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RESEARCH ARTICLE

Effects of Using Calcium Propionate and Trisodium Citrate in Dairy Cows on Daily Milk Yield and Milk Quality

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ABSTRACT

In the current study, 3 groups with similar characteristics were formed in Uşak Cattle Breeders Association Research Farm. A total of 21 cows, which were the third and fourth lactation, were selected and grouped as including 7 Holstein cows in each group. The animals in the first group were given 143 g / kg calcium propionate and the animals in the second group were give 30 mg / kg trisodium citrate orally for two months. The animals in the third group received no additives as a control group. In the farm where the samples were taken, the maintenance and feeding conditions were carried out in optimum conditions. Somatic cell count (SCC), protein, fat, dry matter, lactose, Ph parameters and daily milk yields were examined in the collected raw milk samples. As a result of the quality analysis in the collected milk, in the groups given calcium propionate and trisodium citrate, there was no statistical difference between the groups in all the parameters except for the number of somatic cells ($p>0.05$). However, a very significant difference was observed in the mean number of somatic cells in the cows treated with trisodium citrate ($p<0.001$) and Trisodium citrate administration was found to help reduce the number of somatic cells in milk.

Key words: Trisodium citrate, calcium propionate, milk quality, the number of somatic cells.

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Süt İneklerinde Kalsiyum Propiyonat ve Trisodyum Sitrat Kullanımının Günlük Süt Verimi ve Süt Kalitesi Üzerine Etkileri

ÖZ

Bu çalışmada, Uşak Damızlık Süt Sığırı Yetiştiricileri Birliği Çiftliğinde benzer özellikte 3 grup oluşturulmuştur. Rastgele seçilen üçüncü ve dördüncü laktasyon olan 21 inek belirlenmiş, her bir grupta 7 Holştayn ırkı sığır olacak şekilde gruplandırılmıştır. Birinci gruptaki hayvanlara 143 gr/kg kalsiyum propiyonat, ikinci gruptaki hayvanlara 30mg/kg trisodyum sitrat oral yolla verilmiştir. Üçüncü gruptaki hayvanlar ise kontrol grubu olarak hiçbir ilave verilmemiştir. Preparatlar iki ay boyunca haftada bir kez içirilmiş ve ertesi gün sağımdan sonra çiğ sütteki kalite parametreleri incelenmiştir. Numunelerin alındığı çiftlikte bakım ve besleme koşulları optimum şartlarda uygun olarak gerçekleştirilmiştir. Toplanan çiğ süt numunelerinde somatik hücre sayısı (SHS), protein, yağ, kuru madde, laktoz, Ph parametreleri ve günlük süt verimleri incelenmiştir. Sütte kalite analizlerinde; kalsiyum propiyonat ve trisodyum sitrat verilen gruplarda somatik hücre sayısı hariç tüm parametrelerde grup arasında istatistiksel fark görülmemiştir ($p>0,05$). Ancak Trisodyum sitrat uygulanan ineklerde ortalama somatik hücre sayıları arasında çok önemli farklılık görülmüştür ($p<0,001$) ve Trisodyum sitrat uygulamasının sütlerde somatik hücre sayısının azalmasına yardımcı olduğu tespit edilmiştir.

Anahtar kelimeler: Trisodyum sitrat, kalsiyum propiyonat, süt kalitesi, somatik hücre sayısı.

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INTRODUCTION

The amount and quality of milk produced is affected by many factors. Some of these factors are related to the animal (genetics, age, etc.), others are related to environmental factors (nutrition, climate, hygiene, etc.). It is possible to increase the amount and quality of milk by paying attention to factors that can be taken under control. The amount of milk increases in direct proportion to the age of the dairy cow, but cows in herds that are poorly managed and not adequately cared for can easily get mastitis. Mastitis causes a large amount of somatic cells to pass into the milk. Since the increase in the number of somatic cells in the milk decreases the milk quality, the value of the milk produced decreases and may even cause it to be disposed of before it can be used. Generally, the number of somatic cells in the milk is checked to determine the quality of milk. The increase in the number of somatic cells in milk causes a change in the composition of the milk. While increasing somatic cell count decreases milk lactose, casein and fat ratios, it increases chlorites, sodium, immunoglobins, serum proteins and milk pH (Mbonwanayo, 2013).

Trisodium Citrate; It is used as flavouring, stabilizing agent and acidity regulating component in the food industry. It is highly soluble in water and has a positive effect on flavour. Citric acid loses its protons of three carboxy groups in solution, which makes citric acid an excellent pH control tool with a buffering effect in acid solutions. Available in the form of white granular crystal or white crystalline powder, trisodium citrate dihydrate is odourless and has a pleasant salty taste. Trisodium citrate, which tends to form large crystals in humid air, dissolves easily in water. Biologically fully degradable trisodium citrate is used to maintain pH levels in soft drinks (Anonymous 2020a).

Calcium Propionate; It is an organic salt formed by the reaction of Calcium Hydroxide with propionic acid. Calcium propionate can be produced in both crystal and powder form. Calcium propionate is easily soluble in water. Calcium propionate is very effective in preventing the growth and reproduction of mould, yeast and other microorganisms in bakery products (Anonymous 2020b).

With the onset of lactation in dairy cows, it is necessary to create care and feeding conditions that allow dry matter consumption to reach a sufficient level in a short time. In addition, a metabolic adaptation should be created that enables them to meet their increased energy and calcium needs from body reserves when necessary (Goff, 1999, Goff 2001). Calcium propionate is used to reduce the incidence of hypocalcemia and ketosis in transition periods of dairy cows, since it is both a source of calcium and energy (Defrain et al., 2005; Mandebvu et al., 2003; Stokes and Goff, 2001; Goff et al., 1996, Melendez et al., 2002).

The study aimed to determine effects of using calcium propionate and trisodium citrate in dairy cows on daily milk yield and milk quality traits in Holstein cows.

Properties of Quality Analysed in Milk

In terms of milk quality, the ratios of milk fat, milk protein and non-fat dry matter vary with the quality of the feed given to animals. Generally, the ratios of essential fatty acids from the feed are as follows: 65%acetic acid, 20% propionic acid, 12% butyric acid, 3% other fatty acids. If cows are fed with high density concentrate, low level of roughage and pelleted feeds, the acetic acid decreases, the propionic acid ratio increases and the fat ratio in milk decreases accordingly (Bhoite and Padekar,2002).

Milk protein and non-fat dry matter (NFDM), protein and NFDM levels in milk are affected by environmental factors at a low level. Protein and NFDM rates in milk decrease by 0.2% in case of malnutrition. However, NFDM and protein ratios increase by 0.2% in feeding with 25% more than the requirement (Anonymous 2019).

The protein found in milk is in the form of casein and serum proteins (immuglobulins, α-lactalbumin, β-lactoglobulin, serum albumin and proteose peptones). While caseins are synthesized from amino acids in the blood, immunoglobulins come from the immune system. In addition, 80% of the total protein in milk is in the form of casein. While the rate of immunoglobulins is high at the beginning of lactation, it decreases to a low amount after the 5th day of lactation (Bhoite and Padekar,2002).

Lactose; Acetic acid, butyric acid and propionic acid are taken from rumen feeds. Through the blood, proprionic acid converts into glucose in the liver and reaches the mammary glands through the blood. Glucose reaching the udder is used in the synthesis of lactose. Since the lactose concentration is high in the alveoli, it increases the amount of milk by coming into the water (Anonymous 2019). 99% of the carbohydrates in milk are in the form of lacquer. The amount of lactose in cow's milk is between 48 and 50 g / litre (Hoden and Coulon, 1991).

Somatic Cell Count; In mastitis, after the macrophage cells in the mammary gland stimulate the cow's defence system, the defence system sends the neutrophil cells to the place where the bacteria occupy and the neutrophil cells try to kill the bacteria. For this reason, more than 90% of the somatic cells found in mastitis milk are neutrophil cells (Pamela and Douglas 2002). In other words, we can list the types of leukocytes found in milk with mastitis in a descending order as follows; neutrophils, macrophages and lymphocytes. If there is no intervention for mastitis occurring in the herd, mastitis decreases milk yield and milk quality, causes breast blindness, decreases the value of dairy animals and causes them to be sent to slaughter. In addition, the milk of the mastitis-infected animal harms human

health and the duration of mastitis-infected cows in breeding is shortened.

Generally, the number of somatic cells in milk is used to determine the status of breast inflammation. If the number of somatic cells in the milk is below 200,000 SHS / ml, it means that the milk is not with mastitis, and if it exceeds this limit, the presence of mastitis is considered. However, it is accepted that drinking milk and products made from this milk with the number of somatic cells lower than 400,000 SHS / ml according to the standards of the European Union and lower than 500,000 SHS / ml according to the Turkish Food Codex are not harmful to human health (Çoban et al., 2006).

MATERIAL and METHOD

The research was conducted at the Uşak Cattle Breeders Association Research Farm, following the approval of the Uşak University Animal Experiments Local Ethics Committee (USAKHADYEK 2018 / 1-03). A total of 21 cows, which were the third and fourth lactation, were selected and grouped as including 7 Holstein cows in each group thus a total of 3 groups were formed.

1. 1 litre of aqueous solution containing 680 g of calcium propionate was given orally to each cow for two months, once a week after morning milking.
2. 500 ml of aqueous solution containing 18 g of trisodium citrate was given orally to the cows in the group, once a week after morning milking for two months. Milk samples were taken the next day.
3. No calcium propionate or trisodium citrate was given to the cows in the group. Feeding continued with the same ration as it was before. No intervention was made to the cows in the control group.

The preparations were given once a week between 1 November 2019 and 30 December 2019 for two months, and the quality parameters in raw milk were examined the next day after milking

The cows selected for the study were randomly divided into experimental and control groups. The animals were housed under the existing farm conditions without any special feeding and housing treatments. In addition to the ration in Table 1, vitamin premix, yeast and toxin binders were given to animals milked by machine twice a day, in the morning and in the evening.

Table 1. Example of Ration Given to Cows

Feed Ingredients	Example of ration given to dairy cows (kg)
Corn Silage, 30-35% KM	15,0
Alfalfa Hay	5
Cattle Milk Feed, 21HP, 2750 ME	5
Wheat Straw	1
Sugar Beet Pulp	5
Wheat Bran	2
Rations ME Mcal / kg KM	2,36

Business records were used for daily milk yields. Milk samples were taken into 200 ml sterile containers during milking and divided into two every Monday, the day after calcium propionate and trisodium citrate were given for two months. Somatic cell counts were made by microscopic method in the laboratory of the Uşak Dairy Cattle Breeders Association. After the milk was spread on two areas of 5x20 mm2 on the slide, it was kept in an oven at 37 °C for 1 hour, and leukocytes and epithelial cell nuclei were stained by dripping a dye solution containing methylene blue. Using a 100 immersion objective, 20 fields were counted in each preparation and their averages were calculated. SHS in 1 ml of milk was calculated by multiplying these obtained averages by the microscope factor. In other samples, they were sent to Uşak University Scientific Analysis and Technological Application and Research Center by cold chain, and protein, fat, dry matter, lactose, pH parameters were examined by using Milkotester brand (Master Classic LM2 P1 Model) Milk Quality Analyzer.

Analysis of the data; The data obtained in terms of the properties emphasized were evaluated with the variance analysis technique. Duncan test was used to determine different groups. Variance analysis was conducted in IBM SPSS statistics 20 program package.

RESULTS

As can be seen in Table 2, as a result of the analysis of variance in terms of mean milk yields, the differences in the sampling months and between the groups were found to be insignificant ($p>0.05$).

As a result of the variance analysis in terms of milk fat, sampling time and cow group interaction were not found to be significant ($p>0.05$). Similarly, sampling times and differences between groups were

not found to be significant (p>0.05). Although the difference is not significant, the average milk fat ratios (Calcium Propionate-Trisodium Citrate) measured in the experimental group as a result of the application (4.08 ± 0.77%, 4.63 ± 0.40%) were found to be lower than the average milk fat ratio measured in the control group (4.75 ± 0.31%) (Table 2)

As can be seen in Table 3, as a result of the analysis of variance in terms of milk protein, the differences in the sampling months and between groups were not found to be significant (p>0.05). Although the difference is not significant, the average milk fat ratios (Calcium Propionate-Trisodium Citrate) measured in the experimental group (3.54 ± 0.44%, 3.84 ± 0.36%) as a result of the applications were found to be lower than the average milk fat ratio measured in the control group (3.99 ± 0.22%). In addition as a result of the analysis of variance in terms of dry matter in milk, the differences in the sampling months and between the groups were found to be insignificant (p>0.05). As a result of the application, the mean dry matter ratios (Calcium Propionate-Trisodium Citrate-Control) measured in the experimental group (9.49 ± 0.48%- 9.67 ± 0.34%) were found to be similar to the mean dry matter ratio (9.96 ± 0.22%) measured in the control group.

As can be seen in Table 4, as a result of the analysis of variance in terms of lactose in milk, the differences in the sampling months and between the groups were found to be insignificant (p>0.05). As a result of the application, the mean lactose ratios (Trisodium Citrate) measured in the experimental group (5,16±0,44%) were found to be lower than the lactose ratio measured in the control group (5,31±0,18%). Also as a result of the analysis of variance in terms of Ph in milk, the differences in the sampling months and between the groups were found to be insignificant (p>0.05).

As seen in Table 5, a very significant difference was observed between the mean numbers of somatic cells before and after trisodium citrate was given to the cows (p<0.001). The difference between the mean numbers of somatic cells of the control and experimental groups was significant before trisodium citrate was given (P <0.05), while the difference between the two groups was very significant after trisodium citrate was given (p<0.001). It was found that trisodium citrate administration helped reduce the number of somatic cells in milk.

Table 2. Mean Milk Yields (gr/kg) and Fat (%)

			Yield (gr/kg)			**Fat (%)**		
Groups	N	Parameters	November	December	P	November	December	P
Calcium Propionate	7	Min.	15.70	16.38	-	2.90	3.09	-
		Max.	23.00	23.75		4.78	5.22	
		x̄±Sx̄	20.14±2.87	20.88±2.74		3.73± 0. 69	4.08± 0.77	
Trisodium Citrate	7	Min.	15.50	11.50		3.36	4.05	-
		Max.	22.30	23.38		4.47	4.99	
		x̄±Sx̄	18.90±2.74	19.45±4.66	-	3.97±0.51	4.63±0.40	
Control	7	Min.	13.80	14.63		3.88	4.33	-
		Max.	19.40	19.50		4.51	5.17	
		x̄±Sx̄	16.86±2.24	16.85±2.41	-	4.12±0.24	4.75± 0.31	
Total	21	Min.	11.68	11.85		3.26	3.26	
		Max.	15.05	14.40		3.87	3.87	
		x̄±Sx̄	12.85±1.90	13.35±1.33		3.94±0.32	3.6590±0.34	
			(A)	(A)		(A)	(A)	

p > 0.05; -: n.s; *: P < 0.05; **: P < 0.01; ***: P < 0.001 Capital letters are used to compare the means in the sampling months.

Table 3. Protein (%) and Dry Matter in Milk (%)

			Protein (%)			Dry Matter in Milk (%)		
Groups	N	Parameters	November	December	P	November	December	P
Calcium Propionate	7	Min.	3.03	3.08	-	8.81	9.02	-
		Max.	4.29	4.24		9.98	10.17	
		x̄±Sx̄	3.58±0.46	3.54±0.44		9.26±0.47	9.49±0.48	
Trisodium Citrate	7	Min.	3.61	3.29		8.72	9.15	-
		Max.	4.13	4.17		9.87	10.08	
		x̄±Sx̄	3.85±0.22	3.84±0.36	-	9.44±0.46	9.67±0.34	
Control	7	Min.	3.81	3.80		8.21	9.72	-
		Max.	4.43	4.36		10.05	10.31	
		x̄±Sx̄	4.11±0.23	3.99±0.22	-	9.53±0.75	9.96±0.22	
Total	21	Min.	3.37	3.01		10.99	11.56	
		Max.	3.96	3.99		11.99	11.93	
		x̄±Sx̄	3.57±0.33	3.59±0.51		11.48±0.49	11.74±0.18	
			(A)	(A)		(A)	(A)	

p > 0.05; -: n.s; *: P < 0.05; **: P < 0.01; ***: P < 0.001 Capital letters are used to compare the means in the sampling months.

Table 4. Lactose in Milk (%) and Ph in Milk (%)

			Lactose in Milk (%)			Ph in Milk (%)		
Groups	N	Parameters	November	December	P	November	December	P
Calcium Propionate	7	Min.	5.21	5.29	-	6.58	6.63	-
		Max.	5.54	5.49		6.72	6.77	
		x̄±Sx̄	5.36±0.15	5.38±0.08		6.65±0.05	6.71±0.05	
Trisodium Citrate	7	Min.	3.81	4.37		6.58	6.70	-
		Max.	5.40	5.39		6.85	7.21	
		x̄±Sx̄	5.03±0.69	5.16±0.44	-	6.66	6.82	
Control	7	Min.	5.06	5.02		6.56	6.70	-
		Max.	5.46	5.45		6.68	6.77	
		x̄±Sx̄	5.32±0.16	5.31±0.18	-	6.62±0.05	6.74±0.02	
Total	21	Min.	5.32	5.31		6.61	6.64	
		Max.	5.48	5.50		6.64	6.74	
		x̄±Sx̄	5.39±0.07	5.40±0.09		6.62±0.01	6.67±0.05	
			(A)	(A)		(A)	(A)	

p > 0.05; -: n.s; *: P < 0.05; **: P < 0.01; ***: P < 0.001 Capital letters are used to compare the means in the sampling months..

Table 5. The Number of Somatic Cells in Milk

Group	N	Parameters	November	December	P
Calcium Propionate	7	Min.	163384.60	134182.50	-
		Max.	201949.60	189780.00	
		$\bar{x}\pm S\bar{x}$	180466.92±16157.57 (A)	165675.05±20950.29 (A)	
Trisodium Citrate	7	Min.	130528.80	78922.50	***
		Max.	180360.40	101969.25	
		$\bar{x}\pm S\bar{x}$	154579.96±20410.17 (B)	92204.90±9057.03 (B)	
Control	7	Min.	176313.80	165345.50	-
		Max.	208935.00	200780.00	
		$\bar{x}\pm S\bar{x}$	189107.32±13463.28 (A)	184633.90±15067.61 (A)	
Total	21	Min.	130528.80	134182.50	
		Max.	208935.00	200780.00	
		$\bar{x}\pm S\bar{x}$	174.718±57	147.504±21	
			*	***	
			P<0.05	p<0.001	

p > 0.05; -: n.s; *: P < 0.05; **: P < 0.01; ***: P < 0.001 Capital letters are used to compare the means in the sampling months.

DISCUSSION

Kara et al. (2009) conducted a study to determine the effects of calcium propionate in cattle at the time of retention secundinarum, first oestrus and first insemination, and concluded that there is no difference in terms of milk yield and composition and this finding concurs with the finding of the current study.

Dhillon et al. (1995) reported that when mastitis is detected in water buffaloes, continuous administration of tri-sodium citrate reduces the bacterial content of milk. Prakash et al. (2010) observed that injection of tri-sodium citrate (30 mg/kg) with trimethoprim venously for 7 days once a day effectively cured mastitis in dairy cows. Trisodium citrate reduces the bacterial content in milk, in parallel with decreasing the number of somatic cells, which is consistent with the study. In addition, in the same study by Parakash et al. (2010), the increase in the fat ratio of trisodium citrate in milk differs from the study.

Eyduran et al. (2005) found that lactation order and seasonal factors had a negative effect on the number of somatic cells in a study conducted in August and November in 27 Holstein cows. In the study, the decrease in the number of somatic cells in cattle is due to drinking trisodium citrate, not different factors.

Mbonwanayo et al. (2016) investigated the effect of Trisodium Citrate on somatic cells in cattle and they determined that there was no difference between the two groups in terms of daily milk yield, fat, protein, milk dry matter and milk ORP values and that the number of somatic cells decreased in the group containing Trisodium Citrate, which concurs with the finding of the current study.

A decrease was observed in the number of somatic cells in the calcium propionate group, but the difference was found to be insignificant. Çağdaş et al. (2009) examined the effects of calcium propionate on hypocalcemia, dry matter intake, body condition score, milk production and reproductive disorders in dairy cows and reported that calcium propionate drunk twice was beneficial in the treatment of milk fever and had a preventive effect for metritis when drunk three times. This shows that there are other benefits of drinking Calcium Propionate to cows.

No significant effect of calcium propionate on milk quality was determined in the study. Kara et al. (2010) in his study by dividing 24 dairy cattle into 3 groups in Bursa; calving time, 24 hours after calving and 7 days after calving, three times calcium propionate (0.68 kg calcium propionate was given) was administered. It was determined that the group administered 3 times calcium propionate tended to decrease the serum BHBA concentration and the incidence of subclinical ketosis during the first four

weeks of lactation. This can be counted among the benefits of giving calcium propionate to dairy cattle.

CONCLUSION

In the current study, a decrease in the number of somatic cells was observed in the group that contained calcium propionate, but the difference was found to be insignificant. It was found that calcium propionate did not cause a change in the protein, fat, dry matter, lactose, Ph parameters and daily milk yields in milk samples.

In the study, the ratio of citrate in the alveoli was increased by drinking trisodium citrate to dairy cows. A lower somatic cell count was detected in the experimental group that was given trisodium citrate compared to the control group. In lactating older cows, using trisodium citrate can be an effective and useful way to counter the increasing trend of somatic cell count in milk.

It can be said that the use of trisodium citrate, which is very easy and cheap to supply, can be used in many fields, especially in the preservation of human food, and has no known harmful effects, in lactating dairy animals can be particularly beneficial in keeping the number of somatic cells in milk at a certain level. On the other hand, more detailed studies are needed to demonstrate the possible effects of longer-term use of trisodium citrate on milk quality, milk components and the general physiological state of the animal. The fact that trisodium citrate is easily available, low-cost and has no adverse health effects will facilitate the transfer of this application to the field.

Ethics Committee Information: This study was carried out after obtaining permission from Uşak University Animal Experiments Local Ethics Committee (**USAKHADYEK 2018/01-03**).

Conflict of interest: The authors declare that there is no conflict of interest.

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RESEARCH ARTICLE

The Effect of Bisphenol A on Notch Signaling Pathway in Development of Rat Testis

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ABSTRACT

Bisphenol A (BPA) is the object of great concerns because of its widespread use, due to several millions of tons its production throughout the world each year. BPA is an endocrine disrupting chemical and is classified as a probable human carcinogen. Fetal and perinatal exposure to environmentally relevant doses of BPA induces multigenerational impairments of male fertility. Notch signaling functions to regulate cell-fate by modulating differentiation, proliferation, and survival of cells. Notch signaling system plays a significant role in male germ cell differentiation and survival. In this study, it was aimed to explore the alterations/changes in protein expression of Notch I receptor following BPA treatments during embriyonal (E) days 18 to 21, postnatal (P) days 0 to 3, and P4 to P7 time intervals in the rat testes in vivo. The results of study revealed that the used doses of BPA had'nt effect on Notch 1 protein expressions in rat testes during selected time preiods.
Key words: Bisphenol A, Notch, Rat, Testis

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Bisphenol A'nın Rat Testis Gelişiminde Fonksiyon Gören Notch Sinyal Yolağı Üzerine Etkisi

ÖZ

Endokrin bozucu kimyasallar arasında Bisphenol A (BPA) her yıl dünyada bir kaç milyon ton üretilmesi ve yaygın olarak kullanılmasıyla en çok dikkat çeken bir ajandır. BPA endokrin bozucu bir ajandır ve insanlar için karsinojen olarak sınıflandırılmaktadır. Fötal ve perinatal dönemde çevredeki dozlarla uyumlu olarak BPA'ya maruz kalmak nesiller boyunca erkek infertilitesinin bozulmasını indüklemektedir. Notch sinyal fonksiyonları hücrelerin farklılaşmasını, çoğalmasını ve canlı kalmasını düzenleyerek hücre kaderini belirler. Notch sinyal sistemi erkek germ hücrelerinin farklılaşması ve yaşamasında önemli bir rol oynamaktadır. Bu çalışmada in vivo embriyonal (E) 18-21, postnatal (P) 0-3 ve P4-7. günlerde BPA uygulamasını takiben rat testislerinde Notch I reseptör protein ekspresyonunun değişip değişmediğini araştırmak amaçlandı. Araştırmadan elde edilen sonuçlar, seçilen zaman dilimlerinde kullanılan BPA dozlarının sıçan testislerinde Notch 1 protein ekspresyonları üzerine etkisi olmadığını ortaya koydu.
Anahtar Kelimeler: Bisphenol A, Notch, Sıçan, Testis

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INTRODUCTION

The endocrine disrupting chemicals are of great concerns for causing impairments in endocrine system functions of living organisims and due to their widespread prevalence in environment. Among all endocrine disrupting chemicals, Bisphenol A (BPA) [2,2-bis (4-hydroxyphenyl) propane] is the most noticeable agent due to its widely use in the world, with several millions of tons of production every year (Bigsby et al. 1999, Delfosse et al. 2014, Safe et al. 2001). BPA is a type of plasticizer which is mainly used in the production of polycarbonate plastics, as well as epoxy resins. Because of this feature, it is widely used in many products for both babies and adults (Calafat et al. 2009, Calafat et al. 2008, Calafat et al. 2005, Staples et al. 2000, Vandenberg et al. 2009). People and animals are constantly exposed to these substances on a daily basis through canned food, plastic food containers, baby nursing bottles and many sources. Especially babies are exposed to high levels of BPA due to the passage of BPA from baby food containers into formula and the use of BPA in medical devices (Calafat et al. 2009, Calafat et al. 2008, Carwile et al. 2009). As a result, BPA is classified as a possible carcinogen for humans (Besaratinia and Pfeifer 2007, World Health Organization, 2002). Studies conducted with BPA have shown that it has estrogenic properties and is transmitted to the baby through both placenta and milk (vom Saal et al. 2007, Vandenberg et al. 2009). BPA has also been detected in placental tissue, amniotic fluid, and serum of pregnant women and fetus (Ikezuki et al. 2002, Schönfelder et al. 2002).

While the potential effects of other endocrine disruptors on the development and function of reproductive organs in mammals have been noted (Albert and Je'gou 2014), there are very few studies on the toxicological and epidemiological evaluations of BPA (Rochester 2013). It has been shown that exposure to lower doses of BPA than the current reference dose determined for humans; disrupts the reproductive physiology in mice (Howdeshell et al 1999) and triggers behavioral disorders in rats (Farabollini et al. 1999). It has been emphasized that, exposure to BPA close to the dose present in the environment during perinatal development in mice, disrupts testicular development in puberty and early adulthood (Kabuto et al.2004; Kawai et al. 2003). It has been observed that, exposure to BPA in the uterus suppresses testosterone production in neonatal rats (Tanaka et al. 2006) and changes testosterone production in mice fetal testes in vitro (Huang et al. 2013).

Exposure to BPA during 16-20th days of pregnancy causes morphological changes in the development of Sertoli, Leydig cells and tubulus seminiferus contorts (Horstman et al. 2012). Researchers have also shown that BPA has an effect on the male reproductive system and reduces daily sperm production in rats (Takahashi and Oishi 2003). It has been reported that, in rats exposed to fetal BPA, the effects on the testes on the postnatal 3rd day are different than those who were exposed in the adulthood (Thuillier et al. 2009). In addition, it has been reported that oral exposure to BPA does not cause significant changes in the reproductive system in both generations of rats (Ema et al. 2001).

There are also few studies on the possible consequences of BPA exposure in the uterus on the development of the male urogenital canal. Exposure to endocrine disruptors during fetal and neonatal periods, which are critical periods of development, can affect the development of many systems, especially the reproductive system and the endocrine system. Therefore, recent studies have focused on these periods. The programming of hyphotalamus-hyphophysis-testis path occurs in neonatal period. Previous research reported that rats which exposed to estrogen during neonatal period caused reproductive disorders during early adulthood. Neonatal period is one of the most important phases that has a vital role in shaping of fertility of young individuals (Goyal et al. 2003). Also, just before the parturation, embriyonic days 18-22 is the phase which most important stages of male genital system had formed (Lupien et al. 2006).

In studies conducted so far, it has been determined that BPA exposure causes the most destructive effects on, spermatogenesis and spermiogenesis in men which were observed in the fetal and neonatal stages rather than the adulthood (Aikawa et al. 2004, Toyama et al. 2004, Toyama and Yuasa 2004). Therefore, we aimed to examine the effect of BPA on the "embryonal" and "neonatal" period, which is a critical period in the development of the reproductive system.

The "Notch" gene was first isolated in Drosophila in 1980s. The Notch signaling pathway is an evolutionarily conserved pathway that includes heterodimer transmembrane receptors, consisting of noncovalent intracellular domain (NICD) and extracellular action. These receptors are activated by two distinct families of ligands that bind to a distinct but equally conserved transmembrane. Thus, the Notch signal is generated through the cell to cell junctionss (Artavanis-Tsakonas et al. 1999, Chen et al. 2006).

Notch signaling pathway members play a critical role in the cellular development of mammalian systems during embryogenesis. Notch signal family members receive extracellular signals from the cell surface and regulate gene expression in the nucleus (Baron et al. 2002). The Notch signaling pathway determines the fate of the cell (Lai 2004) and plays an important role in regulating the processes of cell proliferation (Go et al. 1998), differentiation (Mitsiadis et al. 1998) and apoptosis (Shelly et al. 1999).

In mammals, four Notch receptors (Notch 1, 2, 3, 4) and five ligands (Delta-like 1, 3, 4, Jagged 1, 2) had been identified so far. These Notch receptors are being activated after binding to one of the membrane-binding ligands, which are members of Jagged / Serrare / Delta (Baron et al. 2002, Koch and Radtke 2007). When the receptors bind to the ligands, Notch receptors release the NICD and become ready for proteolytic cleavage. NICD enters the nucleus and combines with recombination signal binding protein-JK. This complex regulates the cleavage enhancer (Hes) and Hes-related transcription factors (Hey) of the Notch target genes (Bray 2006, High et al. 2007, Shih and Wang 2007).

Notch signal has been studied in many tissues during development. Notch expression in the male genital system has also been studied by researchers. It was determined that only Notch 2 was expressed in Sertoli cells in neonatal mice (Dirami et al. 2001). It has been shown by different researchers that Notch1, 2, 3 and 4 are expressed in spermatogonia (Hayashi et al. 2001, Mori et al. 2003). In addition, it has been emphasized that the expression of Notch 1 and its ligand Jagged 2 is necessary in spermatogenesis in human and rat testes (Hayashi et al. 2001). Despite these studies, there are very few studies on the development of Notch 1 and BPA in the male genital system.

The Notch signaling system is essential for the proliferation and differentiation of germ cells in the development of the male genital system (Crittenden et al. 1994, Hayashi et al. 2001).

Disruption of Notch signaling pathway by BPA may affect early spermatogenesis in rats. Therefore, in our study, effects of BPA on spermatogenesis in embryonal and neoanatal periods and its relationship with Notch signaling pathway, which plays an important role in spermatogenesis, were investigated.

METHODS

Animal Procedures

All procedures were approved by the Ethical Committee of Afyon Kocatepe University, Turkey (AKÜHADYEK-219-13; 25.04.2013). Fifty, two-month-old female Wistar rats obtained from Afyon Kocatepe University Experimental Animals Unit were used in the study. The rats were cared in a 14-h light/10-h dark cycle (lighting period from 7:00 a.m. to 9:00 p.m.) and were provided ad libitum water in BPA-free glass bottles. The animals were fed with a special soybean-free 5V01 animal feed (PMI Nutrition International, USA) ad-libitum, in order to eliminate possible effects of phyotestrogens.

5 groups were determined for the study. In each group 5 rats were used in experiments and 5 rats were spared as internal control for the same group. 50 mg / kg / day BPA was applied to the experimental groups and vehicle [Sesame oil + ethanol (SE), 9: 1] was applied to the control group. In Group 1, daily intraperitoneal (i.p.) BPA or vehicle were administered to pregnant mothers from the 18th to the 21st day of pregnancy. Neonatal rats in group 2 were subjected to i.p. injections between days P0 and 3. The first injection was made approximately 4 hours after the birth. In Group 3, injections were administered between days P4 and7. Subcutaneous (sc) injections were made in the offsprings at same doses. In the first three groups, after giving the injections in respective time intervals, the rats were euthanized on the post natal 7th day at 17.00. In group 4, pregnant rats were injected with BPA at days E18 to 21 and for the 5th group BPA injections were made on days P3 to 3 in neonatal rats. The pregnant animals in 4th group and the pups in the 5th group were euthanized at 17.00 after their last injection. The doses were named as in Table 1 according to their duration.

Table 1. Experimental design

Groups (a= experiment, b= control)	Drug administered	**Day of euthanasia**
1a (E 18-E 21)	50mg /kg i.p.	P 7
1b (E 18-E 21)	Ethanol/sesame oil i.p.	P 7
2a (P 0-P 3)	50mg /kg s.c.	P 7
2b (P 0-P 3)	Ethanol/sesame oil s.c.	P 7
3a (P 4-P 7)	50mg /kg s.c.	P 7
3b (P 4-P 7)	Ethanol/sesame oil s.c.	P 7
4a (E 18-E 21)	50mg /kg i.p.	E 21
4b (E 18-E 21)	Ethanol/sesame oil i.p	E 21
5a (P 0-P 3)	50mg /kg s.c.	P 3
5b (P 0-P 3)	Ethanol/sesame oil s.c.	P 3

P: Postnatal; E: Embryonic

Pregnant animals were euthanized by cervical dislocation under general anesthesia (21.1 mg / kg of ketamine and 4.2 mg / kg of xylazine) (Ozden-Akkaya et al. 2017) and the offspring were decapitated. Soon after, when the pregnant rats were euthanized, the fetuses were removed from the embryonic sac.

Testes of fetuses and neonatals were fixed in Bouin's solution for 48 h at room temperature and then 5 μm parafin sections were prepared by using routine histological methods.

Immunohistochemistry for Notch 1 was performed using Universal LSAB Kits (Histostain Plus Broad Spectrum #859043; Invitrogen, Frederick, MD) according to the manufacturer's protocol. Briefly, paraffin sections (5 μm) were treated with primary antibodies against Notch 1 (Cell Signalling, D1E11, mümkünse dilüsyonu) for 30 min at 37°C followed by incubation with biotinylated secondary antibody (Histostain Plus Broad Spectrum, Invitrogen, 859043) for 30 min at room temperature (RT). Samples were incubated in streptavidin-HRP (Histostain Plus Broad Spectrum, Invitrogen 859043) for 30 min at RT. Following the sections were incubated in 3,30-diaminobenzidine (DAB) (Vector 4100) for 5 min to reveal positive signals. Counterstaining was carried out with haematoxylin (Merck, 70225752). Sections were placed first in 95% and then in absolute alcohol. In the sequel of dehydration with graded alcohols, sections were cleared in xylene and mounted with Entellan®.

Immunohistochemical evaluations were made by examining whether the target tissue was stained or not. The stained tissue structures and the intensity of the staining were also taken into consideration.

Values from 0 to 3 were given by two independent observers according to the characteristics as non-stained (-), weakly stained (+), moderately stained (++), and intensely stained (+++).

RESULTS

In order to see the fetal and neonatal effects, the effect of BPA administration in 5 different periods on Notch 1 protein in the testis was examined.

Notch 1 protein expression was detected in cytoplasms of cells. Notch 1 immunoreaction was also detected in peritubular myoid cells, vascular endothelial cells and vascular smooth muscle cells in testicular tissues of different periods. When all periods were examined, no reaction was observed in Sertoli cells, spermatogonia and Leydig cells of any experiment groups. The semi-quantitative evaluation results of the severity of Notch 1 immunostaining in the testes of the rats in the control and experimental groups in different periods are presented in Table 2.

E18-21 (7) period: BPA administrations were applied to pregnant mothers at days E18 to21. Testicular tissues of male puppies were examined by immunohistochemistry at postnatal day 7. A moderate Notch 1 immunization was observed in myoid cells and vascular smooth muscle cells. Vascular endothelial cells, on the other hand showed strong immunization in both control and experiment groups. (Figures 1A, 1B).

P0-3 (7) period: After BPA administration, the testicular tissues of the male pups were taken on the postnatal 7th day and the tissue sections were evaluated immunohistochemically. In both control and the experimental groups, a weak Notch 1 reaction was detected in myoid cells and vascular smooth muscle cells while the vascular endothelial cells showed strong positivity (Figures 2A, 2B).

P4-7 (7) period: The testicular tissues were collectedon the postnatal 7th day following the end of the experiment. A weak Notch 1 immunization was found in myoid and vascular smooth muscle cells in control and experimental group samples. In both groups a strong reaction was visible in vascular endothelial cells (Figures 3A, 3B).

E18-21 period: BPA was applied to pregnant rats during embryonal days 18-21. Testicular tissues of male offspring were collected on the embryonal 21st day. In both groups, a weak Notch 1 immunization was observed in myoid cells and vascular smooth muscle cells while a moderate reaction was visualised in vascular endothelial cells (Figures 4A, 4B).

P0-3 period: 0Immunohistochemical evaluation was made by taking testicular tissues on the postnatal 3rd day. A weak Notch 1 immunization was observed in myoid cells and vascular smooth muscle cells. The reaction was strong in vascular endothelial cells (Figures 5A, 5B).

Table 2. When all periods were examined within themselves, there was no difference visible between the groups in terms of Notch 1 staining intensity

	E18-21(7) Group I		P0-3(7) Group II		P4-7(7) Group III		E18-21 Group IV		P0-3 Group V	
	Control	BPA	Control	BPA	Control	BPA	Control	BPA	Control	BPA
Myoid	++	++	+	+	+	+	+	+	+	+
Endothelial	+++	+++	+++	+++	+++	+++	++	++	+++	+++
Myoid cell of the vessels	++	++	+	+	+	+	+	+	+	+

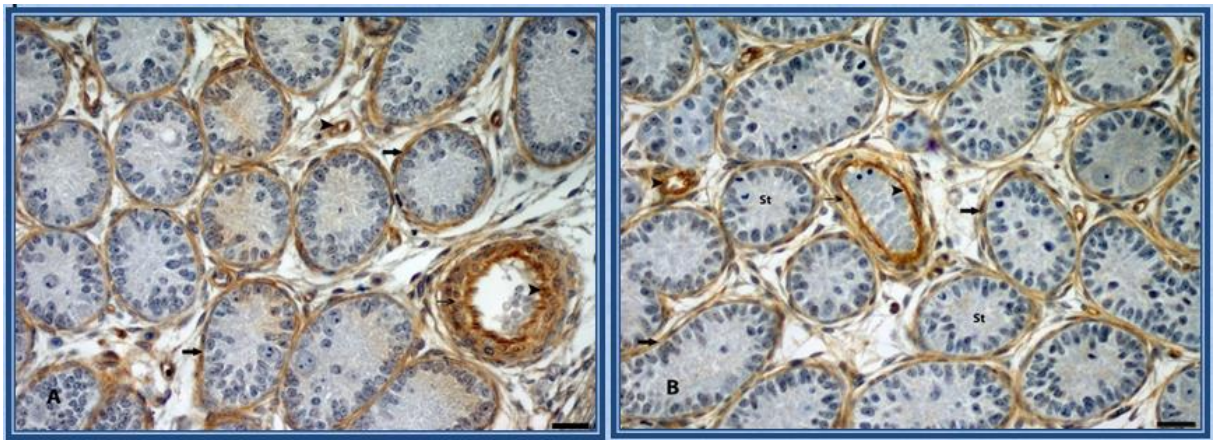

Figure 1: Notch 1 immunreaction of the group 1. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 μm

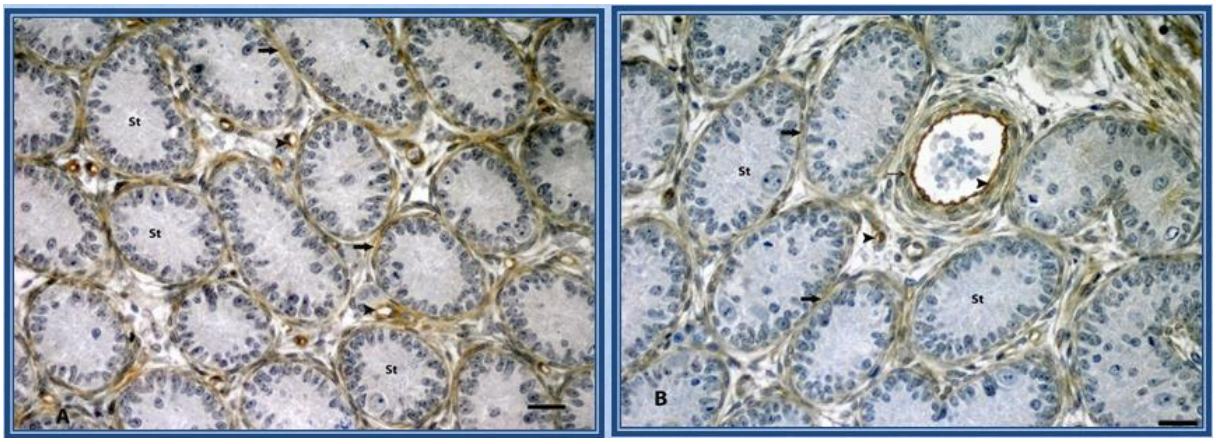

Figure 2: Notch 1 immunreaction of the group 2. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 μm

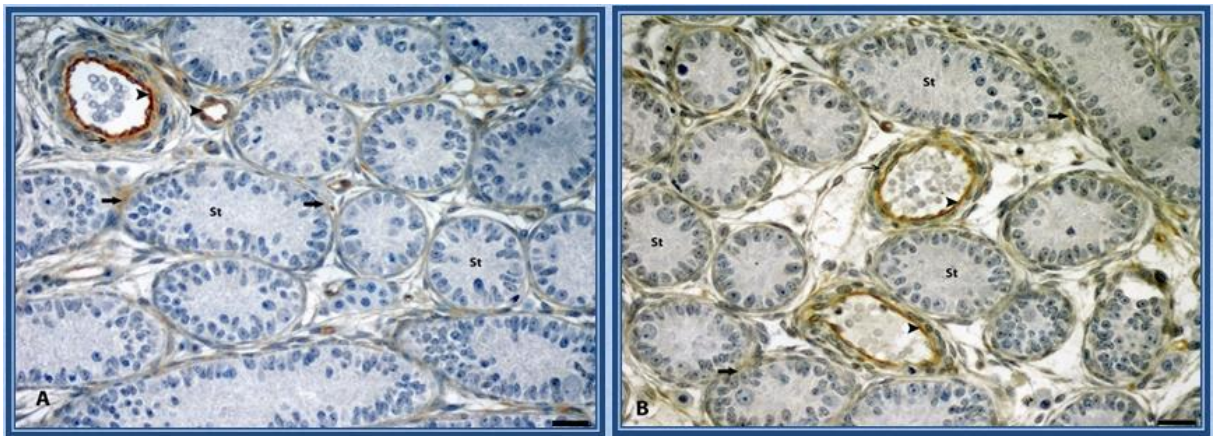

Figure 3: Notch 1 immunreaction of the group 3. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 µm

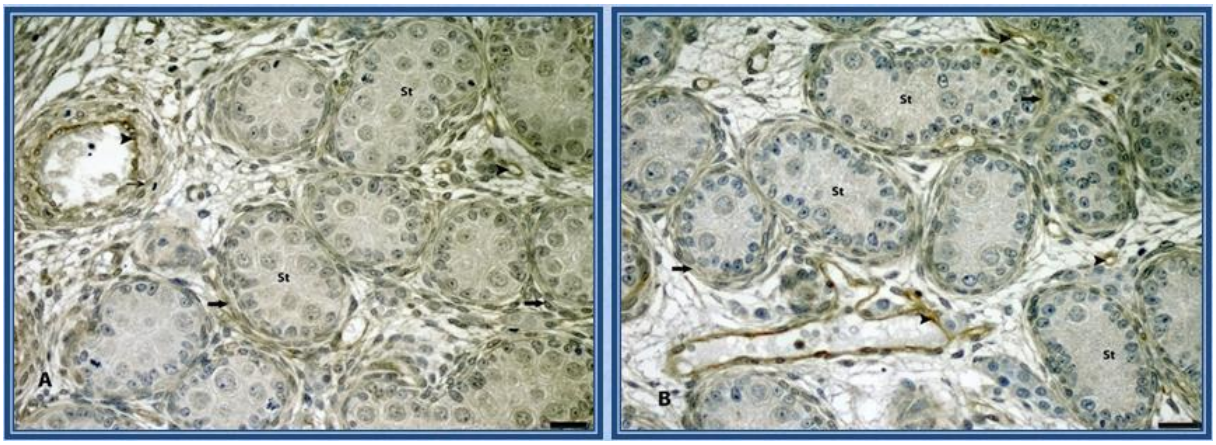

Figure 4: Notch 1 immunreaction of the group 4. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 µm

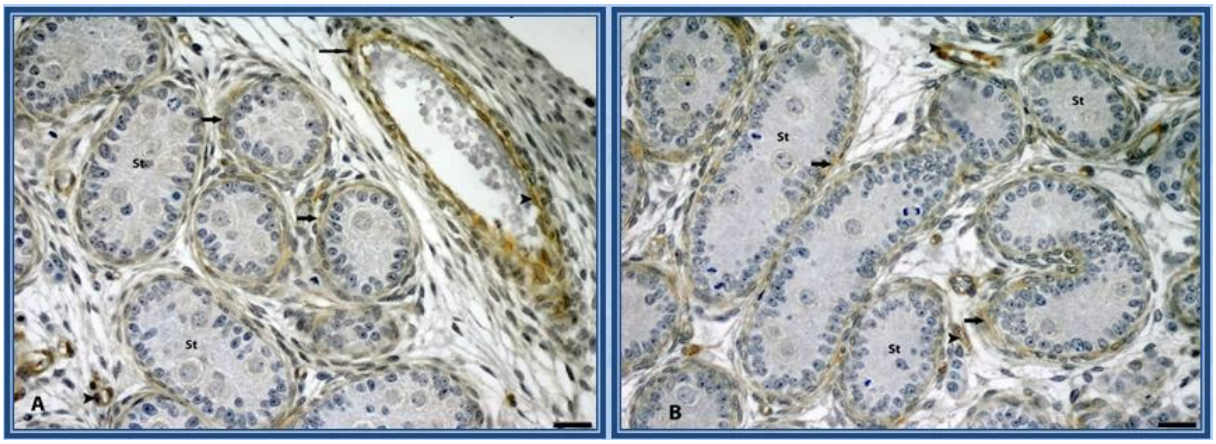

Figure 5: Notch 1 immunreaction of the group 5. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 µm

DISCUSSION

BPA is a conspicuous agent, which is being produced in great amounts annually in the world and is widely used in packaging, medical devices and various plastic materials (Bigsby et al. 1999). BPA has been categorized as an endocrine disruptor and in recent studies it has been elaborated that BPA affects the development of organs which are sensitive to different hormones including gonads (Munoz-de-Toro et al. 2005). BPA, which has estrogenic effects, has been reported to have destructive effects on spermatogenesis and spermiogenesis, especially when exposed in fetal and neonatal stages (Aikawa et al. 2004). Although, studies have been conducted for various effects of BPA on females and males gonads, however, the effects of embryonal and neonatal exposure to BPA are not clear entirely yet.

The Notch signaling pathway is of vital importance in the regulation of processes of cell proliferation (Go et al. 1998), differentiation (Mitsiadis et al. 1998), apoptosis (Shelly et al. 1999) and development of the male genital system (Hayashi et al. 2001). Expressions of different Notch receptors and their ligands in testes had been identified by researchers. However, the effects of embryonal and neonatal exposure of BPA on Notch 1 receptor had not been analyzed. In the recent study, the effects of embryonal and neonatal BPA exposure aimed to be investigated in 5 different stages by demonstrating Notch 1 receptor immunreactivity by immunohistochemical staining.

The Notch 1 immunoreaction was located above the endothelium and in the vessel cells. No reaction was observed in Sertoli cells, spermatogoniums or Leydig cells. No difference was found between the experimental and control groups in terms of testicular morphology. Spermatogonia and Sertoli cells were observed in closed seminiferous tubules and tubules in testes.

It has been reported that the Notch signaling system is induced by the mitotic division of germ cells in Caenorhabditis elegans. In the absence of this induction, the cell leaves the mitotic cell cycle; enters meiosis and completes gametogenesis (Austin and Kimble 1987). On the contrary, excessive induction of Notch causes ectopic germ cell mitosis (Henderson et al. 1997). The first information about Notch signal expression in neonatal and adult rodents and human testes was reported in 2001 (Dirami et al. 2001).

The researchers examined rat testicular tissues for Notch receptors in 3 days intervals until day 28. The first Notch 1 immunoreaction was reported in spermatogenic cells on the 19th day. Moreover, it has been reported that meiotic spermatogoniums and Leydig cells express Notch1 in postnatal 15th day while at the end of the 1st month Notch 1 expression was reported in spermatogoniums and Sertoli cells (Murta et al. 2013). Researchers initially reported only Notch 2 expression in ertoli cells of neonatal mice (Dirami et al. 2001). But, later on Notch 2 expression was also reported in Sertoli cells (Hahn et al. 2009).

Notch 1 expression also had been demonstrated in spermatogonia during adulthood and puberty (Dirami et al. 2001, Hayashi et al. 2001, Murta et al. 2013; Sahin et al. 2005). The activity of Notch 1 reported to be higher in spermatocytes and spermatids (Mori et al. 2003). Notch 1 and its ligand Jagged 2 had been localized in the acrosomal region during the maturation of spermatids especially in the pachetene phase both in rat and human (Hayashi et al. 2001). Positive Notch 1 immunoreactivity was also demonstrated in Leydig cells, elongating spermatocytes, spermatogonia, Sertoli cells and primary spermatocytes in a study conducted in adult rats (Sahin et al. 2005). It was suggested that Notch 1 / Jagged 2 signalling contributes in acrosome formation and Notch 1 expression could have higher correlation in spermiogenesis than germ cell proliferation (Sahin et al. 2005). Since we investigated Notch 1 expression in testis until postnatal 7th day; we could'nt see any expression in Leydig and Sertoli cells in accordance with the previous reports. The expression was only visible in myoid cells, vascular endothelial cells and vascular smooth muscle cells. Myoid cells participate in the formation of the basement membrane and together with Sertoli cells, they maintain the morphology of the seminiferous tubule. While the contractions of the myoid cells are effective in sperm transport, the fluid in the seminiferous tubules flows towards the rete testis (Romano et al. 2005). In addition, myoid cells provide the nourishment of germ cells for the development by secretion and synthesis of the extracellular matrix (Liu et al. 2013).

In the experimental study we conducted, in E18-21 when the effects of BPA were examined, a moderate Notch 1 immunereaction was observed in myoid cells and vascular smooth muscle cells in both groups. The reactions in both groups were strong in vascular endothelial cells. In the E18-21 period, a weak Notch 1 immunization was detected in myoid cells and vascular smooth muscle cells while a moderate expression was detected in vascular endothelial cells in both groups. In addition, a weak Notch 1 reaction was observed in myoid cells and vascular smooth muscle cells meanwhile the expression was found to be strong in vascular endothelial cells in both groups during the P0-3 period and P4-7 period (Calafat et al. 2008). There was no difference in Notch 1 expression between the groups.

Although we studied BPA's effect on Notch 1 expressions in rat testes, investigating other Notch family members for BPA's effect could be beneficial. Also determining different time intervals in order to investigate BPA's effect during whole testis development may provide more comprehensive results.

CONCLUSION

Since Notch 1 reaction was not observed in spermatogenic cells during these periods, it was thought that the possible effects of BPA could be on myoid cells and vascular endothelium where Notch 1 reactions were observed. However, there was no difference between the experimental groups and the control groups in terms of reaction intensity.

It was concluded that the determined dose of BPA had no effect on Notch 1 in the testes during the investigated periods. It is possible that BPA can be effective on spermatogenic cells over other Notch receptors and ligands during these periods.

Conflict of Interest: The authors declared that there is no conflict of interest.

Ethical Statement: All procedures were approved by the Ethical Committee of Afyon Kocatepe University, Turkey (AKÜHADYEK-219-13; 25.04.2013).

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RESEARCH ARTICLE

The Effect of Herbal Extract Mixture (Digestarom) Supplementation to Diets on Duodenum and Ileum Histology in Broiler Chickens

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ABSTRACT

Phytogenic feed additives are used as antibacterial, anti-inflammatory and performance enhancer in the nutrition of broiler chicks. In the present study, it was aimed to investigate the effects of the usage of phytogenic feed additives which are used as an alternative to antibiotics in broilers at different levels in the rations, on the morphology of the duodenum and ileum. For this purpose, 48 male ROSS-308 one-day-old broiler chicks were used in the study. Chicks with a body weight of 43±3 g were randomly divided into 24 replicate sets from three experimental groups, each of which consisted of 8 replicates (2 chicks per house). The other two experimental groups were fed with a basal diet supplemented with 100 mg/kg (DIG100) and 150 mg/kg (DIG150) phytogenic product Digestarom®. In conclusion, it was determined that herbal extract mixture supplementation to broiler chick rations generally increased villus height and villus width, and decreased tunica muscularis thickness. Hence, herbal extract mixture might lead to better digestion and absorption by increasing the intestinal surface area, digestion and absorption levels, also increase body weight gain in broiler chicks by reducing the microbial load.
Keywords: Broiler, crypt depth, muscular thickness, villus height, villus width

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Etlik Piliç Rasyonlarına Bitkisel Ekstrakt Karışımı (Digestarom) Eklenmesinin Duodenum Ve İleum Histolojisi Üzerine Etkisi

ÖZ

Etlik piliç beslemede fitojenik yem katkıları antibakteriyel, antienflamatuvar ve performans arttırıcı olarak kullanılmaktadır. Sunulan çalışmada broylerlerde antibiyotiklerin yerine alternatif olarak kullanılan fitojenik yem katkı maddelerinin rasyonlarda farklı düzeyde kullanılmasının, duodenum ve ileum morfolojisi üzerine etkilerinin araştırılması amaçlanmıştır. Bu amaçla çalışmada 48 adet erkek ROSS-308 bir günlük broyler civciv kullanıldı. Vücut ağırlıkları 43±3 g olan civcivler, her bir deney grubu 8 tekrardan oluşan (her kümeste 2 civciv olmak üzere) üç deney grubundan 24 tekrarlama kümesine rastgele ayrıldı. Kontrol grubu hayvanlar mısır/soya fasulyesi küspesine dayalı bazal diyet ile beslendi. Diğer iki deneme grubu ise 100 mg/kg (DIG100) ve 150 mg/kg (DIG150) fitojenik ürün Digestarom® ile desteklenmiş bazal diyetle beslendi. Sonuç olarak, broyler civciv rasyonlarına bitkisel ekstrakt karışımı takviyesinin genel olarak villus yüksekliği ve villus genişliğini artırdığı, tunika muskularis kalınlığını ise azalttığı belirlenmiştir. Dolayısıyla, bitkisel ekstrakt karışımı bağırsak yüzey alanını, sindirim ve emilim seviyelerini artırarak daha iyi sindirim ve absorbsiyona yol açabilir, ayrıca mikrobiyal yükü azaltarak broyler civcivlerde canlı ağırlık kazancını artırabilir.
Anahtar Kelimeler: Broiler, kas kalınlığı, kript derinliği, villus genişliği, villus yüksekliği

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INTRODUCTION

The additives such as antibiotics, probiotics, prebiotics, organic acids, herbal extracts and enzymes are added to feedstuffs in order to improve the health level and feed efficiency of animals and to improve nutrient utilization (Yörük et al. 2004). The usage of growth-stimulating antibiotics [antibiotic growth promoters (AGPs)] in the poultry industry has been severely criticized by governments and consumers due to the development of bacterial resistance and the harmful effects of these products on human health (Alçiçek et al. 2004). Therefore, the usage of antibiotics as growth stimulators in animal nutrition has been completely banned in the member states of the European Union in 2006 (Vesna et al. 2007). As a result of this situation, feed additive manufacturers have directed to the search for products that may be an alternative to antibiotics. Plant extracts have an important place among the feed additives. The positive effects of plant extracts on animal yield and health have been determined by studies (Du ve Hu 2004, Guynot et al. 2005, Cherian et al. 2013, Zeng et al. 2015, Du et al. 2016, Hafeez et al. 2016). One of the most important features of plant extracts is that they are natural. Due to this feature, their usage in animal nutrition has increased in recent years.

Phytogenic feed additives (PFAs) are plant extracts obtained from various aromatic herbs and spices. As phytogenic feed additives, the whole plant, the seed, fruit, leaf and root parts of the plant also essential oils are used (Çetin and Göçmen 2013). Many different bioactive elements such as alkaloids, flavonoids, mucilage, glycosides, saponins, phenolics, piperine, polyphenols, polypeptide, tannins, terpenoids, cineol, allicin, linalool, capsaicin and thymol can be found in their structures (Upadhaya ve Kim 2017). Phytogenic feed additives have antibacterial, anti-inflammatory, antiviral (Du et al. 2016), antifungal (Guynot et al. 2005), anticoccidial (Du and Hu 2004), antioxidant (Cherian et al. 2013), appetite enhancer, digestive facilitator effects (Hafeez et al. 2016, Erhan 2015). Because of these features, their usage in the feeding of broiler chickens has come into prominence. Also, these substances increase small intestinal villus heights and crypt depths, as well as provide significant increases in the absorption surface (Zeng et al. 2015).

Despite these positive effects of phytogenic feed additives, some studies in broilers did not show any effect in terms of performance, feed consumption, feed conversion rate and small intestine morphology. Lee et al. (2003) found that the additions of thymol essential oil in thyme and cinnamaldehyde essential oil in cinnamon to broiler feeds did not have a positive effect on the growth performance of broiler chickens. Basmacıoğlu et al. (2004) determined that the additions of oregano and rosemary essential oil and their mixtures to broiler feeds did not differ in terms of feed consumption and feed conversion ratio compared to control groups. In another study, Mountzouris et al. (2011) reported that the addition of an essential oil mixture containing carvacrol, anethole and limonene to broiler feeds did not show any difference in performance values compared to the control group at the end of the 42-day feeding period. In studies conducted to evaluate the intestinal morphology, it was found that the additions of clove (Agostini et al. 2012) and oregano essential oil to broiler feeds (Peng et al. 2016) did not have an effect on the villus morphology parameters of broiler chickens. In studies investigating the effects of phytogenic feed additives on growth performance, feed consumption and intestinal morphology, it has been determined that the reasons for not showing positive results may be the type or dosage of phytogenic feed additives, diseases and/or stress factors such as ambient temperature and feeding condition (Basmacıoğlu et al. 2004, Lee et al. 2003, Hong et al. 2012).

The small intestine is an organ that the digestion and absorption of foods take place. To be long of the small intestine provides long-term contact both between foods and digestive enzymes and between digested foods and the absorptive cells of the epithelium. The small intestine consists of three parts as duodenum, jejunum and ileum. Many features of these sections are the same. The inner surface of the small intestine macroscopically shows numerous folds called plica circularis (plicae circulares). When examined microscopically, intestinal villi are seen. These structures are protrusions of the mucosa towards the lumen. Intestinal villi consist of lamina epithelialis and lamina propria. Thanks to these formations, which are only found in the small intestine sections, the absorption surface increases considerably. The lamina epithelialis consists of a single layer of high columnar cells (absorptive cells) and goblet cells between them. The number of goblet cells increases from the duodenum towards the ileum. The main function of these cells is to secrete acid glycoproteins, which are to protect and lubricate the intestinal surface from both chemical and mechanical effects. Among the intestinal villi, there are small openings of simple tubular glands called intestinal glands (crypts or Lieberkühn glands). The epithelium of the villi continues with the epithelium of the glands. Intestinal glands contain undifferentiated cells, absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells. Undifferentiated cells in the crypts differentiate into mucus-secreting goblet cells of the villi epithelium and cylindrical absorptive cells (Tanyolaç 1999, Junqueira et al. 2006).

In this study, it was aimed to investigate the effects of phytogenic feed additives at different levels in rations, which are used as an alternative to antibiotics in broilers, on duodenum and ileum morphologies.

MATERIAL and METHODS

Before starting the presented study, a permit was obtained from the Ethics Committee of Aydin Adnan Menderes University (Aydin, Turkey). In this study, 48 male ROSS-308 one-day-old broiler chicks were used. The animals were obtained from a local commercial hatchery located near Aydin. The experimental area was cleaned and fumigated one week before the experiment, then heated to 32 ºC. The study continued for 42 days. The animals were kept in 24 hours of light and 50-60% relative humidity during the experimental period. The ambient temperature was 32 ºC in the first week. Then, it was set to a constant temperature of 24 ºC by lowering 0.5 ºC per day. Feed and water were given as ad libitum.

Average weight of a chick ranged between 40 to 46 g (43±3 g). Chicks were randomly placed in 24 replicate pens of three experimental groups (8 replicate pens per treatment; 2 chicks in per pen). Control animals were fed a corn/soybean meal-based basal diet (Aviagen 2014). Basal diets were formulated in three different ways, as indicated by Aviagen (2014), starter (days 1-10), grower (days 11-24) and finisher (days 25-42). The other two experimental groups were fed a basal diet supplemented with 100 mg/kg (DIG100) and 150 mg/kg (DIG150) phytogenic product Digestarom® (BIOMIN Holding GmbH, Getzersdorf, Austria). Each kg of Digestarom® contains 20 g of cinnamon, 20 g of cumin, 170 g of peppermint oil, 150 g of garlic oil, 50 g of anise oil, 40 g of fennel oil also SiO2 and NaCl as carriers.

On the 21st and 42nd days of the study, one animal randomly selected from the eight replicate groups in each experimental group was slaughtered. Duodenum and ileum tissue samples from each animal were removed and fixed in neutral buffered formalin (NBF) solution for 24 hours. After routine histological processing, the tissue samples were embedded in paraffin and 6 µm-thick serial sections were taken from the prepared paraffin blocks at 300 µm intervals.

Crossmon's triple staining method (Crossmon 1937) was applied to determine the histological appearance and histomorphological changes in three serial sections. Periodic Acid Schiff Reagent (PAS) staining method (Culling et al. 1985) was applied to the other three sections to reveal the number of goblets. The prepared preparations were examined under a light microscope (Olympus CX41, Japan). Five in each section, a total of 15 villus height, villus width, crypt depth and muscular layer thickness for each animal, interactively with the help of image analysis program (Olympus cellSens Entry, Imaging software, Olympus Corporation, Tokyo 163-0914, Japan) were determined. In addition, goblet cell numbers in the middle 100 µm portion of 15 villi belong to each animal were counted manually.

Statistical Analysis

SPSS (Statistical Package for the Social Sciences, version 18.0) package program was used for statistical analysis of the obtained data. The results were presented as mean ± standard errors. The statistical differences between the groups in terms of villus height, villus width, crypt depth, muscular layer thickness and goblet cell number in tissue samples taken from the duodenum and ileum on the 21st and 42nd days of the study were evaluated with Kruskal-Wallis analysis of variance. Post hoc 97 multiple comparison test was performed to determine which group or groups caused the difference. Values with differences $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***) were considered significant in the statistical analyzes (Conover 1980).

RESULTS

The villus height, villus width, crypt depth, tunica muscularis thickness and goblet cell number in duodenums of all groups are given in Table 1 and Table 2.

On the 21st day of the study, in the histomorphological examination of samples taken from the duodenum, it was determined that the villus height significantly increased in the DIG100 group compared to the control group. There was no statistical difference between the control and DIG150 groups. In the evaluation made in terms of villus width, it was seen that the numerical increases in the DIG100 and DIG150 groups were not statistically significant compared to the control groups (Figure 1). The crypt depth was found as similar in all groups. Although the tunica muscularis thickness decreased in all digestrom-treated groups compared to the control groups, it was determined that this decrease was only statistically significant in the DIG100 group (Figure 2). It was also observed that there was no significant difference in terms of the number of goblet cells between all groups.

