birinin, tek başına kaba yem kaynağı olarak kullanılabilmesi mümkün görülmemekte ancak, diğer kaba yemlerle birlikte bir miktar kullanılmasının mümkün olabileceği sonucuna varılmıştır. Ancak bu atıklar, kaba yemlere alternatif olarak düşünülmemeli, kısmen kaba yemlerin yerine kullanılabileceği dikkate alınmalıdır. *İn vitro* sindirilebilirlikleri ve kaba yem kalite sınıfı bakımından yemler değerlendirildiğinde kurutulmuş cibrenin hayvan beslemede kullanım için diğerlerine göre daha uygun olduğu kanaatine varılmıştır. Üzüm atıklarının katkı maddeleri ilavesiyle peletlenmesi sayesinde, atıkların besleme değerlerinin artırılması, hayvan performansının iyileşmesi, yem saçımının ve tozuşmanın engellenmesi sağlanabilecektir. Bununla birlikte, üzüm atıklarının; yonca gibi yüksek protein içerikli silajlarda protein parçalanmasını önlemek amacıyla katkı maddesi olarak kullanılması ve farklı tanen bağlayıcılar kullanılarak yemlerin sindirilebilirliğini artırmaya yönelik ileri düzeyde *in vivo* çalışmaların yapılması önerilmektedir.

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Research Article

Effects of Thymoquinone on Cortisol Level, Blood Antioxidant Parameters and Capacity in Broiler Chickens under Oxidative Stress

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Abstract

This study was conducted to evaluate the effect of graded doses of thymoquinone (TQ) in broiler chickens exposed to oxidative stress on performance, antioxidant capacity and blood biochemical parameters. A total of 320 one-day-old broiler chicks was used in a completely randomized design. Chicks were divided to two groups, 160 chicks exposed to oxidative stress induced by tert-BHP injection (ip, 0.02 mmol per kg body weight) three in week from week 3 of age and 160 chicks as non-stressed. In each group, thymoquinone injection (ip) at 4 doses of 0, 5, 8 and 11 mg per kg body weight (40 chicks for each dose) were done three in week from week 2 of age. Results showed that TQ at dose of 8 mg per kg body weight increased total body gain and feed intake, but at dose of 11 mg per kg body weight improved feed conversion ratio in non-stressed chickens ($P<0.05$). Administration of TQ significantly increased the enzyme levels of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and the levels of total antioxidant capacity (TAC), and decreased cortisol, enzyme activities of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) and malondialdehyde (MDA) in both non-stressed and stressed chickens. Based on the results of this study, thymoquinone administration at dose of 8 and 11 mg/kg body weight could ameliorate the adverse effect of oxidative stress on growth and enhance the blood antioxidant capacity and antioxidant enzyme activity.

Keywords: *Antioxidant enzyme, Broiler, Oxidative stress, Performance, Thymoquinone*

Oksidatif Stres Altındaki Broiler Tavuklarda Timokuinon Uygulamasının Kortizol Seviyesi, Kan Antioksidan Parametreleri ve Kapasitesi Üzerine Etkileri

Özet

Bu çalışma, farklı dozlardaki timokuinon (TQ) kullanımının oksidatif strese maruz bırakılan broiler tavuklardaki performans, antioksidan kapasite ve kan biyokimyasal parametreler üzerine etkilerini araştırmak amacıyla yürütüldü. Toplam 320 adet bir günlük broiler civcivler rastgele düzen sistemi içerisinde gruplandırıldı. Civcivler iki gruba ayrılarak 160 civciv haftada üç kez ve 3 haftalıktan itibaren olmak üzere tert-BHP enjeksiyonu (ip. olarak 0.02 mmol her bir kg vücut ağırlık) ile oksidatif strese maruz bırakıldı. Diğer 160 civcive stres uygulanmadı. Her bir grup için timokuinon enjeksiyonu ip. olarak 4 doz halinde (her bir kg vücut ağırlığa 0, 5, 8 ve 11 mg dozlarında - her bir doz için 40 civciv olacak şekilde) haftada üç kez ve 2 haftalıktan itibaren olmak üzere uygulandı. Elde edilen sonuçlar her bir vücut ağırlığa 8 mg dozundaki TQ uygulamasının total vücut ağırlık kazanımını ve yem tüketimini arttırırken her bir vücut ağırlığa 11 mg dozundaki TQ uygulamasının ise yem dönüşüm oranını stres oluşturulmayan civcivlerde geliştirdiğini gösterdi ($P<0.05$). TQ uygulanması hem stres uygulanan hem de uygulanmayan civcivlerde anlamlı derecede süperoksit dismutaz (SOD), katalaz (CAT) ve glutatyon peroksidaz (GPx) enzim seviyeleri ile total antioksidan kapasitesini (TAC) artırırken kortizol, alanin aminotransferaz (ALT), alkalin fosfataz (ALP) ve aspartat aminotransferaz (AST) enzim aktiviteleri ile malondialdehit (MDA) seviyesini düşürdü. Çalışmanın sonuçları, her bir kg vücut ağırlığa 8 ve 11 mg dozlarındaki timokuinon uygulamasının büyüme üzerinde oksidatif stresin olumsuz etkilerini azalttığını, kan antioksidan kapasitesini ve antioksidan enzim aktivitesini ise arttırdığı gösterdi.

Anahtar sözcükler: *Antioksidan enzim, Broiler, Oksidatif stres, Performans, Timokuinon*

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INTRODUCTION

Oxidative stress is a condition caused by an imbalance between pro-oxidants and antioxidants. This imbalance is due to increased levels of reactive oxygen species, nitrogen species or decreased antioxidant defense system occurs [1]. An increase in reactive oxygen species causes lipids peroxidation and damage to proteins and then the vital cell functions which finally lead to poor performance in animals [2]. There are two enzymatic and non-enzymatic systems used to protect the body against oxidative damages [3]. Addition of antioxidant supplement to diet is a key non-enzymatic way to increase the antioxidant capacity of the animal body [4-6]. There are many different antioxidants in the plants which are classified in the category of the natural antioxidants and show the protective effects of oxidative stress [6]. Black seed (Nigella sativa) with the major active antioxidant component named thymoquinone (TQ) has been used as a natural antioxidant [4]. Supplementation of TQ in broiler diets showed the better performance, health and lower mortality rate [7]. The administration of TQ in rodents and other lab animals showed the anti-stress, anti-microbial, anti-tumor and immune system stimulant effects [4]. The effects of thymoquinone as an antioxidant on many biological parameters [2,4,8-10] in non-stressed condition have been studied, but its effect on growth and health parameters of broiler chicks in the stress condition remains also unclear. We hypothesized that thymoquinone is capable to increase blood antioxidant capacity and prevent the adverse effects of oxidative stress on performance and blood parameters in broiler chicks. Therefore, this experiment was carried out to evaluate the effects of TQ on performance, antioxidant capacity and blood biochemical parameters in non-stressed and stressed broiler chicks.

MATERIAL and METHODS

Chicks used in this study received human care and the experimental protocol was approved by the Research Committee of Islamic Azad University, Science and Research Branch (approval date: 17.05.2014; no: 18500).

Chemicals

Tert- butyl hydroperoxide (2-Methylpropane-2-peroxol, tert- BHP) was used to induce the oxidative stress. tert-BHP was purchased from Sigma–Aldrich Chemical Company (CAS Registry No. 75-91-2). Thymoquinone (2-isopropyl-5-methyl-1, 4- benzoquinone) was purchased from Sigma-Aldrich Chemical Company (CAS Registry No. 490-91-5). TQ diluted in dimethyl sulfoxide and pure sterile olive oil, and injected intraperitoneally.

Birds and Management

A total of 320 one-day-old broiler chicks (Ross 308) was used in a completely randomized design. Chicks were divided to two groups, 160 chicks exposed to oxidative stress induced by tert-BHP and 160 chicks as non-stressed. The birds belonging to oxidative stressed experimental groups intraperitoneally received 0.2 mmol tert-BHP per kg body weight (BW) intraperitoneally three in week from week 3 of age. A sham operation was conducted in birds belonging to non-stressed groups through injection of normal saline. Non-stressed or stressed chicks were divided to four subgroups with 40 chicks per each. Thymoquinone injected (ip) to subgroups at the levels of 0, 5, 8 and 11 mg per kg body weight three in week from week 2 of age. The study consisted of 8 treatments with 4 replicates of 10 birds per each. Treatments were as C, control; CTQ5, received TQ at 5 mg per kg BW; CTQ8, received TQ at 8 mg per kg BW; CTQ11, received TQ at 11 mg per kg BW; S, exposed to oxidative stress alone; STQ5, stressed chicks received TQ at 5 mg per kg BW; STQ8, stressed chicks received TQ at 8 mg per kg BW; and STQ11, stressed chicks received TQ at 5 mg per kg BW. The chicks were weighed and randomly assigned to experimental units. The rearing temperature was 32°C in the first week rearing to 21°C at the end of the trial. The birds were kept under a 23 h light: 1 h dark and had a free access to fresh water. Body weight and feed consumption were measured and feed conversion ratio (FCR) calculated for overall period.

Blood Sampling and Measurements

On days 28 and 42, 2 chicks randomly selected from each replicate and blood samples were taken from wing's vein and then the blood serum was separated with centrifuge at 2000 × g for 15 min and stored at -20°C. Individual serum samples were analyzed for aspartate aminotransferase (AST), alkaline aminotransferase (ALT), alkaline phosphatase (ALP), catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and Plasma malondialdehyde level was determined using commercial kits (Pars Azmoon, Tehran, Iran) based on method described by Dropper et al.[11]. Antioxidant capacity was measured by Koracevic method [12] using an automated analyzer (Technicon RA-1000, Tarrytown, USA). Serum cortisol level was measured by EIA kit (Mono Bind Co., CA, USA).

Statistical Analysis

Statistical analyses were conducted with the general linear model procedure of SAS for Windows version 9.1 (SAS Institute Inc., Cary, NC) to determine if variables differed between groups. The Kolmogorov-Smirnov test was used to test the normal distribution of the data before statistical analysis was performed. The data were compared between groups by Duncan test. Probability values of less than 0.05 ($P < 0.05$) were considered significant.

RESULTS

Total antioxidant capacity of broiler chickens was shown in *Table 1*. Chickens in CTQ8 and CTQ11 had the highest antioxidant capacities in both days 28 and 42 of

age. TQ increased total antioxidant capacity of serum in non-stressed chicks compared to control group, also in stressed chicks TQ increased this capacity as compared with S group, especially at level of 11 mg per kg BW.

Liver enzymes appeared in serum and MDA level of chicks at days 28 and 42 of age are presented in *Table 2* and *3*. Chicks in S group had the highest and those in CTQ8 and CTQ11 had the lowest levels of ALT, AST, ALP and MDA in both measurement days. In overall, administration of TQ resulted in decrease of liver enzyme and MDA level of serum in non-stressed chicks compared with the control group. Also, TQ decreased these measurements in stressed groups as compared with chicks in S group. The effect of TQ in decrease of ALT, AST, ALP and MDA levels in the serum was lower in stressed compared to non-stressed conditions.

Enzyme activities of SOD, GPx and CAT are presented in *Tables 4* and *Table 5*. Chicks in CTQ8 and CTQ11 had the highest and chicks in S and STQ5 had the lowest enzyme activity of SOD, GPx and CAT in 28 and 42 of age. TQ administration resulted in increase of these enzyme activity in non-stressed and stressed chicks; however, its effect on enzyme activity in non-stressed was higher than stressed conditions.

Table 1. *The effect of Thymoquinone on serum total antioxidant capacity of broilers (mmol per liter)*
Tablo 1. *Timukuinon uygulamasının broiler civcivlerde serum total antioksidan kapasite üzerine etkileri (mmol/L)*

Control Day	Treatments							
	C	CTQ5	CTQ8	CTQ11	S	STQ5	STQ8	STQ11
Day 28	0.64^{bc}	0.83^{b}	1.24^{a}	1.22^{a}	0.32^{d}	0.48^{cd}	0.51^{cd}	0.67^{bc}
Day 42	0.56^{c}	0.79^{b}	1.26^{a}	1.24^{a}	0.41^{d}	0.52^{c}	0.53^{c}	0.71^{b}

Means within columns with different superscript differ significantly (P<0.05); ***C,*** *control;* ***CTQ5,*** *received TQ at 5 mg per kg BW;* ***CTQ8,*** *received TQ at 8 mg per kg BW;* ***CTQ11,*** *received TQ at 11 mg per kg BW;* ***S,*** *exposed to oxidative stress alone;* ***STQ5,*** *stressed chicks received TQ at 5 mg per kg BW;* ***STQ8,*** *stressed chicks received TQ at 8 mg per kg BW; and* ***STQ11,*** *stressed chicks received TQ at 5 mg per kg BW*

Table 2. *The effect of thymoquinone on level of liver enzymes and MDA in serum of broilers at day 28 of age*
Tablo 2. *Timukuinon uygulamasının 28 günlük broiler civcivlerde karaciğer enzim seviyesi ve serum MDA üzerine etkileri*

Treatments	ALT (U/L)	ALP (U/100 mL)	AST (U/L)	MDA (mol/L)
C	9.42±1.14^{d}	758.51±27^{f}	39.11±3.79^{e}	4.11±0.37^{e}
CTQ5	8.26±1.20^{f}	749.95±23^{f}	38.72±3.17^{e}	3.64±0.29^{f}
CTQ8	7.25±1.39^{g}	684.94±24^{f}	33.40±3.52^{f}	3.32±0.33^{g}
CTQ11	8.83±1.18^{e}	928.84±26^{e}	31.56±3.66^{g}	3.28±0.3
S	17.11±1.36^{a}	2451.26±36^{a}	74.28±5.91^{a}	6.89±0.56^{a}
STQ5	12.15±0.97^{c}	2289.13±43^{b}	61.35±5.27^{b}	4.47±0.41^{c}
STQ8	13.91±1.39^{b}	2108.54±36^{c}	57.14±4.37^{c}	4.24±0.31^{d}
STQ11	13.85±1.41^{b}	1962.73±39^{d}	53.54±4.78^{d}	4.27±0.39^{d}

Means within columns with different superscript differ significantly (P<0.05); ***C,*** *control;* ***CTQ5,*** *received TQ at 5 mg per kg BW;* ***CTQ8,*** *received TQ at 8 mg per kg BW;* ***CTQ11,*** *received; TQ at 11 mg per kg BW;* ***S,*** *exposed to oxidative stress alone;* ***STQ5,*** *stressed chicks received TQ at 5 mg per kg; BW;* ***STQ8,*** *stressed chicks received TQ at 8 mg per kg BW; and* ***STQ11****, stressed chicks received TQ at 5 mg per kg BW*

Table 3. *The effect of thymoquinone on level of liver enzymes and MDA in serum of broilers at day 42 of age*
Tablo 3. *Timukuinon uygulamasının 42 günlük broiler civcivlerde karaciğer enzim seviyesi ve serum MDA üzerine etkileri*

Treatments	ALT (U/L)	ALP (U/100 mL)	AST (U/L)	MDA (mol/L)
C	12.41±1.06^{d}	1278.62±41^{e}	36.61±3.46^{f}	5.97±0.81^{d}
CTQ5	11.22±0.87^{e}	1224.08±38^{f}	37.45±3.52^{e}	5.66±0.74^{d}
CTQ8	10.13±1.21^{f}	1127.58±28^{g}	33.36±2.87^{g}	4.76±0.58^{e}
CTQ11	11.40±1.34^{e}	1005.77±34^{h}	32.95±3.13^{g}	3.71±0.92^{f}
S	16.71±1.33^{a}	2187.27±39^{a}	63.05±3.68^{a}	9.38±0.75^{a}
STQ5	11.53±0.98^{e}	1962.68±36^{b}	59.30±4.05^{b}	7.86±1.03^{b}
STQ8	13.64±1.18^{b}	1884.76±43^{c}	54.10±3.79^{c}	6.87±0.92^{c}
STQ11	13.26±1.27^{c}	1665.42±29^{d}	52.82±4.14^{d}	6.79±0.67^{c}

Means within columns with different superscript differ significantly (P<0.05); ***C,*** *control;* ***CTQ5,*** *received TQ at 5 mg per kg BW;* ***CTQ8,*** *received TQ at 8 mg per kg BW;* ***CTQ11,*** *received TQ at 11 mg per kg BW;* ***S,*** *exposed to oxidative stress alone;* ***STQ5,*** *stressed chicks received TQ at 5 mg per kg BW;* ***STQ8,*** *stressed chicks received TQ at 8 mg per kg BW; and* ***STQ11,*** *stressed chicks received TQ at 5 mg per kg BW*

Cortisol levels in the serum of chickens are presented in *Tables 4* and *Table 5*. There were differences for cortisol level among treatment at day 28 of age, but a significant difference was found between chicks in S group and other treatments at days 28 and 42 of age. The highest cortisol level in both days was for S group. Administration of TQ decreased serum cortisol level in stressed condition at days 28 and 42 of age.

Table 6 shows the effects of TQ on body weight gain, feed intake and feed conversion ratio of broiler chickens in overall period. The lowest body weight gain was for

Table 4. *The effect of thymoquinone on Cortisol and activities of antioxidant enzymes in serum of broilers at day 28 of age*
Tablo 4. *Timukuinon uygulamasının 28 günlük broiler civcivlerde kortizol ve antioksidan enzim aktiviteleri üzerine etkileri*

Treatments	Gpx (U/mL)	CAT (U/mL)	SOD (U/mL)	Cortisol (ng/mL)
C	476.83±17.5^{d}	13.15±1.24^{c}	114.34±5.64^{c}	1.52±0.06^{cd}
CTQ5	522.12±23.6^{c}	14.64±1.49^{b}	139.81±5.71^{b}	1.47±0.04^{d}
CTQ8	571.48±31.3^{b}	15.76±1.62^{a}	151.58±6.84^{a}	1.57±0.05^{cd}
CTQ11	596.39±29.4^{a}	15.65±1.34^{a}	144.62±4.94^{a}	1.43±0.06^{d}
S	363.26±28.8^{h}	6.17±0.62^{f}	76.28±4.19^{f}	3.16±0.05^{a}
STQ5	422.63±26.4^{g}	8.92±0.77^{e}	99.41±4.26^{g}	1.97±0.07^{b}
STQ8	438.78±30.3^{f}	9.56±1.07^{d}	113.28±5.53^{e}	1.77±0.04^{bc}
STQ11	458.35±24.1^{e}	9.84±0.86^{d}	107.24±4.76^{d}	1.63±0.07^{cd}

Means within columns with different superscript differ significantly (P<0.05); ***C,*** *control;* ***CTQ5,*** *received TQ at 5 mg per kg BW;* ***CTQ8,*** *received TQ at 8 mg per kg BW;* ***CTQ11,*** *received TQ at 11 mg per kg BW;* ***S,*** *exposed to oxidative stress alone;* ***STQ5,*** *stressed chicks received TQ at 5 mg per kg BW;* ***STQ8,*** *stressed chicks received TQ at 8 mg per kg BW; and* ***STQ11,*** *stressed chicks received TQ at 5 mg per kg BW*

Table 5. *The effect of thymoquinone on Cortisol and activities of antioxidant enzymes in serum of broilers at day 42 of age*
Tablo 5. *Timukuinon uygulamasının 42 günlük broiler civcivlerde kortizol ve antioksidan enzim aktiviteleri üzerine etkileri*

Treatments	Gpx (U/mL)	CAT (U/mL)	SOD (U/mL)	Cortisol (ng/mL)
C	428.57±19.4^{b}	13.87±1.35^{c}	110.24±6.32^{b}	1.55±0.04^{b}
CTQ5	442.18±21.6^{b}	14.92±1.52^{b}	135.42±5.15^{a}	1.42±0.07^{b}
CTQ8	483.74±18.7^{a}	16.76±1.11^{a}	147.27±5.24^{a}	1.51±0.04^{b}
CTQ11	478.36±23.4^{a}	16.84±1.27^{a}	139.36±4.86^{a}	1.39±0.03^{b}
S	291.44±27.6^{e}	9.63±1.77^{f}	85.70±5.55^{c}	2.64±0.06^{a}
STQ5	325.73±20.9^{d}	9.76±1.61^{f}	107.28±7.09^{b}	1.71±0.05^{b}
STQ8	353.72±18.7^{c}	10.28±1.74^{e}	119.13±6.31^{b}	1.64±0.04^{b}
STQ11	488.91±24.2^{b}	11.43±1.43^{e}	111.51±7.51^{b}	1.58±0.06^{b}

Means within columns with different superscript differ significantly (P<0.05); ***C,*** *control; CTQ5, received TQ at 5 mg per kg BW;* ***CTQ8,*** *received TQ at 8 mg per kg BW;* ***CTQ11,*** *received TQ at 11 mg per kg BW;* ***S,*** *exposed to oxidative stress alone;* ***STQ5,*** *stressed chicks received TQ at 5 mg per kg BW;* ***STQ8,*** *stressed chicks received TQ at 8 mg per kg BW; and* ***STQ11,*** *stressed chicks received TQ at 5 mg per kg BW*

Table 6. *Effects of thymoquinone on feed intake, daily weight gain and feed conversion ratio of broilers with and without oxidative stress*
Tablo 6. *Timokuinon uygulamasının oksidatif stres uygulanan ve uygulanmayan civcivlerde yem tüketimi, günlük ağırlık kazanımı ve yem dönüşüm oranı üzerine etkileri*

Treatments	Body Weight Gain	Feed Consumption	Feed Conversion Ratio
C	2320±41.2^{b}	4508±63.2^{b}	1.94±0.09^{ab}
CTQ5	2426±38.4^{ab}	4682±51.8^{a}	1.93±0.11^{ab}
CTQ8	2461±51.2^{a}	4702±48.9^{a}	1.91±0.06^{bc}
CTQ11	2388±62.9^{b}	4502±71.7^{b}	1.88±0.08^{c}
S	2165±52.2^{c}	4380±61.5^{c}	2.02±0.07^{a}
STQ5	2373±48.3^{ab}	4752±56.6^{a}	2.00±0.04^{a}
STQ8	2400±38.4^{ab}	4746±59.5^{a}	1.98±0.03^{ab}
STQ11	2327±54.8^{ab}	4539±67.3^{b}	1.95±0.08^{ab}

Means within columns with different superscript differ significantly (<0.05); ***C,*** *control;* ***CTQ5,*** *received TQ at 5 mg per kg BW;* ***CTQ8,*** *received TQ at 8 mg per kg BW;* ***CTQ11,*** *received TQ at 11 mg per kg BW;* ***S,*** *exposed to oxidative stress alone;* ***STQ5,*** *stressed chicks received TQ at 5 mg per kg BW;* ***STQ8,*** *stressed chicks received TQ at 8 mg per kg BW; and* ***STQ11,*** *stressed chicks received TQ at 5 mg per kg BW*

stressed chicks (S) and the highest was for CTQ8. Chicks in stressed groups received thymoquinone (STQ5, STQ8 and STQ11) had higher body weight gain than chicks in S group. Chickens exposed to oxidative stress had the lowest feed intake. Administration of TQ at doses of 5 and 8 mg/kg BW increased and at dose of 11 mg/kg BW had no significant effect on feed intake in non-stressed chicks. The lowest FCR was for chicks received 11 mg TQ per kg BW in non-stressed group. Chicks in S and STQ5 groups had the highest FCR. Administration of TQ at 5, 8 and 11 mg/kg BW in stressed chicks had no significant effect on FCR.

DISCUSSION

Body weight gain of chicks exposed to oxidative stress reduced compared to control and it reflects the effects of free radicals on chicken's body. Free radicals produced by t-BHP particularly peroxyl radical disrupt many body functions including inter- and intra-cellular enzymes and receptors by splitting the peptide chain, changing electric charges and increasing the sensitivity of proteins to proteolysis enzymes [13-16]. In addition, these products lead to membrane lipids peroxidation and affect to cell membrane and increase of cell membrane permeability and ion transfer from inappropriate places of cell surfaces and their mitochondria [14]. These events lead to wasting a lot of produced energy [20] and disruption of cellular function and body weight in chickens receiving tert-BHP. Most of the free radicals produced by oxidative stress play a secondary role to the occurrence of these events could lead to lower growth and feed intake and worse FCR.

Thymoquinone is a free radical scavenger that makes cleanup superoxide, hydroxyl radical and single oxygen molecule [9]. As seen in *Table 1*, thymoquinone increased total antioxidant capacity of serum. This means that TQ acts as free radical scavenger.

Thymoquinone crosses morpho-physiological barriers and its easy access to subcellular compartments could facilitate the radical scavenging effect [21]. Also, thymoquinone influence on intracellular signaling pathways and increased the secretion of insulin [22], catabolism of glucose and energy production in both conditions with and without received t-BHP [23-25]. These two properties, free radical scavenger and improvement in energy production, resulted in increased body weight gain and feed intake; as observed in our study.

At the time of stress, cortisol releases from the adrenal gland and protects the body against stress by increasing the gluconeogenesis and glucose production and the metabolism of fats, proteins and carbohydrates to produce energy and ultimately the protection of the body against stress [26]. Increase of cortisol level in S group shows the effects of free radicals to create oxidative stress. Differences in cortisol levels between S group and STQ groups are due to the antioxidant effect of TQ on the neutralization of a part of free radicals.

The level of AST, ALT, and ALP increased in S group. These enzymes called liver enzymes, because their levels are high in the liver cells and are low in other tissues. Damages to liver cell membranes as a result of oxidative stress disrupt their performance [18], and thus cause to release these three enzymes into the blood [13,27]. High levels of these enzymes in chickens of S group related to the free radicals come from tert-BHP. The neutralization of free radicals in the groups received TQ prevented the damages to liver cells. The destruction of liver cells is reduced by TQ as antioxidant and then a less amounts of these enzymes released into the blood.

The enzyme activities of glutathione peroxidase and catalase increased in both conditions with and without oxidative stress by increasing the level of TQ. There are a lot of findings [7,10,27] that showed free radicals are able to neutralize these two enzymes. Thus the loss of these enzyme activities in groups under oxidative stress is due to the injection of tert-BHP and the production of free radicals. TQ removed free radicals and thereby prevented its negative effect on the enzyme activities of glutathione peroxidase and catalase. A study [10] showed that TQ prevents the glycosylation of superoxide dismutase enzyme by reducing glucose level in the blood. Glycosylation of this enzyme reduces its activity; therefore, TQ can effects on pancreatic beta cells and reduces the glucose level which leads to increase the activity of SOD. This enzyme cooperates with glutathione peroxidase to create the first barrier to defense of the cells, thus the use of TQ can decreases free radicals and damage to the cells.

Based on the results of this study, in non-stressed condition 8 and 11 mg thymoquinone per kg body weight could enhance the antioxidant capacity and activity and performance of broiler chicks and in stressed condition ameliorate the adverse effect of oxidative stress on growth and enhance the blood antioxidant capacity and antioxidant enzyme activity.

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Research Article

Prevalence and Antimicrobial Susceptibility of *Salmonella* in Rendered Animal Products Used in Poultry Feed in Turkey [1]

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Abstract

The increased prevalence of *Salmonella* contamination in poultry has gained considerable scientific attention during last decades. In this study, a total of 500 samples of rendered animal products (meat meal, meat-bone meal, blood meal, chicken meal and feather meal) were obtained from several feed factories and rendering plants in Turkey and these samples were analyzed for *Salmonella* spp., *Salmonella Enteritidis* and *Salmonella Typhimurium* status. According to the results, 13, 11 and 8 samples obtained from feed factories were determined positive for *Salmonella* spp., *Salmonella Enteritidis* and *Salmonella Typhimurium* respectively. However, all samples obtained form rendering plants were negative. Antibiotic susceptibility profiles of isolates confirmed as positive were determined by using 17 different antibiotics. It was determined that *Salmonella* spp. and *Salmonella Enteritidis* serovars were resistant to amikacin, cephazolin and erythromycin, sensitive to amoxicillin, chloramphenicol, flumoquin, phosphomycin, kanamycin, oxytetracycline, spiramycin, streptomycin, tetracycline, tobramycine and vancomycin and moderate sensitive to gentamicin, linkomycin and rifampicin.

Keywords: *Salmonella, Poultry, Rendered animal products, Antibiotic, Susceptibility*

Türkiye'de Kanatlı Yemlerinde Kullanılan Rendering Ürünlerde *Salmonella* Varlığı ve Bunların Antimikrobiyal Duyarlılığı

Özet

Kanatlılarda artan *Salmonella* bulaşıklığı son on yıldır bilimsel yönden büyük önem kazanmıştır. Bu çalışmada Türkiye'deki çeşitli yem fabrikaları ve üretim noktalarından toplam 500 adet hayvansal rendering ürünü (et unu, et-kemik unu, kan unu, tavuk unu ve tüy unu) toplanmış ve bu örnekler *Salmonella* spp., *Salmonella Enteritidis* and *Salmonella Typhimurium* bakımından analiz edilmiştir. Sonuçlara göre yem farbrikalarından elde edilen 13, 11 ve 8 örnek sırasıyla *Salmonella* spp., *Salmonella Enteritidis* and *Salmonella Typhimurium* bakımından pozitif olarak tespit edilmiştir. Bununla birlikte üretim noktalarından toplanan tüm örnekler ise negatif sonuç vermiştir. Pozitif olan izolatların antibiyotik duyarlılıkları 17 farklı antibiyotik kullanılarak tayin edilmiştir. *Salmonella* spp. ve *Salmonella Enteritidis* serovarlarının amikasin, sefazolin ve eritromisin'e dirençli, amoksisilin, kloramfenikol, flumoquin, fosfomisin, kanamisin, oksitetrasiklin, spiramisin, streptomisin, tetrasiklin, tobramisin ve vankomisin'e duyarlı, gentamisin, linkomisin ve rifampisin'e orta derecede duyarlı olduğu tespit edilmiştir.

Anahtar sözcükler: *Salmonella, Kanatlı, Rendering ürünleri, Antibiyotik, Duyarlılık*

INTRODUCTION

Rendered products such as meat and bone meal (MBM), meat meal (MM), poultry meal (PM), feather meal (FM), blood meal (BM) and fish meal (FM) are important animal derived feedstuffs for poultry nutrition [1]. In Turkey, rendering by-products have been used intensively in poultry diets because of their quality protein, calcium and utilizable phosphorus ingredients. Meat-bone meal, the most produced rendering product, has been used only in poultry and pig diets because of BSE (Bovine Spongioform Ensephalopathy) risk. The use of meat and bone meal for

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livestock feeding was banned in 2002 by the European Union [2]. In Turkey, the use of rendered animal products in poultry nutrition were banned by Republic of Turkey Ministry of Food, Agriculture and Livestock in 01/01/2016. However, this prohibition were delayed until 01/01/2017 [3].

Inadequate hygienic conditions in the production and storage of feedstuffs and primer and secondary factors during the slaughter process of animals may lead to microbiological contamination of the obtained products. The most important subject in the use of rendered animal products is the prevention of microbiological contamination. Beside oil seed meals, European Food Safety Authority (EFSA) reported that animal derived proteins were the major risk feed materials for introducing *Salmonella* contamination to feed mills and industrial compound feed [4]. Salmonella species are gram-negative facultative intracellular bacteria and they have a wide spectrum of diseases [5]. Salmonellosis is one of the most frequent foodnorne diseases, being an important public health problem in almost all industrialized countries [6].

Although the risk of *Salmonella* spp. in rendered animal products always available, the temperatures used during the feed production are normally sufficient to eliminate *Salmonella* spp.[7]. In spite of the fact that *Salmonella* contamination is the potential risk for all feedstuffs, only a few of serotype of 2300 identified *Salmonella* are related to clinical symptoms in animals and humans. Also, *Salmonella* strains are not resistant to physical and chemical agents and they deactivate in 1 h at 55°C or in 15-20 min at 60°C [8].

However, the secondary contaminations during the transportation from rendering plant to feed factories and storage are the major problem in *Salmonellosis*. In a study, it was reported that there were any *Salmonellosis* agent in rendering samples obtained from production point, but 8.7% of transported and stored samples were positive for *Salmonella* spp.[9].

Due to the increase in antibiotic-resistant pathogens, the use of antibiotics in poultry feeds were banned legally in Europe Union and in the present country also accepted this decision by considering human and animal health. However, expanding in the global market for livestock, feed, feed additive and food can still lead to the spread of several *Salmonella* serotypes and lead to the increase in the incidence of *Salmonella* spp. infections [10].

The aim of this study was to investigate the existence of *Salmonella* spp., which is considered as an important risk for poultry and consumer health in the worldwide and also Turkey, in poultry feeds contained potential infected rendered animal products, to determine the types of identified strains serologically by specifying the incidence of *S.* Typhimurium and *S.* Enteritidis, which are the most dangerous species, and to determine the antibiotic susceptibility of these species.

MATERIAL and METHODS

Sample Collection

A total of 500 rendering samples (100 meat meal, 100 meat-bone meal, 100 blood meal, 100 chicken meal, 100 feather meal) were collected from rendering plants and feed manufacturers in several provinces in Turkey. All collected samples were transported to Istanbul University, Faculty of Veterinary Medicine Laboratory under cold-chain procedure and stored at +4°C for further analysis.

Isolation and Identification

Pre-enrichment procedure was applied to samples in non-selective medium (buffered peptone water). After homogenization, all samples were incubated at 37°C for 24 h. For selective enrichment, approximately 0.1 mL of each sample was inoculated to selective enrichment medium (Rappaport Vassiliadis Soy Broth) and all tubes were vortexed before incubation. After the incubation period at 42°C for 24 h, transition to brillant-green phenol-red lactose sucrose agar, a spesific solid medium, was done. Due to the suggestions offered by international procedures, a second specific agar (xylose lysine deoxycholate, XLD agar), was also preferred for parallel study. After selective enrichment, parallel transition was done with Standard plate spreading mehod to both agar. After the incubation of mediums at 37°C for 24 h, chemical tests were applied for identification of typical colonies. Optionally, motility test was also done by using semi indol motility (SIM) agar. A loop of colony from all *Salmonella* spp. positive samples was transferred parallelly to Hektoen Enteric Agar and Bismuth Sulphite Agar. Black "rabbit-eye" colonies with a black zone and metallic sheen surrounding the colony in Bismuth Sulphite Agar were confirmed as *Salmonella* Typhimurium and bluish-grey/dark-grey color colonies were confirmed as *Salmonella* Enteritidis [11].

Serological Identification

Serogrouping of 32 strains, determined as *Salmonella* spp. by microbiological isolation, were performed by plate agglutination method. According to agglutination tests performed by using "Phase 1" and "Phase 2" antiserums, it was determined that 11 and 8 strains pertained to serogroup D1 and B, respectively, and they were serotyped as *Salmonella* Enteritidis and *Salmonella* Typhimurium, respectively. Also, 13 isolates evaluated as *Salmonella* spp. have not reacted positively result with available antiserums [12].

