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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Effect of Propolis Applied to Goat Kids at Weaning Period on Heat Shock Protein Genes

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Abstract: In recent years, studies on the use of natural and organic additives have gained importance in goat breeding in order to prevent offspring losses and to encourage their growth and development by limiting the use of antibiotics. Especially the weaning period is a stressful period for kids and negative effects such as weight loss, increased susceptibility to viral and bacterial infections may be observed as a result of decreased nutrient intake and utilisation during this period. Considering these disadvantages that occur during the weaning period, it was thought that propolis would increase the potential to protect the health and welfare of kids during the weaning period due to its immunomodulatory, antioxidant, and anti-inflammatory effects. Therefore, we aimed to examine the expression levels of HSP27, HSP60, and HSP70 molecular chaperones that modulate the cellular stress response, which partially express the effects of propolis on weaning stress. Saanen kids were divided into propolis treated (n=10) and control (no propolis treatment; n=10) groups. The propolis-treated group received 0.4 cc propolis once a day for two weeks after weaning. Expression levels were calculated by $2^{-\Delta\Delta Ct}$ using the Pfaffl method and statistical significance levels were determined by Student t test. Blood samples were taken on the day of weaning and the following day to determine the effect of weaning stress on HSP27, HSP60, and HSP70 expression levels. The effect of propolis on weaning stress was examined in samples taken after two weeks of propolis treatment. The expression levels of HSP27 and HSP60 increased by approximately 2-fold during weaning stress, while HSP70 increased by 3.35-fold. When 0.4 cc propolis was applied to kids under weaning stress, a statistically significant downregulation of HSP27 level 1.08-fold, HSP60 level 1.56-fold, and HSP70 level 2.12-fold was obtained at the end of 2 weeks compared to the control group. Our study showed that propolis treatment decreased stress protein levels during weaning stress.

Keywords: *Goat kid, Heat shock proteins, Propolis, Weaning stress.*

Sütten Kesim Dönemindeki Oğlaklara Uygulanan Propolisin Isı Şok Protein Genlerine Etkisi

Özet: Son yıllarda keçi yetiştiriciliğinde, yavru kayıplarını önlemek ve antibiyotik kullanımını sınırlandırarak büyüme ve gelişmelerini teşvik etmek amacıyla doğal ve organik katkı maddeleri kullanımına yönelik çalışmalar önem kazanmıştır. Özellikle sütten kesme dönemi oğlaklar için stresli bir dönem olup, bu süreçte besin alımının ve yararlanımının azalması sonucu kilo kaybı, viral ve bakteriyel enfeksiyonlara karşı artan duyarlılık gibi olumsuz etkiler gözlemlenebilir. Sütten kesim döneminde ortaya çıkan bu dezavantajlar düşünüldüğünde propolisin immünmodülatör, antioksidan ve antiinflamatuvar etkilerinden dolayı sütten kesim döneminde oğlakların sağlığını koruma ve refahını arttıracağı düşünülmüştür. Bu nedenle çalışmada propolisin sütten kesme stresi üzerindeki etkilerini kısmen ifade eden, hücresel stres tepkisini modüle eden HSP27, HSP60 ve HSP70 moleküler şaperonların ekspresyon seviyelerini incelemeyi amaçladık. Saanen oğlakları propolis uygulanan (n=10) ve kontrol (propolis uygulanmayan; n=10) gruplarına ayrıldı. Propolis uygulanan gruba sütten kesildikten sonra 2 hafta boyunca günde bir kez 0,4 cc propolis verildi. Ekspresyon seviyeleri Pfaffl yöntemi kullanılarak $2^{-\Delta\Delta Ct}$ ile hesaplandı ve student t testi ile istatistiksel önem düzeyleri belirlendi. Sütten kesme stresinin HSP27, HSP60 ve HSP70 ekspresyon seviyeleri üzerindeki etkisini belirlemek için sütten kesim günü ve ertesi gün kan örnekleri alındı. Propolisin sütten kesme stresi üzerindeki etkisi, 2 haftalık propolis uygulamasından sonra alınan örneklerde incelendi. Oğlaklarda sütten kesme stresi sırasında HSP27 ve HSP60'in ekspresyon seviyeleri yaklaşık 2 kat artarken, HSP70 3,35 kat artmıştır. Sütten kesme stresi altındaki oğlaklara 0,4 cc propolis uygulandığında 2 haftanın sonunda kontrol grubuna göre HSP27 seviyesinde 1,08 kat, HSP60 seviyesinde 1,56 kat, HSP70 seviyesinde ise 2,12 kat istatistiksel olarak anlamlı downregülasyon elde edildi. Çalışmamız propolis uygulamasının sütten kesme stresi sırasında stres protein düzeylerini azalttığını gösterdi.

Anahtar Kelimeler: *Isı şok proteinler, Oğlak, Propolis, Sütten kesim stresi.*

Introduction

The economic success of goat breeding enterprises depends on reducing production costs and minimizing kid losses during birth and the neonatal period. Additionally, increasing the number of kids that reach a healthy and marketable age after weaning is also crucial. In dairy goat breeding, early weaning of goat kids is preferred to increase milk yield, reduce laborand feed costs, and promote functional reticulon-rumen development. However, this process is an imperative and a potential welfare concern. Weaning is a very stressful time involving adaptation to a new diet (Chauhan et al., 2019; Datt et al., 2023).

Stress leads to a decrease in feed utilisation or a negative effect on the efficiency of the gastrointestinal mucosa. By affecting growth rate, several other health problems may occur, such as developmental retardation, reduced weight gain, suppression of the immune system, or reduced function (Durosaro et al., 2023; Khan et al., 2016).

In response to stress, the unfolded protein response of the endoplasmic reticulum and mitochondria, cytosolic heat shock, hypoxic stress, and oxidative stress response occur. Following the response, defense pathways are activated, which initiate the activation of effector mechanisms that protect the animal from stress and repair the damage caused by stress. In this process, there is a decrease in protein translation levels and an increase in protein folding (Durosaro et al., 2023; Sala et al., 2017). Molecular chaperones are a family of proteins that facilitate and regulate the correct folding of proteins (Mogk et al., 2002). These molecular chaperones are present at normal levels in all eukaryotic and prokaryotic cells while maintaining normal biological activities. However, under many stressful conditions, chaperones are required to correctly fold proteins in the stress response and increase chaperone levels. These proteins are also called "stress proteins" because they increase their activities by protecting against the stress factor (Liberek et al., 2008; Öztürk et al., 2009).