In the histomorphological examination of the samples taken from duodenum on the 42nd day of the study, it was determined that the increase in the DIG150 group was statistically significant, although the villus height increased in all digestrom-treated groups compared to control groups. In the evaluation made in terms of villus width, it was found that the increase in the DIG150 group was significant compared to control group. In addition, villus width significantly decreased in DIG100 group compared to control group (Figure 3). The crypt depth was similar in the control, DIG100 and DIG150 groups. There was no significant in terms of tunica muscularis thickness between groups. In addition, it was determined that the number of goblet cells in all groups given digestrom was significantly lower than control group.

In the histomorphological examination of ileum samples on the 21st day of the study, it was

determined that there was no statistically significant difference in terms of villus height between control, DIG100 and DIG150 groups. In the evaluation made in terms of villus width, it was determined that the increase in DIG100 and DIG150 groups was statistically significant compared to control group (Figure 4). The crypt depth was found to be similar in all groups. Tunica muscularis thickness significantly decreased in all digestrom-treated groups compared to control groups, while the difference between DIG100 and DIG150 groups was not significant. In addition, while the number of goblet cells was similar between control and DIG100 groups, the increase in the DIG150 group was statistically significant compared to other groups (Table 3).

On the 42nd day of the study, in the histomorphological examination of ileum samples, it was determined that the villus height in DIG100 and DIG150 groups significantly increased compared to control group. In addition, although the villus width in all digestrom groups increased compared to control groups, the increase in the DIG100 group was only statistically significant (Figure 5). In the evaluation made in terms of crypt depth, tunica muscularis thickness and number of goblet cells it was determined that the difference between the control and digestrom groups was not significant (Table 4).

Table 1. On the 21st day of the study, villus height, villus width, crypt depth, muscular thickness and number of goblet cells in the duodenums of control and digestrom-treated groups (n= 8).

Groups	Villus Height (µm) ($\bar{x} \pm S\bar{x}$)	Villus Width (µm) ($\bar{x} \pm S\bar{x}$)	Crypt Depth (µm) ($\bar{x} \pm S\bar{x}$)	Muscular Thickness (µm) ($\bar{x} \pm S\bar{x}$)	Number of Goblet Cells ($\bar{x} \pm S\bar{x}$)
Control	1154.76 ± 36.13[b]	177.16 ± 3.93	131.34 ± 3.09	197.39 ± 3.76[a]	7.14 ± 0.16
DIG100	1406.53 ± 40.79[a]	179.30 ± 3.68	128.21 ± 3.27	183.52 ± 3.22[b]	7.31 ± 0.15
DIG150	1103.52 ± 32.50[b]	182.45 ± 3.96	127.39 ± 3.15	196.86 ± 3.44[a]	7.26 ± 0.18
p	***	NS	NS	*	NS

[a,b:] Different superscripts in the same column indicate the significant difference. NS: Non-significant, *p<0.05, ***p<0.001.

Table 2. On the 42nd day of the study, villus height, villus width, crypt depth, muscular thickness and number of goblet cells in the duodenums of control and digestrom-treated groups (n= 8).

Groups	Villus Height (µm) ($\bar{x} \pm S\bar{x}$)	Villus Width (µm) ($\bar{x} \pm S\bar{x}$)	Crypt Depth (µm) ($\bar{x} \pm S\bar{x}$)	Muscular Thickness (µm) ($\bar{x} \pm S\bar{x}$)	Number of Goblet Cells ($\bar{x} \pm S\bar{x}$)
Control	974.17 ± 44.77[b]	183.31 ± 4.96[b]	167.08 ± 5.06	250.69 ± 4.62	7.97 ± 0.19[a]
DIG100	1038.01 ± 41.51[b]	167.78 ± 6.02[c]	172.71 ± 4.99	260.48 ± 5.75	6.71 ± 0.18[b]
DIG150	1360.47 ± 33.26[a]	199.53 ± 4.72[a]	159.41 ± 3.81	248.41 ± 5.02	6.28 ± 0.17[b]
p	***	***	NS	NS	***

[a,b,c:] Different superscripts in the same column indicate the significant difference. NS: Non-significant, ***p<0.001.

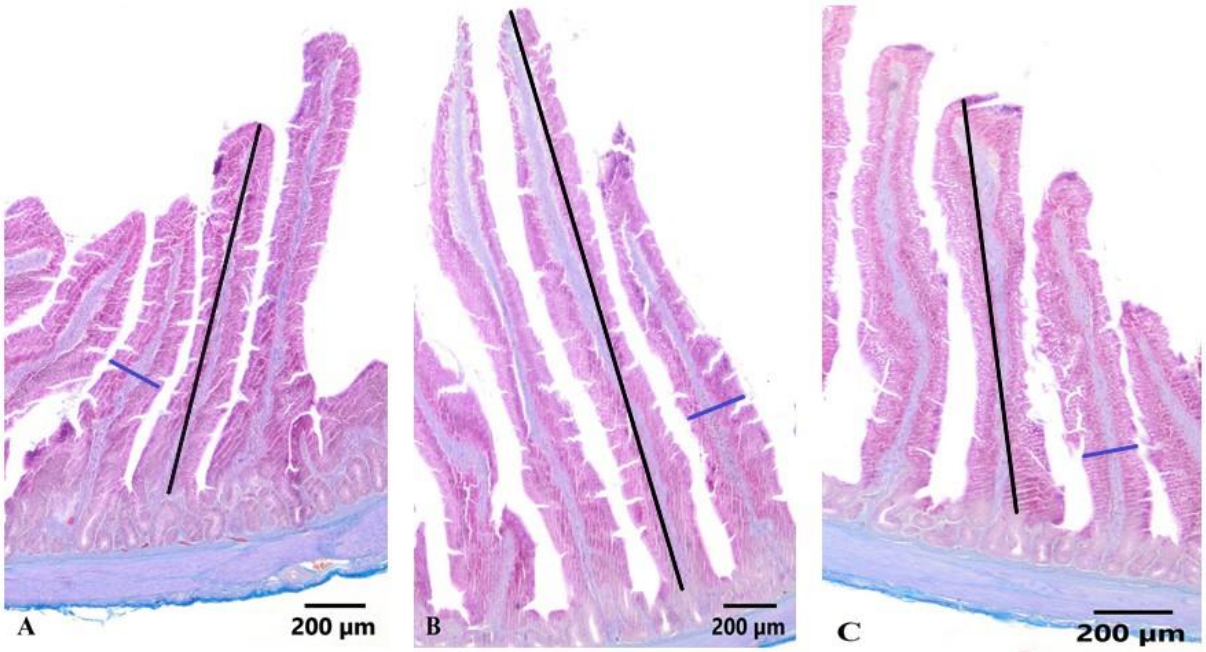

Figure 1: On the 21st day of the study, villus height (black lines) and villus width (blue lines) in the duodenums of control (A) and digestrom100 (B) and digestrom150 (C)-treated groups (n= 8). Crossmon triple staining. Scale bar: 200 µm.

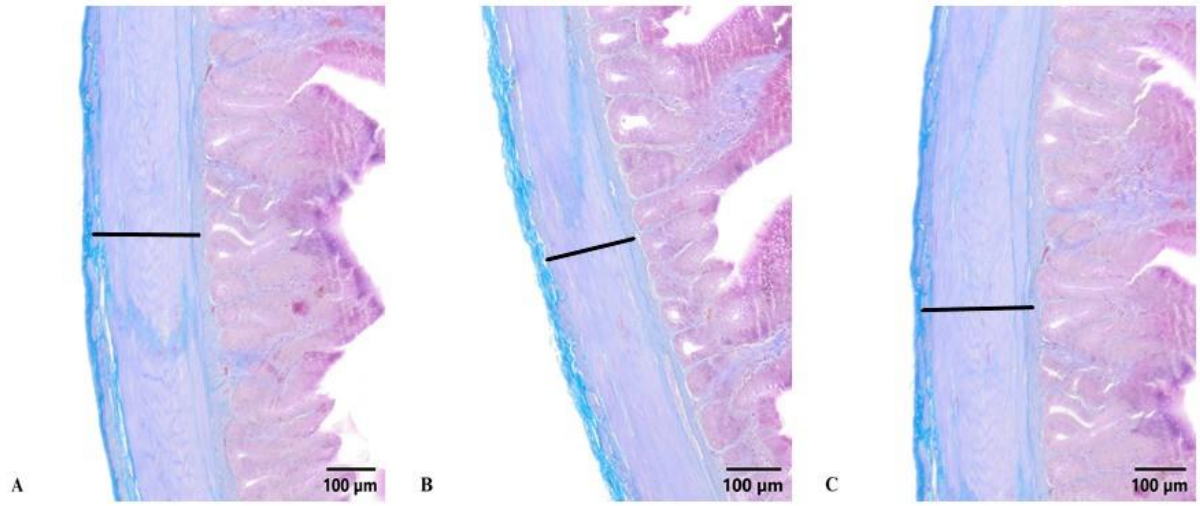

Figure 2: On the 21st day of the study, tunica muscularis thickness (black lines) in the duodenums of control (A) and digestrom100 (B) and digestrom150 (C)-treated groups (n= 8). Crossmon triple staining. Scale bar: 100 µm.

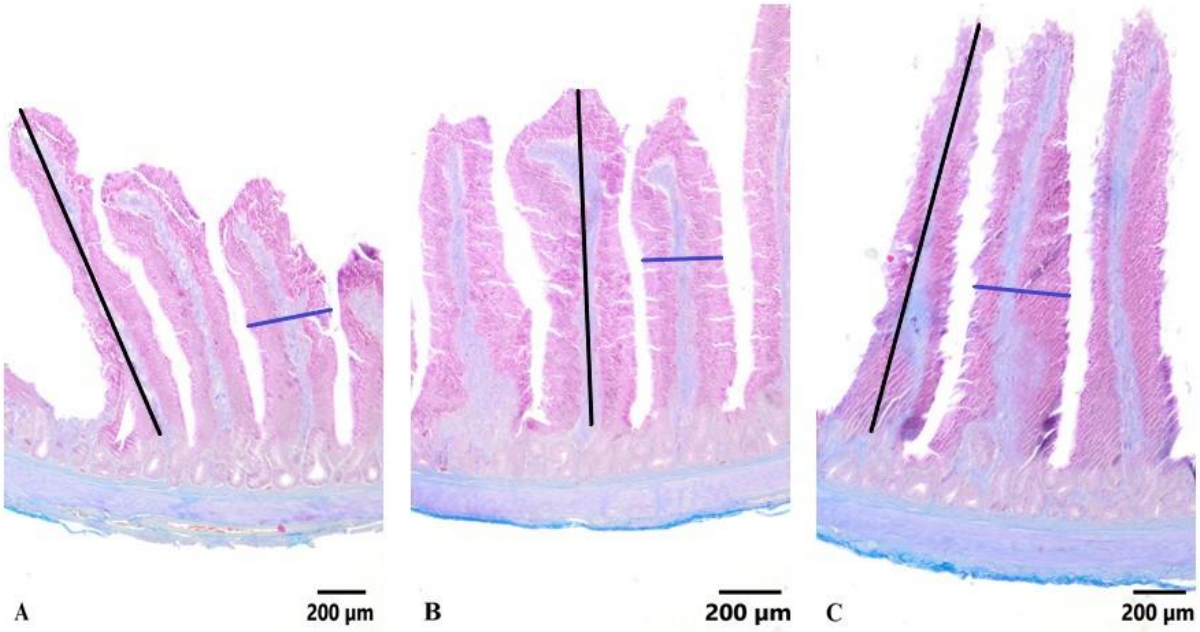

Figure 3: On the 42st day of the study, villus height (black lines) and villus width (blue lines) in the duodenums of control (A) and digestrom100 (B) and digestrom150 (C)-treated groups (n= 8). Crossmon triple staining. Scale bar: 200 µm.

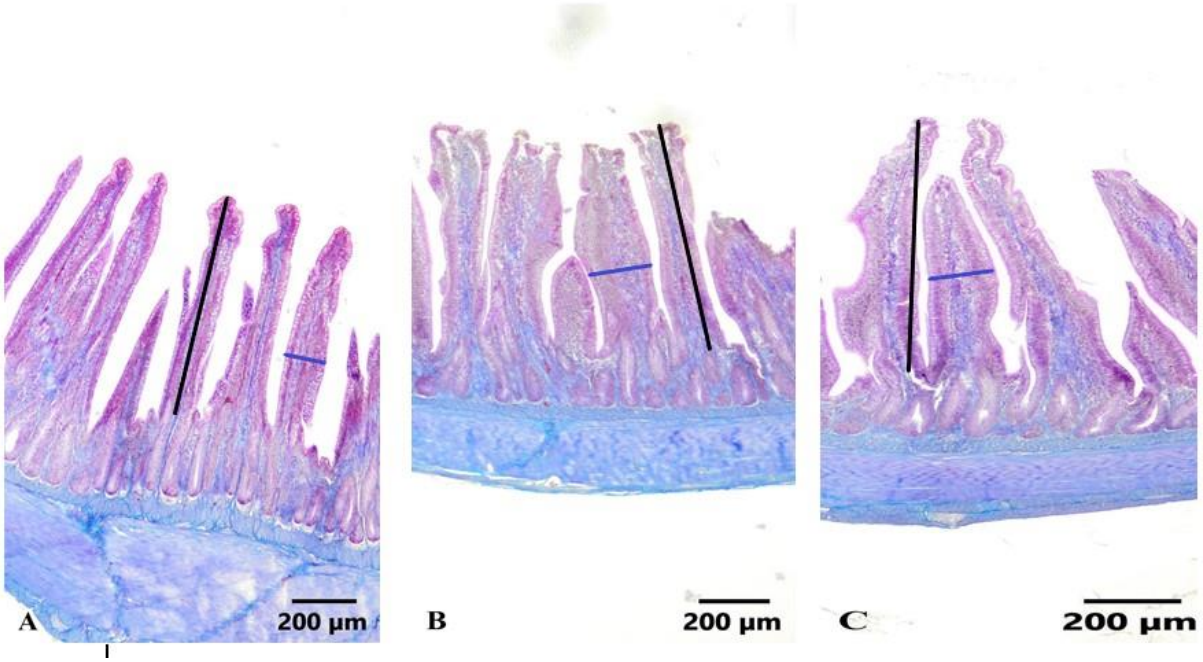

Figure 4: On the 21st day of the study, villus height (black lines) and villus width (blue lines) in the ileums of control (A) and digestrom100 (B) and digestrom150 (C)-treated groups (n= 8). Crossmon triple staining. Scale bar: 200 µm.

Table 3. On the 21st day of the study, villus height, villus width, crypt depth, tunica muscularis thickness and the number of goblet cells in the ileums of control and digestrom-treated groups (n= 8).

Groups	Villus Height (µm) ($\bar{x} \pm S_{\bar{x}}$)	Villus Width (µm) ($\bar{x} \pm S_{\bar{x}}$)	Crypt Depth (µm) ($\bar{x} \pm S_{\bar{x}}$)	Muscular Thickness (µm) ($\bar{x} \pm S_{\bar{x}}$)	Number of Goblet Cells ($\bar{x} \pm S_{\bar{x}}$)
Control	590.14 ± 10.29	176.98 ± 4.74^{b}	160.09 ± 2.84	248.89 ± 5.44^{a}	12.00 ± 0.15^{b}
DIG100	615.97 ± 10.27	191.25 ± 3.89^{a}	160.18 ± 2.53	226.89 ± 4.05^{b}	11.76 ± 0.15^{b}
DIG150	615.76 ± 11.50	187.69 ± 3.65^{a}	155.24 ± 2.66	220.93 ± 4.95^{b}	12.50 ± 0.16^{a}
p	NS	**	NS	***	***

$^{a,b:}$ Different superscripts in the same column indicate the significant difference. NS: Non-significant, *p<0.05, **p<0.01, ***p<0.001.

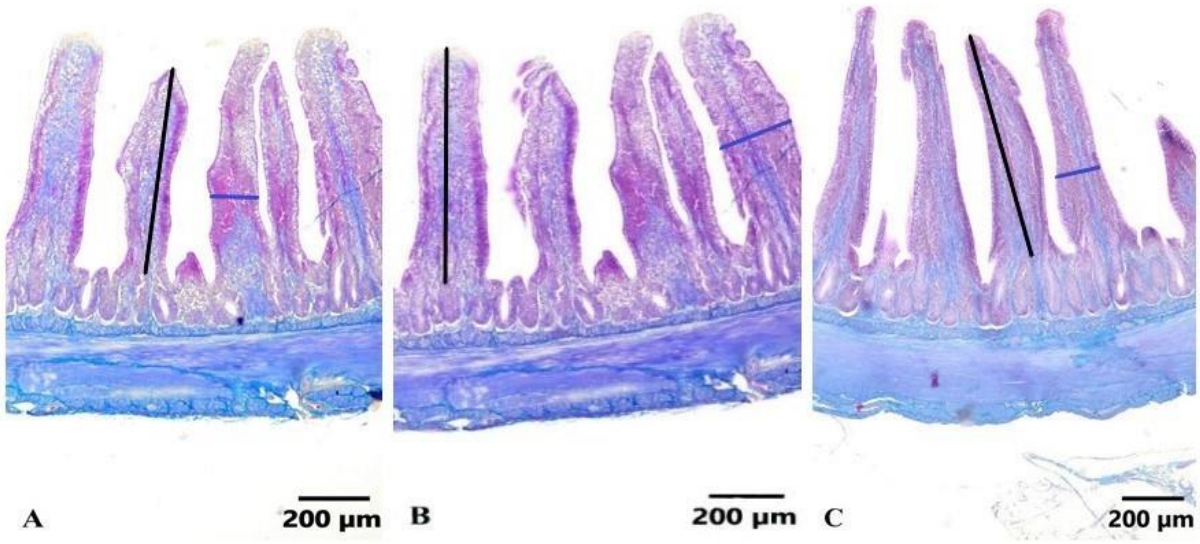

Figure 5: On the 42st day of the study, villus height (black lines) and villus width (blue lines) in the ileums of control (A) and digestrom100 (B) and digestrom150 (C)-treated groups (n= 8). Crossmon triple staining. Scale bar: 200 µm.

Table 4. On the 42nd day of the study, villus height, villus width, crypt depth, tunica muscularis thickness and the number of goblet cells in the ileums of control and digestrom-treated groups (n= 8).

Groups	Villus Height (µm) ($\bar{x} \pm S_{\bar{x}}$)	Villus Width (µm) ($\bar{x} \pm S_{\bar{x}}$)	Crypt Depth (µm) ($\bar{x} \pm S_{\bar{x}}$)	Muscular Thickness (µm) ($\bar{x} \pm S_{\bar{x}}$)	Number of Goblet Cells ($\bar{x} \pm S_{\bar{x}}$)
Control	455.04 ± 12.68^{b}	164.85 ± 4.21^{b}	146. 90 ± 3.79	215.61 ± 2.46	14.06 ± 0.14
DIG100	510.36 ± 14.41^{a}	197.80 ± 5.96^{a}	148.03 ± 4.19	229.06 ± 5.43	13.57 ± 0.18
DIG150	535.10 ± 16.15^{a}	169.55 ± 3.46^{b}	145.75 ± 3.02	238.43 ± 5.96	13.66 ± 0.16
p	***	***	NS	NS	NS

$^{a,b:}$ Different superscripts in the same column indicate the significant difference. NS: Non-significant, ***p<0.001.

DISCUSSION

Murugesan et al. (2015) and Namkung et al. (2004) found that supplementation of herbal extract mixture to broiler chicken rations increased villus height in small intestines, Tavangar et al. (2021) reported that it significantly increased both the villus height and the villus width. Thus, in the absence of inflammation in the intestines, it has been shown that increased villus height and villus width increase surface area, digestion and absorption levels, and provide better digestion and absorption. In the study, herbal extract mixture supplementation to broiler chick rations significantly affected villus height, villus width, tunica muscularis thickness and goblet cell counts in duodenum and ileum sections of 21-day-old chicks. In the presented study, duodenal villus height was found to be significantly higher in DIG100 group at the age of 21 days and in DIG150 group at the age of 42 days compared to control groups as similar to the studies of Murugesan et al. (2015), Namkung et al. (2004) and Tavangar et al. (2021). The height of ileum villus was found to be significantly higher in DIG100 and DIG150 groups at the age of 42 days compared to control group. In addition, duodenal villus width was found to be wider in DIG150 group at the age of 42 days compared to control group. It was determined that ileum villus width was significantly wider in DIG100 and DIG150 groups at the age of 21 days, and in DIG100 group at the age of 42 days compared to control group.

Intestinal crypts are the main source of epithelial cells in the structure of the villus intestinalis. Crypt depth is directly related to the epithelial cell cycle (Markovic et al. 2009). Murugesan et al. (2015) determined that the addition of phytogenic feed additives to chicken rations decreased the crypt depth in the jejunum but there was no difference between groups in duodenum and ileum. Humer et al. (2015) also determined that the addition of phytogenic feed additives to male broiler rations did not affect the crypt depth in ileum. The crypt depth findings of our study are consistent with these studies (Humer et al. 2015, Murugesan et al. 2015).

Tunica muscularis thickness shows the microbe load in the intestines of broiler chicks. Broiler chicks with thin tunica muscularis have a lower germ load, which positively reflects to body weight gain (Gordon and Bruckner-Kardoss 1961). Murugesan et al. (2015) determined that the addition of phytogenic feed additives to broiler chicken rations significantly reduced the thickness of the tunica muscularis in the duodenum and ileum. Humer et al. (2015) also determined that the addition of phytogenic feed additives to male broiler rations did not affect the thickness of the tunica muscularis in ileum. In the present study, duodenum tunica muscularis thickness was found to be lower in the DIG100 group compared to control group at the age of 21 days. The thickness of the ileum tunica muscularis was found to be thinner in DIG100 and DIG150 groups at the age of 21 days. Our findings are in line with the study of Murugesan et al. (2015) but are inconsistent with the study of Humer et al. (2015). The reason for this incompatibility may be the type of phytogenic feed additives or the application doses.

The increase in number of goblet cells per villus is associated with high production of mucins and glycoproteins that bind to pathogenic bacteria and prevent them from attaching to the intestinal mucosa (Chacher et al. 2017). Namkung et al. (2004) and Humer et al. (2015) found that herbal extract mixture supplement to broiler chicken rations did not affect the number of goblet cells in the small intestine. In the present study, herbal extract mixture supplementation to broiler chick rations did not affect goblet cell counts in the duodenum at the age of 21 days and in the ileum at the age of 42 days in accordance with these studies.

But, it was determined that the number of goblet cells in the duodenum decreased in DIG100 and DIG150 groups in the 42-day age compared to control group, and the number of goblet cells in ileum increased in DIG150 group in the 21-day age. Unlike other studies (Namkung et al. 2004) and Humer et al. 2015), the decrease in the number of goblet cells in duodenum and the increase in ileum may be due to the type or application doses of the phytogenic feed additives.

CONCLUSIONS

In conclusion, in the presented study, it was determined that herbal extract mixture supplementation to broiler chick rations generally increased villus height and villus width, and decreased tunica muscularis thickness. Therefore, herbal extract mixture might lead to better digestion and absorption by increasing the intestinal surface area, digestion and absorption levels, also increase body weight gain in broiler chicks by reducing the microbial load.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical Permission: Ethics committee report of the study was obtained from Aydin Adnan Menderes University Animal Experiments Local Ethics Committee with the number of 64583101/2021/094 dated June 24, 2021.

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RESEARCH ARTICLE

Risk of Heavy Metal Contamination in Krill Oils

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ABSTRACT

Different omega-3 sources have been widely used as a portion of supplementary food in recent years. One of the popular sources of omega-3 fatty acids is krill oil. Thus, the aim of the current study was to examine the contents of commercially available krill oils sold in the markets. For this purpose, a total of 11 different krill oil brands randomly selected from different pharmacies. The chemical analysis was carried out in a laboratory accredited by the Turkish Accreditation Agency. Our results indicated that the fatty acid contents of the commercial krill oils tested varied to quite an extent, but within the tolerable limits in 10 out of 11 samples. The peroxide content of the samples differed from 10 to 30 meqO_2/kg-oil. The mercury and cadmium levels were up to the standard limits set by Codex Alimentarius for food supplements. However, all products contained more than the tolerable limits of lead and only 1 sample had arsenic levels measured below acceptable limits. Thus, none of the krill oil samples provided the required European Union standards. It suggests that the manufacturers overlooked some issues while producing krill oils. This may pose a potential threat to public health in the long term.
Keywords: Fatty acid composition, heavy metal contamination, krill oil, peroxide levels

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Kril Yağlarında Ağır Metal Kontaminasyon Riski

ÖZ

Son yıllarda gıda takviyesi olarak farklı omega-3 kaynakları yaygın olarak kullanılmaktadır. Omega-3 yağ asitlerinin popüler kaynaklarından biri de kril yağıdır. Bu nedenle, mevcut çalışmanın amacı, piyasada bulunan kril yağlarının genel özelliklerini incelemektir. Bu amaçla farklı eczanelerden rastgele seçilen toplam 11 kril yağı markasından alınan numunelerin kimyasal analizleri Türk Akreditasyon Kurumu tarafından akredite edilmiş bir gıda analiz laboratuvarında gerçekleştirilmiştir. Elde edilen sonuçlar, test edilen ticari kril yağlarının yağ asidi içeriğinin oldukça büyük ölçüde değişse de 11 numuneden 10'unda tolere edilebilir sınırlar içinde olduğunu göstermiştir. Numunelerin peroksit içeriği, 10 ila 30 meq O_2/kg-yağ arasında bulunmuştur. Civa ve kadmiyum seviyeleri, gıda takviyeleri için Codex Alimentarius tarafından belirlenen standart limitleri içerisindeydi. Öte yandan, test edilen tüm ürünler, tolere edilebilir kurşun sınırlarından daha fazlasını içerirken, yalnızca 1 numunede kabul edilebilir sınırların altında arsenik seviyeleri görüldü. Sonuç olarak, rastgele örnekleme yoluyla toplanan kril yağlarının hiçbiri gerekli Avrupa Birliği standartlarını sağlayamadı. Bu durum üreticilerin kril yağları üretirken bazı konuları gözden kaçırdığını düşündürmektedir. Bu, uzun vadede halk sağlığı için potansiyel bir tehdit oluşturabilir.
Anahtar Kelimeler: Ağır metal kirliliği, kril yağı, peroksit seviyeleri, yağ asidi bileşimi

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INTRODUCTION

Krill" (Euphausia Superba) means "small fry of fish" in the Norwegian language. It is also a term used to describe the shellfish belonging to the Euphausiacea family (Nicol and Endo, 1997; Tou et al., 2007). Krill is a shrimp-like small crustacean that lives in the cold waters of the Antarctic Ocean (Vacchi et al. 2012), and there are about 85 species of krill. Their sizes can range from the smallest millimetric dimensions to 15 cm in length (Nicol and Endo 1997). They have opportunistic features and feed with any possible plankton. They also feed on algae to synthesize omega 3 (Eicosapentaenoic acid-EPA and Docosahexaenoic acid-DHA) for their bodies (Atkinson et al. 2004; Bettina et al. 2009).

Krill is one of the most abundant animal species due to its existence in oceans around the world. As a result, the focus has been on the use of Krill in aquaculture, sport fishing and as a commercial product in the form of bait in the aquariums (Tou et al. 2007; Vacchi et al. 2012). Although widely known as whale food, it is also a food source for seals, seabirds, fish, and, to a lesser extent, humans. Of the different species of krill, only two species of Antarctic (Euphausia Superba) and Pacific (Euphausia Pacifica) Krill are collected as commercial products (Nicol and Endo, 1997). Commercial krill products for human consumption are mostly in the form of frozen raw krill, boiled krill, and peeled krill meat. The use of krill as a food source for humans is expected to increase with the development of new products and technological advances (Tou et al. 2007).

Krill oil for human consumption is a newer product compared to fish oils. Krill oil was started to be used all over the world as a food supplement in the early 21st century (Schiermeier 2010). Krill oil has a better nutritional value in terms of the chemical composition of fatty acids and other antioxidant content than fish oils. There are basically four ingredients that make krill oil superior to other oils: The krill oil is in the form of phospholipids, its ORAC (oxygen radical absorption capacity) value is high, it has a high omega-3 and astaxanthin content (Farooqui and Farooqui 2009; Schuchardt et al. 2011). Thus, the use of krill oils as food and antioxidant supplements to support the physiological processes becoming more popular around the world. However, as with fish oils, heavy metal contamination is one of the biggest doubts about the safe use of krill oils.

The habitat of krill is mostly in the cold oceans of southern Antarctica and Antarctic Krills do not face heavy metals and pollutants like other fish because they live away from industrial areas (Tou et al. 2007). Therefore, it is accepted that krill oil should not contain heavy metals, considering that it is obtained from environments where there are no heavy metal contaminations and toxic wastes. The companies state that they generally obtain krill oil from Antarctic krill, but do not provide any assurance for the heavy metal content in the pharmaceutical products they sell. In this case, it is important to determine the safety and suitability of the conditions of the commercial krill oil products for sale. Thus, the main subject and scope of this research were to investigate whether commercially available krill oils were contaminated with heavy metals and toxic residues.

MATERIAL and METHODS

Commercial krill oils from 11 brands with the largest market share were selected. The selected brands are either directly sold in the European Union (EU) or packaged in Turkey from the krill oils directly acquired from the EU. These commercial krill oil samples were purchased randomly from the pharmacies, and these samples were analysed in the current study. Production dates and storage conditions of the krill oil samples were checked prior to the purchase. The samples were stored in appropriate conditions (refrigerated at +4 ºC) until the analysis. Each sample was measured twice for security purposes. Measurements were carried out at Ege University, Pharmaceutical Development Pharmacokinetics Research and Application Centre, Environment and Food Analysis Laboratory (ARGEFAR). The laboratory has been accredited by the Turkish Accreditation Agency, according to the TS EN ISO/IEC 17025: 2017 standards. The accredited certificate of the institution continues to be valid until March 14, 2023. The acidity values, peroxide levels, fatty acid contents, and heavy metal concentrations were determined according to TS EN ISO 660, TS EN ISO 3960, TS EN ISO 12966-4 and TS EN ISO 12966-2, and TS EN ISO EC 17025 AB-0040-T analyses, respectively.

RESULTS

From the 11 commercial brands tested, total of 25 fatty acids were tested. The types and amounts of tested fatty acids were in Table 1. A total of 12 saturated and 5 omega-3 fatty acids were found in the krill oil samples. Among the saturated fatty acids, Palmitic acid was the highest fatty acid present in 10 out of 11 samples. Among the 11 different brands of krill samples, EPA and DHA were the highest omega-3 fatty acids except for sample X10. Alarmingly, in the 10th sample, EPA and DHA levels were lower compared to the expected levels in krill oils, while the highest omega-3 fatty acid was Linolenic acid (Table 1).

The samples X2 and X5 had the highest level of saturated fatty acids (41.207% and 43.039%, respectively). The lowest saturated fatty acid was in sample X7 (7.547%). Accordingly, the highest EPA and DHA content were in sample X7 (42.454% and 26.993%, respectively). The lowest EPA and DHA content were in sample X10 (4.102% and 3.577%, respectively), and these levels of EPA and DHA were

very low for standard krill oils. The highest level of omega 6 fatty acids was Linoleic acid in all samples tested except for sample X7. Furthermore, the Linoleic acid content of sample X10 was very high (41.722%; Table 1).

The peroxide levels were in Table 2. The overall peroxide levels for the samples tested were high. The lowest levels of peroxide were 10 mEq O2/kg, and the levels raised up to 30 mEq O2/kg in samples X7 and X10.

The heavy metal contents of commercial krill oils tested in the current study were in Table 2. Overall, except for one sample (X7), the arsenic levels tended to be high and varied between 0.028 and 9.824 mg/kg among the tested samples. The mercury levels were highest in X1 (0.041 mg/kg), whereas no traces of mercury were detected in X10. In general, the cadmium levels were low and ranged between 0 to 0.012 mg/kg. The lead levels were very high in all samples tested. The lowest levels of lead were in X8 (0.157 mg/kg), while X4 had the highest levels of lead (0.435 mg/kg).

Table 1. Fatty acid (FA) contents of the commercial krill oils tested in the current experiment.

Types of FA	Names of FA	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
Saturated FA (%)	Caprylic acid (C8:0)	-	-	-	0.319	-	-	0.328	-	0.438	0.004	0.007
	Capric acid (Cl0:0)	-	-	-	0.277	-	-	0.282	-	0.403	0.007	0.004
	Lauric acid (Cl2:0)	0.193	0.248	0.231	0.239	0.277	0.223	0.009	0.116	0.264	0.040	0.102
	Tridecanoic acid (C13:0)	0.050	0.083	0.079	0.056	0.067	0.078	0.002	0.032	0.092	0.014	0.029
	Myristic acid (Cl4:0)	8.875	13.517	12.101	12.842	14.466	11.926	0.268	6.917	12.787	2.256	5.818
	Pentadecanoic acid (C15:0)	0.397	0.558	0.471	0.446	0.516	0.518	0.030	0.279	0.481	0.178	0.294
	Palmitic acid (C16:0)	26.05	27.69	26.72	26.87	26.71	27.07	3.350	18.99	26.32	19.72	18.24
	Heptadecanoic acid (Cl7:0)	-	-	-	-	0.179	-	0.352	0.829	0.245	-	-
	Stearic acid (C18:0)	-	-	-	-	1.920	-	4.197	3.913	-	-	-
	Arachidic acid (C20:0)	0.032	0.031	0.026	0.026	0.109	0.024	0.964	0.351	-	0.339	0.426
	Behenic acid (C22:0)	0.035	0.042	0.048	0.021	0.047	0.041	0.078	0.170	0.062	0.035	0.135
	Tricosanoic acid (C23:0)	-	-	-	-	0.580	-	-	-	0.319	-	-
Total SFA %		34.929	41.207	38.825	39.715	43.093	38.994	7.547	29.821	39.104	21.972	24.054
Omega-3 FA (%)	Linolenic acid (C18:3n-3)	3.479	2.227	2.109	1.990	1.256	2.761	1.132	2.053	2.918	6.165	2.317
	Trans-Linolenic acid (C18:3n6)	0.192	0.235	0.218	0.237	0.189	0.215	0.221	0.307	0.252	0.069	0.323
	cis-8,11,14-Eicosatrienoic acid (C20:3)	0.090	0.090	0.122	0.097	0.132	0.085	0.393	0.192	0.119	0.049	0.283
	cis-11,14,17-Eicosatrienoic acid (C20:3)	0.404	0.379	0.327	0.267	0.243	0.361	0.621	0.257	0.371	0.066	0.298
	Eicosapentaenoic acid (C20:5n3)	28.413	18.532	22.300	21.461	15.734	21.941	42.451	22.181	20.685	4.102	23.379
	Docosahecsaenoic acid (C22:6n3)	13.419	8.524	9.796	8.651	7.157	11.195	26.993	12.387	11.206	3.577	14.601
Omega 3 %		45.311	29.283	34.205	32.102	24.147	35.897	70.576	36.621	34.809	13.844	40.297

Table 1 (Cont). Fatty acid (FA) contents of the commercial krill oils tested in the current experiment.

Types of FA	Names of FA	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
Omega-5 FA (%)	Myristoleic acid (Cl4:1)	0.157	0.208	0.210	0.195	0.203	0.190	0.005	0.101	0.210	0.014	0.076
Omega-6 FA (%)	Linoleic acid (C18:2n6)	2.838	3.777	3.003	3.126	3.922	3.494	1.520	3.332	3.298	41.722	4.169
	Arachidonic acid (C20:4n6)	0.393	0.408	0.411	0.393	-	0.381	3.284	1.400	-	0.273	1.762
Omega-7 FA (%)	Palmitoleic acid (C16:1n7)	4.439	7.774	7.727	8.293	9.546	5.904	1.350	6.219	6.271	2.456	5.748
Omega-9 FA (%)	Oleic acid (C18:1n9)	9.821	14.720	12.451	13.280	15.637	12.832	8.054	17.281	12.282	18.513	18.471
	Gondoic acid (C20:1n9)	0.668	0.898	0.897	0.886	1.114	0.745	4.119	2.692	0.860	0.286	3.199
	Nervonic acid (C24:1)	0.051	0.055	0.048	0.025	-	0.017	-	-	0.121	0.122	0.324

Table 2. Peroxide content and heavy metal levels of the commercial krill oils tested in the current experiment.

Parameters	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
Peroxide (meq O_2/kg-oil)	10.0	10.0	10.0	10.0	10.0	10.0	30.0	10.0	10.0	30.0	10.0
Arsenic (As-mg/kg)	3.348	3.492	1.126	3.509	2.703	9.824	0.028	2.017	7.694	0.572	1.451
Mercury (Hg-mg/kg)	0.041	0.027	0.015	0,010	0.005	0.009	0.002	0.026	0.003	0.000	0.001
Cadmium (Cd-mg/kg)	0.003	0.005	0.001	0.001	0.004	0.012	0.000	0.005	0.003	0.001	0.002
Lead (Pb-mg/kg)	0.364	0.287	0.358	0.435	0.229	0.216	0.191	0.157	0.254	0.184	0.187

DISCUSSION

Today, the characteristic Western diet includes mass amounts of saturated fat, trans-fatty acids, and increased omega-6: omega-3 fatty acid ratio. It has been estimated that the diet of industrialized societies is deficient in omega-3 fatty acids. Moreover, the omega-6:omega-3 ratio is about 15-20:1, instead of 1:1 compared with the diet on which human beings used to have (Simopoulos, 2008). As a result, omega-3 sources such as fish and krill oils are suggested as a supplement for human diets and placed in the market for human use. The current experiment showed that 10 out of 11 commercial krill oil products had EPA and DHA content comparable to the Codex standard for krill oil (Codex Standards 2017; Xie et al. 2019).

The Myristic acid (C14:0), Palmitic acid (C16:0), Palmitoleic acid (C16:1), Oleic acid (C18:1), EPA (C20:5), and DHA (C22:6) are the major fatty acids in krill oils (Phleger et al., 2002; Xie et al., 2017). The Codex standards (2017) reports the common fatty acids and their percentages in krill oil as saturated fatty acids [Myristic (5-13%) and Palmitic acids (17-24.6%)], omega 3 fatty acids [Eicosapentaenoic acid-EPA (14.3-28%) and Docosahecsaenoic acid-DHA (7.1-15.7%], omega 6 [Linoleic acid (0-3%], omega 7 [Palmitoleic acid (2.5-9%)] and omega 9 [Oleic acid (6-14.5%)]. In the current study, the fatty acid contents of the commercial krill oils tested varied to quite an extent. When compared with the Codex standards (Codex Standards 2017), the Myristic acid levels of 4 samples were not within the limits (samples X2, X5, X7, and X10). Only 3 krill oil samples had standard Palmitic acid content (samples X8, X10, and X11). The major omega 3 fatty acids in krill oils, EPA and DHA, were within the Codex standards in 9 out of 11 samples, while the amounts of EPA and DHA were higher in sample X7 and lower in sample X10 compared to the standards (Codex Standards 2017). A total of 8 krill oil samples differed in Linoleic acid contents (samples X2, X4, X5, X6, X8, X9, X10, and X11) against the standards. Nevertheless, except for two samples (samples X7 and X10), these variations were not quite prominent and within the tolerable limits. The 7th sample had very low saturated fatty acid and high omega 3 levels, whereas the 10th sample had very low omega 3 and high omega 6 content compared to standard krill oil (Codex Standards 2017).

Different studies stated that fatty acid content and concentrations differ seasonally due to factors such as sexual maturity, reproduction, water depth, and phytoplankton abundance/quality at different times of the year (Phleger et al. 2012; Reiss et al. 2015; Schmidt et al. 2014). In addition, the storage conditions, transportation processes, and pre-treatment methods of raw materials also have an influence on the lipid composition of krill oil extracted (Sun et al. 2017; Tilseth and Hostmark 2015; Yin et al. 2015). However, very high omega 6 and very low omega 3 contents in the 10th sample were unacceptable since the main reason people buy these kinds of commercial oils for their presumably high omega-3 contents.

Nowadays, most of the people in modern society visualize themselves living a long and healthy life. Therefore, there are plenty of commercially available products that offer longer and healthier life. Among them, krill oils draw attention in recent years due to high and biologically available EPA and DHA content. The positive health effects of omega-3 fatty acids, especially EPA, and DHA, have come to the attention when diets of the Greenland Eskimos were explored (Bang et al. 1971). The Eskimos had high intakes of omega-3 rich seafood diet and very low rates of inflammatory and autoimmune diseases, asthma, cardiovascular disease, and multiple sclerosis. Nowadays we know that omega-3 fatty acids have several beneficial health effects on human health such as psoriasis, cardiovascular disease, cancer, and rheumatoid arthritis (Yashodhara et al. 2009). However, the fatty acid composition in the 10th sample is alarming and suggests that better control of commercial products is needed.

High peroxide levels in oils are one of the parameters that suggest a deterioration in the products. Although peroxide levels are not the only factor to decide deterioration levels of oils, in general, the high peroxide concentrations detected in oils suggest the deterioration and rancidity in the oils. The peroxide levels should be maximum of 5 mEq O2/kg according to the Krill Codex Standards (Codex Standards 2017). However, all tested samples had higher peroxide content than the standards: 9 out of 11 samples contained peroxide levels of 10 mEq

O2/kg, and the remaining 2 samples had peroxide levels of 30 mEq O2/kg. The high peroxide content of tested samples suggested that there could be a deterioration in these products before packaging since there was no problem with the expiration dates of these products.

According to Codex Alimentarius, maximum tolerable limits for arsenic and lead in edible fats and oils are 0.1 and 0.08 mg/kg, respectively. In this case, it is alarming that almost all commercial krill oil products tested contained more than the tolerable limits of arsenic and lead. Allowable limits of mercury and cadmium in food supplements are 0.1 and 1.0 mg/kg, respectively (Codex Alimentarius 2019). All the tested samples contained below the appropriate levels of mercury and cadmium.

Heavy metals in human foods and supplements can and will negatively affect consumers and human foods should be free of heavy metals for health purposes. Normally, krill oil should be extracted from the krill harvested in clean deep-water seas far from industrial developments in the Southern Atlantic Ocean. In addition, krill is near the lowest end of the food chain. Therefore, krill and krill oil should not accumulate heavy metals and other pollutants. However, previous studies showed pesticide residues in krill products (Corsolini et al. 2006; Covaci et al. 2007). Similarly, almost all samples examined in the current study had arsenic and lead contaminations that could be harmful to human health.

Heavy metal contamination of krill oil is an important risk and how these kinds of contaminations took place needs to be studied. One of the possibilities for heavy metal contamination in krill oil is the actual source of krill. The krill used for krill oil production might have caught from the oceans closer to the industrial areas rather than the South Atlantic Ocean or contaminated during the shipping process. Another possibility that explains the contamination was that the products may have been contaminated with these heavy metals during the extraction, production, and/or packaging phases of krill oils in the factories. In both cases, people buy these kinds of supplements to eat right and stay healthy. Therefore, leaving aside commercial concerns, it is inevitable to make the necessary adjustments to create a healthier production environment. The methods and techniques used to obtain oil should be checked again. Factors that may harm human health and cause contamination should be removed from the production lines of factories. A problem that may occur in any link of the food chain affects all living things. People should carefully use heavy metal residues that cannot be easily destroyed in nature, considering other animals. Wastes in production areas should be isolated in a way that does not pollute the environment. The worst scenario would be that humanity already started to pollute Antarctica and therefore started to harm even the creatures living in Antarctica.

Antarctica is supposedly one of the most perfectly conserved regions on the planet (NISTAER 2014). Since there is no industrial area in or near Antarctica, it is unlikely that heavy metal residues will contaminate the krill population. Antarctica has been historically considered a remote and untouched continent. However, long-range transport of pollutants from other continents, local activities in research stations, and the Antarctic tourism industry have introduced many contaminants to the region (Bargagli 2008). Understanding the distribution of trace pollutants such as heavy metals and organic pesticides in Antarctica is considered a top research priority for the next decades (Kennicutt 2015). Paralleled to these concerns, a recent study, unfortunately, reported the presence of pesticides and mercury in mosses, topsoil, and water in Antarctica (Subhavana et al. 2019). Liu et al. (2021) also studied the concentrations of some heavy metals (such as Al, Cr, Pb, Hg, and As) in soil samples collected in East Antarctica. Although the contamination level of these heavy metals was relatively low, the elevated levels of heavy metals compared to the baseline concentrations started to be alarming for this pristine continent (Xu et al. 2020). Areas with high mercury and lead levels corresponded to the station areas that researchers or tourists live and bird colony areas. The rise in mercury and lead around bird population was attributed to the biomagnification of birds' food chain, and various types of anthropogenic activities (Li et al. 2020; Liu et al. 2021).

CONCLUSION

The presence of heavy metals in most of the samples exceeding the WHO and EU standards for oils and food additives poses a significant risk for public health. Furthermore, the varying degrees of alterations in the fatty acid composition from the codex standards for krill oils also create a handicap for food safety. Moreover, the presence of some heavy metals above the tolerable levels in some of the tested krill oil samples indicates that the risk is quite important from a public health point of view. The presence of heavy metals as well as the differences in the fatty acid composition between the statements in companies' prospectus and the values from the analysis is disturbing. These discrepancies suggest that the regulatory mechanisms are required to be more sensitive in monitoring dietary supplements. Thus, more stringent and sustainable control of the compliance of these products with WHO and EU standards is required by the authorities. All food supplements should be strictly controlled whether they comply with the values specified in the packaging and prospectus content. It can also be recommended to develop online control processes for consumers.

Conflict of interest: The authors declared that there is no conflict of interest.

Ethical Approval: : This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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RESEARCH ARTICLE

Detection of Canine Distemper Virus from Ocular Swab and Blood Samples in Dog by Real-Time RT-PCR Method

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ABSTRACT

CDV is a highly contagious, systemic and viral infection encountered all around the world. This study aimed to investigation of the the prevalence of CDV seen in dogs in Burdur. For this purpose, ocular swab and blood samples were taken from 65 CDV pre-diagnosed dogs that were brought into Mehmet Akif Ersoy University Veterinary Faculty clinics. Real-time RT-PCR method was applied on the subject samples in order to get a laboratory diagnosis of CDV. In total, 42 of these, 16 from blood and 26 from ocular swab, were detected as positive by real-time RT-PCR. Nine of them were detected as positive both in ocular swab and blood samples. In addition, 11 of 42 samples (33 animals) detected as positive by real-time RT-PCR were already known to have been taken from dogs vaccinated against CDV previously. In the study, test results of the samples taken from diseased dogs and their relations with vaccine history were evaluated statistically as well. In the light of these results, it is recommended to use ocular swab samples as materials in diagnosing CDV and examining all animals (including those vaccinated) with clinical findings in terms of this infection so as to confine the disease.
Keywords: Canine distemper virus, epidemiology, Real-time RT-PCR

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Real Time RT-PCR Metodu ile Köpeklerin Oküler Swap ve Kan Örneklerinden Canine Distemper Virusunun Tespiti

ÖZ

Canine distemper virus (CDV- köpek gençlik hastalığı) çok bulaşıcı, sistemik ve dünya genelinde yaygın olarak görülen viral bir enfeksiyondur. Bu çalışma ile Burdur'da köpeklerde görülen CDV'nun yaygınlığının araştırılması hedeflenmiştir. Bu amaçla Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi kliniklerine gelen hasta hayvanlardan CDV ön tanısı konulmuş 65 köpekten oküler swap ve kan örnekleri alındı. Söz konusu örneklere CDV laboratuvar tanısı koymak amacıyla real-time RT-PCR metodu uygulandı. 16 adeti kan, 26 adeti oküler swaptan olmak üzere toplamda 42 tanesi real time-RT-PCR ile pozitif olarak belirlendi. Dokuz adeti hem oküler swapta hemde kan örneklerinde pozitif olarak tespit edildi. Ayrıca real-time RT PCR ile pozitif tespit edilen 42 örneğin (33 hayvan) içerisinden 11 tanesinin daha önceden CDV'ye karşı aşılanmış köpeklerden olduğu bilinmekteydi. Araştırmada hasta köpeklerden alınan örneklerin test sonuçları ve aşı geçmişleri ile ilişkileri istatistiki olarak da değerlendirildi. Bu sonuçlar ışığında, CDV'nun teşhisinde materyal olarak oküler swap örneklerinin kullanılması ve hastalığı sınırlandırmak için klinik bulgu gösteren tüm hayvanların (aşılılar dahil) bu enfeksiyon yönünden incelenmesi tavsiye edilmektedir.
Anahtar Kelimeler: Canine distemper virus, epidemiyoloji, Real-time RT-PCR

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INTRODUCTION

CDV belongs to Morbillivirus type taking part in Paramyxovirinae subfamily of Paramyxoviridae family (ICTV, 2019). This virus is a single-stranded RNA virus frequently causing fatal infections in domestic dogs. Its genome is approximately 15.690 nucleotide (nt) long and its nucleoprotein (NP) consists of six gene areas coding phosphoprotein, matrix, fusion, haemagglutinin (H) and large proteins (Yi et al. 2012). CDV is considered the most important reason for various deaths of wild racoons (Procyonlotor), grey foxes (Urocyoncinereoargenteus) and striped skunks (Mephitismephitis), as well as affecting the species in all terrestrial carnivore families, including Canidae, Felidae, Hyaenidae, Mustelidae, Procyonidae, Ursidae and Viverridae (Roscoe, 1993). The mentioned terrestrial carnivores play an important role in the epidemiology of the disease (Deem et al. 2000).

CDV, a multisystemic disease, has a complex pathogenesis that might cause various pathological findings such as pneumonia, enteritis and encephalitis. While mortality rates differ between species, they also differ within species (Appel and Summers 1995, Weckworth et al. 2020). Virulence of the agent depends on the strain of the virus, individual immune state, immune response and age of the animal. The death rate among young ones is higher than that of adult dogs (Lednicky et al. 2004, Martella et al. 2008).

The incubation period of CDV might vary between 1-4 weeks (Beineke et al. 2009). Initially, no clinical findings are observed in 25%-75% of the infected animals, yet as the disease progresses, neurological symptoms become evident and death may occur (Pope et al. 2016). CDV primarily infects respiratory lymphoid tissues. During the first viremic phase, where high viral replication is seen, a systemic transmission appears thanks to lymphoid tissues, and this causes immunosuppression and fever (Martella et al. 2008, Beineke et al. 2009). The second viremic phase appears 6-9 days following the infection and contains the generalized infection of parenchymal and epithelial cells (Martella et al. 2008, Beineke et al. 2009, Kapil and Yeary 2011). 1-3 weeks after clinical findings appear, neurological findings typically occur (Kapil and Yeary 2011).

Acute infected animals spread the virus in all their body fluids regardless of whether they show clinical symptoms or not (Appel and Summers 1995). The virus remains persistent in neurons, urothelium and footpads and continues to spread during 15-90 days after recovering from acute infection (Martella et al. 2008, Kapil and Yaery 2011, Pope et al. 2016).

The clinical diagnosis of distemper disease seen in dogs is difficult, for it has a wide range of symptoms that might be confused with other respiratory and intestinal diseases. Laboratory confirmation is needed for suspicious cases (Elia et al. 2016). Urine (Saito et al. 2006), stool (Tupler et al. 2012), nasal discharge (Kim et al. 2001), blood (Cho and Park 2005) and cerebrospinal fluid (Frisk et al. 1999) can be used as diagnosis materials. In the diagnosis of the CDV disease, various methods such as electron microscopy (Sun et al. 2010), immunoperoxidase (Soma et al. 2001), ELISA (Suzuki et al. 2015) and PCR (Shin et al. 2004) are used.

In this study, we aimed to detect CDV genome presence by real-time RT-PCR test in ocular swab and blood samples of 65 dogs which were brought into Mehmet Akif Ersoy University Veterinary Faculty clinics and most of which had neurological symptoms such as unilateral paralysis, diplegia in legs, seizures, myoclonus, rhythmic tonic and clonic convulsions.

MATERIAL and METHODS

Ocular swab and blood samples were collected from a total of 65 dogs which were brought into Mehmet Akif Ersoy University Veterinary Faculty animal hospital between 2015-2020 with symptoms such as respiratory disorders, cough, neurological disorders, ocular/nasal flow, hardening of nose and plantar and sent to Virology Department for diagnosis.

Trizol®Reagent (Invitrogen/Life Technologies) kit was used for viral RNA extraction of all samples. The obtained viral nucleic acid was subjected to the following thermal cycle program by Applied Biosystems One Step (ABD) device. A Real-time PCR test was performed by targeting the gene area that codes N protein in terms of CDV (Table 2 and 3). An 83 bp fragment of N gene area could be replicated by primer-probe sequences given in Table 1.

RESULTS

Ocular swab and blood samples were sent for diagnosis, and CDV genome presence from 65 samples of dogs clinically suggestive of CDV was searched by real-time PCR method. At the end of the study, a total of 42 samples, 16 blood and 26 ocular swab samples, were detected as positive by real-time RT-PCR (Figure 1). 9 of these had positive genome presence following RT-PCR, both in blood and ocular swab samples. Furthermore, 11 of 42 samples (33 animals) detected as positive by real-time RT-PCR were already known to have been taken from dogs vaccinated against CDV previously.

As a result of the applied chi-square test, a significant difference was found statistically between the vaccinated and unvaccinated animals ($p<0.05$). Accordingly, it was believed that unvaccinated animals had a higher possibility of catching the disease than vaccinated ones (66.7%) and not a significant difference but vaccinated animals also had the possibility of getting sick (33.3%). (Table 4) According to chi-square test performed among blood and ocular samples, no significant difference could be

detected ($p>0.05$), and when evaluated by percentage, ocular samples showed a higher positivity (40%) than blood samples did (24%).

Table 1. Primer/probe sequences (Elia et al. 2006)

Oligonucleotides Sequences (5'-3')	
CDV forward	AGCTAGTTTCATCTTAACTATCAAATT
CDV reverse	TTAACTCTCCAGAAAACTCATGC
CDV probe	FAM-ACCCAAGAGCCGGATACATAGTTTCAATGC-TAMRA

Table 2. Thermal cycle program of real-time PCR

	Temperature	Duration	
Reverse Transcription	50 °C	10min.	1
RT-Inactivation	95 °C	2min.	1
PCR	95 °C	15sec.	50x
	55 °C	30sec.	

Table 3. Concentrations of reaction mixture

Each tube	Master Mix
12.5 µl	4X qRT-PCR Master Mix (Verso-1 step-Thermo)
1.25 µl	Enzyme Mix
1.25 µl	RT-enhancer
1.5 ul (9 mM)	$MgCl_2$ (25mM)
0.50µl (0.4µM)	Forward Primer (20 µM)
0.75µl (0.6µM)	Reverse Primer (20 µM)
1.25 µl (0.25µM)	Probe (5 µM)
0.5 µl µl (0.25µM) (50 nM)	ROX
3.5 µl	H_2O
3 µl	Template RNA
25 µl	**Total reaction mix**

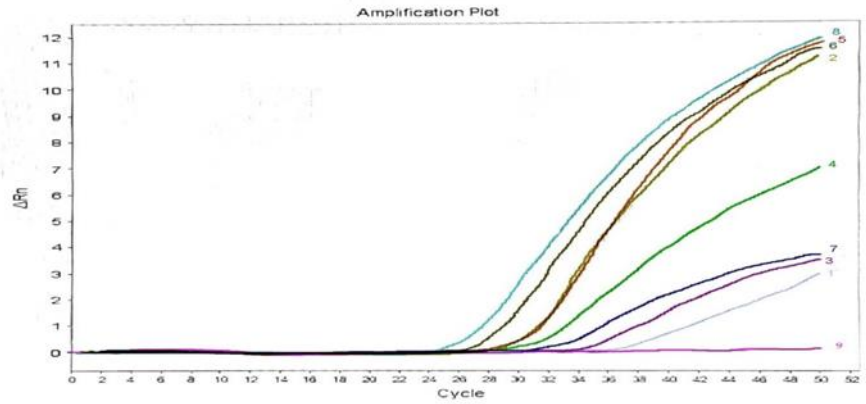

Figure 1: 1,3: Blood sample 2, 4, 5, 6, 7: Ocular swab sample 8: Positive control (MLV vaccine) 9: Negative control Unreal-time PCR results

Table 4. Results of CDV presence by real-time PCR from blood and ocular swab samples of vaccinated and unvaccinated animals.

Sample		**State of vaccination**			
		Vaccinated		**Unvaccinated**	
		n	**%**	**n**	**%**
Blood	**Positive**	3	9,1	13	39,4
	Negative	8	24,2	9	27,3
	Total	11	33,3	22	66,7
Ocular	**Positive**	10	30,3	16	48,5
	Negative	1	3,0	6	18,2
	Total	11	33,3	22	66,7
Statistical values: x^2=9,440 p=0,02					

DISCUSSION

Blood and ocular swab samples collected from 65 dogs suggestive of CDV between 2015-2020 in Burdur province were sent to the virology laboratory for diagnosis. Real-time RT-PCR examined these samples in terms of CDV genome presence, which was detected in 42 samples. 11 of CDV positive samples were reported to belong to vaccinated dogs (according to clinical records). In the studies previously carried out worldwide on this topic, CDV epidemics were reported many times (Laurenson et al. 1998, Nouvellet et al. 2013, Fischer et al. 2016, Ricci et al. 2021). The obtained results were such as to support previous studies. In one of these studies (Budaszewski et al. 2014), CDV positivity was found as 12.2% (19/155) in vaccinated dogs while it was 26.19% (19/155) in our study. Various factors such as suppression of immunity, application of wrong protocols, unsuitable storage conditions, the occurrence of poor immune response and appearance of new antigenic variants capable of escaping from antibodies created by vaccines are among the reasons why vaccinated dogs developed CDV epidemics (Lan et al. 2006). In their study in Brazil, Budaszewski et al. (2014) detected CDV genome presence by PCR test in dogs vaccinated with live vaccine and showing clinical symptoms. The researchers sequenced the obtained isolates and compared them with known CDV vaccine strains. As a result, they revealed that the circulated field strains were different from vaccine strains.

CDV is one of the most important diseases progressing with high morbidity and mortality rates, especially in 3-6-month-old dogs (Feijoo et al. 2019). Although owned dogs are commonly vaccinated, there is no special vaccination program for stray dogs, which constitutes a crucial risk for the disease (Adam et al. 2011). In addition, this study and the previous ones show that CDV cases continuously increase despite vaccination. Therefore, to prevent the spread of the virus and control the number of affected dogs, a timely diagnosis is vital. RT-PCR procedures have recently been developed for fast and reliable diagnosis of CDV (Shin et al. 2004, Yi et al. 2012, Piewbang et al. 2016). In Turkey, its epidemiological presence has been revealed by serological tests such as ELISA (Saltık and Kale 2020) and neutralization tests (Gencay et al. 2004). When we look at the recent studies, even though there are many studies in our country for diagnosing respiratory and digestion system infections (Avci et al. 2016, Aydin et al. 2018, Dinçer 2017, Polat et al. 2019, Timurkan et al. 2018, Timurkan et al. 2021), those about CDV are quite limited (Oğuzoğlu et al. 2018). During these limited amounts of studies, CDV genome presence has been revealed by RT-PCR method. This study is highly significant because that it is one of the first ones in which CDV genome presence has been revealed by real-time PCR.

Although real-time PCR test is an expensive one, it has begun to take the place of conventional PCR since it has a high specificity and sensitivity and requires no process following the reaction; thus the risk of contamination is low (Elia et al. 2006, Pawar et al. 2011). In diagnosing CDV, quite many samples can be taken from animals such as blood (Shin et al. 2004, An et al. 2008), ocular and nasal swab (Saito et al. 2006, An et al. 2008), urine (Shin et al. 2004), vaginal swab (Fischer et al. 2013), lung (Headley et al. 2000, Shin et al. 2004), stomach (Headley et al. 2000), kidney (Namroodi et al. 2013) and bladder (Richter and Motze 1970) tissues. Kim et al. (2006) detected positivity at an important high rate in ocular swab samples of seven dogs infected experimentally. In our study, a higher positivity was also detected similarly in samples collected with an ocular swab (Table 4). They stated that CDV was detected in blood at late stages. Thus blood was not the correct material for early diagnosis, and conjunctiva remained infected for a longer period by being infected earlier than viremia. Furthermore, they associated the reason for this with the exclusion of conjunctiva out of the immune system. Similarly, Shabbir et al. (2011) considered conjunctival swab more reliable than nasal swab and plasma in early diagnosis of CDV. Using a conjunctival swab, Van Drunen et al. (2008) found that the sensitivity and specificity of the immunochromatographic test (IC) was 100% compared to RT-nested-PCR. Lymphocyte and nasal swabs showed low sensitivity and specificity rates. Researchers probably stressed that it was the most suitable sample for early diagnosis of CDV since CDV continuously spread in the eye, which was different from the other samples.

Among the reasons for vaccination failure were various factors such as the quality of the vaccine, the appearing poor immune response and CDV genetic diversity, and the fact that serologically and genetically different variants appeared was considered the basic factor for vaccines not to be able to protect CDV (Martella et al. 2006). Simon- Martinez et al. (2008) stated that H and F proteins, the target proteins in the host immune response, underwent the highest genetic/antigenic changes in CDV. In this study, the fact that vaccinated dogs could also be infected with the disease suggests that gene areas coding H and F proteins of the obtained CDV isolates should be examined in terms of mutations. These isolates should be compared with the existing ones MLV vaccine strains. In this sense, important contributions will be given for developing significant control studies in fighting the disease.

As a result, stray dogs being in the first place, all unvaccinated animals should be considered a potential source of CDV. The disease is mainly characterized by the nervous system and respiratory system symptoms. The infection was also encountered in vaccinated dogs might be ineffective vaccinations and the probability of infection with the

mutant virus. In order to put this forward certainly, advanced phylogenic studies with CDV are needed so that newly circulated variants could be detected and vaccination failures and spreading of the disease could be prevented.

Conflict of Interest: The authors declare that there is no conflict of interest.

Ethical Permission: This study was found in accordance with the principles of the Ethics Committee of Burdur Mehmet Akif Ersoy University with the decision of "Animal Experiments Local Ethics Committee, dated 20.05.2021 and Decision No: 778.

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RESEARCH ARTICLE

Evaluation of the Effect of Panax Ginseng Administration on YKG1 Glioblastoma Cells Before Cisplatin Treatment in Terms of Apoptotic Cell Death and Cell Vitality

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ABSTRACT

In alternative medicine, natural remedies of herbal origin are often used in addition to chemotherapy-like treatments. Panax ginseng one of them and it' s preferred diseases originating from nervous system. The aim of our study, to evaluate Panax ginseng, which was used before cisplatin, in terms of cell death and viability YKG1 cells. Commercially, purchased YKG1 cells were incubated DMEM F12 medium in 5% CO2 incubator divided into 4 groups. Group-1; control, group-2; 48 hours 50 mg/ml Panax ginseng, group-3; 48 hours 50 mg/ml Panax ginseng followed by Cisplatin treatment and group-4; was determined as the only cisplatin-treated group. Cell viability assessed by MTT. Bax/Bcl-2 and Caspase3 primary antibodies, were used for immunocytochemical evaluation. In addition, cells were evaluated by PCR tecnique with using primers Bcl-2 and Caspase-3. According to the immunocytochemistry, while Bax immunoreactivity didn't change into the groups, Bcl-2 decreased in groups 3-4, and Caspase-3 increased in groups 3-4, and PCR results were also supportive with our immunohistochemical results. According to MTT results, cell viability rates decreased in groups 3-4. In our study, it was observed that the use of Panax ginseng, which used as a therapeutic and prophylactic in alternative medicine, didn't change the Bax/Bcl-2 ratio in YKG1 cells, but it could trigger apoptosis increasing Caspase-3 activity together Cisplatin.

Keywords: Bax/Bcl, Caspase 3, Glioblastoma, Panax ginseng, Cisplatin.