PCR Analysis

The primer sequences used in PCR analysis for *Salmonella* spp.[13], *Salmonella* Typhimurium [14] and *Salmonella* Enteritidis [15] are shown in *Table 1*. PCR mix was as follows (final 25 µL); 2 µL DNA samples, 2.5 mM $MgCl_2$, 10 mM Tris–HCl pH 8.0, 5 mM KCl (0.2 mM from each nucleotide), each

Table 1. *The properties of primer sequences designed according to different Salmonella serotypes*
Tablo 1. *Farklı serotiplere göre dizayn edilen primer dizileri ve özellikleri*

Primers (5′ – 3′)	Properties			Target Microorganism
	Gene	Length (bp)	Amp (bp)	
GTGAAATTATCGCCACGTTCGGGCAA	*invA*	26	284	*Salmonella* spp.
TCATCGCACCGTCAAAGGAACC	*invA*	22	284	
CGGTGTTGCCCAGGTTGGTAAT	*fliC*	22	620	*Salmonella* Typhimurium
ACTGGTAAAGATGGCT	*fliC*	16	620	
AGCCAACCATTGCTAAATTGG	*invA*	21	488	*Salmonella* Enteritidis
GCGTAAATCAGCATCTGCAGTAGC	sefA	24	488	

primer (Metabion Inter-national, Martinsried, Germany) 0.8 pmol/mL, 1 U of Taq DNA polymerase (Fermentas, Vilnius, Lithuania). Initial denaturation heat was at 94°C for 5 min. Then the heat treatments, 1 sec at 94°C, 1 sec at 55°C, and 21 sec at 72°C for extension were applied. After 35 cycles, the procedure was completed with 7 min at 72°C heat treatment for last elongation. Amplication products were analyzed in 1.2% (w/v) agarose gel containing 5 µL safe view (Abm, Richmond, Canada).

Antibiotic Susceptibility Testing

Antibiotic sensitivities of isolated *Salmonella* strains were determined by disk diffusion method according to Clinical and Laboratory Standards Institute [16]. For testing, bacterial suspensions were prepared according to McFarland 0.5 turbidity degree and 0.1 mL of suspensions were separated to Muller Hinton Agar and then antibiotic disks (amoxicillin, 15 µg; chloramphenicol, 30 µg; flumoquin, 30 µg; phospho-mycin, 50 µg; kanamycin, 30 µg; oxytetracycline, 30 µg; spiramycin, 100 µg; streptomycin, 10 µg; tetracycline, 30 µg; tobramycine, 30 µg; vancomycin, 30 µg; gentamicin, 10 µg; linkomycin, 10 µg; rifampicin, 5 µg; amikacin, 30 µg; cephazolin, 30 µg; erythromycin, 15 µg) [17] placed on the agar plate. After the incubation of cultures at 37°C for 24 h, diameters of inhibition zones were measured with calliper.

RESULTS

In this study, a total of 500 rendering samples were analysed for *Salmonella* and 32 of samples were positive. While *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium were determined in 13, 11 and 8 samples of positive samples respectively, all samples obtained form rendering plants were negative. *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium contamination in rendering samples obtained from several feed factories and rendering plants were presented in *Table 2* and protocol numbers of 32 positive samples were listed in *Table 3*.

Antibiotic susceptibility status for each isolates were also determined by using 17 different antibiotics. It was determined that *Salmonella* spp. and *Salmonella* Enteritidis serovars were resistant to amikacin, cephazolin and erythromycin, sensitive to amoxicillin, chloramphenicol, flumoquin, phosphomycin, kanamycin, oxytetracycline, spiramycin, streptomycin, tetracycline, tobramycine and vancomycin and moderate sensitive to gentamicin, linkomycin and rifampicin.

Antibiotic suspectibility of strains in 32 positive rendering samples according to their protocol (1-32) numbers were presented in *Table 4* and antibiotic susceptibility percentages of *Salmonella* strains isolated from 32 positive rendering samples were presented in *Table 5*.

DISCUSSION

Almost all by-products transported to rendering plants are mostly contaminated with several pathogenes. It was reported that pathogene contamination in by-products transported to rendering plants were 23% *E. coli* O157:H7, 50% *Salmonella,* 39% *Cryptospodiridium parvum* for cattle origin, 46% *Salmonella*, 49% *Yersinia enterocolitica* for pig origin and 100% *Salmonella* for poultry origin products [18].

Salmonella contamination is the most important microbiological threat in rendering process. Although the application of heat under high pressure during the process is very effective in the elimination of agent, the major risk is the continuation of existence of the agent in the facility by cross-contamination, therefore, re-contamination of end-products by primary and secondary factors such as transportation, storage, factory staffs etc.[19].

Troutt et al.[20] reported that plenty of pathogenes such as Salmonella strains, *Listeria monocytogenes, Campylobacter jejuni* and *Clostridium perfiringens* contamination were detected in raw materilas obtained in pre-processing stage from 17 rendering enterprises in several states of USA. However, in other study by same researchers it was reported that none of these pathogenes were isolated from processed samples obtained from 9 rendering facilities. According to the results it is estimated that treatment of

Table 2. *Salmonella spp., Salmonella Enteritidis and Salmonella Typhimurium contamination in rendering samples obtained from several feed factories and rendering plants*

Tablo 2. *Çeşitli yem fabrikaları ve üretim noktalarından alınan rendering örneklerinde Salmonella spp., Salmonella Enteritidis and Salmonella Typhimurium kontaminasyonu*

Samples Obtained from Feed Factories	Occurence	Incident Rate, %	Isolation and Identification (+)/(-)	PCR Verification (+)/(-)
***Salmonella* spp.**				
Meat meal	4/80	5	4/76	4/76
Meat-bone meal	0/80	0	0/80	0/80
Blood meal	3/80	3.75	3/77	3/77
Chicken meal	6/80	7.5	6/74	6/74
Feather meal	0/80	0	0/80	0/80
Total	13/400	3.25	13/387	13/387
Salmonella Enteritidis				
Meat meal	2/80	2.5	2/78	2/78
Meat-bone meal	0/80	0	0/80	0/80
Blood meal	3/80	3.75	3/77	3/77
Chicken meal	6/80	7.5	6/74	6/74
Feather meal	0/80	0	0/80	0/80
Total	11/400	2.75	11/389	11/389
Salmonella Typhimurium				
Meat meal	2/80	2.5	2/78	2/78
Meat-bone meal	0/80	0	0/80	0/80
Blood meal	1/80	1.25	1/79	1/79
Chicken meal	5/80	6.25	5/75	5/75
Feather meal	0/80	0	0/80	0/80
Total	8/400	2	8/392	8/392
Samples Obtained from Rendering Plants	**Occurence**	**Incident Rate, %**	**Isolation and Identification (+)/(-)**	**PCR Verification (+)/(-)**
***Salmonella* spp.**				
Meat meal	0/20	0	0/20	0/20
Meat-bone meal	0/20	0	0/20	0/20
Blood meal	0/20	0	0/20	0/20
Chicken meal	0/20	0	0/20	0/20
Feather meal	0/20	0	0/20	0/20
Total	0/100	0	0/100	0/100
Salmonella Enteritidis				
Meat meal	0/20	0	0/20	0/20
Meat-bone meal	0/20	0	0/20	0/20
Blood meal	0/20	0	0/20	0/20
Chicken meal	0/20	0	0/20	0/20
Feather meal	0/20	0	0/20	0/20
Total	0/100	0	0/100	0/100
Salmonella Typhimurium				
Meat meal	0/20	0	0/20	0/20
Meat-bone meal	0/20	0	0/20	0/20
Blood meal	0/20	0	0/20	0/20
Chicken meal	0/20	0	0/20	0/20
Feather meal	0/20	0	0/20	0/20
Total	0/100	0	0/100	0/100

Table 3. *Protocol numbers of 32 positive rendering samples*
Tablo 3. *Otuziki pozitif renderin örneğinin protokol numaraları*

***Salmonella spp.* Positive**		***Salmonella Enteritidis* Positive**		***Salmonella Typhimurium* Positive**	
Protocol Number	**Sample**	**Protocol Number**	**Sample**	**Protocol Number**	**Sample**
1	Meat meal	14	Chicken meal	25	Chicken meal
2	Meat meal	15	Chicken meal	26	Chicken meal
3	Chicken meal	16	Chicken meal	27	Chicken meal
4	Chicken meal	17	Chicken meal	28	Chicken meal
5	Blood meal	18	Chicken meal	29	Chicken meal
6	Chicken meal	19	Chicken meal	30	Meat meal
7	Chicken meal	20	Meat meal	31	Meat meal
8	Blood meal	21	Meat meal	32	Blood meal
9	Meat meal	22	Blood meal		
10	Meat meal	23	Blood meal		
11	Blood meal	24	Blood meal		
12	Chicken meal				
13	Chicken meal				

Table 4. *Antibiotic susceptibility of Salmonella strains in 32 positive rendering samples according to their protocol (1-32) numbers*
Tablo 4. *Protokol numaralarına (1-32) göre 32 pozitif rendering örneğinde Salmonella türlerinin antibiyotik duyarlılığı*

Susceptibility	**Amik**	**Amox**	**Ceph**	**Chlo**	**Eryt**	**Flum**	**Phos**	**Gent**	**Kana**	**Link**	**Oxit**	**Rifa**	**Spir**	**Stre**	**Tetr**	**Tobr**	**Vanc**
R	1-13 17-30	-	1-15 18-24 29-32	11 20-21 31	1-6 10-18 20-21 23-27 29-31	11-12 17	-	7-8 17-18	7-8 19	-	-	-	-	-	-	-	-
S	14-16 31-32	1-32	16-17 25-28	1-10 12-19 22-30 32	7-9 19 22 28 32	1-10 13-16 18-32	1-32	9-10 25-26	1-6 9-18 20-32	-	1-32	-	1-32	1-32	1-32	1-32	1-32
MS	-	-	-	-	-	-	-	1-6 11-16 19-24 27-32	-	1-32	-	1-32	-	-	-	-	-

R: *Resistant to antiboitic;* ***S:*** *Sensitive to antibiotic;* ***MS:*** *Moderate sensitive to antibiotic;* ***Amik:*** *Amikacin;* ***Amox:*** *Amoxicillin;* ***Ceph:*** *Cephazolin;* ***Chlo:*** *Chloramphenicol;* ***Eryt:*** *Erythromycin;* ***Flum:*** *Flumoquin;* ***Phos:*** *Phosphomycin;* ***Gent:*** *Gentamicin;* ***Kana:*** *Kanamycin;* ***Link:*** *Linkomycin;* ***Oxit:*** *Oxitetracycline;* ***Rifa:*** *Rifampicin;* ***Spir:*** *Spiramycin;* ***Stre:*** *Streptomycin;* ***Tetr:*** *Tetracycline;* ***Tobr:*** *Tobramycin;* ***Vanc:*** *Vancomycine*

appropriate time-temperature can inactivate large group of food pathogenes during rendering process [20].

In a study carried out by Watkins et al.[21], it was reported that 28 different *Salmonella* strains were isolated from animal feed products and incidence was 18.5%. Pomeroy et al.[22] collected 980 samples of animal feed products from 22 different states in USA and they isolated 43 *Salmonella* strains originated from secondary contaminations in 170 samples. In a recent study, a total of 201 feed ingredient samples (122 animal by-products and 79 plant by-products) were collected from rendering plants and oilseed industry and it eas reported that *Salmonella* were present in 22.9% of samples and animal by-products had a significantly higher *Salmonella* contamination rate (34.4%) than plant by-products [23]. In our study, *Salmonella* serovars (*Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium) were determined in 32 samples of 500 rendered animal products and source of agents were in accord with Watkins et al.[21] and Pomeroy et al.[22]. In this study, salmonella-positive results were only in the samples obtained from feed factories. This finding was associated with secondary contamination sources such as transportation, factory staff and storage conditions and was not in accordance with Ge et al.[23]. Although the revolutionary improvements in food safety have been occured during the last 50 years, still in existence of cross-contaminations in rendered animal products for poultry feed are questionable [24,25].

Proper storage conditions of feedstuffs produced for poultry feeding by using rendering procedures must be kept in mind as the most efficient factor for breaking the contamination chain of *Salmonella*. Sutton et al.[26] reported that *Salmonella* content decreased to the undetectable

Table 5. *Antibiotic susceptibility percentages of Salmonella strains isolated from 32 positive rendering samples*
Tablo 5*. 32 pozitif renderin örneğinden izole edilen Salmonella türlerinin antibiyotiğe duyarlılık yüzdeleri*

Antibiotics	***S. spp.***	***S. Enteritidis***	***S. Typhimurium***
Amikacin	R (100%)	R (72.7%) S (27.3%)	R (75.0%) S (25.0%)
Amoxicillin	S (100%)	S (100%)	S (100%)
Cephazolin	R (100%)	R (81.8%) S (18.2%)	R (50.0%) S (50.0%)
Chloramphenicol	R (7.7%) S (92.3%)	R (18.2%) S (81.8%)	R (12.5%) S (87.5%)
Ertythromycin	R (76.9%) S (23.1%)	R (81.8%) S (18.2%)	R (75.0%) S (25.0%)
Flumoquin	R (15.4%) S (84.6%)	R (9.1%) S (90.9%)	S (100%)
Phosphomycin	S (100%)	S (100%)	S (100%)
Genatmicin	R (15.4%) S (15.4%) MS (69.2%)	R (18.2%) MS (81.8%)	S (25.0%) MS (75.0%)
Kanamycin	R (15.4%) S (84.6%)	R (9.1%) S (90.9%)	S (100%)
Linkomycin	MS (100%)	MS (100%)	MS (100%)
Oxitetracycline	S (100%)	S (100%)	S (100%)
Rifampicin	MS (100%)	MS (100%)	MS (100%)
Spiramycin	S (100%)	S (100%)	S (100%)
Streptomycin	S (100%)	S (100%)	S (100%)
Tetracycline	S (100%)	S (100%)	S (100%)
Tobramycin	S (100%)	S (100%)	S (100%)
Vancomycin	S (100%)	S (100%)	S (100%)

R: *Resistant to antiboitic;* ***S:*** *Sensitive to antibiotic;* ***MS:*** *Moderate sensitive to antibiotic*

levels in meat-bone meal samples exposed to 30 cfu/g *Salmonella* contamination when kept under 28°C for 48 h.

Because there has been growing public health concern over the worldwide emergence of antibiotic-resitant strains of a number of pathogenic bacteria, including *Salmonella* during the past few decades [27], the other parameter investigated in this study was the determination of the susceptibility to several antibiotics of *Salmonella* strains *(Salmonella* spp.*, Salmonella*. Enteritidis and *Salmonella* Typhimurium) isolated from samples of rendered animal products produced for poultry feeding. For this purpose, 17 different antibiotics were used. Medical literatures reported that antibiotic resistances of *Salmonella* strains were variable. The rising of multiple resistance to antibiotics has been making *Salmonella* treatment difficult for last twenty years [28]. It was reported that there were epidemic spread, since 1989, of multiresistant *Salmonella* Typhi [29]. In a study, antibiotic resistance pattern of *Salmonella* spp. from chicken eggs, intestines and environmental samples were investigated and identified serotypes such as *Salmonella* Typhi*, Salmonella* Typhimurium*, Salmonella* Enteritidis, and other serotypes were found 100% sensitive to ceftriazone, ciprofloxacine, cephalexin, gentamycin and chloramphenicol, but strains have shown resistance to co-trimoxazole, nalidaxic acid, ampicilin, tetracyclin and kanamycin [30]. Yildirim et al.[31] reported that resistance of all of the *Salmonella* spp. isolates from raw chicken carcasses, predominant one included *Salmonella* Typhimurium, to penicillin, oxacillin, clindamycin, vancomycin, erythromycin and ampicillin were 100%, 97%, 97%, 92.6%, 89.7% and 85.2%, respectively, also resistance to tetracycline (67.6%), streptomycin (61.7%), neomycin (55.8%) and cephalothin (52.9%) was observed but a small percentage of isolates demonstrate resistance to gentamicin (14.7%), chloramphenicol (10.2%), cefotaxime (2.9%) and amikacin (2.9%). Similarly, Zarakolu et al.[32] reported that resistance of 87 *Salmonella Typhimurium* isolates to ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol were 56%, 90%, 100% respectively, and were sensitive to ciprofloxacin and ofloxacine. Dallal et al.[33] determined that a high percentage of *Salmonella* isolates from chicken and beef meat samples were resistant to nalidixic acid (82%), tetracycline (69%), trimethoprim (63%) and streptomycin (52%) and 68.5% of isolates were multidrug resistant. Similarly, Yan et al.[34] found that *Salmonella* isolates were frequently resistant to sulfamethoxazole (86.4%), sulfamethoxazole/trimethoprim (48.1%), nalidixic acid (30.9%), tetracycline (19.8%), corboxybenzylpenicillin (17.3%), amoxicillin (17.3%) and ampicillin (16.0%) and also multiple resistance was found in 29.6% isolates. In our study, all of the isolated strains were sensitive to amoxicillin and chloramphenicol and were resistant to amikacin, cephazolin and erythromycin. However, isolated *Salmonella* spp. and *Salmonella* Enteritidis serovars were resistant to amikacin, cephazolin and erythromycin, sensitive to amoxicillin, chloramphenicol, flumoquin, phosphomycin, kanamycin, oxytetracycline, spiramycin, streptomycin, tetracycline, tobramycine, vancomycin and moderate sensitive to gentamicin, linkomycin and rifampicin. It was also determined that sensitiveness profiles of isolated *Salmonella* Typhimurium serovars to antibiotics, except for cephazolin, were similar to those

of *Salmonella* spp. and *Salmonella* Enteritidis, but four of *Salmonella* Typhimurium were sensitive and the other four were resistant to cephazolin. Probably, this difference may be incurred because of the agents may have different genetics due to their polymorphic proteins, motile DNA particles such as transposons and plasmids, different intron/exon structures. The other probable cause of the occurence of different resistance characteristics in the same strains may also be due to the ability of motile DNA particles to survive in extracellular region and some microorganisms, such as *Salmonella*, can integrate these particles into their genetic constitutions [15,35].

In conclusion, meat meal, meat-bone meal, blood meal, chicken meal and feather meal samples produced under rendering procedures were analysed for *Salmonella* spp., *Salmonella* Enteritidis and *Salmonella* Typhimurium, and 13, 11 and 8 samples were positive respectively. While there were no any pathogens in the samples obtained from the place of production, some of the samples obtained from feed factories were positive. It is estimated that microbiological quality of rendered animal products are affected by processing technology and trasportation from the place of production to the place of consumption.

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Research Article

Effect of Melatonin on Catalase Enzyme in Mouse Kidney Tissue

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Abstract

In this study, we aimed to immunohistochemically investigate the effects of melatonin administration on catalase in the kidney tissue. In our study, 18 male Swiss albino mice were used and they were divided into three groups as control (n=6), sham (n=6) and experimental (n=6). The experimental group received 10 mg/kg dose of melatonin for 28 days (i.p.). Tissue samples were embedded in paraffin. To examine catalase immunoreactivity, avidin-biotin-peroxidase complex (ABC) technique was performed to tissue sections. Based on the results of the immunohistochemical staining, reaction was showed in proximal tubules in all three groups. The reaction in inner cortical proximal tubules was found to be more intense. There was no immunoreactivity in distal tubules, Malpighian bodies, vascular endothelium and structures in the medulla. Catalase immunoreactivity was determined to be more intense in the experimental group than other groups. Our study supports the idea that melatonin could have a direct effect on the kidney tissue and that it could be used as an antioxidant therapeutic agent by strengthening antioxidant mechanism in oxidative stress.

Keywords: *Catalase, Immunohistochemistry, Kidney, Melatonin, Mouse*

Melatoninin Fare Böbrek Dokusunda Katalaz Enzimi Üzerine Etkisi

Özet

Bu çalışmada, melatonin uygulamasının böbrek dokusunda katalaz üzerine etkisinin immunohistokimyasal olarak araştırılması amaçlandı. Çalışmamızda, 18 adet erkek Swiss albino fare kullanıldı ve bunlar kontrol (n=6), sham (n=6) ve deneme (n=6) olarak üç gruba ayrıldı. Deneme grubuna 28 gün 10 mg/kg dozda melatonin uygulandı (i.p.). Alınan doku örnekleri parafinde bloklandı. Katalaz immunoreaktivitesini incelemek için doku kesitlerine avidin-biotin-peroksidaz kompleks (ABC) tekniği uygulandı. Yapılan incelemeler sonucunda immunohistokimyasal boyamada, üç grupta da reaksiyonun tubulus proksimalislerde olduğu belirlendi. Korteksin iç kısmındaki tubulus proksimalislerde reaksiyonun daha yoğun olduğu görüldü. Tubulus distalislerde, Malpighi cisimciklerinde, damar endotellerinde ve medullada bulunan yapılarda immunoreaktiviteye rastlanmadı. Katalaz immunoreaktivitesinin deneme grubunda, kontrol ve sham grubuna kıyasla daha yoğun olduğu belirlendi. Çalışmamız, melatoninin böbrek dokusu üzerine doğrudan etkisinin olabileceği ve antioksidan mekanizmayı güçlendirerek oksidatif stres durumlarında antioksidan bir terapötik madde olarak kullanılabileceği düşüncelerini desteklemektedir.

Anahtar sözcükler: *Katalaz, İmmunohistokimya, Böbrek, Melatonin, Fare*

INTRODUCTION

Melatonin, first defined by Lerner et al.[1], is an endogenously secreted hormone by pinealocytes in pineal gland [2]. Melatonin has effects on circadian rhythms, sleep, psychology, sexual development and reproduction [3,4]. In addition, melatonin has been reported to affect organs in many systems such as cardiovascular, gastrointestinal, respiratory and renal system [2]. The most important feature discovered in recent years is being a powerful antioxidant [5].

Melatonin was first reported by Ianas et al.[6] to be an antioxidant in 1991. Melatonin is a powerful antioxidant that eliminates hydroxyl (OH) radical, the most harmful radical amongst free radicals. Melatonin is known to be more effective than other antioxidants as it can enter many organelles and cell nucleus and protect DNA from oxidative damage [7,8]. Melatonin shows its antioxidant effects in three ways: Directly binding hydroxyl (OH) radical [9], inhibiting some pro-oxidant enzymes [10] or increasing gene expression and activity of antioxidant enzymes [5,11,12]. Catalase, which is one of the most important antioxidant enzymes, was first isolated in 1937 [8,13]. The task of this enzyme, localized in peroxisomes in the cells, is to transform hydrogen peroxide, which is harmful, into oxygen and water [8,14,15]. This enzyme has been reported to be extensively found

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in hepatocytes in the liver [16] and proximal tubules in the kidney [17]. There are important tasks of the kidney tissue on the oxidative metabolism. Although kidneys constitute less than 1% of total body weight, an average of 10% of total oxygen consumption occurs here [18]. Renal proximal tubular cells exposed to high concentrations glucose and reactive oxygen species (ROS) [19]. For this reason, they are always at risk of oxidative damage. Catalase is found in proximal tubules that is protecting kidney functions efficiently [20,21]. It has been stated that lack of catalase could cause situations that may lead to loss of kidney functions such as oxidative tissue damage and renal fibrosis [18,19]. Melatonin administration has been shown by biochemical methods to increase the levels of antioxidant enzymes such as catalase, glutathione reductase and glutathione peroxidase in liver and kidney [12,22]. Also in a study on pigs, the presence of binding regions of iodomelatonin has been shown at pig kidney cortex [23]. However, there is no study showing the effect of melatonin on catalase in kidney tissue immunohistochemically. In our study, it was aimed to immunohistochemically investigate the effects of exogenous administration of melatonin on catalase in mouse kidney tissues.

MATERIAL and METHODS

This study was performed in accordance with approval from Kafkas University Animal Experimentation Local Ethics Committee (KAÜ-HADYEK/2014-007). In our study, 18 male *Swiss albino* mice were used as experimental animals. Experimental animals were divided into three groups as control (n=6), sham (n=6) and experimental (n=6). The experimental group received 10 mg/kg dose of melatonin (Sigma) dissolved in ethanol and diluted by normal saline for 28 days (i.p.). The same injection procedure applied to sham group, but melatonin was not used. The control group received no applications. At the end of the experimental period, the mice were euthanized and kidney tissues were removed. The tissues were processed with Bouin solution and embedded in paraffin following routine histological phases. 5 µm sections from these blocks were passed through deparaffinization and rehydration process. Triple staining [24] and periodic acid-Shiff (PAS) staining [25] was performed in order to examine the tissues histologically. 3% H_2O_2 application to block endogenous peroxidase activity and microwave application in citrate buffer to unleash antigens were performed in order to examine catalase immunoreactivity. The tissues were washed with PBS (Phosphate buffer solution, 0.1 M, pH 7.2). The tissues were allowed to incubation with anti-catalase antibody (Abcam, 1: 3.000 dilution) for 1 h at room temperature and avidin-biotin-peroxidase complex (ABC) technique [26] was applied. For this, ultravision detection system anti-rabbit, HRP/DAB (Thermo Scientific) kit was used. Finally, counter staining was performed with haematoxylin. Extent of immunoreactivity was assessed semi-quantitatively according to the density of the reaction (No reaction; 0, Weak; 1, Moderate; 2, Strong; 3). Negative control application was performed to determine whether the catalase immunoreactivity was specific. Ten areas in outer cortex and ten areas in inner cortex, total twenty areas of each slide are selected randomly and scored aspect of density of catalase immunoreactivity. One way ANOVA was used to compare catalase immunoreactivity among groups and t-test was used to compare catalase immunoreactivity between inner and outer cortex in SPSS (SPSS version 18.0 for Windows; SPSS Inc.,Chicago, IL, USA).

RESULTS

Histological and Histochemical Results

Similar results were observed all groups regarding histological appearances with triple staining. Nephrons, the functional units of the kidneys, structures forming nephrons, collector tubes and connective tissue areas were observed to be similar *(Fig. 1)*.

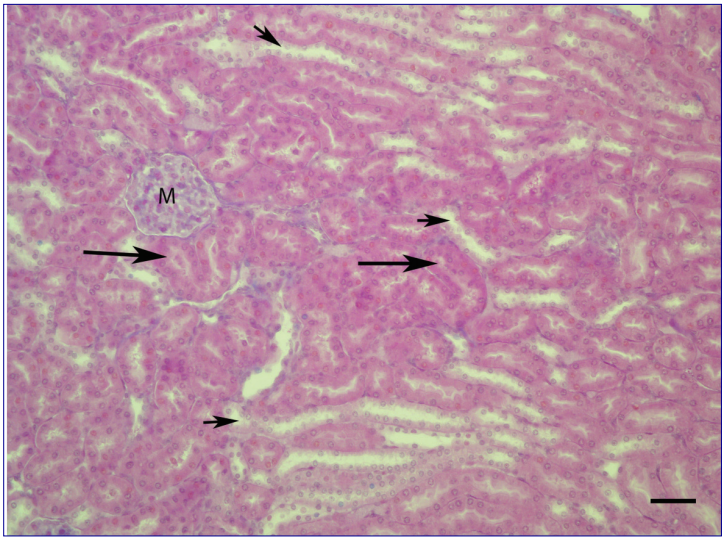


Fig 1. General appearance of the renal tissue. M: Malpighi corpuscle, *long arrow:* tubulus proximalis, *short arrow:* tubulus distalis. Triple staining. Bar: 100 µm

Şekil 1. Böbrek dokusunun genel görünümü. M: Malpighi cisimciği, *uzun ok:* tubulus proksimalis, *kısa ok:* tubulus distalis. Triple boyama. Bar: 100 µm

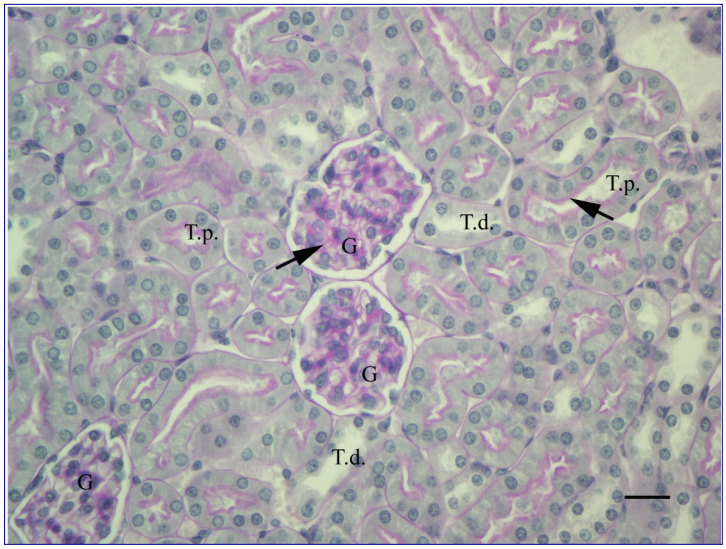
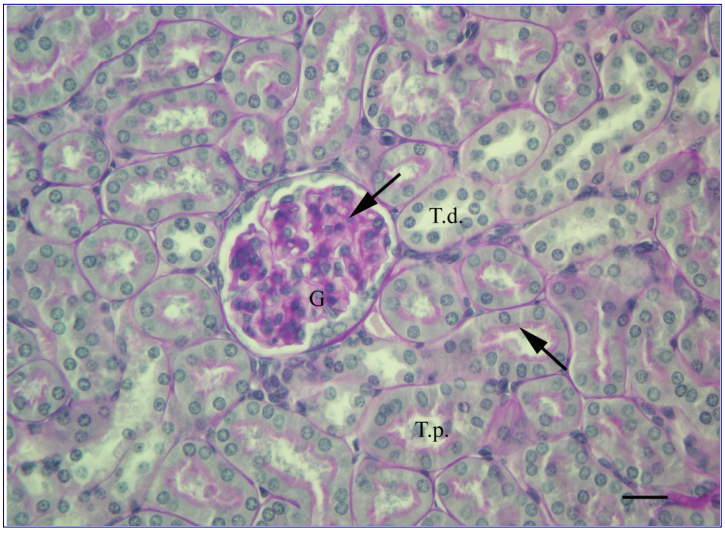


Fig 2. PAS staining in the renal tissue. Control group. G: glomerulus, T.p.: tubulus proximalis, T.d.: tubulus distalis, *arrows:* PAS positive staining. Bar: 50 µm

Şekil 2. Böbrek dokusunda PAS boyama. Kontrol grubu. G: glomerulus, T.p.: tubulus proksimalis, T.d.: tubulus distalis, *oklar:* PAS pozitif boyanma. Bar: 50 µm

Fig 3. PAS staining in the renal tissue. Experimental group. G: glomerulus, T.p.: tubulus proximalis, T.d.: tubulus distalis, *arrows:* PAS positive staining. Bar: 50 µm

Şekil 3. Böbrek dokusunda PAS boyama. Deneme grubu. G: glomerulus, T.p.: tubulus proksimalis, T.d.: tubulus distalis, *oklar:* PAS pozitif boyanma. Bar: 50 µm



There were also no differences between the groups in PAS staining. PAS-positive staining was observed in all groups in glomeruli, Bowman's capsule, apical portions of cells of proximal tubuli and basement membrane. Staining in the inner part of the cortex was determined to be more intense than the outer cortex in proximal tubuli. Strong staining was seen in the basement membrane of distal tubuli *(Fig. 2, Fig. 3)*.

Immunohistochemical Results

Regarding the results of immunohistochemical evaluation, catalase immunoreactivity was observed only in proximal tubuli in all three groups. No immunoreactivity was observed in Malpighian bodies in the cortex, distal tubuli, vascular endothelium and other connective tissues *(Fig. 4a,b)*. No immunoreactivity was also found in the structures in medulla. Immunoreactivity was also observed to be more intense in the inner part of the cortex. These were differences statistically significant for all groups ($P<0.05$). The immunoreactivity was observed usually in the cytoplasm and rarely in the nucleus, and the staining in the cytoplasm was granular in style *(Fig. 4b)*. Catalase immunoreactivity in proximal tubuli was found to be more intense in the experimental group *(Fig. 5)* compared to control *(Fig. 6)* and sham *(Fig. 7)* groups *(Table 1)*. No significant difference was observed between control and sham groups *(Fig. 6, 7)*. The negative control did not show any reaction *(Fig. 8)*.

DISCUSSION

Antioxidant system includes antioxidant enzymes (SOD, CAT, GPX etc.), which can prevent from oxidative stress [8]. Melatonin is an antioxidant that increases the activity of antioxidative enzymes [5,12].

It was determined that the findings from the histological examination of the kidney tissue were consistent with normal histology, in accordance with classical literature [2,27] and that there is similar for all groups.