HSP70 is particularly important in modulating and signalling the stress response within the HSP protein family (Korte et al., 2007). HSP70 increases cell tolerance to stressors, resists apoptosis, and reduces cell peroxidation and inflammatory damage (Ludwig et al., 1999). HSP60 is another important molecular chaperone that is encoded in the nucleus but expressed in the mitochondria (Grundtman et al., 2011). It has protective effects in many cells, exerting similar effects to HSP70 through different mechanisms (Otaka et al., 2006). Depending on changes in environmental conditions and stress factors, HSP60 synthesis increases, and HSP60 is transported into the cytosol and then appears on the cell surface where it acts as a "danger signal" for innate and acquired immunity (Choi et al., 2008; Grundtman et al., 2011). Recent studies have reported an association between extracellular HSP60 and the tissues immune responses and that it is upregulated in the inflammatory response (Grundtman et al., 2011; Liyanagamage and Martinus, 2020). HSP27 enhances antioxidant defenses by neutralizing the toxic effects of oxidized proteins in the cell and reducing the number of free radicals (Rogalla et al., 1999). Therefore, HSP27 levels increase when cells are exposed to oxidative stress (Mehlen et al., 1995). As a result, HSPs are one factor that maintains the balance between survival and an effective immune system in the organism during stress (Dangi et al., 2014). Many natural products such as purple garlic powder, thyme essential oil (Serrano-Jara et al., 2023), piperine (Satitsri et al., 2023) and grape seed meal by-product (Pistol et al., 2023) have been added to livestock diets to support immunity or prevent potential health problems after weaning. In goats, inoculation with rumen fluid (Belanche et al., 2020), palm oil wastewater (Nugroho et al., 2023), and probiotic (Chen et al., 2020) supplementation have been investigated as potential treatment options for immune response, growth performance, oxidative stress, hematological parameters, intestinal health and diarrhea after weaning. In recent years, the use of propolis in the livestock industry has become popular with the restriction of the use of antibiotics and synthetic drugs in this sector. Propolis supplementation was reported to improve growth rate and nutrient digestibility, reduced oxidative stress, and improved antioxidant capacity and immune response under stress conditions (Badawy, 2021; Sarker and Yang, 2010; Shedeed et al., 2019). The use of propolis as a natural alternative to ionophores in ruminants has been proposed by Stradiotti et al. (2004) and Oliveira et al. (2006). The effects of propolis supplementation on antiparasitic (Morsy et al., 2013), antibacterial (Ismael et al., 2019), feed efficiency (Zawadzki et al., 2011), milk yield and quality (Aguiar et al., 2014), in ruminants, have been studied with positive results. Given the positive effects of propolis under stress conditions, it is likely that a similar situation would occur under weaning stress.

The aim of our study was to determine the effect of propolis on changes in the stress system during the weaning period by comparing the gene expression levels of HSP27, HSP60, and HSP70 during the weaning period of goat kids given and not given propolis extract (in ethyl alcohol).

Material and Methods

The experiments were conducted according to the ethical guidelines for laboratory animal research and were approved by the Ethical Committee of Aydın Adnan Menderes University (64583101/2023/10).

Animal Material: The animal material of the study consisted of 20 Saanen goat kids born at the end of March 2023 from goat mothers synchronised in 2022 on a farm in İmamköy Efeler/Aydın. Goat kids rearing feed was used as concentrate feed, hay and alfalfa hay were used as roughage in the ration of weaned kids that weaning on July 2023 (approximately eight weeks old). The Saanen goat kids were divided into two groups: a control group (non-propolis-treated, n=10) and a propolis-treated group (n=10) with an equal ratio of males to females. The goat kids were homogeneously distributed among the groups according to their body weights (control, 10.40 kg ± 1.40; propolis-treated

group, 10.50 kg ± 1.72) and body condition scores (control, 1.84 ± 0.15; propolis-treated group 1.82 ± 0.14).

Study design: In our study, the complete weaning of the goat kids at eight weeks of age was planned by evaluating the literature data (Teh et al., 1984). The propolis group was given 0.4 cc of propolis by syringe once a day for two weeks as a dietary supplement (Manav and Yılmaz, 2023). Propolis extract in ethanol (The contents is given in table 1) was obtained from Idapolis Company (Turkey) within Çanakkale 18 Mart University Technopark.

Table 1. Contents and main constituents of the extract of propolis in ethanol.

Content and main components	Quantity (ppm)
Cumaric acid	19.50
Hydroxybenzoic acid	611.69
Caffeic acid	33.28
Catechin	0.17
Chlorogenic acid	0.35
D-(+) malic acid	71.87
Ellagic acid	18.90
Ferrulic acid	10.54
Gallic acid	2.46
Gentisic acid	0.53
Isorhamnetin	0.74
Isorhamnetin 3-O-glucoside	3.71
Isorhamnetin 3-rutinoside	12.35
Kaempferol	3.94
Myricitrin (Myricetin 3-O-rhamnoside)	1.35
p-hydroxy benzoic acid (4 hydroxy benzoic acid)	278.05
p-cumaric acid	15.36
Protocatechuic acid	4.58
Quercetin (Quercetin 3-O-rhamnoside)	3.63
Quercetin 3-D-xyloside	0.69
Quercetin 3-O-rutinoside hydrate	2.50
Suscinic acid-butanedionic acid	5.97

Goats have a low feed efficiency, a low level of immunity and a high risk of diarrhoea during the weaning period. There are different doses for propolis application in the literature studies, but in our study the 0.4 cc application was preferred due to its effect on both immunity and antidiarrhoeal effect (Manav and Yılmaz, 2023; Sadek et al., 2020).