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Sisplatin Tedavisi Öncesi Panax Ginseng Uygulamasının YKG1 Glioblastoma Hücrelerine Olan Etkisinin Apoptotik Hücre Ölümü Ve Hücre Canlılığı Açısından Değerlendirilmesi

ÖZ

Alternatif tıpta kemoterapi benzeri tedavi yöntemlerine ek olarak sıklıkla bitkisel kökenli doğal ilaçlar kullanılmaktadır. Panax ginseng bunlardan biridir ve sinir sistemi kaynaklı hastalıklarda tercih edilmektedir. Çalışmamızda amaç, sisplatinden önce kullanılan Panax ginsengin YKG1 glioblastoma hücrelerinde hücre ölümü ve hücre canlılığı bakımından değerlendirilmesidir. Ticari olarak satın alınan YKG1 hücreleri, % 5 CO2 inkübatörde DMEM F12 besi yerinde inkübe edildi ve 4 gruba ayrıldı. Grup 1; kontrol, grup 2; 48 saat 50 mg/ml Panax ginseng, grup 3; 48 saat 50 mg/ml Panax ginseng ve ardından Sisplatin tedavisi ve grup 4; sadece Sisplatin uygulanan grup olarak belirlendi. Hücre canlılığı MTT analizi ile değerlendirildi. İmmünositokimyasal değerlendirmede apoptotik belirteçlerden Bax/Bcl-2 ve Caspase-3 primer antikorları kullanıldı. Ek olarak, hücreler PCR tekniği ile Bcl-2 ve Caspase-3 primerleriyle değerlendirildi. İmmünositokimyasal sonuçlara göre Bax immünoreaktivitesi gruplar arasında değişiklik göstermezken, Bcl-2'nin grup 3 ve 4'te azaldığı ve Caspase-3'ün ise grup 3 ve 4'te arttığı görüldü ve PCR sonuçları da destekleyici yönde idi. MTT sonuçlarına göre grup 3 ve 4'te hücre canlılık oranlarının azaldığı görüldü. Alternatif tıpta tedavi edici ve koruyucu olarak kullanılan Panax ginseng'in çalışmamızda, Sisplatin tedavisinden önce kullanılmasının YKG1 hücrelerinde Bax/Bcl 2 oranını değiştirmediği fakat Sisplatinle birlikte Caspase-3 aktivitesini arttırarak apoptozu tetikleyebileceği düşünüldü.

Anahtar Kelimeler: Bax/Bcl, Caspase-3, Glioblastoma, Panax ginseng, Sisplatin.

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GİRİŞ

Glioblastoma (GBM), yüksek morbidite ve mortalite oranlarına neden olan en yaygın ve agresif primer beyin tümörüdür (Coluccia et al. 2018). Tanımlanan başlıca histoplatolojik özellikleri, nekroz ve endotelyal proliferasyon olup, Dünya Sağlık Örgütü (WHO) beyin tümörleri sınıflandırmasında en yüksek derece olan derece IV içinde yer almaktadır (Erkin Özgiray 2021). Standart glioblastoma tedavisi ameliyat, radyoterapi ve alkilleyici kemoterapiyi içerir. Maksimum rezeksiyon, radyasyon tedavisi ve kemoterapiyi takiben bile, ortalama sağ kalım sadece 15 aydır (Komotar, R. J., at all. 2005). Güncel tedavi gelişmeleri, değiştirilmiş sinyal transdüksiyonu, anjiyogenezi baskılama, çeşitli immünoterapi yaklaşımları, kişiye özel alternatif tıp tedavileri de dahil olmak üzere, kötü huylu fenotipi yönlendiren moleküler düzenlemeleri hedeflemeye odaklanmaktadır (Wirsching et al. 2016). Sisplatin (Cis), sitotoksik etkileri iyi tanımlanmış, yaygın kullanılan etkili anti-kanser ilaçlarından biridir ve radyo duyarlılaştırıcıdır (Boeckman et al. 2005, Mjos and Orvig 2014). Bununla birlikte, kan beyin bariyerinden geçmek için yüksek dozlarda Cis kullanımı gereklidir ve genellikle tolere edilemeyen sistemik yan etkileri sebep olmaktadır. Ek olarak, Cis' in yaklaşık %90-%95'i geri dönüşümsüz şekilde plazma proteinlerine bağlanır ve ilaç fraksiyonunun yalnızca serbest kalan % 5-10'nu anti-tümör etkileri gösterecek şekilde uygulanır (Coluccia et al. 2018). Geleneksel Çin tıbbında kullanılan birçok bileşenin tümör oluşumunu önleyebilir, ilaç etkinliği artırabilir ve toksisiteyi azaltabilir, tümör nüksünü ve metastazı azaltabilir, hayatta kalma süresini uzatabilir ve hastaların yaşam kalitesini iyileştirebilir özelliklerinin olduğu bilinmektedir. Paklitaksel-brucea yağı, örneğinde olduğu gibi pazara sunulmuş doğal anti-tümör etkili pek çok aktif bileşen de vardır. Bununla birlikte, şu anda, GBM için geleneksel tıp üzerine nispeten az çalışma bulunmaktadır. (Wang et al. 2019).

Panax ginseng, doğu tıbbında hastalıkların tedavisinde sıklıkla kullanılır ve bitkilerin kralı olarak bilinir. Bitki kökünden kurutularak elde edilen kırmızı ginseng ekstresinde ginsenoidler, polisakkaritler, alkaloidler, glukozitler, fenolik asit ve benzeri birçok aktif bileşenin bulunduğu bildirilmektedir (Wang et al. 2019). Modern farmakolojik çalışmalar, ginsengin anti-adheziv, anti-tümör, anti-diyabetik, yaşlanma karşıtı, nöro-regülasyon, immünomodülasyon gibi bir çok biyolojik aktiviteye sahip olduğunu göstermiştir (Ru et al. 2015). Kırmızı ginsengin endoteliyal hücre invazyonunu ve tüp formasyonunu doza bağlı olarak inhibe ettiği, NF-κB'yi düzenleyerek apoptoza etki ettiği gösterilmiştir (Dai et al. 2017). Tian ve arkadaşları 2015 yılında yaptıkları çalışmada Panax ginseng uygulamasının MNK45 gastrik kanser hücrelerinde Bax/Bcl-2 oranını değiştirerek hücreleri apoptoza sürüklediğini göstermiştir.

Literatür bilgileri tarandığında glioblastoma tümörlerinin tedavisinde Panax ginseng'in apoptotik etkilerini araştıran az sayıda çalışma olduğu görülmektedir. Çalışmamızda, YKG1 hücre hattını kullanarak Sisplatin tedavisinden önce uygulanan Panax ginseng'in hücre canlılığı ve apoptozuna etkilerini immunohistokimyasal olarak ve rtPCR yöntemleriyle incelemeyi amaçladık.

MATERYAL ve METOT

Kimyasalların Hazırlanması

Ticari olarak satin alınan Panax ginseng ekstresi 50µg/ml besi yeri içerisinde hazırlanarak çalışma gruplarına 48 saat boyunca uygulandı. IC50 değeri uygulama dozu daha önce yapılmış uluslar arası makalelerde belirlendiği oranda kullanıldı (Shin et al. 2012). Ticari olarak satın alınan Sisplatin (Sigma-PHR1624) Satıcının talimatlarına göre 1.55µg/ml hazırlandı ve çalışma gruplarına uygulandı (Nakagawa et al. 2007).

Hücre Kültürü

Ticari olarak ATCC'den temin edilen YKG1 hücre hattı kullanıldı. Hücreler, %1 L-glutamin, %1 penicilin-streptomicin, %10 fetal bovine serum içeren DMEM F12 besi yeri içerisinde %70 hücre yoğunluğu elde edilene kadar çoğaltıldı. Dört çalışma grubu belirlendi. Grup 1; kontrol grubu, grup 2, 48 saat boyunca yalnızca Panax ginseng uygulaması, grup 3, 48 saat boyunca Panax+Sisplatin uygulaması ve dördüncü grup 48 saat yalnızca Sisplatin uygulaması yapılacak şekilde tasarlandı. Tüm gruplar için temel besi yeri içerisine Panax ginseng ve sisplatin belirtilen dozlarda ilave edilerek uygulanmıştır.

MTT ile Hücre Canlılığı Tayini

YKG-1 hücreleri 96 gözlü kuyucuklara 1×10^5 hücre olarak ekildi. Her grupta 8 kuyucuk bulunacak şekilde, serum içermeyen besi yerleri uygulama dozlarıyla birlikte 200µL inkübe edildi. Ardından besi yerleri uzaklaştırıldı ve kuyucuklara üreticinin talimatlarına göre hazırlanmış MTT (A3338, BİOMATİK) içeren 110µL besi yeri eklendi 37 °C de 4 saat inkübasyona bırakıldı. İnkübasyon bittiğinde besi yeri uzaklaştırıldı ve kuyucuklara 200µL DMSO ilavesi yapıldı. Yatay karıştırıcıda karanlık ortamda 10 dk inkübe edildi ve 550/650 nm dalga boyu aralığında spektrofotometrede okutuldu (SHIMADZU UV-1601).

İndirekt İmmunositokimyasal Yöntem

%4'lük paraformaldehit ile 30 dk. boyunca hücreler fikse edildi. 3x5 defa PBS (Phosphate Buffered Saline- Fosfat Tampon solüsyonu) ile yıkandı. Permeabilizasyon için %0,1'lik Triton-X 100 15 dk muamele edildi. Ardından %3'lük hidrojen peroksit (H_2O_2) uygulaması ile endojen peroksidaz aktivitesi

inhibe edildi. 3x5 defa PBS ile yıkandı. 1 saat bloking uygulamasından sonra Anti-Caspase 3 (SC-5603, SANTA CRUZ), anti-Bax (SC-526, SANTA CRUZ) ve anti-Bcl-2 (SC- 7283, SANTA CRUZ) antikorları 1:100 oranında dilüe edilerek 1 gece inkübe edildi. 3x5 defa PBS ile yıkama yapıldı. İlk olarak Biotin (30 dk.) ardından Streptavidin (30 dk.) uygulandı ve AEC (TA-125-HA, THERMO) kromojeni ile immunoreaktivitelerin görünürlüğü sağlandı. Kapatma medyumu ile kaplanarak kapatıldı ve fotoğrafları ışık mikroskobu ile çekildi. Gruplardaki hücrelerin immunreaktivite sonuçları boyanma şiddetine göre az, orta ve kuvvetli olacak şekilde sırasıyla (+), (++) ve (+++) şeklinde değerlendirildi ve H-score analizi yapıldı (Numata et al. 2012).

İstatistiksel Analiz

Çalışmanın değerlendirilmesinde tanımlayıcı istatistik olarak medyan, minimum ve maksimum değerler kullanılmıştır (Tablo 1). Grup karşılaştırmalarında Kruskall-Wallis testi, farkı yaratan grup veya grupları belirlemek için çoklu karşılaştırma testlerinden biri olan Dunn's testi kullanıldı. İstatistiksel anlamlılık düzeyi $p<0,05$ olarak alınmıştır. Değerlendirmeler SPSS20.0 paket programında yapılmıştır.

Polimeraz Zincir Reaksiyonu

Hücreler, kültür ortamından pasajlama yöntemiyle kaldırıldı ve RNA izolasyonları yapıldı. Toplanan RNA'lardan cDNA elde edildi. Üreticinin talimatları doğrultusunda SYBR Green Master Mix kit kullanılarak BCL-2, CASPASE-3 ve β-ACTIN genlerinin ekspresyon seviyeleri Real Time PCR yöntemi ile analiz edildi (Biorad CFX 96). CASPASE-3ve BCL-2 genlerinin ifadelenmesinin normalizasyonunu göstermek için β-ACTIN mRNA düzeyleri referans olarak alındı. BCL-2, CASPASE-3ve β-ACTIN mRNA ifade düzeylerindeki farklılıklar "REST (2009 V2.0.13)" istatistik programı ile karşılaştırıldı.

BULGULAR VE SONUÇ

Uygulama Sonrası Hücre Canlılık Oranları

Hücre canlılığının tayini için, daha önce literatür bilgilerinden elde edilen IC50 dozları belirlenerek gruplara uygun olarak uygulandı ve MTT yöntemi kullanıldı. Birinci grup kontrol grubu, ikinci gruba 48 saat boyunca yalnızca panax uygulaması, üçüncü gruba 48 saat boyunca panax+Sisplatin ve dördüncü gruba 48 saat yalnızca Sisplatin uygulaması yapıldı. Çalışma üç kez tekrarlandı ve her tekrarda her gruba ait sekizer adet kuyucuk ortalamaya alındı. Spektrofotometrik sonuçlar değerlendirildiğinde hücre canlılık oranının en yüksek birinci grupta (%81) olduğu görüldü (Tablo 2). Grup 2 de %72 olan canlılık oranı, Grup 3 te % 53, grup 4'te ise %64 tayin edildi (Tablo 2).

İndirek İmmunohistokimyasal Değerlendirme

Avidin-biotin-peroksidaz yöntemi kullanılarak yaptığımız immunohistokimyasal boyama sonuçları H-score yöntemiyle yöntemiyle immunraktivite bakımından değerlendirildi. Caspase-3 immunreaktivitesi için anti-Caspase-3 antikoru kullanıldı. İmmunreaktivite şiddetleri grup 1 de az (+) iken ve grup 2 de orta (++) seviyede görüldü ve grup 3 ve 4'te ise kuvvetli (+++) olduğu görüldü (Figür 1- A, B, C, D).

Sonuçlarımıza göre, anti-Bax antikorunun immunreaktivite seviyesi grup 1, 2, 3 ve grup 4 için az (+) şiddetindeydi (Figür 1- E, F, G, H). Anti-Bcl-2 için gruplardaki immunreaktivite şiddetleri grup 1 ve 2 de şiddetli (+++) seviyede iken, grup 3 ve 4'te ise az (+) seviyesinde kaydedildi (Figür 1- J, K, L, M).

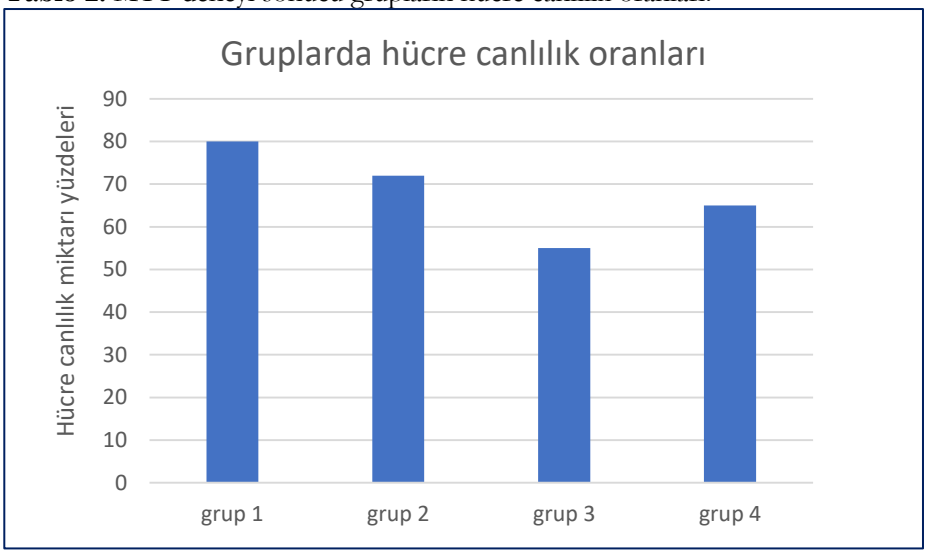
BCL-2, CASPASE-3 Genlerinin Real Time PCR ile Analizi

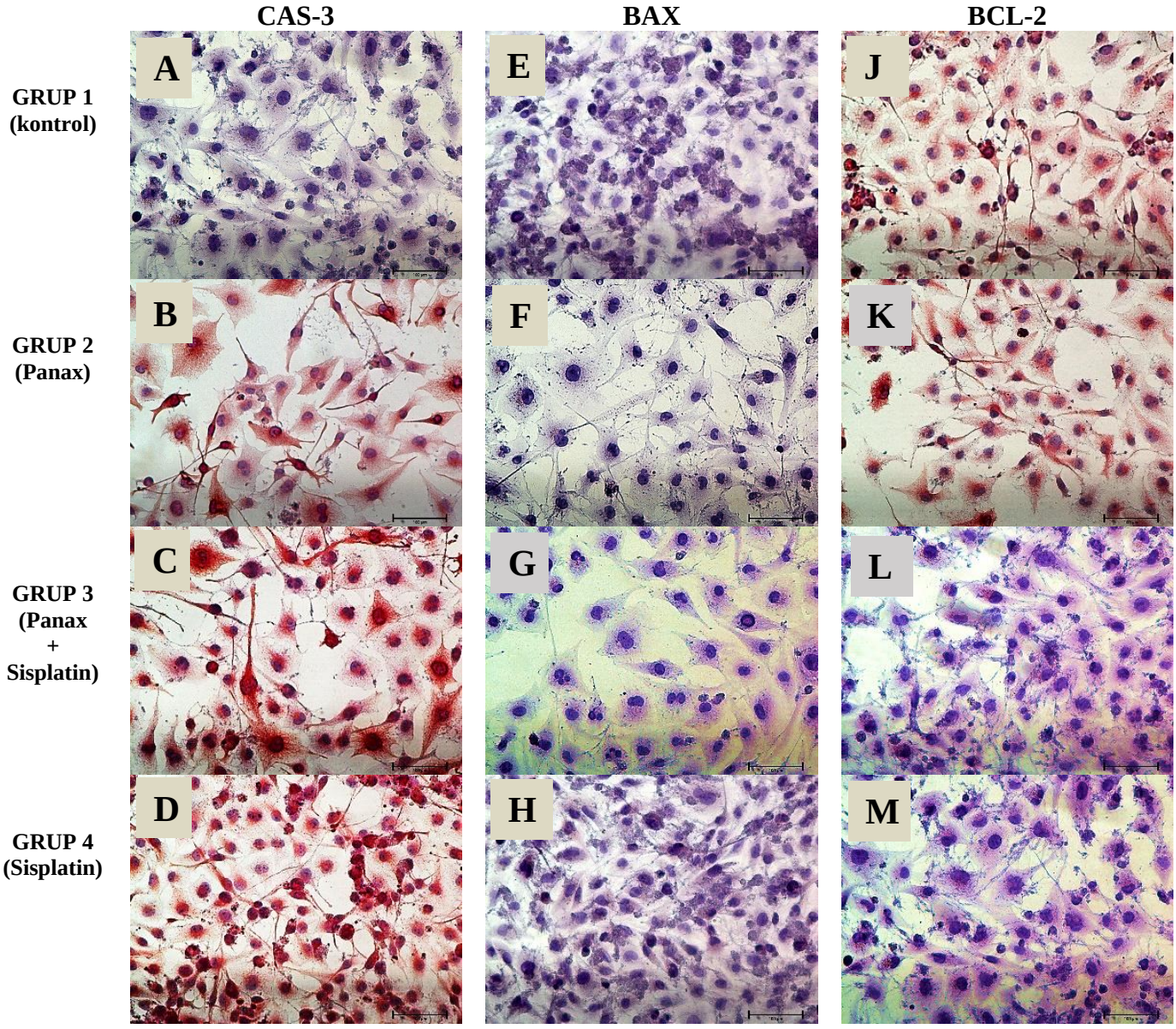
Panax ginseng, Panax+Sisplatin ve Sisplatine maruz bırakılan YKG1 glioblastoma hücre hattında ifade edilen BCL-2, CASPASE-3 genlerinin mRNA seviyelerindeki değişimler kontrol grubu hücrelerine göre belirlendi. Normalizasyon için housekeeping bir gen olan β-ACTIN kullanıldı. YKG1 hücreleri Panax ginseng, Panax+Sisplatin ve Sisplatin ile maruz bıraktıktan sonra BCL-2 geni ekspresyon seviyelerinde kontrole göre up-regülasyon belirlendi (sırasıyla; 34.5, 2.5, 1.9 kat). YKG1 hücrelerini Panax ginseng, Panax+Sisplatin ve Sisplatin ile maruz bıraktıktan sonra CASPASE-3 geni ekspresyon seviyelerinde uygulamalarda yine kontrole göre upregülasyon belirlendi (sırasıyla; 5.1, 10, 10 kat) (Tablo 3).

Table 1. The mean, standard deviation and median values of the groups according to the H-score results.
Tablo 1. H-score sonuçlarına göre grupların orta, standart sapma ve ortanca değerleri.

H_Skor değerleri	Grup	Orta	Std. sapma	Medyan
Caspase-3	Grup 1	106,67	7,572	110,00
	Grup 2	202,00	2,646	201,00
	Grup 3	484,67	4,509	485,00
	Grup 4	486,33	4,726	488,00
Bax	Grup 1	116,00	4,000	116,00
	Grup 2	114,33	5,859	112,00
	Grup 3	116,00	3,606	115,00
	Grup 4	118,00	3,606	119,00
BCL-2	Grup 1	489,00	1,000	489,00
	Grup 2	486,00	5,000	486,00
	Grup 3	110,33	10,693	116,00
	Grup 4	107,33	8,021	108,00

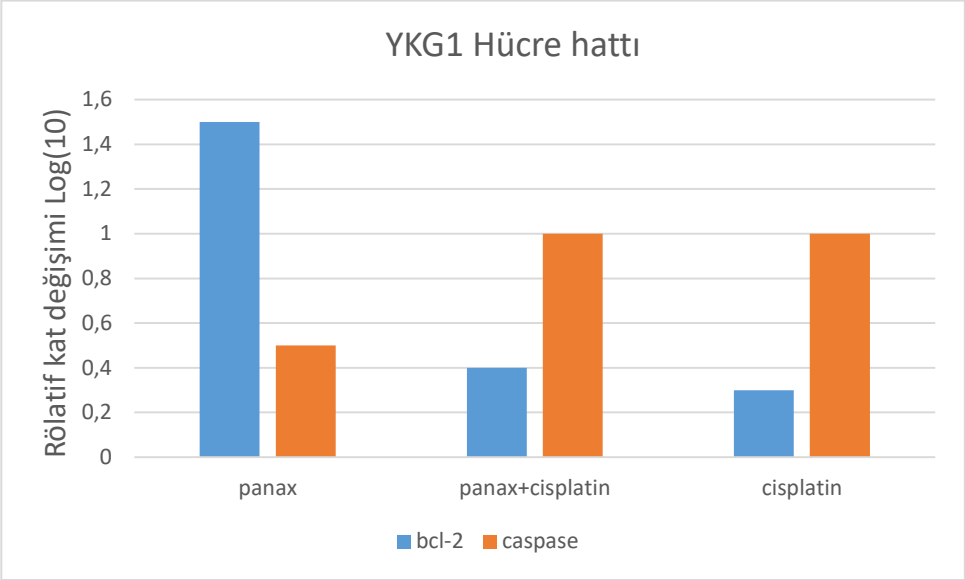
Table 2. Cell viability rates of the groups after MTT experiment.
Tablo 2. MTT deneyi sonucu grupların hücre canlılık oranları.





Şekil 1: İndirek immunohistokimyasal boyama sonucu grupların immunreaktiviteleri. Scale bar-100µm.
Figure 1: Immunreactivities of groups as a result of indirect immunohistochemical staining. Scale bar-100µm.

Table 3. PCR result Gene expression levels in groups.
Tablo 3. PCR sonucu Gruplarda gen ekspresyon seviyeleri.



hücre apoptozunun indüksiyonundan, Bcl-2 proteinleri ise sitokrom C nin sitozole salınmasına

TARTIŞMA

Glioblastoma (GBM), yüksek morbidite ve mortalite oranlarına sahip, tam tedavi sonrası bile 12-15 ay sağ kalım oranı olan ve dünya sağlık örgütüne göre VI dereceden bir kanser türüdür. Düşük sağ kalım oranları ve kanserli hücrelerin tedavi direncinden dolayı adjuvan tedavilere ihtiyaç duyulmaktadır. Panax ginseng'deki saponin bileşeni olan ginsenosidler, kırmızı ginsengin kan akışının iyileştirilmesi, bağışıklık sisteminin güçlendirilmesi, anti-enflamatuar etkileri, yorgunluğun hafifletilmesi ve antioksidan özellikleri gibi biyolojik aktivitelerinden sorumludur (Ham et al. 2019). Ayrıca yapılan pek çok çalışmada Panax ginseng'in kanser hücrelerinin çoğalma hızını ve invazyonunu azalttığı gösterilmiştir (Oh et al. 2019, Yang et al. 2016). Sisplatin halihazırda tedavide kullanılan antikanser bir ilaçtır. Çalışmamızda, YKG-1 insan glioblastoma hücrelerine Sisplatin uygulamasından önce Panax ginseng maruziyetinin hücre canlılığı ve apoptotik sürece olan etkilerini araştırmayı amaçladık. Hücrelere kültüre ortamında 48 saat boyunca Panax ginseng uygulamasının ardından yine 48 saat boyunca Sisplatin tedavisi yapıldı. Uygulamaların ardından hücre canlılığı ve apoptozuna etkileri, MTT, IHC (immunohistokimya) ve rtPCR yöntemleri kullanılarak incelendi.

Apoptoz programlı hücre ölümüdür ve kanserli hücrelerde bu mekanizmayı düzenleyici genlerinde disregülasyon olduğu bilinmektedir. Apoptozu düzenleyici olduğunu bilinen genler ve proteinler arasında Caspase-3, Bax ve Bcl-2 başta gelmektedir. Bax proteinlerinin hücrede, porlar oluşturulması ve mitokondriyal membran geçirgenliğinin arttırılması ve katkı sağlayarak hücrenin yaşam yönünde ilerlemesinden sorumludur. Bax/Bcl-2 oranındaki değişimler mitokondriyal yolak üzerinden hücrelerin apoptozunu direk olarak kontrol eder (Elmore 2007). Çalışmamızda YKG 1 oluşturulan gruplarda uygulama sonrası hücrelerindeki Bax, Bcl-2 ve Caspase-3 proteinlerinin ifadesini immunohistokimyasal yöntem ile ve Bcl-2 ve Caspase-3 gen ekspresyonu değişim oranlarını araştırdık. İmmunohistokimyasal sonuçlarımıza göre Bax immunreaktivite şiddetlerinde, gruplar karşılaştırıldığında anlamlı farklılıkların olmadığı görüldü (p=0,758). Ayrıca Zou ve arkadaşları ve Zhang ve arkadaşları Panax saponinlerinin Bax ifadesini arttırdığını göstermiş (Zou et al. 2019), Tian ve arkadaşları da 2020 yılında yaptıkları çalışmada, Panax ginseng uygulamasının MNK45 gastrik kanser hücrelerinde Bax/Bcl-2 oranını Bax yönünde değiştirerek hücreleri apoptoza sürüklediğini söylemişlerdir (Tian et al. 2020). Çalışmamızda Bax ifadesinde immunohistokimyasal olarak anlamlı (p=0,758) bir artış görülmemiştir. Bu farklılığın Zou ve Tian arkadaşlarının kullandıkları hücre tipinin farklı olmasından ya da Western Blot yöntemini kullanarak Bax ifadesindeki artışı göstermelerinden kaynaklanmış olabileceğini, uygulamalarımızın daha uzun süreli yapılmasıyla bu artışın görülebileceğini düşünmekteyiz. Gruplardaki Bcl-2 değişimleri immunohistokimyasal olarak ve rtPCR ile değerlendirildi. IHC Sonuçlarımıza göre; Bcl-2 proteinlerinin immunoreaktivitesi grup 1 ve grup 2 de şiddetli olarak kaydedilirken uygulama sonrası grup 3 ve grup 4'te az seviyelerinde görüldü ve bu immunoreaktivitedeki azalış kontrol grubu ile

karşılaştırıldığında anlamlıydı (p=0,036) (Figür 1-J, K, L, M). Bcl-2 immunoreaktivitesinde grup 3 ve 4 arasında ise anlamlı bir farklılık görülmedi. Bcl-2 immunoreaktivitesinde grup 3 ve 4 teki anlamlı bir azalış olması panax+Sisplatin uygulamasının ve Sisplatin uygulamasının hücrelerde Bcl-2 ekspresyonunu azalttığını gösterirken yalnızca Panax ginseng uygulanan grup 2'de kontrole göre anlamlı bir değişimin olmaması, Panax ginseng'in Bcl-2 seviyesine tek başına etkisinin olmadığını düşündürmektedir. PCR sonuçları da bu bulgularımızı destekler nitelikteydi ve Bcl-2 ekspresyon seviyesi en düşük grup 3'te kaydedildi. Bu sonuçlar değerlendirildiğinde hücredeki yaşam proteinlerinden biri olan Bcl-2 de ki azalışın Sisplatin gruplarında olması ve Panax ginseg grubunda olmaması panax'ın YKG 1 hücrelerinde Bax/Bcl-2 oranına etkisi olmadığını göstermektedir. Çalışmamızdan elde ettiğimiz sonuçlara göre, 48 saat boyunca YKG1 glioblastoma hücrelerinde Panax ginseng ile uygulama yapılması mitokondriyal yolak üzerinden yani Bax/Bcl-2 oranının değişiminden kaynaklı olarak apoptatik süreci etkilememektedir. Jia D.ve arkadaşları ise 2014 yılında Panax saponinlerinin iskemik rat beyninde nöronlar üzerine anti-apoptotik bir etkisi olduğunu göstermişlerdir (Jia et al. 2014). Sonuçlarımızdaki farklılık Panax ginseng'in sağlıklı hücrelerde anti-apoptotik bir aktiviteye katkı sağlarken kanserli hücrelerde böyle bir etkisi olmama ihtimalini düşündürmektedir.

MTT sonuçlarımıza göre en yüksek hücre canlılığı oranı grup 1 %81 de iken, grup 2'de de %72 olarak tayin edildi. MTT sonuçlarına göre 48 saat yalnız Panax ginseng uygulamasının hücre canlılığını bir miktar düşürdüğü ancak bu oranın glioblastoma hücrelerinde Panax ginseng'in uygulanan doz ve sürede tek başına öldürücü etkiye sahip olmadığını göstermiştir. Ham ve arkadaşlarının 2019 yılında yaptıkları çalışmada Panax ginseng ekstresinin glioblastoma hastalarından elde edilen glioblastoma kök hücrelerinde öldürücü etkiye sahip olduğunu göstermişlerdir (Ham et al. 2019). Çalışmamızda benzeri etki görülmemesinin nedeni olarak kullanılan dozun Ham ve arkadaşlarının uygulama dozlarından daha düşük olması ya da kullandığımız hücrelerin glioblastoma kanser hücreleri olması, Ham ve arkadaşlarının ise kanser kök hücresi üzerinde bu etkileri görmüş olmalarından kaynaklı olabileceği düşünülebilir. Grup 3'te ve 4'te canlı hücre sayısının büyük oranda azalmış olması Panax ginseng uygulamasının tek başına Caspase-3 aktivitesini etkilemediği fakat Sisplatin ile birlikte agonist bir etkiye sahip olabileceğini düşünürmüştür. MTT hücre canlılığı deneyine ilaveten yapılan Caspase-3 immunohistokimyası ve PCR sonuçları da MTT bulgularını destekler niteliktedir. Gruplar arasında Caspase-3 immunohistokimya sonuçları değerlendirildiğinde grup 3 ve 4'te anlamlı bir artış (p=0,024) olduğu görülürken grup 1 ve 2 de ise boyanma şiddetinin aynı olduğu kaydedilmiştir. IHC bulgularında grup 3 ve 4'te Caspase-3 şiddeti artarken, Grup 2 de bir artışın olmamıştır. Bu bulgu, 48 saat Panax ginseng uygulamasının, YKG1 hücrelerinde apoptozu Caspase-3 üzerinden tetiklemediğini göstermiştir. Grup 3 ve 4 kendi arasında değerlendirildiğinde ise gruplar arasında anlamlı bir farklılığın bulunmaması Panax ginseng'in Sisplatin tedavisinden önce ve birlikte uygulanmasının Caspase-3 proteininin varlığı ve ekspresyon seviyesine bir etkisinin olmadığını göstermektedir. Elde ettiğimiz PCR sonuçlarımız da değerlendirildiğinde IHC ve MTT sonuçlarıyla uyumlu olması Panax ginseng'in YKG1 hücrelerinin apoptozuna, uygulanan doz ve sürelerde, katkısı olmadığı sonucunu güçlendirmektedir.

Kırmızı ginsengin endoteliyal hücre invazyonunu ve tüp formasyonunu doza bağlı olarak inhibe ettiği, NF-κB'yi düzenleyerek apoptoza etki ettiği yapılan çalışmalarla gösterilmiştir (Dai et al. 2017). Literatür bilgisi değerlendirildiğinde Panax ginseng'in YKG 1 hücrelerinde apoptozu, NF-κB ya da diğer apoptatik sinyal yolakları üzerinden doz bağımlı olarak ya da uzun süreli uygulama sonucu düzenleyebileceği görülmektedir. Bizim çalışmamızın sonuçlarında da Bax/Bcl-2 oranları sabit iken Caspase-3'ün artmış olması Panax ginseng uygulamasının tek başına ve Sisplatin tedavisi ile kombine olarak kullanılmasının mitokondriyal hücre ölüm yolağını etkilemediğini fakat ölüm reseptörleri sinyali üzerinden hücre ölümüne etkisi olabileceğini göstermektedir. Sonuç olarak alternatif tıpta tedavi edici ve koruyucu olarak kullanılan Panax ginseng'in çalışmamızda kullanılan doz ve sürede YKG1 hücrelerinin apoptozuna etki etmediği görülmüştür. Sisplatin uygulaması öncesinde kullanıldığında ise Sisplatin'in apoptotik etkilerini değiştirmediği görülmüştür. Farklı doz ve süreli uygulamalar ile yapılacak ileri çalışmalarda Panax ginseng' in glioblastoma tedavisinde koruyucu ve tedaviye destek olarak kullanılmasının etkileri detaylı şekilde araştırılmalıdır.

Etik Kurul Bilgileri: Bu çalışma etik standartlarına uygun bicinde hazırlanmıştır ve herhangi bir etik onay gerektirmemektedir.

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RESEARCH ARTICLE

Evaluation of Serum Amyloid-A Levels in Clinically Detected Pneumonia in Goats

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ABSTRACT

In this study, 20 goats between the ages of 0-24 months were used. After clinical, systemic, and hematological examinations, goats were divided into two groups. Clinically diagnosed with pneumonia (n=10) were the study group, whereas healthy goats (n=10) were formed as the control group. Auscultation was performed in the clinical examination. Blood samples from Vena jugularis in the hematological study, White Blood Cell (WBC), Red Blood Cell (RBC), Hemoglobin (Hb), Hematocrit (Hct), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH); Mean Corpuscular Hemoglobin Concentration (MCHC) concentrations were performed. In blood serum, Total Protein (TP), Albumin (ALB), Serum Amyloid-A (SAA) concentrations were measured. As a result, the values of WBC and SAA are statistically higher in goats with pneumonia compared with the control group. As for RBC, Hb, Hct, MCV, MCH, MCHC, TP, ALB concentrations, no statistical difference was found between the two groups. As a result, we think SAA is the most valuable parameter to evaluate the course of the disease and prognosis along with hematologic, biochemical, and clinical findings in veterinary medicine.

Keywords: Pneumonia, Goat, Serum Amyloid-A

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Klinik Olarak Pnömoni Tespit Edilen Keçilerde Serum Amiloid-A Düzeylerinin Değerlendirilmesi

ÖZ

Sunulan çalışmada 0-24 aylık 20 adet keçi kullanıldı. Keçilerin klinik, sistemik ve hematolojik muayeneleri yapıldıktan sonra onarlı iki gruba ayrıldı. Klinik olarak pnömoni teşhisi (n=10) konulan keçiler çalışma grubuna, sağlıklı keçiler ise kontrol (n=10) grubuna dahil edildi. Klinik muayene sırasında oskültasyon yapıldı. Vena jugularisten alınan kan örneklerinden hematolojik muayenede; Lökosit (WBC), Eritrosit (RBC), Hemoglobin (HGB), Hematokrit (HCT), Eritrosit Hacmi (MCV), Eritrosit Ortalama Hemoglobin Miktarı (MCH), Eritrosit Ortalama Hemoglobin Yoğunluğu (MCHC) konsantrasyonları ölçüldü. Kan örneklerinden elde edilen serumlarda; Total Protein (TP), Albumin (ALB) ve Serum Amiloid-A (SAA) konsantrasyonu ölçüldü. Yapılan çalışma sonucunda pnömonili keçilerde WBC ve SAA konsantrasyonları, kontrol grubuna göre istatistiksel açıdan yüksek tespit edildi. RBC, HGB, HCT, MCV, MCH, MCHC, TP, ALB konsantrasyonlarında gruplar arasında bir fark tespit edilmedi. Sunulan çalışma sonuçları dikkate alındığında SAA'nın veteriner hekimlik alanında, hematolojik, biyokimyasal, ve klinik bulgularla beraber hastalığın prognozunun değerlendirilmesinde önemli bir parametre olduğunu düşünmekteyiz.

Anahtar Kelimeler: Pnömoni, Keçi, Serum Amiloid-A

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INTRODUCTION

Respiratory infections are common in livestock. However, pneumonic pasteurellosis is the most common and widely prevalent disease among respiratory system infections in ruminants. Typically, the infection is highly contagious and mostly fatal. Thus, it causes serious economic losses in the livestock sector. The devastating impact of the disease on small ruminant breeding is also evident, and it leads to major deaths due to acute epidemics. Along with this fact, in terms of farm management, the use of chemotherapeutic drugs and intensification of vaccination programs in disease-intensive businesses cause high financial costs almost every year (Davies et al. 1997, Daniel et al. 2006, Mohamed and Abdelsalam, 2008).

Acute Phase Response (APR) is a response that develops following infective cases, inflammatory reactions, immunological problems, traumatic or neoplastic events, and it has been reported that this response develops with systemic and metabolic changes (Gruys et al. 1994, Petersen et al. 2004). Most of the acute phase proteins (APP) have been examined thoroughly for human medicine and are now being used routinely in the diagnosis and prognosis of most diseases. It is considered that APPs can have important usage areas in animal health. In the light of these facts, although APPs have different importance for various animal species, it is a fact that not enough studies have been done in this area, so APPs cannot be used in the field of animal health on the scale of routine tests (Gökçe and Bozukluhan 2009, Eckersall and Bell 2010). Recent studies have presented that Haptoglobin (Hp) and Serum Amyloid A (SAA) are among the essential acute phase proteins for ruminants and α1 acid glycoprotein (AGP) is of moderate importance (Eckersall and Bell 2010). The ratio of APPs in plasma concentrations depends on the severity and activity of the inflammatory reaction, and the determination of the number of APPs in the blood circulation can provide information about the current inflammatory reaction. It has been observed that serum or plasma Haptoglobin (Hp) concentration increases in cattle in natural or experimentally induced infective conditions after inflammatory or traumatic events (Alsemgeest 1994, Heegard et al. 2000, Fisher et al. 2001). Studies have reported that Hp rates in cattle are a very important variable in the diagnosis of bacterial (Skinner et al. 1991) and viral (Höfner et al. 1994) infections and that the concentration of Hp increases substantially in these infections. It has been reported that in some infections such as Bovine Viral Diarrhea (BVD), Mannheimia haemolytica infection, Pasteurella multocida infection, mastitis, metritis, and hepatic lipidosis, changes in serum Hp rates can be an important parameter for APR (Petersen et al. 2004, Eckersall and Bell 2010).

In this study, it was aimed to determine serum amyloid A levels in clinically healthy goats with pneumonia and to determine the relationship between these levels and routine hematological and biochemical parameters.

MATERIAL AND METHOD

Animal Material and Clinical Examinations

0 to 24-month-old 20 goats coming from a private business within the borders of Afyon province were used. Then, the goats with respiratory problems were detected in the businesses, and anamnesis was gathered. Following the anamnesis, the goats diagnosed with pneumonia underwent routine systemic clinic, body temperature, heart and respiratory rate, mucus membrane, and lung auscultation examinations; then, the goats diagnosed with clinical pneumonia formed the disease group (Group P n=10). The Control Group (Group C, n=10) underwent routine systemic clinic, body temperature, heart and respiratory rate, mucus membrane, and lung auscultation examinations and formed by the goats presenting no pathologic conditions. The research was sanctioned by Afyon Kocatepe University Animal Experiments Local Ethics Committee (49533702/145).

Blood samples were obtained from the V. Jugularis of goats in both groups, in accordance with the appropriate technique, into a dry biochemistry tube (red-capped) for serum and EDTA tubes for plasma and hematological assessments. In blood samples, WBC (White Blood Cell), RBC (Red Blood Cell), Hemoglobin (HGB), Hematocrit (HCT), MCV, MCH, and MCHC measurements were made hematologically (Compteur Analyseur d'Hematologie MS9-3). Blood samples were centrifuged at 5000 g for 10 minutes at room temperature. Serums were kept at –20 °C until the measurement phase. Total Protein (BIOLABO SA TP Test Kit), Albumin (BIOLABO SA ALB Test Kit) measurements were analyzed in ELISA Reader (Awareness Technology, Inc. U.S.A. ChemWell) using commercial kits. Serum Amyloid A (Cusabio Biotech CO., LTD. Goat serum amyloid A Test Kit) was measured using commercial kits in ELISA (Chromate 4300 Microplate Reader / Awareness Technology).

In order to statistically analyze the study, the normal distribution test was performed with the Kolmogorov-Smirnov test, and it was observed that the data were not homogeneously distributed. Considering the sampling number, the Mann-Whitney U test was used for intergroup comparisons for each parameter. The level of significance was calculated as $p < 0.05$.

RESULT

Serum Amyloid A (ng/ml) levels were evaluated in the presented study. Serum amyloid A concentration was statistically ($p < 0.001$) higher in the pneumonia group compared to the control group. Serum Amyloid A concentration was determined as 3381.17 ± 178.97 (mean ± SE) in the P group, while it reached up to 719.56 ± 133.01 (mean ± SE) levels in the study group and was higher than the control group. (Table 1)

In the present study, serum T. Protein and Albumin levels were evaluated among biochemical parameters. Serum T. Protein concentration was found as 85.70 ± 11.24 (mean ± SE) in the control group and 90.01 ± 8.05 (mean ± SE) in the pneumonia group. Serum Albumin concentration was 27.00 ± 4.49 (mean ± SE) in the control group and 25.53 ± 4.34 (mean ± SE) in the study group. (Table 1)

In our study, WBC (x109/L) concentration was found to be significantly higher ($p<0.001$) in the Pneumonia group (P:20,71±5.66 x109/L) compared to the control group (F: 10.22 ± 2.46) in the hematological examination. Again, in the P group, MCV (14.08 ± 1.42) and MCH (5.47 ± 0.60) values were statistically significant ($p<0.05$) compared to the Group C (MCV; 12.76 ± 1.08, MCH; 4.95 ± 0.30). (Table 2)

In the evaluation of RBC (x1012/L), HGB (g/dl), HCT (%), and MCHC concentrations, there was no statistically significant difference ($P> 0.05$) in goats with pneumonia compared to the control group. (Table 2)

Table 1. . SAA, TP and Alb Concentrations (Mean±SEM) in Pneumonia and Control Groups

Parameter	Control	Pneumonia	P
SAA	719,56±133,01	3381,17±178,97	P<0.001
T protein	85,70±11,24	90,01±8,05	P>0,05
Albumin	27,00±4,49	25,53±4,34	P>0,05

*P<0.05; **P<0.01; ***P<0.001

Table 2. Hematologic Parameters Concentrations (mean ± SE) in Pneumonia and Control Groups

Parameter	Control	Pneumonia	P
WBC	10,22±2.46	20,71±5.66	P<0.001
RBC	12,79±1,39	12,59±1,84	P>0,05
HGB	6,70±0,75	6,81±0,86	P>0,05
HCT	17,06±2,13	17,05±1,83	P>0,05
MCV	12,76±1,08	14,08±1,42	P<0.05
MCH	4,95±0,30	5,47±0,60	P<0.05
MCHC	39,33±2,38	39,76±3,07	P>0,05

*P<0.05; **P<0.01; ***P<0.001

DISCUSSION

Serum amyloid A (SAA) is among the group of apolipoproteins, the main functions of which are to bind, transport, and excrete lipoproteins. This protein also takes part in the immune response through neutrophil and macrophage activation or elimination of coliform bacteria. Further immune-related functions are to participate in monocyte chemotaxis, to inhibit. Phagocyte oxidative burst, lymphocyte and endothelial cell proliferation, and to stimulate the migration and adhesion of T cells. Furthermore, SAA is in charge of detoxifying endotoxins and regulating phagocytosis during inflammation and infection. The fact that SAA concentration increases in farm animals in case of trauma, viral infections, and physical stress has been confirmed (Murata et al., 2004, Jensen and Whitehead 1998, Bolanos-Garcia and Miguel, 2002). SAA belongs to the large APP group in cattle and

small ruminants (Cray et al.2019). In our study, serum amyloid A concentration was statistically higher ($p < 0.001$) in the pneumonia group in comparison to the control group. Serum Amyloid A concentration was determined as 3381.17 ± 178.97 (mean ± SE) in the P group, while it reached up to 719.56 ± 133.01 (mean ± SE) levels in the study group, which was more elevated compared to the control group.

Acute-phase protein studies are scarce not only on goats but also on sheep, and reference ranges have not been fully determined. In an experimental survey of goats, with subcutaneous turpentine administration, the SAA concentration value, which was 4.88 mg / L before the injection, reached up to 107.8 mg / L level in 24 hours (22-fold increase). Researchers have reported that SAA is a valuable indicator in determining inflammation in goats (Gonzalez et al., 2008). Corynebacterium pseudotuberculosis was applied to goats experimentally; SAA concentration (17.85 ± 0.91 pg / mL) increased more rapidly and significantly (Jeber et al. 2016). The authors reported that this increase is due to the fact that hepatocytes and adipocytes produce SAA that is an acute phase marker that can respond swiftly to infections (Odhah et al., 2018). Gonzalez. et al. (2011) found the changes in haptoglobin level to be significant in their experimental pregnancy toxemia study on goats, while the changes in SAA concentration were not statistically significant. SAA value was determined as 0.92 µg / mL in a study conducted on 55 goats to determine reference ranges of acute-phase proteins in healthy goats. SAA value in goats was determined as 0.46-0.50 mg / L in the measurements carried out with different ELISA kits (Czopowicz M. et al., 2017). In the measurements made in sheep diagnosed with Pasteurella, the SAA level was found higher in comparison to the control group. In their study on goats, they took samples from the farms where goats were vaccinated against Pasteurella and where the vaccination was not applied; both goats with pasteurella and healthy ones from these farms formed the study group. While the SAA was 33.84 pg/ml in the normal vaccinated group and 35.43 pg/ml in the vaccinated pneumonia group; The SAA was 34.12 pg/ml in the normal unvaccinated group and 49.67 pg/ml in the unvaccinated pneumonia group. In our study, SAA concentration was determined as 719.56 ± 133.01 ng/ml in goats in the control group, while the SAA concentration in goats with pneumonia was determined as 3381.17 ± 178.97 ng/ml. As is the case in all other studies, we also think that the high value of SAA concentration in our study was caused by inflammation due to pneumonia in goats. Heegaard et al. (2000) reported that haptoglobin was closely linked to the duration of the acute phase response, the severe clinical findings of pneumonia, and the extend of lung consolidation; however, SAA answered to infection most swiftly.

In the presented study, in terms of serum TP (C: 85,70±11,24, P: 90,01±8,05) and Alb (C: 27,00±4,49, P: 25,53±4,34) concentration, there was no statistically significant ($P>0,05$) increase in Pneumonia group compared to the control group. In the study by Gürgöze and Gökalp (2018), Total protein values were reported as 5.27 ± 1.99 g / dl in Aleppo goats and as 6.75 ± 1.10 g / dl in Ankara Angora goats. In another study conducted on goats, it was reported that the serum TP concentration of healthy goats was in the range of 6.30-8.65 g / dL (İriadam 2004). In a study on Desi goats, TP was 7.29 g/L, and Alb was 4.77 g/L (Balamurugan et al., 2015); in another study on Iranian goats, total protein was 3.294–7.460 g/dl, and albumin was 2.315–3.926 g/dl (Omidi et al., 2018). In other studies, TP and Alb values are quite different for goats. The reason for this is that the goat breeds, ages, genders, and physiological periods used in the study are different (Balamurugan et al., 2015; İriadam, 2004; Omidi et al., 2018; Piccione et al., 2010). In our study, the average values of goats in both groups were determined in this range. In the presented study, it was determined that the increase in serum Alb and TP concentrations in goats in both groups was not statistically significant.

The study found WBC concentration within reference ranges in the control group (Merck Manual, 2021) (WBC: 10,22±2.46) while it was statistically higher ($p<0,001$) in the Pneumonia group (WBC: 20,71±5.66) in comparison to the control group. In our research, WBC concentration was found within the reference ranges in the control group (Merck Manual, 2021). In a field study on Kilis goats, WBC values were determined as 6,92-10,97X103/mm3 (İriadam. 2004). In another study on goats, the WBC value in the control group was found as 10.3X103/µL (Jarikre et al., 2016). These findings are compatible with our control group. In our study, the WBC value in the Pneumonia group (20,71±5.66) was above the normal values. In other studies, it has been reported that WBC count has increased significantly in goats with pneumonia, and leukocytosis, which is caused by a relative increase of neutrophil granulocytes, has happened due to the body's reaction against respiratory system infection (Daramola et al., 2005, Ezeasor and Emikpe., 2015, Maina et al., 2015, Jarikre et al., 2016). In this study, the increase in WBC count in goats who were clinically diagnosed with pneumonia and that there is a statistical significance compared to the control group are consistent with the literature.

In the hematological study conducted on Gaddi goats, Rastog ve and Singh (1990) found RBC value as 20.43xl06/mm3, hemoglobin count as ll.l g/dl, hematocrit value as %31, WBC value as 12.96x 103/mm3, and the percentage of white blood cells as 18-31 of neutrophils, 1-4 of eosinophils, 0-1 of basophils, 55-78 of lymphocytes and 3-7 of monocytes. They calculated the mean red blood cell

volume (MCV) as 18.43, the mean red blood cell hemoglobin (MCH) as 5.1 pg, and the mean red blood cell hemoglobin concentration (MCHC) as 30.4%. In the same study, it was stated that hemoglobin and hematocrit values were characteristically higher than other breeds, and MCV was low. In another study conducted in Turkey, goats within different age ranges (6-month and 3-year-old) were examined haematologically, and the results were found as in the following: RBC value as 10.92-21.50 xl06/mm3, WBC value as 6,92-10.97x 103/mm3, hemoglobin count as 8,40- 10.72 g/dl, hematocrit value as %21.66-37.60, MCV as 19.83-17.48, MCH as 7.69-4.98 pg and MCHC as %37.38-28.45 (İriadam, 2004). In our study, the values for the control and pneumonia groups were found as in the following; RBC C: 12,79±1,39 P: 12,59±1,84 (P>0,05), WBC C: 10,22±2.46 P: 20,71±5.66 (p<0.001), HGB C: 6,70±0,75 P: 6,81±0,86 g/dl (P>0,05), HCT C: %17,06±2,13 P: 17,05±1,83 (P>0,05), MCV C: 12,76±1,08, P: 14,08±1,42 (p<0.05), MCH C:4,95±0,30 P: 5,47±0,60 pg (p<0.05), and MCHC C: %39,33±2,38 P: %37.38-28.45 (P>0,05). Even though there was a statistical difference among the control group in terms of MCV and MCH values, not only they were within the reference ranges (Merck Manual, 2021) but also were parallel to the control groups of other studies on goats (İriadam, 2004, Daramola et al., 2005, Jarikre et al., 2016).

CONCLUSION

Considering the presented study results, it was determined that the increase in SAA concentration in serum obtained from goats in the pneumonia group accompanied the clinical symptoms of naturally infected animals, and SAA was found in minimal concentrations in the control group, which was formed by the healthy animals. Considering the results, it is thought that measuring serum amyloid A concentration as a routine would be beneficial to determine the infection severity, to choose a suitable treatment, to watch the efficiency of the selected treatment method, and would be helpful for detecting animals that show no clinical symptoms and have a subclinical course during the herd health screening in terms of veterinary medicine.

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RESEARCH ARTICLE

Microbiological Quality of Stuffed Mussels Consumed in Istanbul

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ABSTRACT

This study was aimed to determine the microbiological quality of stuffed mussels consumed in Istanbul. A total of 200 stuffed mussels were collected in Istanbul. The samples were investigated for total mesophilic aerobic bacteria (TMAB), total coliform bacteria, *Escherichia coli*, *Staphyloccoccus aureus*, *Vibrio* spp., *Salmonella* spp. and *Listeria monocytogenes*. In the result, TMAB, total coliform bacteria, *E. coli* and *S. aureus* were ranged between 2 x 10^2 cfu/g - 2 x 10^9 cfu/g, < 10^1 - 5 x 10^6 cfu/g, < 10^1 - 5,6 x 10^2 cfu/g and < 10^1 - 9,8 x 10^4 cfu/g, respectively. The mean of TMAB, total coliform bacteria, *E. coli* and *S. aureus* were determined 1.5 x 10^8 cfu/g, 4.5 x 10^4 cfu/g, 2 x 10^2 cfu/g and 6 x 10^3 cfu/g, respectively. On the other hand, *Vibrio spp.* was detected in four stuffed mussel samples. All were identified as *V. alginolyticus*. The results indicated that *Salmonella* spp. and *L. monocytogenes* was not detected but, the hygienic quality of stuffed musses were low. Therefore, it is essential to ensure improving the quality of production technology and developing the sanitation strategies.

Keywords: Public Health, *L. monocytogenes*, Stuffed Mussel, Microbiological Quality, *Salmonella spp.*

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İstanbul'da Tüketime Sunulan Midye Dolmaların Mikrobiyolojik Kalitesinin Belirlenmesi

ÖZ

Çalışmada, İstanbul'da satışa sunulan midye dolmaların mikrobiyolojik kalitesinin ortaya konması amaçlanmıştır. Bu amaçla, İstanbul'da açıkta satışa sunulan 200 midye dolma numunesinin mikrobiyolojik kalitesi incelenmiştir. Örneklerde toplam mezofilik aerob bakteri (TMAB), toplam koliform bakteri, *Escherichia coli*, *Staphyloccoccus aureus*, *Vibrio* spp., *Salmonella* spp. ve *Listeria monocytogenes* araştırılmıştır. Midye dolmalarda TMAB sayısı en az 2 x 10^2 kob/g, en çok 2 x 10^9 kob/g, koliform bakteri sayısı en az < 10^1 kob/g, en çok 5 x 10^6 kob/g, *E. coli* en az < 10^1 kob/g, en çok 5,6 x 10^2 kob/g ve *S. aureus* sayısı en az < 10^1 kob/g, en çok 9,8 x 10^4 kob/g değerlerinde bulunmuştur. Ortalama olarak ise, midye dolmalarda TMAB, koliform bakteri, *E. coli* ve *S. aureus* sayıları sırasıyla 1,5 x 10^8 kob/g, 4,5 x 10^4 kob/g, 2 x 10^2 kob/g ve 6 x 10^3 kob/g olarak belirlenmiştir. Çalışmada *Vibrio spp.* 4 adet örnekte tespit edilmiştir. Yapılan biyokimyasal testlerle, tamamı *V. alginolyticus* olarak identifiye edilmiştir. Sonuç olarak, *Salmonella spp.* ve *L. monocytogenes* tespit edilmemiştir. Fakat örneklerin hijyenik kalitesinin düşük olduğu görülmüştür. Bu nedenle, üretim teknolojisinin kalitesinin iyileştirilmesi ve sanitasyon koşullarının geliştirilmesi gereklidir.

Anahtar kelimeler: Halk Sağlığı, *L. monocytogenes*, Midye Dolma, Mikrobiyolojik Kalite, *Salmonella spp.*

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GİRİŞ

Artan dünya nüfusunun yanı sıra sanayinin gelişmesiyle beraber çalışan birey sayısında artış, yoğun tempoda çalışma gibi etkenler bilindik beslenme alışkanlıklarında değişikliklere yol açarak hazır gıdalara ve alternatif protein kaynaklarına yönelmeye sebep olmaktadır (Kaban ve Kaya, 2006; Güven ve Aysel, 2016).

Yaşadığımız coğrafya, denizler, göl, gölet, baraj gölleri ve akarsular yönünden zengin olduğundan (8,333 km'lik kıyı şeridi ve 177,714 km uzunluğunda akarsu kaynakları) su ürünleri bakımından büyük önem taşımaktadır (Yazıcıoğlu, 2015). Denizlerimiz ve iç sularımız, su ürünleri çeşitlerinin avlanması ve yetiştirilmesi için uygun ekolojik özelliklere sahiptir. Ülkemizde önemi bulunan kabuklu su ürünlerinin başında kara midye (*Mytilus galloprovincialis*) gelmektedir. Diğer türler; kıllı midye (*Modilous barbatus*), akivades (*Tapes decussatus*), kidonya (*Venus verrucosa*), istiridye (*Ostrea edulis*), kum midyesi (*Venus gallina*) ve taş midyesi (*Arca sp.*)'dir. Su ürünleri iyi bir protein kaynağı olmasının yanı sıra çoklu doymamış yağ asitleri, vitamin ve mineraller yönünden de zengindir (Karayücel ve ark, 2004).

Su ürünleri hızlı bozulabilen yapıda olduğu için hazır tüketimde dikkat edilmelidir (Varlık ve ark., 2004). Su ürünlerinde ölüm sonrası oluşan değişimleri belirlemede duyusal tazelik değerlendirmesi, belli kalite dereceleri baz alınarak koku, görünüş, tat ve dokunma duyularının kullanımıyla tespit edilmektedir. Su ürünlerinin taze iken onlara has renk, doku, koku ve lezzet gibi duyusal özelliklerinde ölüm sonrası meydana gelen bu değişimler tazelik kaybının göstergesi olarak kabul edilmektedir. Tazeliğini yitirmiş kabukluların kabukları açık yada hafif aralanmış durumda, hoş olmayan kokuya sahip olduğu, etlerinin ise normal rengini kaybetmiş olduğu belirtilmektedir (Varlık ve ark., 2007).

Su ürünlerinde kontaminasyon, birincil (primer) ve ikincil (sekonder) olarak gerçekleşmektedir. Primer bulaşmada, bulunduğu coğrafya ve çevresel koşullar etkili olmaktadır. Alet – ekipman, personel ve işleme şartları, haşereler, depolama koşulları, paketlendikleri maddeler de sekonder kontaminasyona sebep olmaktadır (Demirel, 2009).

Bu çalışmanın amacı; İstanbul'da açıkta satılan midye dolmaların mikrobiyolojik kalitesinin belirlenmesidir. Bu amaçla farklı semtlerde sokakta satılan midye dolmalardan örnekler toplanmıştır. Toplam mezofilik aerob bakteri (TMAB), toplam koliform bakteri, *Escherichia coli*, *Staphyloccoccus aureus*, *Vibrio* spp., *Salmonella* spp. ve *Listeria monocytogenes* araştırılmıştır.

MATERYAL ve METOT

İstanbul ilinin farklı ilçelerinden, perakende satış noktalarının önlerinde satışa sunulan 200 adet midye dolma numunesi, her bir örnek 5 adet midye olmak üzere toplanarak steril tek kullanımlık poşetlerde, kuru buz ve strafor kutu içerisinde kısa sürelerde soğuk zincir kırılmadan İstanbul Üniversitesi-Cerrahpaşa Veteriner Fakültesi Besin Hijyeni ve Teknolojisi Anabilim Dalı Mikrobiyoloji Laboratuvarı'na ulaştırılmıştır.

Örneklerin Analize Hazırlanması

Laboratuvara getirilen numuneler aseptik şartlarda steril bir cam kap içine alındı ve kabın ağzı kapatıldı. Aseptik şartlarda 10 g alındı ve üzerine 90 ml steril % 0,1'lik peptonlu su ilave edildi. Ana homojenizat solüsyon elde edildikten sonra steril bir pipetle 1 ml çekilerek 9 ml ringer solüsyonu bulunan deney tüplerine aktarıldı. Böylece önce 102'lik, aynı işlemler tekrarlanarak 103, 104 ve 105'lik dilüsyon sıvıları elde edildi (ISO 6887-1, 1999).

Mikrobiyolojik Analizler

TMAB Sayımı

Hazırlanan dilüsyondan Plate Count Agar'a (PCA, CM 0463) dökme ekim yapıldı. 30 °C'de 72 saat inkübasyondan sonra üreyen koloniler sayıldı (ISO 4833, 2003).

Toplam Koliform Bakteri Sayımı

Hazırlanan dilüsyondan Violet Red Bile (VRB) Agar (Merck, 1.01406) besiyerine dökme plak yöntemine göre ekimler yapıldı. Petriler 37°C'de 48 saat inkübasyona bırakıldıktan sonra 1-2 mm çaplı koyu kırmızı renkli koloniler koliform grup bakteri olarak değerlendirildi (APHA, 1992).

E. coli Sayımı

Hazırlanan dilüsyondan TBX Agar (Merck, 1.01406) besiyerine dökme plak yöntemine göre ekimler yapıldı. Petriler 44°C'de 24 saat inkübasyona bırakıldıktan sonra 1-2 mm çaplı yeşil mavi renkli koloniler *E. coli* olarak değerlendirildi (ISO 16649-1, 2001).

S. aureus Sayımı

Hazırlanan desimal dilüsyonlardan içine Egg Yolk Tellurite Emulsion (Oxoid SR054) katılmış Baird Parker Agar (BPA; Oxoid-CM1127) besiyerine yayma plak yöntemine göre 0,1'er ml yayılarak ekimler yapıldı. Ekim yapılan plaklar 37°C'de inkübasyona bırakıldıktan sonra siyah, parlak ve koloni çevresinde temiz zon olan koloniler sayılarak, Rabbit Plasma (Oxoid-R21050) ile doğrulandı. Koagülaz pozitif *S. aureus* olarak değerlendirildi (ISO 6888-1, 2006). Midye dolmalardaki toksin varlığının tespiti amacıyla da SET RPLA test kiti (Oxoid TD900) kullanıldı. Örnekler (10'ar g) 90 ml NaCl solüsyonu (% 0,85) ile karıştırıldıktan sonra santrifüj edildi ve filtrelerden geçilerek stafilokok enterotoksinlerine (A, B, C, D) duyarlı lateks reaktifleri ile muamele edildi. İnkübasyon sonucunda aglütinasyon görülen kuyucuklar pozitif olarak kabul edildi (Crass ve Bergdoll, 1986).

***Vibrio* spp. Sayımı**

Numunelerden 25 g alınarak, üzerine 225 ml % 3 NaCl içeren Alkaline Peptone Water (APW), (Oxoid CM 1028) ilave edildi. 37 °C'de 24 saat inkübasyondan sonra TCBS (Oxoid, CM333) Agar besiyerine öze ile sürme yapılarak ve 35-37°C'de 18-24 saat inkübasyona bırakıldı. Üreyen mavi-yeşil koloniler şüpheli olarak kabul edilerek, doğrulama amacıyla Microbat 24 E (MB1131) ile doğrulandı (ISO 21872-2, 2007).

***Salmonella* spp. Aranması**

Midye dolma numunesinden 25 g alınarak, üzerine 225 ml Buffered Peptone Water (BPW, CM 0509) ilave edildi. 35 °C'de 20 saat inkübasyondan sonra pipetle 1 ml alınarak, Rappaport Vasiliadis Soya Broth (RVS, CM 0866) ekim yapıldı. 42 °C'de 24 saat inkübasyon sonunda, öze ile XLD agara geçildi 37 °C'de 20–24 saat petriler inkübe edildi. XLD agar ortamında koloniler merkezleri siyah pembe koloniler şüpheli olarak kabul edilerek, doğrulama amacıyla üre, TSI (Triple Sugar Iron, CM0277), Lysine Dekarboksilaz Agar (LIA, CM0381), üre (CM0071) ve antiserum testleri (O ve H-Vi polivalan antiserum) yapıldı (ISO 6579, 2002).