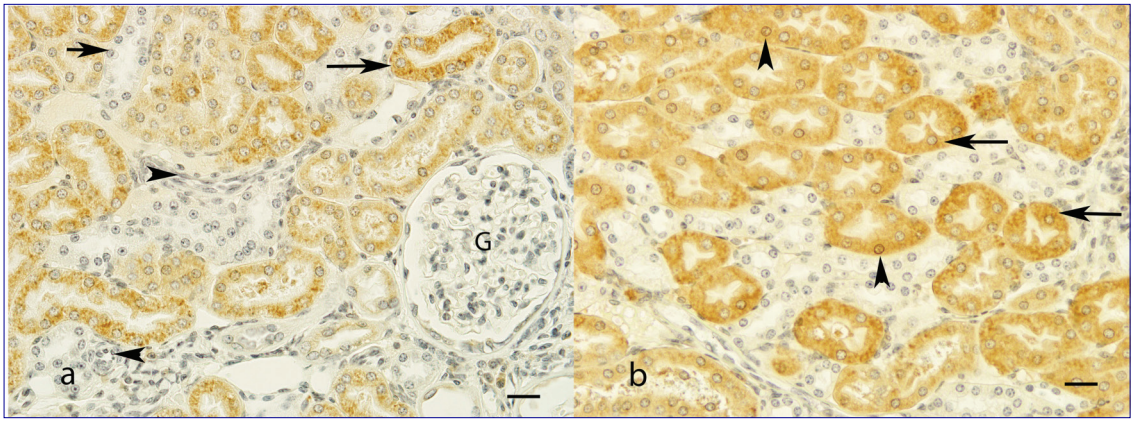
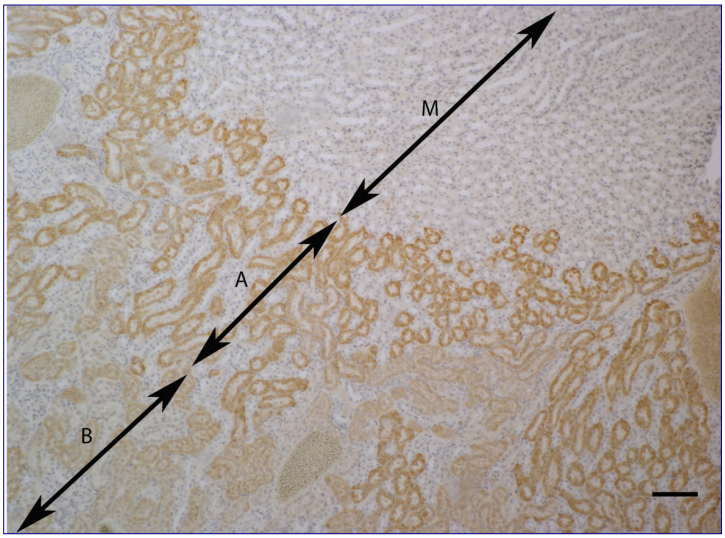


Fig 4. Catalase immunoreactivity in the cortex section of the kidney. Control group. **a)** G: glomerulus, *arrow head:* vascular endothelium, *short arrow:* tubulus distalis, *long arrow:* tubulus proximalis. Bar: 50 µm, **b)** *Arrow:* cytoplasmic immunoreactivity, *arrow head:* immunoreactivity in the cytoplasma and nucleus. Bar: 50 µm

Şekil 4. Böbreğin korteks kısmındaki katalaz immuno-reaktivitesi. Kontrol grubu. **a)** G: glomerulus, *ok başı:* damar endoteli, *kısa ok:* tubulus distalis, *uzun ok:* tubulus proksimalis. Bar: 50 µm, **b)** *Ok:* sitoplazmik immunoreaktivite, *ok başı:* sitoplazma ve çekirdekte immunorektivite. Bar: 50 µm

Fig 5. Catalase immunoreactivity in the renal tissue of the experimental group. M: medulla, A: inner cortex, B: outer cortex. Bar: 200 µm

Şekil 5. Deneme grubu böbrek dokusunda katalaz immuno-reaktivitesi. M: medulla, A: iç korteks, B: dış korteks. Bar: 200 µm



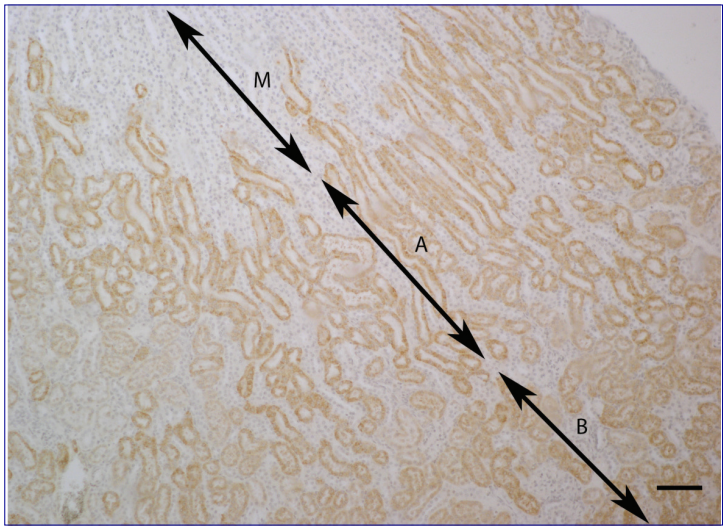


Fig 6. Catalase immunoreactivity in the renal tissue of the control group. M: medulla, A: inner cortex, B: outer cortex. Bar: 200 µm

Şekil 6. Kontrol grubu böbrek dokusunda katalaz immuno-reaktivitesi. M: medulla, A: iç korteks, B: dış korteks. Bar: 200 µm

Ergin and Başaloğlu [28] studied the effect of chronic melatonin injections in kidney tissue and reported that there is no difference histologically, similarly to our findings.

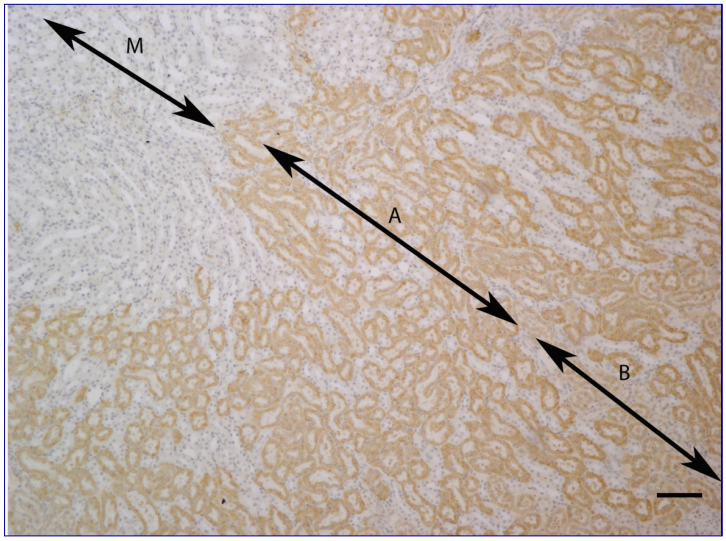


Fig 7. Catalase immunoreactivity in the renal tissue of the sham group. M: medulla, A: inner cortex, B: outer cortex. Bar: 200 µm

Şekil 7. Sham grubu böbrek dokusunda katalaz immunoreaktivitesi. M: medulla, A: iç korteks, B: dış korteks. Bar: 200 µm

Fig 8. Negative control. Control group. Bar: 100 µm

Şekil 8. Negatif Kontrol. Kontrol grubu. Bar: 100 µm

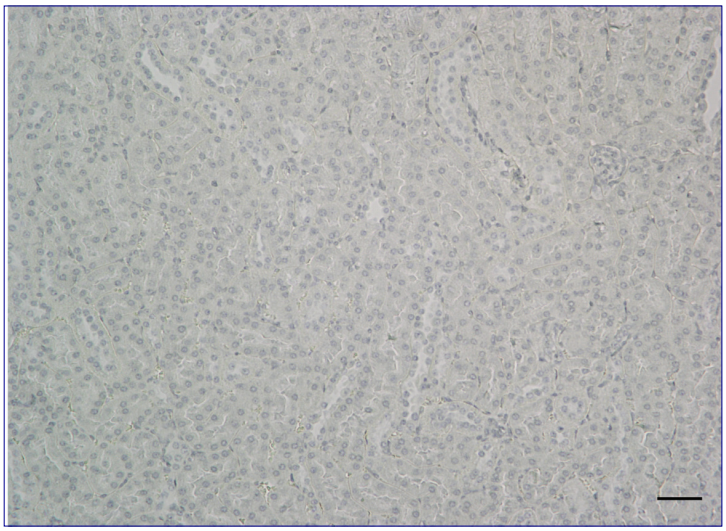


Table 1. *Comparison of catalase immunoreactivity among groups in proximal tubules (P<0.05)*

Tablo 1. *Proksimal tubullerdeki katalaz immunoreaktivitesinin gruplar arası karşılaştırılması (P<0.05)*

Regions of Cortex	Control	Sham	Experimental
Outer cortex	1.250±0.056 [a]	1.166±0.048 [a]	2.033±0.088 [b]
Inner cortex	2.033±0.091 [a]	2.066±0.081 [a]	2.850±0.046 [b]

[a,b] Differences between values having different letters in the same line are statistically significant (P<0.05)

In a study by Bingöl and Kocamış [17] on immunohistochemical examination of catalase enzyme in kidney tissues of healthy and diabetic rats, they have reported that catalase immunoreactivity is intense only in proximal tubuli in the cortex of the kidney and quite weak in the medulla, whereas there is no immunoreactivity in structures such as glomeruli, vascular endothelium, distal tubuli. In the same study, they stated that immunoreactivity in the cortex varies by region and immunoreactivity in the inner part of the cortex in the entire groups is more intense. Similarly in their study, Morikawa et al.[29] studied immunohistochemical localization of catalase in tissues of mammals and they reported that the reaction is in the cytoplasm of proximal tubuli cells of the kidney and that no specific reaction is present in distal tubule epithelium, connective tissue and blood vessels. Zhou and Kang [30] studied immunohistochemical localization of catalase in various tissues in transgenic mice and stated that there is cytoplasmic reaction in proximal tubuli of kidney. They have observed that this present reaction in proximal tubuli is weaker in the outer part of the cortex, stronger in the inner part and not present in distal tubuli, glomeruli, loop of Henle, collector tubes and medulla [30]. Immunohistochemical localization of catalase in geese liver has been reported to be mostly cytoplasmic and that some staining is present in the nuclei of a few hepatocytes [16].

Our findings are parallel to the findings of Bingöl and Kocamış [17], Morikawa et al.[29], Zhou and Kang [30] regarding presence of immunoreactivity in the cortex of the kidney, especially in proximal tubuli in the inner cortex portion and absence of reaction in distal tubuli, vascular endothelium and connective tissue. Kidney and especially proximal

tubular cells always expose to reactive oxygen species (ROS). Catalase is an antioxidant enzyme and locates in peroxisomes [15]. Peroxisomes is the typical organelles of the proximal tubule cells [2], it shows that antioxidant defence and catalase immunoreactivity are seen in these cells intensely. Moreover, in our study, reaction being mostly cytoplasmic and less nuclear is consistent with the findings of similar studies [16,29,30]. However, immunoreactivity in the medulla observed by Bingöl and Kocamış [17] could not be observed in our study.

Bharti et al.[22] have reported that melatonin administration increases catalase, glutathione reductase and glutathione peroxidase in the liver and kidney, thus activates antioxidant mechanisms and has protective and therapeutic effect in oxidative stress. In our study, immunoreactivity being more intense in the experimental group compared to other groups is consistent with the biochemical study of Bharti et al.[22].

In conclusion, our study supports the opinion that melatonin might have a direct effect on the kidney tissue [23]. However, this effect was observed not to cause a change in the histology at light microscopic level. In our study, it was concluded that melatonin administration increases catalase immunoreactivity in kidney. Catalase immunoreactivity being more intense in the experimental group supports the idea that melatonin could be used as a therapeutic agent in oxidative stress by supporting antioxidant mechanism. We believe our study will contribute to studies to be done on renal, melatonin and antioxidant systems.

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Research Article

Somatic Cloning in Cats Using MI or MII Oocytes [1]

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Abstract

Animal production via SCNT provides a unique tool for protection of valuable individuals, conservation of vulnerable and endangered species and production of transgenic animals. A total of 167 MI and 219 MII stage oocytes were used as the material of the study. The oocytes were enucleated at 44 h after in vitro maturation by aspiration of the polar body and the MI or MII plates. Cycling granulosa cells were used for nuclear transfer. Cell fusion was induced with DC pulses of 2.0 kV/cm 60µs, 0.1s apart (2x) delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA). After fusion, the embryos were activated by 1.0 kV/cm 20µs DC pulses 0.1s apart (2x) followed by 2 mM 6-DMAP (6-dimethylaminopurine) incubation in culture medium for 4 h in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38°C. The somatic cell transferred embryos were cultured for 8 days in mSOF medium supplemented with 0.4% BSA in a humidified 5% CO_2, 5% O_2, and 90% N_2 atmosphere at 38°C. After in vitro culture period, all embryos transferred to HSOF containing Hoechst 33342 (5 µg/mL) and the cell numbers were counted under ultraviolet light using a fluorescent microscope. The fusion (66.66 vs 21.55%) and cleavage rates (15.75 vs 11.11%) were significantly higher in MII stage oocytes than MI stage oocytes (P<0.02). While SCNT embryos were developed to morula stage in MII group (14; 9.58%), all the cleaved embryos were arrested at the 2-4 cell stage in MI group. None of the embryos was developed to blastocyst stage in both groups.

Keywords: *Cat, SCNT, In vitro, MI - MII oocytes*

Kedilerde MI ve MII Oositleri Kullanilarak Somatik Klonlama

Özet

Somatik klonlama yoluyla hayvan üretimi, üstün değerdeki bireylerin korunması, savunmasız ve tehlike altında bulunan türlerin korunması ile transgenik hayvanların çoğaltılmasına hizmet eder. Çalışmanın materyalini 167 adet MI ve 219 adet MII dönemdeki oosit oluşturdu. Polar cisimciklerin (MII) ve kromatin setlerin (MI ve MII) enükleasyonu, 44 saatlik in vitro olgunlaştırma periyodunun ardından gerçekleştirildi. Nükleer transfer amacıyla siklik dönemlerdeki granüloza hücreleri kullanıldı. Oosit-somatik hücre komplekslerinde füzyon işlemi, DC akımın sağlandığı 2.0 kV/cm 60 µs, 0.1s ara (2x), BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA) ile gerçekleştirildi. Aktivaston işlemi için ise, 1.0 kV/cm 20µs DC akım 0.1s ara (2x) kullanıldı ve ardından 2 mM 6-DMAP (6-dimethylaminopurine) içerisine alınarak, 38°C'lik sıcaklık ve %5 CO_2, %5 O_2 ve %90 N_2 gaz karışımının sağlandığı inkübatörde 4 saat süresince kültüre edildi. Somatik hücrelerin nakledildiği klon embriyolar daha sonra aynı inkübatör koşullarında %0.4 BSA katkılı mSOF medyumu içerisinde 8 gün boyunca in vitro kültüre bırakıldılar. Ardından klon embriyolar, embriyonik hücre sayılarının tespiti amacıyla Hoechst 33342 (5 µg/mL) içeren HSOF medyumu içerisine alındı ve ultraviyole küplü floresan mikroskobunda değerlendirildi. Sonuçta, füzyon (%66.66-21.55) ve yarıklanma oranlarının (%15.75-11.11) MII dönem oositleri lehine önemli derecede üstün olduğu belirlendi (P<0.02). MII grubunda 14 adet embriyonun (%9.58) morula döneme kadar geliştiği gözlenirken, MI grubunda ise, yarıklanan tüm embriyoların 2-4 hücreli dönemde kaldığı ve her iki gruptan da hiçbir embriyonun blastosist aşamasına ulaşamadığı gözlendi.

Anahtar sözcükler: *Kedi, SCNT, In vitro, MI-MII oosit*

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INTRODUCTION

Most of the wild felid species are described as endangered, rare or vulnerable because of human destruction of natural ecosystems and habitat loss. Domestic cats are commonly used as a model to develop assisted reproductive technologies for the protection of endangered felids and for biomedical research [1]. They may also allow a recipient female for embryo transfer (ET) and recipient cytoplasm for nuclear transfer (NT) from several species of small wild cats [2,3]. Animal production by somatic cell nuclear transfer (SCNT) and interspecies somatic cell nuclear transfer (iSCNT) serves a sole tool for protection of precious individuals, genetically modified animals, conservation of endangered species and a number of opportunities for simple and practised research in human medicine [3-5]. Cats are often used to represent normal physiology and human diseases which are especially located in nervous system and kidneys in studies [4-6]. After the first mammalian Dolly the sheep was produced from a somatic cell from an adult animal [7], studies evolved rapidly and many other cloned animals have been produced by the use of MII oocytes as cytoplasts (sheep [8], ferret [9], dog [10], horse [11], mule [12], cat [13], pig [14], cattle [15], goat [16] and mouse [17]) so far.

In meiotic maturation period, many cytoplasmic and nuclear changes occur that arrange the oocytes for fertilization. During this period, the changes start by activation of mitogen-activated protein (MAP) kinase and maturation-promoting factor (MPF) [18]. In the recipient oocytes, the MPF activity is critical for the reprogramming of nuclei of reconstructed embryos [19]. Also the MPF is thought to be a critic factor in maintaining meiotic resumption [20,21]. The nuclear membrane of donor cells' with low activity of MPF stays stable at the pre-activated oocytes. The DNA synthesis occurs dependently to the stage of the original cell cycle at the time of nuclear transfer [19] and reprogramming of nucleus takes place during the expansion of donor nucleus [22]. After parthenogenetic activation, the nuclear membrane reorganised and DNA synthesis starts [23]. It is stated that MPF activity is greatest at both MI and MII throughout maturation period and because of the high activity of MPF the nuclear membrane of the donor cell is broken down and the chromosomes are condensed [19]. In the amphibian it is found that the most advanced tadpoles generated from MI compared to MII stage oocytes [24]. Moreover in a recent study [25], it is showed that somatic cell transferred porcine MI oocytes are developed to blastocyst stage.

To our knowledge there is no study about the ability of reprograming the somatic cell nuclei of MI stage cat oocytes so far. This study was performed to evaluate the development of domestic cat embryos reconstructed by transferring somatic cells into enucleated MI or MII oocytes *in vitro*.

MATERIAL and METHODS

The experiment was performed in accordance with guidelines for animal research from Istanbul University Ethics Committee on Animal Research (2011/84).

Chemicals

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Collection of Ovaries and Oocyte Recovery

The oocytes were obtained from ovaries of 29 ovariohysterectomised cats at local veterinary clinics. These operations were performed under general anaesthesia and the ovaries were transported to the laboratory in 2-3 h with Phosphate Buffered Saline (PBS) at 35°C. The cumulus oocyte complexes (COCs) were collected by slicing and washing the ovarian surface with oocyte washing medium. The COCs were selected according to their structure of cumulus cells and ooplasm integrity [26].

In Vitro Maturation (IVM)

The maturation medium was Nutrient Mixture F-10 Ham (Ham's F-10) (N-6635). The medium was supplemented with 10 µg/mL FSH (F-2293), 10 µg/mL LH (L-5269), 20 ng/mL EFG (Invitrogen; 13247-051), 0.4% BSA and antibiotics. The selected COCs were maturated in four-well petri dishes (NUNCR, Denmark) in 500 µL maturation medium at 38°C in a humidified 5% CO_2, 5% O_2, and 90% N_2 atmosphere for 44 h. For each experimental group, 30-40 COCs were placed for each perti dish well.

Source of Somatic Cells

Cumulus cells were provided from immature cat oocytes. They were disaggregated mechanically by gentle pipetting in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FCS (v:v) and 75 mcg/mL penicillin G, 50 µg/mL streptomycin, washed by centrifugation and the pellet was suspended in handling DMEM medium supplemented with 15% FCS (v:v). The cumulus cells were cultured *in vitro* until confluent, then were passaged 2 to 3 times before being used for nuclear transfer (NT).

Preparation of Recipient Cytoplasm and Somatic Cell Nuclear Transfer (SCNT)

The *in vitro* maturated oocytes were denuded by vortexing in HSOF medium plus 11.5 mg/mL hyaluronidase for 1 min. Oocytes with an extruded first polar body were considered mature (MII stage; *Fig. 1*) and the oocytes without polar body were separated for an examination under a fluorescent microscope after Hoechst (33342) staining. Oocytes having a MI spindle were selected as MI stage and oocytes with germinal vesicle (GV), germinal vesicle breakdown (GVBD), undetermined nuclear material (UDNM) or degeneration

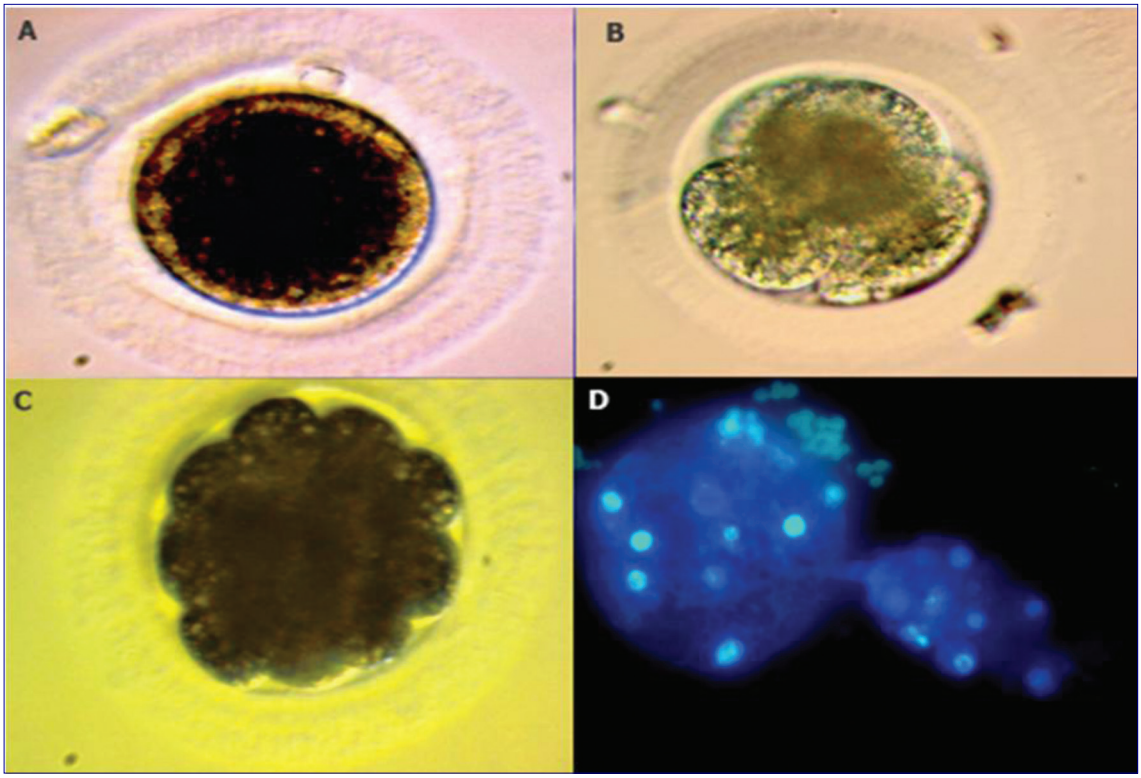

Fig 1. *In vitro* matured cat oocyte and embryos developed after SCNT. **A-** M II stage oocyte with extruded first polar body (x200), **B-** 4-cell stage embryo (x200), **C-** Morula (x200), **D-** Morula stained with Hoechst

Şekil 1. *İn vitro* olgunlaştırılmış kedi oositi ve SCNT sonrası embriyoların gelişimi. **A-** Birinci polar cisimciği ile M II dönemde bir oosit (x200), **B-** 4hücreli dönemde bir embriyo (x200), **C-** Morula (x200), **D-** Hoechst ile boyanmış bir morula (x200)

were discarded. All the selected oocytes (MI or MII) were placed in HSOF media supplemented with 7.5 µg/mL cytochalacin B and were enucleated by aspirating their first polar body and the MI and MII plates with a small volume of surrounding cytoplasm. Successful enucleation was confirmed under ultraviolet light using a fluorescent microscope. After enucleation procedure, the cytoplasts were washed in Calcium free SOF medium and were held in this medium nearly 1 h until somatic cell injection [27]. Micromanipulation was performed by inserting a small size (14-16 µm) individual cumulus cell into the perivitelline space of each enucleated oocyte.

Electrofusion and Activation

The electrofusion and activation protocols were performed according to Yin et al.[27]. Briefly, somatic cell-cytoplast complexes were transferred to a fusion chamber with two electrodes 500 µm apart, filled with fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, and 0.1 mM $MgSO_4$ and 0.05% fatty acid–free BSA) at room temperature. Cell fusion was induced with two DC pulses of 2.0 kV/cm 60µs, 0.1s apart delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA). The fused NT embryos were incubated in HSOF plus 1% essential and 1% non-essential amino acids for 1 h. Then, the cytoplasts were activated by exposure of two 1.0 kV/cm 20 µs DC pulses 0.1 s apart by followed 2 mM 6-DMAP (6-dimethylaminopurine) in culture medium for 4 h in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38°C.

In Vitro Culture

The NT embryos were cultured in mSOF medium supplemented with 0.4% BSA in a humidified 5% CO_2, 5% O_2, and 90% N_2 atmosphere at 38°C with high humidity. At day 3 of culture, all cleaved embryos were transferred to a fresh mSOF medium supplemented with 1.5 mM glucose (G 6152) and cultured for an additional 3 d. After IVC period, all the embryos transferred to HSOF containing Hoechst 33342 (5 µg/mL) for 30 min and the cell numbers were counted under ultraviolet light using a fluorescent microscope *(Fig.1)*.

Statistical Analysis

The experiment was replicated 10 times. Statistical analysis was performed using by a "Mann Whitney U" test by SPSS for Windows version 13.0.

RESULTS

A total of 386 oocytes (167 at MI and 219 at MII stage) were used as the material of the study. The number of somatic cell-cytoplast complexes, the number of cleaved NT embryos after fusion and the developmental stage of

Table 1. *Developmental rate of cat embryos reconstructed by transferring cumulus cells into enucleated M I and MII oocytes*
Tablo1. *Enüklee edilmiş olan MI ve MII dönem oositleri içerisine kumulus hücreleri enjekte edilerek klonlanan kedi embriyolarının gelişim oranları*

Oocyte Stage	No. of Complexes		No. of Cleaved (%)	No. of Embryos Developed to (%)			
	n	Fused (%)		2-4 Cell	4-8 Cell	Morula	Blastocyst
M I stage	167	(21.55)[a] 36	(11.11)[a] 4	(11.11) 4	(0.00) 0	(0.00)[a] 0	(0.00) 0
M II stage	219	(66.66)[b] 146	(15.75)[b] 23	(6.16) 9	(0.05) 3	(9.58)[b] 14	(0.00) 0

[a,b] Different letters indicate statistical differences among columns (P<0.002)

SCNT embryos' are summarized in *Table 1*. The fusion and the cleavage rates of MII stage oocytes were significantly higher than MI oocytes (P<0.02). 14 (9.58%) SCNT embryos were developed to morula stage in MII group. All the cleaved embryos of MI group are arrested at the 2-4 cell stage.

DISCUSSION

Although offspring production from SCNT has been noticed in different mammalian species, the overall success rates are under the expected levels (1-11%). There are too many known and unknown factors and suboptimal situations affecting the production of live healthy animal *in vitro* [28,29]. In cats, it is demonstrated that the fusion (45-66%) and blastocyst (3-8%) rates of cycling fetal fibroblast, adult fibroblast, muscle and cumulus cells were similar [28]. It is stated that the mammalian cell cycle stage of the donor cell nucleus has multiple effects on embryo re-construction and is a main factor in the achievement of NT [19]. There are many different methods for synchronizing cells cycle such as; serum starvation, contact inhibition, chemical cell cycle inhibitors [1,29]. Some researchers achieved pregnancy in sheep [30] and produced calves [31] by transferring both cycling and non-cycling somatic cells and they suggested that cell synchronization by serum starvation is not obligatory. In this study, somatic cells were used without having serum starvation. In cats, it is stated that although the source of the donor nucleus affected the rate of blastocyst development, the cell cycle synchronization method did not [3]. There are some live cloned cat offspring have been acquired after the transfer of embryos reconstituted with cells synchronized by serum-starvation [2,27] and cycling cells [27]. The somatic cells (fibroblasts and cumulus cells) of the cat have a naturally long G0/G1 phase and it is claimed that serum starvation induces the apoptosis process [32].

The fusion and cleavage rates of couplets vary according to the meiotic stage, the quality and the way of maturation of the oocyte, the somatic cell type, the cell synchronization method, the fusion technique and *in vitro* culture conditions [25,28,29]. Both *in vivo* and *in vitro*-matured cat oocytes have been used as recipient cytoplasts for production of cloned embryos and the fusion rates of *in vivo* matured oocytes found higher than the *in vitro* matured oocytes [28]. In this study, *in vitro* matured cat oocytes were used as cytoplast and the fusion rates in MII stage couplets were significantly higher than the MI couplets (21.55 vs 66.66%). The fusion rate of the MII stage couplets were similar to the researchers (45-66%) that they used cycling cumulus cat cells [28], but the MI stage rates (21.55%) were lower than the results in pig results (29-60%) using MI stage oocytes [25]. In the present study, the cleavage rates of couplets were found higher in MII than the MI stage oocytes (15.75 vs 11.11%) and 14/146 of MII stage couplets were developed to morula stage (9.58%). However, all the cleaved MI stage couplets (11.11%) were arrested at early stages (2-4 cell) and others were degenerated. Although Miyoshi et al.[25] showed that porcine MI oocytes have a potential to develop into blastocysts (1.5%) after nuclear transfer of somatic cells, we observed that MI stage cat oocytes have the potential to cleave to early stages (2-4 cell); however, they have no potential to subsequent development. The low developmental rate of MI oocytes in this study can be concluded as a result of difference among animal species.

It is reported that 1 to 11% of cat cloned embryos reached to the blastocyst stage *in vitro*, regardless of synchronization method or the cell type that used [28]. It is known that the highest proportion of mature oocytes is reached between 42 and 45 h of *in vitro* culture [28,33]. Therefore, the oocytes were cultured for 44 h for *in vitro* maturation in this study. However, researchers [34] stated that a prolonged maturation period of 43 h influenced *in vitro* development of reconstructed cat embryos. The extending period leads to lower fusion rates, lower development of embryos to the morula and blastocyst stages. Although MII stage oocytes were cleaved and some of them (9.58%) reached to morula cell stage, they couldn't reach the morula and blastocyst stages in this study. This situation could be related to the length of *in vitro* maturation, and the possible variations of *in vitro* conditions. It is demonstrated that nutrition is important factors on the maturation, fertilization and further development of oocyte and embryos *in vitro* [35]. However, the poor nutritional conditions of the spayed street cats which have been oocyte donors could be another affecting factor.

Although nuclear transfer technique is developed

rapidly, it is not clear how much progress can be attained by optimizing the available procedures. The mechanism of reprogramming somatic cells after nuclear transfer is complex and still unclear. In the present study, it is concluded that the reprogramming events in MI and MII oocytes could have important differences and although using MI oocytes as cytoplasts may provide the opportunity of increasing donor cell numbers. However, it is not supposed to be likely.

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Research Article

Protective Effect of Dandelion *(Taraxacum officinale)* Extract Against Gentamicin-Induced Reproductive Damage in Male Rats [1] [2]

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Abstract

The aim of this study was to investigate the effect of *Taraxacum officinale* extract (TOE) on gentamicin-induced reproductive damage in male rats. Totally, 24 male Sprague Dawley rats were divided into four groups: Group I (n=6); referred as control, physiological saline was intraperitoneally (IP) administered. Group II (n=6); referred as gentamicin (G), 80 mg/kg gentamicin sulphate (GS) was injected IP. Group III (n=6); referred as G + TOE150, 80 mg/kg GS and 150 mg/kg TOE was given IP. Group IV (n=6); referred as G+TOE200 (n=6), 80 mg/kg GS and 200 mg/kg TOE was administered IP. The treatment continued for consecutive 8 days. The cauda epididymal semen samples and testes tissues were collected. Routine semen examinations were performed and oxidative stress levels of testicular tissues were assayed. Reproductive organ weights [total testes weight (TTW) and total cauda epididymal weights (TCEW)] were recorded. GS administration significantly decreased sperm motility ($P<0.01$), glutathione peroxidase (GPx) activity ($P<0.001$) and glutathione (GSH) level ($P<0.05$), and it significantly increased tissue malondialdehyde (MDA) level ($P<0.01$) in comparison with the control group. However, a statistical increase in sperm motility of GT150 ($P<0.01$) group and in GPx activities of both GT150 and GT200 ($P<0.01$) groups as well as a statistical decrease in MDA levels of GT150 and GT200 ($P<0.001$) groups were determined when compared with the G group. In conclusion, short-term administration of GS causes lipid peroxidative damages in testes as well as decreases in sperm motility. However, TOE has a moderate ameliorative effect on sperm motility reductions, but marked improvement effect on lipid peroxidative testicular damages induced by GS.

Keywords: *Taraxacum officinale, Gentamicin, Reproduction, Rat, Oxidative stress*

Erkek Ratlarda Gentamisinin İndüklediği Üreme Hasarına Karşı Karahindiba *(Taraxatum officinale)* Ekstresinin Koruyucu Etkisi

Özet

Bu çalışmanın amacı, gentamisinin erkek ratlarda oluşturduğu reprodüktif hasar üzerine karahindiba ekstresinin (*Taraxacum officinale* extract, TOE) etkilerini araştırmaktı. Toplam 24 adet erkek Sprague Dawley rat dört gruba bölündü: Grup I (n= 6) kontrol grubu olarak adlandırıldı ve hayvanlara intraperitonal yolla fizyolojik tuzlu su uygulandı. Grup II (n=6) gentamisin (G) olarak adlandırıldı ve hayvanlara 80 mg/kg gentamisin sülfat (GS) intraperitonal (IP) yolla enjekte edildi. Grup III (n=6) G + TOE150 olarak adlandırıldı, 80 mg/kg GS ve 150 mg/kg TOE hayvanlara IP yolla enjekte edildi. Grup IV G+TOE200 (n=6) olarak adlandırıldı ve 80 mg/kg GS ile 200 mg/kg TOE hayvanlara IP yolla enjekte edildi. Tedavi ardışık 8 gün boyunca uygulandı. Kauda epididimal sperm örnekleri ve testis dokuları toplandı. Rutin sperma muayeneleri yapıldı ve testis dokularının oksidatif stres düzeyleri ölçüldü. Reprodüktif organ ağırlıkları [toplam testis ağırlığı (TTW) ve toplam kauda epididimis ağırlıkları (TCEW)] kaydedildi. GS uygulaması önemli ölçüde sperm motilitesini ($P<0.01$), glutatyon peroksidaz (GPx) aktivitesini ($P<0.001$) ve glutatyon (GSH) düzeyini ($P<0.05$) düşürdü ve önemli derecede doku malondialdehit (MDA) düzeyini ($P<0.01$) kontrol grubuna kıyasla artırdı. Ancak, istatistiksel olarak G grubuna kıyasla GT150 grubunun sperm motilitesinde ($P<0.01$) ve GT150, GT200 gruplarının GPx aktivitelerinde ($P<0.01$) bir artış olduğu gibi, GT150 ve GT200 gruplarının MDA düzeylerinde ($P<0.001$) bir düşüş belirlendi. Sonuç olarak, kısa süreli GS uygulaması testislerde lipit peroksidasyona sebep olduğu gibi sperm motilitesinde de azalmaya sebep olmuştur. Bununla birlikte, TOE sperm motilitesinin düşüşünde makul oranda iyileştirici etkiye sahiptir, fakat GS indüklü lipid peroksidatif testiküler hasar üzerine belirgin etkisi vardır.

Anahtar sözcükler: *Karahindiba, Gentamisin, Reprodüksiyon, Rat, Oksidatif stres*

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INTRODUCTION

Taraxacum officinale, known as dandelion or lion tooth, is a plant belonging to Asteraceae (composite) [1]. It has been used as protective and curative for many disease and preservative for nutrients [2]. It has been reported that an improvement was observed in the endogenous antioxidant profile by applying roots and leaves of *Taraxacum officinale* to the rats [3-5]. *Taraxacum officinale* extract (TOE) is comprised of beta carotene, provitamin A, xanthophyll, chlorophyll, vitamins C and D, B-complex vitamins, choline, iron, silicon, magnesium, sodium, potassium, zinc, manganese, copper and phosphorus that almost all of these items are known as strong free radical scavengers. It has been found that these structures are strong free radical scavenger and to inhibit lipid peroxidation. The presence of fatty acids, enzymes, vitamins and minerals in various ratios and amounts are demonstrated by the phytochemical studies made upon the *Taraxacum officinale* [2,6]. *Taraxacum officinale* extract particularly ethyl acetate fraction has bioactive phytochemicals effects which can eliminate the reactive oxygen species in vitro medium and it protects DNA against damages of reactive oxygen species (ROS) [7].

Gentamicin is an aminoglycoside antibiotic and effective against various microorganisms [8]. It has been reported that gentamicin adversely affects phosphatase activity and sperm count in male rats [9]. In addition, it was emphasized that gentamicin induced the ascorbic acid mobilization from testis and by this way decrease of the reproductive potential [10].

Gentamycin inhibits cell division of gem cells and protein synthesis in the testis, damages the testicular tissue and reduces sperm count, sperm motility, and sperm viability by increasing the free radical formation and lipid peroxidation and by decreasing antioxidant enzyme levels [11,12]. Oxidative stress is an important factor which influences fertility potential of spermatozoa by lipid peroxidation which may result in sperm dysfunction [13]. Reactive oxygen species (ROS) has pathological roles in male infertility [14].