Collection blood sample: Blood samples were taken from the animals' jugular veins (*vena jugularis*) into EDTA tubes. To determine the effect of weaning stress on HSP levels, goat kids were weaned at 8 weeks of age by blood sampling. Then goat kids were separated from their mothers and blood samples were taken again 24 hours later. After two weeks of propolis treatment, blood was collected from both the propolis-treated group and the control groups. As soon as the samples were collected, they were transported to the laboratory attention to the cold chain.

RNA isolation from blood and cDNA synthesis: Total RNA was isolated from whole blood using a commercial RNA isolation kit (RiboEX, GeneAll, Korea) according to the protocol, and the RNA was obtained RNAs and stored at -80ºC until cDNA synthesis. The concentration of RNA samples was determined at µg/µl level by measuring at 260 nm wavelength in a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher, Finland) and diluted to 1000 ng with DNase/RNase-free water.

RNA samples were converted to cDNA using a cDNA synthesis kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, USA) according to the kit procedure in an Applied Biosystems 2720 Termalcycler (Singapore).

Primer sequences: To determine the effects of propolis applied to goats on heat shock proteins during the weaning process at the gene level, gene expression levels were determined by using primers goat-specific HSP27, HSP60, HSP70, and β-actin genes from the generated cDNAs. Primers for the reference gene β-actin and the reverse primer for the HSP27 gene were designed using the Primer 3 program. The primer sequences for the HSP60 and HSP70 genes were taken from Dangi et al. (2014), and for forward primer HSP27 genes from Tsugami et al. (2023) (Table 2).

Table 2. Primer sequence

Target Gene	Accession number	Primer sequences (5'→3')			Amplicon size
HSP27	XM_018040903.1	F	5'-TCACTCGCAAATACACGCTG-3'	20 bp	142 bp
		R	5'- AAGGTGACGGGAATGGTGAT-3'	20 bp	
HSP60	XM_018061271.1	F	5'-ACTGGCTCCTCATCTCACTC-3'	20 bp	147 bp
		R	5'-TGTTCAATAATCACTGTCCTTCC-3'	23 bp	
HSP70	NM_001285703.1	F	5'-GACGACGGCATCTTCAAG -3'	18 bp	132 bp
		R	5'-GTTCTGGCTGATGTCCTTC-3'	19 bp	
β-actin	NM_001314342.1	F	5'-AGTTCGCCATGGATGATGA-3'	19 bp	54 bp
		R	5'-TGCCGGAGCCGTTGT-3'	15 bp	

Determination of relative gene expression levels: The expression levels of HSP27, HSP60, and HSP70 genes were determined by the qRT-PCR method using the A.B.T.™ 2X qPCR SYBR-Green MasterMix (Turkey) commercial kit in LigthCycler®480 device (Roche, Germany). Each cDNA sample was tested three times, and the cycle threshold (Ct) was determined. The RT-PCR condition was an initial incubation at 95 ºC for 5 minutes, followed by 40 cycles at 95 ºC (15 s) and 60 ºC (30 s). The Ct value obtained for each sample was normalised to β-actin as a reference gene. The results for the target genes in calculating the relative quantification of gene expression were expressed by the formula $2^{-\Delta\Delta Ct}$ using the Pfaffl method (Livak and Schmittgen, 2001).

Statistical Analyses: All statistical analyses and calculations were performed using MS-Excel 2019 and SPSS for Windows Ver. 25.0 (SPSS Inc., Chicago, IL., USA). In statistical decisions, a level of $p<0.05$ was accepted as an indicator of significant difference. Descriptive analyses were performed as mean and standard deviation for all parameters. Continuous variables were assessed for normality using the Shapiro-Wilks test. Comparisons between groups were made using the Student T test for normally distributed data.

Results

The weekly body weight and body condition scores of the goat kids at weaning time and during propolis treatment are summarised in the table (Table 3).

Table 3. Body weight and body condition scores of the goat kids.

		Initial n:10	1. week n: 10	2. week n: 10	*p*
Control group	LW	10.40±1.40[a]	11.43±1.50[b]	11.70±1.60[b]	*
	BCS	1.82±0.14	1.70±0.17	1.86±0.18	IN
Propolis-treated group	LW	10.50±1.72[a]	11.60±1.64[ab]	12.25±1.75[b]	*
	BCS	1.84±0.15	1.75±0.14	1.85±0.16	IN

a, b: Differences between averages with different letters on the same line were statistically (mean ± SE) significant (*p<0.05), IN; insignificant. LW; live weight BCS; body condition score

Effect of weaning stress before propolis application on gene levels: The relative change in HSP27, HSP60 and HSP70 genes due to weaning stress was determined in the 8th week weaning samples. These were taken before weaning and the next day after the kids were weaned. At this stage, propolis application was not started and 20 Saanen kids were included in the calculation in order to increase the number of animals. The levels of expression were determined by the ratio of the data obtained for each HSP protein as a result of the qPCR to that of β-actin. The relative expression levels of each gene were calculated using the Pfaffl method ($2^{-\Delta\Delta Ct}$), with the expression levels of the pre-weaning samples assumed to be 1 (Table 4).

Table 4. Expression levels of HSP proteins in samples taken before and after weaning ($2^{\Delta Ct}$), (ΔCt= Ct (HSP gene) – Ct (β-actin)).

	HSP proteins expression levels			HSP proteins relative expression		
	HSP27	**HSP60**	**HSP70**	**HSP27**	**HSP60**	**HSP70**
Pre-weaning	1,32E-03	2,13E-03	5,63E-02	1	1	1
Post-weaning	2,35E-03	4,95E-03	1,89E-01	1.77	2.32	3.35

The relative expression level of HSP70 protein was detected 3.35-fold up-regulated after weaning 24 h compared to before weaning. In the same sample, the expression levels of HSP27 and HSP60 significantly increased in the 1.77 and 2.32-fold up-regulation, respectively (Figure 1).

To determine the effect of propolis on the weaning stress end of the application, the changes of HSP27, HSP60 and HSP70 genes were examined after weaning compared to the control group. Relative gene expression levels in the samples taken were calculated with Pfaffl method ($2^{-\Delta\Delta Ct}$) accept to expression levels of the control group without propolis application as 1. According to our results, a 1.08-fold significant down-regulation was observed at the HSP27 expression level. At HSP60 and HSP70 expression levels, a 1.56and 2.13 fold downregulation was observed with propolis application, respectively (Figure 2).