***L. monocytogenes* Aranması**

Midye dolma numunesinden 25 g alınarak, üzerine 225 ml One Broth Listeria Selective Supplement (SR0234) katılmış One Broth Listeria Base (CM1066) ilave edildi. 30 °C'de 24 saat inkübasyon sonunda, içerisinde Brillance Selective supplement (SR227) ve Brillance Listeria Differential Supplement (SR228) bulunan Brillance Listeria Agar'a (CM1080) ekim yapıldı. 37 °C'de 24 saat petriler inkübe edildi. Haleli yeşil-mavi renkli koloniler, Microbat 12 L (MB1128) ile doğrulandı (AFNOR, 2009; ISO 11290-1, 2017).

BULGULAR

Bu çalışmada, toplam 200 midye dolma numunesinin mikrobiyolojik kalitesi klasik kültür tekniği kullanılarak analiz edilmiştir. Numunelere ait TMAB, koliform bakteri ve *E. coli* ve *S. aureus* sayıları Tablo 1'de, dağılımları Tablo 2'de, *Vibrio* spp., *Salmonella* spp. ve *L. monocytogenes* varlığı Tablo 3'de verilmiştir.

Tablo 1. Midye dolma örneklerinde tespit edilen TMAB, koliform bakteri ve *E. coli* ve *S. aureus* sayıları
Table 1. The results of TMAB, total coliform bacteria, *E. coli* and *S. aureus* in stuffed mussels

Bakteriler	**Midye Dolma**		
	En Düşük	En Yüksek	Ortalama
TMAB (kob/g)	2×10^2	2×10^9	$1{,}5 \times 10^8$
Koliform Bakteri (kob/g)	$<10^1$	5×10^6	$4{,}5 \times 10^4$
***E. coli* (kob/g)**	$<10^1$	$5{,}6 \times 10^3$	2×10^2
***S.aureus* (kob/g)**	$<10^1$	$9{,}8 \times 10^4$	6×10^3

Tablo 2. Midye dolma örneklerinde tespit edilen TMAB, koliform bakteri, *E. coli* ve *S. aureus* sayılarının dağılımları
Table 2. The dispersion of the number of TMAB, total coliform bacteria, *E. coli* and *S. aureus* in stuffed mussels

Bakteriler (kob/g)	**Bakteri Sayılarının Dağılımı**									
	$<10^1$	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9
TMAB	-	-	9	1	1	27	58	44	56	4
Koliform Bakteri	23	6	35	60	68	7	1	-	-	-
E. coli	47	116	29	8	-	-	-	-	-	-
S.aureus	28	112	33	2	25	-	-	-	-	-

Midye dolmalarda TMAB sayısı en az 2 x 10^2 kob/g, en çok 2 x 10^9 kob/g; koliform bakteri sayısı en az <10^1 kob/g, en çok 5 x 10^6 kob/g, *E. coli* sayısı en az <10^1 kob/g, en çok 5,6 x 10^2 kob/g ve *S. aureus* sayısı en az <10^1 kob/g, en çok 9,8 x 10^4 kob/g değerlerinde bulundu. Ortalama olarak ise, midye dolmalarda TMAB, koliform bakteri, *E. coli* ve *S. aureus* sayıları sırasıyla 1,5 x 10^8 kob/g, 4,5 x 10^4 kob/g, 2 x 10^2 kob/g ve 6 x 10^3 kob/g olarak bulundu. Midye dolma örneklerin 9 adedinde 10^2, 1 adedinde 10^3, 1 adedinde 10^4, 27 adedinde 10^5, 58 adedinde 10^6, 44 adedinde 10^7, 56 adedinde 10^8 ve 4 adedinde 10^9 düzeylerinde TMAB sayısı tespit edildi. Koliform bakteri sayısı, numunelerin 23 adedinde <10^1, 6 adedinde 10^1, 35 adedinde 10^2, 60 adedinde 10^3, 68 adedinde 10^4, 7 adedinde 10^5 ve 1 adedinde 10^6 düzeylerinde bulundu. *E. coli* bakteri sayısı, örneklerin 47 adedinde <10^1, 116 adedinde 10^1, 29 adedinde 10^2 ve 8 adedinde 10^3 düzeylerinde tespit edildi. *S. aureus* sayısı ise, midye dolmaların 28 adedinde <10^1, 112 adedinde 10^1, 33 adedinde 10^2, 2 adedinde 10^2 ve 25 adedinde 10^4 düzeylerinde bulundu. Örneklerin yapılan analizlerinde, Stafilokokal entratoksin tespit edilememiştir.

Tablo 3. Midye dolma örneklerinde *Vibrio* spp., *Salmonella* spp. ve *L. monocytogenes* varlığı
Table 3. Incidence of *Vibrio* spp., *Salmonella* spp. and *L. monocytogenes* in stuffed mussels

Numune	**Adet**	***Vibrio* spp.**	***Salmonella* spp.**	***Listeria* spp.**	***L. monocytogenes***
Midye Dolma	200	4 (% 2,0)	-	3 (% 1,5)	-

Çalışmada *Vibrio* spp. 4 adet örnekte tespit edildi. Yapılan biyokimyasal testlerle, tamamı *V. alginolyticus* olarak identifiye edildi. *Salmonella spp.* ve *L. monocytogenes* hiçbir numuneden izole edilmedi. *Listeria* spp. dört adet midye dolma numunesinde tespit edildi. Bunun 3 adedi *L. gray*, 1 adedi ise *L. ivanovii* olarak bulundu.

Türk Gıda Kodeksinin 29.12.2011 tarihli ve 28157 sayı ile yayınlanan Mikrobiyolojik Kriterler Yönetmeliğinde (TGK, 2011) belirtilen işlenmiş çift kabuklu yumuşakçalar ve tüketime hazır her türlü salata, şarküteri ürünleri ve soğuk mezeler kriterleri açısından midye dolmaları mikrobiyolojik açıdan değerlendirmek mümkündür. İşlenmiş çift kabuklu yumuşakçalar kısmında, örneklerde *Salmonella spp.* ve *L. monocytogenes* olmaması gerektiği ifade edilmiştir. Buna göre, bu çalışmada analize alınan midye dolma örneklerinin hepsi insan tüketimine uygundur. Tüketime hazır her türlü salata, şarküteri ürünleri ve soğuk mezeler kısmında ise, *Salmonella spp.* ve *L. monocytogenes* yanında enterotoksin olmamalı ve *E. coli* sayısı ise 10^1'i geçmemelidir. Buna göre de, toplanan örneklerin 153'ü (% 76,5) *E. coli* açısından tüketime uygun değildir.

TARTIŞMA

Midyelerin avlandıkları suların mikrobiyel kalitelerine göre floraları biçimlenmektedir (Wekell ve ark. 1994). Midyenin hazırlık, depolama, taşıma, satış aşamalarındaki ortam ve personel şartları bulaşmalara sebep olmaktadır (Varlık ve ark., 2004). TMAB sayımı ile gıda işletmelerinde sanitasyonun yeterliliği, gıdanın muhafaza şartları, gıdanın raf ömrü, mikrobiyal kontaminasyon düzeyi hakkında tahmin yapılabileceği belirtilmektedir (Banwart, 1989). Bu çalışmada midye dolmalarda TMAB sayısı, 2 x 10^2 kob/g – 2 x 10^9 kob/g olarak saptanmıştır. Benzer sonuçlar, farklı illerde yapılan çalışmalarda alınmıştır. İstanbul'da Hampikyan ve ark. (2008), midye dolmalarda TMAB sayısını en az 1,2 x 10^3 kob/g ile en çok 2,3 x 10^7 kob/g arasında saptanmıştır. Çolakoğlu ve ark. (2006) Çanakkale'de, TMAB sayısını 9,9 x 10^4 kob/g ile 2,1 x 10^7 kob/g arasında belirlemiştir. Bingöl ve ark. (2008) yaptıkları çalışmada TMAB sayısını en az 1,0 x 10^2 kob/g ile en çok 3,2 x 10^7 kob/g olarak ifade etmiştir. Kök ve ark. (2015), İzmir ve Aydın illerinde yaptıkları çalışmada, TMAB sayısı 10^2 kob/g – 10^6 kob/g düzeyinde belirlemiştir. Yüce (2016) İstanbul'dan topladığı midye dolma numunelerinde TMAB sayısını 10^7 kob/g vermiştir. Tatlısu (2002) yaptığı çalışmada toplanan numunelerin % 63'ünün TMAB değerinin 10^5 kob/g düzeyinde olduğu belirtilmiştir. İzmir'de midye dolmaların iç yüzeylerinden alınan örneklerde sonbahar ve kış aylarında % 16'sında; ilkbahar - yaz aylarında alınan örneklerin % 72'sinde 10^5 kob/g üzerinde değer görülmüştür (Üzgün, 2005). Bu değerlerden daha düşük olarak, Samsun'da satışa sunulan midyelerde yapılan çalışmada TMAB sayısı 1,4 x 10^2 kob/g - 1,5 x 10^3 kob/g olarak tespit edilmiştir (Özçakmak, 1999). Alakavuk (2009), yaptığı mevsimsel çalışmada kış mevsiminde 10^3, yaz mevsiminde ise 10^4 düzeyinde midye dolmalarda TMAB sayısını tespit etmiştir. Ergönül ve ark. (2014), İzmir'de, midye dolmalarda mikrobiyolojik TMAB sayısını 10^1 kob/g ile 10^4 kob/g arasında olduğunu bildirmiştir.

Koliform bakteriler ve *E. coli* indikatör mikroorganizmalar olarak değerlendirilmekte ve bir gıdada indikatör mikroorganizma varlığının belirlenmesi veya bu indikatörün gıdada belirli bir limitin üstünde bulunması, gıdanın patojen ve toksijenik mikroorganizmalarla bulaşabilecek koşullarda üretilip tüketime sunulduğunun bir

göstergesi olarak kabul edilmektedir (Temiz, 1999). Bu çalışmada, koliform bakteri sayısı < 10^1 kob/g, en çok 5 x 10^6 kob/g tespit edilmiştir. Aksu (1996) ve Öner ve Erol (1997) yapmış oldukları çalışmalarda, koliform bakteri sayıları sırasıyla 2,9 x 10^3 kob/g ve 5,9 x 10^2 kob/g olarak saptamıştır. Bu sonuçlardan farklı olarak, Durgun (2013), midye dolma örneklerinde koliform bakteri ve fekal koliform bakteri sayısı <10^1 düzeyinde tespit edilmiştir. Diğer bir çalışmada (Üzgün, 2005), sonbahar-kış döneminde örneklerin iç kısmının % 20'sinde koliform ve % 8'inde fekal koliform bakteri sayısı >2400 MPN/g olarak tespit edilmiştir. 2008 yılında yapılan çalışmada örneklerin % 77'sinde koliform bakteri saptanmıştır (Bingöl ve ark., 2008). 2009 yılında yapılan çalışmada kış mevsiminde 10^3, yaz mevsiminde 10^2 koliform bakteri saptanmıştır (Alakavuk, 2009). Ankara'da satılan 600 midye dolma üzerine yapılan bir çalışmada ise % 30'unda tüketilmeye uygun olmayan düzeyde *E.coli* varlığı tespit edilmiştir (Ateş ve ark., 2011). İstanbul'da koliform bakteri yönünden midye dolmalar incelendiğinde, örneklerin % 52'inde 10^2/g üzerinde tespit edilmiştir (Tatlısu, 2002). Yaptığımız çalışmanın sonuçları, bu araştırmalarda belirtilen değerlerden yüksektir. Bu farklılık, ham maddelerin mikrobiyolojik kalitesinin düşük olmasından ve işleme – muhafaza koşullarının yetersizliğinden olabileceğini düşünülmektedir.

S. aureus, gıdaların hazırlanması aşamasında yetersiz personel hijyeni ortaya koyan önemli bir kriterdir (Aksu, 1996). Bu çalışmada *S. aureus* sayısı en az < 10^1 kob/g, en çok 9,8 x 10^4 kob/g değerlerinde bulunmuştur. Çolakoğlu ve ark. (2006), Kök ve ark. (2015) ve Alakavuk (2009) bulguları, bu çalışmaların sonuçlarına benzerlik göstermektedir. Bingöl ve ark. (2008) midye dolma örneklerinin % 23'ünde *S. aureus* tespit etmiştir. İzmir'de midye dolma örneklerinin 37'sinde *S. aureus* saptanmıştır (Üzgün, 2005). Ankara'da sokakta satılan midye dolmalarla yapılan bir çalışmada ise % 76,6 'inde tüketilmeye uygun olmayan düzeyde *S. aureus* varlığı tespit edilmiştir (Ateş ve ark., 2011). Buna karşılık, İzmir'de yapılan bir çalışmada midye dolmalarda *S. aureus* sayısı <10^1 düzeyinde saptanmıştır (Durgun, 2013). Öner & Erol (1997) ve Aksu (1996) ise, midye dolma örneklerinde *S. aureus*'a rastlamadığını ifade etmiştir. Çalışmalarda tespit edilen konsantrasyonlar arasındaki farklılıklar, numune alma periyotları ve çalışmaların yapıldığı yerlerden kaynaklanmış olabilir.

Midyeler, yaşadıkları ortamdaki büyük miktarda suyu filtre ederek *Vibrio* spp. gibi patojen bakteriler ile kontamine olmaktadır (Cook ve ark., 2002). Bu çalışmada, *Vibrio* spp. 4 adet örnekte tespit edilmiştir ve hepsinin *V. alginolyticus* olduğu belirlenmiştir. Numunelerde, *V. parahaemolyticus* ve *V. cholera* saptanmamıştır. Aynı şekilde, Tatlısu (2002) ve Alakavuk (2009) İstanbul'da yaptıkları çalışmalarda, midye dolma örneklerinde *V. parahaemolyticus* ve *V. cholera* bulamadıklarını bildirmişlerdir. Özçakmak (1999), 35 örneğin hiçbirinde *V. parahaemolyticus* 'a rastlamadıklarını ifade etmiştir. Buna karşılık, Normanno ve ark. (2006) İtalya'da araştırdıkları 600 midye örneğinin 64'ünde (% 10,6) *Vibrio* spp. pozitif bulmuştur. Baffone ve ark. (2000) 37 midye örneğinin 7'sinde *Vibrio* spp. izole etmişler ve bunların 6'sında V. alginolyticus, 1'inde ise *V. parahaemolyticus* saptamışlardır. Cavallo ve ark. (2002) midyelerde *V. vulnificus*, *V. cincinnatiensis*, *V. orientalis*, *V. anguillarum*, *V. marinus*, *V. hollisae* izole ettiklerini ileri sürmüşlerdir. Üzgün (2015) bir çalışmada 6 midye dolma örneğinin kabuk kısmında >140 MPN/ml *Vibrio* spp. tespit etmiştir. Ripabelli ve ark. (1999), 62 midye örneğinin % 48,4'ünde *Vibrio* spp. izole etiklerini bildirmişlerdir. Çalışmaların sonuçlarında ortaya konulan çeşitlilikler, numune alma ve izolasyon metotlarından ileri gelebilir.

Midye tüketimiyle insanlara geçen en önemli patojen bakterilerin başında gelen *Salmonella* spp. kontaminasyonunun kaynağı midyelerin avlandıkları kirli sular olarak belirtilmektedir (Şeker ve ark., 2003). Midye dolma örneklerinin hiçbirinde *Salmonella* spp. tespit edilememiştir. Benzer sonuçlar, Alakavuk (2009), Papadopoulou ve ark. (2007) ve Yüce (2016) tarafından yapılan çalışmalarda alınmıştır. Buna karşılık, incelenen örneklerin Normanno ve ark. (2006) % 0,16'sında; Şeker ve ark. (2003) % 4,8'inde ve Bayizit ve ark. (2003) % 58'inde *Salmonella* spp. izole etmişlerdir. İstanbul'da İkiz ve ark. (2016) tarafından yapılan bir çalışmada ise, 200 midye örneğinin 17'sinde *Salmonella* spp. tespit edildiği bildirilmiştir. Midyeler üzerine yapılan diğer çalışmalarda, Fas'ta Setti ve ark. (2009) % 10 ve Meksika'da Simental ve Martinez – Urtaza (2008) % 7,4 oranında *Salmonella* spp. saptamışlardır. *Salmonella* spp. varlığı ve kontaminasyon düzeyi bakımından çalışmalar arasındaki farklılık, alınan örneklerin orijinleri, muhafaza koşulları, satışa sunuluş şekli (ambalaj yada açık) ve örnekleme teknikleri arasındaki farklılıktan ileri gelebilir.

Bu çalışmada incelenen 200 adet midye dolma numunesinin 4'ü *Listeria* spp. açısından pozitif bulunmuştur. Bunların 3 tanesinde (% 1,5) *L. gray* ve 1 tanesinde (% 0,5) *L. ivanovii* tespit edilmiştir. Numunelerin hiçbirinde *L. monocytogenes* izole edilmemiştir. Listeria ve türlerin midye dolmalarda olmaması yada düşük düzeylerde tespit edilmesi, *Listeria* spp.'nin sularda nadir olarak bulunması şeklinde açıklanmıştır (Kılınç, 2001). Fuchs ve Sirvas (1991), su ürünlerindeki düşük tuz konsantrasyonunun ve laktik asit birikmesi sonucu düşen pH'ın inhibitör etkisinin olabileceğini belirtmiştir. Bu çalışmada saptanan bulgulara benzer değerler Türkiye'de (Terzi ve ark., 2015; Yüce, 2016) ve Yunanistan'da (Soultos ve ark., 2014) yapılan çalışmalarda ortaya konmuştur. Diğer taraftan, % 7,6 (Embarek, 1994), % 5 (İkiz ve ark., 2016) ve % 10 (Göksoy ve ark., 2006) düzeylerinde *L. monocytogenes* tespit etmişlerdir. Kuanberg (1988), su ürünlerinin *Listeria* spp. ile ancak işleme sırasında bulaşabileceğini ileri sürmüştür. Loncarevic (1998) ise, işletmelerde

uygulanan sıcak su yâda sıcak buhar ile yapılan temizlik ve dezenfeksiyon işlemlerinin *Listeria* spp. üzerinde oldukça etkili olacağını vurgulamıştır.

SONUÇ

Midye dolmaların mikrobiyolojisi hakkında kapsamlı araştırma sayısı oldukça azdır. Bu çalışma ile İstanbul'da satışa sunulan midye dolmaların mikrobiyolojik kalitesinin ortaya konması amaçlanmıştır. Sonuç olarak, İstanbul'da tüketime sunulan midye dolma numunelerinin yapılan mikrobiyolojik analizlerinde *Salmonella* spp. ve *L. monocytogenes* tespit edilmemiştir. Fakat örneklerin hijyenik kalitesinin düşük olduğu görülmüştür. Çalışmanın İstanbul'da yapılması en önemli neden olarak görülmektedir. Çünkü İstanbul ülkemizin kentsel anlamda en gelişmiş ve dolayısıyla da endüstriyel manada en büyük kentidir. Bu faktörler yaşam kaynaklarının daha fazla kirlenmesine neden olmaktadır. Midyelerin özellikle mikrobiyolojik yükü, yaşadıkları veya yakalandıkları ortamın mikrobiyal kalitesi ile bağdaşmaktadır. Bu ortamların, kanalizasyon ile bağlantısı ve çöplerle yüksek kirliliği varsa tüketilmeleri halk sağlığı açısından büyük riskler getirmektedir (Wekell ve ark., 1994). İstanbul'da artan nüfusu ile birlikte deniz kirliliğine en fazla maruz yerleşim birimlerindendir. Ayrıca midye dolma, geleneksel yöntemlerle üretilmektedir. Kendisine özgü bir teknolojisi yâda işleme ve muhafaza aşamalarında standardizasyonu bulunmamaktadır. Bu da, gerek üretimde kullanılan alet ve malzemelerin gerekse, üretimde görevli personelin temizlik ve dezenfeksiyon işlemlerinin tam uygulanmamasına neden olmaktadır. Diğer taraftan, tedarikçi kontrollünün de olmaması nedeniyle midye dolma yapımında kullanılan ham maddelerin (pirinç, baharat ve diğer malzemelerin) kalitesi düşük olmaktadır.

Su ürünleri çok çabuk bozulabilen gıda kaynakları olarak bilinmektedir (Cennet, 2007). Ürünlerin özellikle kabukluların toplanma sonrasında ve satışa sunulurken soğuk muhafaza altına alınması gerekmektedir (Aydın ve Büyükünal, 2004). Isıl işlemi uygulanmış ve çiğ ürünlerin aynı alanlarda depolanması engellenerek çapraz kontaminasyonlar engellenmektedir. Böylece, ürünlerin raf ömrü korunmuş olmaktadır ve gıda zehirlenmelerinin önüne geçilmektedir. Fakat, genellikle midye dolma üretiminde çalışan personelin hijyen açısından yeterli bilgiye sahip olmaması ve midye dolmaların açıkta satılması nedeniyle uygun olmayan sıcaklıklara maruz kalmaktadır. Lucca ve Torres (2004), açıkta satışa sunulan gıdaların bozulmasına neden olan en önemli etkenlerin uygun olmayan kaplarda depolanması, çöp kutularının ağzının açık olması ve satış yapılan ortamlarda hayvan ve böceklerin varlığı olarak bildirmişlerdir.

Uygun hammadde seçimi, etkili ısıl işlem uygulanması, alet, ekipman ve personel hijyenine dikkat edilmesi, satışa ambalajlı şekilde sunulması gibi önlemler midye dolmaların kalitesi ve tüketici sağlığı açısından büyük önem arz etmektedir. Güvenli gıda için GMP (Good Manufacturing Practices, İyi Üretim Uygulamaları), GHP (Good Hygienic Practices, İyi Hijyen Uygulamaları) ve HACCP (Hazard Analysis Critical Control Points, Tehlike Analizleri ve Kritik Kontrol Noktaları) gibi yönetim sistemlerini üretiminin her noktasında uygulamak tüketici sağlığı açısından için gereklidir.

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RESEARCH ARTICLE

Isolation of Avipoxvirus from Pigeons

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ABSTRACT

In this study, it was aimed to isolate pox virus which is the causative agent of pox disease which is commonly seen and cause serious lossess in pigeons. In the study, a total of 20 nodule samples from leisons that are clinically which are evaluated as pox nodules clinically were collected. Samples were from four different pigeon flocks each consisting of five samples. Inoculums were prepared from collected tissue samples and inoculated on the chorioallantoic membrane (CAM) of a 10 day old fertilised embryonated chicken eggs. After six to eight days after incubation, pocks were produced by the virus on CAM. Two passages were made further in order to increase the titer of the virus. DNAs were extracted from both nodules and CAM samples and isolated DNAs were amplified by PCR primers for specific for p4b gene. The presence of 578 bp amplified DNA band was regarded as positive in PCR. Nodule samples from infected pigeons were collected and for the first time pox virus from pigeons was isolated and identified molecularly in Turkey.
Keywords: Pox, Pigeon, Isolation, PCR

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Güvercinlerde Çiçek Virusu İzolasyonu

ÖZ

Bu çalışmada, güvercinlerde yaygın olarak görülen ve en çok kayıp oluşturan hastalıklardan biri olan çiçek virusunun izole edilmesi amaçlandı. Çalışmada, klinik muayenede çiçek nodülü olarak değerlendirilen lezyonlar bulunan 4 farklı güvercin kümesinin her birinden 5 hayvan olmak üzere toplam 20 adet güvercinden nodül örnekleri toplandı. Toplanan doku örneklerinden inokulum hazırlanarak 10 günlük embriyolu tavuk yumurtalarında (ETY) koryoallantoik membrana (CAM) ekimler yapıldı. Yumurtalarda 6-8 gün inkubasyondan sonra CAM üzerinde poklar görüldü ve yine ETY'de 2 kez pasaj yapılarak virusun yoğunluğu artırıldı. Çiçek virusunu doğrulamak amacıyla gerek nodül örneklerinden, gerekse CAM'da görülen pok örneklerinden DNA izolasyonu yapılarak p4b geninin varlığı açısından PCR ile test edildi ve 578 bp'lik bantların görülmesi pozitif olarak değerlendirildi. Güvercinlerde görülen çiçek enfeksiyonlarında hastalardan toplanan nodül örneklerinden güvercin çiçek virusu Türkiye'de ilk kez izole edildi ve etken moleküler olarak doğrulandı.
Anahtar Kelimeler: Çiçek, Güvercin, İzolasyon, PCR

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GİRİŞ

Kanatlı çiçeği her yaştan evcil ve yabani kuşların bulaşıcı bir hastalığı olup, 200'den fazla kuş türünde rapor edilmiştir (Adebajo ve ark. 2012, Ha ve ark. 2013). Tüm kanatlı çiçek virusları dünya çapında yayılım gösteren ve DNA karakterinde genetik materyal taşıyan Poxviridae familyasının Avipox virus cinsine dahildir (Jordan ve Pattison 1996, Tripathy 1993, Tripathy ve Reed 2008, Luschow ve ark. 2004).

Kanatlı çiçek etkenleri immünolojik yönden birbirine yakın dört suşa sahip olup patojenite yönünden farklılık göstermektedir. Güvercin çiçek virusu; tavuk, hindi ve güvercinler için patojen bir suştur. Güvercin çiçek virusu diğer çiçek viruslarıyla antijenik ve immünolojik yakınlığından dolayı aşı üretiminde kullanılabilir (Arda ve ark. 1994).

Çiçek virusuna karşı güvercinler cinsiyet olarak aynı duyarlılığa sahip olmalarına karşılık yavrular erginlere göre daha duyarlıdır ve hastalığı daha şiddetli geçirirler. Hastalığın inkübasyon süresi hayvanların bağışıklık, direnç ve immun sistemine göre 5-15 gün arasında değişiklik gösterebilmektedir (Arda 2007).

Bulaşma, kanatlı hayvanların tüysüz bölgelerinde infekte derideki lezyon veya kabukların düşerek yine deri veya mukoza yoluyla gerçekleşir. Özellikle fazla hayvan barındırılan kümeslerde hayvanların tüysüz deri bölgelerinde meydana gelen sıyrık veya yaralar, virusun vücuda girmesinde önemli rol oynar. Sağlam deri ve mukozalardan virus vücuda giremez (Eleazer ve ark. 1983, Docherty ve ark. 1991, Arda ve ark. 1994, Jordan ve Pattison 1996, Tripathy ve Reed 2008). Sivrisineklerin hastalığın mekanik vektörleri olarak rol oynadığı bildirilmektedir (Van-Riper ve Forrester 2007).

Klinik olarak etkilenen kuşlar bağışıklık ve duyarlılığına göre farklılık göstermekle beraber deri formu, difterik form ve sistemik form olmak üzere üç formu vardır (Arda 2007, Kulich ve ark. 2008, Atkinson ve ark. 2010).

Bu çalışmada, güvercinlerde yaygın olarak görülen ve en çok kayıp oluşturan hastalıklardan biri olan çiçek virusunun izole edilmesi amaçlandı.

MATERYAL ve METOT

Çalışmada Harran Üniversitesi Veteriner Fakültesi Mikrobiyoloji anabilim dalına teşhis ve tedavi amacıyla getirilen güvercinlerden klinik olarak çiçek teşhisi konulan hayvanlardan tedavi esnasında alınan yara kabukları kullanıldı. Bu amaçla 4 farklı güvercin kümesinden, her bir kümesten 5 hayvan olmak üzere toplam 20 adet güvercinden, klinik muayenede yüz ve göz çevresinde bulunan ve çiçek nodülü olarak değerlendirilen doku örnekleri toplandı. Numuneler steril bir pens yardımı ile alınarak virus transport medyum (0,5 ml) bulunan eppendorf tüplerine alındı. Numuneler virus izolasyonu amacıyla inokulumun hazırlanmasına kadar -20°C'de saklandı. Bu kümeslerdeki toplam güvercin sayıları Tablo 1'de verilmiştir. Çalışma için, 20.06.2016 tarih 2016/18 sayılı izin belgesi, DOLLVET Hayvan Deneyleri Yerel Etik Kurulu'ndan alınmıştır. Ayrıca yazarlar Araştırma ve Yayın Etiğine uyulduğunu beyan etmişlerdir.

Tablo 1. Güvercin sayıları
Table 1. The numbers of pigeons

Kümes Coop	Toplam Güvercin sayısı Total number of pigeons	Çiçek şüpheli lezyon tespit edilen güvercin sayısı Numbers of pigeons with suspicious pox lesions	Numune alınan güvercin sayısı Number of pigeons sampled
A	35	8	5
B	27	9	5
C	28	5	5
D	30	11	5
Toplam	120	33	20

Virus İzolasyonu

Çiçek lezyonlarından toplanan örnekler, her işletmeden alınan numuneler bir araya toplanarak 4 farklı örnek havuzunun her birinden 10'ar adet ETY'ye ekim yapıldı. Çiçek viruslarını üretmek için, çiçek aşısı yapılmayan ve geçmişte çiçek görüldüğüne dair bilgi bulunmayan bir işletmeden ticari olarak temin edilen 2 günlük ETY' leri kullanıldı. İnokulasyon yapılana dek ETY' leri 37°C ve %55 nem olan kuluçka makinasında tutuldu ver her gün canlılık kontrolleri yapıldı. CAM'a ekimlerin yapılması

amacıyla öncelikle doku örneklerinden inokulumlar hazırlandı. Bu amaçla her örnek havuzundaki doku örnekleri tartıldı ve steril bir havanda steril kum ile ezilerek steril PBS ile %20 oranında süspansiyon haline getirildi. Doku homojenatı 1500 rpm'de 15 dakika santrifüje edildi. Hazırlanan doku süspansiyonu süpernatantına bakteriyel kontaminasyonu önlemek amacıyla penisilin (10000 IU/ml) ve streptomisin (10000 µg/ml) ilave edilerek 37°C'de 45 dakika bekletildi (Gilhare ve ark. 2015, Kabir ve ark. 2015).

Hazırlanan inokulumdan, canlılık muayenesi yapılan 10 günlük ETY'lere ekim yapıldı. Bu amaçla ETY'nin hava boşlukları bir kurşun kalemle işaretlenerek orta kısmından bir yumurta delici ile uygun büyüklükte bir delik açıldı. Steril 1 ml'lik bir tüberkülin enjektörü kullanılarak 0.5 ml hazırlanan inokulumdan CAM üzerine damlatıldı ve iğne ile CAM çizildi. Daha sonra açılan delikler kapatılarak yumurtalar 37 °C'de 6-8 gün inkube edildi. Bu arada her gün 2 kez yumurtaların canlılık kontrolleri yapıldı. İnkubasyon sonunda yumurtalar buzdolabında +4°C'de 2 saat tutularak embriyolar öldürüldü. Daha sonra hava keseleri tarafından kabuklar açılarak CAM çiçek poklarının varlığı açısından değerlendirildi (Kabir ve ark. 2015).

ETY'lerine pozitif kontrol için ticari olarak temin edilen canlı çiçek aşısı (NOBILIS) ile CAM'a inokulasyon yapıldı. Negatif kontrol olarak ise steril fizyolojik tuzlu su inokule edildi. Pozitif ve negatif kontrol ETY'ler diğerleri ile aynı koşullarda inkubasyona kaldırıldı.

Moleküler Tanı

DNA İzolasyonu

Gerek toplanan nodüllerden, gerekse yapılan ekimler sonucunda pokların görüldüğü CAM örneklerinden ticari olarak temin edilen QIAamp DNA Mini Ekstraksiyon kiti (Qiagen, Hilden, Almanya) uygulama protokülüne göre kullanılarak genomik DNA izole edildi. Testte pozitif kontrol olarak kullanılmak üzere, ticari bir tavuk çiçeği aşısından da (NOBILIS) DNA aynı kit kullanılarak DNA izolasyonu yapıldı.

PCR Analizi

Bu amaçla kanatlı çiçeği virusu HP444 suşunun p4b geni için spesifik olan ve Lee ve Lee (1997) tarafından bildirilen primerler kullanılarak PCR'da 578 bp'lik bir DNA amplikasyonu hedeflendi. Kullanılan primerler (forward 5'-CAGCAGGTGCTAAACAACA-3' ve reverse primer olarak 5'-CGGTAGCTTAACGCCGAATA-3') ticari olarak (Invitrogen, ABD) temin edildi ve kullanım için 10 pmol olacak şekilde hazırlandı.

PCR, Kabir ve ark. (2015)'nın bildirdiği yönteme göre gerçekleştirildi. Bu amaçla her örnek için toplam 25 µl çalışma hacmi olacak şekilde reaksiyon karışımı 2,5 µl 10X LA buffer (TaKaRa, Japonya), 1,0 µl 50mM $MgCl_2$ (Bio-Rad, ABD), 1,5 µl 10mM dNTP (Bio-Rad, ABD), 0,2 µl LA taq (TaKaRa, Japonya), 1,5 µl spesifik forward primer, 1,5 µl reverse primer, 10 µl viral DNA ve 6,8 µl dH_2O eklenerek hazırlandı. Tüpler thermocycler (Thermo Scientific) gözlerine yerleştirilerek 94°C'de 5 dakika ilk denatürasyondan sonra 35 döngü 94°C'de 45 saniye denatürasyon, 48°C'de 90 saniye bağlanma, 60°C'de 120 saniye uzama ve son olarak da 60°C'de 10 dakika final uzaması olacak şekilde gerçekleştirildi. PCR sonunda elde edilen amplikonlar %2' lik agaroz jelde 100 Voltta koşturuldu ve elektroforez sonucu ayrılan bantlar UV transilluminatorde görüntülendi.

BULGULAR

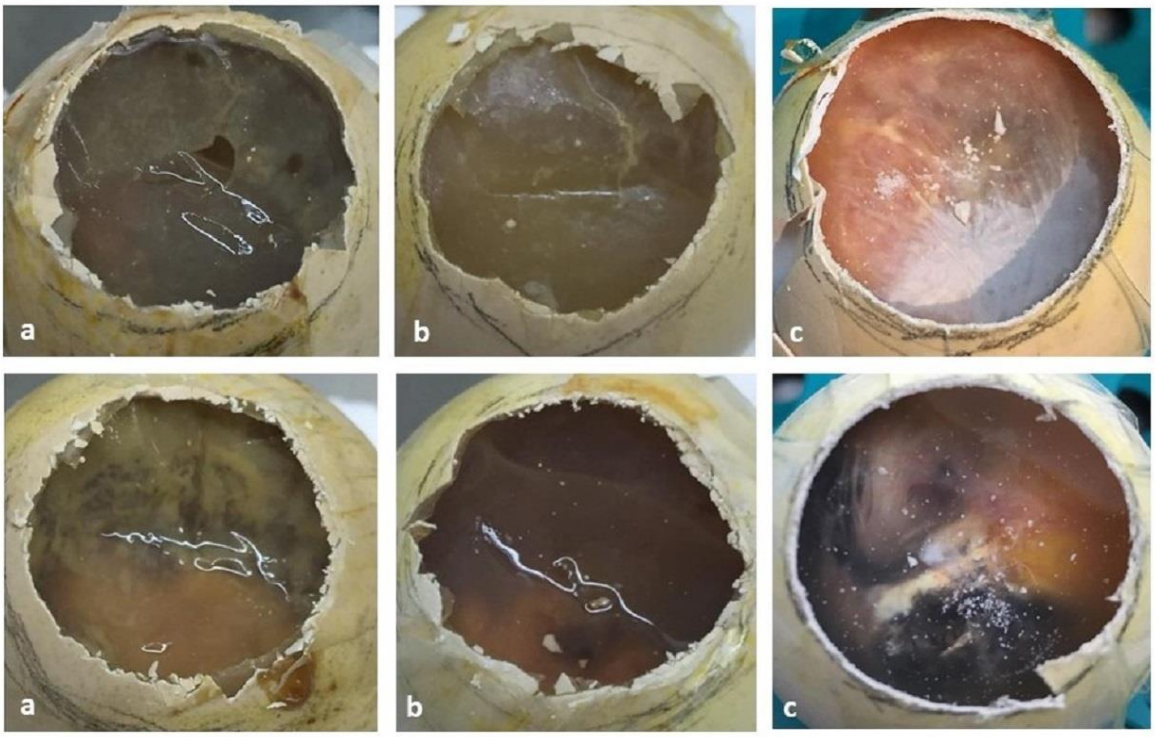
Virus İzolasyonu

Çiçek lezyonlarından ETY'lerin CAM'ına ekimler yapıldıktan sonra inkubasyonun ilk 24 saatinde veya inkubasyon süresince her hangibir embriyoda ölüm görülmedi. 6-8 günlük inkobasyon süresi sonunda yumurtalar buzdolabında +4°C'de 2 saat tutularak embriyolar öldürüldü. İnokulasyon yapılan ETY'lerinin CAM'ları, pozitif ve negatif kontrol ETY'lerin CAM'ları ile karşılaştırılarak CAM üzerinde pokların oluşması veya kalınlaşma görülmesi çiçek virusu açısından şüpheli olarak değerlendirildi. Virus titresinin arttırılması için CAM örneklerinin bir kısmından inokulum hazırlanarak yeniden ETY'nin CAM'ına ekimler yapılarak 2 kez pasajlandı. Her bir işletmeye ait örnek havuzlarından yapılan ekimlerin hepsinde CAM'da çiçek virusu bulguları olarak kabul edilen kalınlaşma ve pokların oluşumu saptandı

Moleküler Tanı

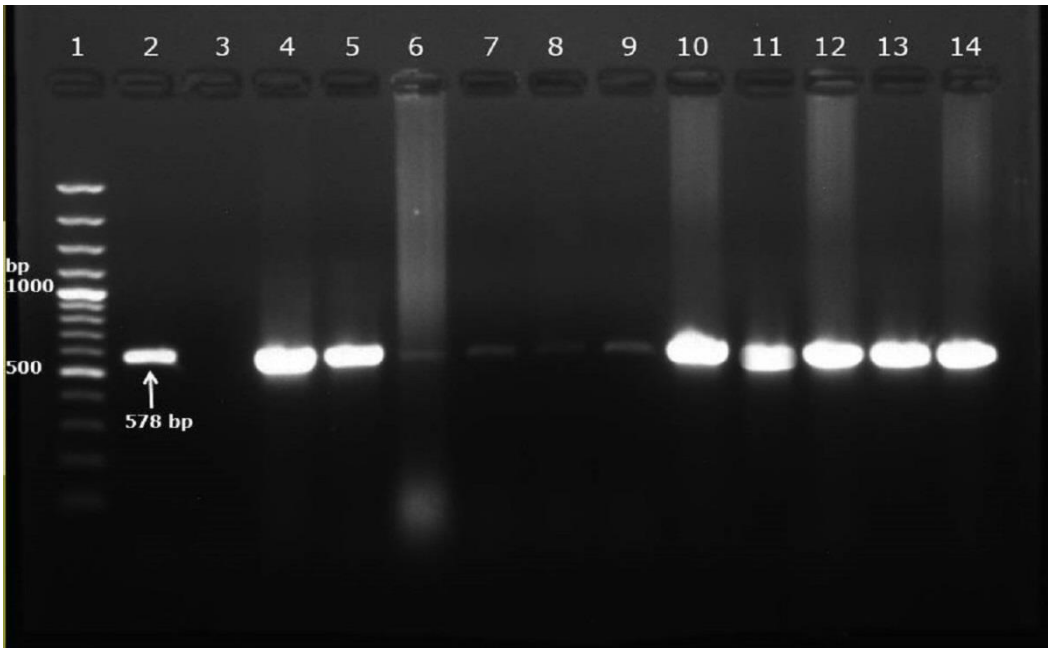
Bu amaçla direkt doku örneğinden ve daha sonra pozitif olarak değerlendirilen CAM örneklerinden yapılan birinci ve ikinci pasajlar olmak üzere her 3 aşamada pozitif olarak saptanan CAM örneklerinde PCR için DNA izolasyonu yapılarak virusun varlığı moleküler olarak doğrulandı.

Moleküler tanı için belirtilen koşullarda elde edilen PCR ürünleri agaroz jelde koşturularak 578 bp bantların varlığı yönünden değerlendirildi. Buna göre gerek direkt dokudan izole edilen DNA örnekleri, gerekse birinci ve ikinci pasajda pozitif olarak değerlendirilen CAM numunelerinden izole edilen DNA örnekleri olmak üzere her 4 işletmeden elde edilen örneklerin tamamında 578 bp'lik amplikonlar yönünden pozitif olarak değerlendirildi. Negatif sonuç elde edilmedi.



Şekil 1. a: Nodüllerden yapılan ekim sonucunda oluşan pok lezyonları, b: Birinci pasaj sonucunda oluşan pok lezyonları, c: İkinci pasaj sonucunda oluşan pok lezyonları.

Figure 1: a: Pocks formed after inoculation from nodules, b: Pocks formed after first passage, c: Pocks formed after second passage.



Şekil 2: Güvercinlerden izole edilen çiçek viruslarına ait PCR ürünlerinin agaroz jeldeki spesifik bantlarını gösteren elektroforez sonuçları. L1= 100 bp DNA Marker, L2= Pozitif kontrol, L3= Negatif kontrol, L4-5= Nodül dokusundan direkt DNA izolasyonu ile PCR, L6-9= İlk ekimde CAM örneklerinden DNA izolasyonu ile PCR, L10-11= Birinci pasajda CAM örneklerinden DNA izolasyonu ile PCR, L12-14= İkinci pasajda CAM örneklerinden DNA izolasyonu ile PCR.

Figure 2: Agarose gel electorophoresis of DNA products generated by PCR. L1= DNA molecular weight marker (100 bp), L2= Positive control, L3= Negative control, L4-5= DNA extraction from skin lesions, L6-9= DNA extraction from CAM after first inoculation, L10-11= DNA extraction from CAM after first passage, L12-14= DNA extraction from CAM after second passage.

TARTIŞMA

Güvercin çiçeği, aşılanmamış duyarlı güvercinlerde yaygın olarak görülen viral bir hastalıktır.

Bilinen yaklaşık 9000 farklı kuş türünden 232 türde doğal çiçek enfeksiyonu rapor edilmiştir. (Audarya ve ark. 2018).

Lee ve Lee (1997)'de, tavuk çiçeğinin tanısında PCR tekniğinin uygulanması için 3'ü ticari aşı suşu ve 3'ü de lokal izolat olan 6 farklı suş ile 4b gen sekansını belirlemek üzere dizayn edilen primerleri kullanarak 578 bp uzunluğunda amplikonları saptayan bir PCR uygulamışlardır. Araştırıcılar çalışmada elde ettikleri verilere dayanarak uyguladıkları PCR tekniğinin tavuk çiçeğinin saptanmasında hızlı, spesifik ve yüksek derecede duyarlı bir teşhis aracı olarak kullanılabileceğini bildirmişlerdir.

Prukner-Radovcic ve ark. (2006)'nın Hırvatistan'da tavuk, güvercin ve hindilerde görülen çiçek vakalarında deri lezyonlarından virusu izole etmek için 11 günlük ETY'lerin CAM'ına inokule etmişler, 7 gün inkubasyondan sonra beyaz renkli fokal pok lezyonlarının veya membranda yaygın bir kalınlaşmanın olup olmadığını değerlendirmişlerdir. Daha sonra bu lezyonlardan DNA ekstarksiyonu yaparak PCR 4b geni yönünden 578 bp'lik amplikonların görülmesini pozitif olarak değerlendirmişlerdir.

Manorolla ve ark. (2010)'nın güvercinlerin de içinde yer aldığı farklı kanatlılarda çiçek virusunun moleküler biyolojik karakterizasyonu için yaptıkları çalışmada 11 günlük ETY'lerin CAM'ına ekim yaparak 7 gün inkubasyondan sonra değerlendirmişlerdir. Deri lezyonlarından DNA izolasyonu yaparak p4b geni açısından PCR uygulamışlardır. Yapılan jel elektroforezde 578 bp'lik bant görülen örnekler pozitif olarak değerlendirilmiştir.

Abdallah ve Hassanin (2013)'nin, Mısır'da farklı kanatlı türlerinden izole edilen APV'lerin saptanması ve moleküler karakterizasyonu için yaptıkları çalışmada tavuk, hindi ve güvercinlerden izolasyon için aşılanmamış bir sürüden elde edilen ETY'lerde CAM'a ekim yaparak ilk ekimde veya ikinci pasajda CAM üzerinde pokların görüldüğünü, bu poklardan ve direkt olarak deri lezyonlardan DNA izolasyonu yapılarak p4b geni açısından PCR yapılarak 578 bp bantların görülmesini pozitif olarak değerlendirildiğini bildirmişlerdir.

Offerman ve ark. (2013)'nın yaptıkları filogenetik çalışmada, Güney Afrika'da güvercinlerin de aralarında bulunduğu farklı kanatlı türlerinden avipox virusları 10-11 günlük ETY'lerin CAM'ında üretmişler ve p4b genini saptayan primerleri kullanarak PCR yapmışlardır.

Abd El-Samie ve ark. (2015), Mısır'ın farklı bölgelerinde çiçek lezyonu bulunan güvercinlerden topladıkları deri lezyonlarından 11 günlük ETY'lerde CAM'a ekim yapmışlar ve 2. pasajda tipik pokların gözlemlendiğini bildirmişlerdir. İzole edilen suşlarda p4b genini saptamaya yönelik olarak PCR uygulamışlar ve 578 bp'lik bantları saptayarak sonucu çiçek yönünden pozitif bulmuşlardır.

Kabir ve ark. (2015), Bangladeş'te görülen bir salgında tavuk ve güvercinlerden çiçek virusunu izole etmek için salgın görülen bölgenin 6 farklı noktadan lezyon görülen 40'ar adet hayvandan örnek toplamışlardır. Araştırıcılar 10 günlük ETY'lerde CAM'a ekimler yapmışlar ve 5-6 günlük inkubasyondan sonra CAM'da karakteristik pokların uluştuğunu, pasajlarla virus konsantrasyonunun artırıldığını belirtmişlerdir. Araştırıcılar poklardan DNA izolasyonu yapmış ve p4b genini saptamak açısından PCR uygulamışlardır. 578 bp'lik bantların görülmesi ile güvercinlerde incelenen 40 örnekten 35'inde pozitiflik belirlemişlerdir.

Masola ve ark. (2015), evcil güvercinlerden avipox virus izolasyonu için 17 güvercinden örnek alarak 10 günlük ETY'lerde CAM'a ekimler yapmışlar ve 5-7 gün inkube ettikten sonra CAM'ları, tipik pokların oluşumu, membranda kalınlaşma ve hemoraji yönünden değerlendirmişlerdir. Ayrıca moleküler olarak virusu belirlemek amacıyla p4b genini belirlemeye yönelik primerler kullanarak 578 bp bant veren örnekleri pozitif olarak değerlendirmişlerdir.

Rahman ve ark. (2019)'nın tavuk, hindi ve güvercinlerde görülen doğal salgın olaylarından nodüler lezyon örnekleri toplayarak virus izolasyonu için 10-12 günlük ETY'lerde CAM'a ekimler yapmışlar 5-6 günlük inkubasyondan sonra CAM'da kalınlaşma ve pokların oluşumu incelenmiş, ayrıca pasajları yapılarak virus yoğunluğu arttırılmıştır. Moleküler tanı için virusun p4b genini saptamaya yönelik primerler kullanarak 578 bp'lik bantların görüldüğü örnekleri pozitif kabul etmişlerdir. Araştırıcılar elde ettikleri saha suşlarının hastalığa karşı etkili bir aşı hazırlanmasında kullanılabileceğini, ayrıca elde edilen PCR ürünlerinin de sekanslama ve filogenetik analiz için kullanılabileceğini bildirmişlerdir.

Mosad ve ark. (2019), tavuk, güvercin ve hindilerde avipoxvirusların saptanmasında agar jel presipitasyon, indirekt immunperoksidaz ve PCR tekniklerini karşılaştırmışlardır. Araştırıcılar PCR tekniğinin teşhiste kullanılabilecek en yeterli test olduğunu bildirmişlerdir.

Aboul Soud ve ark. (2020), PCR ile p4b genini saptayarak doğruladıkları lokal bir kanatlı çiçeği virusunun antijenik ve genomik karekterizasyonunu yaptıkları çalışmada virusu 4 kez pasajlamışlar ve her pasajda virusun titresinin yükseldiğini ve oluşan pokların daha belirgin hale geçtiğini bildirmişlerdir.

Abd El Hafez ve ark. (2021), güvercin çiçeğinin izolasyonu ve moleküler olarak saptanması için yaptıkları çalışmada güvercinlerin baş bölgesi ve vücudun tüysüz bölgelerinden topladıkları nodül örneklerinden izolasyon için ETY'lerinde CAM'a inokulasyon yapmışlar ve CAM'da oluşan pok lezyonlarından DNA izolasyonu yapmışlardır. Araştırıcılar p4b geninin varlığını 578 bp'lik bantların varlığı ile göstermişlerdir.

Yapılan bu çalışmada da, yukarıda bildirilen araştırıcıların sonuçlarıyla uyumlu olarak ETY'lerde CAM'a ekimler yapılarak virus izole edilmiştir. CAM'da kalınlaşma veya pokların oluşumu yapılan pasajlarla daha belirgin olacak şekilde saptanmıştır. Yine araştırıcıların verileriyle paralel olarak gerek deri lezyonlarından, gerekse pok lezyonları bulunan CAM örneklerinden DNA izolasyonu yapılarak p4b geninin saptanması için uygulanan PCR sonucunda 578 bp'lik bantlar saptanmıştır. Ayrıca araştırıcıların Abdallah ve Hassanin (2013) ve Kabir ve ark. (2015)'nın bildirdiği verilere paralel olarak bu çalışmada da, gerek CAM'da oluşan pokların sayısı ve gerekse moleküler olarak saptanan bantların belirginliğinden anlaşılacağı üzere pasajlama ile virus yoğunluğunun artmış olduğu belirlenmiştir.

Sanchez ve ark. (2012), güvercin çiçeğine karşı, ticari olarak bulunan heterolog tavuk çiçek aşısının koruyucu etkinliğini homolog aşılarının koruyucu etkinliği ile karşılaştırmak için yaptıkları çalışmada, güvercinlerde güvercin çiçek virusu suşu ile hazırlanan homolog aşıların, ticari heterolog tavuk çiçeği aşılarına göre çok daha iyi koruma sağladığını bildirmişlerdir. Bu nedenle büyük bir evcil güvercin popülasyonu bulunan Türkiye'de, henüz ruhsatlı bir ticari güvercin çiçek aşısının da bulunmadığı göz önüne alındığında, bu çalışmada Türkiye'de ilk kez izole edilen güvercin çiçek virusunun yerli bir aşı hazırlanması açısından önemi büyüktür.

Ayrıca elde edilen suş ile yapılacak filogenetik çalışmalar da diğer kanatlı çiçek virusları ile yakınlıklarının belirlenmesi açısından önemlidir.

SONUÇ

Sonuç olarak güvercinlerde görülen çiçek enfeksiyonlarında hastalardan alınan nodül örneklerinden ETY'lerde güvercin çiçek virusu Türkiye'de ilk kez izole edilmiş ve moleküler olarak etken doğrulanmıştır. Daha sonra yapılacak etkili bir koruyucu aşı hazırlanması ve etkenin filogenetik özelliklerinin belirlenebilmesi çalışmaları için virusunun izole edilmiş olması önem taşımaktadır.

Çıkar Çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

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RESEARCH ARTICLE

Somatic Cell Count and Bacteriological Evaluation of Milk Obtained from Clinically Healthy Goat

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ABSTRACT

In this study, it was aimed to analyze goat milk samples in terms of somatic cell number, isolation of aerobic bacteria, to determine the in vitro susceptibility of isolated *S. aureus* strains to various antibiotics and to reveal methicillin resistant *S. aureus* strains. The mean number of somatic cells of 313 milk samples examined was $3.25x10^3$, $1.9x10^6$ for 214 (68.3%) milk samples with subclinical mastitis, and $1x10^6$ cells/mL for 32 (27.6%) milk samples with *S. aureus* isolated. Bacteria were isolated from 116 (54.2%) milk samples by culture method. Coagulase negative staphylococci from 46 (39.6%) and *S. aureus* from 32 (27.6%) isolated. Of the 32 *S. aureus* strains tested, 27 (84.3%) were resistant to penicillin G, 17 (53.1%) to amoxicillin/clavulanic acid and cefaprezone, 15 (46.9%) to marbofloxacin, 13 (34.3%) to enrofloxacin, 9 (28.1%) to ceftiofur and 8 (25.0%) to gentamicin. Eight of the *S. aureus* strains (25.0%) had methicillin-resistant *S. aureus* and 5 (62.5%) had multi-antibiotic resistance. As a result, it was determined that i) the mean somatic cell count was low, ii) *S. aureus* strains were highly resistant to penicillin G, amoxicillin/clavulanic acid and cefaperozone, iii) methicillin-resistant *S. aureus* was found to be 25% of the rate.

Key Words: Goat, Isolation, Mastitis, Methicillin resistance *Staphylococcus aureus*, Somatic cell count

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Klinik Olarak Sağlıklı Görünen Saanen Keçilerden Alınan Sütlerin Somatik Hücre Sayısı ve Bakteriyolojik Yönden İncelenmesi

ÖZ

Bu çalışmada keçi sütü örneklerinin somatik hücre sayısı yönünden analizi, aerobik bakterin izolasyonu, izole edilen *S. aureus* suşlarının çeşitli antibiyotiklere in vitro duyarlılıklarının saptanması ve metisilin dirençli *S. aureus* suşlarının ortaya konulması amaçlandı. Toplam 313 süt örneğinin ortalama somatik hücre sayısı $3,25x10^3$, subklinik mastitisli 214 (%68,3) süt örneğinin $1,9x10^6$, *S. aureus* izole edilen 32 (%27,6) süt örneğinin ise $1x10^6$ hücre/ml olarak belirlendi. Kültür yöntemiyle sütlerin 116 (%54,2) adetinden bakteri izole edildi. Örneklerin 46'sından (%39,6) koagulaz negatif stafilokoklar, 32'sinden *S. aureus* (%27,6) izole edildi. Toplam 7 farklı antibiyotiğin test edildiği bu çalışmada, 32 adet *S. aureus* suşundan 27'si (%84,3) penisilin G'ye, 17'si (%53,1) amoksisilin/klavulonik asit ve sefaprezone, 15'i (%46,8) marbofloksasine, 13'ü (%40,6) enrofloksasine, 9'u (%28,1) seftiofura ve 8'i (%25) ise gentamisine dirençli bulundu. *S. aureus* suşlarından 8'inin (%25,0) metisilin dirençli *S. aureus* ve 5'i (%62,5), çoklu antibiyotik dirence sahipti. Sonuç olarak, i) ortalama somatik hücre sayısının düşük olduğu ii) *S. aureus* suşlarının penisilin G, amoksisilin/klavulonik asit ve sefaperozone yüksek düzeyde dirençli olduğu, iii) metisilin dirençli *S. aureus* oranının %25 olduğu görüldü.

Anahtar Kelimeler: İzolasyon, Keçi, Mastitis, Metisilin dirençli *Staphylococcus aures,* Somatik hücre sayısı

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GİRİŞ

Hayvanların meme bezlerinden salgılanan süt, yavruların gelişmesi için gerekli olan önemli besin maddelerini içeren bir vücut salgısıdır. Süt, biyolojik öneminin yanında, insan beslenmesinin farklı dönemlerinde gerekli olan temel besin maddelerini içermesi yönünden de oldukça önemli bir gıdadır. Süt proteinleri küçük çocuklarda kemik gelişimi; kalsiyum, magnezyum ve iyot ise erişkin insanlarda kemik gelişimiyle birlikte, yaşlılarda kemik erimesinin önlenmesinde önemli roller üstlenmektedir (Givens, 2020).

Meme bezinin yangısı olan mastitis, çeşitli patojen mikroorganizmalar tarafından oluşturulan bir enfeksiyonu olup, süt aracığıyla bazı patojen mikroorganizmaların insanlara bulaşmasına aracılık ederek, halk sağlığı açısından da önemli bir sorun oluşturmaktadır (Geary ve ark. 2012, Abebe ve ark. 2016). Mastitis vakaları genel olarak klinik ve subklinik bir seyir izlemektedir. Subklinik mastitisler, belirgin bir klinik bulgu göstermeden uzun süre devam ederek insan, çevre ve diğer hayvanları kontamine etmeleri yanında, önemli düzeyde süt kaybına neden olmaları nedeniyle, klinik mastitislere göre daha önemli bir meme problemi olarak kabul edilmektedir (Juozaitiene ve ark. 2006). Subklinik mastitis etkenleri epidemiyolojik olarak değerlendirildiğinde, primer ve sekonder olmak üzere iki gruba ayrılmaktadır (Cervinkova ve ark. 2013). Primer etkenler arasında *S. aureus, S. agalactiae, T. pyogenes* ve *Mycoplasma* spp. çevresel patojenler arasında ise *E. coli*, *S. dysgalactiae*, *S. uberis, Klebsiella* spp. ve bazı diğer enfeksiyon etkenleri sayılmaktadır (Harmon 1994; Rzewuska ve ark., 2019). Oldukça önemli bir gıda patojeni de olan *S. aureus*, gerek klinik gerekse subklinik mastitislerin en önemli bakteriyel etkeni olarak kabul edilmektedir (Bahraminia ve ark. 2017). Özelikle 1980'li yıllarda metisilin dirençli *S. aureus* (MRSA) suşlarının tespit edilmesiyle, halk sağlığı açısından *S. aureus*'un önemi daha da iyi anlaşılmaya başlamıştır (Burnett ve ark. 2016). Ruminantlarda, özellikle de ineklerde subklinik mastitislerin erken tanısına yönelik çeşitli testler uygulanmaktadır. Bu testlerden biri olan ve sütteki somatik hücre sayısını (SHS) saptamaya yönelik olarak geliştirilen yöntemin, önemli bir indikatör test olduğu bildirilmektedir (Franzoi ve ark. 2020). Yüksek SHS değeri, çeşitli gıda patojenlerinin sütte bulunabileceğini işaret ederken, süt ve ürünlerinin raf ömrünü olumsuz etkilemenin yanında, önemli işleme ve aroma problemlerine de neden olabilmektedir (Rovai ve ark. 2015, Troendle ve ark. 2016). Süt kalitesinin göstergelerinden biri olarak kabul edilen SHS, sağlıklı inek sütü için üst sınır olarak $1x10^5$ hücre/ml olarak kabul edilmektedir (Barrett 2002). İnek sütüyle karşılaştırıldığında, keçi sütü SHS değerinin daha yüksek olduğu bildirilmektedir (Jimenez-Granado ve ark. 2014). Bu durum, keçilerdeki süt salgısının apokrin olması nedeniyle, sitoplazmik parçacıkların da sütte daha yoğun bulunmasıyla açıklanmaktadır. Avrupa Birliği (AB) Otoritesi sağlıklı keçi sütü SHS değerini $>4x10^5$ (EC, 1992), Amerikan Gıda ve İlaç Dairesi (FDA) ise üst sınır olarak $1,5x10^5$ (FDA, 2017) hücre/ml değerini kabul etmektedir. Çalışmamızda AB değeri kriter alınarak, sağlıklı keçi sütlerindeki SHS üst sınırı $>4x10^5$ hücre/ml olarak temel alındı. Bu çalışmada, klinik olarak sağlıklı görünen keçilerden alınan sütlerin SHS yönünden analiz edilmesi, subklinik mastitisli örneklerden aerobik bakteri izolasyonu, izole edilen *S. aureus* suşlarının çeşitli antibiyotiklere in vitro duyarlılıklarının ortaya konulması ve izolatlar arasında MRSA suşlarının belirlenmesi amaçlandı.

MATERYAL ve METOT

Materyal

Çalışmada, Balıkesir ilinde 8 farklı aile işletmesinde ekstansif olarak yetiştirilen Saanen ırkına ait, 1-3 yaşlarında toplam 313 adet sağmal keçiden alınan aynı sayıdaki süt örneği materyal olarak kullanıldı. Anamnezde, hayvanlara bu laktasyon döneminde gerek mastitise gerekse diğer enfeksiyonlara yönelik herhangi bir tedavinin uygulanmadığı bildirildi. Süt örnekleri, klinik olarak sağlıklı görünen hayvanlardan alındı. Örnek almak amacıyla öncelikle hayvanların meme bölgesi su ile temizlendi ve takiben %70'lik etanolle dezenfekte edildi. Bölgenin kurumasını takiben, ilk 3-5 sağım dökülüp, orta sağım sütlerden steril plastik tüplere, her iki memeden olacak şekilde yaklaşık 10-15 ml süt örneği alındı. Örnekler soğuk zincirde ve kısa sürede, Balıkesir Üniversitesi Veteriner Fakültesi Doğum ve Jinekoloji, Besin Hijyeni ve Teknolojisi ile Mikrobiyoloji Anabilim Dalı laboratuvarlarına ulaştırıldı.

SHS Değerinin Belirlenmesi

Suklinik mastitisli sütlerin saptanması amacıyla örnekler, akış sitometrisi tabanlı lazer tarayıcı otomatize SHS cihazıyla (Combi FTS, Bentley®, ABD) test edildi. Çalışmada AB kriter alınarak, SHS değeri $>4x10^5$ hücre/ml olan sütler, subklinik mastitisli olarak kabul edildi.

İzolasyon

Subklinik mastitisli olarak değerlendirilen 214 adet süt örneğinden %7 defibrine koyun kanlı agar (Merck, 1.10886, Darmstadt, Germany) ve MacConkey agara (Merck, 105464) ekimler yapılarak, 37°C'de ve aerobik ortamda, 42-72 saat inkübe edildi. Üreyen etkenler makroskobik morfoloji, hemoliz özelliği, Gram boyamada mikroskobik morfoloji, mannitol salt agarda (Lab, LAB007, Lancashire, UK) üreme özelliği, katalaz, oksidaz, koagulaz, DNaz (BD Difco, 263220, Le Pont de Claix, France), hareket, trehaloz, mannitol, ksiloz, glukoz, arabinoz, maltoz, dulsitol, laktoz, sakkaroz, O/F, nitrat redüktaz, üreaz ve H_2S

testlerine göre identifiye edildi (Arda 1985, Quinn ve ark. 2011, İlhan 2018).

İn vitro Antibiyotik Duyarlılığı ve MRSA Suşlarının Belirlenmesi

S. aureus olarak identifiye edilen 32 adet suş, in vitro antibiyotik duyarlılıklarının saptanması amacıyla brain heart infusion brotta (CM1135, Oxoid, Basingstoke, England), 37°C'de ve aerobik atmosferde üretildi (McFarland Standart Tüp No: 0.5). Antibiyogram testi Mueller-Hinton agarda (Merck, 1.05437), Kirby-Bauer Disk Difüzyon yöntemine göre yapıldı (Bauer ve ark. 1966). Teste penisilin G (P 1) (1U, Oxoid), amoksisilin/klavulonik asit (AMC) (30 µg, Oxoid, UK), sefaperazon (CFP) (75 µg, Oxoid), marbofloksasin (MAR) (5 µg, Mast Diagnostic, UK), enrofloksasin (ENR) (5 µg, Oxoid), seftiofur (AFT) (30 µg, Oxoid) ve gentamisin (CN) (10 µg, Oxoid) diskleri kullanıldı. Sonuçlar, besi yerleri 37°C'de 24 saat inkübe edildikten sonra değerlendirildi (CLSI, 2010a; CLSI, 2010b). MRSA suşların belirlenmesinde oksasilin (OX) (1 µg, Oxoid) kullanıldı (CLSI, 2008).

BULGULAR

SHS Değerleri

SHS yönünden analiz edilen toplam 313 adet süt örneğinin ortalama SHS değeri $3{,}25x10^3$, subklinik mastitisli olarak kabul edilen 214 adet süt örneğinin $1{,}9x10^6$ ve *S. aureus* izole edilen 32 süt örneğinin ise $1x10^6$ hücre/ml olarak belirlendi (Tablo 1).