There was no data in literature about protective effect of dandelion *(Taraxacum officinale)* extract against gentamicin-induced reproductive damage in male rats.

The present study aimed to evaluate the protective effect of *Taraxacum officinale* extract against gentamicin-induced reproductive damage in male rats.

MATERIAL and METHODS

The approval of Atatürk University Animal Experimentations Local Ethics Committee (Approval number: 2013/132) was taken before starting the study.

Plant Material

T. officinale L. samples were collected in September 2013 from Erzurum (Turkey) and identified by Saban KORDALI (Atatürk University, Faculty of Agriculture, Department of Plant Protection, Erzurum). A voucher specimen has been deposited in the Herbarium of Ataturk University, Erzurum (Turkey).

Preparation of the Samples

Plant materials were dried under shade and powdered coarsely before extraction. The dried *T. officinale* L samples were powdered in a blender and then 100 g of sample was extracted individually with 500 mL ethanol at room temperature. The extract was filtered and evaporated to dryness in a vacuum at 40°C with a rotary evaporator after 48 h. Filtration, the organic solvents were evaporated under reduced pressure and temperature. The dried extracts were stored at 4°C until used. The extract was dissolved in 0.5% aqueous carboxymethylcellulose (CMC) suspension in distilled water prior to intraperitoneally administration to animals by using needle eight days.

Animals and Experimental Procedure

In the study, 24 male Sprague Dawley rats aged eight weeks old and weighted 250-300 gr, were used. The animals were obtained from Atatürk University Experimental Research Centre and housed in standard laboratory conditions. Commercial pellet chow and fresh drinking water were available *ad libitum*. Rats were divided into four groups. I. group (n= 6) referred as control group and physiological saline was intraperitoneally (IP) administered. II. group (n=6) referred as Gentamicin (G) and 80 mg/kg gentamicin sulphate (GS) was injected to animals. III. Group (n=6) referred as G + TOE150, 80 mg/kg GS and 150 mg/kg TOE were injected IP to the animals. IV. Group, referred as G+TOE200 (n=6), 80 mg/kg GS and 200 mg/kg TOE were injected IP to the animals. The treatment continued for consecutive 8 days. The animals were tranquilized (xylazine, 10 mg/kg IP) and sacrificed at the end of the 8th day of study.

Collection of Samples

Following decapitation procedure, the testes and cauda epididymidis of the rats were removed from the body and cleaned from adipose or connective tissues with anatomical scissors and tweezers. Cauda epididymal semen samples and testes tissues were collected. Routine semen examinations were performed and oxidative stress levels of testicular tissues were assayed. Reproductive organ weights [total testes weight (TTW) and total cauda epididymides weights (TCEW)] were recorded.

Semen Evaluation

One of cauda epididymidis was used to obtain semen

sample for each animal. For this purpose, randomly selected cauda epididymidis was minced in Petri dish including 5 mL of physiological saline. To provide the migrations of spermatozoa from cauda epididymidis to fluid, the solution-tissue mixture was incubated in a warmed stage at 35°C for 5 min. Following the incubation period, cauda epididymidis residue was removed by using anatomical tweezers from the Petri dish. The fluid remaining in the Petri dish was used as semen sample. Evaluation of semen was conducted using routine spermatological parameters including motility, dead sperm rate and morphological examination of spermatozoa. To evaluate the percentage of sperm motility, light microscope (Primo Star; Carl Zeiss, Oberkochen, Germany) equipped with the heated stage was used. Briefly, a slide was placed on a heated stage warmed up to 35°C placed on a conventional light microscope. Approximately 20 µL of semen sample was dropped on the slide. The percentage of sperm motility was detected by visual investigation of the sample. To estimate the sperm motility, randomly selected three different fields from each sample were evaluated. The average of three field estimations was calculated as the final motility score of the sample [15,16].

To determine the percentage of morphological abnormality of spermatozoa, the method (with a little modification by using only eosin dye instead of eosin-nigrosin dye) described by Turk et al.[15] was used. Briefly, two slides for each semen sample were stained with eosin dye. Then, the slides were evaluated under light microscope at 400x magnification with the help of immersion oil (immersion oil for microscopy type A, no: 1.515; Nikon, Tokyo, Japan). Two hundred spermatozoa from each slide were examined and the numbers of spermatozoa with abnormal head were expressed as percentage.

Sperm viability was evaluated with light microscope at 400x magnification with the help of immersion oil (immersion oil for microscopy type A, no: 1.515; Nikon, Tokyo, Japan) after eosin nigrosin staining [17]. The smear was prepared for counting. A total of 200 cells were counted and the results are presented as percentages.

Biochemical Evaluations of Testicular Tissues

For assaying the levels of MDA, GSH and the activities of SOD, CAT, the homogenates were centrifuged for 15 min at 1000 g at +4°C while to assay the GPx activity of testicular homogenates were centrifuged for 20 min at 9.000 g at +4°C. Following the centrifugation process, the obtained supernatant was subjected to enzyme assays as soon as possible. The homogenisation of testicular tissues was carried out in Teflon-glass homogenizer with a buffer containing 1.15% KCl to obtain 1:10 (w/v) whole homogenate.

The malondialdehyde (MDA) level of testicular tissues was measured by the thiobarbituric acid reaction method of Placer et al.[18]. The values of MDA were expressed as nmol/g^{-1} tissue. The CAT activity of testicular tissue was determined according to the method of Goth [19]. The values of CAT were expressed as kU/g^{-1} protein. The SOD activity of testes was measured as the level of decrease in the absorbance at 560 nm and SOD values of testicular homogenates were expressed as EU/mg protein. To assay superoxide dismutase (SOD) activity of testicular tissues, the method of Sun et al.[20] was used. The GPx activity of testes was determined using the method of Matkovics et al.[21]. The GPx activity of testicular homogenates was expressed as U/mg protein. The GSH content of testicular homogenates was determined at 412 nm according to the method; described by Ball [22], Fernandez and Videla [23]. GSH levels were expressed as mmol/ g^{-1} tissue. The protein content of the testicular tissues was measured according to the method described by Lowry et al.[24].

Statistical Analysis

Statistical comparisons of data were analysed using General Linear Model/Repeated Measures (SPSS, Version IBM 20.0 Microsoft, Chicago, IL, USA) in-group comparisons. Data were expressed as mean±standard error of the mean (SEM). Differences were considered significant when $P<0.05$.

RESULTS

In *Table 1*, motility of G group was significantly lower when compared to control group ($P<0.01$). There were no differences in terms of dead sperm rate, viable sperm rate, total abnormality, TTW and TCEW among all groups ($P>0.05$). In *Fig. 1*, MDA level of G group was significantly higher than in control and treatment groups ($P<0.01$). In *Fig. 2-3*, glutathione peroxidase (GPx) and glutathione (GSH) levels in G group were lower than the other groups

Table 1. *The values (Mean ±SEM) of reproductive (spermatological and testicular) parameters in male rats used*
Tablo 1. *Kullanılan erkek ratların reprodüktif (spermatolojik ve testiküler) parametre değerleri (Ortalama± SEM)*

Groups	Motility (%)	Viability Rate (%)	Dead Rate (%)	Abnormal Head Rate (%)	Testis Weight (g)	Cauda Epididymal Weight (g)
Control	62.30±1.10^{c}	29.80±0.93	41.78±1.76	13.08±1.27	2.91±0.06	0.41±0.01
G	44.03±1.78^{a}	27.58±0.16	37.58±2.56	13.65±1.41	2.85±0.01	0.42±0.02
GT150	52.80±1.26^{b}	26.53±0.14	45.68±2.57	12.00±0.80	2.75±0.08	0.39±0.03
GT200	48.48±1.69^{ab}	26.96±0.92	37.81±1.84	13.73±1.65	2.77±0.07	0.41±0.02
P	**	--	--	--	--	--

*$^{a-c}$ The values represented by different letters within the same row are significantly different from each other, ** P<0.01*

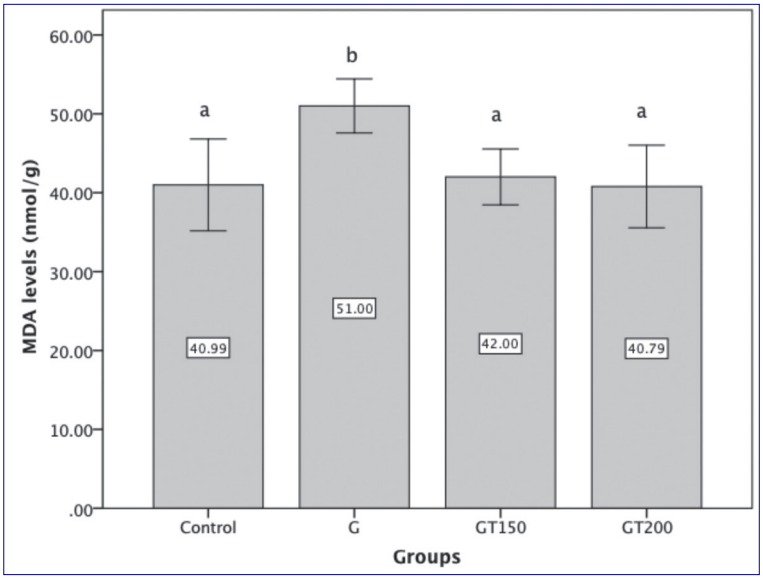


Fig 1. Mean malondialdehyde values (±SEM) of the groups studied

(a-b): Different letters on the columns indicate the statistical differences (P<0.01); G: Gentamicin; GT150: 80 mg/kg gentamicin sulphate and 150 mg/kg *Taraxacum officinale* extract; GT200: 80 mg/kg gentamicin sulphate and 200 mg/kg *Taraxacum officinale* extract

Şekil 1. Çalışılan gruplardaki ortalama malondialdehit değerleri (±SEM)

(a-b): Sütunlardaki farklı harfler istatistiksel farklılıkları belirtir (P<0.01); G: Gentamisin; GT150: 80 mg/kg gentamisin sulfat ve 150 mg/kg *Taraxacum officinale* ekstrakt; GT200: 80 mg/kg gentamisin sulfat ve 200 mg/kg *Taraxacum officinale* ekstrakt

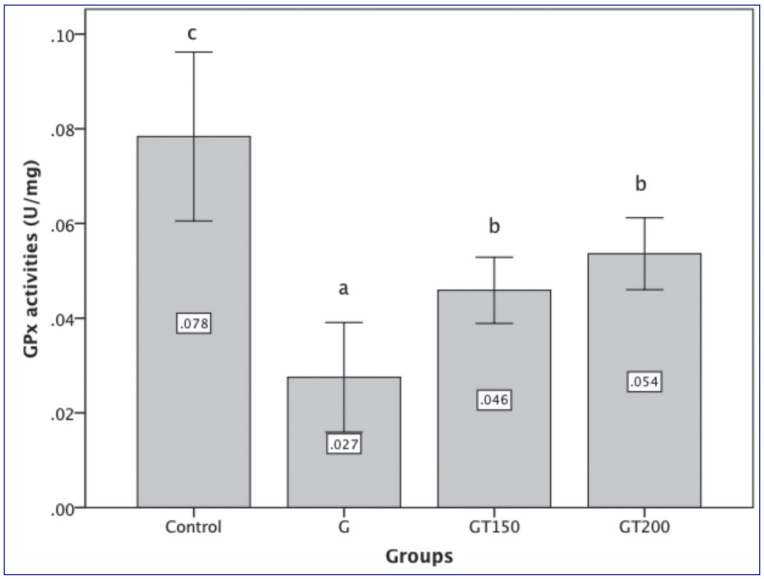


Fig 2. Mean glutathione peroxidase values (±SEM) of the groups studied

(a-b): Different letters on the columns indicate the statistical differences (P<0.01); G: Gentamicin; GT150: 80 mg/kg gentamicin sulphate and 150 mg/kg *Taraxacum officinale* extract; GT200: 80 mg/kg gentamicin sulphate and 200 mg/kg *Taraxacum officinale* extract

Şekil 2. Çalışılan gruplardaki ortalama glutatyon peroksidaz değerleri (±SEM)

(a-b): Sütunlardaki farklı harfler istatistiksel farklılıkları belirtir (P<0.01); G: Gentamisin; GT150: 80 mg/kg gentamisin sulfat ve 150 mg/kg *Taraxacum officinale* ekstrakt; GT200: 80 mg/kg gentamisin sulfat ve 200 mg/kg *Taraxacum officinale* ekstrakt

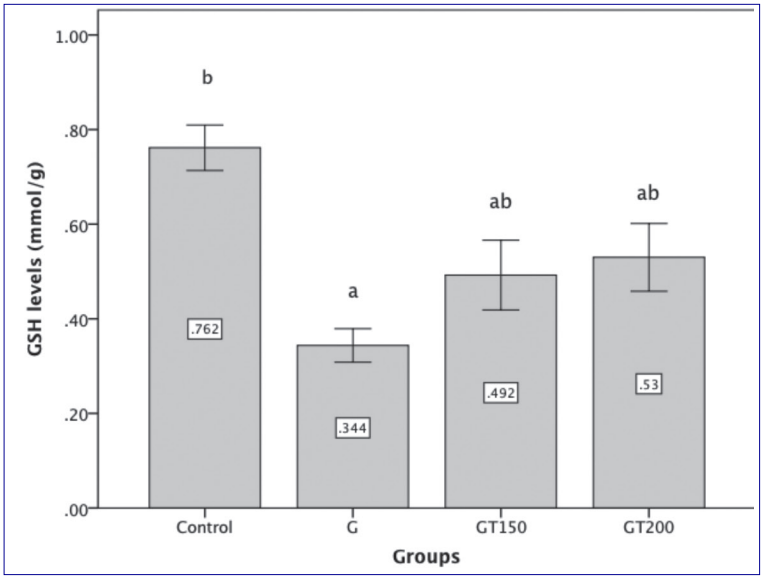


Fig 3. Mean glutathione values (±SEM) of the groups studied

(a-b): Different letters on the columns indicate the statistical differences (P<0.01); G: Gentamicin; GT150: 80 mg/kg gentamicin sulphate and 150 mg/kg *Taraxacum officinale* extract; GT200: 80 mg/kg gentamicin sulphate and 200 mg/kg *Taraxacum officinale* extract

Şekil 3. Çalışılan gruplardaki ortalama glutatyon değerleri (±SEM)

(a-b): Sütunlardaki farklı harfler istatistiksel farklılıkları belirtir (P<0.01); G: Gentamisin; GT150: 80 mg/kg gentamisin sulfat ve 150 mg/kg *Taraxacum officinale* ekstrakt; GT200: 80 mg/kg gentamisin sulfat ve 200 mg/kg *Taraxacum officinale* ekstrakt

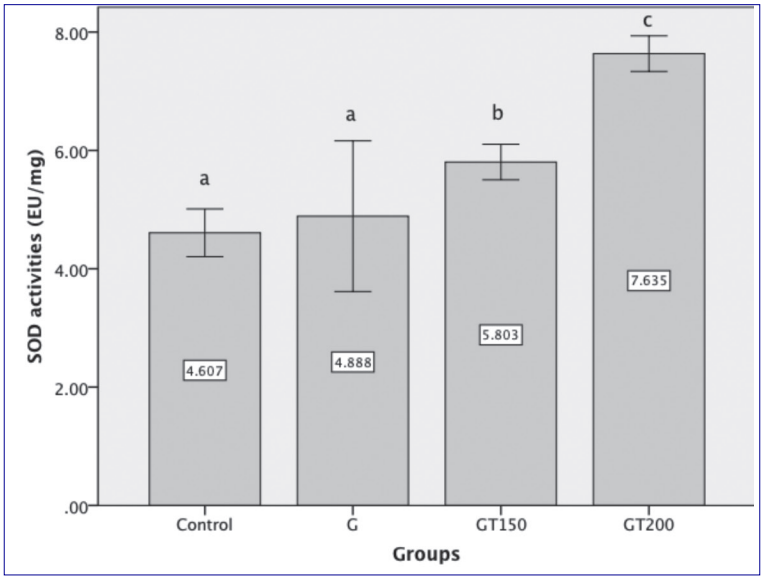


Fig 4. Mean superoxide dismutase values (±SEM) of the groups studied

(a-b): Different letters on the columns indicate the statistical differences (P<0.01); G: Gentamicin; GT150: 80 mg/kg gentamicin sulphate and 150 mg/kg *Taraxacum officinale* extract; GT200: 80 mg/kg gentamicin sulphate and 200 mg/kg *Taraxacum officinale* extract

Şekil 4. Çalışılan gruplardaki ortalama süperoksit dismutaz değerleri (±SEM)

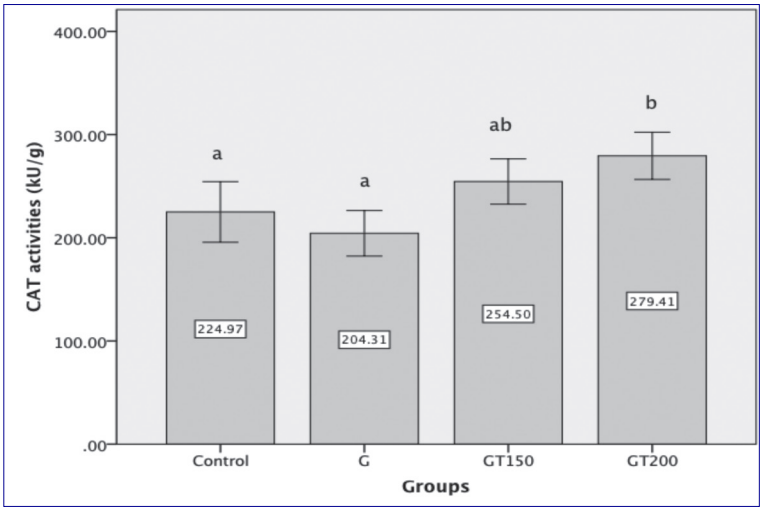
(a-b): Sütunlardaki farklı harfler istatistiksel farklılıkları belirtir (P<0.01); G: Gentamisin; GT150: 80 mg/kg gentamisin sulfat ve 150 mg/kg *Taraxacum officinale* ekstrakt; GT200: 80 mg/kg gentamisin sulfat ve 200 mg/kg *Taraxacum officinale* ekstrakt

Fig 5. Mean catalase values (±SEM) of the groups studied

(a-b): Different letters on the columns indicate the statistical differences (P<0.01); G: Gentamicin; GT150: 80 mg/kg gentamicin sulphate and 150 mg/kg *Taraxacum officinale* extract; GT200: 80 mg/kg gentamicin sulphate and 200 mg/kg *Taraxacum officinale* extract

Şekil 5. Çalışılan gruplardaki ortalama katalaz değerleri (±SEM)

(a-b): Sütunlardaki farklı harfler istatistiksel farklılıkları belirtir (P<0.01); G: Gentamisin; GT150: 80 mg/kg gentamisin sulfat ve 150 mg/kg *Taraxacum officinale* ekstrakt; GT200: 80 mg/kg gentamisin sulfat ve 200 mg/kg *Taraxacum officinale* ekstrakt



(P<0.001, P<0.05; respectively). In *Fig. 4*, the lowest SOD levels were found in control and G groups while the highest level was in GMT200 group (P<0.001). In *Fig. 5*, the CAT activity was found lower in G group than the all other groups (P<0.001).

DISCUSSION

It has been shown that *Taraxacum officinale* extract obtained from its flower inhibits the damage resulting from reactive oxygen species, protects DNA from ROS and has influence on nitric oxide damage [25,26].

There are limited literature data demonstrating the efficacy of *Taraxacum officinale* extract on reproductive parameters in rats. Due to restricted literature data our findings generally were interpreted by comparison with different texture parameters.

In a study conducted by sublethal dose of dandelion extract, Tahtamouni et al.[27] carried out the aqueous extract of *Taraxacum officinale* orally to adult male rats for 60 days in two different sublethal doses; 1/10 LD50 as high dose and 1/20 LD50 as low dose (n=11). The average percentage of sperm showing progressive motility showed a marked decrease in both the low and high dose-receiving groups (33.9±7.8% and 30.7±7.8%, respectively) in comparison to the control group (85.1±6.2%) ($P \leq 0.0001$). In addition, the percentage of sperm with normal morphology decreased significantly in the low- and high dose-receiving groups (85.3±7.6% and 78.9±6.1%, respectively) as compared to the control group (95.2±6.1%) ($P \leq 0.0001$).

In this study, GS decreased sperm motility in comparison with the control group. However, 150 mg/kg but not 200 mg/kg TOE administration to GS-treated rats reversed the reduction in sperm motility when compared with the G group. Our results demonstrated gentamicin produces increase in MDA levels (P<0.01) but decreases the activities of SOD, GPx, catalase and GSH levels. So, it is concluded gentamicin induces functional defect of spermatozoa. Besides, it is thought that the elimination of the reactive

oxygen species is related to antioxidant activity of TOE. On the other hand, it can be expressed that 200 mg/kg TOE administration has toxic effect on sperm motility.

The most important product of lipid peroxidation is MDA. MDA occurs as a result ofthe peroxidation of fatty acids comprising three or more double bonds. MDA acts on the ion-exchange through the cell membranes, leads to crosslinking of the compounds in the membrane and provokes the adverse consequences such as alteration of the ion permeability and the enzyme activity [28].

Sumanth and Rana [29] determined that alcohol extract of the *Taraxacum officinale* roots in dose of 100 mg/kg orally, significantly decreased the level of MDA compared to toxicity group ($P<0.01$). Furthermore it was observed that when toxicity group compared to the control group, the level of MDA significantly elevated ($P<0.001$). Similarly, in the present study, while GS significantly increased the MDA level in comparison with the control group, both doses of TOE significantly decreased the increments in MDA level induced by GS when compared with the G group. Because of gentamicin induced oxidative stress in connection with the ascorbic acid mobilization from testis, it can be mentioned the increasing of free radical formation and lipid peroxidation [10-12]. In spite of that, due to the elimination of the reactive oxygen species by TOE [7], it was observed that the decreasing of MDA level.

GSH wards off the radical species such as superoxide radicals and hydrogen peroxide and protects the membrane protein thiols [30]. Sumanth and Rana [29] reported that 100 mg/kg, but not 50 mg/kg *Taraxacum officinale* roots application provided significant increase in GSH level when compared with the toxicity group. Despite of the dose and mode of administration differences, in terms of effectiveness, the current study is in agreement with this result.

GPx is one of the metalloenzymes (glutathione peroxidase) that capable of removing hydrogen peroxide by converting the oxidized glutathione to reduced glutathione and containing selenium and partly availability in cell membrane. GPx also can restrict the chain reaction of lipid peroxidation by removing the lipid hydroperoxides from cell membrane [31,32].

It was observed that 100 mg/kg dose of alcohol extract from *Taraxacum officinale* roots significantly increase the GPx level compared to the toxicity group ($P<0.001$). In addition, when toxicity group compared to the control group, it was observed significantly decreased in GPx level ($P<0.001$) [29]. There is further study about the increasing level of GPx by the using of hepatoprotective plants [33]. In other study, It was determined that significant increasing in the level of glutathione peroxidase of dandelion group when control group compared to the group of aqueous extract of dandelion leaves [34]. As for our study, groups of GT150 and GT200 ameliorated the GPx level compared to the group of applied solely gentamicin.

CAT convert the harmful hydrogen peroxide to water and oxygen and protects tissues from highly reactive hydroxyl radical [35]. In a study of the application of water extract of dandelion leaves [34], the increasing of CAT level in dandelion groups is in agreement with current study.

As the prime antioxidant enzymes, SOD, can prevent oxidative stress and preventing free radical-induced cellular damage though catalyzing the dismutation reaction of reactive oxygen species (ROS) into oxygen (O_2) and H_2O_2 in biological systems [36-38].

In a study, it was observed that alcohol extract of the *Taraxacum officinale* roots in dose of 100 mg/kg, significantly increased the level of liver SOD compared to toxicity group ($P<0.001$) and in dose of 50 mg/kg, increased at the rate of $P<0.01$. Furthermore it was determined that when toxicity group compared to the control group, the level of SOD significantly decreasing ($P<0.001$) [29]. In the present study, treatment group improved the level of SOD significantly, compared to the groups of gentamicin and control.

Gentamicin have negative effects on testis architecture and germinal cells damages in rats [39]. In our study, reduction of the motility by gentamicin correspond to result of Khaki et al.[39]. Otherwise, gentamicin also caused a significant ($P<0.05$) alteration in plasma and liver enzymatic (catalase, glutathione and super oxygen dehydrogenises) and non-enzymatic (glutathione and vitamin C) antioxidant indices with concomitant increase in the malondialdehyde content; however, there was a significant ($P<0.05$) restoration of the antioxidant status coupled with significant ($P<0.05$) decrease in the tissues' malondialdehyde content, following consumption of diets containing garlic [40].

In nephrotoxicity studies, gentamicin (80 mg/kg - IP, 100 mg/kg - IP; respectively) elevated the serum level of the MDA in the renal tissue, while it decreased CAT, SOD activities and GSH levels in rats [41,42]. Although studied in different tissues, obtained results support our findings in terms of efficacy.

Ghosh and Dasgupta [10], determined that gentamicin induced the ascorbic acid mobilization from testis and by this way decrease of the reproductive potential. By the way, ascorbic acid is one of the water-soluble antioxidants that present in citrus fruits, vegetables and strawberries [43]. As known, ascorbic acid has a protective role against toxic agents -induced histological changes in tissues such as liver, kidney, lungs and testis, bone marrow in rats [44].

In conclusion, short-term administration of GS causes lipid peroxidative damages in testes as well as decreases in sperm motility. However, TOE has a moderate ameliorative effect on sperm motility reductions, but marked improvement effect on lipid peroxidative testicular

damages induced by GS. It is thought that TOE could be an alternative administration to drugs which have common features with TOE but including side effects. It is expected that the obtained results will contribute to the literature.

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Research Article

Comparison Between Goose *(Anser anser)* and Chicken *(Gallus gallus domesticus)* Eggshells During Embryonic Development by Scanning Electron Microscopy [1] [2]

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Abstract

The purpose of this study was to examine the comparative differences of eggshell structure between geese and chickens during embryonic development by SEM. Goose eggs and chicken eggs were used for the study. Eggs of each species were divided into three groups: The eggs within first group were used subsequent to collection, those of second group were collected during the mid-point of incubation and those of third group were collected at the end of the incubation. The eggshells samples with dimensions of 0.5 cm^2 were taken from the equatorial region of eggshells. After these samples were passed through routine process, they were examined via SEM for structural changes of eggshells and rate of elements. In the examination, there were not changes of eggshell structure between two species for days of the incubation. Eggshells thickness of each species and the rate of calcium, oxygen, nitrogen and carbon of eggshells were not statistically different among groups. It was concluded that structural changes of eggshell did not have significant effect on low yield for hatching of goose.

Keywords: *Calcium, Chicken, Eggshell, Embryo, Goose*

Kaz *(Anser anser)* ve Tavuk *(Gallus gallus domesticus)* Yumurta Kabuğunun Embriyonik Gelişim Sırasında Taramalı Elektron Mikroskop ile Karşılaştırılması

Özet

Bu çalışmada, taramalı electron mikroskop kullanılarak embriyonik gelişim sırasında kaz ve tavukların yumurta kabuğundaki değişiklikleri karşılaştırmak amaçlanmıştır. Bu çalışmada kaz ve tavuk yumurtası kullanılmıştır. Her iki türe ait yumurtalar üç gruba ayrıldı. Birinci gruba ait yumurta kabukları kuluçkaya konulmadan, ikinci gruba ait yumurta kabukları kuluçka süresinin ortasında ve üçüncü gruba ait olanlar ise kuluçkanın son günü olan çıkım gününde toplandı. Her bir yumurta kabuğunun ekvator kısmından 0,5 cm^2 ebatında örnekler alındı. Bu örnekler rutin prosedürlerden geçirildikten sonra taramalı elektron mikroskobunda yapısal değişiklikler ve element oranı bakımından incelendi. Elektron mikroskop incelemesinde, kaz ve tavuk türlerinin kuluçka günlerine bağlı olarak yumurta kabuğu katmanlarında belirgin bir değişikliğin olmadığı tespit edildi. Her iki türün kendi içinde kuluçka günlerine göre yumurta kalınlığında ve kabuklardaki elementlerin oranlarında istatistiksel olarak benzerlik olduğu görüldü. Yumurta kabuğunun embriyonik gelişim boyunca uğradığı yapısal değişikliklerin kaz kuluçka veriminin düşük olmasında önemli bir etkiye sahip olmadığı sonucuna varıldı.

Anahtar sözcükler: *Embriyo, Kalsiyum, Kaz, Tavuk, Yumurta Kabuğu*

INTRODUCTION

Poultry lay and incubate their eggs in the nest which is made by them on the ground or in a tree in dry environments. Growth of the embryo is completed in the eggshell during the development process [1]. Poultry eggs have a specific structure and function which prevent the embryo from external infection, exchanges of heat and

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physical condition, and provide what the embryo needs to develop. This complex structure both regulates exchanging water and metabolic gases, and provides calcium for the embryo [2]. It is required that poultry eggs are rotated during the development stages. Any mistakes during this process may cause of a decrease in oxygen uptake, delayed extra embryonic membranes, vascularization and embryonic development [3,4]. Generally, it is known that the yield of incubation rate of waterfowl is lower than that of chicken. High embryonic mortality rate is seen during incubation in geese [5]. The yield of incubation in geese was between 22% and 29% in the Kars region [6]. Conversely, the yield of incubation in chickens is between 83% and 97% [7]. Studies on incubation technique include preheating before placing eggs in the incubator [8], disinfected eggshell with ultra-violet pre and during incubation [9], and the effects of temperature and humidity [8,10]. Eggshell consists of some layers of calcium carbonate ($CaCO_3$). Elements of $CaCO_3$ were based on different sources. Calcium is the most common element in the eggshell structure [11,12]. Carbon and oxygen in this compound are obtained from digestion material rather than stored in tissue [13]. It is shown clearly that eggshell of during embryonic development would be used to determine oxygen and carbon source of species [14]. There are limited studies about nitrogen in eggshells but it is known that nitrogen is found in eggshell [15]. Interaction between organic and inorganic compounds of eggshell has been revealed with regard to the structure and function. Eggshells include four layers in sequence from the inside of the eggshell mammillary layer, palisade layer, vertical crystal layer and cuticle [16]. The mammillary layer is a basic layer of calcium source to support the embryo by providing calcium during the embryonic development. Nearly 80% of calcium, which is needed by embryo during incubation, is provided by the eggshell. While the mammillary layer consists of a lot of structures which are similar to cones, the palisade layer is extended vertically on the mammillary layer. It is known that palisade layer organization is an important index in the strength and the hardness of eggshells [17]. The vertical layer is narrow and vertical, and extends from the palisade layer to the cuticle. Cuticle, which can be seen clearly from out of eggs, is the outermost layer of an eggshell [17,18]. The rate of egg defection changes between 7% and 11% during incubation, collecting and packing the eggs [12]. The eggshell quality is the most important problem in egg production [12,19] because low eggshell quality always causes yield losses of between 5% and 8% [20].

The purpose of this study was to reveal whether there is an important effect of eggshell structure in geese hatching by examining the comparative differences of eggshell structure between geese (a species of waterfowl); with a low yield for hatching, and chickens; with a high yield for hatching, during embryonic development by scanning electron microscopy (SEM).

MATERIAL and METHODS

This study was approved by Kafkas University, Local Ethics Commission of Experimental Animals (Decision no: KAU-HADYEK/2011-14).

In this study, a total of 15 geese eggs and 15 chickens eggs were used; each egg belongs to a different goose and a different chicken, and all of the eggs were obtained from local farms in Kars. The eggs of each species were divided into three groups. Eggs within the first group were used subsequent to collection and therefore not placed in an incubator, those of the second group were collected during the mid-point of incubation (the 16th day of study for goose and the 11th day of study for chicken) and those of the third group were collected at the end of the incubation process (the 31st day for goose and the 21st day for chicken). The eggshells were soaked in distilled water and the shell membranes were removed and then peeled from the samples. Following the removal of the shell membrane remnant, each sample was immersed in 6% of sodium hypochlorite, 4.12% of sodium chloride and 0.15% of sodium hydroxide solution overnight [21]. Later, the eggshells were immersed and removed from distilled water and were left to dry for 24 h. The eggshells samples with dimensions of 0.5 cm^2, were taken from the equatorial region of each eggshell. These samples were positioned on stubs and placed in the Coater, forming a platinum coating. These samples were examined via SEM (Zeiss/ Supra 55 FE-SEM) for structural changes of eggshells and the same samples were examined via BRUKER QUANTAX EDS (Energy Dispersive X-ray Spectroscopy) in SEM for determining the percentage of elements in eggshell. SEM images of all samples were obtained at 5kV, at 500X and 15.000X magnifications, and at 11 mm working distance. The thickness of the eggshell was measured by using ImageJ software (ImageJ 1.46r). To calculate the percentage of each element was used by following formula because of removing rate of platinum which is used for coating;

Final Percentage of the Element = (Percentage of the Element X 100)/(100 - Percentage of the Platinum)

All data of elements rate and eggshells thickness were statistically analyzed using SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). One-way ANOVA was used for determining differences and P-value was < 0.05.