In addition, it is thought that changes in the expression levels of HSP proteins may occur during the weaning process of the control group. Therefore, HSP protein levels were compared in samples taken from the control group after weaning and in samples taken two weeks later. The relative gene expression level of HSP70 showed a 1.03-fold change, while the relative expression levels of HSP27 and HSP60 genes were 1.07 and 1.08, respectively. When the samples from the group allocated for propolis treatment after weaning and at the end of propolis treatment were compared, 2.20-fold down-regulation was detected in HSP70 level and 1.16 and 1.69 changes in HSP27 and HSP60 genes, respectively.

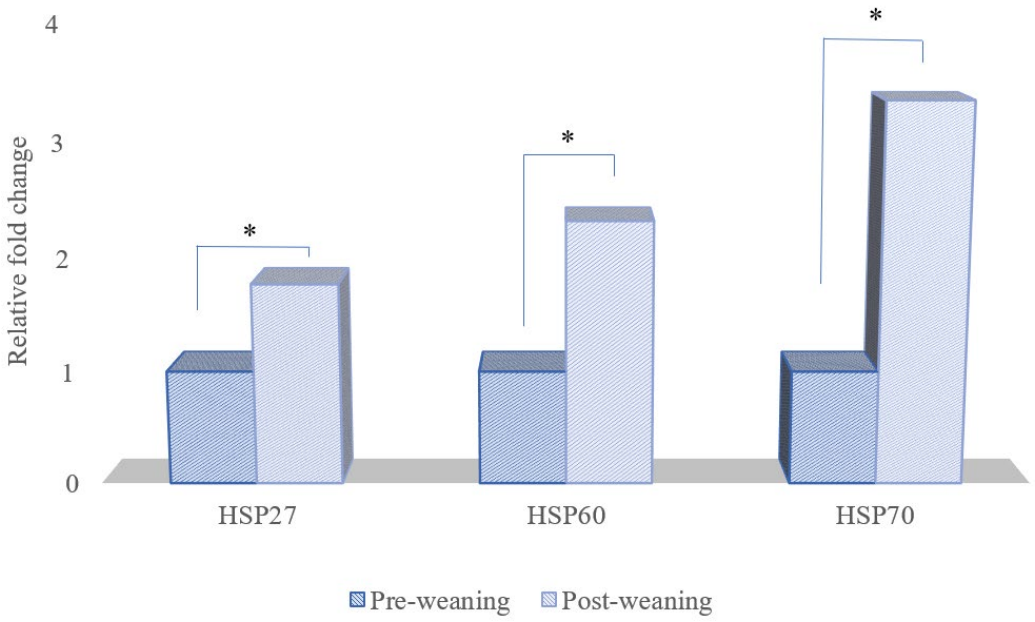

Figure 1. Effect weaning stres on HSP27, HSP60 and HSP70 genes levels (*p <0.001; n=20).

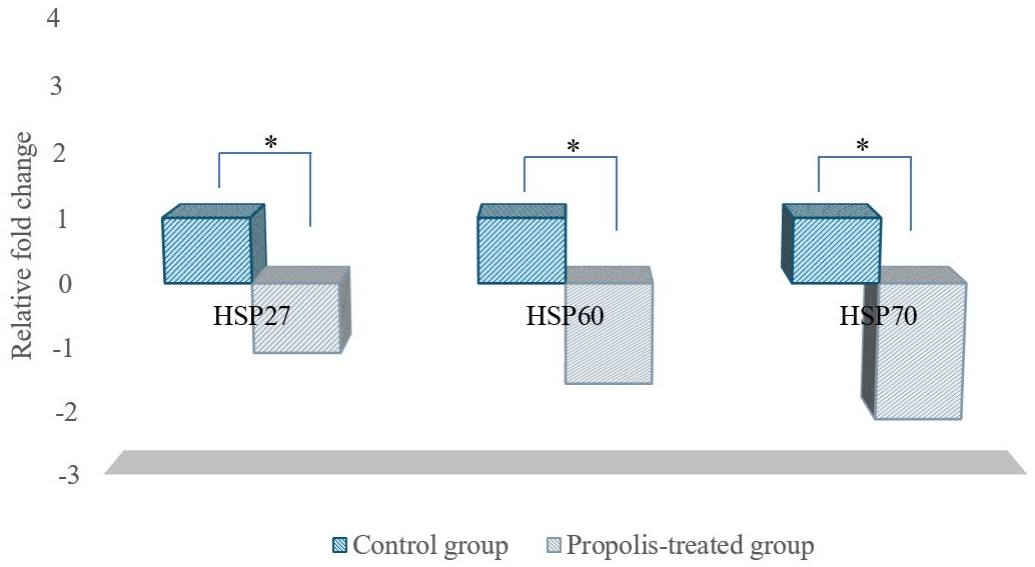

Figure 2. The effect of propolis supplementation on HSP27, HSP60, HSP70 levels in goat kids under weaning stress (*p <0.001; n=10).

Discussion and Conclusion

In goat husbandry, many factors in the breeding process affect animal health and create conditions that can cause stress. It is complicated for animals to adapt to changes in feed intake, diet form and composition, nutrient digestibility, and social and physical environment during the weaning process (Degroote et al., 2020). During the weaning process, the care needs of the kid increase. Weaning is another critical stage in ruminant production, where losses increase after the neonatal period (Baldwin et al., 2004; Khan et al., 2016). Therefore, some measures taken between these periods will prevent offspring losses and contribute significantly to the offspring's quality of life and welfare (Datt et al., 2023). In recent years, studies to minimize the disadvantages of weaning practices in different species have attracted great interest (Cox and Cooper, 2001; Jeppesen et al., 2000). Many studies have addressed problems such as weight loss, decreased feed conversion, and susceptibility to viral and bacterial infections during weaning (Baldwin et al., 2004; Belanche et al., 2020; Chen et al., 2020). However, studies determining the changes that may occur in heat shock proteins due to weaning stress are limited.