İzolasyon

Çalışma kapsamında incelenen 214 adet süt örneğinin 116 (%54,2) adetinden kültür pozitif sonuç alınırken, 98 (%45,8) örnekte ise herhangi bir bakteriyel üremenin olmadığı görüldü. Örneklerden en yüksek oranda (%39,6) KNS ve *S. aureus* (%27,6) izole edildi (Tablo 2).

İn vitro Antibiyotik Duyarlılığı ve MRSA

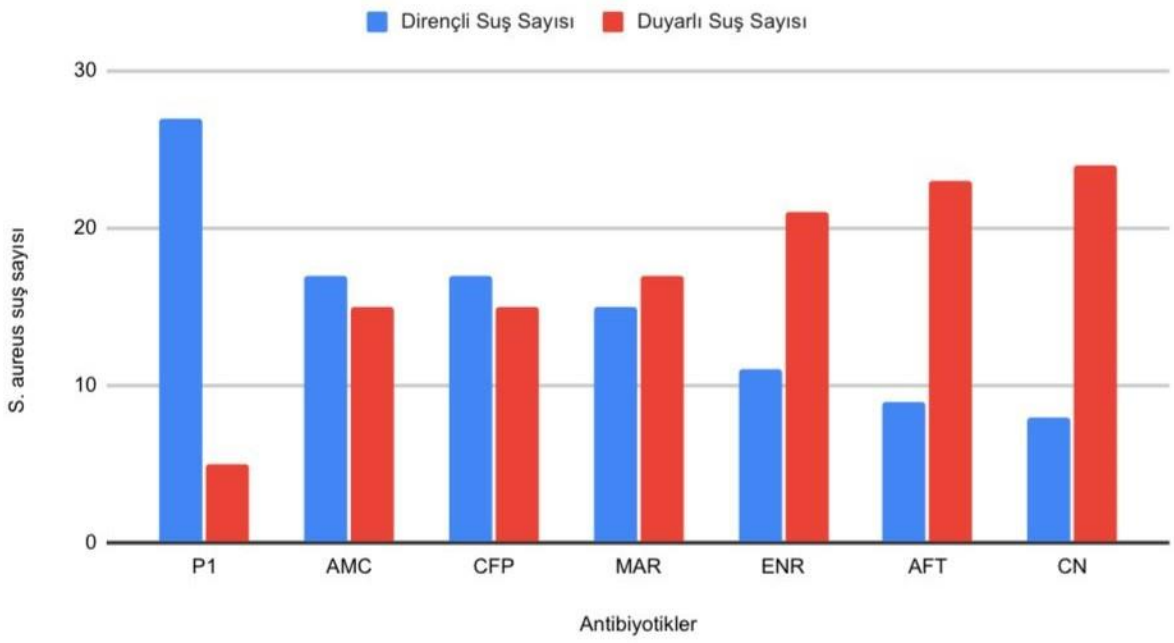
Toplam 7 farklı antibiyotiğin test edildiği bu çalışmada, en yüksek direncin penisilin G'ye (%84,3), en yüksek duyarlılığın ise gentamisine (%75) karşı olduğu saptandı (Resim 1). Sadece 3 (%9,3) adet suşun antibiyotiklerin tamamına aynı anda duyarlı oldukları görüldü. Test edilen 32 adet *S. aureus* suşundan 8'inin (%25) MRSA oldukları belirlendi. MRSA suşlarından 5'inin (%62,5) aynı zamanda çoklu antibiyotik dirençli oldukları tespit edildi. Bunlardan 2 adet suş dört, 3 adet suş ise 7 antibiyotiğe karşı çoklu direnç gösterdi.

Tablo 1. Süt örneklerine ait somatik hücre sayısı (hücre/ml) ve yüzdesi
Table 1. Somatic cell count (cell/ml) and percentage of milk samples

Toplam Süt		**Subklinik Mastitisli Süt** (>4x10^5 hücre/ml)			***S. aureus* İzole Edilen Süt**		
N	Ortalama SHS (hücre/ml)	n	(%)	Ortalama SHS (hücre/ml)	n	(%)	Ortalama SHS (hücre/ml)
313	**3,25x10^3**	**214**	**68,3**	**1,9x10^6**	**32**	**27,6**	**1,1x10^6**

Tablo 2. Subklinik mastitisli keçi sütlerinden izole edilen bakteriler
Table 2. Bacteria isolated from goat milk with subclinical mastitis

Bakteri	**İzolat Sayısı**	**%**
KNS	46	39,6
S. aureus	32	27,6
Corynebacterium spp	15	12,9
E. coli	10	8,6
Enterobacter spp	4	3,4
C. pseudotuberculosis	3	2,6
Streptococcus spp	2	1,7
P. multocida	2	1,7
Candida spp	1	0,9
Toplam	**116**	**100**



Resim 1: Subklinik mastitisli keçi sütlerinden izole edilen *S. aureus* suşlarının antibiyotik direnç profilleri
Figure 1: Antimicrobial resistance profiles of *S. aureus* isolated from goat milk with subclinical mastitis
P1:penisilin G, AMC: amoksisilin/klavulonik asit, CFP: sefaperazon, MAR: marbofloksasin, ENR: enrofloksasin, AFT: seftiofur, CN: gentamisin

TARTIŞMA

Laktasyon dönemindeki hayvanların en önemli sağlık sorunlarından biri olarak kabul edilen mastitis süt verimin azalması, sütün yapısı ve kalitesinin bozulması, memenin körelmesi, tedavi edilmediği bazı vakalarda annenin ölümü, diğer hayvanlar ve özellikle de süt emen yavrulara çeşitli enfeksiyöz ajanların bulaşmasına neden olabilen ve tüm dünyada yaygın olarak görülen, ekonomik yönü fazla olan bir enfeksiyondur (Stuhr ve Aulrich 2010). Önemli bir halk sağlığı sorunu da olan mastitis, daha çok subklinik mastitisler başta olmak üzere çeşitli zoonoz enfeksiyonların insanlara bulaşmasına da neden olabilmektedir (Sah ve ark. 2020). Diğer yandan yüksek SHS değerine sahip sütlerde, mikrobiyolojik kalitenin bozulması yanında, süt ve süt ürünlerinde ekşime, acılık, oksidasyon ve burukluk gibi duyusal bozukluklar da görülmektedir (Santos ve ark. 2003).

Keçilere ait subklinik mastitisli sütlerin saptanmasında uygulanan SHS yöntemiyle ilgili bazı çalışmalar yapılmıştır. Kaygısız (2020), tank sütü örneklerinde yaptığı çalışmada ortalama SHS değerini $7,8x10^5$, Marcinkoniene ve Ciprovica (2020) $1,5x10^3$, Margatho ve ark. (2018) $2,5x10^3$, Park ve Humphrey (1989) ise $9,8x10^5$ hücre/ml olarak rapor etmişlerdir. Gerçekleştirilen bu çalışmada elde edilen ortalama SHS değerinin ($3,25x10^3$), bazı çalışmalardan düşük (Park ve Humphrey 1989, Kaygısız 2020), bazılarından ise yüksek (Margatho ve ark. 2018, Marcinkoniene ve Ciprovica 2020) olduğu görüldü. Diğer yandan bu çalışmaya ait ortalama SHS değerlerin AB (1992) ve FDA (2017) tarafından bildirilen keçi sütü SHS üst sınırı değerlerinden de düşük olduğu görülmektedir. Bu durum; hayvanların ırkı, sağlık durumu, beslenme şekli, laktasyon dönemi ve süt sağım zamanlarının farklı olmasıyla ilgili olabilir.

Keçi mastitis vakalarından çeşitli bakteriyel etkenler izole edilmektedir (Baştan ve ark. 2015). Konya'da subklinik mastitsli 60 adet keçi sütünün bakteriyolojik analizinde, örneklerin 34'ünden (%56,6) büyük çoğunluğu Gram pozitif olan çeşitli bakteriyel etkenlerin ürediği bildirilmiştir (Çiftçi ve ark. 1996). Benzer şekilde Van'da subklinik mastitisli 148 adet keçi sütünün materyal olarak kullanıldığı bir çalışmada, örneklerin 69'undan (%46,6) çeşitli aerobik bakterilerin izole edildiği rapor edilmiştir (İlhan ve ark. 2011). Balıkesir'de gerçekleştirilen bu çalışmada, incelenen 214 adet süt örneğinin kültür pozitif sonuç veren 116 (%54,2) adetinin 46'sından (%39,6) KNS, 32'sinden (%27,6) *S. aureus*, 15'inden (%12,9) *Corynebacterium* spp, 10'undan (%8,6) *E. coli*, 4'ünden (%3,4) *Enterobacter* spp, 3'ünden (%2,6) *C. pseudotuberculosis*, 2'sinden (%1,7) *Streptococcus* spp, 2'sinden (%1,7) *P. multocida* ve 1'inden (%0,9) ise *Candida* spp izole edildi (Tablo 2). Mastitislerle ilgili çalışmalarda farklı oranlarda, değişik cins ve türlerde etkenlerin izole edilmesi, hastalığın multi-faktöriyel bir enfeksiyon olmasıyla açıklanabilir.

Gerek Türkiye' de gerekse Dünya' nın farklı bölgelerinde keçi mastitislerinden izole edilen bakteriyel etkenlerin, antibakteriyellere in vitro duyarlılıklarının saptanmasına yönelik çeşitli çalışmalar yapılmıştır. Cantekin ve ark. (2016), Hatay'da 220 adet keçi sütünün 28'sinden (%12,73), 30 farklı türden bakteri izole ettiklerini bildirmişlerdir. Araştırıcılar izolatlardan 21' ini (%70) penisilin G'ye, 19'unu (%63,3) amoksisiline dirençli bulunurken; 30'unu (%100) gentamisine, 28'ini (%93,3) enrofloksasine, 28'ini (%93,3) oksitetrasikline ve 27'sini (%90) ise amoksisilin/klavulanik asite duyarlı bulunmuştur. Aydın'da yapılan ve 152 adet kıl keçisinden alınan süt örneklerinin materyal olarak kullanıldığı bir çalışmada, 102 (%67,1) örnekte bakteri üremesinin olduğu bildirilmiştir. Antibiyogram testinde *S. aureus* suşları amoksisilin/klavulanik asite %100 duyarlı bulunurken; penisiline %100, kanamisin ve oksasiline ise %90 düzeyinde dirençli bulunmuştur (İşnel ve Kırkan 2012). Benzer şekilde Öztürk ve ark. (2019), Burdur'da mastitis problemi yaşayan keçilerden aldıkları 466 adet süt örneğini bakteriyolojik yönden inceleyerek, 122 (%26,18) örnekten pozitif sonuç aldıklarını bildirmişlerdir. Araştırıcılar *S. aureus* suşlarından 7'sinin disk difüzyon, 5'inin ise minimal inhibisyon yöntemiyle MRSA olduğunu rapor etmişlerdir. Etiyopya'da subklinik mastitisli 47 keçi sütü örneğinin bakteriyolojik analizinde, çeşitli cins ve türden bakterilerin izole edildiği bildirilmiş ve yapılan antibiyogram testinde stafilokok türlerinin penisilin G (%81,8), ampisilin (%90,9), sefoksitin (63,6%), siprofloksasin (90,9%), klindamisin (72,7%) ve vankomisine (100%) yüksek düzeyde dirençli oldukları bildirilmiştir (Haftay ve ark. 2016). Benzer şekilde Kenya'da subklinik mastitisli 110 keçiden alınan aynı sayıdaki süt örneğiyle yapılan izolasyon çalışmasında, değişik cins ve türden 169 farklı bakterinin izole edildiği bildirilmiştir. Disk difüzyon yöntemiyle yapılan değerlendirmede, *S. aureus* suşları gentamisin (%100), kloramfenikol (%100), streptomisin (%100), tetrasiklin (%75) ve norfloksasine (%75) duyarlı bulunurken; penisilin G'ye ise %100 dirençli olduğu rapor edilmiştir (Mahlungu ve ark. 2018). Toplam 7 farklı antibiyotiğin test edildiği bu çalışmada, incelenen 32 adet *S. aureus* suşu penisilin G'ye %84,3, amoksisilin/klavulonik asit ve sefaprezone %53,1, marbofloksasine %46,9, enrofloksasine %34,3, seftiofura %28,1 ve gentamisine ise %25 düzeyinde dirençli bulundu. Gerçekleştirilen bu çalışma ve diğer çalışmalara ait antibiyogram sonuçları incelendiğinde (İşnel ve Kırkan 2010, Haftay ve ark. 2016, Cantekin ve ark. 2017, Mahlungu ve ark. 2018), en dikkat çekici ortak bulgunun, penisiline karşı gelişen yüksek direnç olduğu görülmektedir. Bakterilerin antibiyotiklere karşı sahip oldukları direnç profilleri coğrafi bölgelere

göre, ülkelere, uygulanan yöntem ve antibakteriyellere göre değişiklik gösterdiğinden, penisilin dışındaki antibiyotiklerle ilgili veriler diğer çalışmaların sonuçlarıyla tartışılmamıştır.

S. aureus tüm Dünya'da primer etken olarak hem beşeri hem de veteriner hekimlikte oldukça önemli enfeksiyonlara neden olmaktadır. Konuyla ilgili kaynaklar incelendiğinde, halk sağlığı açısından önem arz eden MRSA enfeksiyonlarının epidemiyolojisinde, antibiyotiklere karşı giderek artan bir direncin geliştiği dikkati çekmektedir (Grema ve ark. 2015, Ismail ve ark. 2021). Türkiye'de MRSA ile ilgili mastitisli ineklerde bazı çalışmalar (Türkyılmaz ve ark. 2010, Sayın ve ark. 2016) yapılmış olmakla birlikte, keçilerde yeteri düzeyde çalışmanın olmadığı görülmektedir. Aras ve ark. (2012), mastitisli keçi sütlerinden izole ettikleri 42 adet *S. aureus* suşundan 2'sinin (%4,8), Öztürk ve ark. (2019) ise 43 adet *S. aureus* suşundan 7'sinin (%16,2) MRSA olduklarını rapor etmişlerdir. Konuyla ilgili Dünya'nın farklı bölgelerinde yapılan çalışmalar incelendiğinde, MRSA izolasyon oranının yaklaşık %2-18,8 arasında değiştiği görülmektedir (Cortimiglia ve ark. 2015, Altaf ve ark. 2020). Bu çalışmada *S. aureus* suşlarının %25'inin, MRSA oldukları saptandı. Bu oranın, gerek Türkiye'de (Aras ve ark. 2012, Öztürk ve ark. 2019), gerekse diğer ülkelerde (Cortimiglia ve ark. 2015, Altaf ve ark. 2020) yapılan çalışmalara ait verilerden daha yüksek olduğu görülmektedir. Bu durum, uygulanan yöntemlerin farklı olması, MRSA prevalansının bölge ve ülkelere göre değişiklik göstermesiyle ilgili olabileceği gibi, bilinçsiz ve yoğun antibiyotik kullanımına bağlı olarak ülkemizde de MRSA prevalansının giderek artmasıyla ilgili olabilir.

Sonuç olarak bu çalışmada, klinik olarak sağlıklı görünen keçi sütlerinin ortalama SHS değerinin genel olarak düşük olduğu, izole edilen bakterilerin tür ve oran olarak konuyla ilgili çalışmalara benzer sonuçlar verdiği, MRSA oranının ise yüksek olduğu görüldü. İnek, koyun ve keçi gibi çiftlik hayvanlarında çeşitli antibiyotiklerin bilinçsiz ve yaygın olarak kullanımı, hayvanlardaki bakteriyel direncin artmasına neden olmakla kalmayıp, bu hayvanlarla doğrudan temas eden bakıcılar başta olmak üzere, çeşitli hayvansal ürünlerle de birçok patojenin insanlara bulaşmasına aracılık ederek, halk sağlığı açısından önemli bir sorun olabileceği düşünüldü.

Çıkar çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Etik izin: Bu çalışma "Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmelik" Madde 8 (k) gereği HADYEK iznine tabi değildir.

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RESEARCH ARTICLE

Effect of Crating Position During Transport on Welfare and Oxidative Stress in Laying Hens

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ABSTRACT

This study was carried out to investigate the effects of crating position of laying hens during transport on live weight loss, mortality rate, plasma biochemical, and oxidative stress parameters under commercial transport conditions in Afyonkarahisar. A commercial multi-decked and fixed-crate-type poultry transport trailer was used for bird transportation. The hens were transported in 9 selected loading positions on the trailer's front, center, rear sections, and top, middle, and bottom rows. Both pre-transport and post-transport hens were weighed individually, and blood samples were taken. Live weight loss, mortality rate, glucose, MDA, AOA, and cortisol concentrations increased, triglyceride and cholesterol concentrations decreased in the hens transported between farm and slaughterhouse. The hens loaded in the bottom row and rear section were most adversely affected by transportation and, the welfare loss of these hens was dramatic. It has been observed that the microclimate conditions of the transport crates in the bottom row are more unfavorable than those in the other positions. The results showed that transport crates positioned in the bottom row and rear section of the trailer were more stressful for laying hens. In conclusion, the potential stress profile of the position of the animal crates during transport can contribute to the strategic solutions to be developed to reduce the welfare losses of laying hens.
Key Words: Crate position, Laying hens, Oxidative Stress, Transport, Welfare

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Yumurtacı Tavuklarda Nakil Kasası Pozisyonunun Hayvan Refahı ve Oksidatif Stres Üzerine Etkisi

ÖZ

Bu araştırma Afyonkarahisar'da ticari nakil koşullarında yumurtacı tavukların nakil aracındaki taşınma pozisyonunun canlı ağırlık kaybı, ölüm oranı, serum biyokimyasal ve oksidatif stres parametreleri üzerine etkisini araştırmak için yapılmıştır. Tavukların taşınması için ticari birçok katlı ve sabit hayvan kasalı kanatlı hayvan nakil kamyonu kullanılmıştır. Tavuklar, kamyon dorsesinin ön, orta ve arka bölümleri ile üst, orta ve alt katlarında belirlenen 9 yükleme pozisyonunda taşınmıştır. Hem nakil öncesi hem de nakil sonrası tavuklar bireysel olarak tartılmış ve kan örnekleri alınmıştır. Çiftlik ile kesimhane arasında taşınan yumurtacı tavuklarda canlı ağırlık kaybı, ölüm oranı ile glikoz, MDA, AOA ve kortizol konsantrasyonları artmış, trigliserit ve kolesterol konsantrasyonları azalmıştır. Nakilden en fazla alt katta ve arka bölümde taşınan tavuklar etkilenmiş ve bu tavukların refah kaybı dramatik olmuştur. Alt katta bulunan nakil kasalarının mikro iklim koşullarının diğer pozisyonlardaki nakil kasalarına göre daha olumsuz olduğu gözlemlenmiştir. Sonuçlar kamyonun alt katında ve arka bölümünde bulunan nakil kasalarının yumurtacı tavuklar için daha stresli olduğunu göstermiştir. Nakil kasası pozisyonuna ait potansiyel stres profilinin yumurtacı tavukların refah kayıplarını azaltmak için geliştirilecek stratejik çözümlere katkı sağlayabileceği sonucuna varılmıştır.
Anahtar Kelimler: Kasa pozisyonu, Nakil, Oksidatif stres, Refah, Yumurtacı tavuk

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INTRODUCTION

Studies to increase animal welfare have accelerated in the last few decades, especially in industrialized and developing countries. As in the EU, awareness, and demands of consumers, citizens, and non-governmental organizations on animal welfare are increasing rapidly in Turkey (Moura et al. 2006, Makdisi and Marggraf 2011, Rezai et al. 2012). So, Council Regulation (EC) No 1/2005 for protecting animals during transport has been transposed into national legislation in Turkey (Passantino 2006, Bozkurt 2018).

The complex production cycle of an intensive animal production system focused on increasing economic efficiency makes processing poultry more stressful and challenging. Transporting animals is also a stressful and traumatic process (Kettlewell and Mitchell 1994). Poor transport conditions such as rough capture, carrying and handling and, restraint (Voslarova et al. 2007), high loading density (Waas et al. 1997), long transport (Ondrašovičová et al. 2008), and unfavorable microclimatic circumstances (Vecerek et al. 2016) enhance welfare losses during the transport of poultry.

Rough handling of animals and environmental stressors causes physiological and biochemical deviations and causes an increase in anxiety and fear, and decreases welfare in animals (Minka and Ayo 2009, Piccione et al. 2013). Poor transportation conditions reduce poultry meat quality, as scientifically verified (Nijdam et al. 2005). Unfriendly animal handling and adverse transport conditions can cause muscle trauma, fractures, and ultimately death in chickens (Wolff et al. 2019). Regulations, recommendations, and guidelines have been entered into force to protect animals during transport, prevent economic losses resulting from welfare losses, and improve food quality in the EU. However, studies report severe welfare losses when transporting food animals by road (Minka and Ayo 2009). Welfare problems related to transportation are more mentioned for spent hens than broiler chickens and other poultry species. These statements bring to mind the extent to which the legal and administrative sanctions aimed at protecting chickens during transport realize their purpose in commercial conditions.

Although detrimental effects of transportation on animals have been well explained, the responses of different animal species to stressors or detailed results regarding their capacity to cope with stress have still not been confirmed (Kettlewell and Mitchell 1994, Onmaz et al. 2011, Vecerkova et al. 2019). Studies reported different tenderness and mortality rates against transport stress for poultry species (Machovcova et al. 2017, Al-obaidy et al., 2020). Researches have been realized to prevent or reduce harmful effects ranging from distress to transport death in animals. Still, consistent, reliable, and applicable solutions in commercial conditions have not been reached yet (Ondrašovičová et al., 2008).

Transportation is very stressful for laying hens when they are delivered to slaughterhouses or processing plants. Careless handling, long-distance transport in fixed crates (Kettlewell and Mitchell 1994, Weeks et al. 2012, Vecerkova et al. 2019), the low number of processing plants that accepting laying hens that reached end-of-lay period due to their low economic value, and insufficient financial support to improve animal well-being (Petracci et al. 2006, Lara and Rostagno 2013) are cause poor welfare in chickens.

Regulations monitoring animal welfare are being implemented, but new scientific studies are needed to support the regulations' standards or to develop these standards. However, it has been reported that research on the welfare of end-of-lay hens during transport is also limited (Bozkurt 2018). More research is needed to develop appropriate strategies to control of environmental conditions to increase knowledge about the negative aspects of transport to laying hens to prevent transport-induced stress and damage, and to improve the welfare of laying hens during transport (Al-obaidy et al. 2020, Machovcova et al. 2017, Vecerkova et al. 2019).

This study was carried out to examine the effects of crating position of hens during transport on live weight loss, mortality rate, plasma biochemical, and oxidative stress parameters in laying hens under commercial transport conditions in Afyonkarahisar.

MATERIALS AND METHODS

Materials and Experimental Design

The study was held under commercial poultry transport conditions in Afyonkarahisar, which has approximately 20% of the laying hen population in Turkey. A total of 2304 white laying hens (64-weeks-old) were used. The hens were transported by a commercial poultry transport trailer with a 12-decked, fixed crated, and open-sided (without a protective cover).

The animal-based assessment was carried out only for the hens that were crated on nine positions in the truck's trailer (the crates located on rows 1, 6, and 12 from bottom to top in each of the truck trailer's front, center, and rear sections). After unloading the flock, dead hens were counted in 18 crates on the same positions and symmetrically located on both sides of the trailer. The width, length, and height were 95 cm, 95cm, and 19 cm respectively of fixed crates with flat sheet floor. An electronic digital scale with a precision of 10 g was used when weighing the birds.

Implementation and Transport

The laying hens housed in multi-tiered aviary system were starved for 6 hours before transport. After removing from the multi-tier cages, the hens were individually numbered, weighed, and taken blood samples. The hens were carried and loaded into the

trailer by hand up to 5-6. Loading density was applied as 12 hens per crate to comply with the relevant legislative requirements (Regulation on welfare and protection of animals in transportation, 24 December 2011, number: 28152) and avoid the effects of high loading stress. The loading of hens started at 14.00 and finished at 17.45 in the same afternoon. The average truck speed was 50 km/h during the journey. The journey lasted an hour on 40 km between the farm and the slaughterhouse. The hens have been waited in the crates for a further 3 hours after arrival at the slaughterhouse because of the workload in the slaughterhouse. No feed or water has been given to the birds during this lairage time onto the trailer. After unloading, hens were weighed, and blood samples were taken with the same procedure applied before the transport. After unloading, the dead birds were counted in the crates and the mortality rate was calculated as the percentage of birds dead on arrival (DOA).

The research was carried out in the winter season (January 2020). Air temperature values have hourly measured during the whole transport process in the day, and the max and min. air temperature values were 8 and 3°C. Excluding the process of blood sampling, numbering, and weighing, all animal transport processes such as catching, carrying by hand, loading, and unloading the hens were performed professionally by employees of the commercial poultry transport company operating in the province.

Blood Sampling and Biochemical Analyzes

The blood samples were drawn from the wing vein before and after transport. Vacuum tubes without anticoagulants were used for blood samples. Blood samples were centrifuged at 3000 rpm for 15 minutes. Subsequently, the plasma samples were collected and stored in a - 80°C freezer until laboratory analysis. Plasma glucose, triglyceride, and cholesterol levels were measured on a Siemens Centaur CP analyzer by Siemens assay kits (Siemens Centaur CP kits). The chemiluminescent microparticle immunoassay (CMIA) method of plasma cortisol analysis was conducted on Abbott Architect Diagnostics I2000 analyzer using the Architect/Abbott kits. The concentrations of Malondialdehyde (MDA) and Antioxidant capacity (AOA) in plasma were determined using poultry Elisa kits (BT-lab ELISA kits) (Draper and Hardley 1990, Koracevic et al. 2001). The ELISA measurements were performed using the BioTek EX 800 Absorbance Microplate Reader. Afyon Kocatepe University Animal Research Ethics Committee approved this research with the reference number AHUHADYEK-154-20.

Statical Analysis

One-way analysis of variance (One-way ANOVA) was used to evaluate the differences between the crating position groups in terms of cortisol, glucose, triglyceride, cholesterol, MDA, and AOA in the plasma and live weight and weight loss. A paired t-test (paired-sample T-test) was applied to compare the plasma values taken before and after transportation for live weight, cortisol, glucose, triglyceride, cholesterol, and oxidative stress parameters in each loading position. The non-parametric test (kruskal-wallis test) was adopted to compare the crating position groups for mortality rates (DOA percentage) during transport. SPSS 21.0 (IBM Company, USA) was used for the analyses.

RESULTS

The results on the live weight, weight loss, and mortality rate in crate position groups are shown in Table 1. The effect of the position of the transport crates on the trailer on the pre-transport live weight was insignificant. Although the impact of crate row on the trailer on live weight after transport was negligible, the post-transport live weight averages were significantly different for crate sections ($p<0.05$). The transport position of the hens had a significant effect on transport-induced weight loss. Weight loss was similar in hens positioned on different trailer rows but differed significantly in weight loss between hens transported on the front, center, and rear sections ($p<0.001$). The mortality rate was not statistically affected by the crating position on the trailer.

The results related concentrations of plasma cholesterol, triglyceride, and glucose before and after transport are presented in Table 2. Significant differences were determined between the plasma cholesterol and triglyceride values determined before and after transportation ($p<0.05$, $p<0.001$). In general, cholesterol and triglyceride mean values were 143.10 mg/dl and 1160.67 mg/dl before transport, and those values were 99.08 mg/dl and 612.10 mg/dl after transport, respectively. Transport increased the concentration of plasma glucose levels in all position groups, but these increases were significant only for hens transported in the middle row and rear section.

Means of plasma cholesterol and triglyceride before transport did not differ significantly between transport position groups.

Though the concentration of plasma cholesterol after transportation in different trailer sections was similar, transport of the birds in the middle and top rows affected plasma cholesterol levels ($p<0.001$). Plasma triglyceride concentration was not affected by crate row. Still, it was higher in chickens transported in the center section of the trailer than in chickens transported in the front and rear sections ($p<0.001$).

The results of cortisol, malondialdehyde, and antioxidant capacity regarding the crate's position are given in Table 3. There was a significant increase in plasma cortisol levels due to transportation in all position groups ($p<0.001$, $p<0.01$).

Table 1. The results of live weight, weight loss, and mortality rate regarding the crate's position on the trailer during transport

Crate position			Live weight (kg)					Weight loss (%)		Mortality rate (%)			
			Before transport		After transport		t						
			n	$\bar{X} \pm Sx$	n	$\bar{X} \pm Sx$	P	n	$\bar{X} \pm Sx$	n	$\bar{X} \pm Sx$	chi-square	P
Row	Top		36	1.87 ± 0.03	36	1.66 ± 0.04	***	36	11.75 ± 0.61	3	2.50		
	Middle		36	1.82 ± 0.04	33	1.65 ± 0.04	***	33	11.60 ± 0.79	3	5.33		
	Bottom		36	1.83 ± 0.03	31	1.61 ± 0.03	***	31	12.63 ± 0.92	3	7.17	5.054	NS
Section		Front	36	1.87 ± 0.03	34	1.70^{a} ± 0.03	***	34	9.73^{c} ±0.60	3	4.33		
		Center	36	1.81 ± 0.04	34	1.61^{ab} ± 0.04	***	34	11.84^{b} ±0.72	3	4.50	0.940	NS
		Rear	36	1.84 ± 0.04	32	1.59^{b} ± 0.04	***	32	14.51^{a} ±0.76	3	6.17		
ANOVA													
Row				NS		NS			NS				
Section				NS		*			***				

***p<0.001 *p<0.05 NS: Not significant

*p<0.05 ^{a,b,c} There are significant differences between groups with different letters(p<0.05)

Table 2. The results of cholesterol, triglyceride, and glucose regarding the crate's position on the trailer during transport

Crate position				Cholesterol (mg/dl)			Triglyceride (mg/dl)			Glucose (mg/dl)		
				Before transport	After transport	P	Before transport	After transport	P	Before transport	After transport	P
			n	$\bar{X} \pm Sx$	$\bar{X} \pm Sx$		$\bar{X} \pm Sx$	$\bar{X} \pm Sx$		$\bar{X} \pm Sx$	$\bar{X} \pm Sx$	
Row	Top		16	140.94 ± 8.22	$81.25^{b} \pm 2.93$	***	1169.94 ± 106.34	674.17 ± 43.50	***	201.75 ± 2.62	206.13 ± 3.88	NS
	Middle		18	139.44 ± 11.69	$122.06^{a} \pm 10.87$	NS	1168.33 ± 90.38	533.19 ± 41.04	***	198.94 ± 3.13	212.56 ± 3.51	**
	Bottom		18	148.67 ± 8.99	$91.94^{ab} \pm 3.42$	***	1144.78 ± 117.54	635.83 ± 53.20	***	202.06 ± 2.66	203.83 ± 2.75	NS
Section		Front	17	142.24 ± 6.72	96.20 ± 4.46	***	1335.71 ± 103.55	$552.08^{b} \pm 38.82$	***	199.29 ± 2.66	204.42 ± 3.71	NS
		Center	18	133.78 ± 9.77	96.56 ± 7.97	*	1030.28 ± 87.40	$732.80^{a} \pm 51.88$	**	199.83 ± 2.92	205.44 ± 3.17	NS
		Rear	17	153.83 ± 11.92	104.62 ± 10.79	**	1123.71 ± 110.89	$544.31^{b} \pm 37.59$	***	203.59 ± 2.84	212.94 ± 3.24	*
ANOVA												
Row				NS	***		NS	NS		NS	NS	
Section				NS	NS		NS	**		NS	NS	

***p<0.001 **p<0.01 *p<0.05 NS: Not significant

*p<0.05 ^{a,b} There are significant differences between groups with different letters(p<0.05)

Table 3. The results of cortisol, malondialdehyde, and antioxidant capacity regarding the crate's position on the trailer during transport

Crate position				Cortisol (ug/dL)			Malondialdehid (MDA) (nmol/ml)			Antioxidant capacity (AOA) (nmol/l)		
				Before transport	After transport	P	Before transport	After transport	P	Before transport	After transport	P
			n	$\bar{X} \pm Sx$	$\bar{X} \pm Sx$		$\bar{X} \pm Sx$	$\bar{X} \pm Sx$		$\bar{X} \pm Sx$	$\bar{X} \pm Sx$	
Row	Top		16	1.13 ± 0.03	1.33 ± 0.06	***	$1.40^{b} \pm 0.04$	1.74 ± 0.06	***	7.01 ± 0.18	7.47 ± 0.18	*
	Middle		18	1.17 ± 0.02	1.41 ± 0.06	**	$1.47^{ab} \pm 0.05$	1.64 ± 0.05	***	7.06 ± 0.18	7.52 ± 0.23	*
	Bottom		18	1.17 ± 0.03	1.34 ± 0.05	*	$1.59^{a} \pm 0.04$	1.80 ± 0.05	***	7.36 ± 0.14	7.53 ± 0.15	NS
Section		Front	17	1.15 ± 0.02	$1.50^{a} \pm 0.19$	*	1.45 ± 0.04	1.74 ± 0.06	***	6.91 ± 0.17	7.30 ± 0.19	*
		Center	18	1.18 ± 0.04	$1.35^{b} \pm 0.21$	*	1.45 ± 0.03	1.68 ± 0.05	***	7.10 ± 0.13	7.54 ± 0.21	*
		Rear	17	1.15 ± 0.02	$1.24^{b} \pm 0.13$	NS	1.57 ± 0.06 *	1.76 ± 0.04 -	**	7.45 ± 0.18	7.67 ± 0.13	NS
ANOVA												
Row				NS	NS		*	NS		NS	NS	
Section				NS	**		NS	NS		NS	NS	

***p<0.001 **p<0.01 *p<0.05 NS: Not significant

*p<0.05 ^{a,b} There are significant differences between groups with different letters(P<0.05)

As an oxidative stress parameter, MDA was also increased in transported hens ($p<0.001$, $p<0.01$). Similarly, transport of the birds resulted in an increase in AOA averages except for the hens transported on bottom rows and rear sections.

There was no significant difference between crate positions in terms of plasma cortisol concentration before transplantation. Post-transport plasma cortisol level was not affected by the crate row, but it was significantly affected by the crate section. The increase in cortisol concentration exchanged more pronounced as the position in which the hens were crated onto the trailer changed from rear to front.

Differences among the top, middle and bottom rows regarding plasma MDA means were significant ($p<0.05$) before transportation but were insignificant after transport. The highest and lowest MDA means were found in hens transported to the bottom and top rows, respectively. Neither before nor after transport AOA concentrations were not affected by crate row. The transport did not affect plasma MDA and AOA levels in hens transported in the crates positioned onto the trailer's front, center, and rear sections.

DISCUSSION

Pre-slaughtering transport of old laying hens resulted in body weight loss and deaths (DOA) in the study. These results were not surprising, as many researchers reported that transporting poultry is a stressful manufacturer's operation (Minka and Ayo 2009, Ajakaiye et al. 2010, Miranda-de la Lama et al. 2012). In line with the results of this study, Ondrašovičová et al. (2008) reported that factors related to animal transport such as catching, handling, and journey time significantly affect body weight loss during transport in broiler chickens.

The mortality rates determined in the study were between 2.50 and 7.17%. These rates indicated that the transport conditions in the study were unfavorable and stressful, and the old laying hens were quite sensitive to these conditions. These mortality rates are higher than the DAO percentages in Europe reported for broilers (0.31-1.64%)(Voslarova et al. 2007, Vecerek et al. 2016, Jacobs et al. 2017) or turkeys (0.15-0.38%)(Petracci et al. 2006, Machovcova et al. 2017). The mortality in this study was higher than the values (0.72 - 1.22 %) reported in the limited number of studies examining the transport stress in layer hens (Petracci et al. 2006, Vecerkova et al. 2019). These results were attributed to hand catching and moving hens to the trailer, rough animal handling during loading, and poor microclimatic conditions in animal crates during lairage before unloading (Nijdam et al. 2005, Voslarova et al. 2007, Langkabel et al. 2015). Also, considering that the research was conducted in the winter season, it was argued that cold stress may have been very effective in increasing chicken deaths. Because the protective cover was not used and the surrounding of the trailer was open during the journey. Moreover, the studies on the transport of broiler and spent hens in the winter season reported higher DOA percentages than those poultry transports has been done in other seasons (Vecerek et al. 2016, Vecerkova et al. 2019). In addition, the high mortality rate was also associated with the age of laying hens (64 weeks of age) that were used in this study as farm animal species. Because spent hens are very fragile, and rough and careless handling during catching, loading, transportation, lairage, and unloading may have played a role in the increase in DOA percentages due to internal organ, muscle or bone traumas. Benincasa et al. (2020) reported that microclimatic conditions in the animal transport crates during animal transporting and lairage processes caused stress and decreased bird welfare.

The blood profile also confirmed the stress status related to the transport position of the hens. So, there was a decrease in cholesterol concentration and an increase in cortisol and glucose concentrations in transported chickens. In a similar study, Nijdam et al. (2005) reported that corticosterone and glucose levels increased during the capture and transport of broiler chickens. In addition, the decrease in triglyceride concentration in this study is further evidence of the stress response of chickens to transport. Also, Al-obaidy et al. (2020) reported similar results to these findings. Remage-Healey and Romero (2001) said that plasma triglyceride levels decrease during stress as a dimension of lipid regulation in poultry. Again, the MDA and AOA levels determined in this study indicate the oxidative damage in the transported hens (Onmaz et al. 2011, Pamok et al., 2019). The crate row position slightly affected the bodyweight loss of transported hens, but the crate section strongly influenced bodyweight loss. Bodyweight loss was increased linearly as the position of the transport crates went away from front to rear on the trailer. Similar results were found for the mortality rate, but the differences among the crate position groups were statistically insignificant. Bodyweight loss and mortality increased slightly as the crate position replaced from top to bottom rows.

The obtained results in the study showed that the position of the chicken transport crate on the trailer affected animal welfare. The stress conditions of the transport crates located in different sections or rows on the trailer were also different. Nevertheless, the impact of the trailer section was more meaningful on transport stress than trailer rows. Moreover, weight

loss and cortisol and triglyceride concentrations in transported hens exhibited significant differences among crate sections. It has been observed that the hens transported in the rear section of the trailer were adversely affected by transport than those transported in other sections. The low triglyceride concentration, slightly elevated glucose, and oxidative stress markers were emphasized to the higher stress in hens transported in the back of the trailer. The obtained results suggest that transporting hens in the rear section of the trailer may be more stressful, and animal welfare may decline, resulting in even death (Nielsen et al. 2011, dos Santos et al., 2017).

The reason for this may be that the rear section of the trailer may be more affected by the cold weather conditions, and the hens transported in this section may have been exposed to acute hypothermia. Because twelve hens were loaded in each crate to avoid high loading density according to legal sanctions, this may have caused the effect of cold stress to be greater than expected, especially in the rear section (Marahrens et al. 2011). So, It has already been reported that the climatic conditions of the immediate environment in which the animals are placed affect animal welfare (Benincasa et al. 2020). Dos Santos et al. (2017) reported that the temperature and humidity were higher in the trailer's rear section in animal transports during the warm season. Therefore, the transport stress was higher in the rear section. Also, the vibrations depending on the driving speed of the truck and the road's surface condition may have negatively affected the chickens transported in the rear of the trailer. Zhou et al. (2015) reported that transport vibration levels increased with truck speed, road conditions, load level, and overloading.

In general, the hens transported in the bottom row and rear section of the trailer were most adversely affected, and the welfare losses of these birds were higher. Waas et al. (1997) determined that deer transported in the rear and center sections of the trailer had higher heart rate and plasma lactate levels than those transported in the front section, in parallel with the results of this study. The researchers explained that the trailer's rear section might have been shaken more due to the truck's speed or the high transport vibration during the journey, and those loaded on the rear section may have had to do more physical activity to stay in balance. However, Vignola et al. (2008) and Dos Santos et al. (2017) reported that transport, season, or handling had a significant impact on animals transported than their position on the trailer.

It was determined that there was higher mortality and relatively slightly higher MDA and live weight loss in the bottom crate. These results show that the crates' microclimate on the bottom rows (insufficient oxygen, polluted air, high temperature, humidity, etc.) are adverse compared to the crates on the middle and top rows. It was thought that the fact that the side protection covers of the trailer bed were closed during the journey may have prevented the ventilation in the bottom rows to some extent. Also, Vecerkova et al. (2019) emphasized that old layer hens transported to the slaughterhouse may be more susceptible to transport stress and poor transport conditions than other poultry types. Also, Mitchell and Kettlewell (1994) reported that inadequate ventilation causes heterogeneous temperature and humidity in the transport vehicle and increases the risk of heat stress. Ajakaiye et al. (2010) said that high-temperature stress causes weight loss in chickens.

CONCLUSIONS

Consequently, Live weight loss, mortality rate, glucose, MDA, AOA, and cortisol levels were increased, while triglyceride and cholesterol levels decreased in laying hens transported between farm and slaughterhouse. The results showed that transport crates located in the bottom row and rear section of the trailer are very stressful and have a high risk for poor welfare for laying hens. The potential stress and risk profile of the animal crate's position on the trailer can instruct strategic solutions to reduce the welfare losses of the laying hens during transport.

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RESEARCH ARTICLE

Effect of Zeolite Use on Na^+, K^+ and Cl^- Ions in Neonatal Calves with Diarrhea

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ABSTRACT

In this study, the effects of zeolite applied in addition to routine treatment protocols on Na^+, K^+, and Cl^- levels in calves with diarrhea were investigated. 1-32 days old twelve calves with diarrhea, and 6 healthy controls brought to Sefa Veterinary Clinic in Viranşehir district were enrolled for the study. The responsible bacteria were *Escherichia coli* in nine of the diarrhea calves and *Criptosporodium parvum, Corona virus* and *Clostridium perfiringens* were in the others. Calves with diarrhea were divided into three groups in equal numbers (n=6) as negative control (healthy animal+1 g/kg day oral zeolite), positive control (routine treatment in animals with diarrhea), zeolite group (routine treatment in animals with diarrhea + zeolite (1g/kg/day, oral)). The application was made according to the prospectuses of İsotonic 0.9% NaCl (Polifleks®, Polifarma, Turkey), sodium bicarbonate (Bikarvil®, Vilsan, Ankara), Vitamin, Amino acid, Electrolyte, Dextrose (Duphalyte®, Zoetis, Spain) + Vitamine C (İnjacom C®, Ceva, Istanbul) + Marbofloxacin (Marbox®, Ceva, Istanbul) were administered daily to the positive control and zeolite groups. During the treatment procedure, death was observed on the 6th day in a calf with *Corona* virus. During the treatment, Na^+, K^+, and Cl^- ions were measured in the serum on the 1st, 3rd, and 5th-days. There was no statistical difference between the groups in terms of Na^+ and Cl^- ions on the 1st, 3rd, and 5th days ($p>0.05$). However, the 1st and 5th-day K^+ levels were found to be higher in the zeolite group compared to the other groups ($p<0.001$). The reason for this difference is thought to be due to both the cellular damage due to diarrheal agents and the increase in K^+ absorption from the environment by zeolite. However, it was observed that the healing process of diarrhea was faster in the zeolite group compared to the other groups.

Keywords: Diarrhea, Ion, Neonatal calves, Zeolite

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İshalli Buzağılarda Zeolit Kullanımının Na^+, K^+ ve Cl^- İyonları Üzerine Etkisi

ÖZ

Bu çalışmada ishalli buzağılarda rutin tedavi protokollerine ek olarak uygulanan zeolitin Na^+, K^+ ve Cl^- seviyeleri üzerine etkileri incelenmiştir. Çalışma için Viranşehir ilçesi Sefa Veteriner Kliniğine getirilen 1-32 günlük 12 adet ishalli ve sağlıklı 6 adet buzağı kaydedildi. İshalli buzağıların 9 tanesinde *Escherichia coli*, diğerlerinde ise *Criptosporodium parvum, Corona virus* ve *Clostridium perfiringens* etken tespiti yapıldı. İshalli buzağılar eşit sayıda olacak (n=6) şekilde negatif kontrol grubu (sağlıklı hayvan +1g/kg /gün, oral zeolit), pozitif kontrol grubu (ishalli hayvanda rutin tedavi), zeolit grubu (ishalli hayvanda rutin tedavi + zeolit (1g/kg /gün, oral)) olarak üç gruba ayrıldı. Pozitif kontrol ve zeolit grubuna günlük olarak izotonik 0.9% NaCl (Polifleks®, Polifarma, Türkiye), sodyum bikarbonat (Bikarvil®, Vilsan, Ankara), vitamin, amino asit, elektrolit, dekstroz (Duphalyte®, Zoetis, İspanya) + Vitamin C (İnjacom C®, Ceva, İstanbul) + Marbofloksasin (Marbox®, Ceva, İstanbul) uygulaması prospektüslerine göre yapıldı. Tedavi prosedürü içerisinde *Corona virüs* etkenli bir buzağıda 6. günde ölüm görüldü. Tedavi süresince 1. 3. ve 5. günlerde serumda Na^+, K^+ ve Cl^- iyonları ölçümü gerçekleştirildi. 1. 3. ve 5. günlerde Na^+ ve Cl^- iyonu bakımından gruplar arasında istatistikî olarak fark görülmedi ($p>0.05$). Ancak, çalışmada zeolit grubunda diğer gruplara göre 1.ve 5. gün K^+ düzeyleri daha yüksek bulunmuştur ($p<0,001$). Bu farklılığının nedeninin hem ishal etkenlerine bağlı oluşan hücresel hasardan hem de zeolitin ortamdan K^+ emilimini arttırmasından ileri geldiği düşünülmektedir. Bununla birlikte, zeolit grubunda diğer gruplara göre ishalin iyileşme sürecinin daha hızlı olduğu görülmüştür.

Anahtar kelimeler: Diyare, İyon, Neonatal Buzağı, Zeolit

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GİRİŞ

Dünya sığırcılığında buzağı kayıpları, önemli üretim kayıplarından biridir. Ülkemizde de yıllık ortalama %10-15 arasında buzağı kayıpları yaşandığı bilinmektedir (Şahal ve ark. 2018). Buzağı ishalleri gelişme performansında azalma, hastalığın tedavisinde birden fazla ilaç kullanımı ve hastalığa bağlı ölümle ciddi ekonomik kayıplara sebep olmaktadır. Sığırcılık işletmelerinde neonatal dönemde görülen buzağı ishallerini ortaya çıkaran etmenleri en aza indirgemek ve koruyucu önlemler almak kritik öneme sahiptir (Şen ve ark. 2013). Ortamda hijyenin sağlanamaması ve buzağıların yetersiz kolostrum almasına bağlı olarak antikor ve beslenme yetersizliği ishalin başlıca nedenlerini oluşturur (Aksoy 2016, Şen ve ark. 2013). Buzağılarda ishal birtakım enfeksiyöz (bakteri, parazit, protozoal, mantar ve virus) ve nonenfeksiyöz ajanlar (alerjik, alimenter, stres, toksik ve hazırlayıcı etmenler) ile ortaya çıkmaktadır. Buzağılarda ishal ile seyreden enfeksiyöz hastalıklar Kolibasillozis, Salmonellozis, Enterotoksemi, Rotaviruslar, Koronaviruslar, Adenoviruslar, Bovin Viral Diyare, Cryptosporidiozis'dir (Kaya ve Coşkun 2018, Şen ve ark. 2013, Yazıcı 1993, Yeşilbağ ve ark. 2012). *Salmonella, Camplyobacter spp ve Escherichia coli (E. coli*) gibi etkenler, dokulara zararlı bağırsak toksinleri salgılarlar. Bu toksinler, canlının yaşamsal fonksiyonlarının bozulmasına ve K^+ (potasyum), Na^+ (sodyum), Cl^- (klor) iyon salgısını artırır. Ayrıca buzağılarda ishale ciddi dehidrasyon, hipokloremi, metabolik asidozis, hiponatremi ve hipokalemi eşlik etmektedir (Akyüz ve ark. 2017).

İshal, metabolizmada genel olarak plazma Na^+ değerlerini çoğu vakada orta derecede azaltır veya normal sınırlardadır. Plazmada K^+ değeri belirgin şekilde yükselir, kan inorganik fosfor ve üre nitrojeni değerlerindeki artış renal yetmezliğin göstergesidir (Akyüz ve ark. 2017). Buzağılarda bulunan HCO_3^- (bikarbonat), K^+, Na^+ ve Cl^- kaybının büyük bir kısmı ishal ile gerçekleşmektedir. Sağlıklı bir buzağıda Na^+ miktarı: 60-150 mmol/L, K^+ miktarı: 4-20 mmol/L ve Cl^- miktarı: 40-130 mmol/L'dir. İshalli buzağılarda kan pH'sı, Na^+ ve Cl^- ve plazma $HCO3^-$ değerleri azalır. Elektrolit dengesinde ortaya çıkan bu değişiklikler metabolik asidoz ve hiperkalemi şekillenmesine neden olmaktadır (Aksoy 2016). Rutin tedavide diyet tedavisi, kemoterapötik ajanlar, mide-bağırsak antiseptikleri, dehidratasyon tedavisi, büzüştürücüler (tannik asit, meşe kabuğu), adsorbanlar (aktif kömür), iz element ve mineralller (Cu, Co, Mn, Zn, Se, Ca, P, Mg ve Fe), vitaminler (A, B, C, D, E), probiyotik ve prebiyotikler kullanılmaktadır (Bakhshi ve ark. 2006, Aksoy 2016). İshal tedavisinde rutin ilaç uygulamalarıyla tedavi masrafı, antibakteriyel ilaç kullanımıyla bakteriyel dirence ve çevre kirliliği oluşturması söz konusudur (Şen ve ark. 2013, Vidal ve ark. 2020). Antibiyotik kullanımını azaltacak madde ve uygulamaların araştırılması ülke ekonomisine katkısı bakımından son derece önemlidir (Waldenstedt 2003). Ülkemizde yoğun şekilde bulunan zeolit hayvan besleme ve sağlık alanlarında çeşitli amaçlarla kullanılmasının (Papaioannou ve ark. 2005, Şentürk Demirel ve ark. 2010) yanı sıra endüstri, madencilik, uzay teknolojileri, enerji, sağlık, tarım, hayvancılık, kirlilik kontrolleri ve kâğıt-deterjan sanayisi gibi alanlarında da geniş bir kullanım alanı bulmuştur (Köktürk 1995, Melenova ve ark. 2003). Zeolitler toz haline getirilip oral alındığında gıda veya vücut sıvılarıyla silikatlar gibi reaksiyon göstermezler. Doğal zeolit olan klinoptilolit mineralinin sindirim mekanizmasında emilimi düşük ve içerisinde Na^+,K^+, kalsiyum ve magnezyum gibi iyonları barındırmaktadır. Yapısında belirgin bir farklılaşma olmaksızın katyon değişimi, su kaybetme ve kazanma özelliğine sahiptir (Akdağ ve Ulutaş 2014). Zeolit kullanımı ekonomik olması ve beraberinde çevreye zarar vermemesi (Martínez ve ark. 2004); fare ve sıçanlarda 6-12 ay boyunca uygulanan zeolit mineralinin hiçbir zehirli etkisi ile karşılaşılmaması gibi avantajlara sahiptir (Pavelic ve ark. 2002). Zeolitin sığırlarda hipokalsemi ve abomasum deplasmanını azalttığı, yüksek verimli ineklerde süt ateşi, ketozis ve ağır metal zehirlenmesine karşı koruyucu olduğu, rumen aktivitesini ve iştahı arttırdığı (Aksoy ve ark. 2018, Thilsing-Hansen ve ark. 2002, Thilsing-Hansen ve Jørgensen 2001); zeolit içeren balon kataterin üriner sistemde (*Pseudomonas aeruginosa, Staphylococcus aureus* ve *E. coli*) ve diş hekimliğinde zeolit içeren pastanın (*Streptococcus mutans ve Streptococcus immitis*) antibakteriyel özellik gösterdiği (Hotta ve ark. 1998), dişte plak oluşumunu azalttığı (Akdağ ve Ulutaş 2014), in vitro çalışmada nazokomiyal solunum yolu enfeksiyonlarına karşı (*Candida albicans, Staphylococcus aureus* ve *Pseudomonas aeruginosa*) güçlü etkili olduğu belirlenmiştir (Matsuura ve ark. 1997). Zeolit domuz ve buzağıların rasyonuna ilave edilmesiyle ishalin görülme sıklığını, süresini ve şiddetini azaltmaktadır (Rodríguez-Fuentes ve ark. 1997, Papaioannou ve ark. 2005). Ayrıca yeni doğanlarda zeolit immunglobulin emilimini arttırdığı tespit edilmiştir (Stojic ve ark. 1995). Bu çalışmada ishalli buzağılarda zeolit uygulanmasının Na^+, K^+ ve Cl^- iyonları üzerindeki etkisi araştırılmıştır.

MATERYAL METOT

Hayvan Materyalinin Seçimi

Araştırmada Harran Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu tarafından 12.03.2019 tarih ve 2019/03 nolu oturum 01 nolu kararınca etik kurul iznine gerek olmadığı onayı alınmıştır. Mevcut çalışma tedavi amacıyla Viranşehir ilçesi Sefa Veteriner Kliniğine getirilen 1-32 günlük 12 adet ishalli ve 6 adet sağlıklı olan toplamda 18 adet buzağıyla yapılmıştır. İshalli buzağılar rastgele 2 gruba (n=12) ayrılmıştır. Ateşleri ateş ölçer (dijital derece) ile ölçüldü. İshal etkenleri buzağı hızlı ishal tanı kitlerinden (5'li tanı kiti, CEVA, Belçika) dışkı

muayenesi ile belirlendi. Buzağılardan steril dışkı çubuğuyla alınan dışkı numunesinin, kit içerisine yerleştirilmesi suretiyle etken pozitifliği değerlendirildi. Dehidratasyon derecesi buzağıların göz çukurluğuna ve deri elastikiyetine bakılarak klinik olarak belirlendi.

Tedavi Protokolü

Mevcut çalışmada her iki ishalli gruptaki buzağılara rutin tedavi protokolü uygulanmıştır. Rutin sağaltım protokolü olarak Bikarbonat ihtiyacı (mmol) = Vücut Ağırlığı (kg) x Baz Açığı (mmol/L) x0.5; Gerekli olan sodyum miktarı (GSM) (mmol/L) = [125-Ölçülen sodyum miktarı (mmol/L)] x [(0.6 x vücut ağırlığı (kg)] (Şentürk 2001) göre hesaplanarak intravenöz 0.9% NaCl (Polifleks®, Polifarma, Türkiye), sodyum bikarbonat (Bikarvil®, Vilsan, Ankara), vitamin, amino asit, elektrolit, dekstroz (Duphalyte®, Zoetis, İspanya) enjeksiyonluk çözelti; Vitamin C (İnjacom C®, Ceva, İstanbul) prospektüslerinde bildirilen doz ve sıklıkta kullanıldı. Marbofloksasin (Marbox®, Ceva, İstanbul) 1ml/50 kg 3 gün süreyle kas içi verilmiştir (Grandemange ve ark. 2002). Negatif kontrol grubu (Sağlıklı hayvanlar, n=6) bu hayvanlara oral yolla 1 g/kg zeolit (Nat- Min-9000) uygulaması; Pozitif kontrol grubu (ishalli hayvanlar, n=6) gruba yukarıda açıklanan rutin tedavi uygulaması; Zeolit grubu (ishalli hayvanlar, n=6) yukarıda açıklanan rutin tedavi prosedürüyle beraber 1 g/kg (Zarkovic 2003) oral yolla zeolit uygulaması yapılmıştır. Sağlıklı buzağılara yem olarak anne sütü canlı ağırlığının %5'i olacak şekilde sabah akşam verilmiştir. Buzağılardan venöz kan örnekleri 1. 3. ve 5. günlerde kan gazı cihazının kitlerine ait heparinli şırıngalara 1 ml kan alınmıştır (Torsein ve ark. 2011). Alınan kanların bulunduğu enjektörler, direkt olarak kan gazı kitlerine yerleştirilerek kan gazı ölçüm cihazıyla (Edan i15 Vet, Edan Instruments, Çin) analiz edilmiştir. Alınan serumlarda Na^+, K^+ ve Cl^- iyon değerleri mmol/L olarak ölçülmüştür. İstatistiki değerlendirme SPSS 21.0 paket programı kullanılmıştır. Sonuçlar ortalama ± standart hata olarak verilmiştir. Verilerde parametrik test varsayımlarını (homojenlik ve normallik) sağladığından gruplar arasındaki istatistiksel farkları belirlemede ANOVA testi kullanılmıştır. Gruplar arasındaki farklılığı ifade etmek için post-testte Duncan testi kullanılmıştır. İstatistiksel anlamlılık için $p<0,05$ olarak dikkate alınmıştır.

BULGULAR

Çalışma başlangıcında her iki grupta *E. coli, Corona virus, Cl perfiringes* ve *Criptosporidium parvum* nedenli klinik belirti olarak ishal ve dehidratasyon belirlendi. Tedavi protokolü sürecinde kontrol grubunda *Corona virüs* etkenli bir buzağıda, 6. günde ölüm görüldü.

Çalışmamızda kullanılan ishalli buzağılara ait yaş, vücut ısısı, ishal etkeni, dehidratasyon derecesi Tablo 1' de verilmiştir.

Tablo 2 'de sağlıklı ve ishalli buzağılara ait 1, 3 ve 5. günlerde alınan kan örneklerinde Na^+, K^+ ve Cl^- miktarlarının istatistiki değerlendirme tablosu verilmiştir. Yapılan çalışmada Na^+, K^+ ve Cl^- değerleri bakımından 1., 3. ve 5. günlerde gruplar içi istatistiki fark belirlenmemiştir.

Na^+ ve Cl^- iyonu miktarı bakımından gruplar arasında ve 1., 3. ve 5. günler arasında istatistiki açıdan bir farklılık görülmemiştir ($p>0.05$).

K^+ iyon değeri 1. ve 5. günde üç grupta birbirinden farklı bulunmuştur en yüksek değer zeolit uygulanan gruba aittir ($p<0.001$). K^+ değeri 3. günde zeolit ile pozitif kontrol grupları birbirine benzer, sağlıklı buzağılarda farklılık söz konusudur ($p<0.001$).

Bu çalışmada *E. coli* etkenli ishalli buzağılarda zeolit grubunda klinik olarak diğer gruplara göre daha hızlı iyileşme (genel bir canlanma, göz yuvarlağının görünümü ve deri elastikiyetinde düzelme) görülmüştür.

Tablo 1. İshalli buzağıların klinik bulguları
Table 1. Clinical signs of diarrhea calves

Yaş/gün	Ateş/^{o}C	Etken	Dehidratasyon Derecesi
5	36,7	*E.coli*	%4
3	38,5	*E. coli*	%4
4	38,5	*E. coli*	%4
17	38,3	*E. coli*	%4
32	37,3	*E. coli*	%6
11	36,9	*E. coli*	%6
9	37,6	*E. coli*	%6
17	36,8	*Cripto*	%6
9	37,8	*Cl.perfiringes*	%6
8	35,7	*Corona* virus	%8
9	36,2	*E. coli*	%6
10	37,7	*E. coli*	%6

Tablo 2. Buzağılara ait 1., 3. ve 5. günlerde alınan kan örneklerinin Na^+, K^+ ve Cl^- miktarları
Table 2. Na^+, K^+ and Cl^- amounts of blood samples taken from calves on the 1st, 3rd and 5th days.

Gruplar	n	Negatif Kontrol (Sağlıklı hayvan+ zeolit)	Pozitif Kontrol (İshalli hayvanda rutin tedavi)	Zeolit (ishalli hayvanda rutin tedavi+ zeolit)	P
Na^+_1	18	132 ±6.00	126,96 ± 9,09	130,52± 8,16	Ö.D
Na^+_3	18	132,83 ±5,70	128± 8,61	127,58± 9,47	Ö.D
Na^+_5	18	132,66 ±5,35	132,66± 7,77	132,46± 7,82	Ö.D
K^+_1	18	4,10 ± 0,28^{c}	5,31± 0,74^{b}	5,98± 0,37^{a}	***
K^+_3	18	4,08 ± 0,29^{b}	5,54 ±0,84^{a}	6,27± 0,60^{a}	***
K^+_5	18	4,05 ± 0,33^{c}	5,49± 0,88^{b}	6,31 ±0,59^{a}	***
Cl^-_1	18	102,83±5,67	96,05± 5,33	97,05 ± 7,48	Ö.D
Cl^-_3	18	103,00 ±4,77	97,78 ± 5,45	98,11 ±6,14	Ö.D
Cl^-_5	18	103,50±5,12	98,27 ± 5,58	98,62 ± 6,27	Ö.D

Na^+_1: 1. gün Na^+ değeri, Na^+_3: 3. gün Na^+ değeri, Na^+_5: 5. gün Na^+ değeri; K^+_1: 1 gün; K^+ değeri, K^+_3: 3. gün K^+ değeri, K^+_5: 5. gün K^+ değeri; Cl^-_1: 1. gün Cl^- değeri, Cl^-_3: 3. gün Cl^- değeri, Cl^-_5: 5. gün Cl^- değeri. Veriler, ortalama ± standart hata olarak verilmiştir. ÖD: İstatistiki önem yok, ***: $p<0.001$ farklılık istatistik açıdan önemlidir. a, b, c: Aynı satırdaki farklı harf taşıyan değerler arasındaki fark istatistiksel açıdan önemlidir.
Na^+_1: Day 1 Na^+ value, Na^+_3: Day 3 Na^+ value, Na^+_5: Day 5 Na^+ value; K^+_1: 1 day; K^+ value, K^+_3: Day 3 K^+ value, K^+_5: Day 5 K^+ value; Cl^-_1: Day 1 Cl^- value, Cl^-_3: Day 3 Cl^- value, Cl^-_5: Day 5 Cl^- value. Data are given as mean ± standard error. ÖD: No statistical significance, ***: $p<0.001$ difference statistically significant. a, b, c: The difference between values with different letters in the same row is statistically significant.

TARTIŞMA

Son yıllarda buzağı ishallerinin sağaltım ve profilaksideki gelişmelere rağmen, gelişme gerilikleri, tedavi-laboratuvar giderleri ve ölüm ile ekonomik kayıplara sebep olmaktadır (Izzo ve ark. 2015, Torsein ve ark. 2011). Zeolitin biyoyararlanımı ve zehirliliği ile ilgili az sayıda çalışma bulunmakla birlikte ülkemizde de yapılmış çalışmalar sınırlı sayıdadır (Filya ve ark. 1999, Thilsing-Hansen ve Jørgensen 2001, Toledano-Magaña ve ark. 2015, Ural ve ark. 2017). 2001 yılında Avrupa Komisyonu tarafından zeolitin çiftlik hayvanları yemlerinde katkı maddesi olarak kullanılmasına izin verilmiştir (Anonim 2013). Zeolitin ishal önleyici, antimikrobiyel, antiviral ve toksin bağlayıcı olarak kullanılabileceği belirtilmiştir (Sadeghi ve Shawrang 2008). Yeni doğanlarda rasyona uygun miktarda katılan zeolit türevleri ile ishalin süresi, şiddeti ve yoğunluğunun azalttığına dair çeşitli çalışmalar yapılmıştır (Natalija ve ark. 2005, Rodríguez-Fuentes ve ark. 1997). Farklı miktarda zeolitin sağlıklı (5g/L zeolit) ve ishalli (1g/kg zeolit) buzağılarda pasif immuniteyi arttırdığıyla ilgili çeşitli çalışmalar yapılmıştır (Natalija ve ark. 2005, Sadeghi ve Shawrang 2008). Bu çalışmada 1 g/kg miktarında zeolit kullanımının buzağılarda zararlı olmadığı ve zeolit grubundaki hayvanlarda klinik iyileşmeyi olumlu etkilediği görülmüştür. Farklı bir çalışmada da buzağılarda doğumdan sonraki ilk 10 gün oral klinoptilolit uygulamasının intestinal antikor absorbsiyonunu arttırarak *E. coli*'ye bağlı ishal görülme sıklığını azaltabileceği belirtilmektedir (Pourliotis ve ark. 2012). İshalli yavru domuzlarda *E. coli* enfeksiyonuna karşı 100 g/hayvan başı zeolitin ishali tedavi ettiği belirlenmiştir (Martínez ve ark. 2004). Mevcut çalışmada *E. coli* etkenine bağlı ishali bulunan ve zeolit uygulanan buzağılarda ishalin diğer etken ve kontrole göre erken iyileştiği tespit edilmiştir. Bunun sebebi zeolitin bağırsaklarda *E. coli* ye karşı antikor emilimini arttırmasından ileri geldiği düşünülmektedir.