RESULTS

From the eggshells of the first and second group; mammillary layer, palisade layer, vertical layer and cuticle were seen normal view with the SEM. For each species, on the ends of the mammillary knobs in the eggshells of the third group were seen to be partially flattened. In the SEM examination of goose and chicken eggshells

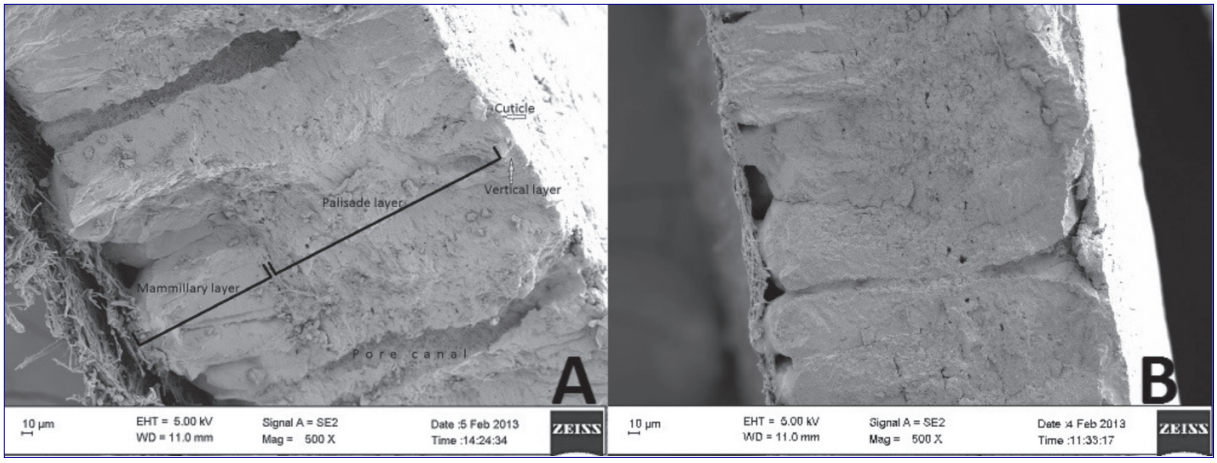
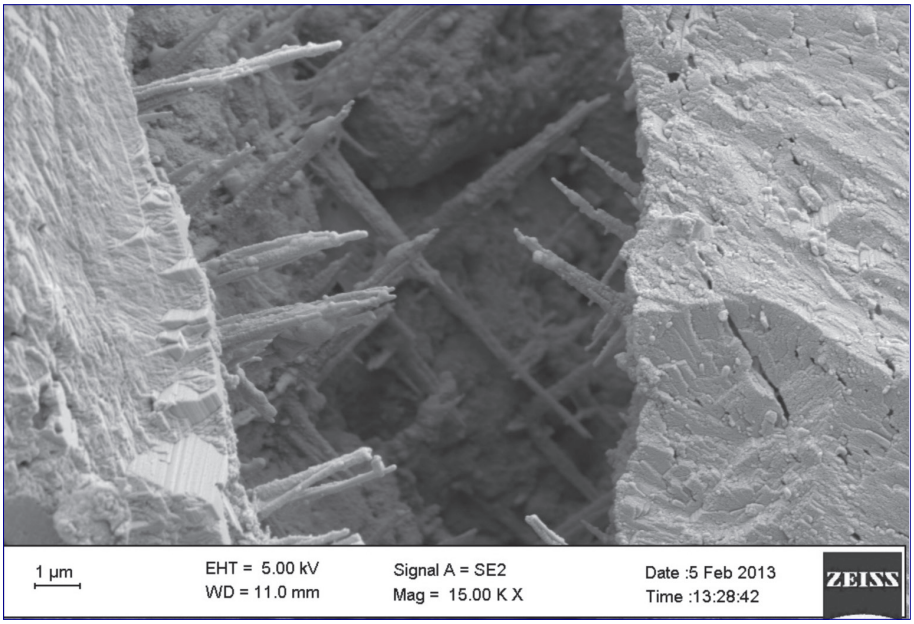


Fig 1. Cross-sectional SEM views of a goose (A) and a chicken (B) eggshells, before incubation. x500, Bar 10 µm
Şekil 1. Kuluçkaya konulmadan alınan kaz (A) ve tavuk (B) yumurtası kabuğu enine kesitinin SEM görünümü. x500, Bar 10 µm

Fig 2. A micrograph of the goose eggshell before incubation, a pore canal and some spire calcite structures which taper to center of pore canals. x15.000, Bar 1 µm

Şekil 2. Kuluçkaya konulmadan alınan kaz yumurtası kabuğundaki kanalın içine uzanan dikensi kalsiyum çıkıntıları mikrografı. x15.000, Bar 1 µm



were observed that the structure views of both species eggshells were similar to each other during embryonic development. The existence of pore canals in both goose and chicken eggshells drew attention to ultrastructural view in the cross section *(Fig. 1)*. It was found that the pore canals extended between the inner and the outer surface of both goose and chicken eggshells. The spire calcite structures which taper to center of the pore canals in the palisade layer of geese eggshells were visible *(Fig. 2)*. However, they were not seen in those of the chicken eggshells. In both species and in each group, the pore canals orifices on the inner surface of the eggshells were observed ultrastructural *(Fig. 3)* but those of outer the surface of the eggshells were usually coated by cuticle which is the outermost layer of the eggshell *(Fig. 4)*.

It was observed that palisade layer of the eggshells in both species contained many hollow vesicles disorderly in all groups *(Fig. 1)*. It was found that at the mid-point of the incubation and at the end of the incubation process in both geese and chickens eggshell membrane was able to be peeled from eggshell easier than that of first group of them *(Fig. 5)*. When examined with SEM, the inner surface of basal caps of mammillary layer was partially flattened in the eggshell at the end of the incubation process in both species. Apart from mammillary layer, the other layers of the eggshell were similar in appearance in all species from before incubation until the end *(Fig. 6)*. After analysis, it was found that the ratio of calcium, carbon and oxygen was not significantly different in both the mammillary and palisade layers in terms of incubation days among all groups *(Table 1)*. It was found that the ratio of nitrogen in both mammillary and palisade layers of geese and chickens eggshells were at a level of approximately 5% when result of EDS analysis in the SEM was considered. In these eggshell layers of both species, there was not significantly difference when

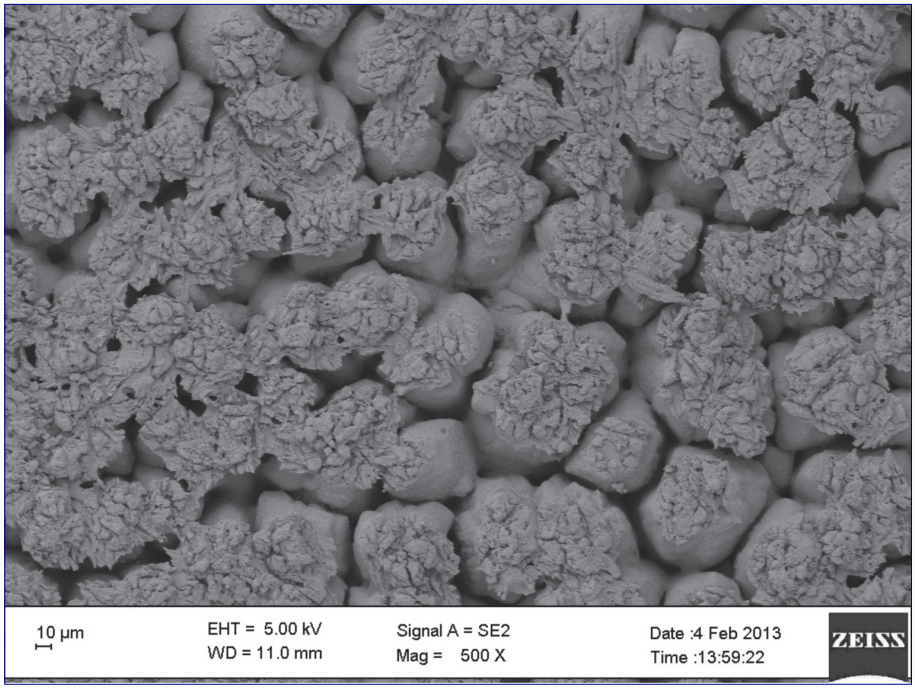
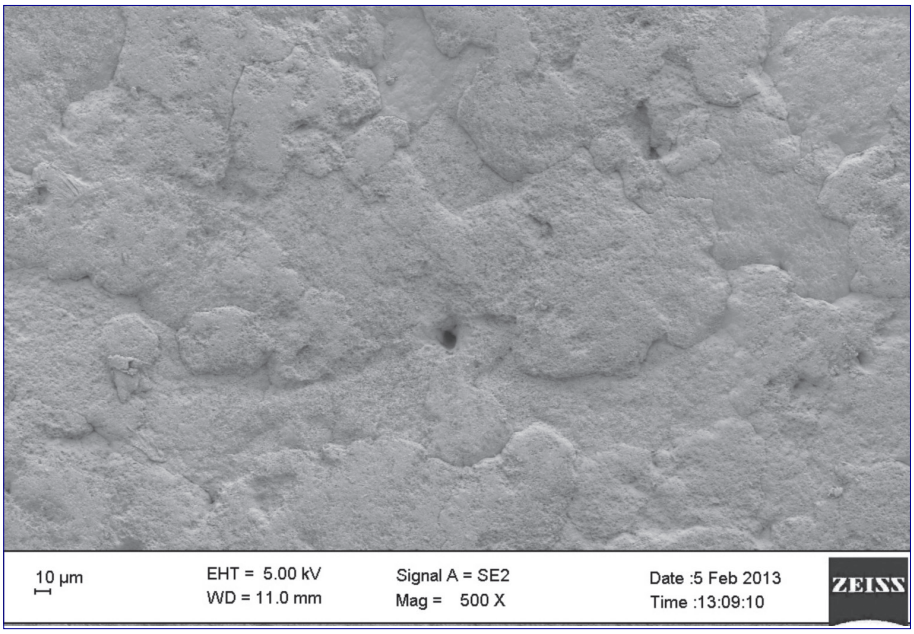


Fig 3. An SEM view of pore canals in inner surface of a chicken eggshell on the 11th day of incubation. x500, Bar 10 µm

Şekil 3. Kuluçkanın 11. gününde tavuk yumurta kabuğunun iç yüzünde gözenek kanallarının SEM görünümü. x500, Bar 10 µm

Fig 4. An SEM view of the outer surface of a chicken eggshell on 21st day of incubation. A few outer orifices of pore canals are seen in the micrograph. x500, Bar 10 µm

Şekil 4. Kuluçkanın 21.gününde tavuk yumurta kabuğunun dış yüzünün SEM görünümü. Mikrografta bir kaç gözenek kanalının girişleri görülmektedir. x500, Bar 10 µm



nitrogen ratio was analysed statistically for incubation days *(Table 1)*. Elemental analysis of the eggshell showed that the percentage of magnesium and sodium was less than 1% in both the mammillary and the palisade layers of both species for all groups. Furthermore, it was identified that the mammillary and the palisade layers of eggshells included calcium, oxygen, carbon, nitrogen and traces of magnesium and sodium in this study. It was calculated that the average thickness of chicken eggshell was 340.8±26.2 µm and that of goose eggshell was 495.8±22.2 µm. It was found that the thickness of chicken eggshells was not statistically significantly different among chicken groups for incubation days and that of geese eggshells was not significantly different in terms of statistics among geese groups for incubation days *(Table 2)*.

DISCUSSION

The purpose of this study was to examine and compare the differences in the structure of eggshells between geese; with low yield for hatching, and chickens; with high yield for hatching, during embryonic development by using SEM.

Parsons [22] reported that eggshells contained; shell membrane, mammillary layer, palisade layer, vertical crystal

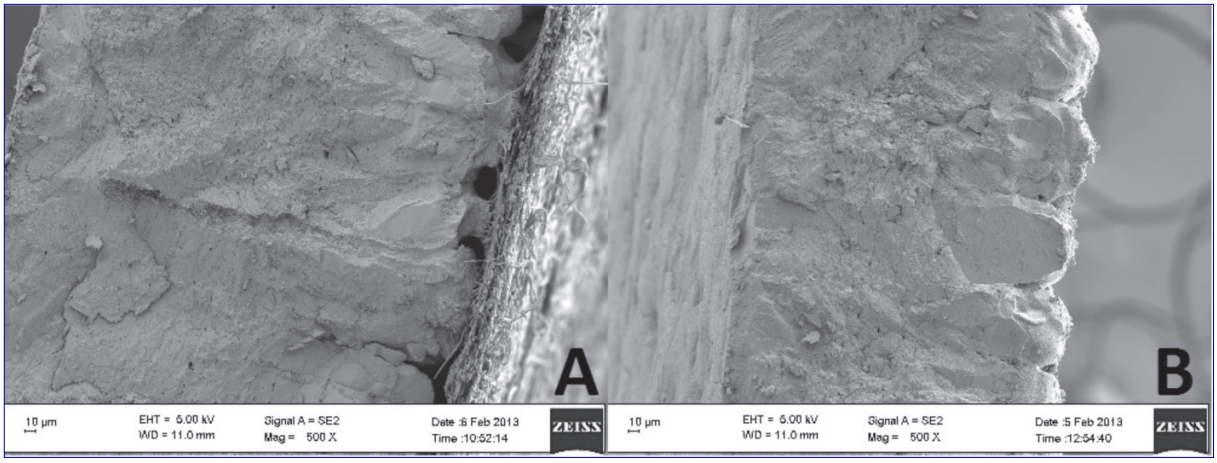
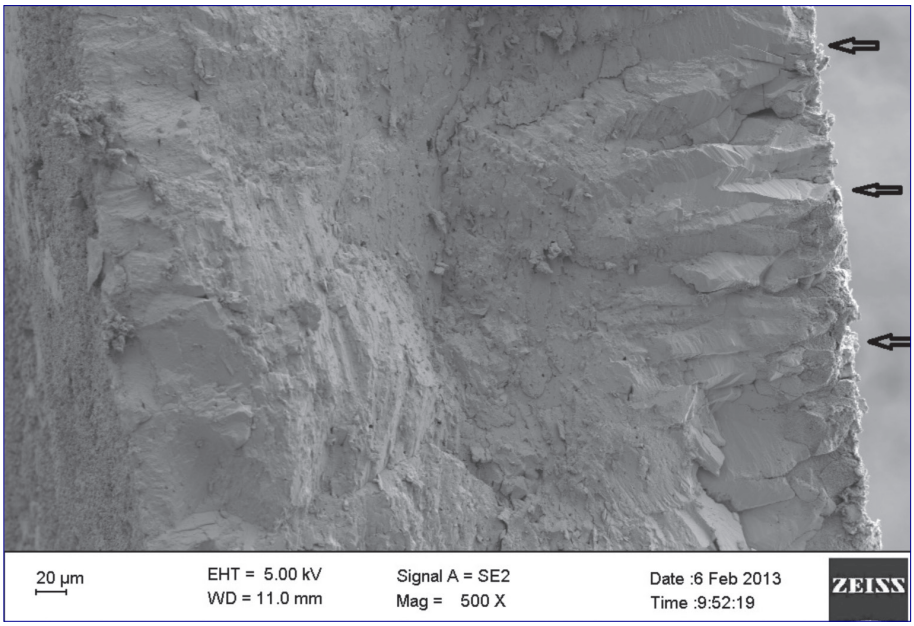


Fig 5. SEM views of cross-section of a goose (A) on the 16th day and a chicken (B) eggshell on the 11th day of incubation. x500, Bar 10 µm

Şekil 5. Kuluçkanın 16.gününde ki kaz (A) ve 11.gününde ki tavuk (B) yumurta kabuğunun enine kesitinin SEM görü-nümü. x500, Bar 10 µm

Fig 6. An SEM view of a goose eggshell at the end of incubation. It is seen that the inner surface of mammillary layer was partially flattened *(arrows)*. x500, Bar 20 µm

Şekil 6. Kuluçkanın sonunda kaz yumurta kabuğunun SEM görünümü. Mammillari katmanının kısmen düzleştiği *(oklar)* görül-mektedir. x500, Bar 20 µm



layer and cuticle. According to Abdel-Salam et al.[23], embryo consumes calcium and other elements from the mammillary layer during embryonic development in the incubation period. Ruiz and Lunam [18] reported that the eggshell quality was not significantly decreased during the hatching period. In the present study, we have identified that the basal caps of the mammillary layer on the last days of incubation were partially flattened. Besides that, it was observed that in all layers of eggshell during incubation there were not any major differences between goose and chicken eggshells. Simons and Wiertz [24] reported that in the outer orifice of the pore canal that traversed the eggshell was filled with cuticle material. In this study, it was drawn to attention that there were pore canals which extended between the mammillary layer and cuticle in both species. It was observed that the inner orifice of the pore canal was open, however the outer orifice was filed with cuticle material in all groups as mentioned earlier. Carnarius et al.[25] mentioned that the palisade layer was the most important layer for the eggshell in terms of strength. Although the spire calcite structures, taper to the center of the pore canals in the palisade layer, were observed in geese eggshell, they were not seen in those of chicken eggshell. These structures within the pore canal were seen in samples before incubation, so we considered that they were a feature of goose species and related to the strength of the eggshell and they were not related to the incubation period. Bönner et al.[26] reported that the thickness of average of goose eggshell was 530 µm, Gualhanone et al.[27] stated that the thickness of average of chicken eggshell was 370 µm and Yamak et al.[28] informed that the thickness of average of chicken eggshell was 380 µm. In this study, there was a big difference between the thickness of average of goose eggshells and that of chicken so we compared the thickness of an

Table 1. *Comparison levels of per element in both the mammillary and the palisade layers of eggshells in relation to six groups of incubation days (P<0.05)*
Tablo 1. *Kuluçka günlerine gore altı gruba ait yumurta kabuklarının mammillari ve palisade katmanlarında her bir elementin düzeylerinin karşılaştırılması (P<0.05)*

Species	Incubation Days (D)	n	Calcium (%) ± Std. Dev	F	Oxygen (%) ± Std. Dev	F	Carbon (%) ± Std. Dev	F	Nitrogen (%) ± Std. Dev	F
Mammillary Layer										
Chicken	0th D	5	64.64±8.5	1.19	22.58±9.1	1.14	9.14±3.4	0.60	6.57±0.7	1.47
	11th D	5	54.20±10.4		31.66±10.7		9.64±1.8		5.13±0.9	
	21st D	5	59.83±10.0		27.91±6.8		8.49±2.1		5.44±1.1	
Goose	0th D	5	65.73±12.3		21.00±10.2		7.90±3.1		5.31±0.7	
	16th D	5	56.44±7.3		28.74±7.2		9.75±1.6		4.92±1.3	
	31st D	5	62.59±6.4		23.90±6.6		8.13±0.7		5.34±1.0	
Palisade Layer										
Chicken	0th D	5	57.04±11.6	1.03	29.00±10.0	0.85	10.96±3.0	1.53	5.82±0.3	1.08
	11th D	5	49.98±2.0		34.65±2.9		10.22±0.7		5.53±1.4	
	21st D	5	52.81±4.1		32.19±4.7		9.64±0.5		4.87±1.1	
Goose	0th D	5	57.78±7.2		27.35±7.1		9.69±1.4		5.13±0.8	
	16th D	5	52.66±12.9		31.67±12.7		11.15±1.4		4.46±1.4	
	0th D	5	57.04±11.6		29.00±10.0		10.96±3.0		5.82±0.3	

Table 2. *Comparison of thickness of eggshells among groups of each species for incubation days (P<0.05)*
Tablo 2. *Grup içinde kuluçka günlerine göre yumurta kabuğu kalınlığının karşılaştırılması (P<0.05)*

Species	Incubation days (D)	n	Thickness of Eggshell (µm)	Std. Deviation	F
Chicken	0th D	5	349.80	30.4	0.40
	11th D	5	336.00	13.4	
	21st D	5	336.60	33.9	
Goose	0th D	5	482.60	31.9	1.45
	16th D	5	501.00	16.9	
	31st D	5	504.00	8.9	

average eggshell statistically among the groups within each species. The average thickness of goose eggshells was found to be 495 µm and that of chicken eggshells was found to be 340 µm when they were calculated by adding the three groups of each species. It was found that the thicknesses of goose and chicken eggshells were not significantly different among the groups of each species for incubation days. Turkyilmaz et al.[29] reported that eggshell thickness did not have a significant effect on the incubation period. Nys and Gautron [30] and Marie et al.[31] mentioned that approximately 95% of eggshell was made from $CaCO_3$ and the rest of eggshell was made from organic material which consisted of eggshell membrane. Nakano et al.[15] identified that there was less nitrogen ratio in eggshell than other elements. It was considered that the ratios of calcium, oxygen and carbon in EDS analysis were higher than those of other elements in an eggshell due to the fact that they take place in the $CaCO_3$ compound in eggshells. In addition, it was observed that the ratio of nitrogen in the mammillary and the palisade layer of geese and chickens eggshells were at a level of approximately 5%, and there was not statistically significant difference in each layer between species during the incubation period in terms of nitrogen ratio.

We concluded that when the indication of SEM results of goose eggshells were compared with those of chicken eggshells, the structural changes of an eggshell in SEM, identified elemental distribution in eggshells, and the thickness of eggshells did not have a clear significant effect on low yield for the hatching of goose eggs during embryonic development.

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Research Article

Morphometric Evaluation of Chinchillas *(Chinchilla lanigera)* Femur with Different Modelling Techniques

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Abstract

Together with technological developments, different methods in anatomic and morphometric studies have been started to be used. In fact, in this study, more than one method was used and the reliability of these methods was presented. This study was performed in the aim of getting 3D model with three dimensional (3D) reconstruction and photogrammetric methods obtained from multidetector computerized tomography (MDCT) images of femur of chinchilla by mimics program, comparing the data of both methods and presenting whether there was a difference between genders as well as left and right sides. For this purpose, 6 chinchillas of both sexes were used. First of all, MDCT images of animals were taken under general anaesthesia and 3D reconstruction was obtained after transferring the data to personal computer as DICOM format. After taking MDCT images, the femurs of the animals killed by the conventional methods were cleaned of muscle and fats by boiling and their 3D models was formed by using images via photogrammetric methods. The measurements were taken from the models of two methods and volume and surface area of femur were found significant at a level of $P<0.05$ as a result of statistical processes. According to this, a statistically difference was not found in morphometric measurement values except volume and surface area. Moreover, while a statistical difference was not found between right and left femur of both sexes, a difference was determined between sexes and it was observed that the measurement values of females were higher than males. As a result, since 3D models obtained by both methods had almost same values, it was considered that these methods could be used in anatomic and morphometric studies.

Keywords: *Chinchilla, Femur, Three dimensional reconstruction, Photogrammetry, Morphometry*

Chinchilla *(Chinchilla lanigera)* Femur'unun Farklı Modelleme Teknikleri ile Morfometrik Değerlendirilmesi

Özet

Teknolojik gelişmelerle birlikte anatomik ve morfometrik çalışmalarda farklı yöntemler kullanılmaya başlanmıştır. Hatta bir çalışmada birden fazla yöntemle çalışılarak, bu yöntemlerin güvenilirliği ortaya konulmaya çalışılmaktadır. Sunulan bu çalışma şinşilla'nın femur'u üzerinde multidedektör bilgisayarlı tomografi (MDBT) görüntülerinden mimics programı ile elde edilen üç boyutlu (3B) rekonstrüksiyon ve fotogrametrik yöntemlerle elde edilen 3B model üzerinden gerçekleştirerek, her iki yöntem verilerini karşılaştırmak ve cinsiyetler ile sağ ve sol taraf arasında fark olup olmadığını ortaya koymak amacıyla gerçekleştirilmiştir. Bu amaçla her iki cinsiyetten 6'şar şinşilla kullanılmıştır. Önce hayvanların genel anestezi altındayken MDBT görüntüleri çekilerek, veriler DICOM formatında kişisel bilgisayara aktarılarak daha sonra 3B rekonstrüksiyonu yapılmıştır. MDBT görüntüleri alındıktan sonra usulüne göre hayvanların ölümü gerçekleştirilerek, maserasyon yöntemi ile femur elde edilmiştir. Kas ve yağlardan kaynatılarak temizlenen femur'un fotografları çekilerek, görüntülerden fotogrametrik yöntemlerle 3B modeli oluşturulmuştur. Her iki yöntemle oluşturulan modeller üzerinden ölçümler alınmış ve istatistiki işlemler yapılarak, femurun hacmi ve yüzey alanı $P<0.05$ düzeyinde anlamlı bulunmuştur. Buna göre hacim ve yüzey alanı dışında morfometrik ölçüm değerlerinde istatistiki bir fark görülmemiştir. Ayrıca her iki cinsiyetin de sağ ve sol femur'u arasında istatistiki farklılık yokken, cinsiyetler arasında farklılık tespit edilmiştir ve dişilerde ölçüm değerleri erkeklerden fazla olduğu görülmüştür. Sonuç olarak her iki yöntemle oluşturulan 3B modeller hemen hemen aynı değerlere sahip olmasından dolayı, anatomik ve morfometrik çalışmalarda bu yöntemlerin kullanılabileceği düşünülmektedir.

Anahtar sözcükler: *Şinşilla, Femur, Üç boyutlu rekonstrüksiyon, Fotogrametri, Morfometri*

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INTRODUCTION

Since femur that is the longest bone in the body binds many muscles and tendon on itself and joins in the structure of many joints and it is the biggest bone in the body, it's quite important. For these reasons, the studies related with femur were focused on presenting the biometric differences between races and sexes [1].

Many morphometric studies were performed on femur in human and veterinary medicine. The measurements were taken from computerized tomography (CT) images of femurs belonging to Caucasus adults in Anatolia and their morphometric variations depending on sex and age were presented [2]. Some authors studied on the morphometric measurements of tibia and femur belonging to African and European societies [3]. Sex and right-left differences of femoral condyles in Greek society were also measured by digital caliper and then compared [1]. Whether there was a difference between left and right sides as well as parameters such as gender and age or not was presented by 3D model formed via CT images belonging to femur and radius of human [4].

Morphometric measurements of hind limb bones of New Zealand rabbits were carried out to compare the right and left sides [5]. After taking several morphometric measurements belonging to the thoracic and hind limb bones of New Zealand rabbits and guinea pigs, whether there was a difference between sexes as well as right and left sides was presented as a result of measurements performed by caliper [6,7]. The measurements were taken on X-ray images of the whole skeleton of chinchilla and the differences between sexes were determined [8]. The hind limb skeleton anatomy was presented by X-ray images of chinchilla [9].

Recently, some anatomic and morphometric studies have been based on comparison of the data obtained via many methods instead of just a single method. Related with this, in a study, 3D reconstruction obtained from CT images of human femur was stated both manually and automatically, then the data were compared [10]. Moreover, the morphologies of hind limb skeleton of rodents of different races were presented by using X-ray images of and 3D reconstruction [11].

Analysis and morphometry of human or animal bones are quite difficult especially irregular surfaces. In order to obtain 3D models of substances having different shapes and properties, different approaches are present. Photogrammetry, which has low cost, no exposure to radiation for patients and is an appropriate model to get measurement values from the formed model, is considered as a reliable method that can be preferred for the evaluation and investigation of bones [12,13]. Photogrammetry is a branch of technology and science where reliable information can be obtained about substances and environment as a result of recording, measurement and interpretation processes of photographic images shaped by radiating beams from substances and their environment as well as their radiated electromagnetic energy. This method is used to determine, measure and interpret the form and characteristic properties of living or non-living things with high accuracy [12].

In addition to photogrammetric methods, 3D reconstruction was carried out in anatomic studies by using technology in terms of some software programs of 2D MDCT images. Thus, anatomic structures can be clearly displayed by this model and morphometric measurements can be taken [14]. Except 2D CT images, reconstructions obtained by X-ray radiography and laser scanners were same as the data obtained from the models formed via CT images and therefore they are trusted methods in morphological studies [15].

In the literature survey, the studies related with chinchilla femur were limited just with 2D images and they were not transferred to 3D structure. In this study which was performed by taking the advantage of computer technology and photograph technology, the morphometric measurements were taken from models belonging to chinchilla femur obtained by mimics program and photogrammetric methods, the data of both methods were compared and determination of whether there was a difference between genders as well as right and left sides was carried out.

MATERIAL and METHODS

This study was accepted by the ethics committee of the Veterinary Faculty of Selcuk University on 29.01.2016 (Decision number: 2016/15).

In the study, a total of 12 adult chinchillas *(Chinchilla lanigera)* of both sexes weighing from 500 to 600 g. were used. Throughout this study, 3D models of femur were obtained one by one with mimics program and photogrammetric methods.

In order to obtain 3D reconstruction via mimics program, MDCT images of femur were obtained at high resolution. The animals of which the images would be taken were anesthetized with a mixture of 60 mg/kg ketamin (ketalar) amd 6 mg/kg xylazine (Rompun) intravenously [16]. Under anesthesia, MDCT images of animals in prone position were taken. The parameters of MDCT instrument (Somatom Sensation 64; Siemens Medical Solutions, Germany) were adjusted as; physical detector collimation, 32 x 0.6 mm; final section collimation, 64 x 0.6 mm; section thickness, 0.50 mm; gantry rotation time; 330 msec; kVp; 120; mA, 300; resolution, 512 x 512 pixel; resolution range, 0.92 x 0.92. Dosage parameters and scannings were performed by taking standard protocols and literature [17,18] into consideration. Thus, radiometric resolution (MONOCHROME2; 16 bits) was obtained at the lowest radiation level and with optimum

image quality. The axial images were stored in the format of DICOM and then they were transferred to a personal computer having 3D modeling program Mimics 13.1 (Multimodal Immersive Motion rehabilitation with Interactive Cognitive Systems).

In the first stage of automatic segmentation process, the limits of femur were determined. Section cleaning process was applied one by one to places outside these limits with computer mouse and the femur was colored after cleaning these places. The images the limits of which were determined were overlapped and reconstruction of femur was carried out by 3D transformer component of Mimics 13.1 program.

In 3D model formation via photo-grammetric method, first of all, identification of objects to be evaluated with photogrammetric evaluation should be done in a known coordination system during taking photos or coordinates should be given to some points on the object. Since measuring 3D coordinates of points on the object by traditional methods is almost impossible, a new setup was designed and coded targets were used for control points *(Fig. 1)*.

Coded targets can be used to identify a local coordinate system and model scale to make correct match-up picture alignment process. Determination and match-up of coded targets in the pictures were carried out automatically by the software.

Picture taking geometry has great importance in taking photogrammetric images. In the study, a picture scale depending on the size of the object was determined by taking the digital camera into consideration and B/H (Base/Height) ratio suitable for this picture scale as well as picture shooting plan. The pictures belonging to object surfaces were taken with Sony DSC-W570 digital camera having 16 megapixels optical resolution, equal distances to the object with 70% overlapping ratio, Base/Height ratio of 1/3 and by rasing the camera with approximately 30° angle *(Fig. 2)*.

In our study, Photoscan software was used in order to make photogrammetric evaluation. Photogrammetric evaluation process stages of photoscan software constituted of picture alignment, formation of dense point cloud, formation of 3D model grid network, model tissue covering and place referencing.

During picture alignment process, the software automatically perceives coded target points on pictures and moreover finds common points outside these coded target points after match-up between pictures and forms connection points. By means of these points, the location

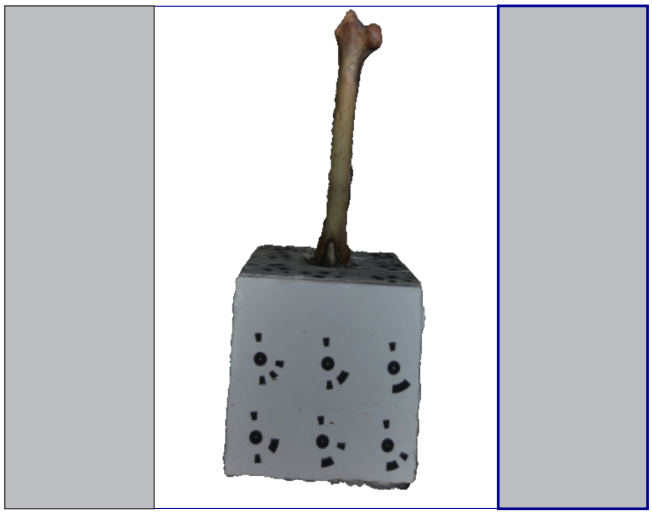

Fig 1. Setup used for taking photos and coded target points
Şekil 1. Resim çekiminde kullanılan düzenek ve kodlu hedef noktaları

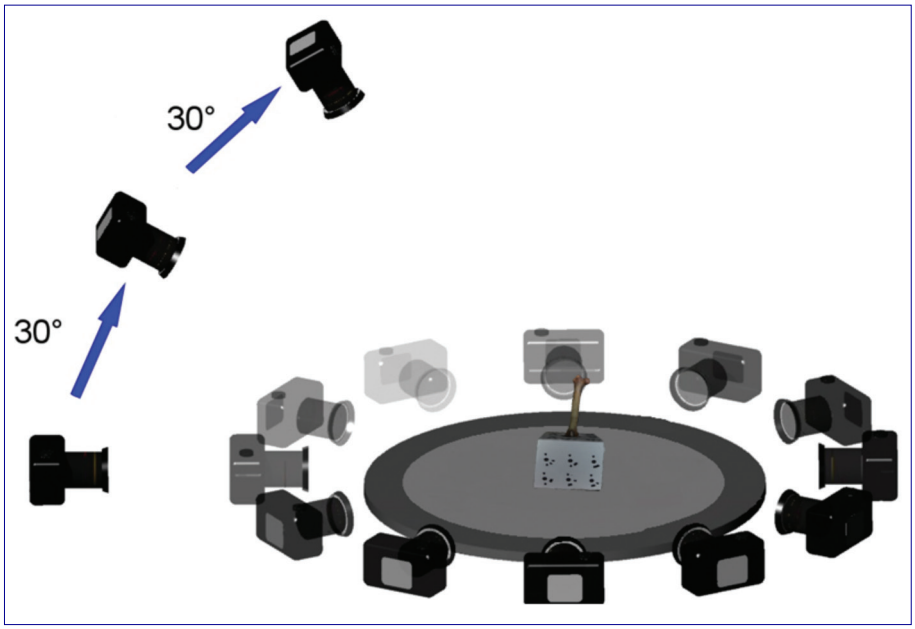

Fig 2. Simulated picture taking process

Şekil 2. Simule edilmiş resim alım işlemi görüntüsü

Fig 3. 3D model of femur obtained by photogrammetric methods
a. Caudal view, **b.** Lateral view, **c.** Cranial view, **d.** Medial view
Şekil 3. Fotogrametrik yöntemlerle elde edilen femur'un 3B modeli
a. Kaudal görünüm, **b.** Lateral görünüm, **c.** Kranial görünüm, **d.** Medial görünüm

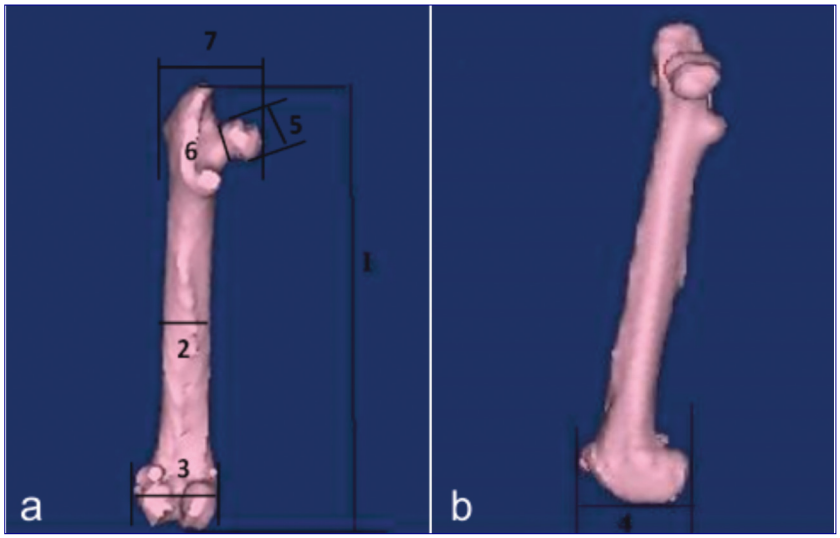

Fig 4. Measurement points taken from femur
a. Caudal view of femur, **b.** Medial view of femur
1. Maximum femoral length, **2.** Mid-shaft transverse diameter, **3.** Distal width, **4.** Medial condylar depth, **5.** Femoral head diameter, **6.** Narrowest neck width, **7.** Proximal width

Şekil 4. Femur'dan alınan ölçüm noktaları
a. Femur'un kaudal görünümü, **b.** Femur'un medial görünümü
1. Femur'un maksimum uzunluğu, **2.** Gövde ortasının transvers çapı, **3.** Distal genişlik, **4.** Medial kondiler derinlik, **5.** Fermur başı'nın çapı , **6.** Boynun en dar kısmının genişliği, **7.** Proksimal genişlik

of camera and directions can be calculated during every picture talking process. After this process, dense point match-up was carried out between stereo picture pairs and point cloud model was formed.