Under normal conditions, HSP acts as a chaperone molecule, is increased expressed under stress conditions, and plays a role in maintaining cell homeostasis (Kresnoadi et al., 2020). It is known that HSP expressions are affected by endogenous physiological factors (Ehrenfried et al., 1995) and environmental factors (Yılmaz et al., 2018). In addition, changes in HSP expression have been determined in situations such as exercise, weaning, transport, high temperature, and exposure to toxins (Hussain et al., 2021). It has been reported that HSP expressions change in the gastro-intestinal system with weaning and expression may be affected depending on the stage after weaning and changes in the gastrointestinal system (Arvans et al., 2005). Apart from the gastrointestinal tract, changes in HSP expression levels have been detected in various tissues, such as the myocardium, kidney, and longissimus dorsi muscle (Li et al., 2018). There is no literature on the changes in HSP proteins in circulation during weaning in ruminants. Therefore, in our study, we planned to take samples before and 24 hours after weaning to determine the effect of weaning stress on the expression of HSP proteins. When the studies conducted in ruminates were examined, Although the intensity of weaning stress varies between species, it has been reported that both behavioral and physiological stress is more pronounced in the first 24-48 hours following an abruptly weaned (Lynch et al., 2019; Kazemi et al., 2023; Vickery et al., 2023). Especially the first 24 hours were considered because cortisol levels affect the expression of HSPs (LeBlanc et al., 2012).

There was no difference between the groups in the goat kids whose weekly live weights were measured during the study period. When the literature information was evaluated, Abd-Allah and Daghash (2019) reported that the weaning weights of calves fed with 50 mg propolis / head / day in addition to the ration were 7.7 kg more than the control group. Cécere et al. (2021) added 150 µl/day propolis to the milk of lambs for 42 days and reported a difference of approximately 3 kg in live weight. The reason for the increase in live weight in our study is that our application period may have been short in comparison to other studies.

In our results, an increase in the level of HSP27, HSP60, and HSP70 was observed. Li et al. (2018) investigated the effect of weaning age on HSP proteins in piglets and determined HSP27 level was unaffected by weaning stress, while the HSP70 level increased. High phenolic content additives inhibit HSP27 and HSP70 (Roussou et al., 2004). Since propolis has a high phenolic content, it is thought that HSP27 and HSP70 may determine its effects during the weaning process. However, Cécere et al. (2021) and Shedeed et al. (2019) proved that propolis application reduced serum ROS levels and increased antioxidant levels according to a study in sheep. It is thought that propolis plays a role in balancing the oxidative stress caused by stress in this period and causes a positive effect on stress proteins.

Weaning stress causes an increase in inflammatory molecules and affects intestinal health. Gut microbiota changes, intestinal dysfunction occurs, antioxidant system is inhibited (Hussain et al., 2021). Studies report that polyphenols positively affect the changes at the molecular level mediated by the intestinal microbiota under the influence of weaning stress (Moreno-Indias et al., 2016; Mosele et al., 2015; Selma et al., 2009;). Many studies have documented successful feeding strategies with weaning stress and polyphenol supplementation in pigs (Fiesel et al., 2014). Unfortunately, there is no such extensive literature on buffalo, cattle, goats and sheep. Growth and development parameters and immunoglobulins were evaluated as immune responses in calves treated with propolis during weaning (Sarker and Yang, 2010).

Our results show that propolis, which has been used in human health for centuries due to its strengthening the immune system, accelerating the healing and regeneration of tissues, antioxidant and anti-inflammatory effects, is suitable for animal husbandry. Many studies have investigated its use in treating diarrhoea, as an alternative to antibiotics, and its effects on growth and development parameters. The effect of propolis during the weaning process, which is one of the critical growth periods of goat kids, has not been investigated. In addition, the results of our study indicate the importance of weaning stress and possible dietary interventions with polyphenols to improve offspring growth and production in ruminants.

Although we do not know which molecular pathway the application of propolis, rich in polyphenols, affects the level of HSP proteins, we can say that it can minimize the effects of weaning stress with the decrease in HSP levels. Supplementation of feed with propolis is known to reduce HSP70 levels in animals. A similar effect was reported in our study to be effective in weaning stress. However, further studies are needed to define the effect of propolis application on the rumen microbiome, its interaction with different diets and its long-term effects on animal productivity during weaning.

Conclusion

During the weaning period, some disadvantages may occur on the immune system and general health status of kids. During this period, the immune system may weaken due to stress and nutritional changes, disease resistance may decrease and growth performance may be adversely affected. In this context, considering the immunomodulatory, antioxidant and anti-inflammatory effects of propolis, it can be recommended to be used to support the health of kids during the weaning period. Thanks to these effects of propolis, it can be aimed to alleviate the negative effects encountered during the weaning period and to improve the general health of animals. In accordance with the aforementioned data, the administration of propolis during the weaning period has been demonstrated to facilitate the healthy development of goats.

Limitations

In this study, circulating levels of HSPs could not be determined, only changes in gene levels were measured. In addition, the effects of weaning age were not examined to better understand the effect of propolis on weaning stress.

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Conflict of Interest

The authors stated that they did not have any real, potential or perceived conflict of interest.

Ethical Approval

This study was approved by the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee (19.01.2023, 64583101/2023/10 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Strongylidae ile Enfekte Atlarda, İvermektin ve Praziquantele Karşı Antelmentik Direnç Durumunun Tespiti