Yeni doğanlarda ishal ve kolostrum alımının azalmasına bağlı vücutta elektrolit seviyelerinde azalmaya neden olur. Özellikle ishal serum Na^+ seviyesini azaltır (Şen ve ark. 2013). 60 adet (1 aydan küçük) ishalli buzağı ve 10 adet sağlıklı buzağının kan Na^+ miktarlarını ölçüldüğü çalışmada, ishalli hayvanlarda Na^+ miktarının sağlıklı olan hayvanlara göre düşük olduğu tespit edilmiştir (Abbas ve ark. 2019). Mevcut çalışmada, zeolit grubunun kan örneklerinde Na^+ iyonu miktarı 1. günde diğer günlere göre az olduğu; 3 ve 5. günlerde ise Na^+ iyonu miktarının 1. güne göre yüksek olduğu tespit edilmiştir. Golbeck ve ark. (2018) ishalli buzağılarda Marbofloksasin, oral yolla elektrolit çözeltisi (1 litrede 20.1 g dekstroz, 3.9 g NaCl, 3 g $KHCO_3$ ve 3 g Na^+propiyonat) ve damar içi hipertonik (8.4%) sodyum bikarbonat uygulaması sonrası kontrol grubuna göre tedavi sonrası Na^+ iyonunun arttığını tespit etmişlerdir. Tedavi öncesi Na^+ miktarı 130.74 ± 9.44 mmol/L'den 134.18 ± 4.23 mmol/L olacak şekilde arttığı tespit edilmiştir. Mevcut çalışmada hem pozitif kontrol hem de zeolit uygulanan gruptaki kan Na^+ miktarı arttırmıştır. Zeolit ve pozitif kontrol grubunda Na^+ değerindeki artış bakımından gruplar arasında istatistiki olarak bir fark belirlenmemiştir. Çalışmamızda Na^+ değerindeki bu artışın ishalin azalmasından; ayrıca ishalli buzağılara ilk gün uygulanan rutin tedavinin etkisinin ortaya çıkmasından kaynaklandığı düşünülmektedir.

Mevcut çalışmada pozitif kontrol ve zeolit uygulanan gruplarda 1, 3 ve 5. günlerde K^+ seviyesi artışı belirlenmiştir. Zeolit grubu ile diğer gruplar arasında 1. ve 5. günlerde fark istatistiki olarak yüksek çıkmıştır ($p<0.001$). Ancak bu yükseliş normal değerler arasında (4-10 meq/L) (Naylor ve ark. 1990) olduğu görülmüştür. Trefz ve ark. (2013) serum $K^+>$ 5.8 mmol/L olması durumunu hiperkalemi olarak yorumlaması gerektiğini ve bunun nedenin ise dehidratasyondan kaynaklanmış olabileceğini bildirmişlerdir. Mevcut çalışmada hem pozitif kontrol hem de zeolit grubunda K^+ seviyeleri>5.8 mmol/l'den yüksek olduğu tespit edilmiştir. Hiperkalemi oluşum nedenlerinin başında hücresel hasar gelmektedir (Tras 2013) ve çeşitli bakteriyel, viral ve paraziter hastalıklarında bağırsak hücrelerinde hasara neden olduğu bilinmektedir (Akan 2001, İzgür 2001).

Zeolitin K^+ üzerine etkisi ile ilgili yapılan farklı çalışmalarda yetişme alanlarına zeolit eklenmesinin bitkiler tarafından alınan potasyum miktarını arttırdığı belirlenmiştir (Gül ve ark. 2006). Çalışmamızda zeolit grubunda K^+ miktarının yüksek bulunmasının nedeni hem ishal etkenlerine bağlı oluşan hücresel hasardan hem de zeolitin ortamdan K^+ emilimini arttırmasından ileri geldiği düşünülmektedir. Hiperkalemi özelikle 7-8 mmol/ L'nin üzerinde belirlendiğinde yaşamı tehdit eden ciddi kalp problemlerine (bradikardi ve aritmi gibi) neden olduğu bilinmektedir (Basoglu ve Aydogdu 2013). Ancak, çalışmamızda K^+ seviyesinin arttığı buzağılarda kalp ile ilgili herhangi bir belirti ile karşılaşılmadı. Grove-White ve Michell (2001) ishalli buzağılarda sıvı tedavisi sırasında K^+ seviyesinin azaldığını belirlemişlerdir. Trefz ve ark. (2013) serum K^+ miktarının yeni doğan ishalli buzağılarda farklı olabileceğini ve mevcut K^+ depo seviyelerini yansıtamayacağını vurgulamışlardır.

Omole ve ark (2001) 21 sağlıklı ve 21 ishalli buzağıda elektrolit, glikoz ve glisin tedavisi sonrası serum Na^+, K^+ ve Cl^- ölçümlerinin yapıldığı bir çalışmada, sağlıklı hayvanlara göre ishal görülen hayvanların serumlarında Na^+, K^+ ve Cl^- miktarının arttığı tespit edilmiştir. Ancak, bu değerlerin normal değerlerden daha düşük olduğu görülmüştür. Mevcut çalışmada ishal tanısı konulan 12 hayvanda da K^+ miktarının sağlıklı hayvanlardaki normal değerlerden yüksek olduğu Na^+ ve Cl^- miktarları bakımından da ishalli buzağılar ile sağlıklı buzağılar arasında istatistiki olarak bir fark belirlenmemiştir ($p<0.001$). Lee ve ark.

(2020) 75 günden küçük 180 adet Kore yerli ırkı ishalli buzağıda kan serum Na^+ (104.0- 167.0 mmol/L), K^+ (2.0- 9.0 mmol/L) ve Cl^- (75-140.0 mmol/L) seviyeleri ile mevcut çalışmadaki pozitif kontrol, negatif kontrol ve zeolit grubundaki buzağıların kan serum seviyeleri ile benzer olduğu tespit edilmiştir.

SONUÇ

Zeolitin antibakteriyel etkili olmasının yanı sıra ekonomik olması ve çevreye zarar vermemesi gibi avantajları; suda çözünmemesi, ishalli buzağılarda emme refleksinin azalmasıyla yapılan uygulama hataları aspirasyon pnömonisine yol açabileceğinden uzman kişilerce uygulanma zorunluluğu dezavantajlarını oluşturmaktadır. Bu çalışmada *E. coli* nedenli enfeksiyona sahip ishalli buzağıların bulunduğu zeolit grubunda tedavinin klinik olarak diğer gruplara göre daha hızlı iyileşme gösterdiği görülmüştür. Araştırmamızda zeolit grubunda diğer gruplara göre 1.ve 5. gün K^+ düzeyleri daha yüksek bulunmuştur ($p<0.001$). Bu farklılığının nedeninin hem ishal etkenlerine bağlı oluşan hücresel hasardan hem de zeolitin ortamdan K^+ emilimini arttırmasından ileri geldiği düşünülmektedir. İshal tedavisinde zeolitin rutin tedaviye eklenmesi önerilse de Na^+, K^+ ve Cl^- miktarları yönünden hayvanlar mutlaka kontrol edilmelidir. İshal tedavisinin masraflı ve zor bir süreç olması nedeniyle koruyucu hekimlik (buzağıların yaşadığı yerde biyogüvenliği sağlaması, yeterli kolostrum tüketilmesi, iyi beslenme koşulları ve hayvan refahı) öncelikle yapılması tavsiye edilmektedir.

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RESEARCH ARTICLE

Clinicopathological Characteristics of Cats with Obstructive Lower Urinary Tract Disease in the Aydın Province (Turkey)

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ABSTRACT

The aim of this study was to retrospectively investigate the clinicopathological characteristics of cats with obstructive FLUTD in Aydin Province (Turkey) and the relationship of these features with short- (<36 hours) and long-term (≥36 hours) obstructions. A total of 27 adult cats of different breeds and ages with obstructive FLUTD were included in the study. Of these, 19 had short-term (Group 1), and 8 had long-term (Group 2) urethral obstruction history and clinical findings. Most of the cats with urethral obstruction were male, a mean age of 4.32±0.6 and cross-breed. Regarding the baseline characteristics, no obvious differences between the two groups. Besides localized symptoms such as dysuria, stranguria, oliguria/anuria, polysystemic findings such as dehydration, vomiting, and lethargy were statistically more frequent in the Group 2 cats compared to the Group 1. Pyuria, haematuria and proteinuria were the most common findings in the urinalysis. In cats with long-term obstruction (Group 2), azotemia and hyperkalemia were relatively severe and common. In conclusion, this study demonstrated that clinicopathological changes in cats with urethral obstruction are related to the duration of the obstruction. Considering the severity of both clinical findings and metabolic changes, emergency intervention in cats with urethral obstruction is of great importance.

Keywords: *Azotemia, Clinicopathology, Feline lower urinary tract disease, Urethral obstruction*

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Aydın İlinde (Türkiye) Obstrüktif Alt İdrar Yolu Hastalıklı Kedilerin Klinikopatolojik Özellikleri

ÖZ

Bu çalışmanın amacı, Aydın ilinde (Türkiye) obstrüktif FLUTD'li kedilerin klinikopatolojik özelliklerini ve bu özelliklerin kısa (<36 saat) ve uzun süreli (≥36 saat) tıkanıklıklarla ilişkisini retrospektif olarak araştırmaktı. Çalışmaya obstrüktif FLUTD'li farklı ırk ve yaşlarda toplam 27 yetişkin kedi dahil edildi. Bunlardan 19'u kısa süreli (Grup 1) ve 8'i uzun süreli (Grup 2) üretral obstrüksiyon öyküsü ve klinik bulgularına sahipti. Üretral obstrüksiyonu olan kedilerin çoğu erkek, genç-orta yaşlı (ortalama 4.32±0.6) ve melezdi ve iki grup arasında temel özellikler açısından belirgin bir fark yoktu. Disüri, strangüri, oligüri/anüri gibi lokalize semptomların yanı sıra dehidratasyon, kusma ve letarji gibi polisistemik bulgular Grup 2'deki kedilerde Grup 1'deki kedilere göre istatistiksel olarak daha sıktı. . Piyüri, hematüri ve proteinüri idrar tahlilinde en sık görülen bulgulardı. Uzun süreli obstrüksiyonu olan kedilerde (Grup 2) azotemi ve hiperkalemi nispeten şiddetli ve yaygındı. Sonuç olarak, bu çalışma üretral obstrüksiyonu olan kedilerde klinikopatolojik değişikliklerin obstrüksiyonun süresi ile ilişkili olduğunu göstermiştir. Hem klinik bulguların hem de metabolik değişikliklerin ciddiyeti dikkate alındığında üretra tıkanıklığı olan kedilerde acil müdahale büyük önem taşımaktadır.

Anahtar Kelimeler: *Azotemi, Klinikopatoloji, Kedi alt idrar yolu hastalığı, Üretral obstrüksiyon*

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INTRODUCTION

Feline lower urinary tract disease (FLUTD) is one of the common diseases in cats and includes disorders affecting the urinary bladder and/or urethra (Neri et al. 2016, Lew-Kojrys et al. 2017). Urinary tract infections, urethral plugs, uroliths, neoplasms, malformations and idiopathic diseases (ie, feline idiopathic cystitis) can result in FLUTD (Segev et al. 2010, Sævik et al. 2011). Regardless of the cause, it can have life-threatening consequences in cats. Urethral obstruction, a common (18-58%) consequence of FLUTD, is reported as a potentially life-threatening emergency in veterinary practice (Gerber et al. 2008, Segev et al. 2010, Fröhlich et al. 2016).

Although the most common causes of obstructive FLUTD are physical factors such as urethral plaques and uroliths, detrusor and urethral dysfunction can also cause it (Joseph et al. 1996, Segev et al. 2010). Clinical findings of obstructive FLUTD vary considerably depending on several factors, including the degree of urine outflow reduction, duration of the obstruction, and presence of secondary bacterial infection (Joseph et al. 1996). Obstructive FLUTD may cause localized lower urinary tract symptoms such as dysuria, hematuria, pollakiuria, and pain, as well as polysystemic findings associated with uremia and electrolyte disturbances, such as loss of appetite, vomiting, dehydration, and lethargy. Even the cases with delayed intervention may result in death. (Segev et al. 2010, Fröhlich et al. 2016). For all that, these findings in cats with obstructive FLUTD are often reversible with appropriate treatment and management (Neri el al. 2016). Survival is related to the clinician's immediate intervention by anticipating the vital metabolic and hemodynamic disturbances that may occur in the patient.

The clinicopathological features of cats with obstructive and/or non-obstructive FLUTD have been presented in regional studies (Gerber et al. 2005, Neri et al. 2016, Lew-Kojrys et al. 2017, Kovarikova et al. 2020). However, it is well known that climatic, environmental, dietary and behavioural factors play a role in the etiopathogenesis of this disease (Sumner and Rishniw 2017). To the best of our knowledge, no study examining the clinicopathological features of cats with obstructive FLUTD under considering the duration of obstruction in Turkey. This study aimed therefore to describe the signalment, clinical findings, and results of the urinalysis and blood biochemical analysis of cats with obstructive FLUTD in the Aydın province (Turkey) and to evaluate the clinicopathological characteristics under the short- (<36 h) and the long-term (≥36 h) urethral obstruction.

MATERIAL and METHODS

Animals

This study was carried out retrospectively using the data records of the cats treated for obstructive FLUTD in Aydin Adnan Menderes University Veterinary Teaching Hospital during the period 2018-2021. Urethral obstruction was diagnosed on the basis of clinical history of strangury, pain at abdominal palpation (central hypogastric region), and distension of the urinary bladder associated with ischuria. Obstruction was also diagnosed when the bladder size was normal, but calculi were seen in the urethra, or obvious resistance was experienced with urethral catheterization. Cats were excluded from the study if they had other known concurrent diseases such as diabetes mellitus, liver disease or chronic kidney disease, or had been treated with any medication such as steroids or diuretics. A total of 27 cats with obstructive FLUTD were assessed in this study. The cats enrolled in the study were evaluated as for the effect of the duration of obstruction on clinical and laboratory parameters and divided into 2 groups. According to the medical history and clinical findings, cats with signs of urethral obstruction for up to 36 hours were defined as Group 1 (n=19), and cats with signs of obstruction for more than 36 hours as Group 2 (n=8).

Procedures

Medical history data including the signalment and complaints (pollakiuria, dysuria, hematuria, stranguria and vomiting) of the cats obtained from the owner, were recorded. Vital signs (dehydration, lethargy, and collapse) of the patient were evaluated with a detailed physical examination. All the evaluation procedures were performed without any sedation or anaesthesia.

Urine and blood samples were collected on the patient admission, with initial IV catheter placement and prior to medical intervention. Urine samples were taken with a urinary catheter from all cats in the Group 1. Except for 3 cats with cystocentesis, urine samples were obtained by urinary catheter in the Group 2 cats. Urinalysis included determining urine specific gravity (SG) by refractometer, dipstick chemistry (Combur-Test; Roche Diagnostics, Germany), and urine sediment cytology. In the cytological examination of the urine sediment, epithelial cells, struvite crystals and bacteria were noted.

Blood samples were taken into a 2 ml serum separation tube (Vacutainer, Beckton, Dickenson) and into a 2 ml heparinized blood gas injector during IV catheterization. Blood gas analysis was performed with Irma Trupoint Blood Analysis System (USA). These blood gas analysis outputs obtained the blood potassium (K) data discussed in this study. Blood samples in serum separation tubes were centrifuged after clot retraction at 2000 x g for 10 min to obtain sera. Serum creatinine (CREA) and blood urea

nitrogen (BUN) concentrations were measured with the Spotchem EZ SP-4430 device (Japan). Azotemia was defined as mild (BUN 30–50 mg/dL and CREA 2-3 mg/dL) and moderate-severe (BUN ≥ 50 mg/dL and CREA ≥ 3 mg/dL). Hyperkalemia that can be seen in cats with obstruction was also classified as mild (5–6 mmol/L) and moderate-severe (>6 mmol/L) (Mauro et al. 2020).

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 19.0 (IBM Corporation, Armonk, USA). All the graphics were drawn using GraphPad 8.0.2 (San Diego, CA, USA). The distributions of all continuous variables were evaluated by the Shapiro–Wilk test. Accordingly, urine SG and pH, and blood Na and K values were normally distributed, while serum BUN and CREA concentrations were not normally distributed despite log transformation. Means, standard error of means (SEM), medians and interquartile ranges (IQR) for each continuous parameter were calculated using descriptive statistics. Independent sample *t*-test or Mann–Whitney test was used to compare continuous variables between the Groups. Categorical variables (clinical signs and results of urinalysis) were described using percentages (%). The Chi-Square test (Fisher's exact test or Pearson Chi-Square test) was used to compare these variables. All analyses were considered statistically significant at $P< 0.05$.

RESULTS

The baseline characteristics, clinical findings, urinalysis results and azotemia-hyperkalemia evaluations of cats with obstructive FLUTD examined in this study are presented in Table 1, Table 2, Table 3 and Table 4, respectively. The blood biochemical results of the study groups, including BUN (A), CREA (B), and K (C), are shown in Figure 1.

A total of 27 cats with obstructive FLUTD were included in the study. Of these, 19 had short-term (Group 1), and 8 had long-term (Group 2) urethral obstruction history and clinical findings. Twenty-three (85.18%) of the cats were male. Although the ages of the cats evaluated in this study ranged from 8 months to 10 years, obstructive FLUTD was more common in cats aged 2-6 years. Thirteen (48.1%) of 27 patients examined were cross-breed followed by Persian cats with 14.81% (Table 1). There were no significant differences between the Group 1 and Group 2 regarding age, sex and breed.

All cats with obstructive FLUTD had dysuria, stranguria, oliguria/anuria, abdominal pain, and distended bladder findings in the medical history and clinical examination. Additionally, polysystemic findings such as dehydration, vomiting, and lethargy were statistically more frequent in the Group 2 cats compared to the cats of Group 1. Only 1 of the Group 2 cats died, and thus the total mortality was determined as 3.70% (Table 2).

Urinary catheterization was used to obtain the urine sample as part of the therapeutic protocol in cats with obstructive FLUTD. However, necessary cystocentesis was performed in 3 cats in Group 2 as part of the emergency response. The urine specific gravity ranged from 1.005 to 1.060 (mean 1021.29±2.94). The urine pH ranged from 6 to 9 (mean 8.01±0.13). Different degrees of positive reaction for protein (96.29%), blood (96.29%) and leukocytes (100%) in the urinary dipstick was seen in almost all urine samples. Pyuria was seen more intensely in the urine of the Group 2 cats compared to the Group 1 cats. Microscopic examination of urine sediments revealed epithelial cells in 85%, bacteria in 74% and struvite crystals in 14% (Table 3).

Serum BUN, CREA and whole blood K concentrations were measured in terms of the risk of postrenal azotemia and hyperkalemia. Accordingly, of the 27 cats with obstructive FLUTD evaluated, 14 were azotemic (51.85%). Moderate-severe azotemia was determined in almost all cats (7; 87.5%) in the Group 2, while it was present only 1 (3.70%) of the cats in the Group 1. Mild hyperkalemia in five (4 in Group 1 and 1 in Group 2) and moderate-severe hyperkalemia in four cats (all in Group 2) of the 27 patients with obstructive FLUTD were determined (Table 4.) Blood BUN, CREA and K concentrations of the Group 2 cats were found to be higher ($P<0.001$) than those of the cats in the Group 1 (Figure 1).

Table 1. Baseline characteristics of cats with obstructive FLUTD in this study.

	Total	Group 1	Group 2
Number of cats (%)	27	19	8
Age (mean ± SEM)	4.32±0.6	4.96±0.82	3.56±0.84
Sex			
Males	23 (85.1%)	15 (78.9%)	8 (100%)
Females	4 (14.81%)	4 (21.05%)	0
Breed			
British Shorthair	3 (11.11%)	1 (5.2%)	2 (25%)
Persian	4 (14.81%)	2 (10.52%)	2 (25%)
Scottish Fold	3 (11.11%)	2 (10.52%)	1 (12.5%)
Siamese	3 (11.11%)	3 (15.78%)	0
Angora	1 (3.70%)	1 (5.2%)	0
Cross-breed	13 (48.1%)	10 (52.6%)	3 (37.5%)

Group 1 and Group 2 describe cats with FLUTD showing signs of short (<36 hours) and long-term (≥36 hours) urethral obstruction, respectively.

Table 2. The clinical signs of cats with obstructive FLUTD in this study.

	Total	Group 1	Group 2	P
Number of cats (%)	27	19	8	
Dysuria	27 (100%)	19 (100%)	8 (100%)	-
Oliguria/anuria	27 (100%)	19 (100%)	8 (100%)	-
Pollakiuria	7 (25.92%)	6 (31.57%)	1 (12.5%)	0.301
Vomiting	10 (37.03%)	4 (25.05%)	6 (75%)	**0.014**
Haematuria	22 (81.48%)	15 (78.94%)	7 (87.5%)	0.528
Dehydration	15 (55.55%)	8 (42.10%)	7 (87.5%)	**0.038**
Lethargy	9 (33.33%)	4 (25.05%)	5 (62.55%)	**0.049**
Inappetence	20 (74.07%)	12 (63.15%)	8 (100%)	0.057
Mortality	1 (3.70%)	0	1 (12.5%)	-

Group 1 and Group 2 describe cats with FLUTD showing signs of short (<36 hours) and long-term (≥36 hours) urethral obstruction, respectively.

Table 3. The results of the urinalysis of cats with obstructive FLUTD in this study.

	Total	Group 1	Group 2	P
Number of cats (%)	27	19	8	
SG (mean±SEM)	1021.29±2.94	1022.36±3.27	1018.75±6.46	0.585
PH (mean±SEM)	8.01±0.13	7.92±0.12	8.25±0.35	0.273
Dipstick – protein	26 (96.29%)			0.815
0	1 (3.70%)	1 (5.25%)	0	
1+	3 (11.11%)	2 (10.52%)	1 (12.5%)	
2+	11 (40.74%)	8 (42.10%)	3 (37.5%)	
3+	12 (44.44%)	8 (42.10%)	4 (50%)	
Dipstick – blood	26 (96.29%)			0.810
0	1 (3.70%)	1 (5.25%)	0	
1+	7 (25.92%)	5 (26.31%)	2 (25%)	
2+	7 (25.92%)	5 (26.31%)	2 (25%)	
3+	12 (44.44%)	8 (42.10%)	4 (50%)	
Dipstick- leukocytes	27 (100%)			**0.006**
0	0	0	0	
1+	1 (3.70%)	1 (5.25%)	0	
2+	6 (22.22%)	5 (26.31%)	1 (12.5%)	
3+	20 (74.07%)	13 (68.42%)	7 (87.5%)	
Urine sediment				
Epithelial cells	23 (85.18%)	16 (84.21%)	7 (87.5%)	0.663
Struvite crystals	4 (14.81%)	2 (10.52%)	2 (25%)	0.337
Bacteria	20 (74.07%)	13 (68.42%)	7 (87.5%)	0.301

Group 1 and Group 2 describe cats with FLUTD showing signs of short (<36 hours) and long-term (≥36 hours) urethral obstruction, respectively.

Table 4. Evaluation of azotemia and hyperkalemia in cats with obstructive FLUTD in this study.

	Total	Group 1	Group 2
Number of cats (%)	27	19	8
Azotemia	14 (51.85%)	5 (26.31%)	8 (100%)
Mild	6 (22.22%)	4 (21.05%)	1 (12.5%)
Moderate-severe	8 (29.62%)	2 (10.52%)	7 (87.5%)
Hyperkalemia	9 (33.3%)	4 (21.05%)	5 (62.5%)
Mild	5 (18.51%)	4 (21.05%)	1 (12.5%)
Moderate-severe	4 (14.81%)	0	4 (50%)

Group 1 and Group 2 describe cats with FLUTD showing signs of short (<36 hours) and long-term (≥36 hours) urethral obstruction, respectively.

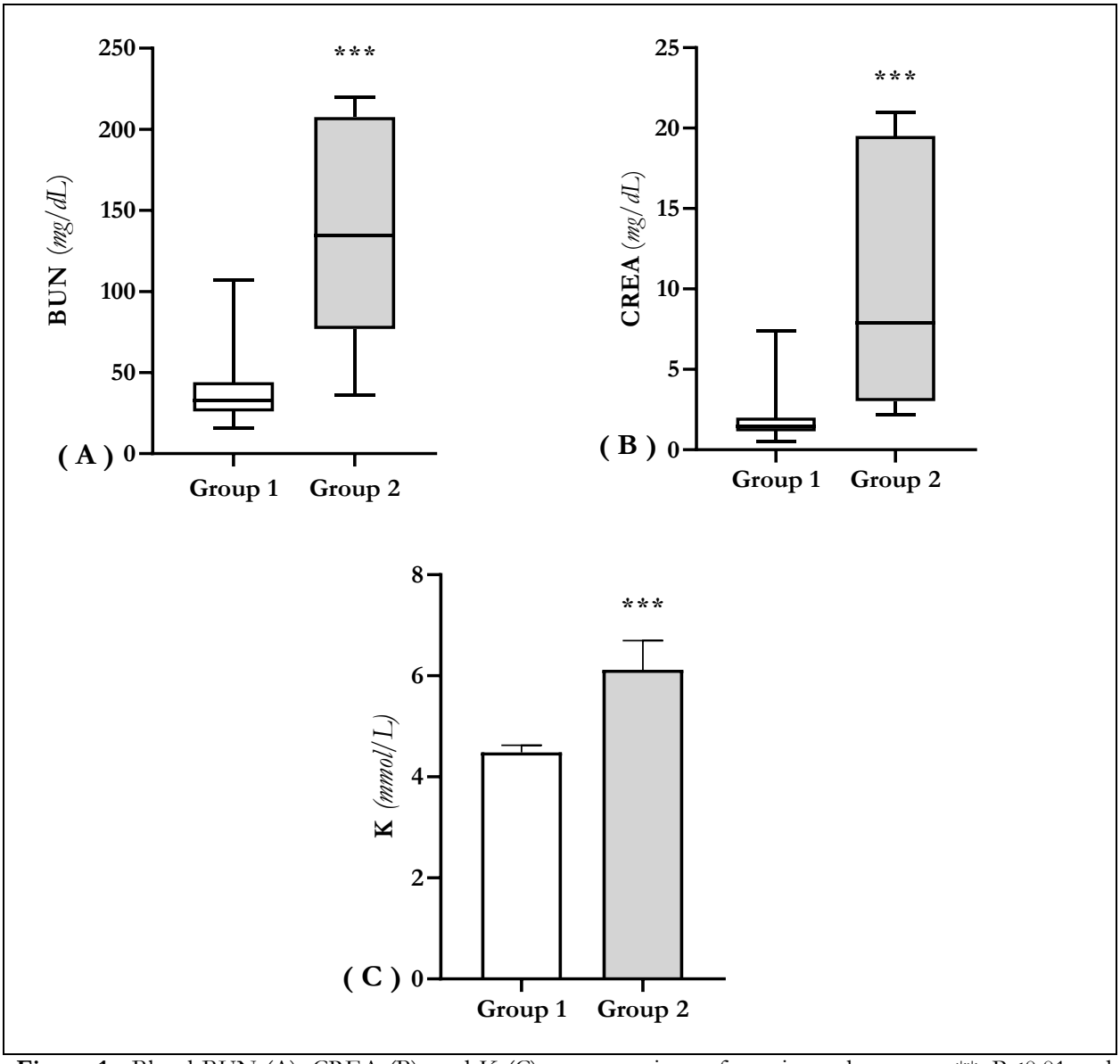

Figure 1: Blood BUN (A), CREA (B), and K (C) concentrations of cats in study groups. **: P<0.01 and ***:P<0.001

DISCUSSION

Urethral obstruction is a life-threatening complication reported to occur in 18-58% of cats with FLUTD (Gerber et al. 2005, Gerber et al. 2008). In this study, clinicopathological characteristics of cats with obstructive FLUTD in Aydın province (Turkey) and the relationship of these features with short (<36 h) and long-term (≥36 h) obstructions were evaluated retrospectively.

The incidence and causes of FLUTD are affected by age, sex, and breed. A gradual decrease in the frequency of idiopathic cystitis and urethral obstruction and an increase in the frequency of urinary tract infections with age are reported (Lekcharoensuk et al. 2001, Segev et al. 2011, Kovarikova et al. 2020). The fact that most of the cats evaluated in this study consisted of young and middle-aged (2-6 years) is consistent with previous data (Lekcharoensuk et al. 2001, Segev et al. 2011). On the other hand, in a study (Mauro et al. 2020) evaluating post-mortem urinary tract changes in cats with urethral obstruction, the mean (7.4 years) age was older and this is attributable to the fact that chronic diseases such as chronic kidney disease and cystitis may increase the risk of urethral obstruction. The age-related difference between the studies may be explained by the variety in the cat population evaluated for the purposes. In this context, cats with diseases such as chronic kidney disease or diabetes mellitus that are considered increasing risk factor for urethral obstruction was not enrolled in our study. Urethral obstruction are reported almost exclusively in male cats due to their relatively long and narrow

urethra, similar to our results in Table 1 (Lee and Drobatz 2003, Segev et al. 2011, Sumner and Rishniw 2017). The most cat breed affected by obstructive FLUTD in this study were cross-breed which are the most common cats in Turkey. The second was Persian cats. Lekcharoensuk et al. (2001) described that the risk of FLUTD may be higher in Persian and Himalayan cats, although no breed predisposition has been determined (Lekcharoensuk et al. 2001, Saevik et al. 2011, Kovarikova et al. 2020). There are no obvious differences in the baseline characteristics of cats between the Group 1 and Group 2 in this study (Table 1). The duration of obstruction may be related to the owner's interest and early recognition of the signs rather than the baseline characteristics of the cats.

Clinical findings in cats with obstructive FLUTD vary depending on the extent of inflammation of the urinary system, the degree and duration of the obstruction (Joseph et al. 1996). Affected cats may present with symptoms localized to the urinary system such as dysuria, stranguria, oliguria/anuria, distended bladder and abdominal pain, and/or polysystemic findings such as dehydration, vomiting, lethargy, and collapse (Segev et al. 2011, Neri et al. 2016). The clinical signs observed in all the study cats were in agreement with previous above-mentioned reports. Polysystemic findings such as dehydration, vomiting, and lethargy were higher proportion in the Group 2 cats compared to the Group 1 (Table 2). In fact that no patient died in the Group 1, while one cat of the Group 2 died due to collapse. One explanation for this may be that the clinical severity of the disease and mortality are closely related to the duration of the obstruction.

Hematuria, proteinuria, and pyuria are the most observed findings in urinalysis (Fischer et al. 2009, Kovarikova et al. 2020). The pressure in the bladder increases because the urine flow cannot be fully ensured in urethral obstruction. Hematuria may be associated with submucosal bleeding due to high intravesical pressure. Submucosal haemorrhage and necrosis of the epithelium within 10 hours after obstruction occur (Fischer et al. 2009, Kovarikova et al. 2020). The origin of the proteinuria in cats with obstructive FLUTD is most likely post-renal (Segev et al. 2011). Inflammatory processes both as a cause and a consequence of the obstruction result in varying degrees of pyuria (Segev et al. 2011). The most common findings in the urinalysis of this study cats (Table 3) were haematuria (96.29%), proteinuria (96.29%) and pyuria (100%). The changes and frequency observed in urinalysis in our study are in concordance with previous studies and can be explained by similar reasons. However, it must be noted that dipsticks may cause false readings, especially in the presence of severe hematuria. Therefore, the high proportion of proteinuria and pyuria should be interpreted with caution. These parameters in urine were analyzed only by dipstick.

Obstructive FLUTD is the most common and vital cause of post-renal acute kidney injury. Acute kidney injury results from increased pressure within the renal pelvis and ureter that decreases renal blood flow and glomerular filtration rate. Loss of renal function and acute renal injury have been reported within 24 hours of the obstructive event. The primary biochemical changes of acute kidney injury are azotemia and hyperkalemia (Joseph et al. 1996, Fischer et al. 2009). Previous studies have highlighted these biochemical changes associated with acute renal injury in cats with obstructive FLUTD. In a retrospective study (Kyles et al. 2005), 83% of cats with 163 urethral stones had azotemia, and 35% had hyperkalemia. Similarly, Segev et al. (2011) reported that the most common biochemical abnormalities in cats with urethral obstruction are azotemia (85%), hyperkalemia (48%), hypercalcemia (56%), and hyponatremia (55%). However, the evaluation in these studies has not been performed by the duration of urethral obstruction. The changes in serum BUN and CREA, and blood K concentration are shown in Figure 1. Azotemia was found to be 26% and 100% of cats with FLUTD showing signs of short-term (Group 1) and long-term (Group 2) obstruction, respectively. Similarly, moderate-to-severe hyperkalemia was not detected in any of the cats in Group 1, whereas moderate-severe hyperkalemia was detected in half of the cats in Group 2. More severe and polysystemic clinical manifestations in cats with long-term obstruction are closely related to azotemia and hyperkalemia, as noted in previous studies (Joseph et al. 1996, Fischer et al. 2009, Neri et al. 2016).

CONCLUSION

This is the first study to assess the clinicopathological features of cats with obstructive FLUTD in Aydın province. The clinicopathological changes in cats with urethral obstruction for more than 36 hours were more severe compared to the cats with urethral obstruction for less than 36 hours; however, these changes are mostly reversible with early intervention and appropriate treatment. Therefore, it is crucial to predict the complications that may occur in cats short and long term urethral obstructions and to intervene immediately by veterinary clinicians.

Conflict of Interest: The authors declare that there is no conflict of interest for this article and no financial support has been received

Ethical Permission: This study was carried out retrospectively using the data records of the cats that were treated for obstructive FLUTD in Aydın Adnan Menderes University Veterinary Teaching Hospital. No animal material was used.

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RESEARCH ARTICLE

Eye Injuries in Cats with Head Trauma

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ABSTRACT

Head trauma may lead to damage of soft tissue and bones of skull by an external force. The modified Glasgow coma scale (mGCS) is used to evaluate the prognosis and classify the neurological status of cats and dogs with head trauma. The aim of this study is determine the ophtalmic pathologies and the incidence of these pathologies as a result of head trauma in cats. In addition, any possible correlation between intraocular pressure (IOP) and mGCS will be investigated in traumatized cats. The animal material of the study consisted of 18 cats with acute head trauma. The animals were properly restricted so that mGCS scores were recorded. Systematic eye examination was performed as far as the clinical condition of the patient allowed during the clinical examination and treatment of the traumatized cats. Proptosis was observed in 4 cats (22.22%) as unilaterally in 3 cats and bilaterally in 1 cat. Subconjunctival hemorrhage was observed in 7 cats (38.88%) as bilaterally in 2 cats and unilaterally in 5 cats. Unilateral hyphema was observed in 2 cats (11.11%). Three cats had anisocoria (16.66%), 2 cats had unilateral nystagmus (11.11%), and 1 cat had unilateral Horner's syndrome (5.55%). In cats with glaucoma the median mGCS was 8 (score 4-15), while non-glaucoma the median mGCS was 9 (score 6-14). However, no statistical correlation was found between mGCS and glaucoma ($p>0.05$). The results of this study indicate that glaucoma, subconjunctival hemorrhage and proptosis are the most common pathologies in cats with head trauma and these pathologies should not be ignored during the intervention of the head trauma patients. In addition, it is thought that irreversible loss of vision can be prevented by frequently repeated IOP measurements, regardless of the mGCS score, in cases with head trauma.
Key Words: Cat, head trauma, eye injury, mGCS, trauma.

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Kafa Travmalı Kedilerde Göz Yaralanmaları

ÖZ

Kafa travması, harici bir kuvvetle yumuşak doku ve kafatası kemiklerinin hasar görmesine neden olabilir. Modifiye Glasgow koma skalası (mGCS), kafa travmalı kedi ve köpeklerin prognozunu değerlendirmek ve nörolojik durumlarını sınıflandırmak için kullanılır. Bu çalışmanın amacı, kedilerde kafa travması sonucu oluşan oftalmik patolojileri ve bu patolojilerin görülme sıklığını belirlemektir. Ayrıca travma geçirmiş kedilerde göz içi basıncı (GİB) ile mGCS arasındaki olası ilişki araştırılacaktır. Araştırmanın hayvan materyalini akut kafa travmalı 18 kedi oluşturmuştur. Hayvanlar uygun şekilde kontrol altına alınıp, mGCS skorları kaydedildi. Travma geçiren kedilerin klinik muayenesi ve tedavisi sırasında hastanın klinik durumunun izin verdiği ölçüde sistematik göz muayenesi yapıldı. Proptozis 4 kedide (%22.22); 3 kedide unilateral, 1 kedide bilateral olarak gözlendi. Subkonjonktival kanama 7 kedide (%38.88); 2 kedide bilateral, 5 kedide tek taraflı olarak gözlendi. 2 kedide (%11.11) tek taraflı hifema görüldü. Üç kedide anizokori (%16.66), 2 kedide unilateral nistagmus (%11.11) ve 1 kedide tek taraflı Horner sendromu (%5.55) görüldü. Glokomlu kedilerde medyan mGCS 8 (skor 4-15), glokom olmayan kedilerde medyan mGCS 9 (skor 6-14) olarak kaydedildi. Ancak mGCS ile glokom arasında istatistiksel bir ilişki bulunamadı ($p>0.05$). Kafa travmalı kedilerde glokom, subkonjonktival kanama ve proptozis patolojilerinin en sık görülen patolojiler olduğu ve kafa travmalı hastaların müdahalesinde bu patolojilerin göz ardı edilmemesi gerektiği sonucuna varıldı. Ayrıca kafa travmalı olgularda mGCS skoru ne olursa olsun göz içi basınç ölçümlerinin sık sık tekrarlanmasıyla geri dönüşü olmayan görme kaybının önlenebileceği düşünülmektedir.
Anahtar Kelimeler: Göz yaralanması, kafa travması, kedi, mGCS, travma.

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INTRODUCTION

Head trauma may lead to damage of soft tissue (including brain, skin and eyes) and bones of skull by an external force. Rapid diagnosis and response to the trauma patient is required to ensure the best prognosis (Kuo et al. 2018). Approximately 50% of cats and dogs are exposed to head trauma due to more frequently high-energy blunt traumas such as motor vehicle accidents and contusion injuries. Falls from a height, gunshot wounds, intentional/unintentional attacks from other animals and humans also are other common causes of trauma (Platt 2008, Dewey and Fletcher 2015). Traumatic pathologies tend to encountered in more than one body system and often including the head region in feline trauma patient with a small size of body. Extremity injuries are the most common pathologies in traumatized cats, followed by head injuries (Rochlitz 2004).

The modified Glasgow coma scale (mGCS) is used to evaluate the prognosis and classify the neurological status of cats and dogs with head trauma. This scale; divided into three neurologic examination categories that assess level of consciousness, motor activity, and brainstem reflexes. Each category is scored from 1 to 6 with 1 indicate to more severe dysfunction. The obtained scores from each category added together to establish coma score. The patient's condition is classified as grave (3-8), guarded (9-14), or good (15-18) according to the established coma score. High scores tend to with the best prognosis, while low scores associated with more than 50% mortality rate over the 1st 72 hours. This scoring system provides information about patient assessment, prediction of treatment response, evaluation of therapeutic options, and the prognosis (Platt et al. 2001, Platt and Olby 2004, Dewey and Fletcher 2015).

The first ophtalmic pathology associated with forebrain damage as a result of trauma in cats and dogs is myosis of the pupils. Due to the progression of forebrain and/or brainstem damage, the pupils are observed as mydriatic and response to light stimuli. These clinical signs could be associated with brain herniation accompanied by altered consciousness and cardiorespiratory patterns (Platt 2008, Sande and West 2010, Opperman 2014, Dewey and Fletcher 2015). Glaucoma represents a diverse group of diseases. In all species, it results in vision loss caused by characteristic optic nerve and retinal pathology. Glaucoma is therefore generally considered a neurodegenerative disease. The most important and only risk factor for the development of glaucoma is increased intraocular pressure (IOP) (McLellan and Miller 2011). The TonoVet® rebound tonometer is more accurate, especially at IOP >30 mmHg; does not require topical anesthetic, well tolerated by cats. Normal IOP is 20.74±0.5 mmHg with the TonoVet® rebound tonometer in cats (Rusanen et al. 2010). Secondary glaucomas, constituting for 95-98% of glaucoma cases in cats, are associated with alter aqueous humor dynamics due to uveitis, neoplasia, trauma, and intraocular hemorrhage (Blocker and van der Woerdt 2001). Underlying Depending on the pathogenesis, they may be unilateral or less frequently bilateral in presentation and are most common in adult cats (McLellan and Miller 2011).

The aim of this study is determine the ophtalmic pathologies and the incidence of these pathologies as a result of head trauma in cats. In addition, any possible correlation between IOP and mGCS will be investigated in traumatized cats.

MATERIALS and METHODS

Animals

The animal material of the study consisted of 18 cats (different breeds, ages and genders) presented to Selcuk University, Faculty of Veterinary Medicine, Surgery Clinic and Emergency Unit with acute head trauma. Care was taken to ensure that any drug therapy such as fluid-electrolyte (mannitol, diuretic, etc.), analgesics and corticosteroids were not used in the cats included in the study. Cats with head trauma within 24 hours selected for the study inclusion.

Clinical Examination

Cats with head trauma evaluated primarily for life-threatening abnormalities. As in every trauma patient, evaluation of ABC (airway, breathing and circulation) is very critical for emergency interventions. However, the determination of hypovolemia and hypoxemia also was performed (Sande and West 2010). After the all of these evaluations, an IV catheter was placed in the cephalic vein of the forelimb and routine clinical examinations were performed with mGCS at the same time (Platt and Olby 2004, Dewey and Fletcher 2015). The animal was properly restricted so that mGCS scores were recorded. Each animal was graded as grave (3-8), guarded (9-14) or good (15-18), with adding each score in the three categories including motor and brainstem reflexes and level of consciousness. In addition, systemic hematological examinations (blood gas analysis (GEM Premier 3000, USA) and hemogram (MS4e, France)) were performed in the Central Laboratory, Faculty of Veterinary Medicine, Selcuk University.

Treatment Protocol

In order to maximize venous drainage from the brain and minimize the increase in intracranial pressure (ICP), the traumatized cat positioned with head elevated 30o angle of body level. Also, compression of the jugular vein was avoided since this leads to increase in ICP. Initially, supplemental oxygen (50-100 ml/kg/min) administered via face-mask to the cats over 15 minutes and also care was taken to ensure that aggressive hyperventilation used for short

periods. The mask was changed periodically to prevent rebreathing of carbon dioxide. This flow-by oxygen was delivered in a stress-free manner to prevent ICP increase due to fluttering. Fluid and electrolyte administration were performed considering clinical and hematological evaluations and mGCS results. Depending on severity of the clinical condition of the cat, one-third of the shock dose (15-20 ml/kg/hour) 0.9% sodium cloride (NaCl) or lactated Ringer's solutions were administered by IV infusion over 15 to 30 minutes. An osmotic diuretic, mannitol (1 g/kg, IV, over 15-30 minutes) was administered to suspected cats with increase ICP. Vaso-active agents such as dopamine (2-10 mcg/kg/min, IV infusion) were used for arterial blood pressure support in the presence of arterial hypotension despite fluid resuscitation. Clinically stabilized cats as result of treatments were prepared for tomographic examination. Also, in case of post-traumatic epileptic disorder, levatiracetam (20 mg/kg, PO, q8) and phenobarbital (7.5 mg, IM, q12) were used (Platt 2008, Sande and West 2010, Dewey and Fletcher 2015).

Computed Tomographic (CT) Examination

Cats with acute head trauma were included in CT examination according to their clinical status and in severe trauma cases, examination was postponed. Examination was performed after the administration of butorphanol (0.1 mg/kg, IV, Butomidor®, Interhas) and medetomidine HCl (40-80 mcg/kg, IM, Domitor®, Zoetis), which are routinely used in trauma patients for pain management and restriction. In cases of sedative drugs were not sufficient, anesthesia was provided with propofol (4-6 mg/kg, IV, Propofol-PF, Polifarma) according to the clinical condition of the animal. For tomographic examination (Asteion, Toshiba Medical Systems Corporation, Japan), 120 kV, 100 mA and 2 mm slice thickness values were selected and performed in helical cranial scanning mode.

Eye Examination

Systematic eye examination was performed as far as the clinical condition of the patient allowed during the clinical examination and treatment of the traumatized cats. Pupillary light reflexes and oculocephalic reflexes were noted with direct inspection. Schirmer tear test and florescein staining were performed in the presence of corneal lesion. Cornea, iris, lens and fundus were examined with a direct ophthalmoscope (Hasvet, Turkey). Intraocular pressure (IOP) measurement (Icare Tonovet Plus, Finland) was performed. In cases of the fundus was suspected, examination was performed with the retinal camera (ClearView® 2, United Kingdom). Ultrasonographic (Edan Dus U50, CHINA) examination was performed in cases of lens and fundus examination could not be performed due to pathologies in the anterior chamber (hyphema, etc.). Conditions that required operative treatment such as traumatic proptosis and hyphema were kept waiting until the cat became clinically more stable to being anesthetized.

Statistics

SPSS 25 (IBM Corp, 2017) statistical software was used to evaluate the data. Due to the small sample size and nonparametric data, the results were presented as median (min-max). Chi-Square Test was utilized to determine the relationship between mGCS and glaucoma (left and/or right eye). Statistical significance was accepted as $p<0.05$.

RESULTS

Eighteen cats of different breeds, genders and ages (1 Siamese, 1 Persian, 2 British Shorthairs, 1 Scotish Fold and 13 Crossbreeds; 9 females and 9 males, mean age 12±1 months) with head trauma were included in the study. In the trauma history of 18 cats; It was determined that the cause of trauma of 6 cats due to motor vehicle accidents and 9 cats due to fall from height. For the other 3 cats, cause of trauma was not known by their owners (Table 1).

The mGCS was evaluated in 3 categories as good (15-18), guarded (9-14) and grave (3-8). Out of 18 cats, 9 (50%) were grave, 8 (44.44%) were guarded, and 1 (5.56%) was good. While 4 of the traumatized cats died (mGCS grave), 14 cats (mGCS guarded-good) survived.

As a result of tomographic examination; maxillary and mandibular symphysial separation in 2 cats (Fig 1), mandibular symphyseal separation and os temporale fracture in 1 cat, mandibular symphyseal separation in 2 cats and temporomandibular joint (TMJ) luxation in 1 cat were detected. In addition, hard palate fracture was observed in 4 cats. In our study, there was no indication to require decompressive surgery in cats with head trauma. Mandibular separations and hard palate fractures were repaired with surgical procedures. Fixation was performed with cerclage in cats with symphysial separation. One cat with TMJ luxation were treated with close reduction followed by applied muzzle bandage. In animals with hard palate fractures, palatography was performed and a feeding tube was placed by using the pharyngeostomy method. The tube was kept in place for 7-12 days.

As a result of eye examinations performed in cats with head trauma, proptosis was observed in 4 cats (22.22%) as unilaterally in 3 cats and bilaterally in 1 cat. In these cases, tarsorrhaphy was performed and the treatment was continued for 10-14 days. The lateral or medial canthus left slightly open to the facilitate the application of topical ophthalmic drugs such as moxifloxacin (Vigamox®, Alcon) and artificial tears (Refresh®, Allergan) during administration. In a case of bilateral proptosis (case 14), keratoconjunctivitis sicca and corneal ulceration

occured in the 2nd week of tarsorraphy (Fig 2). In the 3 cats with unilateral proptosis (cases 1, 6 and 17), there were not any complication after the tarsorraphy (Fig 3). Subconjunctival hemorrhage was observed in 7 cats (38.88%) as bilaterally in 2 cats (cases 4 and 16) and unilaterally in 5 cats (cases 2, 5, 7, 8 and 12). Unilateral hyphema was observed in 2 cats (11.11%) (cases 8 and 15). In these cases 1% prednisolone acetate (1 drop/eye, 4-6 times daily) (Norsol® Forte, Bilim Pharmaceuticals) as topical corticosteroid with good corneal penetration and tropicamide (1 drop/eye, twice daily) (Tropamid®, Bilim Pharmaceuticals) as mydriatic were administered. Coagulation was observed in a cat with hyphema (case 8) and the clot was removed by anterior chamber puncture directly from the limbus to prevent the resulting increase in IOP (Fig 4). Three cats had anisocoria (16.66%) (Fig 5), 2 cats had unilateral nystagmus (11.11%), and 1 cat had unilateral Horner's syndrome (5.55%). In addition, pupils were observed as mydriatic in 5 cats (27.77%) and miotic in 10 cats (55.55%). Retinal examination revealed retinal edema in 1 cat (Fig 6). After treatments for head trauma in cats anisocoria, nystagmus and Horner's syndrome resolved spontaneously. In seven cats (38,88%), IOP was found to be above 25 mmHg. In cases with increased IOP, timolol maleate (1 drop/eye, twice daily) (Timosol®, Bilim Pharmaceuticals) was administered and IOP measurements were repeated frequently (every 2-4 hours) during the treatment. Dorzolamide HCl (1 drop/eye, 3 times daily) (Cosopt®, Merck Sharp Dohme) was additionally administered in cats without normalization of IOP with treatment. In cats with glaucoma (cases 1, 4, 5, 6, 8, 14 and 17), the median mGCS was 8 (4-15), while non-glaucoma (cases 2, 3, 7, 9, 10, 11, 12, 13 and 18) the median mGCS was 9 (6-14). However, no statistical correlation was found between mGCS and glaucoma ($p>0.05$) (Table 2).

Table 1. Diagnostic evaluations of cats with head trauma

Case	Trauma	mGCS Total score	IOP (mmHg)		Eye Injuries	
			Left	Right	Left	Right
1	Motor vehicle accident	4 (Grave)	42	8	Ocular proptosis	-
2	Fall from height	9 (Guarded)	11	11	-	Subconjunctival hemorrhage
3	Fall from height	7 (Grave)	20	25	Horner's syndrome	-
4	Unknown	8 (Grave)	34	34	Subconjunctival hemorrhage, nystagmus	Subconjunctival hemorrhage
5	Fall from height	12 (Guarded)	28	32	Anisocoria	Subconjunctival hemorrhage
6	Motor vehicle accident	10 (Guarded)	7	29	-	Ocular proptosis
7	Motor vehicle accident	12 (Guarded)	16	4	-	Subconjunctival hemorrhage, nystagmus
8	Unknown	8 (Grave)	19	29	-	Subconjunctival hemorrhage, hyphema
9	Fall from height	6 (Grave)	17	22	-	-
10	Motor vehicle accident	14 (Guarded)	9	14	-	-
11	Motor vehicle accident	6 (Grave)	14	10	Anisocoria	
12	Fall from height	12 (Guarded)	12	10	Subconjunctival hemorrhage	-
13	Fall from height	8 (Grave)	13	9	-	-
14	Unknown	5 (Grave)	48	39	Ocular proptosis	Ocular proptosis
15	Motor vehicle accident	11 (Guarded)	13	14	-	Hyphema
16	Fall from height	12 (Guarded)	13	12	Subconjunctival hemorrhage	Subconjunctival hemorrhage
17	Fall from height	15 (Good)	29	22	Ocular proptosis	-
18	Fall from height	8 (Grave)	13	11	Anisocoria	

mGCS: Modified Glasgow coma scale, IOP: Intraocular pressure

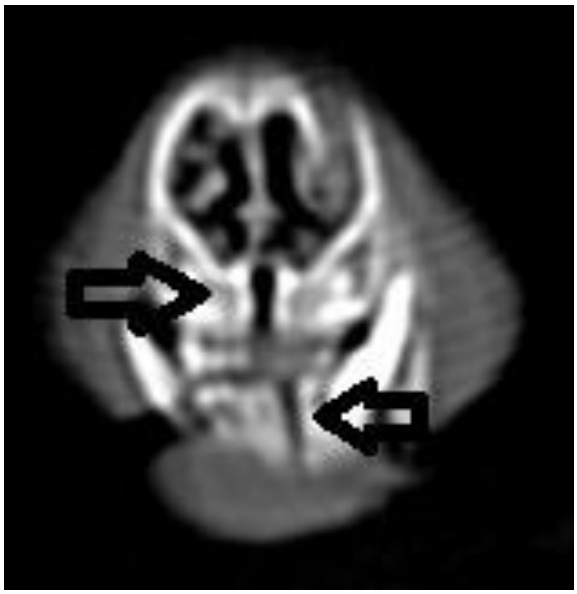

Figure 1: Case 9; maxillary and mandibular symphysial separation (black arrow).

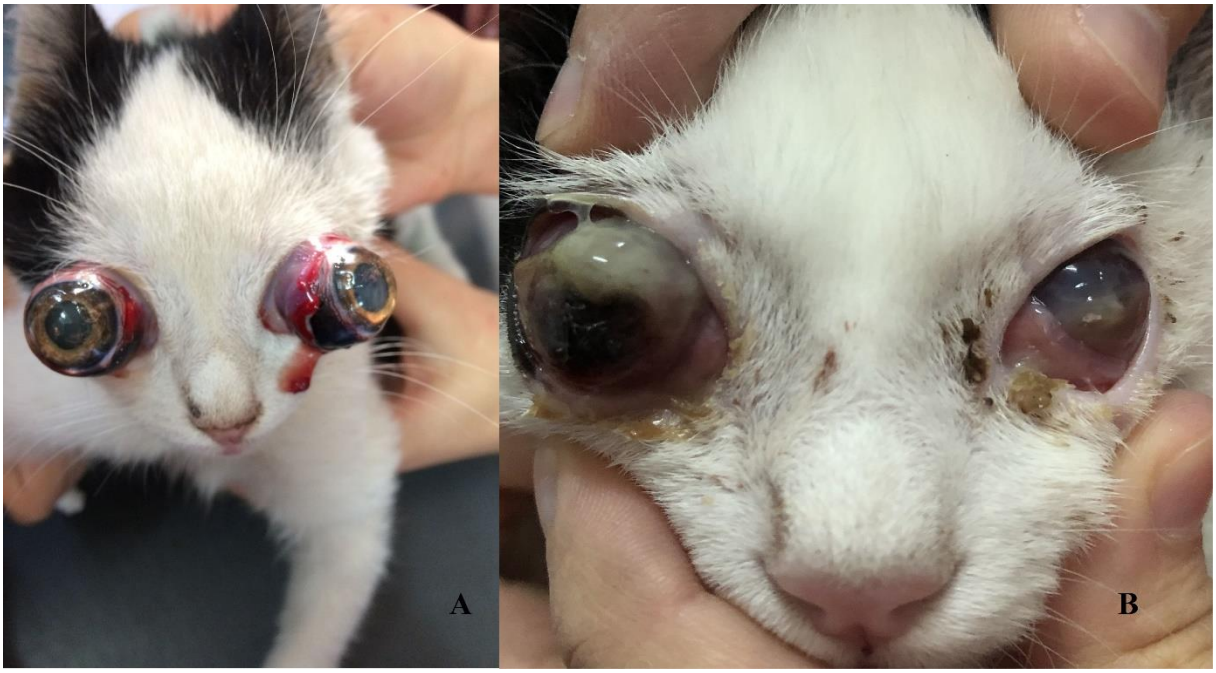

Figure 2: Case 14; bilateral ocular proptosis (A), post-op (tarsorrhaphy) 2nd week, keratoconjunctivitis sicca and corneal ulceration (B).

Figure 3: Case 17; ocular proptosis on the left eye (A), post-op (tarsorrhaphy) 4th week (B).

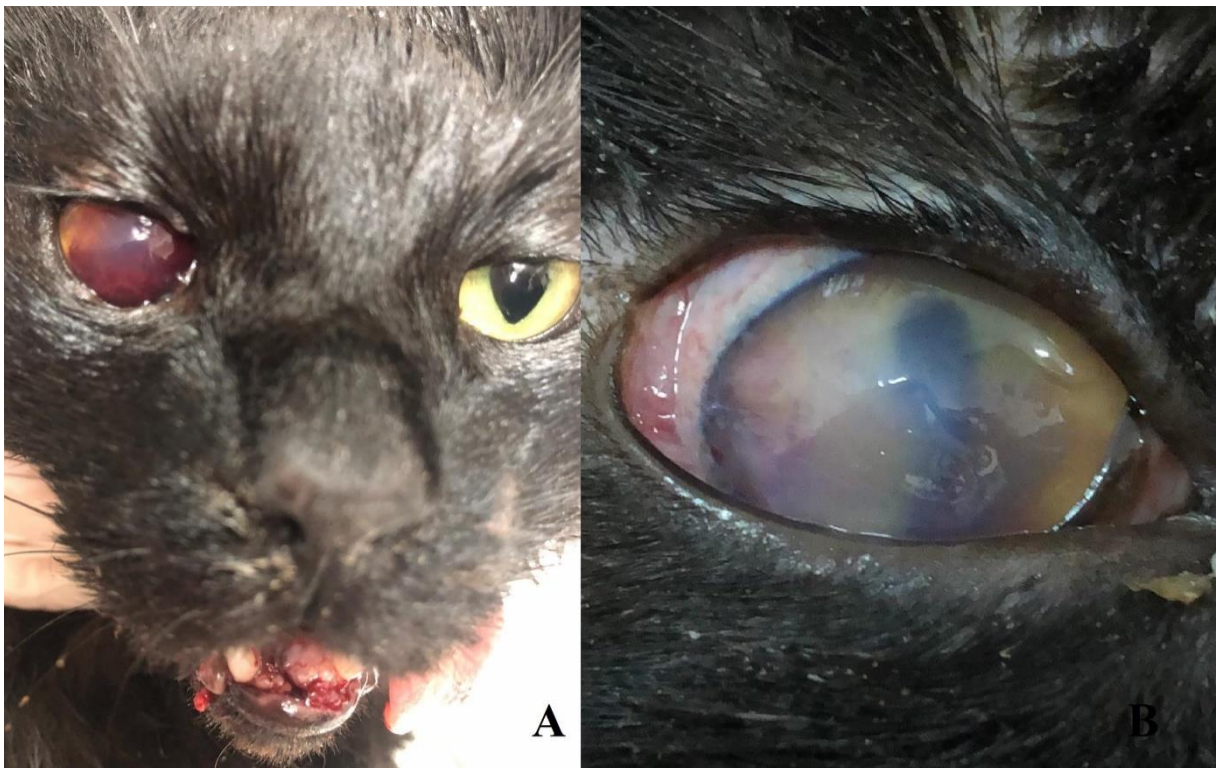

Figure 4: Case 8; unilateral hyphema (A), clot was removed by anterior chamber puncture (B).

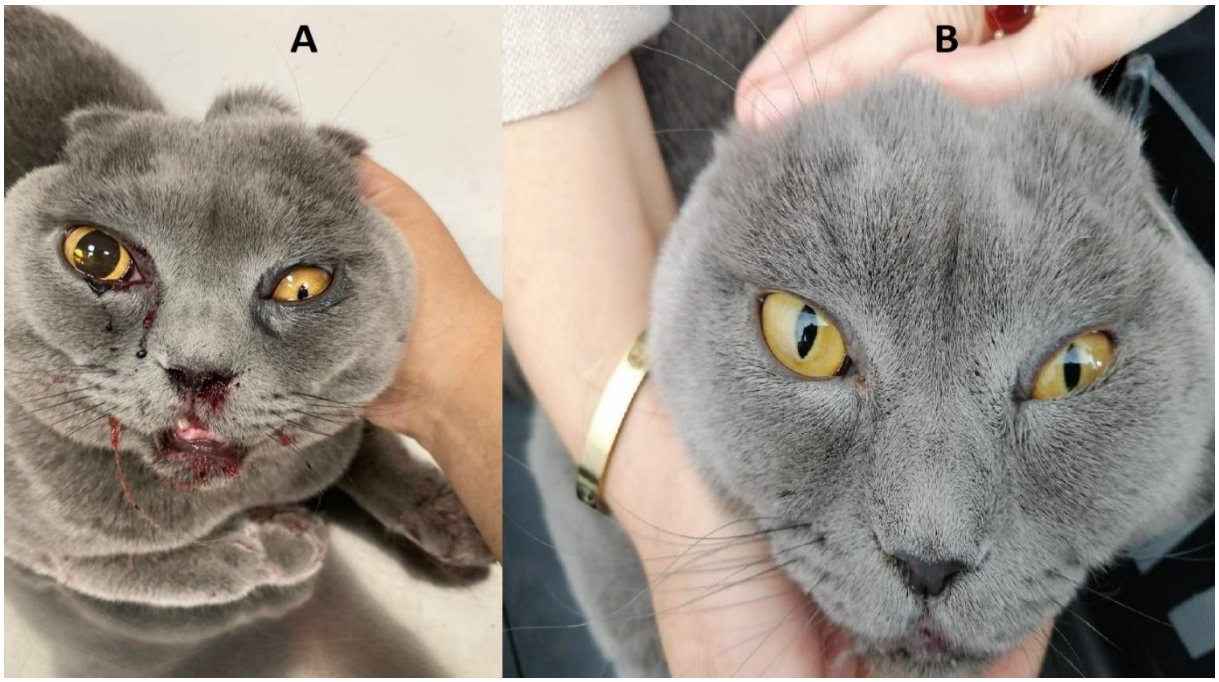

Figure 5: Case 18; Anisocoria (A), post treatment 3rd week (B).

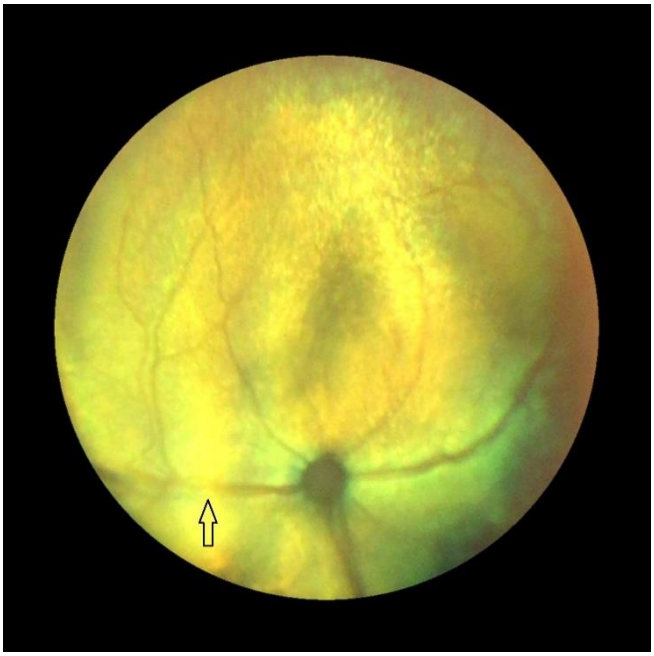

Figure 6: Case 12; retinal edema in the retinal examination (black arrow).

Table 2. Statistical correlation between mGCS and glaucoma.