Georeferencing of the model was performed by at least three points whose real earth coordinates were known by transforming with 7 parameters (3 parameters of translation, 3 parameters of version, 1 parameter of scale). Since just real dimensions of the model were required in our study, transformation was carried out by scaling the model after using the distances between coded target points.

After georeferencing of the model, 3D grid network was formed from dense point cloud, after formation of numerical surface model, 3D real model of the object was obtained by covering this model picture tissue *(Fig. 3)*. These processes were carried out for each bone and 3D of all bones were obtained.

Various morphometric measurement values, surface areas and volumes were taken from 3D models belonging to femur of chinchilla formed by photogrammetric and mimics programs. While taking measurements of femur, literatures such as [1,2,19] were taken into consideration *(Fig. 4)*.

Measurements

1. Maximum femoral length: Maximum distance from the uppermost margin of the head of the femur to the lowest margin of the medial condyle.

2. Mid-shaft transverse diameter: Transverse diameter at the middle of the shaft.

3. Distal width: The maximum distance across the femoral condyles in the transverse plane.

4. Medial condylar depth: The maximum anteroposterior diameter of the medial femoral condyle.

5. Femoral head diameter: Width of caput ossis femoris.

6. Narrowest neck width: Narrowest width of the collum ossis femoris.

7. Proximal width: The maximum distance between the head of femur to greater trochanter.

8. Volume: Volume of the femur.

9. Surface area: Surface area of the femur.

10. Index: (Mid-shaft transverse diameter/Maximum femoral length) x100

The average values and standard deviations of morphometric measurements obtained by mimics program and photogrammetric methods in terms of right and left sides as well as in terms of sexes were calculated. The materiality control of differences between average values was carried out via SPSS 16.00 software program and independent t- test.

RESULTS

Several morphometric measurement values were obtained from 3D reconstruction formed by using mimics program of MDCT images belonging to chinchilla femur and from the model obtained by photogrammetric methods. The statistical results of volume and surface area were found significant at the level of P<0.05 *(Table 1, Table 2)*.

In *Table 1*, it was observed that some measurement values belonging to chinchilla femur obtained by mimics program and photogrammetric methods (maximum femoral length, mid-shaft transverse diameter, distal width, medical condylar depth, femoral head diameter, narrowest neck width, proximal width and index) were not different statistically in both right and left sides. However,

Table 1. *Statistical results of morphometric measurements of chinchilla femur obtained by mimics program and photogrammetric methods (Mean±SD)*
Tablo 1. *Şinşilla femur'unun mimics programı ve fotogrametrik yöntemlerle elde edilen morfometrik ölçüm değerlerinin istatistiki sonuçları (Ortalama±SS)*

Measurements	Right		Left	
	Mimics (n=12)	Photogrammetry (n=12)	Mimics (n=12)	Photogrammetry (n=12)
Maximum femoral length (mm)	55.64±2.31	55.00±2.16	55.50±2.21	55.01±2.17
Mid-shaft transverse diameter (mm)	5.54±0.43	5.32±0.35	5.55±0.43	5.24±0.41
Distal width (mm)	9.33±0.34	9.13±0.36	9.38±0.36	9.13±0.36
Medial condylar depth (mm)	8.74±0.32	8.58±0.39	8.70±0.31	8.62±0.33
Femoral head diameter (mm)	5.57±0.50	5.35±0.34	5.63±0.42	5.35±0.27
Narrowest neck width (mm)	3.52±0.25	3.36±0.22	3.52±0.27	3.31±0.25
Proximal width (mm)	12.01±0.57	11.89±0.72	12.09±0.61	11.90±0.66
Volume (cm^3)	1471.54±158.65 [a]	1105.83±144.50 [b]	1456.38±153.30 [a]	1107.50±135.31 [b]
Surface (cm^2)	1698.03±144.88 [a]	1106.08±145.15 [b]	1685.44±141.33 [a]	1109.75±123.34 [b]
Index	9.94±0.55	9.67±0.53	10.00±0.62	9.51±0.55

[a,b] Different letters in the same line are statistically significant (P<0.05)

Table 2. *Statistical results of morphometric measurements of chinchilla femur obtained by mimics program and photogrammetric methods for females and males (Mean±SD)*
Table 2. *Şinşilla femur'unun mimics programı ve fotogrametrik yöntemlerle elde edilen morfometrik ölçüm değerlerinin birlikte erkek ve dişilerde istatistiki sonuçları (Ortalama±SS)*

Measurements	Right		Left	
	Male (n=12)	Female (n=12)	Male (n=12)	Female (n=12)
Maximum femoral length (mm)	54.04±1.04 [a]	56.60±2.36 [b]	54.04±0.99 [a]	56.48±2.36 [b]
Mid-shaft transverse diameter (mm)	5.25±0.24 [a]	5.61±0.45 [b]	5.20±0.25 [a]	5.59±0.51 [b]
Distal width (mm)	9.04±0.24 [a]	9.42±0.37 [b]	9.03±0.28 [a]	9.48±0.33 [b]
Medial condylar depth (mm)	8.43±0.35 [a]	8.90±0.17 [b]	8.47±0.28 [a]	8.85±0.23 [b]
Femoral head diameter (mm)	5.21±0.23 [a]	5.71±0.46 [b]	5.30±0.21 [a]	5.68±0.42 [b]
Narrowest neck width (mm)	3.29±0.12 [a]	3.59±0.25 [b]	3.24±0.17 [a]	3.58±0.25 [b]
Proximal width (mm)	11.52±0.31 [a]	12.38±0.60 [b]	11.64±0.34 [a]	12.35±0.67 [b]
Volume (cm^3)	1191.16±212.57 [a]	1386.21±230.35 [b]	1176.91±188.83 [a]	1386.96±220.18 [b]
Surface (cm^2)	1287.18±310.02 [a]	1516.93±329.07 [a]	1288.66±292.23 [a]	1506.52±323.53 [a]
Index	9.71±0.55 [a]	9.91±0.55 [a]	9.63±0.53 [a]	9.89±0.70 [a]

[a,b] Different letters in the same line are statistically significant (P<0.05)

the measurement values of volume and surface areas belonging to both right and left femur were statistically different between two methods (P<0.05).

In *Table 2*, it was observed that a statistical difference was found in the values of maximum femoral length, mid-shaft transverse diameter, distal width, medical condylar depth, femoral head diameter, narrowest neck width, proximal width and volume of chinchilla in both right and left femur between genders. However, there wasn't a significant difference in terms of statistics in surface and index values between sexes. Moreover, a statistically difference was not found between right and left femur of both male and female chinchillas.

DISCUSSION

The values of maximum femoral length, mid-shaft transverse diameter, distal width, medial condylar depth, femoral head diameter, narrowest neck width, proximal width and volume of chinchilla indicated a statistically significant difference between sexes and they were higher in females than males. According to the results obtained from rontgen images of chinchilla femur performed by Lammers et al.[8], the determination of femur length that was longer in females than males was in accordance with our results obtained via photogrammetric and Mimics program. The rate of femur volume to body height in human beings is higher in males than in females [10].

It was observed that there wasn't a difference in surface and index values of chinchilla between sexes. A statistical difference was also not indicated in any morphometric measurement values obtained from right and left femurs of both female and male chinchillas. Pazvant and Kahvecioğlu [7] stated that right and left morphometric values (maximum femoral length, mid-shaft transverse diameter, distal width, proximal width, femoral head diameter, index) of long bones of thoracic and hind limbs in guinea pigs were very close to each other and did not have homotypical variation in terms of statistics, however, they indicated that sex was an important factor. A statistical difference was not found between right and left sides of maximum femoral length, mid-shaft transverse diameter, distal width, proximal width, femoral head diameter values of New Zealand rabbits [5]. Moreover, there was not also a difference in New Zealand rabbits between sexes and it was observed that maximum femoral length was longer in males than in females [6]. But in broilers statistical difference was found between the sexes in femoral morphology [20].

In some biomedical researches, it was stated that a statistical difference was not found between sexes in terms of the bone sizes of permanently used *Callithrix jacchus* [21].

In human beings, while maximum femoral length was statistically different between females and males, there was no difference in mid-shaft transverse diameter. Moreover, these values were higher in males than in females in contrast to chinchilla [2]. In the morphometric study of Terzidis et al.[1] which was performed on femur of Greek people, while a statistical difference was recorded between women and men, a difference was not found between right and left sides in both men and women. According to the measurements of the model obtained from CT images, it was determined that while there was a statistical difference between sexes in terms of femoral head diameter and narrowest neck width values, a difference was not found between right and left sides. Moreover, it was observed that the values belonging to men were higher than those of women [4].

It's typical that there are differences in surface area and volumes of 3D model obtained via mimics software and 3D model obtained via photogrammetric method. In mimics software, the limits of femur are determined, 3D model of the object is formed by overlapping the images the limits of which are determined. In photogrammetric method, on the other hand, the camera is calibrated, after the errors caused by the camera (e.g. distortion) are corrected, dense point cloud is formed by means of common points in the images. 3D models are obtained from this point cloud. The density of this formed point cloud is approximately 300 points per square millimeter. Moreover, the measurement sensitivity of used control points is 0.05mm.

In the production of numerical surface model aiming to represent the object with a surface that is defined mathematically, distribution and frequency of points are the most effective factors in providing the suitability of the model for the real shape of the object. From so many points (so much point density it has) a 3D surface is produced, so much it approaches to the real surface. As the number (density) of points increases, the accuracy obtained from numerical surface model also increases.

As a consequence, according to the values obtained by both methods, sexual dimorphism was observed in femur of chinchilla. The measurement values obtained by mimics program and photogrammetric methods were not statistically different except volume and surface area. This indicated that both methods can be used in anatomic and morphometric studies confidingly. Morover, 3D re-construction of rigid materials such as bones and cartilages transferred to anatomy laboratory via photogrammetric techniques may be useful for forensic medicine and/or diagnostic purposes.

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Research Article

Role of Antibiotic in Drug Resistance and Integrons Prevalence in *Escherichia coli* Isolated from Human and Animal Specimens

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Abstract

It is believed that high antibiotic consumption and horizontal transfer of resistance genes are two major causes of antibiotic resistance in bacteria. To confirm or reject this belief, we studied the role of drug administration in antimicrobial resistance (AMR), prevalence of Class I and II integrons and integron-mediated resistance in *Escherichia coli* isolated from human and animal specimens. Recording demographic data, *E. coli* from different specimens including human, chicken, cattle and sheep was isolated followed by phenotypic antibiotic susceptibility testing and detection of Class I and II integrase genes. The correlation between integrons and resistance (P value) was evaluated using SPSS software. According to demographic records, chickens received the highest dose and variation of antibiotics. As expected, the most prevalent MDR strains and integrons were found in chicken strains. Chi square analysis showed a significant correlation between integrons and resistance pattern mostly in *E. coli* strains isolated from chicken rather than other specimens. Our survey confirmed that the use of antibiotics is strongly associated with the prevalence of antimicrobial resistance and integrons in commensal *E. coli*. Such results confirm that high doses of antibiotics and selection pressure may remove susceptible intestinal microorganisms followed by resistant ones.

Keywords: *Antimicrobial resistance, Cattle, Chicken, Escherichia coli, Integrons*

İnsan ve Hayvan Örneklerinden İzole Edilen *Escherichia coli*'nin İlaç Direnci ve İntegronların Yaygınlığında Antibiyotik Rolü

Özet

Yüksek antibiyotik kullanımı ve dayanıklılık genlerinin yatay transferinin bakterilerde antibiyotik direncinin iki önemli nedeni olduğuna inanılmaktadır. Bu inancı onaylamak veya reddetmek için, insan ve hayvan örneklerinden izole edilen *Escherichia coli*'nin antimikrobiyel direnci (AMR), Sınıf I ve II integronları ve integron-aracılı direnç prevalansında ilaç uygulamasının rolünü araştırdık. Demografik verileri kaydetmek için; fenotipik antibiyotik duyarlılık testi ve Sınıf I ve II integraz genlerinin tespiti sonrası insan, tavuk, sığır ve koyun gibi farklı örneklerden *E. coli* izole edildi. İntegronlar ve direnç (p değeri) arasındaki korelasyon SPSS yazılımı kullanılarak değerlendirildi. Demografik kayıtlara göre, tavuklar en yüksek doz ve değişimli antibiyotik aldı. Beklenildiği gibi, en yaygın MDR suşları ve integronları tavuk suşlarında bulundu. Ki kare analizi, başka örnekler yerine çoğunlukla tavuktan izole edilen *E. coli* suşlarının integronları ve direnç modeli arasında anlamlı bir korelasyon gösterdi. Araştırmamız, antibiyotik kullanımının ortakçı *E. coli*'de antibiyotik direnci ve integronların yaygınlığı ile sıkı ilişkili olduğunu doğruladı. Bu sonuçlar, yüksek doz antibiyotik ve seçim baskısının dirençli olanların ardından duyarlı bağırsak mikroorganizmalarını ortadan kaldırabildiğini doğrulamaktadır.

Anahtar sözcükler: *Antimikrobiyel direnç, Sığır, Tavuk, Escherichia coli, İntegronlar*

INTRODUCTION

Antibiotics are widely used for therapy and control of bacterial infections as well as to promote growth in animals [1-4]. However, over the past decades a rapid increase in the number of antibiotic-resistant clinical isolates has been observed as well as a low rate of development and introduction of new antimicrobial agents have been occurred [5-7].

There are three principal types of antibiotic resistance, namely, intrinsic, acquired, and adaptive antibiotic resistance [8]. However, antibiotic maladministration stabilizes resistance, in other words selection pressure mediated by antibiotics can shift normal micro flora to antibiotic-

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resistant microorganisms to increase the resistance gene transferring rate [5].

Followed by taking inadequate antibiotics, the emergence of *E. coli* isolates with multiple antibiotic-resistant phenotypes, involving co-resistance to four or more unrelated families of antibiotics, has been previously reported and it has been suggested that resistance in bacterial populations may spread from one ecosystem to another by lateral gene transfer, specifically integrons [9,10]. Integrons are bacterial genetic elements that allow the shuffling of smaller mobile elements called gene cassettes; they have been termed a genetic construction kit for bacteria [11-13]. The main components of an integron are an integrase enzyme *(IntI)*, a recombination site *(attI)* and a promoter located upstream of the integration site. Integrons are involved in the evolution and spread of antibiotic-resistance genes in enteric bacteria. There are various classes of integrons [14], according to their integrases and associated cassettes [15,16]. Since many gene cassettes of integrons contain AMR genes in Gram-negative bacteria, the horizontal transfer of integrons through plasmids and transposons has been found to play an important role in the dissemination of AMR genes and the development of multiresistance [16]. As a result of the variation of antibiotics taken by human, chicken, cattle, and sheep, and selection mediated by such drugs, it is probable to observe different patterns of resistance in *E. coli* strains isolated from different specimens.

In fact, in current study the role of drug administration in AMR, prevalence of Class I and II integrons and integron mediated resistance in *E. coli* isolated from human and animal specimens were evaluated.

MATERIAL and METHODS

Ethics

Human stool (faeces) samples were collected in accordance with the bioethics organizations in Iran (including The Ministry of Health and Medical Education, Office of Study for Humanistic and Islamic Science on Medicine and Medical Ethics). For human stool samples, IRB approval was obtained from Tehran University of Medical Sciences. For animal samples, Permission was obtained from Alborz University of Veterinary Sciences and Institutional Animal Care and Use Committee (IACUC) approved this specific study. To collect samples, written information about the study was given to the owners of breeding farm and facility and Informed consent was obtained. A questionnaire including sex, age, antibiotic diet and enteric disease was filled and signed by the human participants and consent form was recorded by the authors.

Samples Collection

This study was performed from August 2015 to October 2015. The authors collected the samples from four sources: Healthy volunteer human not using any antibiotic for 2 weeks before sampling from Amini Medical Laboratory located in Alborz province, large intestine swabs from chicken, cattle, and sheep faeces. Faecal samples were collected from living animal and so no animal was sacrificed. Chicken enteric specimens were obtained from private animal breeding farm Qadir in Karaj city (suburb of Alborz province with geographic coordinate of 35.8840059, 50.9716793), while sheep and cattle enteric samples were collected from private facility Raeesi located in Zavareh city (suburb of Isfahan province with geographic coordinate of 33.449974, 52.490830).

To collect samples, oral and written information about the study was given to each human participant and owners of breeding farm and facility and an informed consent was obtained. A questionnaire about information pertaining to the sex, age, antibiotic diet and enteric disease was filled by the human Participants.

Bacterial Isolates

To isolate *E. coli*, faecal samples were inoculated to Lauryl Sulphate Tryptose (LST) Broth (Merck, Germany) broth followed by EC broth (Merck, Germany) at 44.5°C and streaked on EMB agar (Merck, Germany). Colonies showing metal sheen were considered as presumptive *E. coli* isolates and underwent IMV*i*C test for final confirmation [17].

Demographic Data Collection

While collecting faecal samples, the antibiotic consumption of each participant was recorded. According to this data, highly different patterns of antibiotics were used, among which chicken prescribed intensive antibiotic diets were including soltrim (trimetoprima sulfametoxazol), fozbac, tetracycline, doxycycline, chloramphenicol, enrofloxacin, gentamicin, furazolidone and colistin. Tetracycline, trimetoprima and penicillin had been administrated for cattle, while sheep were fed with oxytetracycline, chlortetracycline, neomycin sulphate, ceftiofur sodium and spectinomycin. However, human participants included in this study were healthy volunteers who had not taken any antibiotic for at least two weeks before sampling.

Antimicrobial Susceptibility Testing

Phenotypic antibiotic susceptibility was tested applying Pad tan Teb (Tehran, Iran) disks by Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates according to the guidelines of Clinical and Laboratory Standards Institute [18]. A panel of 24 antibiotic discs containing 7 categories listed in *Table 3-6* were tested and *E. coli* ATCC 25922 was used as quality control strain. For antimicrobial susceptibility testing, the inoculum of *E. coli* was suspended in the sterile saline solution (0.85% NaCl) with a sterile swab to adjust turbidity to match the 0.5 McFarland standards, and streaked evenly on Mueller-Hinton agar plates. Plates were

incubated inverted at 35°C for 18 h and the inhibition zone diameters were measured [18].

PCR Assay for Detection of Resistance Genes

To extract the genomic DNA, bacterial cells were centrifuged at 2.500 round per min (rpm) for 15 min. Discarding the supernatant, following manufacturer's protocol (Bioneer's AccuPrep Genomic DNA Extraction Kit), DNA from all isolates was extracted.

To detect Class I and II integrase genes, two sets of primers were designed (listed in *Table 1)* a new duplex PCR was developed to detect both genes simultaneously.

The gene amplification protocol was performed by ABI verity 96 well thermal cycler in reaction mixture at a final volume of 25 µL consisting of 12.5 µL, 2x CinnaGen PCR master kit containing *Taq* DNA Polymerase (recombinant), PCR buffer, $MgCl_2$, dNTPs, in addition to 2 µ DNA template and specific pmols of each set of primers. The temperature profile for integrase genes amplification was as follows: initial denaturation (94°C for 5 min), followed by 30 cycles of denaturation (94°C for 20 sec), annealing (30 s at 60°C), and extension (72°C for 1 min); and then a final extension (72°C for 10 min).

PCR products were evaluated by electrophoresis in 1% agarose gel containing SYBR green and visualized by a Gel DOC™ XR+ (BIORAD) and analysed by Image Lab™ 4.0 software.

Statistical Analysis

The SPSS software (version 19) was used for statistical analysis. The association between presence of integrons and antibiotic resistance was determined by $\chi 2$ or Fisher's exact test. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Specimens

Considering the questionnaires, human taking antibiotics within 2 weeks were excluded; hence 50 specimens (isolates) from 29 male and 21 female, aged 17-43 years participated in the study. In addition to human isolates, 50 faecal samples were collected from each three animal species and thus 200 isolates were included in this study.

Antimicrobial Resistance

Antibiotic susceptibility results showed no extensively drug-resistant (XDR isolate); however, 186 (93%) isolates were MDR (resistant to at least 1 category) and 14 isolates were susceptible to 1 category. Of the 200 *E. coli* isolates from different specimens, the overall rates of resistance to antimicrobial agents were higher in chicken isolates than in the others since among the isolates, chicken isolates showed the highest resistance to all categories while the number of isolates resistant to 2, 3, 4, 5 and 6 categories were 3, 9, 10, 17 and 11, respectively *(Table 2)*.

Overall results showed that the highest and the lowest resistance to cephems belongs to cephalothin and ceftazidime while for aminoglycosides, amikacin and tobramycin showed the highest and the lowest resistance, respectively *(Table 3, Table 4)*. Penicillins, were also used in this study among which piperacillin had the best anti-

Table 1. *Primers used for amplification of Class I and II integrase genes*
Tablo 1. *Sınıf I ve II integraz genlerinin amplifikasyonunda kullanılan primerler*

Gene	Sequence (5′ → 3′)	Product Size (bp)	Annealing (Temperature)
Int 1	Forward: 5′TCTCGGGTAACATCAAGG3′ Reverse: 5′GTTCTTCTACGGCAAGGT3′	287	60
Int 2	Forward: 5′ CACGGATATGCGACAAAAAGGT 3′ Reverse: 5′ GTAGCAAACGAGTGACGAAATG 3′	789	60

Table 2. *Source and number of resistant isolates to antimicrobial categories*
Tablo 2. *Antimikrobiyel kategorilerine dirençli izolatların kaynak ve sayısı*

No. of Resistant Categories	No. of Chicken Isolates	No. of Human Isolates	No. of Cattle Isolates	No. of sheep Isolates	Sum
Resistant to 7 categories	0	0	0	0	0
Resistant to 6 categories	11	0	1	1	13
Resistant to 5 categories	17	6	0	0	23
Resistant to 4 categories	10	12	7	4	33
Resistant to 3 categories	9	22	23	13	67
Resistant to 2 categories	3	10	17	20	50
Resistant to 1 category	0	0	2	12	14
Resistant to 0 category	0	0	0	0	0

microbial activity since just one chicken isolate showed resistance *(Table 5)*.

Detection of Integrons

Of the 200 *E. coli* isolates tested, 55 (27.5%) carried *Int I* and 19 (9.5%) carried *Int II* while 9 (4.5%) isolates carried both classes. Class I integrase gene was detected in 50% (25/50), 38% (19/50), 6% (3/50) and 16% (8/50) while Class II integrase gene was detected in 26% (13/50), 8% (4/50), 0% (0/50), and 4% (2/50) of chicken, human, cattle and sheep isolates, respectively. Among the isolates, 14% (7/50) of chicken and 4% (2/50) of sheep harbored both classes of integrase genes simultaneously.

To analyse the relationship between the prevalence of integrase gene and resistance, the software SPSS 19.0 was used to evaluate the *p* value of Fisher's exact test. In some strains, integron carriage significantly caused correlated resistance including Class I integron and CTX ($P<0.001$), CRO ($P<0.001$), SXT ($P<0.001$), AMC ($P=0.001$), AMP ($P=0.009$), S ($P=0.009$), NOR ($P=0.023$), NA ($P=0.027$) and LEV ($P=0.05$) resistance in chicken originated strains, SXT ($P<0.001$) in cattle originated strains, SXT ($P=0.003$) and GM ($P=0.02$) resistance in sheep originated strains and AN ($p=0.016$) resistance in human originated strains. However less prevalence of Class II integron caused less correlated antibiotic resistance including, S ($P<0.001$) resistance in chicken

Table 3. *Antimicrobial resistance of E. coli isolates from human and animals*
Tablo 3. *İnsan ve hayvanlardan izole edilen E. coli antimikrobiyel direnci*

Specimen	No. (%) of Isolates Resistant to Antimicrobial Agents						
	Cephems						
	CAZ* (30 µg)	CTX* (30 µg)	CRO* (30 µg)	CT* (30 µg)	CF* (30 µg)	CZ* (30 µg)	CN* (30 µg)
Human	1 (2%)	19 (38%)	5 (10%)	2 (4%)	47 (94%)	5 (10%)	19 (38%)
Chicken	3 (6%)	28 (56%)	3 (6%)	5 (10%)	47 (94%)	5 (10%)	14 (28%)
Cattle	0 (0%)	5 (10%)	0 (0%)	0 (0%)	50 (100%)	0 (0%)	0 (0%)
Sheep	1 (2%)	9 (18%)	0 (0%)	2 (4%)	47 (94%)	2 (4%)	18 (36%)
Total	5 (2.5%)	61 (30.5%)	8 (4%)	9 (4.5%)	191 (95.5%)	12 (6%)	51 (25.5%)

** **CAZ:** ceftazidime, **CTX:** cefotaxime, **CRO:** Ceftriaxone, **CT:** ceftizoxime, **CF:** cephalothin, **CZ:** cefazolin, **CN:** cephalexin*

Table 4. *Antimicrobial resistance of E. coli isolates from human and animals*
Tablo 4. *İnsan ve hayvanlardan izole edilen E. coli antimikrobiyel direnci*

Specimen	No. (%) of Isolates Resistant to Antimicrobial Agents					
	Aminoglycosides					
	S* (10 µg)	TOB* (100 µg)	AN* (30 µg)	K* (30 µg)	N* (30 µg)	GM* (10 µg)
Human	5 (10%)	46 (92%)	0 (0%)	3 (6%)	8 (16%)	1 (2%)
Chicken	7 (14%)	36 (72%)	0 (0%)	8 (16%)	11 (22%)	9 (18%)
Cattle	0 (0%)	41 (82%)	0 (0%)	2 (4%)	0 (0%)	0 (0%)
Sheep	2 (4%)	21 (42%)	0 (0%)	4 (8%)	8 (16%)	1 (2%)
Total	14 (28%)	144 (72%)	0 (0%)	17 (8.5%)	29 (14.5%)	11 (5.5%)

** **S:** Streptomycin, **TOB:** Tobramycin, **AN:** Amikacin, **K:** Kanamycin, **N:** Neomycin, **GM:** Gentamycin*

Table 5. *Antimicrobial resistance of E. coli isolates from human and animals*
Tablo 5. *İnsan ve hayvanlardan izole edilen E. coli antimikrobiyel direnci*

Specimen	No. (%) of Isolates Resistant to Antimicrobial Agents			
	Folate Pathway Inhibitors	Penicillins		
	SXT* (25 µg)	AMC* (20/10 µg)	PRL* (100 µg)	AMP* (10 µg)
Human	0 (0%)	16 (32%)	0 (0%)	12 (24%)
Chicken	5 (10%)	34 (68%)	1 (2%)	42 (84%)
Cattle	0 (0%)	7 (14%)	0 (0%)	10 (20%)
Sheep	1 (2%)	8 (16%)	0 (0%)	10 (20%)
Total	6 (3%)	65 (32.5%)	1 (0.5%)	74 (37%)

** **SXT:** Trimothoprim/Sulfamethoxazole, **AMC:** Amoxacillin/Klavulanic Acid, **PRL:** Piperacillin, **AMP:** Ampicillin*

Table 6. *Antimicrobial resistance of E. coli isolates from human and animals*
Tablo 6. *İnsan ve hayvanlardan izole edilen E. coli antimikrobiyel direnci*

Specimen	No. (%) of Isolates Resistant to Antimicrobial Agents						
	Quinolones				Chloramphenicol	Tetracyclines	
	NOR* (10 µg)	CIP* (5 µg)	NA* (30 µg)	LEV* (5 µg)	C* (30 µg)	TE* (30 µg)	DOX* (30 µg)
Human	7 (14%)	4 (8%)	12 (24%)	3 (6%)	7 (14%)	25 (50%)	7 (14%)
Chicken	23 (46%)	8 (16%)	40 (80%)	11 (22%)	23 (46%)	24 (48%)	20 (40%)
Cattle	2 (4%)	0 (0%)	6 (12%)	0 (0%)	6 (12%)	21 (42%)	10 (20%)
Sheep	1 (2%)	1 (2%)	2 (4%)	1 (2%)	8 (16%)	10 (20%)	13 (26%)
Total	33 (16.5%)	13 (6.5%)	60 (30%)	15 (7.5%)	44 (22%)	80 (40%)	50 (25%)

* ***NOR:*** *Norfloxacin,* ***CIP:*** *Ciprofloxacin,* ***NA:*** *Nalidixic Acid,* ***LEV:*** *Levofloxacin,* ***C:*** *Chloramphenicol,* ***TE:*** *Tetracycline,* ***DOX:*** *Doxycycline*

originated strains, SXT ($P<0.001$) resistance in cattle originated strains, K ($P<0.078$) and GM ($P<0.001$) resistance in sheep originated strains and SXT ($P=0.003$) resistance in human originated strains *(Table 6)*.

DISCUSSION

Nowadays, usage of antibiotics in farm animals is quite prevalent and widespread, and has been a typical practice of farmers all around the world [19]. The majority of drugs are fed to animals as feed additives to promote their growth in factory farms and in veterinary hospitals and as pharmaceuticals for animals. There are great amounts of data suggesting that consistently administering antibiotics to farm animals has caused an increase in the antibiotic-resistant bacteria especially in human [20]. Bacteria in the human microbiome can learn how to resist against more drugs because human are exposed to slight amounts of antibiotics than animals [21,22].

To check the role of drugs in bacterial resistance, we chose a common bacterium exposed to different antibiotics in different specimens and tested the resistance both phenotypically and genetically. Our results demonstrated that chicken isolates were highly resistant to antimicrobial agents commonly used as feed additives or therapeutics. In other words, chicken which were prescribed soltrim (trimetoprima-sulphametoxazol), fozbac, tetracycline, doxycycline, chloramphenicol, enrofloxacin, gentamicin, furazolidone and colistin showed high resistance almost to all antibiotics specifically CTX (56%), CF (94%), TOB (72%), NOR (46%), NA (80%), AMC (68%), AMP (84%), C (46%), TE (48%) and DOX (40%) and higher integrons (Class I and II) prevalence rather than other sources *(Table 6)*.

Regarding the results listed in *Table 3*, considerable resistance to cefotaxime, kanamycin, gentamicin, sulpha-methoxazole/trimethoprim, norfloxacin, ciprofloxacin, nalidixic acid, levofloxacin, amoxicillin-clavulanate, ampicillin, chloramphenicol among *E. coli* isolates of chicken origin can be observed in comparison to other origins. Besides the above-mentioned results, the chicken was the only source, which took SXT and Gentamicin. It can be observed that 10% and 18% of the chicken-derived isolates were resistant to these agents, which urges the hypothesis of antibiotic mediated resistance. Just 5 chicken and 1 sheep isolates showed resistance to folate pathway inhibitors, suggesting that SXT is an efficient antimicrobial agent. Like other categories, resistance to quinolones was more prevalent in chicken isolates, while ciprofloxacin had the most efficiency and nalidixic acid proved to be a weak antibiotic.

Our results confirm Kang *et al.*[23] survey in which commensal *E. coli* strains isolated from enrofloxacin and norfloxacin medicated chicken were compared to *E. coli* from swine which were not fed by the mentioned above agents, whereby they found the *E. coli* of chicken origin to be much more resistant than the swine ones.

In addition, Ojeniyi [24] tested 3444 *E. coli* isolates from battery hens (received antibiotic) and 2284 isolates from free-range (antibiotic free) chickens and found all isolates from battery hens as MDR, while no free-range chicken was MDR.

Van den Bogaard *et al.*[25] followed a similar study; they compared the AMR of laying hens (which were seldom given any antibiotics), broilers and turkey (with high dose drug). Finally they found the prevalence and degree of antibiotic resistance for nearly all antibiotics tested was significantly higher in the turkey and broiler population, as compared to that of the laying hens.

Sáenz *et al.*[26] investigated AMR of 474 *E. coli* isolates recovered from animal faeces (broilers, pigs, pets, bulls, and horses), human stool (patients and healthy volunteers) and food products of animal origin. They found different patterns of AMR, for example Sáenz found high frequency of nalidixic acid, ciprofloxacin and gentamicin resistance in *E. coli* isolates from broilers (88, 38 and 40%, respectively), and from foods (53, 13 and 17%, respectively). High levels of resistance to trimethoprim-sulphamethoxazole and tetracycline have been found in *E. coli* isolates from broilers, pigs, and foods. Regarding the results, they believed

there should be a significant association between drug consumption and attributed AMR, which is in accordance with our results.

Besides the phenotype results, we gained expectable genetic results, since 50% and 26% of chicken origin isolates harboured Class I and II integrase genes, respectively, while 7 isolates carried both of them. Six of 7 isolates carrying both *int I* and *II* were resistant to 6 categories indicating the role of integrons in resistance.

As a result of universal intensive chicken antibiotic feeding, the rates of AMR and integrons prevalence are to some extent similar. For example Cavicchio *et al*.[27] isolated 299 *E. coli* from avian source and found 49.8% of the isolates carried Class I and 10.4% carried Class II.

Ponce-Rivas *et al*.[28] studied *E. coli* isolates from chicken litter and found Class I integron genes in 52.63% of the isolates, which is compatible to our results. The results of these mentioned studies are in accordance to our investigation. Vasilakopoulou *et al*.[29] evaluated the prevalence of Class I integron in *E. coli* of poultry and human origin and they found the integron carriage rate for poultry isolates was 49.2%, for hospital isolates was 26.2% and for healthy people was 11.1%. Vasilakopoulou survey confirms our results (poultry integron rate) and shows a difference between AMR of hospitalized and healthy people which can be attributed to drug consumption by hospitalized patients.

Kang *et al*.[23] conducted a project to compare the integron prevalence in *E. coli* isolates with different antibiotic patterns from poultry, clinical isolates from human, healthy human and commensal isolates from swine while he found the prevalence as follow: 44%, 33%, 23% and 13%, respectively, which means the lowest and the highest incidence of integrons belongs to healthy people and poultry, respectively.

Of course the prevalence of Class I integron investigated by Oosterik *et al*.[30] is half of our report since they found *int I* gene just in 21.6% of *E. coli* isolated from poultry faeces; however, in their report there is no demographic record of antibiotic prescription to explain such difference.

Over several decades, to varying degrees, bacteria causing common infections have developed resistance to each new antibiotic, and AMR has evolved to become a worldwide health threat. This study showed that the use of antibiotics is strongly associated with the prevalence of AMR in commensal *E. coli*. Class I integrons were found to be widely disseminated among *E. coli* isolates from chicken with intensive antibiotic prescription and play pivotal role in resistance mediating. Regarding the demographic data, we can come to the conclusion that high doses of antibiotic make selection pressure and remove the susceptible microorganisms permitting the resistant to stay and reside, such phenomenon can be accelerated by lateral transfer of integrons.

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Competing Interests

The authors of this article declare that there is no competing interest.