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Özet: Günümüzde at yetiştiriciliğinde, parazitlerde antelmentik ilaçlara karşı gelişen direncin artması, at sağlığı ve antiparaziter amaçla yapılan tedavilerde ciddi sorunlar oluşturabilmektedir. Bu çalışma, atların sindirim sistemi helmintlerinden olan *Strongylus* spp. etkenlerine karşı, ivermektin ve praziquantelin etki düzeylerinin belirlenmesi ve bu maddelere karşı gelişebilecek bir antelmentik direnç durumunun tespit edilmesi amacıyla yapılmıştır. Şanlıurfa, Mersin ve Adana illerinde, safkan İngiliz atı yetiştiriciliğinin yapıldığı 30 çiftlikten, farklı yaşlarda bulunan 60 attan, dışkı örnekleri alındı ve Strongylidae yumurtaları yönünden McMaster tekniği ile incelendi. Örneklerin 20'sinde (%33,33) Strongylidae etkenlerinin yumurtalarının varlığı belirlendi. Strongylidae ile enfekte oldukları belirlenen atlara, ticari ivermektin ve praziquantel kombinasyonu sırasıyla 0,2 ve 1,5 mg/kg dozlarda oral yoldan uygulandı. Uygulamadan sonraki 14. gün, atlardan ikinci kez dışkı örnekleri alındı ve yapılan tüm analizler tekrarlandı. Kullanılan ilaç kombinasyonunun, Strongylidae etkenlerine karşı %100 oranında etkili olduğu, eş zamanlı olarak yapılan Real-Time PCR analiz sonuçlarının hem tedavi öncesi hem de sonrasında, konvansiyonel analiz sonuçları ile bire bir uyumlu olduğu belirlendi.
Sonuç olarak, safkan İngiliz atlarında belirlenen Strongylidae enfeksiyonlarında, ivermektin ve praziquantel kombinasyonu uygulamasının ardından antelmentik direnç durumu kaydedilmedi. Bununla birlikte, antelmentik ilaç direncinin oluşma olasılığı göz önünde bulundurularak, antiparaziter ilaç kullanımı konusunda daha dikkatli olunması gerektiği düşünülmektedir.
***Anahtar Kelimeler*:** *Antelmentik direnç, At, Real Time PCR, Strongylidae, Strongylus spp.*

Determination of Anthelmintic Resistance to Ivermectin and Praziquantel in Strongylidae Infected Horses

Abstract: The growing resistance of parasites to anthelmintic drugs in horse breeding has the potential to cause significant challenges in horse health and antiparasitic treatments. The objective of this study was to ascertain the efficacy of ivermectin and praziquantel against *Strongylus* spp., which are digestive system helminths of horses, and to determine the potential for anthelminthic resistance to develop against these agents. Fecal samples were collected from 60 horses of varying ages from 30 thoroughbred British horse breeding farms in Şanlıurfa, Mersin, and Adana provinces. The samples were then examined using the McMaster technique for Strongylidae eggs. The presence of Strongylidae eggs was identified in 20 samples (33.33%). Horses infected with Strongylidae were treated orally with a combination of commercial ivermectin and praziquantel at 0.2 and 1.5 mg/kg, respectively. On the 14th day following administration, fecal samples were collected from the horses for a second time, and all analyses were repeated. It was determined that the drug combination used was 100% effective against Strongylidae agents. Furthermore, the results of the Real-Time PCR analysis, which was conducted simultaneously with the conventional analysis, both before and after treatment, demonstrated the same efficacy.
In conclusion, no anthelmintic resistance was observed in Strongylidae infections in thoroughbred British horses following the combination of ivermectin and praziquantel. However, in light of the potential for anthelmintic drug resistance, greater caution should be exercised in the use of antiparasitic drugs.
***Keywords*:** *Anthelmintic resistance, Horse, Real-Time PCR, Strongylidae, Strongylus spp.*

Giriş

Atlarda helmint enfeksiyonlarının tedavisinde kullanılan ilaçlara karşı parazitlerde antelmentik direnç oluşumunun meydana gelmesi; at popülasyonu, yetiştiriciliği ve sağlığı için ciddi bir tehdit durumu oluşturabilmektedir. Yarış amaçlı yetiştirilen atlarda paraziter enfeksiyonlar bu hayvanların gelişimlerini ve performanslarını etkileyebilmektedir (Seyoum ve ark., 2017). Gastrointestinal sistem parazitlerinin çoğunluğu, Cyathostomes ya da small Strongylidaes adı verilen bir grup nematoddan oluşur (Ionita ve ark., 2013). Cyathostominler, atlarda tüm yaş gruplarında görülebilmekte olup, atların bu parazitlerin 15-25 farklı türü ile enfekte olması durumu yaygın olarak görülebilmektedir (Bellaw ve Nielsen, 2020). Bugüne kadar, atların gastrointestinal nematodlarını tür düzeyinde tanımlamak için konvansiyonel ve qPCR (quantitative polymerase chain reaction), RFLP (restriction fragment length polymorphism) ve RLB (reverse line blot) analizleri dahil olmak üzere çeşitli moleküler teknikler kullanılmıştır (Courtot ve ark., 2023; Ghafar ve ark., 2023).

Atlarda antelmentik amaçlı kullanılan ticari preparatların içeriğinde Benzimidazol, Tetrahidropirimidin veya Makrosiklik Lakton (ML) grubu etken maddelerinden biri bulunmaktadır (Matthews, 2014). Bu etken maddelerin ticari olarak kolay bulunması ve nispeten maliyetlerinin düşük olmasından dolayı yoğun bir şekilde tercih edilerek kullanılmaktadır (Janicki, 2016). Yaygın ilaç kulanımı neticesinde, parazit enfestasyonları ile ilgili önemli sağlık sorunları azalmış olmakla birlikte, aşırı ve kontrolsüz ilaç kullanımından dolayı, antelmentik direnç ve bunun gibi birçok olumsuz etkilerinde görülmesine sebep olmuştur. İvermektin etken maddesi ile tedavi edilen atlarda, tedavinin ardından geçen uzun bir süreden sonra, bu hayvanlarda parazit istilası belirtilerinin görülmeye başlaması ve bu belirtilerin devam etmesi ile antelmentik direncin geliştiği tespit edilmiştir (Coles ve ark., 2006). Antelmentiklerin sık ve gelişigüzel kullanımı neticesinde, benzimidazoller, tetrahidropirimidinler ve ML dahil olmak üzere yaygın olarak kullanılan antelmentiklere karşı Ascaridlerde ve Cyathostominlerde antelmintik direnç gelişmesi durumu görülebilmektedir (Macdonald ve ark., 2023; Nielsen, 2022). Son dönemlerde atların gastro intestinal nematod enfeksiyonlarında moksidektin tedavisini takiben Cyathostominlerde gelişen direncin giderek daha fazla sayılarda rapor edildiği bildirilmektedir (Abbas ve ark., 2021; Flores ve ark., 2020; Lignon ve ark., 2021; Martins ve ark., 2021; Nielsen ve ark., 2020). Yakın gelecekte ve günümüzde atlar için antiparaziter tedavide kullanılan antelmentik müstahzarların, direnç gelişmiş olan atlardaki parazitlere karşı etkisiz kalabileceği kaygısı, hayvan sahipleri ve Veteriner Hekimler için bir endişe kaynağıdır. Bu nedenle, mevcut antelmentikler daha stratejik olarak kullanılmalı, yeni larvisitler ve antiparaziter ilaçlar geliştirilmelidir.