Parameter	**Cats with glaucoma (n:7)**	**Cats without glaucoma (n:9)**	**P value**
Modified glascow coma scale (mGCS)	Score 8 (Score 4-15)	Score 9 (Score 6-14)	>0.05

DISCUSSION

Head trauma management should be aimed at restoring cerebral perfusion pressure, thus maintaining cardiopulmonary circulation, preventing increased ICP and minimizing increases in cerebral metabolic rate (Adamantos and Garosi 2011, Garosi and Adamantos 2011, DiFazio and Fletcher 2013). Therefore, treatment protocols; fluid-electrolyte (mannitol, hypertonic saline, lactate Ringer's, crystalloids, colloids), oxygenation, pain control (anesthetics, analgesics and sedatives), antibacterials, anticonvulsants and decompressive surgeries in cats with head trauma were managed according to clinical and hematological evaluations. In the study, morbidity and mortality rates were evaluated in cats with head trauma, and it was thought that the urgent sequential systemic treatment protocol was crucial. It has been reported that epistaxis, eye traumas, mandible fractures, symphysial separation, tooth fracture and TMJ luxation are the most common clinical injuries encountered in cats with head trauma (Bonner et al. 2012). Knight and Meeson (2019) evaluated 75 cats with head injury. As a result of the study, they reported that mandibular fractures and mandibular symphysial separations were the most common injuries (Knight and Meeson 2019). In a study conducted in 45 cats with head trauma, a high rate of mandibular symphysial separations (25/45) was reported (Tundo et al. 2019). In our study, it was determined that the most common orthopedic problem in 18 cats with head trauma was mandibular symphysial separation (n=5, 27.77%). This finding in our study is consistent with the aforementioned literature.

It has been reported that ophthalmoscopic examination should be performed first for the diagnosis of subconjunctival hemorrhage, which is an important finding in patients with head trauma due to it may reflect cerebral and/or meningeal hemorrhage (Laffey and Kavanagh 2002, Stocchetti et al. 2005). In this study we observed that glaucoma followed by subconjunctival hemorrhage (38.88%) as the most common eye disorders. In addition, no retrospective study has been found that on subconjunctival bleeding in cats. Saastamoinen et al (2019) evaluated 147 dogs with subconjunctival hemorrhage and they reported that 119/147 dogs had traumatic bleeding and motor vehicle accidents (47/119) were the most common causes of trauma. In our study, subconjunctival hemorrhage was observed in 7 cats (38.88%) and the cause of the trauma was found to fall from a height (4/7). However, the findings of the current study do not support that study (Saastamoinen et al. 2019). Possible explanation for this controversy may be the lack of adequate number of cases and differention of the species. However, subconjunctival hemorrhage occured in both animal species is due to the trauma (motor vehicle accident, fall from a height) that those species are the most exposed.

Head trauma can cause intracranial hemorrhage or increased pressure. In both cases, a compression can occur in the brain. Parasympathetic dysfunction may occur as a result of compression at the oculomotor nerve which is the third of the cranial nerves and arises from the midbrain. The symmetry of the trauma causes both pupils to be constricted. While asymmetrical, only the pupil on the affected side becomes constricted and resulting in anisocoria. If the compression on the brain cannot be prevented, the constricted pupils become dilated and the pupillary light reflexes disappear. This clinical sign reports that the prognosis of the patient is poor (Barnes Heller and Bentley 2016). As a result of the evaluation in this study, 3 out of 5 cats with dilated pupils were died. It has been observed that dilated pupils in cats with head trauma show the presence of serious damage on the brain and that the mortality rate may be high in these cases, which is consistent with the literature. Therefore, urgent treatment options focused to brain damage should be evaluated in animals with dilated pupils.

In head traumas, increased IOP may occur, usually secondary to cases of anterior uveitis, hyphema and proptosis. It has been reported that post-traumatic IOP increase occurs when the trabecular network is occluded by clots, inflammatory or red blood cells (Kaur et al. 2014). In our study, proptosis was found in 4 of the 7 cats with increased IOP, and hyphema was found in 1 cat (case 8). In the other cat with hyphema (case 15), IOP was measured at normal range. This situation can be explained by the fact that the hyphema was partial and coagulation was not occured, and the trabecular meshwork was not occluded, which is consistent with the literature. However, in the presence of severe hyphema and coagulation, the need for surgical intervention should not be ignored.

Common findings in cases with mild proptosis were mild exophthalmos, conjunctival inflammation, mild corneal damage, and normal or reduced pupillary light reflex. In addition, these patients may have different degrees of loss of vision (Mandell and Holt 2005). In cases with more severe proptosis, exophthalmos is more prominent, there is significant tearing of the ocular muscles and optic nerve, hyphema may have formed, severe drying of the ocular structures, corneal rupture and/or rupture of the bulbus oculi, and severe pain. The prognosis is poor in terms of restoring of loss of vision in these patients. Potential complications associated with proptosis include persistent strabismus, ulcerative keratitis, keratoconjunctivitis sicca, neuroretinal degeneration, and phthisis bulbus (Wheler et al. 2001, Mandell and Holt 2005). In our evaluation, mild proptosis was formed in 1 cat (case 17) and pupillary light reflex was weak. No postoperative complications were observed as a result of tarsorraphy. However, severe proptosis

was observed in 1 cat (case 14). There was no the pupillary light reflex and pupillary dilatation was observed in this cat. With the guidance of the patient owner, bilateral tarsorraphy was performed instead of enucleation, and keratoconjunctivitis sicca and ulcerative keratitis occured in the 2nd postoperative week. These complications were observed in accordance with the literature, and the poor prognosis in severe proptosis cases made us think that the enucleation option should be evaluated.

CONCLUSION

The results of this study indicate that glaucoma, subconjunctival hemorrhage and proptosis are the most common pathologies in cats with head trauma and these pathologies should not be ignored during the intervention of the head trauma patients. In cases of the eye problems such as anisocoria, Horner's syndrome and nystagmus were not resolve spontaneously during treatment, the treatment protocol should be reassessed. In addition, it is thought that irreversible loss of vision can be prevented by frequently repeated IOP measurements, regardless of the mGCS score, in cases with head trauma.

Conflict of interest: The authors declare that there is no actual, potential or perceived conflict of interest for this article.

Ethical statement: This study was approved by the Selcuk University, Faculty of Veterinary Medicine, Laboratory Animal Production and Research Center Ethics Committee (SUVDAMEK) (2019/83). Patient owners of all cases signed an informed consent form.

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RESEARCH ARTICLE

The Effect of Carnosic Acid on Semen Freezability in Malaklı Shepherd Dogs

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ABSTRACT

The objective of the study was to determine the effect of carnosic acid, to Malakli Shepherd dog semen motility, morphology, viability and membran integrity parameters after frozen-thawed prosess. Five animal were used in this study and the sperm rich portion of the ejaculates were mixed and divided in to five equal groups. Each group extended with either tris as a control group and containing carnosic acid 10 μM, μM, 100 μM, 200 μM. Following equilibration for one and a half hour, the straws were frozen in nitrogen vapor and then stored in liquid nitrogen. Later, the frozen straws were thawed in a water bath for motility, morphology, viability and membran integrity parameters examination. The highest motilities and HOST-Eosin test results were detected in 10 μM group at 65.00±2.23 % and 69.83±0.70 % respectively, the lowest abnormal sperm rate were detected in 10 μM group at 9.50±1.52 % and the differences in these groups ($p < 0.05$) were statistically significant compared to the control group. With regard conclusion, it was determined that the doses of 10 μM of carnosic acid added to extender in freezing of Malakli dog sperm showed a protective effect compared to the control group in terms of motility, spermatozoon morphology, membrane integrity and viability.
Keywords: Carnosic acid, cryopreservation, Malaklı shepherd dogs, sperm

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Malaklı Köpeği Spermasının Dondurulabilirliği Üzerine Karnosik Asitin Etkisi

ÖZ

Sunulan çalışmanın amacı, dondurulmuş çözdürülmüş Malaklı Çoban Köpeği spermasında karnosik asitin motilite, morfoloji, canlılık ve membran bütünlüğü parametrelerine etkisini belirlemektir. Bu çalışmada beş baş Malaklı köpeğine ait ejakülatların spermatozoon bakımından zengin kısmı kullanıldı. Ejakülatlar birleştirildi ve beş eşit gruba bölündü. Kontrol grubu temel Tris sulandırıcısıyla sulandırıldı ve diğer gruplar 10 μM, 50 μM, 100 μM, 200 μM karnosik asit içeren gruplar oluşturuldu. Bir buçuk saat ekilibrasyonun ardından, payetler azot buharında donduruldu ve daha sonra sıvı azot içinde depolandı. Ardından dondurulmuş payetler 37°C sıcak suda çözdürüldü ve motilite, morfoloji, canlılık ve membran bütünlüğü parametreleri değerlendirildi. En yüksek motilite ve HOST-Eozin değerleri 10 μM'lık grupta elde edildi ve sırasıyla %65,00±2,23 ve %69,83±0,70 olarak belirlendi, en düşük anormal spermatozoon oranı ise 9,50±1,52 ile yine10 μM'lık gruptan elde edildi ve bu gruplardaki fark ($p<0.05$) kontrol grubuna göre istatistiki olarak önemli bulundu. Sonuç olarak Malaklı köpeği spermasının dondurulmasında 10 μM karnosik asitin, motilite, spermatozoon morfolojisi, membran bütünlüğü ve canlılığı açısından kontrol grubuna kıyasla koruyucu bir etki gösterdiği belirlenmiştir.
Anahtar Kelimeler: Karnosik asit, kriyoprezervasyon, Malaklı köpeği, sperma

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GİRİŞ

Çoban köpekleri Türkiye'de Akbaş ve Karabaş isimleri altında sınıflandırılabilir. Kangal ve Malaklı Karabaş grubunun en tanınan iki üyesidir **(**Atasoy, 2010). Karabaş tipi Malaklı köpekler başta Aksaray, Nevşehir ve Şereflikoçhisar olmak üzere Türkiye'nin farklı bölgelerinde yetiştirilmektedir. Üreme özellikleri henüz ırka spesifik olarak ortaya koyulmamasına rağmen, Anadolu'ya has diğer bir ırk olan Kangal çoban köpeklerine benzemektedir. Dişileri ortalama olarak 7-8 aylık olduklarında, erkekler ise çoğunlukla dişilerden 2 ay sonra 9-12 aylık iken cinsel olgunluğa (Puberta) ulaşırlar ve bir yaşından sonra yetiştirmeye alınırlar (Cupps, 1991). Dişiler 6-8 ayda bir kızgınlık gösterirler ve iyi bakım besleme koşullarında bir defada 6-8 yavru doğurup 8-10 yaşına kadar yavru verebilirler. Erkekler ise yıl boyunca seksüel aktivite gösterirler, mevsimsel etkiler minimum düzeyde görülmektedir (Özgüneş ve Çiftçi, 1993, Özbeyaz, 1994).

Suni tohumlama tekniğinden faydalanılmak istendiğinde uzun süre fertilite yeteneğini koruyabilen spermaya ihtiyaç duyulmaktadır. Spermanın metabolik işlevlerini durdurularak fertilizasyonda önemli bir kayba yol açmadan süresiz bir şekilde muhafaza edilmesi spermanın dondurularak saklanması ile yapılabilir (Lemma, 2011). Bilimsel ve teknolojik olarak canlı hücre dondurma işlemleri, Polge ve arkadaşlarının 1949 yılında kriyoprotektan özelliği olan gliserolün bulması ile başlamıştır ve ilk dondurulan hücreninde spermatozoon olduğu bilinmektedir (Leibo ve Brandley, 1999).

ROS (reaktif oksijen türleri) spermatozoonların fertilizasyon kabiliyetlerinde fizyolojik olarak rol oynamakta olup özellikle spermatozoonların zona pellusidaya bağlanmasını, akrozom reaksiyonu geçirmesini ve zona pellusida içerisinden geçerek oositin membranı ile birleşmesini sağlamaktadır. ROS düşük konsantrasyonda bulunduğunda normal spermatozoon fonksiyonları için arabulucu gibi davranırken üretimi gerekenden daha fazla olursa o zaman hücreler için toksik hale gelmektedir (Griveau ve Le Lannou, 1997).

Organizmada bulunan birçok savunma mekanizması sayesinde serbest oksijen radikallerinin oluşumu ve meydana getirdikleri hasarın önlenebileceği, bunların da antioksidanlar olarak adlandırılabileceği bildirmiştir (Akkuş, 1995). Spermatozoonlar seminal plazmasında bulunan antioksidanlar vasıtasıyla kendini oksidatif strese karşı korumaya çalışmaktadır (Kim ve Parthasarathy 1998). Antioksidanların ilk etkileri, hücre membranı yapısında bulunan lipid peroksidasyona (LPO) karşı korunmasıdır. Bunun neticesinde, başlangıçta antioksidanlar LPO'u engelleyen moleküller olarak tanımlanmışlardır. Bugün ise antioksidan tanımı lipitlerin yanı sıra proteinler, nükleik asitler ve karbonhidratlar gibi diğer hedef molekülleri koruyucu etkilerini de içerecek şekilde genişletilmiştir. Böylece antioksidanlar hedef moleküllerdeki oksidan hasarı engelleyen veya geciktiren maddeler olarak nitelenmekte ve buna bağlı olarak antioksidanların etkileri değişik şekillerde olabilmektedir (Rangan ve Bulkley 1993).

Rosmarinus officinalis L.'nin güçlü antioksidan aktivitesinden sorumlu olan, karnosik asit (CA), karnosol ve rosmarinik asittir (RA) (Zanganeh ve ark, 2013). Abietatrien türevi diterpenler karnosik asit ve karnozol biberiyenin antioksidan etkisinin %90'ından sorumludur. Biberiye ekstresi ve onun polifenolleri CA ve RA, apoptosisin indüksiyonuna ve azalmış hücre sağ kalımına yol açan spesifik yolakları hedeflemek için kimyasal maddeler olarak kullanılabilir. Sulu biberiye ekstraktında bulunan CA ve diğer fenolik bileşiklerin, zar lipid düzenini artırarak membranı sertleştirdiği ve antioksidan etkinliğini bu yolla gösterebileceği bildirilmiştir (Prez-Fons ve ark, 2006).

Sunulan bu çalışmada Malaklı köpeği spermasının dondurularak saklanmasında sulandırıcıya katılacak olan karnosik asitin dondurma-çözdürme sonrası motilite, anormal spermatozoon oranı ve HE (HOST-Eozin) test üzerine etkileri, çözdürme sonrası meydana gelebilecek hasarların en aza indirilmesinde karnosik asitin etkinliğinin belirlenmesi amaçlanmıştır.

MATERYAL METOT

Araştırma Afyonkarahisarda bulunan ticari bir işletme ile Afyon Kocatepe Üniversitesi Veteriner Fakültesi Dölerme ve Suni Tohumlama Anabilim Dalı Araştırma Laboratuvarında gerçekleştirildi.

Sunulan çalışma için Afyon Kocatepe Üniversitesi Veteriner Fakültesi Etik Kurulu'ndan gerekli izin (AKÜHADYEK-456-l5-Referans nolu araştırma) alınmış ve 3-5 yaşlı 5 baş ergin Malaklı köpeğinden alınan spermalar kullanıldı. Köpeklerin bakım ve beslemesi özel bir işletme şartlarında ve standart yetiştirme koşullarında yapıldı.

Spermanın Alınması ve Değerlendirilmesi

Köpeklerden sperma, kızgın bir dişi olmadan deneyimli bir araştırmacı tarafından elle masaj yöntemiyle alındı. Erkek köpeklere sağ tarafından yaklaşılarak prepusyum üzerinden penis ve bulbus glandise masaj yapıldı. Bulbus glandis şişmeye başladığında prepusyum çekilerek penis prepusyumdan sıyrıldı ve bulbus glandis köküne masaja devam edildi. Ardından penis arka bacaklar arasından geriye çevrilerek spermatozoondan zengin olan ikinci fraksiyon alındı (Akçay, 2000, İnanç ve ark. 2018a).

Ejakulatlar, haftada bir kez, 6 tekrar yapılarak alındı. Alınan ejakulatlardan uygun özellik (sperma yoğunluğu ≥ 400×10^6 spermatozoa/ml; motilite ≥%80) gösterenler miks yapılarak antioksidan içermeyen (kontrol) ve 4 farklı konsantrasyonda

karnosik asit (10 μM, 50 μM, 100 μM ve 200 μM) içeren 5 farklı çalışma grubu oluşturuldu.
Spermaların sulandırılmasında, Tris (Hidroksimetilaminometan) (297,58 mM), Sitrik asit (96,32 mM), Fruktoz (82,66 mM), %20 yumurta sarısı ve %5 gliserol içeren sulandırıcı kullanıldı.
Köpeklerden alınan ejekulatlar makroskobik ve mikroskobik yönden değerlendirildi ve normospermi özelliği gösteren ejakulatlar çalışmada kullanıldı. Birleştirilen ejekülatlar 10 μM, 50 μM, 100 μM, 200 μM ve kontrol (0 μM) karnosik asit içeren temel tris yumurta sarısı sulandırıcısı ile yoğunluğu $200x10^6$/ml olacak şekilde sulandırıldı. Ardından payetlere çekildi, uçları polivinil alkol ile kapatıldı ve +4°C' de 1,5 saat ekilibrasyona bırakıldı. Ekilibrasyon sonrası sperma grupları sıvı azot buharı (-100 °C) yüzeyinin 11cm üzerinde 15 dakika tutularak donduruldu. Daha sonra dondurulmuş spermalar azot tankı içerisine (-196°C) alındı. Çalışmada; dondurma-çözdürme sonrası spermatolojik muayeneler; motilite, morfolojik muayene ve membran bütünlüğü ve ölü canlı spermatozoonları belirleyen HE test kullanılarak spermatolojik muayeneleri yapıldı.

Çözüm Sonu Spermatolojik Parametrelerin Değerlendirilmesi

Spermatozoa Motilitesi
Dondurma sonrası, spermatolojik muayeneler için, dondurulmuş payetler 37^0C' de 25 saniyede çözdürüldü. Isıtma tablalı faz kontrast mikroskobun (Olympus CX 31) 37^0C'ta, 20X büyütmesinde lam-lamel arasına alınan sperma numunesinde en az 3 mikroskop sahası incelenerek, sahalardaki motilite değerlerinin ortalaması % motilite oranı olarak kaydedildi (Demirci, 2002, Tekin, 1994).

Anormal Spermatozoa Oranı
Anormal spermatozoon oranı Giemsa boyama yöntemiyle boyanan slaytlar immersiyon objektif altında (100X) incelenerek çeşitli spermatozoon kısımlarına (baş, orta kısım ve kuyruk) ait bozukluklar ve bunların görülme oranları tespit edildi (Watson, 1975). Her bir slayttan 200 hücre sayıldı ve % olarak kaydedildi (Watson 1975).

HE Test
Sperma numunelerinde ölü-canlı spermatozoon oranını ve membran bütünlüğünü birlikte değerlendiren HE test kullanıldı (Ducci ve ark., 2002; Gündoğan ve ark., 2010). Ependorf tüpler içerisine su banyosunda 37 °C'deki 100 mOsm'luk HOS solüsyonundan 1 ml alınarak üzerine sperma numunesinden 10 μl eklendi. Daha sonra eozin boyası ilave edilip karışım 37 °C'lik su banyosunda 30 dk. inkübasyona bırakıldı. İnkübasyon sonrası lam üzerine bu karışımdan alınıp frotileri çekilerek çok kısa bir sürede kuruması sağlandı. Hazırlanan frotilerde 400'lük büyütmede 200 spermatozoon baş kısmının tamamın ya da bir bölümünün boya alıp almayışına ve kuyruktaki kıvrılma veya şişme olup olmayışına göre dört tipte sınıflandırıldı. Kuyruğu şişmiş, baş boya almamış (H+/E-), kuyruğu şişmemiş, baş boya almamış (H-/E-), kuyruğu şişmiş, baş boya almış (H+/E+), kuyruğu şişmemiş, baş boya almış (H-/E+) şeklinde değerlendirildi.

İstatistiksel Analiz
İstatistiksel analizde en az 6 farklı uygulamadan elde edilen verilerin ortalamaları kullanıldı. Karnosik asit içeren ve içermeyen sulandırıcı grupların karşılaştırılmasında SPSS 16.0 programında Varyans analizi, aralarında önemli farklılık bulunan ikiden fazla grubun karşılaştırılmasında ise çoklu karşılaştırma testlerinden Duncan Testi kullanıldı. Farkın $p<0.05$ düzeyde olması önemli kabul edildi.

BULGULAR

Dondurma-Çözdürme Sonrası Motilite Bulguları
Aksaray Malaklısı Çoban köpeklerinde dondurma çözdürme sonrası grupların motilite oranları Tablo 1'de sunulmaktadır. Buna göre subjektif motilite yönünden 10 μM, 50 μM ve 100 μM karnosik asit içeren grupların, kontrol grubuna göre istatistiki bir üstünlük ($p<0.05$) sağladığı gözlendi.

Dondurma-Çözdürme Sonrası Anormal Spermatozoon Oranları
Araştırmada çözüm sonrası anormal spermatozoon oranları ile ilgili elde edilen bulgular Tablo 2'de verilmiştir. Buna göre kontrol grubuile kıyaslandığında 10 μM karnosik asit katılan grupta belirgin bir derecede düşüş saptanmış ve istatistiki olarak fark ($p<0.05$) önemli bulunmuştur.

Dondurma-Çözdürme Sonrası HE test parametreleri
Dondurma-çözdürme sonrası HE test sonucunda elde edilen bulgular Tablo 3'te verildi. Modifiye HOS Test yönünden değerlendirdiğimizde, canlı ve membran bütünlüğü oranı açısından kontrol grubuna göre 10 μM ve 100 μM karnosik asit eklenen gruplarda kontrol grubuna göre istatistiki olarak farkın ($p<0.05$) önemli olduğu belirlendi.

Tablo 1. Dondurma-çözdürme sonrası motilite değerleri (X ± SEM, n:6).

Gruplar	Motilite %
Kontrol	46.66±5.57[b]
CA 10 µM	65.00±2.23[a]
CA 50 µM	61.66±3.07[a]
CA 100 µM	60.00±2.58[a]
CA 200 µM	55.00±4.28[ab]
p	*

a-b: Aynı sütundaki farklı harfler taşıyan ortalamalar arası farklılıklar önemlidir. (*: p<0.05).

Tablo 2. Dondurma-çözdürme sonrası anormal spermatozoon oranları (X±SEM, n:6).

Gruplar	Anormal Spermatozoon Oranı %
Kontrol	18.33±2.17[a]
CA 10 µM	9.50±1.52[b]
CA 50 µM	12.50±1.83[ab]
CA 100 µM	14.33±1.17[ab]
CA 200 µM	12.33±3.10[ab]
p	*

a-b: Aynı sütundaki farklı harfler taşıyan ortalamalar arası farklılıklar önemlidir. (*: p<0.05).

Tablo 3. Dondurma-çözdürme sonrası HE-test parametreleri (X ± SEM, n:6).

Gruplar	HE-Test (%)			
	H+/E-	**H-/E-**	**H+/E+**	**H-/E+**
Kontrol	62.33±1.33[cd]	24.00±1.28[a]	6.00±1.09[b]	7.67±0.67[b]
CA 10 µM	69.83±0.70[a]	15.83±1.16[bc]	7.83±0.60[b]	6.50±0.50[b]
CA 50 µM	65.33±2.31[bc]	22.66±1.81[a]	5.66±0.80[b]	6.33±0.49[b]
CA 100 µM	67.16±0.60[ab]	18.66±1.12[b]	7.16±0.40[b]	7.00±1.06[b]
CA 200 µM	59.33±1.20[d]	14.17±0.87[c]	15.33±0.56[a]	11.16±0.48[a]
p	*	*	*	*

a-d: Aynı sütundaki farklı harfler taşıyan ortalamalar arası farklılıklar önemlidir. (*: p<0.05).

TARTIŞMA

Spermanın dondurulması esnasında soğuk şokuna karşı spermatozoonlarda görülen aşırı hassasiyet, hasarı artırarak çözüm sonrası in vitro ve in vivo spermatolojik parametreleri olumsuz yönde etkilemektedir. Yüksek oranda doymamış yağ asiti bulunan spermatozoon membranında, LPO'a karşı duyarlı hale gelmektedir. Spermatozoonun soğutulması ve dondurulması ve çözdürülmesi geri dönüşümsüz faz değişimine bağlı hasarlara ve oksidatif strese neden olmakta ve meydana gelen oksidatif stres ve oluşan sitotoksik aldehitler spermatozoonlarda fonksiyon kayıplarına sebep olmaktadır (Holt 2000, Watson 2000). Bu nedenle sulandırıcılara katılan kriyoprotektif ve antioksidan özellikli katkı maddeleri sayesinde oksidatif stres ve soğuk şoku hasarı en aza indirilebilmektedir. Kriyoprotektan özellikler gösterebilen antioksidan bileşiklerin de bulunması, bu maddelerle dondurulan spermalardan daha iyi sonuçlar alınmasını sağlamaktadır (Aitken 1994, Alvarez ve Storey 1995, Bucak ve Tekin 2007, Bucak ve ark 2010, Agarwal 2014).

Touazi ve ark. (2018), horozlarda diyete ekledikleri düşük yoğunluklu biberiye esansiyel yağının kısa süreli saklanan spermada lipid peroksidasyonu ve soğuk şokunun etkilerini azalttığını ve yüksek motilite oranları olduğunu bildirmişlerdir. Yüksek dozlarda ise etkilerin yıkıcı olduğunu ve bunun yüksek dozların hücre zarları ve akrozom bütünlüğü olmak üzere farklı spermatozoa yapılarına verdiği zarardan kaynaklanabileceğini bildirmişlerdir. Heidari-Vala ve ark. (2013) gastrik gavajla 60 gün süreyle 50 veya 100 mg/kg biberiye ekstraktı verdikleri ratlarda her iki dozda da serum testosteron dozunu azalttığı, toplam spermatozoon sayısı, motilite ve canlılığın değişmediğini bunun yanında Rosmarinus officinalisin her iki dozda spermatogonia, 50 mg/kg dozda leydig hücresi ve spermatid, 100 mg/kg dozlarında ise spermatid sayısını artırdığını bildirmişlerdir. Bu çalışmalar biberiye ekstraktı ile yapılmış ve karnosik asit içeren çalışmalardır ve sonuçları çalışmamız ile uyumlu bulunmuştur.

Rosmarinus officinalis, karnosik, rosmarinik asitler ve kafur gibi molekülleri içeren zengin bir polifenol ve uçucu yağ kaynağı olarak bilinir (Cuvelier, 1996, Frankel E.N, 1996, Richheimer, 1996, Rašković, 2014). Bu bileşikler, antioksidan, antienflamatuar ve antikarsinojenik özellikler dahil olmak üzere çeşitli biyolojik aktiviteler gösterir. Yaşlı horozlarda, Borghei-Rad ve ark. (2017) biberiye yaprağı ekstraktı ile yapılan besin takviyesinin, oksidatif stres hasarlarıyla mücadele ederek sperma kalitesini ve fertilite düzeylerini önemli ölçüde iyileştirdiği bildirmiştir.

İnanç ve ark. (2018b), farklı yaşlarda Malaklı köpeklerinde yaptıkları çalışmada dondurma çözdürme sonrası elde ettikleri total motilite değerleri çalışmamızda elde ettiğimiz motilite değerleri ile uyumlu bulunmuştur. Yine aynı çalışmada elde ettikleri anormal spermatozoon oranlarında elde ettikleri değerler çalışmamızda bulduğumuz değerlerden yüksektir. Bu fark spermaya katılan maddeler, boyama yöntemi ve analiz yönteminden kaynaklanmış olabilir.

Zanganeh ve ark. (2013), geyik sulandırıcısına ilave ettikleri %4'lük biberiye ekstraktının çözüm sonu motilite, canlılık ve membran bütünlüğü parametrelerine pozitif etki gösterdiğini bildirmişlerdir. Malo ve ark. (2010), domuz sperma sulandırıcısına düşük, orta ve yüksek yoğunlukta biberiye ekstraktının eklenmesi ile motilite ve canlılık ve HOST değerleri üzerinde olumlu etki yaptığını bildirmişlerdir. Daghigh-Kia ve ark. (2014), boğa spermasına rozmarinik asit ilavesinin canlı spermatozoa yüzdesi, motilite ve membran bütünlüğünü koruduğunu tespit etmişlerdir. Biberiye özündeki bu maddelerin belirgin olarak yangı önleyici, sitotoksik ve antioksidan, özellikleri bulunmaktadır. Daghigh-Kia ve ark. (2014), glutatyon (5 mM glutatyon + 10 g L^{-1} biberiye ekstraktı) ile kombinasyon halinde ve yalnız biberiye ekstaktı (10 g L^{-1}) ilavesinin boğa spermasında ROS üretimine karşı hücre içi imminolojik sistemin önemli ölçüde iyileştirdiğini, ayrıca Malo ve ark. (2010), domuz spermasında malondialdehit (MDA) üretiminin, dondurma-çözdürme sonrası sulandırıcıya ilave edilen biberiye ekstraktı ile değiştiği belirtmiştir. Motlagh ve ark. (2014) koçlarda biberiye ekstaktının %4 ve %6 lık konsantrasyonlarının çözüm sonu yüksek motilite, membran bütünlüğü ve canlı spermatozoon oranları gösterdiklerini bildirmişlerdir. Sunulan bilimsel çalışmaların sonuçları incelendiğinde, biberiye ekstraktının spermatolojik verilere yaptığı olumlu etkinin çalışmamızda özellikle 10 µl'lik grupta elde ettiğimiz sonuçlar ile benzerlik gösterdiği görülmüştür.

Yeni ve Avdatek (2018), kısa süreli sakladıkları epididimal manda spermasına kattıkları karnosik asit sonrası 12.5, 25 ve 50 µg/ml gruplarda motilite değerlerinin kontrol grubuna göre yüksek olduğunu, yine aynı çalışmada en düşük toplam anormal spermatozoon oranının 12.5 µg/ml'lik grupta elde edildiğini ve bunun kontrol grubuna göre istatistiksel olarak anlamlı ($p<0.05$) olduğunu ve en yüksek H+E-oranının ise 62.5±4.46 ile 25 µg/ml'lik grupta elde edildiğini bildirmişlerdir. Yazarların elde ettikleri sonuçlar çalışmamızda bulduğumuz sonuçlar ile uyumlu bulunmuştur.

Güngör ve ark. (2019), koç spermasına dondurma esnasında 0.05 ve 0.2 mM düzeyinde karnosik asit ekledikleri çalışmalarında total motiliteyi sırasıyla 35.0 ± 7.15 ve 31.0 ± 4.45 olarak belirlemişlerdir. Bu sonuçları kontrol grubuna göre istatistiki olarak fark göstermemiştir. Kullandıkları dozları çalışmamızda kullandığımız 50 ve 200 µM lık gruplarımız ile aynı dozdadır. Elde ettikleri değerlerin bizim değerlerimizden düşük olmasının hayvan türlerinin farklı olmasından kaynaklanabileceği

değerlendirilmiştir. Yine aynı çalışmada plazma membran ve akrozom bütünlüğü (PMAI) ve çalışmamızdaki membran bütünlüğü (HE) değerleri açısından 50 ve 200 µM'lık dozlarda her iki çalışmada da kontrol gruplarına göre fark bulunamamıştır.
Çalışmamızda elde edilen verilere göre tüm parametreler değerlendirildiğinde tris sulandırıcısına 10 µM karnosik asit eklemenin köpek spermasının çözüm sonrası motilite, morfoloji ve mebran bütünlüğü-canlılığı üzerine olumlu etkileri olduğu sonucuna varılmıştır.

Çıkar çatışması: Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

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RESEARCH ARTICLE

Dose-dependent Apoptosis and Antiproliferative Activity of Lung Cancer Cells (A-549) Treated with Navitoclax (Abt-263)

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ABSTRACT

Lung cancer is an aggressive type of tumor with a high mortality rate. The use of agents targeting molecular abnormalities that regulate resistance to apoptosis is seen as a promising approach for the treatment of this disease. BH3 mimetic antagonists such as ABT-737 and its derivative ABT-263 (navitoclax) have been developed to block the function of pro-survival Bcl-2 family members. Navitoclax is a potent small molecule inhibitor of the Bcl-2 family, and numerous studies have reported that Navitoclax has therapeutic effects against many types of cancer, including lung cancer, acute lymphoblastic leukemia, and ovarian cancer. Bcl-2 family proteins are central regulators of mitochondrial apoptosis and validated anti-cancer targets. ABT-263 (Navitoclax), an inhibitor of the Bcl-2 family, is used in clinical trials for cancer treatment. However, the anticancer mechanisms of ABT-263 have not been fully elucidated. Overall, our work highlights the need to target anti-apoptotic Bcl-2 proteins that activate the mitochondrial cell death program to kill cancer cells. In this study, the effects of ABT-263 on human lung cancer cells were investigated in vitro. In our study using the lung cancer cell line as a model, the antiproliferative effect and apoptosis activity of navitoclax concentrations of 0.1, 0.5, 1, 5, 25 μM after 48 hours was demonstrated. Combined use of chemotherapeutic drugs with Navitoclax suggests that it may bring new approaches to the treatment of lung cancer.

Keywords: ABT-263, A-549 Cell Line, Apoptosis, Lung Cancer, Navitoclax.

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Navitoklax (ABT-263) ile Muamele Edilmiş Akciğer Kanser Hücrelerinin (A-549) Doza Bağlı Apoptoz ve Antiproliferatif Aktivitesi

ÖZ

Akciğer kanseri, ölüm oranı yüksek agresif bir tümör türüdür. Apoptoza direnci düzenleyen moleküler anormallikleri hedefleyen ajanların kullanılması, bu hastalığın tedavisi için ümit verici bir yaklaşım olarak görülmektedir. ABT-737 gibi BH3 mimetik antagonistleri ve bunun türevi ABT-263 (navitoclax), hayatta kalma yanlısı Bcl-2 aile üyelerinin fonksiyonunu bloke etmek için geliştirilmiştir. Navitoclax, Bcl-2 ailesinin güçlü bir küçük molekül inhibitörüdür ve çok sayıda çalışma, navitoclaxın akciğer kanseri, akut lenfoblastik lösemi ve yumurtalık kanseri dahil olmak üzere birçok kanser türüne karşı terapötik etki gösterdiğini rapor etmiştir. Bcl-2 ailesi proteinleri, mitokondriyal apoptozun merkezi düzenleyicileri ve doğrulanmış anti-kanser hedefleridir. Bcl-2 ailesi inhibitörü olan ABT-263 (Navitoclax), kanser tedavisi için klinik olarak denemelerde kullanılmaktadır. Ancak ABT-263'ün antikanser mekanizmaları tam olarak aydınlatılmamıştır. Genel olarak çalışmamız, kanser hücrelerini öldürmek için mitokondriyal hücre ölümü programını etkin hale getiren anti-apoptotik Bcl-2 proteinlerinin hedeflenmesine olan ihtiyacı vurgulamaktadır. Bu çalışmada ABT-263'ün insan akciğer kanseri hücreleri üzerindeki etkileri in vitro olarak araştırılmıştır. Çalışmamızda bir model olarak akciğer kanser hücre hattını kullanarak, navitoclaxın 0.1, 0.5, 1, 5, 25 μM konsantrasyonlarının 48 saat sonunda antiproliferatif etkisi ve apoptoz aktivitesi gösterilmiştir. Navitoclax ile birlikte kemoterapik ilaçların kombine edilerek kullanılması, akciğer kanseri tedavisine yeni yaklaşımlar getirebileceğini düşündürmektedir.

Anahtar Kelimeler: ABT-263, A-549 Hücre Çizgisi, Akciğer Kanseri, Apoptoz, Navitoklax.

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GİRİŞ

ABT-263'ün antikanser mekanizmaları tam olarak aydınlatılmadığından, çalışmamızda ABT-263'ün insan akciğer kanseri hücrelerinin antiproliferasyonu üzerindeki etkisi, apoptoz temelinde değerlendirilmiştir. Bu çalışmamızın, BH3 taklidinin anti-kanser mekanizmasına ve akciğer kanseri olan hastaların tedavisinde potansiyel uygulamalarına yeni bir bakış açısı sağlaması beklenmektedir. Hayatta kalmayı sürdüren Bcl-2 ailesi üyelerinin (Bcl-2, Bcl-xL ve Mcl-1) aşırı ekspresyonu, yaygın olarak tümör bakımı, ilerlemesi ve kemorezistans ile ilişkilidir. İnsan tümör hücrelerinde ABT-263, Bax translokasyonunu, sitokrom c salınımını ve ardından apoptozu indükler. ABT-263'ün tek başına oral uygulaması, küçük hücreli akciğer kanseri ve akut lenfoblastik löseminin ksenograft modellerinde tam tümör gerilemelerini indükler (Tse ve ark. 2008). B hücreli lenfoma 2 (BCL-2) familyası proteinleri öncelikle programlanmış bir hücre ölümü düzenleyicisi olarak çalışır, bu sayede aralarındaki çoklu etkileşimler hücrenin hayatta kalmasını belirler. Bu, anti-apoptotik ve pro-apoptotik proteinler olan iki ana BCL-2 protein sınıfını açıklar (Hwang ve ark. 2021). Anti-apoptotik proteinler, intrinsik apoptotik yolun artmasıyla sonuçlanan BCL-2 ailesi inhibitörleri için çekici hedeflerdir. BCL-2 ailesi inhibitörleri, çeşitli kanser türlerinde, fibrotik hastalıklarda, yaşlanmayla ilgili ve ayrıca otoimmün hastalıklarda yeni hedefli tedaviler için kapsamlı bir şekilde incelenmiştir. Navitoclax bunlardan biridir ve BCL-2, BCL-W ve B-hücreli lenfoma-ekstra-büyük dahil olmak üzere BCL-2 anti-apoptotik proteinlere karşı yüksek bir afiniteye sahip olduğu keşfedilmiştir (Hamdy ve ark. 2020). Navitoclax'ın tek bir ajan olarak veya diğer ilaçlarla kombinasyon halinde tümör progresyonunu ve fibrozis gelişimini başarılı bir şekilde iyileştirdiği gösterilmiştir. Navitoklaks bugüne kadar faz I ve faz II klinik çalışmalarına girmiştir. Navitoclax tek başına küçük hücreli akciğer kanserini ve akut lenfositik lösemiyi güçlü bir şekilde tedavi ederken, katı tümörler için kombinasyon terapisinde diğer kemoterapötik ajanların terapötik etkisini arttırmaktadır (Anuar ve ark. 2020).

Akciğer kanseri, vakaların % 15'ini temsil eden ve ABD'de her yıl 30.000 yeni vakayı oluşturan kansere bağlı ölümlerin en yaygın nedenidir (Jackman ve Johnson 2005). Akciğer kanseri için son 40 yılda tedavilerde önemli gelişmeler görülmemiştir ve hedefe yönelik tedavi onaylanmamıştır (Sato ve ark. 2007). Terapötik direncin üstesinden gelmek için ümit verici bir yaklaşım olarak, apoptoza direnci düzenleyen moleküler anormallikleri hedefleyen ajanların kullanılması düşünülmektedir (William ve Glisson 2011).

Anti-apoptotik (Bcl-2, Bcl-XL, Bcl-w, Mcl-1) ve pro-apoptotik (Bax, Bak, Bim, Bid, Puma, Bad, Noxa, Bik, Bmf ve Hrk) proteinleri, bir hücrenin çeşitli sitotoksik saldırılardan sonra yaşayıp yaşamayacağını belirler (Hanahan ve Weinberg 2000). Kanserde hayatta kalma yanlısı proteinlerin aşırı ifadesi genellikle kötü prognoz ve kemoterapiye direnç ile ilişkilidir.

Apoptotik kontrol noktalarından kaçınmak için, kanser hücreleri genellikle anti-apoptotik Bcl-2 proteinlerini aşırı ifade eder (Korsmeyer, 1992). Bcl-2 kontrollü apoptotik yolun aktivasyonunun çoğu, kemoterapötik maddelerin etkinliği için kilit nokta olması nedeniyle, Bcl-2 aile üyeleri terapötik gelişim için çekici hedefler olarak ortaya çıkmıştır.

Bcl-2 ailesinin anti-apoptotik üyelerinin antikanser ilaçların neden olduğu hücre ölümünü baskıladığı bulunmuştur. Bu nedenle, Bcl-2 aile üyelerini inhibe eden tedaviler, kanser ile savaşmak için potansiyel bir strateji olabilir (Adams ve Cory 2007; Yip ve Reed 2008). Ayrıca yapısal ve fonksiyonel çalışmalar, hem anti- hem de pro-apoptotik üyelerin, korunmuş bir Bcl-2 homoloji (BH3) alanı tarafından düzenlendiğini ve böylece kanser tedavisi için çeşitli BH3 mimetiklerinin geliştirilmesiyle sonuçlandığını göstermiştir (Llambi ve Green 2011; Weyhenmeyer ve ark. 2012). Çalışmamız, anti-kanser terapötikleri olarak geliştirilen küçük moleküllü BH3 mimetik bileşiklerinin etkilerine dayanmaktadır. BH3 mimetikleri, hayatta kalma yanlısı Bcl-2 proteini için yüksek bağlanma özgüllükleri gösterir. Bu durum olası yan etkileri sınırlandırdığından, bu ilaçların diğer hastalık alanlarında da faydalı olabileceği tahmin edilebilir. Burada Bcl-2, Bax ve Kaspaz-3' ün fonksiyonel önemi araştırıldı. Navitoclax'ın konsantrasyonlarının akciğer kanser hücrelerinin sağkalımında hayatta kalma yanlısı etkilerinin üstesinden nasıl gelebileceği araştırıldı.

MATERYAL ve METOT

Hücre Kültürü

Sunulan çalışmada kullanılan A-549 insan akciğer kanser hücreleri (CRL-1571TM, ATCC®) Afyonkarahisar Sağlık Bilimleri Üniversitesi Tıp Fakültesi Biyokimya Anabilim Dalı'ndan temin edildi. Hücreler, içinde % 10 Fetal Bovine Serum (FBS) (Gibco Qualified, 10500-064, USA), % 1 L-glutamin (Gibco 25030-024) ve % 1 penisilin-streptomisin (Gibco 15140-122, USA) karışımı içeren RPMI 1640 (Capricorn Scientific, cp19-2782, Germany) içinde % 5 CO^2 içeren ve 37 ^{0}C'ye ayarlı inkubatörde (Thermo Heracell, 51026282, USA) kültüre edildi.

Reaktif

Navitoclax (ABT-263; Selleckchem) deneylerden önce DMSO içinde seyreltilmiştir (Lee ve ark. 2018). 1 mg ABT-263, 1.0261 ml DMSO içinde seyreltilerek stok elde edilmiştir. Stoktan dilusyon yapılarak diğer dozlar hazırlanmıştır.

MTT

Akciğer kanser hücrelerine uygulanan Navitoclax'ın (Sellect chemicals, S100126) çeşitli konsantrasyonlarının hücre canlılığı üzerindeki etkileri

MTT testi ile çalışılmıştır. Büyüyen hücreler, 96 oyuklu plakalara ekilmiştir ve yaklaşık % 70-80 konflue olana kadar kültürlenmiştir. Hücreler, belirtilen konsantrasyonlarda test bileşikleri ile işleme tabi tutulmuştur. Daha sonra oyuklara 5 mg/mL MTT içeren solüsyon ilave edilmiştir ve 3 saat 37 °C de inkübe edilmiştir. Formazanı çözündürmek için 200 µL DMSO ilave edilmiştir ve karanlıkta 10 dakika çalkalanmıştır. 570 nm'deki absorbans, bir mikroplaka okuyucusu (Bio Tek Epoch, USA) ile kaydedilmiştir.

Toplam RNA Extraksiyonu ve cDNA Sentezi

Üreticinin talimatlarına göre RNA sentez kiti (ABT 102-01-10, Türkiye) kullanılarak hücreler lize edildi ardından etanol ile çökeltildi. RNAase free bir ortam oluşturabilmek adına RNA izolasyonu esnasında kullanılacak bütün malzemeler uygun çözeltilerle (RNA away) önceden temizlenerek hazır edildi. Nanodrop ile ölçüm, izole edilen RNA'ların saflığını ve miktarını belirledi. OD260/280 oranı 1.7-2.1 aralığında olan RNA'lar cDNA sentezinde kullanıldı (White ve Kaestner 2009). RNA izolasyonu işlemi baştan sona buz üzerinde gerçekleştirildi. cDNA kiti (Thermo Fisher 4368814, USA) ve 4 µL 5 × RT primer kullanılarak 10 ng toplam RNA cDNA'ya dönüştürüldü. 20 µL reaksiyonları bir termocycler içinde 15 dakika 55 °C de, 5 dakika 85 °C de inkübe edildi ve 4 °C de tutuldu.

Kantitatif Real-Time PCR analizi

qPCR, Brightgreen Universal 2x qPCR (Abt, 204454, Türkiye) kiti kullanılarak gerçekleştirildi. 10 µL PCR reaksiyonu 0,6 µL Forward, 0,6 µL Reverse, nükleaz içermeyen su ve kitteki diğer ürünler kullanılarak reaksiyon 96 oyuklu plakada 95 °C de 10 dakika süreyle inkübe edildi, ardından 40 döngü 95 °C de 15 saniye ve 60 °C de 60 saniye süreyle inkübe edildi. Tüm reaksiyonlar, üç kez gerçekleştirildi. Her bir transkripsiyon analizi için primerler spesifik dizayn edildi ve literatürdeki çalışmalar referans alındı (Çizelge 1). Kantitatif döngü (Ct), numunenin floresansının tanımlanan eşikten geçtiği kesirli bir döngü olarak tanımlandı. RT-qPCR verilerinin analizi $2^{-\Delta\Delta Ct}$ yöntemi ile hesaplandı.

Tablo 1. Real-time PCR reaksiyonlarında kullanılan primer sekansları (Sentebiolab, Türkiye)
Table 1. Primers sequens used for real-time PCR reactions

Primers	Forward	Reverse
B-actin	CACCCCAGCCATGTACGTTC	ACCATCGCTATCTGAGCAGC
Bax	CGCCTCACTCACCATCTGGAA	CCTCAAGACCACTCTTCCCCA
Bcl-2	GAGGGGCTACGAGTGGGATGC	GGAGGAGAAGATGCCCGGTGC
Casp-3	GGAAGCGAATCAATGGACTCTGGA	CCTGAGGTTTGCATCGAC

İstatistik Analizler

Elde edilen verilerin istatistiksel analizi amacıyla SPSS 22.00 paket programı kullanıldı. Sonuçlar ortalama ± standart hata olarak sunuldu. Verilerin normal dağılıma uygunluğu Shapiro-Wilk testi kullanılarak yapıldı. MTT sitotoksisite analizlerinde, normal dağılım göstermeyen gruplar arası farklılık Kruskall-Wallis varyans analizi ile değerlendirildi. Farkın hangi grup veya gruplardan kaynaklandığını belirlemek için Bonferroni düzeltmeli Mann-Whitney U testi uygulandı. Normal dağılım gösteren gruplar arası farklılık tek yönlü varyans analizi (ANOVA) ile, farkların önem kontrolü ise post hoc Duncan testi ile yapıldı. Gen ekspresyon analizlerinde, normal dağılım göstermeyen gruplar arası farklılık Mann-Whitney U testi ile normal dağılım gösteren gruplar arası farklılık student's t-testi ile gerçekleştirildi. Yapılan istatistiksel analizlerden elde edilen sonuçlardan $p<0.05$, $p<0.01$ ve $p<0.001$ olan değerler önemli kabul edildi.

BULGULAR ve TARTIŞMA

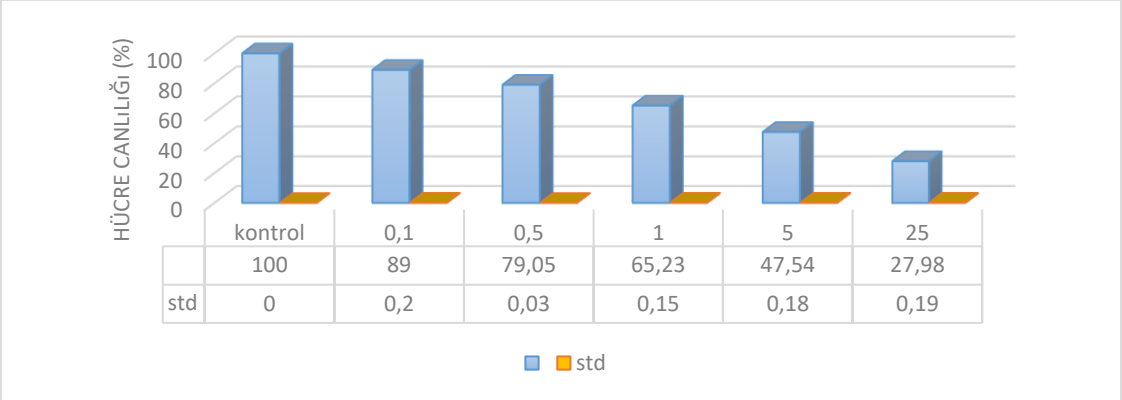
Navitoclax'ın A-549 Hücreleri Üzerine Antiproliferatif Etkisi

Kontrol ve farklı konsantrasyonlarda Abt-263 uygulanan gruplarda A-549 hücrelerindeki hücre canlılığı (%) grafik 1 de gösterilmiştir. Kontrol grubu olarak çalışılan hücreye 0 µM ilaç uygulaması yapılmıştır. Letal doz uygulaması yapılmamıştır, kontrol grubuna ilaç uygulaması yapılmadığı için canlılık % 100 kabul edilir. 0.1, 0.5, 1, 5 ve 25 µM Navitoclax uygulanan gruplarda (Lock ve ark. 2008) hücre canlılığı (%) kontrol grubu ile karşılaştırıldığında (Şekil 1) Navitoclax uygulanan gruplarda istatistiksel olarak bir azalma tespit edildi ($p<0.001$). 25 µM Navitoclax uygulanan gruptaki hücre canlılığının (%) kontrol ve 1 µM Navitoclax uygulanan gruba göre daha düşük olduğu ($p<0.001$), 5µM Navitoclax uygulanan grup ile benzer olduğu ($p>0.05$) belirlendi. 5 µM Navitoclax uygulanan gruptaki hücre canlılığının (%) ise kontrol, 0.1, 0.5 ve 1 µM Navitoclax uygulanan gruba göre anlamlı düzeyde azaldığı ($p<0.001$), 25 µM Navitoclax uygulanan grup ile istatistiksel bir fark olmadığı ($p>0.05$) görüldü (Çizelge 2).

Tablo 2. A549 hücre hattında MTT testi ile Navitoclax'ın sitotoksik aktivitesinin değerlendirmesi
Table 2. The cytotoxic activity of Navitoclax by MTT test in A549 cell line

Abt-263 konsantrasyonu (µM)	**Hücre Canlılığı (%) ($\bar{x} \pm S\bar{x}$)**
Kontrol (0 µM)	100 ± 0.00^{a}
0.1	89 ± 0.2^{b}
0.5	79.05 ± 0.03^{bc}
1	65.23 ± 0.15^{c}
5	47.54 ± 0.18^{de}
25	27.98 ± 0.19^{e}
p	***

a,b,c,d,e Aynı sütunda farklı harf taşıyan veriler arasındaki fark istatistiksel açıdan önemlidir.
***: $p < 0.001$, $\bar{x}$: Ortalama, $S\bar{x}$: Standart Hata

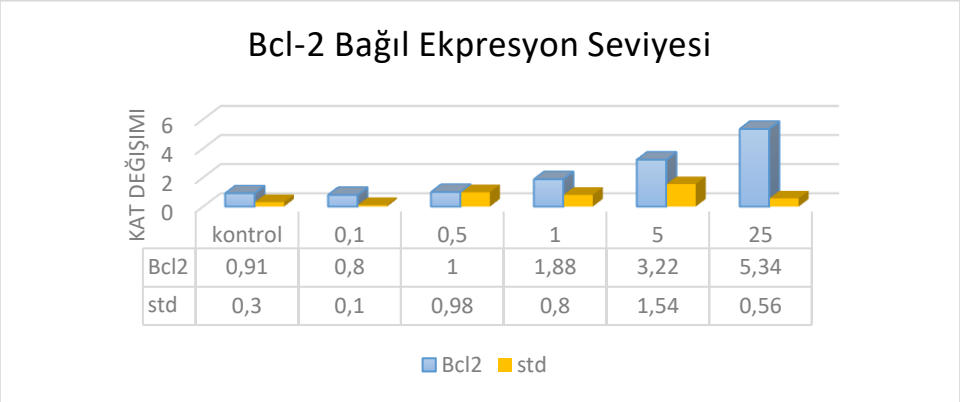


Şekil 1. Kontrol ve farklı konsantrasyonlarda (0.1, 0.5, 1, 5, 25 µM) Navitoclax uygulanan gruplarda hücre canlılık yüzdeleri
Figure 1. Cell viability percentages in control and Navitoclax applied groups at different concentrations (0.1, 0.5, 1, 5, 25 µM)

Apoptoz İlişkili Proteinlerin Ekspresyonları
Apoptoz ile ilişkili Bcl-2 ve Bax proteinlerinin ekspresyonu açısından kontrol grubu istatistiksel olarak karşılaştırıldığında (Şekil 2, Şekil 3) 25 µM Navitoclax uygulanan grupta görülen artışların önemli olduğu belirlendi ($p < 0.001$) (çizelge 3, çizelge 4).

Tablo 3. Kontrol ve farklı konsantrasyonlarda(0.1, 0.5, 1, 5, 25 µM) Navitoclax uygulanan gruplarda Bcl-2 ekspresyon değişimlerinin değerlendirmesi
Table 3. Evaluation of bcl-2 expression changes in control and Navitoclax at different concentrations (0.1, 0.5, 1, 5, 25 µM)

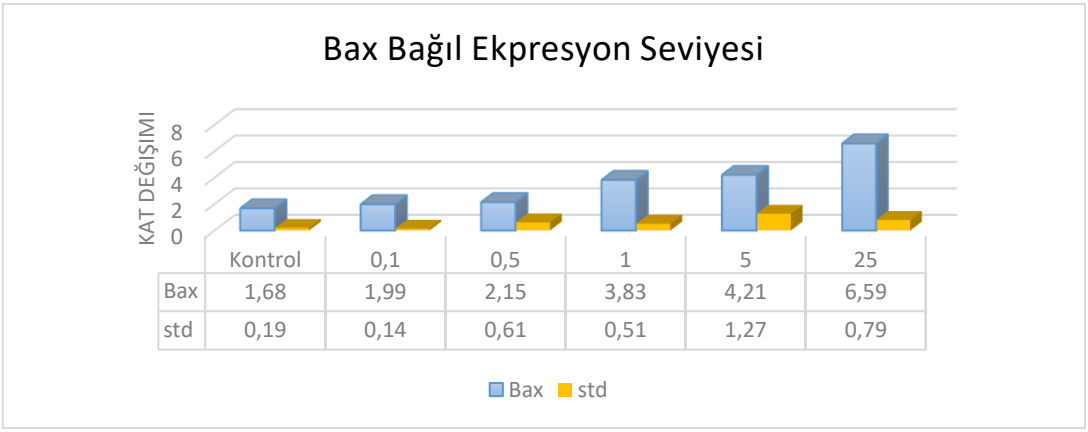
Abt-263 konsantrasyonu (µM)	**Bcl-2 Ekspresyon Seviyesi ($\bar{x} \pm S\bar{x}$)**
Kontrol (0 µM)	0.91 ± 0.3^{a}
0.1	0.8 ± 0.1^{ba}
0.5	1.0 ± 0.98^{bc}
1	1.88 ± 0.8^{cd}
5	3.22 ± 1.54^{de}
25	5.34 ± 0.56^{e}
p	***



Şekil 2. Kontrol ve farklı konsantrasyonlarda (0.1, 0.5, 1, 5, 25 µM) Navitoclax uygulanan gruplarda Bcl-2 ekspresyon değişimleri
Figure 2. Bcl-2 expression changes in control and Navitoclax applied groups at different concentrations (0.1, 0.5, 1, 5, 25 µM)

Tablo 4. Kontrol ve farklı konsantrasyonlarda (0.1, 0.5, 1, 5, 25 µM) Navitoclax uygulanan gruplarda Bax ekspresyon değişimlerinin değerlendirmesi
Table 4. Evaluation of Bax expression changes in control and Navitoclax at different concentrations (0.1, 0.5, 1, 5, 25 µM)

Abt-263 konsantrasyonu (µM)	**Bax Ekspresyon Seviyesi ($\bar{x} \pm S\bar{x}$)**
Kontrol (0 µM)	1.68 ± 0.19^{a}
0.1	1.99 ± 0.14^{ba}
0.5	2.15 ± 0.61^{bc}
1	3.83 ± 0.51^{c}
5	4.21 ± 1.27^{de}
25	6.59 ± 0.79^{e}
p	***

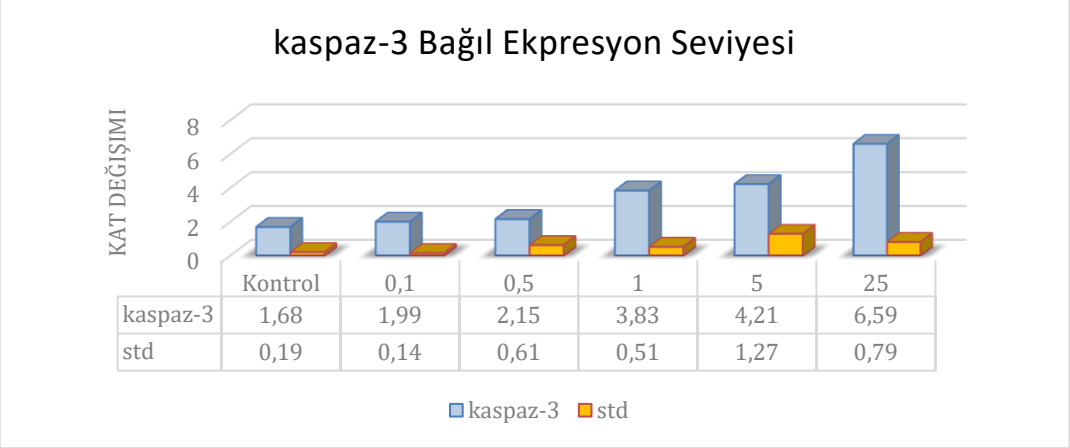


Şekil 3. Kontrol ve farklı konsantrasyonlarda (0.1, 0.5, 1, 5, 25 µM) Navitoclax uygulanan gruplarda Bax ekspresyon değişimleri
Figure 3. Bax expression changes in control and Navitoclax applied groups at different concentrations (0.1, 0.5, 1, 5, 25 µM)

Kaspaz-3 Aktivitesi
Kontrol grubu ile farklı konsantrasyonlarda Navitoclax uygulanan gruplarda kaspaz aktivasyonları yönünden istatistiksel olarak karşılaştırıldığında (çizelge 5), 25 µM Navitoclax uygulanan grupta kontrol grubuna göre Kaspaz-3 aktivasyonunun arttığı tespit edildi ($p < 0.001$). 0.1 ve 0.5 µM Navitoclax uygulanan gruplar (şekil 4) istatistiksel olarak benzerlik göstermişlerdir ($p > 0.05$).

Çizelge 5. Kontrol ve farklı konsantrasyonlarda (0.1, 0.5, 1, 5, 25 µM) Navitoclax uygulanan gruplarda Kaspaz-3 ekspresyon değişimlerinin değerlendirmesi
Table 5. Evaluation of Caspase-3 expression changes in control and Navitoclax at different concentrations (0.1, 0.5, 1, 5, 25 µM)

Abt-263 konsantrasyonu (µM)	**Kaspaz-3 Ekspresyon Seviyesi ($\bar{x} \pm S\bar{x}$)**
Kontrol (0 µM)	1.28 ± 0.12^{a}
0.1	1.09 ± 0.32^{b}
0.5	1.15 ± 0.43^{bc}
1	2.00 ± 0.16^{cd}
5	3.21 ± 1.19^{de}
25	5.03 ± 1.23^{e}
p	***



Şekil 4. Kontrol ve farklı konsantrasyonlarda (0.1, 0.5, 1, 5, 25 µM) Navitoclax uygulanan gruplarda Kaspaz-3 ekspresyon değişimleri
Figure 4. Caspase-3 expression changes in control and Navitoclax applied groups at different concentrations (0.1, 0.5, 1, 5, 25 µM)

Apoptoz, hücre ölümüne yanıt olarak sergilenen ana mekanizmalardan biridir ve tümör hücrelerinde apoptozun indüklenmesi, kanser tedavisi için potansiyel bir hedef anlamına gelir. Bcl-2 ailesi proteinleri, apoptotik yolun düzenlenmesinde anahtar rol oynar. Bcl-2 aşırı ekspresyonu genellikle meme kanseri, prostat kanseri, B hücreli lenfomalar ve kolorektal adenokarsinomlar dahil olmak üzere çeşitli kanserlerle ilişkilidir (Gonsalves ve ark. 2018). Bu nedenle, Bcl-2 dünya çapında tıbbi kimyagerleri çeken yeni bir anti-kanser hedefidir. Bcl-2 hedefinin altında yatan araştırmalar, 'BH3-mimetikleri' olarak adlandırılan küçük molekül inhibitörlerinin üretilmesiyle sonuçlanmıştır (Wang ve ark. 2018). Bu ilaçlar, kanser hücrelerinin apoptozunun aktivasyonu ile sonuçlanan hayatta kalma yanlısı Bcl-2 proteinlerine bağlanma gösterir. Yapı bazlı ilaç tasarımı ve Nükleer Manyetik Rezonans (NMR) bazlı taramanın bir sonucu olarak keşfedilen ilk BH3 mimetikleri, bir N-asilsülfonamid analoğu olan ABT-263 dir. Küçük moleküller olarak birkaç Bcl-2 inhibitörü klinik geliştirme aşamasındadır ve sonuçlar, bu moleküllerin tek başına veya kombinasyon halinde kanser tedavisinde potansiyel uygulama olabileceğini göstermiştir (Suvarna ve ark. 2019).
Hücre ölümünden kaçınmak, kanser için ayırt edici özelliklerden biridir (Hanahan ve Weinberg 2011). Kontrolsüz hücre döngüsü ilerlemesini yönlendiren onkojenik dönüşüm, genellikle apoptozu tetikleyen doğuştan gelen tümör baskılayıcı kontrol noktalarını etkinleştirir (Lowe ve ark. 2004). Apoptotik kontrol noktalarını ortadan kaldırmak için, kanser hücreleri sıklıkla, yukarı regüle edilmiş BIM ve PUMA'yı ayıran anti-apoptotik Bcl-2 ailesi proteinlerini aşırı ifade eder. Bu nedenle, birçok kanser hücrelerinde Bcl-2/Bcl-x yer değiştirir (Anderson ve ark. 2014). Kanser tedavisi geliştirme stratejilerinden biri, içsel programlanmış hücre ölümünü teşvik etmektir. Anti-apoptotik proteinlerin mitokondriyal zarlar üzerindeki yürütücü proteinlerle etkileşimini bloke etmek, bu yaklaşımın temeli olmuştur. Bu nedenle, Bcl-2 ailelerinin hayatta kalma yanlısı proteinleri, bu terapötik müdahale için potansiyel hedeflerdir. Birkaç Bcl-2 ailesi inhibitörü, anti-kanser ilaçları olarak araştırılmıştır ve bunlardan biri Navitoclaxtır. Bir çalışma, anti-apoptotik proteinlerin BH3 alan bağlama proteinleri ile etkileşimini bozarak kanser hücresi apoptozunu indüklemek için navitoclaxın mekanizmasını bildirmiştir.
Antikanser ilaçlara karşı direncin altında yatan ana mekanizmalardan biri, B hücreli lenfoma-2 (Bcl-2) familyası proteinlerinin değişmiş ifadesidir (Martinou ve Youle, 2011). Böylece, Bcl-2 ailesi proteinleri, tümör başlangıcını, büyümesini ve radyoterapinin yanı

sıra kemoterapiye direnci kolaylaştıran apoptozun merkezi kontrolörleri olarak gelişmiştir (Oltersdorf ve ark. 2005). Mevcut kanıtların ortaya koyduğu gibi, Bcl-2, Bax gibi pro-apoptotik moleküllerin aracılık ettiği apoptozu inhibe eder. Bu nedenle, Bcl-xl/Bcl-2'nin veya herhangi birinin bağlanma kabiliyetini bozan moleküller diğer anti-apoptotik ila pro-apoptotik moleküller, kanser yönetiminde terapötik öneme sahip olacaktır (Shangary ve Johnson 2002).