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Research Article

B-mode Echotexture Analysis and Color Doppler Sonography in Canine Mammary Tumors

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Abstract

Forty-one mammary gland tumors from twenty eight bitches were used for the study. Ultrasonographic examinations of tumor masses were performed before surgical excision and a quadratic region-of-interest (ROI) was chosen randomly on B-mode tumor images for the echotexture analyses. All tumors were evaluated histopathologically after surgery. Contrast (CONT), Mean Gradient (MG), Mean Value (MV), Homogeneity (HOM), Entropy (ENTR) and Gray Value (GV) parameters were used for the texture analyses of ultrasonographic images. Ultrasonographic image characteristics were additionally evaluated by the following macroscopic patterns: tumor shape, invasion of tumor to surrounding tissue, tumor border sharpness, echogenicity of tumor, hyperechogenic artifact, anechogenic artifact, and shadow around tumor. After B-mode ultrasonographic examination, Pulsatility Index (PI), Resistive Index (RI), Peak Systolic Flow Velocity (Vmax) and Number of Color Pixel (CP) parameters were evaluated by means of color Doppler sonography. Statistical analysis of the HOM and GV parameters indicated that there was a significant difference between benign (3.10 and 1.14) and malignant tumors (1.54 and 0.57; P<0.01). Besides, a significant difference was found between images of Malignant-Mixed Tumors (MMT) and Benign-Mixed Tumors (BMT) with regard to CONT and HOM (p< 0.001). In addition, MV was significantly higher in malignant tumors in comparison to the benign cases (P<0.05). A significant negative correlation was found between tumor size and MV in malignant tumor and adenocarcinoma cases (-0.991/ P<0.05; -0.999/P<0.01, respectively). On the other hand, there was a positive correlation between tumor size and GV (0.961/P<0.05) in malignant tumors.

Keywords: *Canine, Mammary tumor, Doppler, Echotexture analysis, Ultrasound*

Köpek Meme Tümörlerinde B-Mode Ekodesen Analizi ve Renkli Doppler Ultrasonografi

Özet

Çalışma için 28 dişi köpeğe ait olan 41 meme tümörü dokusu kullanılmıştır. Cerrahi eksizyon öncesi tümörlü kitleler B-Mode ultrasonografik muayene ile incelenmiş, görüntüler digital olarak kayıt edilmiş ve ekodesen analizi için bu görüntüler üzerinde rastgele olarak dörtlü inceleme alanları (Region of Interest) seçilmiştir. Tüm tümörlü dokular, cerrahi eksizyon sonrası histopatolojik olarak incelenmiştir. Ultrasonografik resimlerin yapısal analizleri için, Kontrast (CONT), Ortalama Gradyan (MG), Ortalama Değer (MV), Homojenite (HOM), Entropi (ENTR) ve Gri Değer (GV) parametreleri kullanılmıştır. Ultrasonografik resimler ek olarak tümör kitlesi, tümör şekli, tümörün çevre dokulara invazyonu, tümör sınır keskinliği, tümörün ekojenitesi, hiperekojenik artefakt, anekojenik artefakt ve tümör etrafındaki gölgelenme gibi makroskopik parametreler açısından da değerlendirilmiştir. B-Mod ultrasonografik muayenenin ardından, renkli Doppler ile Pulzatil İndeks (PI), Rezistif İndeks (RI), Pik Sistolik Akım Hızı (Vmax) ve Renkli Piksel Sayısı (CP) parametreleri değerlendirilmiştir. İstatistiki analizler sonucunda HOM ve GV parametreleri açısından, benign (3.10 ve 1.14) ve malign (1.54 ve 0.57; P<0.01) tümörler arasında önemli farklar bulunmuştur. Ayrıca CONT ve HOM parametreleri açısından Malign Miks Tümörler (MMT) ve Benign Miks Tümörler (BMT) arasında önemli farklar (P<0.001) bulunmuştur. Ek olarak, MV malign tümörlerde, benign tümörlere göre önemli düzeyde (P<0.05) yüksek bulunmuştur. Tümör büyüklüğü ve MV arasında, malign tümörlerde ve adenokarsinomlarda önemli düzeyde negatif korrelasyon saptanmıştır (sırası ile -0.991/P<0.05; -0.999/P<0.01). Diğer yandan malign tümörlerde tümör büyüklüğü ve GV arasında pozitif korrelasyon saptanmıştır (0.961/P< 0.05).

Anahtar sözcükler: *Dişi köpek, Meme tümörü, Doppler, Ekodesen analizi, Ultrason*

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INTRODUCTION

Mammary tumors are one of the most common tumor types reported in female dogs [1,2]. Incidence of malignant mammary tumors among all mammary tumors is within a range of 41 to 68 per cent [3-6]. Histopathological examination is required for the diagnosis of tumor type [7], however B-mode and color Doppler ultrasonography were studied to discriminate between benign and malignant tumors [8]. Studies conducted with two dimensional B-mode ultrasonography showed that classifying malignant and benign mammary tumors was not possible in veterinary medicine [9,10]. In contrast, Marquardt et al.[11] compared B-mode ultrasonographic images including shape, size, anechogenic areas and echogenicity of surrounding tissue with histologic findings and determined that some ultrasonographic parameters may have an important role in classifying malignant tumors but diagnosing tumors with low malignancy might be impossible [12]. To obtain further assurance, more detailed examinations are necessary. Improvements of the differentiability of the different changes of canine mammary tumors can be achieved by refined and improved ultrasonographical examination technologies [13]. Color Doppler ultrasonography is used in order to assess tumor vascularity in human medicine for the purpose of antivascular therapy [14,15]. Studies performed in human medicine revealed a significant correlation among echogenicity, echostructure and mamma sonographic findings [16]. Echostructure analysis is carried out by examination of regions of interest (ROI) by digital B-mode ultrasonography and consecutive calculation of special parameters with a computerized programme [17].

Computed echostructure analysis was performed on acquired B-mode ultrasonographic images for diagnostics purposes in humans previously [18,19]. Garra et al.[20] and Bader et al.[16] investigated the differences between mammary tumors and other tissue types by using texture analysis and echogenicity parameters.

The objective of the present study was to compare B-mode image echostructure and color Doppler ultrasonographical analyses with histopathologic findings to figure out, whether it is possible to discriminate between benign and malignant canine mammary tumors.

MATERIAL and METHODS

Animal Grouping and Image Acquisition

Twenty eight mongrel bitches with a total of 41 mammary tumors or tumor-like lesions brought to the Clinic of Obstetrics and Gynaecology, Faculty of Veterinary Medicine, University of Ankara (TR) were used in the study. The age of the bitches was in the range of 5 to 14 years.

The clinical status of all bitches was determined according to standard procedures. The general condition of the bitches with mammary tumors was determined to be moderately or severely affected. Detailed examination of mammary glands was performed by inspection and palpation. The absence or presence of lung metastases was evaluated by thoracic radiography. The mammary masses were examined by B-mode ultrasound (Esaote AU5; 7.5 MHz, linear transducer). Mastectomy was performed under general anesthesia. The animals were premedicated with 0.045 mg/kg of atropine sulphate (Belladone®, Alke, İstanbul, Turkey) and sedated with 2 mg/kg of xylazine HCL (Alfazyne® %2, Egevet, İzmir, Turkey). Following sedation, 10 mg/kg ketamine HCL (Alfamine® %10 Egevet, İzmir, Turkey) was applicated i.v. The tumor and mammary tissue were sent to the pathology laboratory for routine processing (Department of Pathology, Faculty of Veterinary Medicine, University of Ankara, Tr). Tissue specimens were immediately fixed in formalin (10%) and were embedded in paraffin using standard techniques as described by Luna, (1968) [21]. Tissue sections (5-6 µm) were cut and stained with haematoxylin-eosin (HE). Canine mammary tumors were classified as benign and malignant tumors according to Moulton [22]. Malignant tumors were divided into two groups: Adenocarcinomas and malignant mixed tumors (MMT).

B-mode ultrasonographical images were inspected macroscopically according to the method described by Marquardt et al.[12] and Gonzalez de Bulnes et al.[23]. Tumor shape (regular or irregular), invasion of tumor to surrounding tissue (clear or not clear), tumor border sharpness (sharp or not sharp), echogenicity of tumor (hypoechogenic, hyperechogenic, anechogenic, or mixed), echo display of tumor (homogeneous or heterogeneous), hyperechogenic artifact (present or absent), anechogenic artifact (present or absent), and shadow around tumor (present or absent) were examined.

Tumor dimensions were divided into three groups by using a formula for an area of an ellipse (AE; 3.14 X a/2 X b/2; a= Longer axis; b= Shorter axis) [24] on ultrasonographical images: Group 1 = ≤ 200 mm^2, group 2 = > 200 mm^2 - ≤ 500 mm^2, group 3 = > 500 mm^2

Analysis of ultrasound images was performed using a series of custom-developed computer algorithms optimized for ultrasonography (Synergyne©, Version 2.8, WHIRL, Saskatoon, Sask., Canada) on a Sun Sparc Station 20 computer (Sun Microsystems, Mt. View, CA, USA). Digitized images of mammary tumors were divided into four equal quadrants. On each quadrant, a quadratic region-of-interest (ROI) was chosen randomly on B-mode tumor images and echotexture analyses were done using a customized program (PEPE v1.0, German Cancer Research Center, Heidelberg, Germany) as described by Schmauder et al.[25]. For the computer-assisted analysis of B-mode imaging, echotexture of the tissues using the parameters mean gray level (MGL), mean gradient (MG), homogeneity (HOM), entropy (ENTR), contrast (CONT) and gray value (GV) was evaluated *(Fig. 1)*. These parameters were defined

by Allison et al.[26] and Moss et al.[27] as: *Mean Gray Level* (Arithmetical average grey level of all pixels in picture, defines the brightness), *Mean Gradient* (Variations in grey values of neighbor pixels, defines microtexture of sample), *Homogeneity* (Uniformity of grey value combination of neighbor pixels in defined matrix, defines either micro- or macrotexture of sample), *Entropy* (A measure of the uniformity of matrix values), *Contrast* (A measure of how many large grey-level differences are present in the ROI), *Gray Value* (The brightness of pixels in a digitized image).

The largest sections of tumors were visualized and measured on B-mode ultrasonography for the best evaluation. The assessment of material was done according to number of tumors as some dogs suffered from more than one mass. Computer-assisted analyses were therefore performed on 286 ROI (regions of interest) from malignant, 48 ROI from benign and 118 ROI from adenocarcinomas of 86 tumor images from 28 bitches. A total of 14 bitches were examined by power Doppler due to vascularization of masses and parameters of RI, PI and Vmax (n=21) were investigated. Moreover, 58 Color Doppler images were obtained from 28 dogs by monitoring the highest colorful blood velocities.

The blood flow of tumor vessels was quantified by the Doppler pulsatility index (PI), resistive index (RI) and peak systolic flow velocity (Vmax) (Esaote AU5; 7.5-10 MHz, linear transducer) *(Fig. 2)*. While PI is defined as (peak systolic velocity-end diastolic velocity)/time averaged velocity, RI is defined as (peak systolic velocity - end diastolic

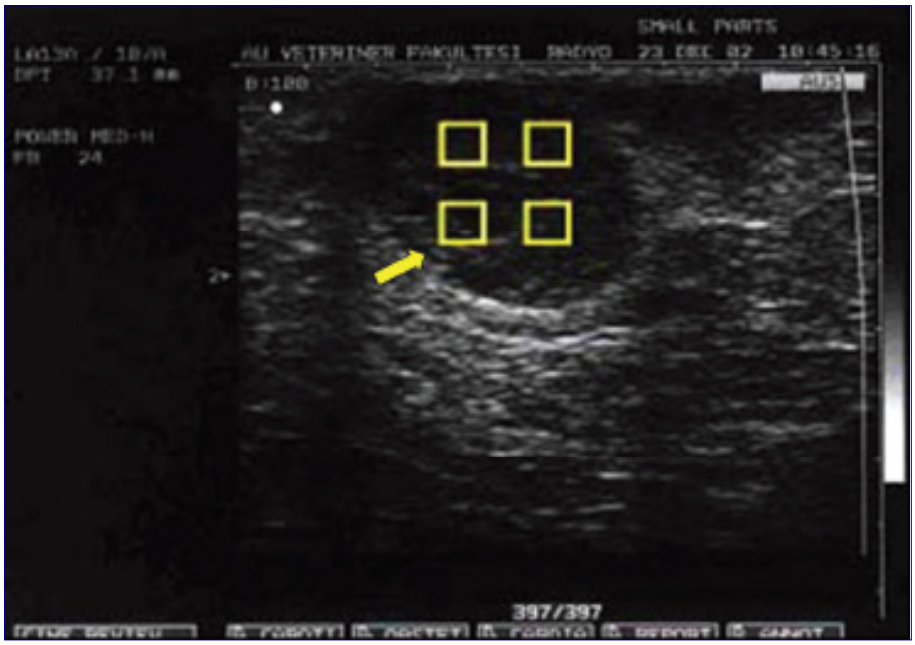
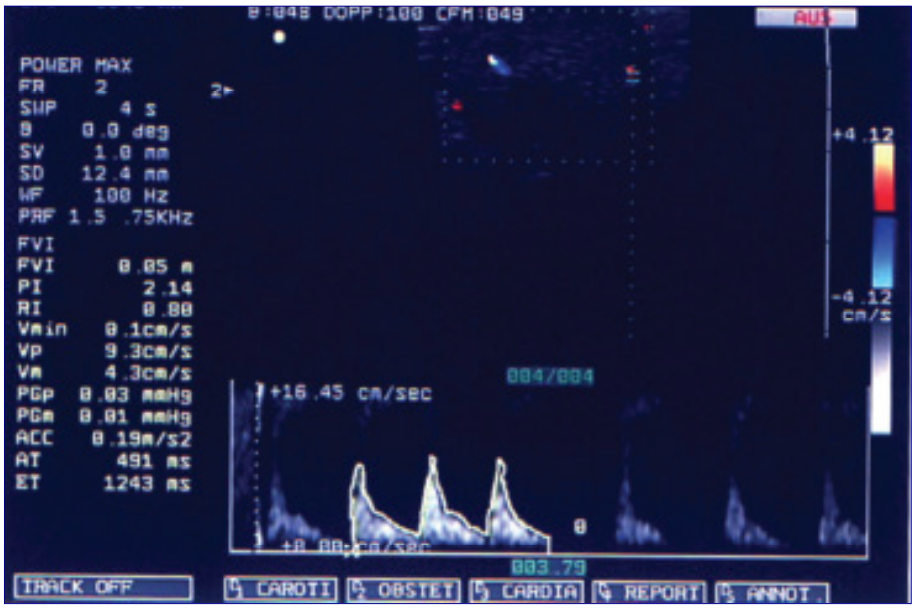


Fig 1. Computer-assisted analysis of B-mode imaging

Şekil 1. B-Mod görüntülemede bilgisayar destekli analiz

Fig 2. The blood flow analysis of a tumor vessel by color Doppler

Şekil 2. Tümör kan damarında renkli Doppler ile kan akış analizi



velocity)/peak systolic velocity [8]. The vascularization of the mammary tumors was visualized in Color-Angio-Mode and quantified using a computer program (Adobe Photoshop software 5.0, Adobe Systems) to determine the number of color pixels [28].

Statistical analysis was performed by SPSS® (Version 17.0, SPSS Inc, Chicago, USA). All data are given as means ± standard deviation descriptive statistic; the normality and homogeneity of variances were assessed for all variables tested by means of a "Shapiro-Wilks" test and "Bartlett-Box"test. For normally distributed data, differences between groups were compared using "one-way ANOVA". For not normally distributed data, the "Mann–Whitney-U" test was used for comparison between two groups. "Kolmogorov-Smirnov Z-test" was used for the difference between more than two groups.

RESULTS

The results of histopathological examination and numbers of tumors are given in *Table 1*.

Based on the histopathological diagnosis, the tumors were divided into 2 groups: Benign tumors (n=5) and malignant tumors (n=36). A total of 286 regions-of-interest (ROI) from malignant tumors and 48 ROI from benign tumors were obtained. Echotexture parameters are represented on *Table 2*.

Significant differences in echostructure parameters between the different groups of tumors were found *(Table 2)*. HOM, GV and MV were significantly higher in malignant than in benign tumors (P<0.01 and P<0.05). Similarly in MMT, the average CONT and HOM were significantly higher than in BMT (P<0.001 each), and among the malignant tumors, adenocarcinomas had significantly higher GV than the MMT (P<0.001). But there was no significant difference between tumor types concerning the Doppler parameters (P>0.05) *(Table 3)*.

In *Table 4*, the relation between malignant tumor sizes and echostructure parameters is given.

In malignant cases, a negative correlation was found between tumor size and MV (r=-0.995; P<0.05), and a positive correlation between tumor size and GV (r=0.961, P<0.05) *(Table 4)*. No significant difference was calculated when the average values were compared between groups. In *Table 5*, the relation between tumor size of AC and echostructure parameters is given.

In adenocarcinoma cases, a negative correlation was determined between tumor size and MV (r= -0.999; P<0.01), whereas in benign tumors, there was no significant correlation between tumor size and any echostructure parameters.

Table 1. *Postoperative histopathological diagnosis and numbers of mammary tumors*
Tablo 1. *Postoperatif histopatolojik teşhis ve meme tümörü sayısı*

Tumor Type	Benign Tumors	Malignant Tumors	
		Adenocarcinomas	Malignant Mixed Tumors
Benign mixed tumor	4		
Fibro-mixo-lipo adenoma	1		
Tubular adenocarcinoma		2	
Tubulopapillary adenocarcinoma		1	
Complex adenocarcinoma		1	
Papillary cystic adenocarcinoma		3	
Solid adenocarcinoma		2	
Malignant mixed tumor			27
N	5	9	27

Table 2. *Echostructure analysis of regions of interest (ROI) on B-Mode images of malignant, benign, malignant and benign mixed tumors.*
Tablo 2. *Malign, benign, malign ve benign karma tümörlere ait B-Mod resimler üzerindeki inceleme alanlarının (ROI) ekodesen analizi*

Tumor Pathology	CONT (X ± SD)	MG (X ± SD)	MV (X ± SD)	HOM (10^{-3}) (X ± SD)	ENTR (X ± SD)	GV (X ± SD)
Malignant (N=286)	98.43±65.10	31.76±12.60	77.39±46.27	3.10±2.08	2.65±0.49	1.14±1.06
Benign (N=48)	162.72±154.62	27.58±18.08	65.28±44.78	1.54±1.25	2.41±0.69	0.57±0.46
P	>0.05	>0.05	<0.05	<0.01	>0.05	<0.01
MMT (N=168)	101.11±67.46	31.73±12.33	75.74±43.04	3.15±2.98	2.64±0.43	0.70±0.58
BMT (N=48)	162.72±154.62	27.58±18.08	65.28±44.78	1.54±1.25	2.41±0.69	0.57±0.46
P	<0.001	>0.05	>0.05	<0.001	>0.05	>0.05
MMT (N=168)	101.11±67.46	31.73±12.33	75.74±43.04	3.15±2.98	2.64±0.43	0.70±0.58
AC (N=118)	94.61±61.68	31.80±13.04	79.74±50.60	2.42±2.32	2.66±0.57	1.49±1.41
P	>0.05	>0.05	>0.05	>0.05	>0.05	<0.001

***MMT:** Malignant mixed tumor, **BMT:** Benign mixed tumor, **AC:** Adenocarcinoma, **CONT:** Contrast, **MG:** Mean Gradient, **MV:** Mean Value, **HOM:** Homogeneity (X 10^{-3}), **ENTR:** Entropy, **GV:** Gray Value (X 10^{-3}); P<0.05 indicates statistically significant difference*

Table 3. *Relationship between tumor types and Doppler parameters (PI, RI, Vmax, CP)*
Tablo 3. *Tümör tipi ve Doppler parametreleri (PI, RI, Vmax, CP) arasındaki ilişki*

Tumor Type	PI (X ± SD) (n)	RI (X ± SD) (n)	Vmax (X ± SD) (n)	CP (X ± SD) (n)
Malignant	1.85±0.30 (16)	0.68±0.12 (16)	7.15±4.34 (16)	64910.53±51431.88 (51)
Benign	1.83±0.34 (5)	0.63±0.10 (5)	6.64±2.76 (5)	62386.69±52217.46 (13)
P	>0.05	>0.05	>0.05	>0.05
MMT	1.83±0.34 (11)	0.69±0.13 (11)	6.53±3.50 (11)	65718.72±39947.3 (32)
BMT	1.83±0.34 (5)	0.63±0.10 (5)	6.64±2.76 (5)	62386.69±52217.46 (13)
P	>0.05	>0.05	>0.05	>0.05
MMT	1.83±0.3 (11)	0.69±0.13 (11)	6.53±3.50 (11)	65718.72±39947.3 (32)
AC	1.93±0.23 (5)	0.67±0.13 (5)	8.52±6.06 (5)	207293.30±234725 (19)
P	>0.05	>0.05	>0.05	>0.05

PI: *Pulsatility index,* ***RI:*** *Resistive index,* ***Vmax:*** *Maximum systolic flow (cm/s),* ***CP:*** *Number of color pixel;* ***MMT:*** *Malignant mixed tumor,* ***BMT:*** *Benign mixed tumor,* ***AC:*** *Adenocarcinoma; P<0.05 indicates statistically significant difference*

Table 4. *Relationship between malignant tumor sizes and echostructure parameters*
Tablo 4. *Malign tümör büyüklükleri ve ekodesen parametreleri arasındaki ilişki*

Groups	MV (X ± SD)	MG (X ± SD)	CONT (X ± SD)	HOM (x10^{-3}) (X ± SD)	ENTR (X ± SD)	GV (X ± SD)
Group 1 (n=56); ≤200 mm^2 106.53±52.59	88.28±40.76	32.27±9.81	117.73±70.15	4.95±4.54	2.68±0.30	0.50±0.25
Group 2 (n=48); > 200 mm^2 ≤ 500 mm^{2}303.30±58.64	78.12±41.84	30.70±13.21	90.76±60.70	3.64±3.48	2.54±0.48	0.52±0.33
Group 3 (n=64); > 500 mm^2 760.84±398.25	62.36±43.27	31.71±13.80	89.80±68.03	7.30±7.01	2.51±0.45	1.52±1.51
R	-0.995; P< 0.05	-0.133; P< 0.05	-0.750; P> 0.05	0.791; P> 0.05	-0.734; P>0.05	0.961; P< 0.05

n: *number of B-mode images of each tumor;* ***AE:*** *area of an ellipse (mm^2);* ***CONT:*** *Contrast,* ***MG:*** *Mean Gradient,* ***MV:*** *Mean Value,* ***HOM:*** *Homogeneity (X 10^{-3}),* ***ENTR:*** *Entropy,* ***GV:*** *Gray Value (X 10^{-3}); P<0.05 indicates statistically significant difference*

Table 5. *Relationship between adenocarcinoma tumor sizes and echostructure parameters*
Tablo 5. *Adenokarsinom tümör büyüklükleri ve ekodesen parametreleri arasındaki ilişki*

Groups	MV (X ± SD)	MG (X ± SD)	CONT (X ± SD)	HOM (x10^{-3}) (X ± SD)	ENTR (X ± SD)	GV (X ± SD)
Group 1 (n=18); ≤200 mm^2 128±29.82	147.40±35.55	38.58±4.64	109.27±22.95	1.93±1.24	2.84±0.13	0.75±0.22
Group 2 (n=8); > 200 mm^2 ≤ 500 mm^2 386.3±0.0	137.41±20.18	43.81±2.58	197.60±33.84	1.01±0.43	3.08±0.05	2.61±0.64
Group 3 (n=92); > 500 mm^2 1830.27±677.24	61.49±38.60	29.43±13.66	82.78±59.90	1.87±1.68	2.50±0.60	1.53±1.51
R	-0.999; P<0.01	-0.873; P>0.05.	-0.568; P>0.05	0.319; P>0.05	-0.844; P>0.05	0.048; P>0.05

AE: *area of an ellipse (mm^2);* ***CONT:*** *Contrast,* ***MG:*** *Mean Gradient,* ***MV:*** *Mean Value,* ***HOM:*** *Homogeneity (X 10^{-3}),* ***ENTR:*** *Entropy,* ***GV:*** *Gray Value (X 10^{-3}); P<0.05 indicates statistically significant difference*

Results of the comparison between benign and malignant tumors according to macroscopical B-mode image evaluation are given in *Table 6*.

Macroscopical evaluation of B-mode ultrasonographic images revealed no significant differences between benign and malignant tumors (P>0.05).

DISCUSSION

Mammary tumors are one of the most common neoplasms in bitches. Reports indicate that the incidence of mammary neoplasms comprises a range of 22.9 to 52 per cent of all canine tumors [2,29]. Histopathological examination is essential to exhibit criteria of tumor dignity. Nevertheless there is no consensus on classification of canine mammary tumors due to variety of mammary tumors, though there are many proposals for histological and histogenetical classification [7,30,31]. Researchers mostly use the classification of canine and feline mammary tumors prepared by the World Health Organization (WHO) and adapted from human tumor classification systems [32,33]. However, the fact that myoepithelial cells in canine mammary tumors contribute to neoplastic proliferation

Table 6. *Comparison of benign and malignant tumors according to macroscopical B-mode image evaluation*
Tablo 6. *Makroskopik B-Mode resim değerlendirmesine göre, benign ve malign tümörlerin karşılaştırılması*

USG Appearance	Parameters B-mode Pictures	Benign (n=12) n; (%)	Malignant (n=74) n; (%)	P
Tumor shape	Regular	12/6 (50)	74/37(50)	>0.05
	Irregular	12/6 (50)	74/37 (50)	>0.05
Invasion of tumor to surrounding tissue	Clear	12/4 (33.3)	74/32 (43.2)	>0.05
	Not clear	12/8 (66.7)	74/42 (56.8)	>0.05
Tumor border sharpness	Sharp	12/9 (75)	74/50 (67.6)	>0.05
	Not sharp	12/3 (25)	74/24 (32.4)	>0.05
Echogenicity of tumor	Mixed	12/8 (66.7)	74/48 (64.9)	>0.05
	Hypoechogenic	12/2 (16.7)	74/13 (17.6)	>0.05
	Anechogenic	12/0 (0)	74/5 (6.8)	>0.05
	Hyperechogenic	12/2 (16.7)	74/8 (10.8)	>0.05
Structure of tumor	Homogeneous	12/4 (33.3)	74/26 (35.1)	>0.05
	Heterogeneous	12/8 (66.7)	74/48 (64.9)	>0.05
Hyperechogenic artifact	Existent	12/1 8.3)	13 (17.6)	>0.05
	Absent	12/11 (91.7)	61 (82.4)	>0.05
Anechogenic artifact	Existent	12/3 (25)	10 (13.5)	>0.05
	Absent	12/9 (75)	64 (86.5)	>0.05
Shadow existence around tumor	Existent	12/0 (0)	4 (5.4)	>0.05
	Absent	12/12 (100)	70 (94.6)	>0.05

USG *= ultrasonographical*

differs canine tumors from tumors of other animals and humans [34]. In this study, pathological findings including tubular adenocarcinoma, tubulopapillary adenocarcinoma, solid adenocarcinoma and malignant mixed tumor were evaluated as malignant tumors, and fibro-myxo-lipo adenomas as benign tumors. Since the aim of the study was to create a supportive method which might help to make a decision for surgery, malignant and benign tumors were compared, as well as malignant and benign mixed tumors. Besides tumors diagnosed as tubular adenocarcinoma, tubulopapillary adenocarcinoma and complex mammary adenocarcinoma were classified under the heading of "adenocarcinoma".

Histopathological examination to diagnose mammary gland tumors is obligatory [2]. Nevertheless, other diagnosis techniques should be taken into consideration [6,35].

In human studies, differences between mammary tumor and other types of tissues (necrosis in adipose tissue, proliferative mastopathies and cysts) detected by means of B-mode echotexture analysis were reported [16,20]. Tumors were classified as benign or malignant tumors dependant on the appearance of tissues. Because of this differentiation, a significant decrease in the number of breast biopsies was achieved [20].

In veterinary medicine, computer-assisted texture analysis programs were developed to evaluate changes in the ovarium and endometrium during the estrous cycle [25,36]. Morphological and echotexture attributes were correlated with CL function, and the luteal tissue heterogeneity correlated to circulating progesterone concentrations [37].

Results of the present study indicate that based on homogeneity and gray value, computer-assisted texture analysis may be a helpful diagnostic method to differentiate benign from malignant tumors among the here investigated tumor types. Homogeneity defines the level of uniformity in ROIs on B-Mode ultrasound images. Presence of lower gray value combination accompanied with equal distribution means an increase in homogeneity, the contrary means a decrease [38]. Due to the presence of bone, cartilage and fatty tissues in benign mixed tumors [7], relative high gray value combination occurs on B-Mode ultrasound images that appears to be more heterogeneous. However, lower gray value combination and more homogeneous distribution on ultrasound images were observed when malignant tumor types were evaluated without further categorization, since a malignant mixed tumor is formed by mesenchymal or epithelial components; this might coincide with cyst formation in cases of papillary cystic adenocarcinoma, in case of solid carcinoma little stroma is present [30,33]. Some studies in human medicine demonstrated that fibrocystic lesions, fat necrosis and cystic structures can be differentiated from malignant tumors by using echostructure analysis [16].

Previous studies demonstrated a correlation between canine mammary tumor size, tumor type and prognosis [8,39]. Nyman et al.[8] reported that malignant tumors were larger than benign ones. In the present study, no correlation between echostructure parameters and the size of benign tumors was found, however, between MMT and adenocarcinomas, echostructure parameters based on tumor size differentiated. Therefore, the fact that echostructure parameters do not change in larger masses might be a characteristic for diagnosis of benign tumors.

In this study, in malignant tumors, the mean value parameter decreased with increasing size of tumors, whereas the gray value increased. Mean value parameters in adenocarcinomas showed similar changes. In benign tumors, no correlation was found between tumor size and echotexture parameters, therefore particularly mean value and gray value might be important parameters to differentiate benign from malignant tumors.

Rapid increase in canine mammary tumor size may be a malignancy criterion [22,40]. This feature is similar to human mammary tumors [41,42]. Decrease in mean value parameters is observed together with a decrease in image brightness. Mean gray value parameters varied by tumor size and resolution of images [43,44] that leads to a non-homogenous appearance. Rapidly grown malignant tumors (adenocarcinoma, sarcoma) are reported to have morphologically irregular surfaces with a bluish color and nodular composition on palpation contrary to histologically benign tumors (adenoma, fibroma, mixed tumors) [45]. Schoenrock [46] reported that the incidence of spongy nodules or spongy smooth structures was 10 per cent. These clinical findings explain the heterogeneous echostructure appearance of malignant tumors.

The results of the present study obtained from both Doppler parameters and morphological analysis of ultrasonographical images demonstrated that no differences were found between malignant and benign tumors ($P>0.05$). It has been reported in some studies that ultrasonographical noninvasive methods were unable to provide useful information for differentiation between benign and malignant tumors [9,10,13]. On the other hand Marquardt et al.[11] indicated that shape, relation with surrounding tissue, echogenic rim, internal echogenicity, internal echographic pattern, posterior acoustical enhancement, sound attenuation, shadowing were important criteria for the evaluation of canine mammary gland tumors. Marquardt et al.[12] reported that the percentage of preoperative accurate diagnosis was 77.4 in malignant tumors, and 91.9 in benign tumors. Nyman et al.[8] demonstrated that echogenicity, tumor border shape, acoustical shadowing, number of vessels to the tumor and the total vascular flow were important diagnostic criteria for discrimination between malignant and benign canine mammary tumors. Bastan et al.[47] reported that tumor size, shape, border irregularity, echotexture, internal echogenicity and acoustic transmission parameters were useful for evaluation of canine mammary gland tumors. Different results of many studies performed by visual analysis of B-mode ultrasonographical images may be due to ultrasonographical technique, categorization, the number of material, individual differences in evaluation. However, the facts that medullar carcinomas might be comprehended as benign tumors [48,49], and sonographical dorsal or ventral shadowing were missed by certain authors [50,51] show that B-mode ultrasonography does not provide enough information on tumor characteristics.

In humans, radical mastectomies remained the standard until the 1970s, when a new understanding of metastasis led to perceiving cancer as a systemic illness as well as a localized one, and more sparing procedures were developed that proved equally effective. Mammary tumours, clinically not yet apparent, were sometimes not detected by ultrasonographical examination. The demonstration of primary multiplicity of canine mammary tumours via ultrasound is only possible for already clinically diagnosed tumours. A final diagnosis of the primary multiplicity in an early stage is only possible when using histological examination. A differentiation between benign and malign tumours was not possible with ultrasonographical examination. An exact diagnosis could only be made by histological examination [13]. Our findings revealed that tumor shape, invasion of tumor to surrounding tissue, anechogenic artifact, hyperechogenic artifact and other parameters were ultrasonographically detectable in both malign and benign tumors, therefore these parameters proved not to be useful for differentiation between benign and malign mammary tumors.

Studies with color Doppler sonography showed remarkable differences in peak systolic flow velocity between malignant and benign tumors [52,53]. In a study conducted in humans [54], preoperatively carcinoma diagnosis with color pixel intensity was achieved in 60% of cases and accurate diagnosis was possible in 91.9%. However, carcinoma diagnosis was correct in 92% and diagnosis was accurate in 78% of cases when analyzed by color Doppler sonography. There was no difference concerning microvasculature structures between non-metastatic malignant and benign canine tumors [55]. Both B-Mode ultrasonography and color Doppler sonography techniques were inadequate to diagnose tumor characteristics [56]. In practice, there are some structural differences between human and canine mammary tumors. In human medicine, epithelial-myoepithelial carcinoma tumor types and benign-malignant canine myoepithelial proliferations do not appear [57]. The ineffectiveness in tumor differentiation by using ultrasonography and color Doppler presumably might be due to this anatomical and histological diverseness of canine mammary tumors.

In conclusion, these results demonstrate that it is possible to differentiate between benign and malignant

tumors by means of echostructure analysis, whereas B-mode or Doppler ultrasonography techniques are insufficient to distinguish malignancy from benignity in canine mammary tumors. Furthermore, echostructure analysis corresponding to B-mode image acquisition might be an oncoming perspective.

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Short Communication

Complete Genome Sequence of a Novel Duck Parvovirus Isolated in Fujian, China

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Abstract

In the present study, we sequenced and analysed the complete genomes of a novel duck parvovirus (NM100) isolates derived from Muscovy ducks in Fujian, Southeast China. According to the phylogenetic analysis, based on the complete genome and VP1 gene showed that novel duck parvovirus strain NM100 belong to the MDPV clusters, whereas the VP3 gene showed that strain NM100 belong to the GPV clusters. Two putative genetic recombination events were detected using similarity plots analysis. These findings suggest that a novel duck parvovirus circulating in Muscovy duck flocks with recombination in nature, which enable us to understand the molecular characteristics and evolutionary diversity of waterfowl parvoviruses.