Çalışmamızda atlar için en patojen ve önemli sindirim sistemi helmintlerinden birisi olan *Strongylus* spp. parazitlerine karşı, ivermektin ve praziquantelin etkilerinin belirlenerek, bu maddelere karşı parazitlerde bir antelmentik direnç durumunun gelişip gelişmediğinin belirlenmesi amaçlanmıştır.

Materyal ve Metot

Örnekler: Bu çalışma, Temmuz-Ağustos 2019 tarihleri arasındaki dönemde Şanlıurfa, Mersin ve Adana ilinde sportif amaçlı safkan İngiliz at üretimi ve/veya yetiştiriciliği yapılan çiftliklerde yürütülmüştür. Araştırmada, 30 farklı çiftlikteki safkan İngiliz ırkı, yaş aralıkları 1 ile 18 arasında değişen, 35 dişi ve 25 erkek olmak üzere toplam 60 attan, dışkı örnekleri rektumdan taze olarak alındı.

Dışkı muayenesi: Her attan alınan taze dışkı örneği, yabancı maddelerle kontamine olmayacak şekilde ayrı ayrı vida kapaklı kaplar içerisine alınarak numaralandırıldı. Dışkı örnekleri McMaster tekniği ile *Strongylus* spp. etkenlerine ait yumurtalar yönünden incelendi. Her örnek için, gram dışkıdaki parazit yumurtası sayıları (EPG (number of eggs per gram of feces)) belirlenerek kaydedildi (MAFF, 1986). Sayımlarda, yumurta tespiti alt limiti "50" olacak şekilde belirlendi. McMaster tekniğinde negatif olan dışkı örnekleri, doymuş tuzlu su kullanılarak flotasyon yöntemi ile analiz edildi. İlgili parazitin analizi açısından, hiç yumurta görülmeyen ve en az 1 adet yumurta görülen dışkı örneklerinin EPG değerleri sırasıyla; "0", ve "<50" olarak değerlendirildi (MAFF, 1986).

Dışkıda yumurta sayısı azaltma testi (Faecal egg count reduction test (FECRT)) ve Antelmentik etkinin hesaplanması: FECRT analizi antelmentik dirençliliğinin tespiti amacıyla dünyada en yaygın olarak kullanılan metottur. Dünya Veteriner Parazitolojiyi Geliştirme Derneği (World Association for the Advancement of Veterinary Parasitology (WAAVP) tarafından da kullanımı tavsiye edilmektedir. FECRT analizi, dışkı örneğinde, antelmentiğin kullanıldığı gün belirlenen EPG ile tedaviden 10-14 gün sonraki EPG değerlerinin karşılaştırılması esasına dayanmaktadır. Bahsedilen 2 farklı zamanda, belirlenen parazit yumurta sayısındaki azalmanın %95'ten daha az bir oranda olması durumunda, test edilen nematod popülasyonunda, seçilen antelmentiğe karşı, bir antelmentik direncin geliştiği şeklinde kabul edildiği bildirilmektedir (Köse, 2014).

İkinci dönemde yapılan antelmentik uygulamanın etki yüzdesi kontrol grubu olmaksızın, Dışkıda Yumurta Sayısı Azalım Testine (Fecal Egg Count Reduction Test-(FECRT)) göre hesaplandı. Antelmentik direnç etki yüzdesi (%): tedavi öncesi EPG sayısından, tedavi sonrasındaki EPG sayısı çıkartılarak bulunan değer, tedavi öncesi EPG sayısına bölünüp, 100 ile çarpılması formülü ile hesaplandı. Hesaplama ile antelmentik direnç etki yüzdesi ancak %90'ın altında tespit edilir ise antelmentik direnç durumunun geliştiği bildirilmektedir (Çırak ve ark., 2010). İlgili literatürde verilen formül ve hesaplamalara göre değerlendirmeler yapıldı.

Moleküler analizler: Dışkı örnekleri, Strongylidae ile enfekte oldukları belirlenen atlardan, ivermektin ve praziquantel etken maddeleri ile tedavi edilmeden önce (0.

gün), tedavinden sonraki 14. gün ve 28. gün olmak üzere üç farklı zamanda alındı. Alınan bütün dışkı örneklerinden total genomik DNA izolasyonları, ticari bir kit (QIAamp Fast DNA Stool Mini Kit (Cat. No. / ID: 51604 Qiagen, Hilden, Germany)) kullanılarak ve ticari kit protokolü takip edilerek elde edildi (Kaspar ve ark., 2017).

Dışkı örneklerinden elde edilen total genomik DNA izolatları, Real-Time PCR analizleri ile *Strongylus* spp. nematodları yönünden incelendi. Real-Time PCR analizleri için, *Strongylus* spp. etkenlerinin ITS-2 genine spesifik 172 bp.lik bir PCR ürününü amplifiye eden Sv-F (5'-GTA TAC ATT AAA TAG TGT CCC CCA TTC TAG-3')ve Sv-R (5'-GCA AAT ATC ATT AGA TTT GAT TCT TCC G-3') spesifik primer çiftleri, aynı gen bölgesi üzerinde bu primer çiftlerine spesifik olan Sv-Probe (5'-FAM-TGG ATT TAT TCT CAC TAC TTA ATT GTT TCG CGA C-BHQ-3') hidroliz probu kullanılarak gerçekleştirildi. Real Time PCR analizleri, LightCycler 480 (Roche Diagnosctics, Mannheim, Germany) cihazında uygulandı. Mastermix karışımı için genomik DNA izolatlarından 5 µl alındı ve ticari kit protokolü takip edilerek toplam hacim 20 µl olacak şekilde hazırlandı. Real-Time PCR analizlerinde, uygulanan termal döngü ticari kit protokolü (LightCycler 480 Probe master kiti Cat: 04707494001 Roche Diagnosctics, Mannheim, Germany) doğrultusunda, ilk denatürasyon 95 °C'de 10 dakika 1 döngü, akabinde 95 °C'de 15 sn denatürasyon, 60 °C'de 1 dakika (tek nokta ölçüm) okuma olmak üzere 40 döngü olarak uygulandı (Kaspar ve ark., 2017).