Apoptozdan kaçınma, kanserin bir özelliğidir ve kemorezistans ile ilişkilidir (Pommier ve ark. 2004). Bcl-2 ailesi, intrinsik yoldan apoptoza katılır (Brunelle ve Letai 2009). Bcl-2, ER pozitif tümörlerin yaklaşık %85'inde, HER2 pozitif tümörlerin %50'sinde, üçlü negatif meme kanserlerinin % 41'inde ve bazal benzeri tümörlerin % 19'unda aşırı eksprese edilir (Dawson ve ark. 2010; Oakes ve ark. 2012b; Vaillant ve ark. 2013). Hayatta kalma yanlısı Bcl-2 ailesi ekspresyonu, kemoterapötik ajanlara dirençle ilişkilidir (Fiebig ve ark. 2006).

Apoptoz yolunun aktivasyonu, ya kanser hücresi ölümünü ya da yerleşik sitotoksik maddelere duyarlılığı ya da intrinsik hücre ölüm yolunun aktivasyonu yoluyla işlev gören radyasyon terapisini indükleyen hedeflenen kemoterapiler için en yaygın mekanizmadır (Zhu ve ark. 2016). Anti-apoptotik alt ailesini (Bcl-2, Bcl-xL, Bcl-w, Mcl-1 ve A1), pro-apoptotik alt ailesini (Bax, Bak ve Bok) ve yalnızca BH3'ü içeren Bcl-2 ailesi proteinler (Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa ve Puma), kemoterapi sırasında hücre ölümünün düzenlenmesinde merkezi bir rol oynar (Adams ve Cory 2007; Youle ve Strasser 2008). Anti-apoptotik proteinler, hücreleri çeşitli sitotoksik koşullardan korurken, pro-apoptotik proteinler hücre apoptozunu destekler. Özet olarak, verilerimiz, ABT-263 tedavisinin in vitro kanser hücresi apoptozunu indüklemede tümör büyümesini ve ilerlemesini inhibe etmede güvenli ve oldukça etkili olduğunu göstermektedir.

Navitoclax ile etkili tedavi için bir ön koşul, anti-apoptotik Bcl-2 proteininin tümör hücrelerinde ekspresyonudur. Bu amaçla, A-549 hücre hattında bu proteinlerin ekspresyonu araştırıldı. Bu çalışmanın amacı, Bcl-2 inhibitörü Navitoclax'ın akciğer kanseri hücrelerinde, hücre büyümesi inhibisyonu ve apoptoz indüksiyonu üzerindeki tedavisinin etkinliğini araştırmaktı. Sonuçlarımız, tüm konsantrasyonların anti-apoptotik Bcl-2 proteinini ifade ettiğini ve A-549 hücre hatlarının Navitoclax'a duyarlı olduğunu göstermektedir. Navitoclax apoptoz indüksiyonu yoluyla hücre çizgisi canlılığını daha da azaltmıştır.

SONUÇ

Sonuç olarak çalışmamızda insan akciğer kanser hücrelerinde in vitro Navitoclax uygulamasının apoptozisi indüklediği ve hücre canlılığını azalttığı tespit edilmiştir. Navitoclax' ın hücre canlılığını azaltıcı etkisinin hücrelerde apoptozisi indükleyici etkisinden kaynaklanabileceğini göstermektedir. Yeni yapılacak çalışmalarda in vivo denemelerle Navitoclax' nın etkisine yönelik mekanizmaların daha iyi anlaşılabileceği düşünülmektedir. Ayrıca ileride yapılacak olan çalışmalarda Navitoclax ile birlikte kemoterapik ilaçların kombine edilerek kullanılması, akciğer kanseri tedavisine yeni yaklaşımlar getirebileceğini düşündürmektedir.

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RESEARCH ARTICLE

Hepatoprotective effect of pomegranate (Punica granatum l.) in a rabbit model of steatohepatitis

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ABSTRACT

The study aimed to exhibit liver protection, activity, and probable side effects of LPJE on a steatohepatitis rabbit model. In this study, 48 rabbits were used. The first group (Group 1) rabbits were fed a standard diet (SD), while the second group (Group 2) rabbits were fed a high-fat diet (HFD). In two groups, three different doses of LPJE (25 mg/kg, 50 mg/kg, and 100 mg/kg) were given simultaneously to the rabbits for eight weeks. Results showed that the serum albumin concentration was significantly different in the LPJE group (100 mg/kg) than in the positive control group. The cholesterol concentration of the LPJE (50mg/kg) administered subgroup of Group 2 (96.71 ± 12.03) was found to be the lowest. According to histopathological examination results, Group 2 did not display a statistical difference; however, the lowest liver fat deposition degree was 26.49 ± 8.64% in the LPJE (50 mg/kg) subgroup. As a result of this study, the lyophilized pomegranate extract has hepatoprotective activity. The most effective dose was 50 mg/kg on the non-alcoholic steatohepatitis (NASH) rabbit model. No side effects were determined up to 100 mg/kg pomegranate juice extract on rabbits.

Keywords: Antioxidant activity, bioactivity, non-alcoholic steatohepatitis, punica granatum.

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Bir tavşan steatohepatit modelinde narın (Punica granatum l.) hepatoprotektif etkisi

ÖZ

Bu çalışmada, bir steatohepatit tavşan modelinde Liyofilize nar suyu ekstresinin (LPJE) karaciğer koruması, aktivitesi ve olası yan etkilerinin gösterilmesi amaçlandı ve 48 tavşan kullanıldı. Birinci grup (Grup 1) tavşanlara standart bir diyet (SD), ikinci grup (Grup 2) tavşanlara ise yüksek yağlı bir diyet (HFD) verildi. Her iki gruptaki üçer alt grup tavşanlara sekiz hafta boyunca eş zamanlı olarak üç farklı dozda LPJE (25 mg/kg, 50 mg/kg ve 100 mg/kg) verildi. Pozitif ve standart kontrol grubu tavşanlara ise 1 ml cmc solüsyonu içirildi. Sonuçlar; serum albümin konsantrasyonunun LPJE grubunda (100 mg/kg) pozitif kontrol grubundan önemli ölçüde farklı olduğunu gösterdi. En düşük kolesterol konsantrasyonu, Grup 2'nin (96.71 ± 12.03) LPJE (50mg/kg) uygulanan alt grubunda bulundu. Histopatolojik inceleme sonuçlarına göre Grup 2 istatistiksel olarak farklılık göstermedi; ancak en düşük karaciğer yağlanma derecesi LPJE (50 mg/kg) alt grubunda %26.49 ± 8.64 olarak ölçüldü. Sonuç olarak, liyofilize nar ekstresinin hepatoprotektif aktivite gösterdiği ve alkolsüz steatohepatit (NASH) tavşan modelinde en etkili dozun 50 mg/kg olduğu saptandı. Ayrıca tavşanlarda 100 mg/kg nar suyu ekstresine kadar herhangi bir yan etki tespit edilmedi.

Anahtar Kelimeler: Alkolsüz steatohepatit, antioksidan aktivite, biyoaktivite, punica granatum

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INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a deterioration of the liver functions without alcohol and histopathological alcohol-induced hepatic impairment (Ludwig et al. 1980). The golden standard for the diagnosis of NASH is liver biopsy (Marchesini and Marzocchi 2007, Neuschwander-Tetri 2005). Steatosis, inflammatory infiltration, ballooning degeneration in hepatocytes, and fibrosis are the main histopathological findings of NASH (Brunt 2002, Sonsuz and Baysal 2011). The primary objective in the treatment of NASH is to prevent and treat metabolic syndrome. Presently, there are no effective drugs used in NASH treatment (Adorini et al. 2012, Pfohl et al. 2020). However, lipid/cholesterol reducers, insulin sensitizers, alpha-glucosidase inhibitors, non-steroidal anti-inflammatory drugs, and cholinesterase inhibitors are used for NASH treatments. (Scarpini et al. 2003, Takahashi et al. 2015).

Pomegranate is used for protection from diseases in folk medicine, particularly in the Middle East countries (Gurib-Fakim 2006). Pomegranate juice contains active compounds such as vitamin C, anthocyanins, punicalagin, ellagic and gallic acids (El-Nemr et al. 1990). Previous evidence suggests that pomegranate had chemopreventive, cardioprotective, antihyperlipidemic, and hepatoprotective effects (Afaq et al. 2005, Sumner et al. 2005, Fuhrman et al. 2005, Osman et al. 2011). Moreover, the phenol content of pomegranate products, such as extracts and juice, is abundant. Thus, dietary pomegranate products showed protective effects against inflammation-related disorders in different human clinical trials (Pfohl et al. 2020). A clinical trial showed that a pomegranate extract reduced complications linked with obesity in overweight and obese individuals (Hosseini et al. 2016). Pomegranate is considered to be effective against NASH owing to its antihyperlipidemic activity (Fuhrman et al. 2005, Pagano et al. 2002, Çolak and Tuncer 2010).

In the present study, the hepatoprotective effect of lyophilized pomegranate fruit extract obtained from fresh pomegranate on the livers of rabbits fed with HFD was investigated. Also, its antioxidant properties and possible side effects were evaluated.

MATERIALS AND METHODS

Plant Material

Pomegranate extracts were prepared from the fresh pomegranate whole fruits growing in the Mediterranean Region of Turkey. Fresh pomegranate fruits were squeezed with a juicer, and the juice was lyophilized. "Lyophilized pomegranate juice extract" was obtained and administered per os to the test animals in three different doses [(25 mg/kg; 50 mg/kg; and 100 mg/kg in 1 ml 0.5% carboxymethyl cellulose (CMC)].

Animals

Forty-eight non-pathogenic, five-month-old, male New Zealand breed rabbits were used in the present study. The research was conducted on a total of two groups. One of them was the main control group (Group 1), and the other was the experimental group (Group 2). Each group was formed by four subgroups (n=6). The experiments in all groups were initialized after a one-week acclimatization process. The rabbits in Group 1 were fed a Standart diet (SD) ad libitum, while the ones in Group 2 were fed a high-fat diet (HFD), for all groups were free to reach the water. The animals were kept under standard conditions for animal care.

Standard Control Group (SCG) in Group 1 and Positive Control Group (PCG) in Group 2 were formed for intergroup comparisons. Instead of pomegranate extracts, 1 mL of 0.5% CMC was given daily to the rabbits in those groups (placebo).

Group 1 was formed by a total of four subgroups, one of which was the standard control groups (1 mL of 0.5% CMC/day) and lyophilized pomegranate juice extract at 25 mg/kg, 50 mg/kg, and 100 mg/kg doses were given daily to the animals in the other three subgroups.

For setting the steatohepatitis model, an HFD (20% of corn oil and 1.25% (w/w) of cholesterol addition to the SD) was given ad libitum to the experimental group (Group 2) for eight weeks (Ogawa et al. 2010). During the model formation, pomegranate extracts were given simultaneously to the rabbits in group 2 in three doses (25, 50, and 100 mg/kg) for eight weeks.

During the experiment, daily live weight gain and food intake of the rabbits were recorded.

Sampling and Analyses

At the end of the experiment, blood samples from central ear arteries were obtained with 22-gauge intravenous catheter from the rabbits into serum tubes and anticoagulant tubes from all the groups. Serum samples were stored at -20°C until the measurement time.

In the serum samples, Alanine Transaminase (ALT), Aspartate Transaminase (AST), Gamma-Glutamyl Transferase (GGT), Albumin (ALB), Total Protein (TP), Total Bilirubin (TBIL), Glucose (GLU), Cholesterol (TC), Triglycerides (TG), High-Density Lipoprotein (HDL), and Low-Density Lipoprotein (LDL) values were measured with a Mindray BS 120 biochemistry analyzer. NEFA (Diametra, REF: FA115, LOT: 288862) and insulin levels (Diametra, REF: DKO076, LOT: 3118) were measured with ELISA kits. Very Low-Density Lipoprotein (VLDL) value was calculated with TG/5 formula (Civelek et al. 2011).

For the evaluation of the antioxidant activity (AoA), Glutathione (GSH), Malondialdehyde (MDA), and

Nitric Oxide (NO) levels were measured. MDA and NO were examined in the liver tissue samples.

After the procedure, both groups (Group 1 and 2) were euthanized under anesthesia (Xylazine 5 mg/kg + Ketamine 35 mg/kg) by administering 150 mg/kg of intravenous thiopental sodium. Then, the rabbit's tissues were collected, livers were removed, and wet weights were measured. To standardize body weight on the liver scale, the formula of body weight/liver weight was used. The samples were sent to the laboratory in 10% Ca-formaldehyde for histopathological examination.

The tissue samples obtained from the liver were kept in neutral buffered formaldehyde solution for 48 hours. Sections in 6-8 μm thickness were made using Cryostat and stained with oil red O staining technique (Lillie and Ashnburn 1943). The preparation was closed with aqueous adhesives and examined under a light microscope.

Statistical Analyses

For Group 1 and Group 2, the animals' live weights were taken as covariate variables in the last week. The covariance analysis (ANCOVA) method was utilized in the comparisons within the groups. The Mann-Whitney U test was employed in the comparison of the control groups (SCG and PCG). To analyze the disparities between live weight gain and food intake of the subgroups of Group 1 and Group 2, the Kruskal-Wallis H test was utilized. For those statistical analysis methods, MedCalc 15, SPPS 13 for Windows, and Microsoft Excel 2010 packages were used.

The research procedure was carried out with the approval of the Institutional Ethic Committee of the Faculty of Veterinary Medicine, Afyon Kocatepe University (No. 2012/234).

RESULTS

Biochemistry, oxidant/antioxidant parameter measurements, and pathologic evaluation results of the present study were showed in Table 1-4.

Table 1. Biochemical analysis results (Group 1 and Group 2)

Groups	Mean ± SEM (n=6)												
	Dose (mg/kg)	**ALB (g/dL)**	**AST (U/L)**	**ALT (U/L)**	**GGT (U/L)**	**TBIL**	**TP (g/dL)**	**TG (mg/dL)**	**TC (mg/dL)**	**HDL (mg/dL)**	**LDL**	**NEFA**	**VLDL (mg/dL)**
Standard Control Group (SD)	-	4.32 ±0.086	41.01 ±4.60	35.40 ±6.73	12.76 ±1.82	0.15 ±0.012	6.46 ±0.21	61.15 ±11.59	59.37 ±11.25	32.20 ±5.18	23.21 ±7.79	0.08 ±0.01	12.22 ±2.32
P. granatum liyophilized extract (LPJE)	25	4.50 ±0.079	47.22 ±4.20	37.00 ±6.15	11.17 ±1.66	0.17 ±0.011	6.67 ±0.19	55.36 ±10.58	52.13 ±10.27	27.06 ±4.73	23.72 ±7.11	0.07 ±0.01	11.07 ±2.12
	50	4.59 ±0.079	51.88 ±4.20	44.00 ±6.15	12.19 ±1.66	0.16 ±0.011	6.96 ±0.19	45.87 ±10.58	60.73 ±10.27	33.46 ±4.73	26.19 ±7.11	0.08 ±0.01	9.17 ±2.12
	100	4.47 ±0.079	46.72 ±4.20	44.67 ±6.16	12.08 ±1.66	0.15 ±0.011	6.58 ±0.19	34.92 ±10.60	44.14 ±10.29	29.71 ±4.74	15.20 ±7.12	0.08 ±0.01	6.98 ±2.12
P	**-**	**0.176**	**0.408**	**0.648**	**0.931**	**0.505**	**0.336**	**0.374**	**0.664**	**0.789**	**0.723**	**0.912**	**0.374**
Positive Control Group (HFD)	-	4.46 $\pm 0.10^{b}$	53.28 ±26.18	49.75 ±11.24	32.32 ±4.19	0.17 ±0.016	7.04 ±0.23	104.66 ±22.58	118.52 ±12.03	43.56 ±4.86	57.44 ±7.86	0.11 ±0.014	20.93 ±4.52
P. granatum liyophilized extract (LPJE)	25	4.16 $\pm 0.10^{ab}$	41.13 ±26.96	61.00 ±11.57	26.70 ±4.31	0.19 ±0.016	6.46 ±0.24	162.89 ±23.25	125.47 ±12.39	42.21 ±5.00	51.36 ±8.09	0.12 ±0.014	32.57 ±4.65
	50	4.39 $\pm 0.10^{ab}$	69.82 ±26.17	69.18 ±11.23	23.49 ±4.19	0.20 ±0.016	6.66 ±0.23	94.37 ±22.58	96.71 ±12.03	44.90 ±4.86	40.46 ±7.85	0.07 ±0.014	18.87 ±4.52
	100	4.04 $\pm 0.10^{a}$	96.95 ±26.39	64.07 ±11.33	24.99 ±4.22	0.18 ±0.016	6.10 ±0.23	92.14 ±22.76	114.95 ±12.13	44.53 ±4.90	49.45 ±7.92	0.08 ±0.014	18.42 ±4.55
P	**-**	**0.019**	**0.504**	**0.662**	**0.483**	**0.626**	**0.057**	**0.146**	**0.406**	**0.981**	**0.509**	**0.109**	**0.146**

[a]and [b] describe difference between groups.

Table 2. Biochemical analysis results (Group 1 and Group 2)

Groups	Mean ± SEM (n=6)												
	Dose (mg/kg)	**GLU (mg/dL)**	**İnsulin (U/L)**	**Liver weight (BW/LW)**	**LS (%) Mean**	**Food Consumption (g)**	**Live weight gain(g)**	**MDA (nmol/mL)**	**dMDA (nmol/mL)**	**NO (µmol/L)**	**dNO (µmol/L)**	**GSH (mmol/L)**	**AoA (mmol/L)**
Standard Control Group (SD)	-	107.46 ±7.99	0.14 ±0.08	2.51 ±0.19	0.01 ±3.62	7732.20 ±1646.24^{ab}	780.40 ±177.75	3.95 ±0.29	2.20 ±0.19	11.57 ±2.01	7.53 ±1.30^{ab}	26.62 ±3.82	6.37 ±0.24
P. granatum liyophilized extract (LPJE)	25	111.23 ±7.30	0.31 ±0.07	2.61 ±0.18	3.38 ±3.31	8536.50 ±804.73^{a}	793.00 ±331.22	4.05 ±0.26	2.59 ±0.18	14.67 ±1.84	7.26 ±1.19^{a}	26.41 ±3.48	6.65 ±0.22
	50	113.86 ±7.30	0.22 ±0.07	2.88 ±0.18	3.37 ±3.31	7394.33 ±969.32^{ab}	702.50 ±320.13	3.48 ±0.26	2.18 ±0.18	14.02 ±1.84	11.90 ±1.19^{b}	19.40 ±3.48	6.45 ±0.22
	100	119.69 ±7.30	0.17 ±0.07	2.35 ±0.18	6.57 ±3.31	6885.66 ±364.77^{b}	617.50 ±230.90	3.22 ±0.26	2.44 ±0.18	16.03 ±1.84	10.53 ±1.19^{ab}	22.51 ±3.49	6.40 ±0.22
P	**-**	**0.713**	**0.406**	**0.231**	**0.627**	**0.047**	**0.764**	**0.134**	**0.347**	**0.449**	**0.036**	**0.440**	**0.805**
Positive Control Group (HFD)	-	125.86 ±6.64	0.03 ±0.06	2.73 ±0.13	49.50 ±9.51	4720.33 ±529.31	619.33 ±231.69	4.75 ±0.40	2.46 ±0.26	11.64 ±1.57^{ab}	12.58 ±1.83	13.08 ±2.85	7.01 ±0.15
P. granatum liyophilized extract (LPJE)	25	132.91 ±6.84	0.22 ±0.07	2.46 ±0.14	44.44 ±8.94	4187.16 ±402.27	508.00 ±250.06	4.90 ±0.41	2.73 ±0.27	9.85 ±1.62^{b}	8.64 ±1.88	19.65 ±2.93	6.88 ±0.16
	50	117.24 ±6.64	0.11 ±0.06	2.68 ±0.13	26.49 ±8.64	4637.50 ±971.37	589.16 ±275.93	4.95 ±0.40	2.81 ±0.26	10.31 ±1.57	15.82 ±1.83	23.24 ±2.85	6.83 ±0.15
	100	109.16 ±6.69	0.09 ±0.06	2.56 ±0.13	27.81 ±8.70	4992.16 ±616.94	569.66 ±191.50	4.60 ±0.40	3.16 ±0.26	16.14 ±1.58^{a}	12.05 ±1.84	15.53 ±2.87	7.07 ±0.15
P	**-**	**0.112**	**0.277**	**0.519**	**0.148**	**0.209**	**0.904**	**0.928**	**0.335**	**0.046**	**0.092**	**0.093**	**0.690**

[a]and [b] describe difference between groups.

Table 3. Biochemical analysis results (Standard Control Group and Positive Control Group)

Groups		Mean ± SEM (n=6)											
	Dose (mg/kg)	**ALB (g/dL)**	**AST (U/L)**	**ALT (U/L)**	**GGT (U/L)**	**TBIL**	**TP (g/dL)**	**TG (mg/dL)**	**TC (mg/dL)**	**HDL (mg/dL)**	**LDL**	**NEFA**	**VLDL (mg/dL)**
Standard Control Group (SD)	-	4.32 ±0.31	41.00 ±8.46	35.40 ±6.99	12.76 ±1.99	0.15 ±0.019	6.46 ±0.64	61.10 ±37.13	59.24 ±28.99	32.12 ±7.61	23.16 ±24.86	0.084 ±0.042	12.22 ±7.42
Positive Control Group (HFD)	-	4.47 ±0.23	51.33 ±17.35	48.67 ±13.97	31.92 ±13.35	0.17 ±0.050	7.04 ±0.76	104.53 ±25.74	118.23 ±24.64	43.12 ±11.56	59.79 ±17.75	0.108 ±0.037	21.55 ±5.47
P	**-**	**0.360**	**0.201**	**0.143**	**0.006**	**0.169**	**0.201**	**0.045**	**0.018**	**0.201**	**0.047**	**0.709**	**0.047**

Table 4. Biochemical analysis results (Standard Control Group and Positive Control Group)

Groups	Mean ± SEM (n=6)												
	Dose (mg/kg)	**GLU (mg/dL)**	**Insulin (U/L)**	**Liver weight (BW/LW)**	**LS (%) Mean**	**Food Consumption (g)**	**Live weight gain(g)**	**MDA (nmol/mL)**	**dMDA (nmol/mL)**	**NO (µmol/L)**	**dNO (µmol/L)**	**GSH (mmol/L)**	**AoA (mmol/L)**
Standard Control Group (SD)	-	107.40 ±27.13	0.14 ±0.11	2.51 ±0.22	0.00 ±0.00	7732.20 ±1646.24	780.40 ±177.75	3.95 ±0.94	2.20 ±0.50	11.56 ±3.62	7.52 ±0.66	26.60 ±7.83	6.37 ±0.66
Positive Control Group (HFD)	-	126.50 ±23.34	0.04 ±0.00	2.73 ±0.38	50.00 ±29.15	4720.33 ±529.31	619.33 ±231.69	4.77 ±0.60	2.45 ±0.45	11.55 ±3.24	12.61 ±6.07	13.20 ±8.23	7.01 ±0.17
P	**-**	**0.784**	**0.006**	**0.170**	**0.005**	**0.009**	**0.329**	**0.100**	**0.410**	**1.000**	**0.017**	**0.036**	**0.094**

In biochemical measurements, no statistical difference was determined in the ALB value of Group 1. At the same time, a statistically significant decrease was detected in LPJE (100 mg/kg) subgroup when compared to the LPJE (25 mg/kg) subgroup of Group 2 in intergroup comparisons ($p < 0.05$). In the comparison of the control groups, no statistical difference was found.

When ALT, AST, GGT, TBIL, and TP levels were evaluated, no statistically significant differences were detected in Group 1 and Group 2. In comparing the control groups, a statistical difference was seen only in the GGT value (SCG, 12.76 ± 1.99; PCG, 31.92 ± 13.35, $p = 0.006$).

Although no statistically significant difference was detected between Group 1 and Group 2 in terms of TG values, a numerical difference was found between the SCG and LPJE (100 mg/kg) subgroup of Group 1. In comparing control groups, the increase of TG in Group 2 was considered statistically significant (SCG, 61.10 ± 37.13, PCG, 104.53 ± 25.74, $p = 0.045$).

In the statistical evaluation of the TC measurements, no statistical difference was detected in Group 1 and Group 2. In comparing the control groups, the increase of TC in Group 2 was found to be statistically significant (SCG, 59.24 ± 28.99; PCG, 118.23 ± 24.64, $p = 0.018$).

No statistical differences were found in HDL, LDL, NEFA, and VLDL values in Group 1 and Group 2. In the comparison of the control groups, no statistically significant differences were detected in HDL and NEFA values, but the increase in LDL (SCG, 23.16±24.86; PCG, 59.75±17.75 $p = 0.047$) and VLDL (SCG 12.22 ± 7.42; PCG, 21.55 ± 5.47, $p = 0.047$) values in Group 2 were considered as significantly detected.

Even though there were no statistical differences in serum GLU and insulin levels between Group 1 and Group 2, a numerical difference was detected in serum insulin concentration in SCG when compared to the LPJE (25 mg/kg) subgroup of Group 1 and in PCG compared to LPJE (25 mg/kg) subgroup of Group 2. Where there was not a statistical difference in GLU level in the comparison of the control groups, the decrease in the insulin level in Group 2 (SCG, 0.14 ± 0.11; PCG, 0.04 ± 0.00, $p = 0.006$) was found to be statistically significant.

In evaluating wet liver weight (LWW) and liver steatosis rates (LS%) in Group 1 and Group 2, no statistically significant differences were found in none of the groups. Moreover, although no statistical difference was detected in Group 2 in terms of LS%, a numeric difference was found in LPJE (50 mg/kg) subgroup (26.49 ± 8.64) and LPJE (100 mg/kg) subgroup (27.81 ± 8.70) of Group 2 when compared to PCG (49.50 ± 9.51). In comparing the control groups (SCG, 0.00 ± 0.00; PCG, 50.00 ± 29.15, $p = 0.005$), a statistically significant difference in liver steatosis was detected.

In terms of the food consumption during the study, there was a statistically significant difference between the LPJE (25 mg/kg) subgroup of Group 1 (8536.50 ± 804.73) and the LPJE (100 mg/kg) subgroup of Group 1 (6885.66 ± 364.77) ($p = 0.047$). In comparing the control groups (SCG, 7732.20 ± 1646.24; PCG, 4720.33 ± 529.31, $p = 0.009$), the food consumption of Group 2 was dramatically lower than Group 1. No difference was detected in the food consumption rate within Group 2.

During the statistical evaluation of the differences in the animals' live weights between the beginning and the end of the study, no statistical difference was determined in Group 1 and Group 2.

In comparing oxidant and antioxidant concentrations of tissue and serum, a statistical difference was detected in NO level in Group 1 ($p = 0.036$). No statistical difference was detected in any other oxidant and antioxidant concentrations of tissue and serum. In Group 2, only the NO value increased at a statistically significant PCG rate compared to the LPJE (100 mg/kg) subgroup of Group 2 ($p = 0.046$). In the comparison of the control groups, there was a statistical difference in dNO (SCG, 7.52 ± 0.66; PCG, 12.61 ± 6.07, $p = 0.017$) and GSH (SCG, 26.60 ± 7.83; PCG, 13.20 ± 8.23, $p = 0.036$) values.

DISCUSSION

HFD, obesity, insulin resistance, and oxidative stress are the main factors of NASH. Unlike many other studies, an HFD was preferred in the present study rather than the chemicals to induce the experimental model. Due to its rich antioxidant content and preliminary studies on its bioactivity, the hepatoprotective effect of pomegranate was investigated in the present study.

The first symptom of NASH is the increase in liver enzymes. However, high levels of these enzymes do not always indicate liver fibrosis (Angulo et al., 1999; Gören & Fen, 2005). When positive and standard control groups were compared in the study, although a numerical increase was detected in the liver enzymes in Group 2, only the increase in GGT was statistically significant. The present research findings were found to be in parallel with the previous studies (Ogawa et al., 2010; Bayan et al., 2004). The rise in GGT suggests that damage in bile ducts might develop due to HFD (Ideo et al. 1972). It was reported in a study by Patel et al. (2019) that the use of pomegranate extract enriched with punicalagin even in such high doses like 600 mg/kg/day did not lead to an increase in ALT, AST, and GGT values. Haber et al. (2007) reported that pomegranate extract given in the dose of 1420 mg/day did not increase liver enzymes. Like the other studies, it was determined in the present study that the pomegranate extract given to the rabbits in Group 1 did not cause an increase in AST, ALT, and GGT levels in any of the doses. The researchers have reported that

different pomegranate extracts dramatically lower the rises in ALT, AST, and GGT in liver injury (Osman et al. 2011, Celik et al. 2009, Ashoush et al. 2013, Zou et al. 2014). Unlike those studies, in the present report, no statistically significant differences were detected in ALT, AST, and GGT levels in Group 1 and Group 2. Unlike the study presented in these studies, while different pomegranate components were used, the extract of pomegranate fruit was used in our research.

A toxicity study demonstrated that pomegranate extract did not affect serum ALB level in male rats. On the other hand, pomegranate in the dose of 60 mg/kg/day led to a statistically significant increase in female rats' serum albumin level (Patel et al. 2008). In the present study, male rabbits were used, and similarly, there was no significant difference found in serum ALB level. In several other studies, a decrease in the ALB level in blood due to liver injury was reported to bring to the normal range by administering the pomegranate extract (Osman et al. 2011, Shaban et al. 2013). In this report, although 100 mg/kg pomegranate extract given to Group 2 decreased serum ALB limits compared to the control group, the results were within the reference limits.

In the present research, it was discovered that pomegranate did not change serum TP levels in Group 1 and Group 2. A statistical difference was not detected in comparing the control groups (SCG and PCG), yet a numerical increase was observed in Group 2. In the subgroups of Group 1, no statistically significant difference was seen. Some other research reports are similar to Vidal et al. (2003) and different from Patel et al. (2008) our findings. Evaluating the concentrations of serum TP in Group 2, a decrease was observed in all subgroups compared to the positive control group numeric decrease was in the LPJE (100 mg/kg) subgroup of Group 2. However, this difference was only numerical and not statistically significant ($p = 0.057$). In another study, pomegranate was reported to increase the low level of serum TP. Since ALB consists of 50% of plasma proteins (Kaysen 1998), the present study's decrease could be due to the reduction of the ALB level.

In the present study, pomegranate extract did not significantly affect the TG level in Group 1 and Group 2. However, a numerical decrease was found in the serum TG concentration of LPJE (100 mg/kg) subgroup of Group 1 compared to SCG. In our opinion, it seems a dose-dependent effect. The TG level of Group 2 was higher than the TG levels of control rabbits (SCG and PCG 250mg). The previous reports are consistent with our results (Zou et al. 2014, Lei et al. 2007). Lei et al. (2007) reported that pomegranate peel extract (at 400 and 800 mg/kg/day doses) provided a statistically dramatic decrease in the animals' TG level fed an HFD. Despite not being statistically significant in the present study, LPJE (100 mg/kg) numerically decreased the TG level. According to the previous studies, pomegranate peels' TG lowering effect could be due to the tannin content (Zou et al. 2014, Squilacci and Di Maggio 1946).

Although cholesterol is used in many functional tasks in the body, high levels significantly increase heart and blood vessel diseases (Mamurekli et al. 2000). When the control groups (SCG and PCG) were compared, TC level increased significantly in Group 2, although there was no statistical difference. The results of the present study were found to be in accord with previous results (Ogawa et al. 2010, Zou et al. 2014, Zhang et al., 2010). In their research on obese rats, Huang et al. (2005) determine that pomegranate lowered plasma TC level. Similarly, concentrated pomegranate juice was reported to decrease the TC level in patients with hyperlipidemia and diabetes (Zhang et al. 2010, Esmaillzadeh et al. 2006). It was reported that the pomegranate extract enriched with punicalagin led to statistically significant decreases in the values of the liver and serum TC, particularly in high doses (150 mg/kg) in the Non-alcoholic fatty liver disease (NAFLD) model (Zou et al. 2014). Lei et al. (2007) reported that pomegranate extract lowered serum TC level in the doses of 400 and 800 mg/kg. However, the decrease was not statistically significant. In the mentioned studies in which a decrease was observed in TC, pomegranate extracts were enriched with punicalagin. The decrease in TC level was not significant might have resulted from the punicalagin amount of the extract.

Our study determined that pomegranate extract affects the levels of HDL in Group 1 and Group 2. However, compared with the control groups (SCG and PCG), a numerical increase was detected in the serum HDL concentrations in the animals fed an HFD. The mentioned results may be related to the high fat and cholesterol levels of the diet. The researcher reported no significant dietary pomegranate effect on the lipid profile, consistent with our results (Esmaillzadeh et al. 2006, Rashidi et al. 2013). Zhang et al. (2010) reported that pomegranate extract given in the dose of 400 mg/kg lowered serum HDL concentration, but no difference occurred between the groups in 800 mg/kg.

The present report determined that pomegranate extracts did not affect the LDL levels in any of the groups. Compared with the control groups, it was observed that the LDL levels were statistically significant in the rabbits fed an HFD than the ones on an SD. Thus, our findings were compatible with the outcome of a previous study by Ogawa et al. (2010). It was reported in the conducted studies that various pomegranate extracts lowered the level of serum LDL when compared to the control group. Concentrated pomegranate extracts were used in both of the studies (Zou et al. 2014, Esmaillzadeh et al. 2006). The present study determined that pomegranate did not affect the level of serum LDL for treatment groups. It has been considered that this

might be related to the different active substance contents of different extracts.

In our research, no statistical difference was detected in the VLDL levels in Group 1 and Group 2. Comparing with the control groups (SCG and PCG), VLDL levels of the rabbits on an HFD were measured higher than those on SD, and a statistically significant difference was found. The obtained results were parallel with the findings of Ogawa et al. (2010). In another study conducted on patients with steatohepatitis, no change in the patients' VLDL levels was reported (Bayan et al. 2004). Aviram et al. (2000) did not find a difference in serum VLDL levels of the healthy people who drank 50 ml/day of pomegranate juice every day for two weeks.

Huang et al. (2005) gave pomegranate juice to the standard and diabetic rats for five weeks and reported that the value of NEFA dropped in both of the groups. On the contrary, in our study, no statistical difference was detected in the level of NEFA in Group 1 and Group 2.

In other previously published studies investigating the toxicity of pomegranate in healthy animals, it was reported that pomegranate did not affect blood glucose (Patel et al. 2008, Xu et al. 2009). Herein, the pomegranate extract raised the GLU level, but that rise was statistically insignificant. Although several studies suggest that pomegranate does not affect the blood glucose level (Huang et al. 2005a, Rashidi et al. 2013, Huang et al. 2005b, Jelodar et al. 2007, Rock et al. 2008), some other researchers have stated that it has hypoglycemic activity (El-Nemr et al. 1990; Parmar and Kar, 2008, Hontecillas et al. 2009). In our study, while there was a decrease in the serum glucose level in the LPJE (100 mg/kg) subgroup of Group 2 compared to PCG, it was statistically insignificant. It was thought that these differences might be due to the different sugar contents of the pomegranate fruit.

The HFD leads to insulin resistance (Hancock et al. 2008). It has been reported that pomegranate does not affect hepatic insulin sensitivity, yet it increases peripheral tissues' insulin sensitivity (Vroegrijk et al. 2011). In the present study, when we compared Group 1 and Group 2, serum insulin level decreased significantly in Group 2. Similarly, Pagano et al. (2002) reported that the patients' insulin levels with NASH were lower than the control group in a statistically significant manner. In another study by Gonzalez-Ortiz et al. (2011), it was reported that pomegranate did not affect insulin level. This was similar to the findings of the present study. The intergroup examination results demonstrated that pomegranate did not affect the level of insulin in either of the groups.

Zou et al. (2014) reported that pomegranate extract enriched with punicalagin lowered liver weight. On the contrary, in the present study, no statistical difference was detected in the comparisons of Group 1, Group 2, and the control groups (SCG and PCG) in terms of the wet liver weights.

The results suggest that pomegranate did not side affect the level of liver in any of the doses in Group 1. While the level of steatosis in SCG was 0%, it was 6.57% in the LPJE (100 mg/kg) subgroup of Group 1. No previous literature about the effects of pomegranate on liver histopathology was revealed. The present study is original in that sense. We suggest that the numerical increase in dose-dependent steatosis ratio might be associated with the high glucose/energy level. The results of the histopathologic examination in Group 2 demonstrated no statistical difference within the groups. Besides, numerical differences were detected within the groups in our research. While the ratio of steatosis was 49.50% in PCG, the ratio was 44.44% in LPJE (25 mg/kg) subgroup, 26.49% in LPJE (50 mg/kg) subgroup and 27.81% in LPJE (100 mg/kg) subgroup. Although a numerical decrease was observed in the rates of hepatic steatosis, no statistically significant difference emerged that might have resulted from the small number of samples. Zou et al. (2014) reported that hepatic steatosis dramatically lowered in the group with 150 mg/kg of pomegranate extract than the animals fed an HFD. In another study, it was reported that pomegranate extract decreased the accumulation of liver TG at a statistically significant rate. However, it did not affect the TG level of the liver (Xu et al. 2009). The pomegranate's lowering effect on hepatic steatosis might be a result of its inhibitory effect on the pancreatic lipase (Zhang et al. 2010). It was also reported that pomegranate significantly decreased TNF-α, IL1-β, IL-4, and IL-6, playing an important role in the pathogenesis of NASH (Non-alcoholic steatohepatitis) and NAFLD (Non-alcoholic fatty liver disease) (Zou et al. 2014). Examining the rates of hepatic steatosis in our study, a statistically significant increase was observed in the group fed an HFD compared to the group fed an SD.

That increase confirms the accuracy of the model. There are several studies conducted on the effects of pomegranate on live weight (Patel et al. 2008, Zou et al. 2014, Xu et al. 2009, Vroegrijk et al. 2011). Along with the studies reporting that pomegranate lowers live weight gain (Patel et al. 2008, Zou et al. 2014, Vroegrijk et al. 2011), other studies suggest that it does not affect the live weight (Ashoush et al. 2013, Xu et al. 2009). In this study, no effect of the pomegranate on live weight was detected.

In their study on pomegranate's oxidant/antioxidant paramaters on rats, Moneim (2012) determined a statistically insignificant numeric increase in serum NO level compared to the control group. In our study, there was a statistically significant increase in serum NO in Group 1 and the level of dNO in Group 2. Combining with O2 (superoxide) which has a higher oxidant property than itself, NO converts to ONO2− e (peroxynitrite), which was less harmful (Besson-Bard et al. 2008). Kaur et al. (2006) reported that pomegranate extract swept O2- anion up to 53%.

The increase seen in our study might be related to pomegranate's lowering effect on the level of O2-.
Several studies have investigated the effects of pomegranate juice and pomegranate extracts in various polarities on the levels of antioxidant parameters in the serum and tissues. However, the results obtained from these studies are not consistent (Osman et al. 2011, Celik et al. 2009, Ashoush et al. 2013, Moneim 2012; Faria et al. 2007, Yüce and Aksakal 2007, Türk et al. 2008). In our study, no statistically significant changes were determined in the levels of MDA, GSH, and AoA in Group 1 and Group 2. This change might be related to pomegranate juice, whole pomegranate extract, or pomegranate extracts in various polarities and different preparation methods (Osman et al. 2011, Celik et al. 2009, Ashoush et al. 2013, Moneim 2012, Faria et al. 2007, Yüce and Aksakal 2007, Türk et al. 2008).

CONCLUSION

Consequently, depending on the Group 1 data, it can be stated that the use of lyophilized whole pomegranate extract in various doses has no adverse effects on the general metabolism. In the present study, lyophilized whole pomegranate extract was evaluated using the NASH rabbit model for hepatoprotective activity. The active dose was found to be 50 mg/kg. It was ascertained that the use of pomegranate up to the dose of 100 mg/kg had no significant side effects on the parameters examined in this study.

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RESEARCH ARTICLE

The İncidence of Neospora Caninum in Dairy Cows with Abortion and İnfertility Problems in Aksaray Providence

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ABSTRACT

The contribution of animal husbandry to the national economy cannot be ignored. The causes of infertility are various and complex. Economic losses are increasing in dairy cattle breeding due to infectious and non-infectious causes. The percentage of abortion due to infectious agents is not fully known, but infectious agents lie in about 90% of the cases whose etiology can be determined. *Neospora caninum* is considered to be one of the most important abortion factors of cattle. Although, this protozoa is always ignored. It causes abortion in cows. In this study, it was aimed to detect *N. caninum* from the blood serum obtained from 137 dairy cattle brought to Aksaray University Faculty of Veterinary Medicine between 2017-2019 and suffering from abortion and infertility problems. For serological diagnosis, ELISA (Enzyme-Linked ImmunoSorbent Assay) test was performed.
As a result, *N. caninum* agent was detected in 37.22 % (51/137) of the samples that belong to cattle with abortion and infertility problems. It was concluded that economic loss due to infertility in dairy cattle and protozoa, which are one of the infection factors, should be given importance.
Keywords: Abortion, Dairy Cow, Infertility, *Neospora caninum*.

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Aksaray İlinde Abortus ve İnfertilite Problemi Olan Süt İneklerinde *Neospora Caninum* İnsidansı

ÖZ

Hayvancılığın ülke ekonomisine katkısı yadsınamaz. İnfertilitenin nedenleri çeşitli ve karmaşıktır. Süt sığırcılığında bulaşıcı ve bulaşıcı olmayan nedenlerle ekonomik kayıplar artmaktadır. Enfeksiyöz ajanlara bağlı abortların yüzdesi tam olarak bilinmemekle birlikte, etiyolojisi belirlenebilen olguların yaklaşık %90'ında enfeksiyöz ajanlar bulunmaktadır. *Neospora caninum* sığırların en önemli abortus faktörlerinden biri olarak kabul edilmektedir. Bununla birlikte, bu protozoa her zaman göz ardı edilir. İneklerde abortusa, köpeklerde ise ölümcül sinir-kas hastalıklarına neden olur. Bu çalışmada, Aksaray Üniversitesi Veteriner Fakültesi'ne 2017-2019 yılları arasında getirilen abort ve infertilite sorunu yaşayan 137 süt sığırından elde edilen kan serumundan *N. caninum'un* saptanması amaçlanmıştır. Serolojik tanı için ELISA (Enzyme-Linked ImmunoSorbent Assay) testi yapıldı. Sonuç olarak abortus ve infertilite sorunu olan sığırlara ait örneklerin %37,22'sinde (51/137) *N. caninum* etkeni tespit edilmiştir. Süt sığırlarında infertiliteye bağlı ekonomik kayıplara ve enfeksiyon faktörlerinden biri olan protozoalara önem verilmesi gerektiği sonucuna varıldı.
Anahtar Kelimeler: Abortus, İnfertilite, *Neospora caninum*, Süt İneği

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INTRODUCTION

The causes of fertility loss are diverse and complex. Problems that cause low productivity and infertility in dairy cattle farms are grouped under four main headings. These are as follows: functional disorders, infectious disorders, congenital-acquired structure anomalies and care-nutrition disorders. The causes of abortion in cows may be classified as non-infectious and infectious factors. Non-infectious causes are; hereditary or chromosomal disorders, nutritional problems, toxic conditions, and hormonal imbalances. Infectious causes are reported as bacterial, fungal and protozoal agents. The percentage of abortion due to infectious agents is not known, but infectious agents underlie approximately 90% of the cases whose etiology can be determined. While bacterial agents such as *Brucella, Campylobacter, Leptospira, Chlamydia, Salmonella, Listeria* cause bacterial infertility and abortions in cows; viruses such as Bovine viral diarrhea virus (BVDV) and protozoa such as *Trichomonas, Neospora, Toxoplasma, Sarcocystis* species cause infertility and abortions (Yoo, 2010). Neospora caninum, which is a protozoa agent, is one of the infectious causes that has been increasing in importance in cow reproduction in recent years. Infestation occurs by ingestion of food contaminated with an infected dog or fox feces containing the eggs (oocysts) of *N. caninum*. This protozoa, causes high abortion rates in cows. Dogs become infected when they consume infected cattle abortion material or placenta. Eggs are then produced in the dog's digestive tract and shed in the feces, remaining stable in the environment for about six or eight months. Eggs can be present in fresh or conserved forages such as grass, silage, hay and any other feeds. *Neospora caninum* is considered as one of the most important abortion factors of cattle. Neosporosis has been recognized one of the most important reproductive diseases in cattle all over the world. This protozoa, which has a widespread transmission feature, 90% of the cows in herds can be infected (Barber and Trees, 1996; Wouda, 2000; Dubey, 2003; Dubey et al., 2007; Dubey and Schares, 2011; Khan et al., 2020).

Whether Neosporosis is a problem for humans is still a question to be answered. Hence infected dogs and cattle may be major risk factors for transmission of the infection to humans especially in farm and slaughterhouse workers. Although the presence of protein structures or specific antibodies of the causative agent has been demonstrated in humans, there is no evidence that it can lead to abortion or other clinical findings. The other questions that are eagerly awaited about Neosporosis are; the reactivation mechanism of the protozoa during pregnancy, abortion mechanism, protozoa immunology, and drug treatment options. (Dubey, 2007; McCann et al., 2008).

Neospora caninum is an important cause of infectious abortion, infertility, early fetal death, stillbirth, decrease in milk yield-reproductive performance, earlier culling, decrease in post-weaning growth and feed efficiency in cattle world-wide (VanLeeuwen et al., 2010). Infection is common. Serological surveys suggesting that from 5-66 % of cows are seropositive, and the protozoa is passed by vertical transmission from cattle to calf with no signs of the disease. There is an increase in abortions world-wide, especially in European countries. In the examinations, it is determined that it is of *Neospora* origin. Seropositivity in dairy cows has been reported as 7-33% as a result of studies conducted in different regions in Turkey, which acts as a bridge between Europe and Asia (Kul et al., 2009; Dubey and Schares, 2011; Yıldız. and Gökpınar, 2017; Erol et al., 2019).

This suggests that the causative agent may cause diseases that cause significant economic losses in dairy cows. *N. caninum* is estimated to cause cattle producers losses exceeding a billion dollars each year world-wide. The annual economic loss in dairy, and beef cattle in Australia is estimated to be 85 million dollars, and 25 million dollars, respectively. In New Zealand, this loss is 17.8 million dollars annually in the dairy industry. In a study conducted in the USA, it was determined that there is a loss of approximately 1 kg per day in milk production in cows carrying *N. caninum* antibodies. In the Netherlands, the loss per farm is estimated at 249 €, on a country basis of 19 million €, and in Switzerland the average annual loss is 9.7 million €. It is estimated that up to 42% of cows abort due to Neospora caninum and the economic damage is directly related to the value of the fetus (Häsler et al., 2006; Dubey et al., 2007).

Several laboratory methods are now available for the detection of *N. caninum* infection in animals. Serologic examinations are proposed to detect *N. caninum* antibodies. Among serologic methods, ELISA technique is adequately reliable in terms of definingspecific antibodies titers to *N. caninum*. Furthermore, it has greater applicability for epidemiologic researchs (Dubey et al., 2007; Guido et al., 2016).

In this study, it was aimed to detect *N. caninum* from the blood serum obtained from 137 dairy cattle brought to Aksaray University Faculty of Veterinary Medicine between 2017-2019 and suffering from abortion and infertility problems. Thus, the presence of Neosporosis which is becoming increasingly important as a cause of abortion in dairy cows in the world, and in Turkey, will be determined in Aksaray. The study was planned with the thought that it would constitute an important source of data for the country's livestock and future studies.

MATERIAL and METHOD

1. Blood Serum Samples

In the study, blood serums obtained from a total of 137 dairy cattle with abortion and infertility problems brought to Aksaray University Veterinary Faculty for diagnosis between 2017 and 2019 were taken for study. Blood serums were centrifuged at 3000 rpm for 5 minutes, and stored. The resulting supernatant was inactivated at 56°C for 30 minutes and stored at -20°C until use.

2. Test Kit and ELISA Reader

A commercially available ELISA assay kit (IDEXX Neospora Ab Test, Cat No. 99-09566) was used in the study. The OD results obtained as a result of the tests performed with the ELISA kit were evaluated with the Biotek ELx800 ELISA reader.

Method

Double ELISA (Enzyme-Linked Immuno Sorbent Assay) test was performed on the samples whose serums were taken for serological examination. Serum samples were applied with the commercial ELISA kit for the presence of IgG against *N. caninum* as specified by the company and the results were obtained.

It was examined with a spectrophotometer at a wavelength of 630 nm. Serum samples causing ≤ 30% inhibition were considered positive. All blood serums were studied in duplicate.

In the study, 2 groups were formed in the light of the information collected from the sick animals and/or samples that came to the veterinary faculty (Tables 1 and 2). Group formation was based on whether the animals gave birth and how many times they gave birth. According to the information in the inventory, it was observed that the animals in group one had repeat breeder complaints. In Group 2, it was determined that there was an abortion problem.

Group 1 (n=35); animals that have never given birth (nulliparous), Group 2 (n=102); defined as the group of animals that primipar, and/or multipar. According to these criteria, a total of 137 blood serum samples formed the first group.

The second group was divided into 4 groups (Lactation I-II-III and IV) according to their lactation status (Table 2). The blood sample numbers in the four groups were 27, 38, 24, and 13, respectively. In this way, homogeneity was tried to be achieved between the groups according to the number of births per animal.

RESULTS

In the results obtained in the study, *N. caninum* agent was detected in 37.22% (51/137) of the total samples. Their distribution is given in tables the below (Tables 3 and 4).

Accordingly, in table 3; No causative agents were detected in group 1. In group 2, the causative agent was detected in 50% (51/102). This determination gives us information about the distribution of *N. caninum* in the herd. There is a direct ratio between the increasing age of the animal and the contamination.

In table 4, according to the lactation status (I, II, III, and IV, respectively) of the agent detected from the samples in group 2, 7.41% (n=2/27); 60.53% (n=23/38); 70.83% (n=17/24) and 69.03% (n=9/13) were detected.

Table 1. Animal groups.

Groups	Maternal status	n
1	Nullipar	35
2	Primipar and Multipar	102

Table 2. Distribution of animals in group 2 according to their lactation status.

Lactation status	n
I	27
II	38
III	24
IV and more	13

Table 3. Distribution of sera with antibodies to *N. caninum* by groups.

Groups	*N. caninum*
1	0 % (0/35)
2	50 % (51/102)

Table 4. Distribution of the presence of antibodies detected in animals in Group 2.

Lactation status	n
I	7.41 % (2/27)
II	60.53 % (23/38)
III	70.83 % (17/24)
IV and more	69.03 % (9/13)

DISCUSSION

Neospora caninum has been recognized world-wide, first all as a disease affecting only dogs, then as an important cause of infection abortions in cattle. It is a protozoa that was first recognized as a cause of abortion in infected cows (Barber and Trees, 1996; Dubey, 2003).

Neosporosis is responsible for abortion and neonatal deaths in cattle (Barber and Trees, 1996; Dubey, 2003; Ocal et al., 2014). Therefore; this suggests that the causative agent may cause diseases that cause significant economic losses in dairy cows. *N. caninum* is estimated to cause cattle producers world-wide losses exceeding a billion dollars each year (Reichel et al., 2013). The increase in abortions originating from *Neospora caninum* draws attention in European countries and of course in Turkey, which acts as a bridge between Europe and Asia (Akca et al., 2005; Vural et al., 2006; Ica et al., 2006; Kul et al., 2009; Kurtdede and Ural, 2009;Ocal et al., 2014).

Seropositivity in dairy cows in our country varies between 7% and 33%. In *N. caninum* screening studies conducted in cattle from different regions in Turkey: 5.5% in Kırıkkale region (n: 200; Yıldız and Gokpınar, 2017), 7.2% in Kars region (n: 1100; Mor and Akca, 2012), 5-33% in Central Anatolia region, 21.03% in Afyonkarahisar region (n:485; Celik et al., 2013), and 23.4% in Kırşehir Çiçekdağı region (n:116; Yıldız et al., 2017) are reported as seropositive. Some researchers have detected a seropositivity rate of 32.35% (n:986) in the field surveys they have conducted in different provinces (Erol et al., 2019). Acıcı et al., (2019) reported that they detected 49.9% *N.caninum* DNA in their study on waste fetuses. Although 37.2% (51/137) seropositive results detected serologically in our study are higher than in some regions mentioned above, it is consistent with the rate found in studies conducted especially in Turkey.

The gradual increase in Neosporiasis in Turkey is also noteworthy. In our study, *N. caninum* was detected in Aksaray province. The presence of the agent was found to be 50% (n:51/102) in group 2 in aborted dairy cows. In this study, it was concluded that the high seropositive rate was due to the detection of the agent on the blood serums brought with the complaint of abortion. Since other reported studies are in the form of field scanning, it is seen that the seropositivity does not exceed 30% (Celik et al., 2013; Erol et al., 2019).

As a result, the presence of *N. caninum* in dairy cows was detected in Aksaray province. It was concluded that the protozoa should be found, and the studies should be more comprehensive, and concentrated throughout the province. It was concluded that animal owners and veterinarians should be informed about this infection and the study should be expanded more comprehensively. We believe that these results will be a source for future studies. It was concluded that *N. caninum* infection may also be responsible for abortion and economic losses in dairy farms in Aksaray province.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical Permisson: This study was made from blood samples brought to Aksaray University Veterinary Faculty Virology Department and is not subject to HADYEK's permission in accordance with Article 8 (k) of the Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees. In addition, the authors declared that they comply with the Research and Publication Ethics.

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CASE REPORT

Treatment of Rectocutaneous Fistulae Caused by Bite Wound in a Cat

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ABSTRACT

Recto-cutaneous fistulas, which are common in humans, rarely occur in companion animals. In this case report, we aimed to provide information to our colleagues about the diagnosis and treatment of recto-cutaneous fistula in a cat. A one and a half months old British shorthair kitten, weighing 600 g, was brought to Afyon Kocatepe University Veterinary Health Application and Research Center Surgery Clinic by its owner because of an open wound on the side of the tail for a week, and feces coming from this wound. Recto-cutaneous fistula was diagnosed on clinical examination. Under general anesthesia, the defect was sutured with simple pattern. Since the skin sutures were opened, the wound was treated with rifamiycine, nitrofurazone ointment and bephanthene cream after irrigation with rivanol %0.1. In conclusion, recto-cutaneous fistula, which is the most important complication of rectal perforation for any reason, is rarely seen in cats and dogs. Accurate diagnosis and treatment are important for rapid recovery.

Keywords: Cat, bite wound, recto-cutaneous fistula

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Bir Kedide Isırık Yarası Kaynaklı Rektokutanöz Fistül Tedavisi

ÖZ

İnsanlarda sıklıkla görülen rektokutanöz fistüller, evcil hayvanlarda nadiren ortaya çıkar. Bu olgu sunumunda, yavru bir kedide karşılaşın rektokutanöz fistül olgusunun tanı ve tedavisi hakkında klinisyen meslektaşlarımıza bilgi vermek amaçlanmıştır. Biritish shorthair ırkı 1,5 aylık 600 gr ağrılığındaki bir kedi, kuyruğunun yanında içinden dışkı gelen bir yara şikayetiyle Afyon Kocatepe Üniversitesi Veteriner Sağlık Uygulama ve Araştırma Merkezine getirildi. Klinik muayene yapılarak rektokunatöz fistül tanısı konuldu. Genel anestezi altında rektumdaki defekt belirlendi ve basit ayrı dikiş uygulaması yapılarak kapatıldı. Deri dikişleri açıldığı için %0,1'lik rivanol ile irrigasyon sağlandıktan sonra rifamisin ampul, furasin pomad ve bepanten krem ile yara tedavisi yapıldı. Sonuç olarak, herhangi bir sebeple oluşan rektum perforasyonunun en önemli komplikasyonu olan rektokutanöz fistül kedi ve köpeklerde nadiren görülen bir durumdur. Doğru teşhis ve tedavi iyileşmenin hızlı şekillenmesinde önemlidir.

Anahtar kelimeler: Kedi, ısırık yarası, rektokutanöz fistül

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INTRODUCTION

A fistula is defined as an abnormal connection in the form of a canal between two epithelial-layered surfaces. Gastrointestinal fistulas can be congenital or acquired, and they can form between the intestine and another organ or skin (Pickhardt et al. 2002).

Perirectal abscesses and fistulas are common in humans, but similar conditions are rarely seen in animals (Łojszczyk-Szczepaniak et al. 2014). Enterocutaneous fistulas are pathological tunnels between the intestines and the skin, usually occur in large animals due to complications of umbilical hernia or their treatment (Byeon et al. 2008). Enterocutaneous fistulas are rarely encountered pathologies in small animals (Tsioli et al. 2009).

Rectal perforations and recto-cutaneous fistulas can be difficult to treat, sometimes despite early diagnosis and treatment, as they are prone to complications such as dehiscence, infection, and delayed healing. In dogs and cats, rectal perforation has been described most often in association with pelvic fractures. Overall, rectal tears are very rare (<1%) in cases of pelvic trauma, and urethral or bladder perforation is more common in dogs with pelvic fractures. Other causes of rectal perforation include penetrating bite wounds, perineal herniorrhaphy or anal sacculectomy, trauma from swallowed foreign bodies or intraluminal foreign bodies entering through the anus, iatrogenic tears during rectal examination, misuse of enema tubes, gunshot injuries, abscesses at the base of the tail, and rectal perforation. stab wounds can be counted (Kılıç and Yaygıngül, 2010). Recto-cutaneous fistula is a potential complication of any rectal perforation, including perianal surgery. Most rectal perforations described in dogs and cats occur in the most caudal 4 cm of the rectum (Muir 1998; Byeon et al. 2008; Frasson 2008; Łojszczyk-Szczepaniak et al. 2014).

Its clinical manifestations are drainage of feces from the fistula through the perianal defect and pain and swelling at the mouth of the fistula due to an inflammatory reaction. Diagnosis of recto-cutaneous fistula in dogs and cats is easily made by drainage of feces and detection of fecal contamination of the area (Frasson 2008).

In this case report, we aimed to provide information to our colleagues about the diagnosis and treatment of recto-cutaneous fistula in a cat.

CASE HISTORY

A one and a half months old British shorthair kitten, weighing 600 g, was brought to Afyon Kocatepe University Veterinary Health Application and Research Center Surgery Clinic by its owner because of an open wound on the side of the tail for a week, and feces coming from this wound. In the anamnesis taken from the patient's owner, it was learned that he had been taken to another clinic before and the wound was treated, but the healing did not occur.

As a result of the physical examination, an infected wound was seen on the craniolateral part of the anus. A thermometer was advanced from the anus and perforation of the rectum was determined and a diagnosis of recto-cutaneous fistula was made. In the present case, it was understood that the recto-cutaneous fistula was formed as a result of abscess due to the bite wound. The animal was anesthetized with medetomidine HCl (40 mcg/kg, IM, Domitor, Zoetis) and ketamine HCl (5 mg/kg, IM, Ketasol, Richer pharma), after providing asepsis-antisepsis, the defect in the rectum was determined and a simple separate sutured with polydioxanone (PDS, 4/ 0), and an appropriately sized tube lubricated with vaseline was inserted into the rectum (Muir 1998). The skin was closed routinely with non-absorbable suture material propylene (Prolene, 4/0). A penrous drain was placed in the wound. In the postoperative period, cefdinir suspension (10 mg/kg, PO, Cempes, Sanovel) was prescribed twice a day for 7 days. Two days later, the drain and tube in the rectum were removed. Since the skin sutures were opened, the wound was treated with rifamiycine (Rif ampoule 125 mg, Koçak Farma), nitrofurazone ointment (Furacin pomade, Sanofi İlaç) Dexpanthenol (Bephanthen cream, Bayer) after irrigation with ethacridine lactate %0.1 (Rivanol toz 1 g, İstanbul İlaç). After 14 days the animal had fully recovered (Fig. 1).

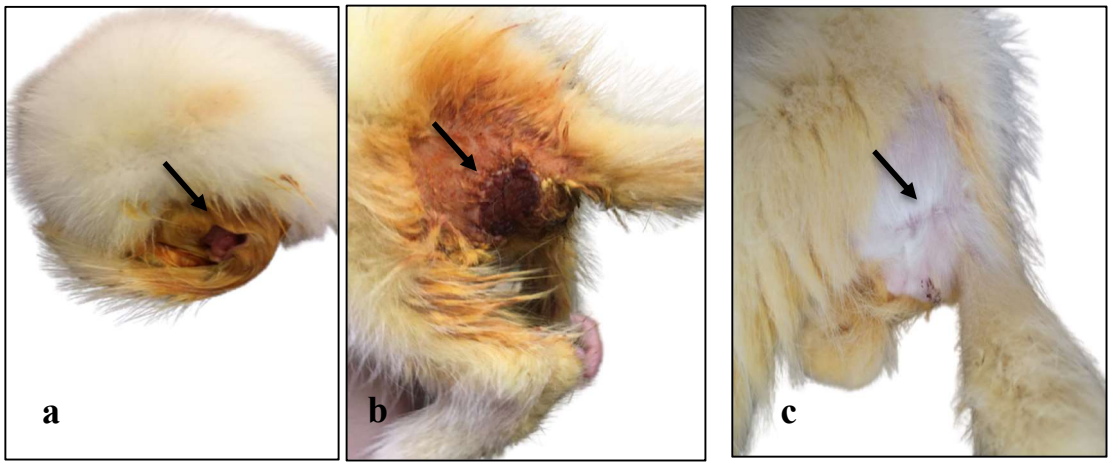

Figure 1: a. Rectocutaneous fistula (black arrow), **b.** Clinical appearance of the wound on Postoperative 7th day (black arrow), **c.** Complete recovery of the wound on Postoperative 14th day (black arrow).
Resim 1: a. Rektokutanöz fistül (siyah ok), **b.** Postoperatif 7. gündeki yaranın klinik görünümü (siyah ok), **c.** Postoperatif 14. gündeki tam iyileşme (siyah ok).

DISCUSSION and CONCLUSSION

Rectal perforations in cats and dogs usually occur as a result of fractures in the pelvis (Tsioli et al. 2009). Recto-cutaneous fistulas are one of the rare pathologies in cats (Kılıç and Yayıngül 2010).

In this case, the recto-cutaneous fistula in the kitten was formed as a result of abscess caused by the bite wound. Bite wounds are involved in the etiology of recto-cutaneous fistula and are consistent with literature data (Muir 1998).

Diagnosis is quite easy with stool draining from the fistula through the perineal defect and clinical signs such as pain and swelling at the mouth of the fistula. In the present case, feces were seen coming from the defect located on the craniolateral side of the anus and a diagnosis of recto-cutaneous fistula was made (Frasson 2008).

Kılıç and Yayıngül (2010) conservatively treated a recto-cutaneous fistula associated with anal sac surgery in a cat. In our case, the defect in the rectum was repaired with operative intervention and the skin was closed routinely. Two days later, the skin wound was closed with second degree healing as the stitches on the skin were opened. There was no complication related to the fistula in the control performed three months later.

In conclusion, recto-cutaneous fistula, which is the most important complication of rectal perforation for any reason, is rarely seen in cats and dogs. Accurate diagnosis and treatment are important for rapid recovery.

Conflict of Interest: The authors declared that there is no conflict of interest.

Ethical Approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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ERRATUM

A Retrospective Study on Wild Orphan Animals in Afyon Kocatepe University Wildlife Rescue Rehabilitation, Training, Practice and Research Center (AKUREM)

Afyon Kocatepe Üniversitesi Yaban Hayatı Kurtarma Rehabilitasyon Eğitim Uygulama ve Araştırma Merkezindeki (AKUREM) Yabani Öksüz Yavrular Üzerine Bir Retrospektif Araştırma

Emine Hesna KANDIR*, Tuğrul Çağrı GÜL

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