Keywords: *Duck parvovirus, Recombination, Phylogenetic analysis*

Çin'in Fujian Bölgesinde İzole Edilen Yeni Bir Ördek Parvovirus'unun Tüm Genom Sekansı

Özet

Bu çalışmada, Fujian, Güneydoğu Çin'de Muscovy ördeklerinden türetilmiş yeni bir ördek parvovirus izolatlarını (NM100) sıralayıp tam genomlarını analiz ettik. Tam genom ve VP1 genine dayalı filogenetik analize göre, yeni ördek parvovirus suşu NM100'ün MDPV kümelerine ait olduğunu, oysa VP3 geni, NM100 suşunun GPV kümelerine ait olduğunu gösterdi. Benzerlik alanları analizi kullanılarak, iki olası genetik rekombinasyon olayları tespit edildi. Bu bulguların, doğada Muscovy ördek sürülerinde rekombinasyon yoluyla dolaşan yeni ördek parvovirusu olduğu değerlendirilmektedir ki, bunlar da su kuşu parvovirusunun moleküler özelliklerini ve evrimsel çeşitliliğini anlamamızı sağlar.

Anahtar sözcükler: *Ördek parvovirus, Rekombinasyon, Filogenetik analiz*

INTRODUCTION

Waterfowl parvoviruses can cause diseases with high mortality and morbidity to goslings and Muscovy ducklings. Genomic analysis and antibodies neutralization test revealed that the waterfowl parvoviruses could be divided into two groups: the goose parvovirus (GPV) group and Muscovy duck parvovirus (MDPV) group. GPV can cause highly contagious and fatal disease in goslings and Muscovy ducklings; whereas MDPV only cause disease with Muscovy ducklings. The MDPV is highly related to GPV, exhibiting more than 80.0% nucleotide sequence identity [1-4]. Recently, GPV were detected in swan, Cherry Valley ducks and *Anser cygnoides* in China [5-7].

The genome of GPV and MDPV are about 5.1 kb in length, single-stranded DNA and contain two major open reading frames (ORFs). The left-hand side of the genome encodes the non-structural protein, while the right-hand of the genome encodes the capsid proteins (VP1, VP2 and VP3). The VP2 and VP3 are contained within the carboxyl terminal portion of VP1, deriving from the same gene with differential splicing [1].

In this study, we isolated sequenced and analysed the complete genome of a novel duck parvovirus strain NM100. Derivation of the genomic sequences of novel duck parvovirus strain NM100 implied that the virus had two putative genetic recombination events with

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recombination in nature between MDPV and GPV, which provides insights with the genome characterization and aetiology for waterfowl parvoviruses circulating in China.

MATERIAL and METHODS

Case History

A commercial Muscovy duck flock was experienced elevated mortality associated with typical MDPV syndromes, such as locomotory dysfunctions, weight loss, buccal respiration, and watery ocular discharges, at the spring of 2012 in Fujian, China. Most of the sick Muscovy ducklings were younger than 21-day-old and the mortality was nearly 45%.

Virus Isolation and Nucleic Acid Extraction

The virus (designated NM100) was isolated described previously [7], using 10-day-old Muscovy duck embryos by suspension into the allantoic cavities. All Muscovy duck embryos were collected from commercial Muscovy duck farms, which had no previous history with MDPV or GPV infections, and no MDPV or GPV vaccines used before. The virus was harvested after three passages of infected Muscovy duck embryos. Five Muscovy duck embryos were used as control in order to make sure that no vertical transmission waterfowl parvoviruses were detected by the waterfowl parvoviruses universal primers described by us before [8]. Genomic nucleic acids were extracted using the Total DNA/RNA Isolation Kit (Omega Bio-Tek, GA, USA) according to the manufacture's instructions. Duck-origin viral pathogens were tested by using PCR (RT-PCR) technology, only waterfowl parvoviruses universal primers were detected positive for the isolated virus.

Genome Sequencing

The strain NM100 genome were amplified by polymerase chain reaction (PCR) according to the similar strategy described previously [4], with overlapped fragments encompassed the completely GPV and MDPV genome. The PCR products were purified and then cloned. In each case, five positive clones were randomly selected and sequenced (Sangon Biotech, Shanghai, China) to both directions using an ABI model 3730 automatic DNA sequencer (ABI, CA, USA). We connected the overlapped gene fragments into the NM100 full-length genome with software Lasergene (DNAStar, v7.1, Madison, WI, USA), and submitted to GenBank.

Genomic Characterization, Homologous Recombination Analysis and Phylogenetic Analysis

For comparative studies, the complete genome sequences of waterfowl parvoviruses strains were retrieved from GenBank *(Table 1)*. Four strains of the virus (P [10], P1 [11], PT [12,13] and D [13]), which only had the NS and VP1 gene coding region sequences isolated from Muscovy ducks in Fujian, were subjected to phylogenetic analysis. Sequence comparison and genomic homology was determined using the ClustalW method. Phylogenetic analysis was performed by MEGA 6.0 using the neighbour-joining method with the maximum-likelihood model. Bootstrap scores were generated from 1000 replicates.

Table 1. *Virus description and Genbank accession numbers for waterfowl parvoviruses sequences used in this study*
Tablo 1. *Bu çalışmada kullanılan su kuşu parvovirus dizileri için virus açıklamaları ve Genbank erişim numaraları*

Accession Number	Strain	Host	Date	Region	Reference
U25749	B	goose	1960s	Hungary	[1]
EU583390	82-0231	goose	1982	TW, China	[2]
KC178571	Y	Muscovy duck	2011	AH, China	[3]
KC478066	SHFX1201	swan	2012	SH, China	[5]
KT343253	SDLC01	cherry valley duck	2015	SD, China	[6]
KC996729	SYG61V	a	a	JS, China	[9]
KT232256	FJ01	*Anser cygnoides*	2013	FJ, China	[7]
U22967	FM	Muscovy duck	1994	Hungary	[1]
JF926697	P	Muscovy duck	1988	FJ, China	[10]
JF926698	P1	a	a	FJ, China	[11]
KM093740	MDPV-GX5	Muscovy duck	2011	GX, China	[15]
KC171936	SAAS-SHNH	Muscovy duck	2012	SH, China	[14]
JF926695	PT	Muscovy duck	1997s	FJ, China	[12,13]
JF926696	D	a	a	FJ, China	[13]
KU641556	NM100	Muscovy duck	2012	FJ, China	TS

*Anhui, **AH;** Fujian, **FJ;** Jiangsu, **JS;** Shanghai, **SH;** Taiwan, **TW**. a means the vaccine candidates not writing the host and date. **TS;** this study*

The genome recombination events were detected using the Simplot 3.5.1, the GPV vaccine strains SYG61v [9] and MDPV virulent strain FM [1] were used for detection the similarity plots analysis. A multiple comparison-corrected P-value cut-off of 0.01 was used throughout.

RESULTS

Genomic Organization

The genome of NM100 was found to be 5073 nucleotides in length. The non-structural protein (NS) encodes 627 aa (nt 518-2401), the VP1 encodes 732 aa (nt 2420-4618), the VP3 encodes 534 aa (nt 3014-4618), respectively. The inverted terminal repeats (ITRs) was found to be 387 nt in length, which was present at the 5′ and 3′ terminal ends of the genome. The complete genome sequences has been submitted to GenBank under the accession No.KU641556.

Phylogenetic Analysis

The phylogenetic tree based on the NS and VP1 gene coding region (NM100, position nt 518-4618) sequences *(Fig. 1-1)* and VP1 gene coding region (NM100, position nt 2420-4618) sequences *(Fig. 1-2)* indicate that NM100 was at the same genetic evolution clades with the Muscovy parvovirus recombinant strains (SAAS-SHNH) [14], which belonged to the MDPV and N-MDPV cluster. GPV isolates (except for GPV-PT strain and its deviated vaccine strain D) were all at the GPV genetic evolution clades.

The phylogenetic tree based on the VP3 gene coding region (NM100, position nt 3014-4618) sequences *(Fig. 1-3)* indicate that NM100 shared different genetic evolution clades with typical MDPV isolates (FM, P and P1), which belonged to the GPV and N-MDPV cluster, near to the GPV clusters rather than typical MDPV cluster. However, GPV isolates (except for GPV-PT strain and its deviated vaccine strain D) were all at the GPV genetic evolution clades and typical MDPV isolates (FM, P and P1) were at the MDPV cluster.

Sequence Comparison

The NM100 genome shared 93.7% nucleotide sequence identities with MDPV strain FM, compared with other reported MDPV isolates SAAS-SHNN and MDPV-GX5 [15], the NM100 genome shared 99.5% and 95.0% nucleotide sequence identities, respectively. Compared with GPV isolates, the NM100 genome shared 84.8%-85.9% nucleotides sequence identities, respectively.

For VP1 coding region sequences nucleotides homology analysis, the NM100 strain shared 89.1% to 89.5 0% nucleotide sequence identities with typical MDPV isolates (FM, P and P1), respectively. The NM100 strain shared 99.9% nucleotide sequence identities with SAAS-SHNN, 99.5% and 99.3% with GPV-PT and its deviated vaccine, 98.9% with MDPV-GX5. Nucleotide identities of the VP1 of GPV isolates varied between 87.9%-89.6%, respectively.

For VP3 coding region sequences nucleotides homology analysis, the NM100 strain shared 85.5% to 85.9% nucleotide sequence identities with typical MDPV isolates (FM, P and P1), respectively. The NM100 strain shared 99.8% nucleotide sequence identities with SAAS-SHNN, 99.4% and 99.3% with GPV-PT and its deviated vaccine, 98.6% with MDPV-GX5. Nucleotide identities of the VP1 of GPV isolates varied between 91.2%-93.1%, respectively.

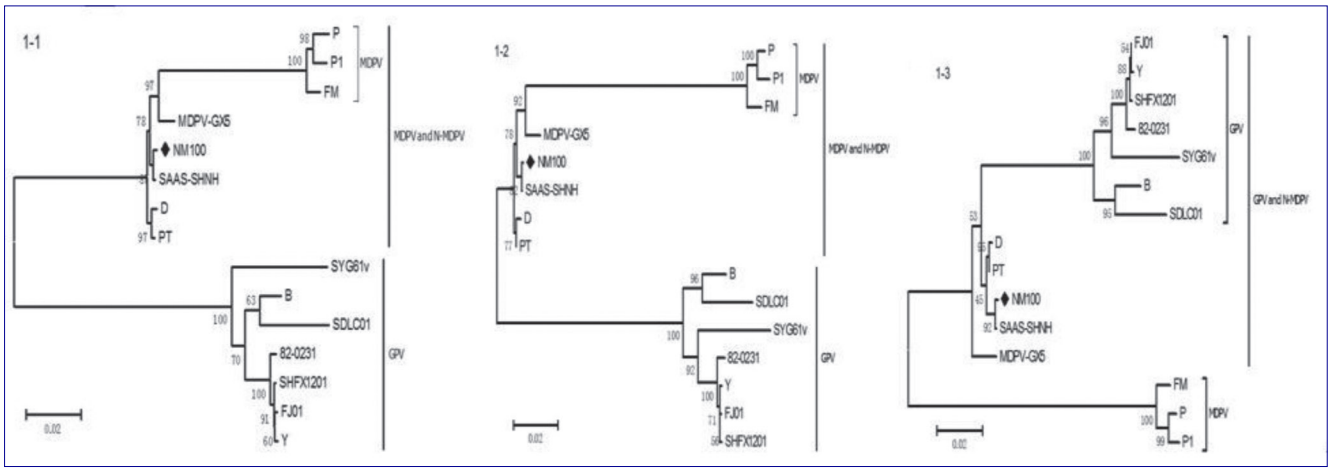

Fig 1. Phylogenetic analysis based on NS and VP1 coding region *(1-1)*, VP1 coding region *(1-2)* and VP3 coding region *(1-3)*

The phylogenetic tree shows that the novel duck parvovirus NM100 NS and VP 1coding region *(1-1)*, VP1 coding region *(1-2)* closely with MDPV clusters, whereas the VP3 coding region belonged to the GPV clusters *(1-3)*.

Şekil 1. NS ve VP1 kodlama bölgesine *(1-1)* dayalı filogenetik analiz, VP1 kodlama bölgesi *(1-2)* ve VP3 kodlama bölgesi *(1-3)*

Materyal ve Metot kısmında tarif edildiği üzere, komşu-birleştirme yöntemiyle oluşturulan ağaç. Öz yükleme puanları 1.000 tekrarla elde edildi. Filogenetik ağaç, yeni ördek parvovirusu NM100 NS ve VP kodlama bölgesini *(1-1)* ve MDPV kümelerine yakın VP1 kodlama bölgesini *(1-2)* gösterirken, VP3 kodlama bölgesi ise GPV kümelerine ait idi *(1-3)*.

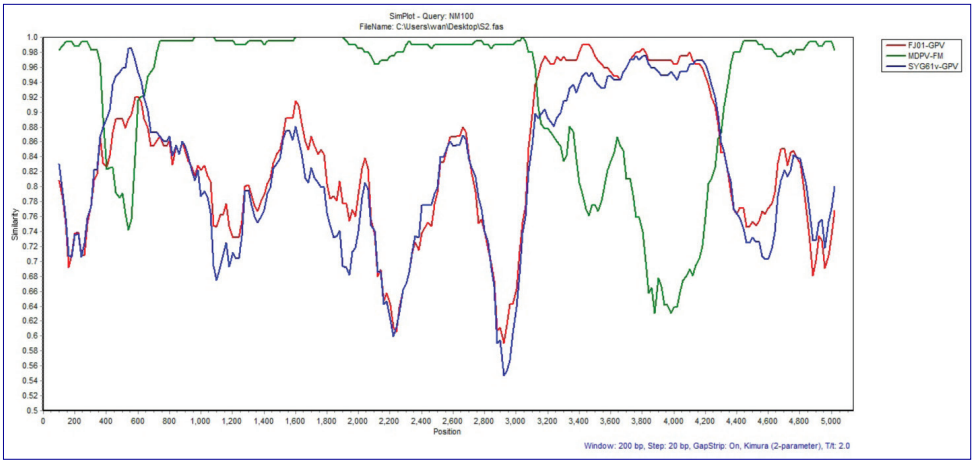

Fig 2. Similarity plots of the full-length sequence of novel duck parvovirus NM100

The schematic drawing demonstrated to denote the recombination position

The GPV virulent strain FJP01 and vaccine strain SYG61v were shown in red and blue, and MDPV virulent strain FM was shown in green. Two recombination breakpoints were observed, one is about 200 base-pair between 3′ ends of the inverted terminal repeat region and parts of 5′ ends of the NS region (position 419 to 610), and the other is nearly 1.150 base-pair (position 3.116 to 4.241) at the VP3 coding region.

Şekil 2. Yeni ördek parvovirusu NM100'ün tam-uzunluk dizisinin benzerlik çizimleri

Şematik çizim rekombinasyon konumunu belirtmek için gösterildi. GPV virulent FJP01 suşu ve aşı suşu SYG61v kırmızı ve mavi olarak, MDPV virulent FM suşu ise yeşil olarak gösterildi. İki adet rekombinasyon kesme noktası gözlendi: birisi, ters çevrilmiş terminal tekrar bölgesinin 3′ uçları ile NS bölgesinin (pozisyon 419-610) 5′ uçlarının parçaları arasındaki yaklaşık 200 baz-çifti, diğeri ise VP3 kodlama bölgesindeki yaklaşık 1.150 baz-çifti (pozisyon 4.241 ile 3.116)

Homologous Recombination Analysis

Using Simplot 3.5.1 software, two putative recombination breakpoints in the nucleotide sequences extending from nt 419 to 610 and from nt 3116 to 4241 *(Fig. 2)*. The first recombination event occurred between strain FM and the GPV strain SYG61v in the 426-611 nt region. The second recombination event occurred between strains FM and SYG61v in the 3127-4249 nt region. Strain FJ01 (GPV) used as GPV virulent control, which was isolated in Fujian, the same as strain NM100.

DISCUSSION

Previous studies had found that DNA viruses have a wide range of genome recombination, especially the parvoviruses under Family *Parvoviridae* [16]. Genus Bocaviruses has a large number of natural recombination phenomena [17]. Regarding waterfowl parvovirus genome recombination, which can change the pathogenic types, U.S. researchers have found new parvovirus strains in Muscovy ducks. The whole genome of this strain (PSU-31010) and its main coding region have not been completely established, but the homology rate between the known gene fragments and classic MDPV and GPV in this region was 84.5% and 84.6%, respectively [18]. Further, its genetic evolution tree belongs to the MDPV subset, and relates to clades different than the classic strain of the MDPV virus. Wang et al.[12] reported Muscovy duck origin GPV (GPV PT strain), which had the VP1 unique region the same features as MDPV. Recombinant waterfowl parvovirus (SAAS-SHNH) was subsequently found among Muscovy Ducks in the Shanghai area, with a genome structure the same as the MDPV genomic structure [14]. Recently, Cheery Valley duckling-origin GPV, which cause beak atrophy and dwarfism syndrome (BADS), genomic characteristics, showed that the virus is close to European GPV isolates, but separated from Asian GPV isolates, which had not reported in China before [6].

In our study, we isolated the NM100 with 10-day-old Muscovy duck embryos, the phylogenetic tree created from NS and VP1 region, the VP1 region and the VP3 region showed more evolution diversity between waterfowl parvoviruses. From the NS and VP1 region, the VP1 region, the NM100 shared closer with MDPV. Whereas, phylogenetic tree based on the VP3 region, the NM100 shared closer with GPV. Also, MDPV isolates (designated by the sequences submitter) (MDPV-GX5, GVP-PT and its deviated vaccine D) shared the same evolution phylogenetic with NM100. The two putative recombination breakpoints in the nucleotide sequences extending from nt 419 to 610 and from nt 3116 to 4241, especially the right recombination regions in the novel MDPV isolates NM100's VP3 coding region.

In summary, a novel duck parvovirus (N-MDPV), designated NM100 was obtained, sequenced, and characterized. It is clear from the multiple sequence alignments and phylogenetic analysis that NM100 represents

a distinct member of the MDPV related parvoviruses. These results suggest that recombination between GPVs and MDPVs may play significant roles in viral infectivity, host range, and pathogenicity. Further investigation of the pathogenicity of this virus on other commercial waterfowl species and the recombinant routes of this virus remain pressing questions for future research.

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Conflict of Interest

The authors declare that they have no any competing interests.

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Letter to the Editor

Echocardiographic, Magnetic Resonance Angiography and Pathologic Results of an Iliac Arterial Thromboembolism (Saddle Thrombus) in a Cat [1]

(Bir Kedide İliak Arteryal Tromboembolizmin (Saddle Trombus) Ekokardiyografik, Magnetik Rezonans Anjiografi ve Patolojik Bulguları)

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[1] *This case has been presented as poster in "15th National Congress Veterinary Surgery (1st International Turkey Veterinary Surgery Congress), Erzurum, Turkey, 11th-14th May, 2016"*

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Dear Editor,

Arterial thromboembolism (ATE) is a common mortal complication of the myocardial diseases in cats [1-3]. The different forms of the cardiomyopathy cause the ATE, and underlying cardiac etiology is the hypertrophic cardiomyopathy (HCM) in 13% of encountered cases [1,2]. Endothelial dysfunction associated with slowing down of the circulation in left atrium leads to local thrombus formation. Thrombus origins to left atrium and cause the ATE and ischemia at the bifurcation of the distal abdominal aorta, which is named as "saddle thrombus". Diagnosis of the disease usually determined based on the clinical examination results and prognosis is poor, if the ischemia localizes the hind limbs bilaterally [1,3]. Here, echocardiographic (ECHO), magnetic resonance angiography (MRA) and pathologic results of an iliac ATE in a cat were presented for veterinary practitioners in a cat.

Scottish fold breed, a 2 year-old, male cat was presented with sudden onset of bilateral hind limb paresis. Clinically, bilateral painfully hind limbs, lack of the femoral pulsation, cyanotic pulvinuses and paraparesis were detected. There was no abnormality on the radiographs of the vertebral column. Electrocardiography pointed out the sinus rhythm; however, ECHO examinations revealed the HCM *(Fig. 1)*. These results suspected the ATE; thus, a MRA was planned to investigate the distal branches of the aorta. The MRA images demonstrated that there was a narrowing abdominal aorta at the level of kidneys and the contrast medium (omnipaque 10 ml, iv.) was not progressing caudally at the level of iliac arteries *(Fig. 2)*.

Based on the ECHO and MRA results an ATE (saddle thrombus) due to HCM was diagnosed.

Medically, a therapy protocol including prednisolone (1 mg/kg, iv.), heparin (0.1 ml, iv.), ranitidine (2.2 mg/kg orally) and enalapril (2.5 mg, oral) were started daily, and aspirin (100 mg, orally) was added this protocol after a day. However, the cat died in a week even though the care and therapy regimen provided.

In the necropsy, a "Y" shaped, 3-4 cm long, fragile, heterogenic thrombus was determined at the caudal abdominal aorta, which was extending to bifurcation of the iliac arteries. It was also attached to vessels lumens *(Fig. 3a)*.

Microscopically, a fibrin thrombus attached to intima of the vessel was observed *(Fig. 3b)*.

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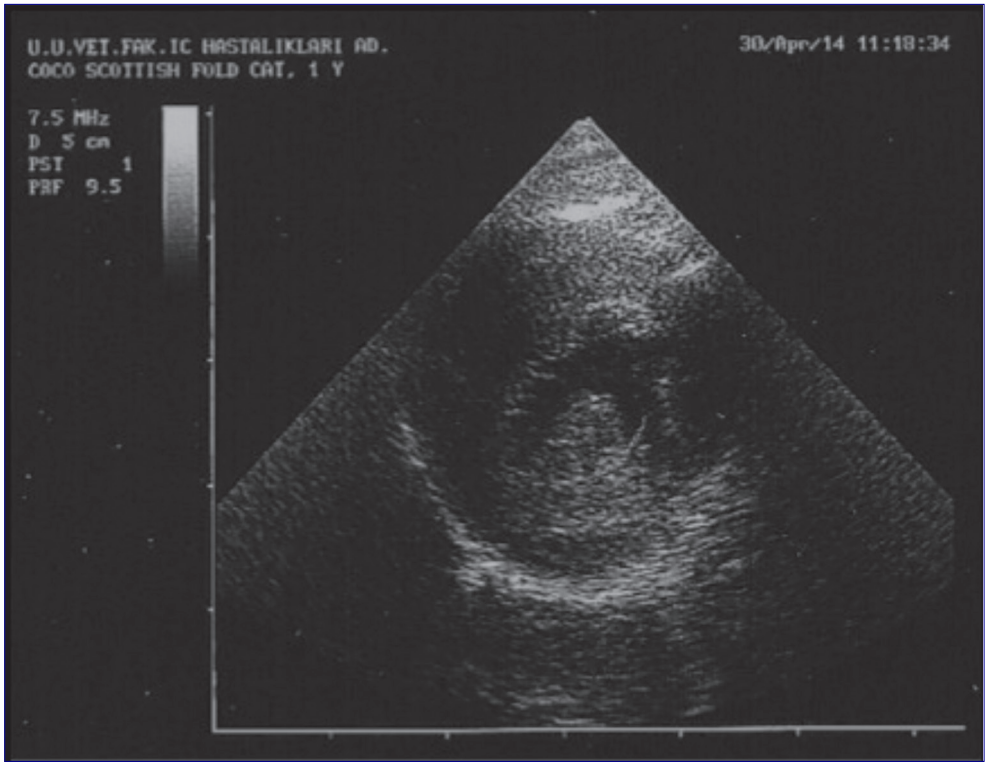

Fig 1. Hypertrophic cardiomyopathy diagnosed in Echo view

Şekil 1. EKO'da tanınan hipertrofik kardiyomyopati

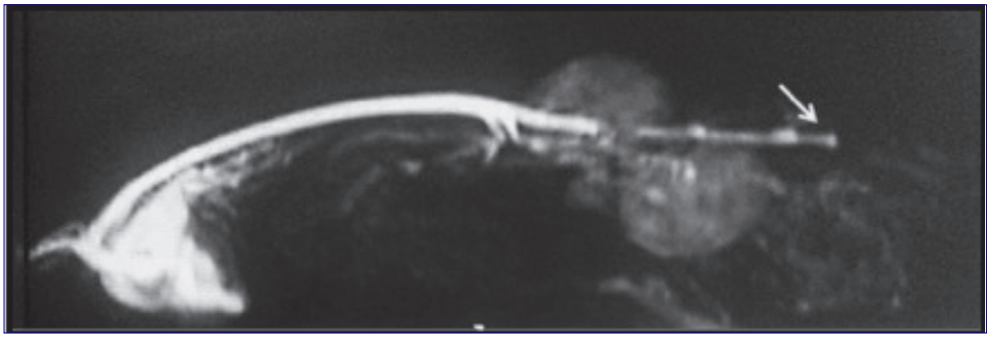

Fig 2. MRA view. The point of arterial thromboembolism observed in the abdominal aorta *(arrow)*

Şekil 2. MRA görüntüsü. Abdominal aortada görülen arteryal tromboembolizmin bulunduğu nokta *(ok)*

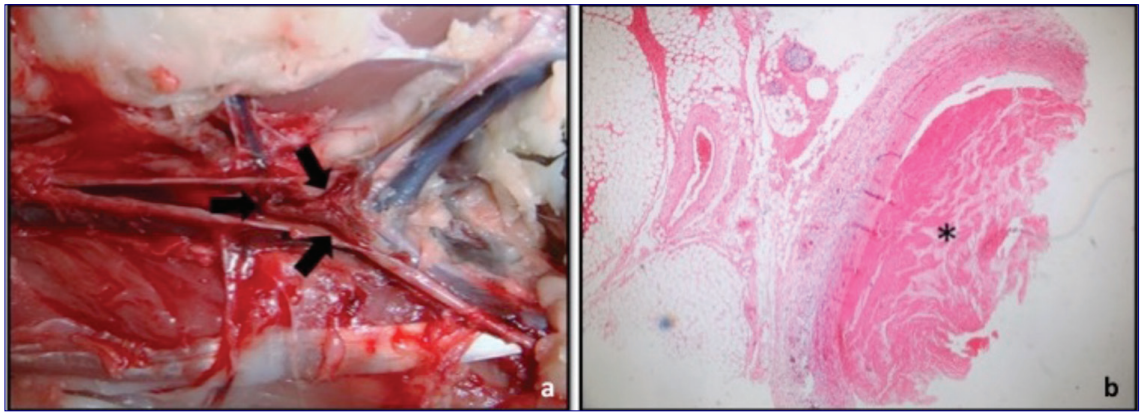

Fig 3. a- "Y" shape thrombus determined at the caudal abdominal aorta and bifurcation of the iliac arteries, **b-** a mostly fibrin thrombus attached to intima of the vessel intima *(asterix)*, H&E x40

Şekil **3. a-** Caudal abdominal aortada, iliak arterlerin bifurkasyosunda saptanan "Y" biçimindeki trombus (oklar), **b-** çoklukla damar intimasına yapışık fibrin trombusu *(asterisk)*, H&E x40

YAZAR DİZİNİ - 2016
(AUTHOR INDEX - 2016)

YAZIM KURALLARI

1- Yılda 6 (Altı) sayı olarak yayımlanan Kafkas Üniversitesi Veteriner Fakültesi Dergisi'nde (Kısaltılmış adı: Kafkas Univ Vet Fak Derg) Veteriner Hekimlik ve Hayvancılıkla ilgili (klinik ve paraklinik bilimler, hayvancılıkla ilgili biyolojik ve temel bilimler, zoonozlar ve halk sağlığı, hayvan besleme ve beslenme hastalıkları, hayvan yetiştiriciliği ve genetik, hayvansal orijinli gıda hijyeni ve teknolojisi, egzotik hayvan bilimi) orijinal araştırma, kısa bildiri, ön rapor, gözlem, editöre mektup ve derleme türünde yazılar yayımlanır. Dergide yayımlanmak üzere gönderilen makaleler Türkçe, İngilizce veya Almanca dillerinden biri ile yazılmış olmalıdır.

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 şekil ve tablo gibi görsel öğelerin 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 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 oluşur ve toplam (metin, tablo, şekil vs dahil) 12 sayfayı geçemez. Yabancı dilde yazılmış makaleler yabancı dilde başlık, yabancı dilde özet ve anahtar sözcükler, Türkçe başlık, Türkçe özet ve anahtar sözcükler dışında Türkçe makale yazım kurallarında belirtilen diğer bölümlerden oluşur. Türkçe ve yabancı dilde özetlerin her biri yaklaşık 200±20 sözcükten oluşmalıdır.

Kısa Bildiri, konu ile ilgili yeni bilgi ve bulguların bildirildiği fakat orijinal araştırma olarak sunulamayacak kadar kısa olan yazılardır. Kısa bildiriler, orijinal araştırma makalesi formatında olmalı, fakat özetlerin her biri 100 sözcüğü aşmamalı, referans sayısı 15'in altında olmalı ve 6 sayfayı aşmamalıdır. Ayrıca, en fazla 4 şekil veya tablo içermelidir.

Ön Rapor, kısmen tamamlanmış, yorumlanabilecek aşamaya gelmiş orijinal bir araştırmanın kısa (en çok 4 sayfa) anlatımıdır. Bunlar orijinal araştırma makalesi formatında yazılmalıdır.

Gözlem (Olgu Sunumu), uygulama, klinik veya laboratuar alanlarında ender olarak rastlanılan olguların sunulduğu makalelerdir. Bu yazıların başlık ve özetleri orijinal makale formatında yazılmalı, bundan sonraki bölümleri Giriş, Olgunun Tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalı ve 4 sayfayı geçmemelidir.

Editöre Mektup, bilimsel veya pratik yararı olan bir konunun veya ilginç bir olgunun resimli ve kısa sunumudur ve 2 sayfayı geçmemelidir.

Derleme, güncel ve önemli bir konuyu, yazarın kendi görüş ve araştırmalarından elde ettiği bulguların da değerlendirildiği özgün yazılardır. Bu yazıların başlık ve özet bölümleri orijinal araştırma makalesi formatında yazılmalı, bundan sonraki bölümleri Giriş, Metin, Sonuç ve Kaynaklar bölümlerinden oluşmalı ve 12 sayfayı geçmemelidir.

5- Makale ile ilgili gerek görülen açıklayıcı bilgiler (tez, proje, destekleyen kuruluş vs) makale başlığının sonuna üst simge olarak işaret konularak makale başlığı altında italik yazıyla belirtilmelidir.

6- Kaynaklar, metin içinde ilk verilenden başlanarak numara almalı ve metin içindeki kaynağın atıf yapıldığı yerde parantez içinde yazılmalıdır.

Kaynak dergi ise, yazarların soyadları ve ilk adlarının başharfleri, makale adı, dergi adı (orijinal kısa ad), cilt ve sayı numarası, sayfa numarası ve yıl sıralamasına göre olmalı ve aşağıdaki örnekte belirtilen karakterler dikkate alınarak yazılmalıdır.

Örnek: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

Kaynak kitap ise yazarların soyadları ile adlarının ilk harfleri, eserin adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı olarak yazılmalıdır.

Editörlü ve çok yazarlı olarak yayınlanan kitaptan bir bölüm kaynak olarak kullanılmışsa, bölüm yazarları, bölüm adı, editör(ler), kitap adı, baskı sayısı, sayfa numarası, basımevi, basım yeri ve basım yılı sırası dikkate alınarak aşağıdaki örneğe göre yazılmalıdır.

Örnek: **McIlwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI numarası bulunan kaynaklarda bu bilgi ilgili kaynak künyesinin sonuna eklenmelidir.

Online olarak ulaşılan kaynaklarda web adresi ve erişim tarihi, kaynak bilgilerinin sonuna eklenmelidir.

Diğer kaynakların yazımında bilimsel yayın ilkelerine uyulmalıdır.

Kaynak listesinde "et al." ve "ve ark." gibi kısaltmalar yapılmaz.

7- Bakteri, virus, parazit ve mantar tür isimleri ve anatomik terimler gibi latince ifadeler orijinal şekliyle ve italik karakterle yazılmalıdır.

8- Editörlük, dergiye gönderilen yazılar üzerinde gerekli görülen kısaltma ve düzeltmeleri yapabileceği gibi önerilerini yazarlara iletebilir. Yazarlar, düzeltilmek üzere yollanan yazıları online sistemde belirtilen sürede gerekli düzeltmeleri yaparak editörlüğe iade etmelidirler. Editörlükçe ön incelemesi yapılan ve değerlendirmeye alınması uygun görülen makaleler ilgili bilim dalından bir alan editörü ve iki raportörün olumlu görüşü alındığı takdirde yayımlanır.

9- Yayınlanan yazılardan dolayı doğabilecek her türlü sorumluluk yazarlara aittir.

10- Yazarlara telif ücreti ödenmez.

11- Resim ve baskı masrafları için yazarlardan ücret alınır. Ücret bilgileri *http://vetdergi.kafkas.edu.tr/* adresinden öğrenilebilir.

12- Yazarlara 50 adet ayrı baskı ücretsiz olarak yollanır.

INSTRUCTIONS FOR AUTHORS

1- The Journal of the Faculty of Veterinary Medicine, University of Kafkas (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly, covers original papers, short communication and preliminary scientific reports, case reports, observation, letter to the editor, and review and on all aspects of veterinary medicine and animal science (clinical and paraclinical sciences, biological and basic sciences, zoonoses and public health, animal feeding and nutritional diseases, animal breeding and genetics, hygiene and technology of food from animal sources, exotic animal science). Manuscripts submitted for publication should be written in Turkish, English or German.

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table must clearly be written in both Turkish and foreign language and their appropriate position should be indicated in the text.

The manuscript and its supplementary (figure etc.) should be submitted by using online manuscript submission system at the address of *http://vetdergi.kafkas.edu.tr/*

During the submission, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the copyright transfer agreement form signed by all the authors should be send to the editorial office.

3- Authors should indicate the name of institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, editorial board may also request the official document of the ethical commission report.

4- Original (full-length) Manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, Introduction, Material and Methods, Results, Discussion, and References and it should not exceed 12 pages including text, tables and illustrations. Abstract should contain 200±20 words.

Short Communication Manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but each of the abstracts should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages. Additionally, they should not contain more than 4 figures or tables.

Preliminary Scientific Reports are short description (maximum 4 pages) of partially completed original research findings at interpretable level. These should be prepared in the format of full-length original articles.

Case Reports describe rare significant findings encountered in the application, clinic and laboratory of related fields. The title and summary of these articles should be written in the format of full-length original articles and the remaining sections should follow Introduction, Case History, Discussion and References without exceeding the total of 4 pages.

Letters to the Editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases without exceeding 2 pages.

Reviews are original manuscripts gather the literature on current and significant subject along with the commentary and findings of the author on the particular subject. The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should follow Introduction, Text, Conclusion, and References without exceeding 12 page.

5- The necessary descriptive information (thesis, projects, financial supports etc) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of title.

6- References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: **Gokce E, Erdogan HM:** An epidemiological study on neonatal lamb health. *Kafkas Univ Vet Fak Derg,* 15 (2): 225-236, 2009.

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: **Mcllwraith CW:** Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4^{t}h ed. ,339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must be complied with the other references.

Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

7- The Latin expression such as species names of bacterium, virus, parasite and fungus and anatomical terms must be written in italic character keeping their original forms.

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