Antelmentik uygulamalar: Antelmentik uygulaması öncesi atların ağırlıkları, otomatik tartım aleti veya kilo tahmin şeridi kullanılarak hesaplandı (Çırak ve ark., 2010). Antelmentikler atlara, üretici firmaların prospektüs bilgileri doğrultusunda uygulandı. Tüm atların, verilen ilacı tam olarak alıp almadığı kontrol edildi.

1.Dönem: Bütün atlardan ayrı ayrı dışkı numuneleri alındı ve *Strongylus* spp. yumurtaları yönünden muayene edildi. *Strongylus* spp. pozitif olarak belirlenen örneklerde yumurta sayımları yapıldı. Araştırmanın bu kısmında Strongylidae etkenlerinde ivermektin ve/veya praziquantel direncinin olup olmadığının belirlenmesi amacıyla, *Strongylus* spp. ile enfekte oldukları tespit edilen atlara, ticari bir preparatta (EQUIMAX® Oral Paste, Virbac) bulunan ivermektin ve praziquantel maddeleri sırasıyla 0,2 ve 1,5 mg/kg dozlarında oral yol ile verildi.

***2. Dönem*:** *Strongylus* spp. etkenleri ile enfekte olduğu tespit edilen atlara, ivermektin ve praziquantel tedavisi yapıldıktan sonraki 14. ve 28. günlerde, ilaç uygulaması yapılan bütün atlardan ayrı ayrı dışkı örnekleri alınarak *Strongylus* spp. yumurtaları yönünden incelendi ve EPG sayıları kaydedildi.

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

Bulgular

Birinci aşamada, farklı işletmelerde bulunan İngiliz ırkı, yaşları 1 ile 18 arasında değişen, 35 dişi ve 25 erkek olmak üzere toplam 60 safkan İngiliz attan alınan dışkı örneği, McMaster tekniği ile Strongylidae yumurtaları yönünden incelendi. Örneklerin 20'sinde (%33,33) Strongylidae yumurtaları tespit edildi ve EPG miktarları kaydedildi. Strongylidae yumurtaları görülen 13 dişi ve 7 erkek olmak üzere toplam 20 safkan İngiliz ırkı at, çalışma materyalini oluşturdu. Erkek olan 7 atın 2'sinde *Strongylus* spp. etkenleri ile birlikte *Parascaris equorum* etkenlerinin miks enfeksiyon şeklinde görüldüğü tespit edildi (Tablo 1).

Tablo 1. Atlarda, dışkı örneklerinde ivermektin-praziquantel uygulamasından önce belirlenen analiz sonuçları.

Örnek	Cinsiyet	Yaş	Renk	Etken	EPG	Real Time PCR (*Strongylus* spp.)	Strongylidae enfeksiyonu
1	Erkek	8	Kır	*Strongylus* spp.	6000	Pozitif	Pozitif
2	Erkek	6	Doru	*Strongylus* spp.	100	Pozitif	Pozitif
3	Erkek	6	Doru	*Strongylus* spp.	7000	Pozitif	Pozitif
4	Dişi	11	Al	*Strongylus* spp.	8000	Pozitif	Pozitif
5	Dişi	18	Al	*Strongylus* spp.	100	Pozitif	Pozitif
6	Erkek	3	Doru	*Strongylus* spp.	1700	Pozitif	Pozitif
7	Erkek	2	Al	*Strongylus* spp., *Parascaris equorum*	1500, 400	Pozitif	Pozitif
8	Dişi	3	Al	*Strongylus* spp.	5400	Pozitif	Pozitif
9	Dişi	2	Al	*Strongylus* spp.	5300	Pozitif	Pozitif
10	Erkek	7	Al	*Strongylus* spp., *Parascaris equorum*	2400, 100	Pozitif	Pozitif
11	Dişi	8	Doru	*Strongylus* spp.	200	Pozitif	Pozitif
12	Dişi	11	Doru	*Strongylus* spp.	200	Pozitif	Pozitif
13	Erkek	2	Al	*Strongylus* spp.	400	Pozitif	Pozitif
14	Dişi	18	Doru	*Strongylus* spp.	200	Pozitif	Pozitif
15	Dişi	2	Doru	*Strongylus* spp.	300	Pozitif	Pozitif
16	Dişi	11	Doru	*Strongylus* spp.	200	Pozitif	Pozitif
17	Dişi	12	Al	*Strongylus* spp.	100	Pozitif	Pozitif
18	Dişi	1	Doru	*Strongylus* spp.	100	Pozitif	Pozitif
19	Dişi	1	Doru	*Strongylus* spp.	100	Pozitif	Pozitif
20	Dişi	7	Doru	*Strongylus* spp.	150	Pozitif	Pozitif

EPG: Gram dışkıdaki parazit yumurtası sayısı (number of eggs per gram of feces).

Tablo 2. Atlarda, ivermektin-praziquantel uygulamasından sonraki 14. ve 28. günlerde belirlenen analiz sonuçları.

Örnek	Cinsiyet	Yaş	Renk	Etken		EPG		Real Time PCR (*Strongylus* spp.)		Antelmentik Direnç Durumu
				14. gün	28. gün	14. gün	28. gün	14. gün	28. gün	
1	Erkek	8	Kır	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
2	Erkek	6	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
3	Erkek	6	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
4	Dişi	11	Al	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
5	Dişi	18	Al	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
6	Erkek	3	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
7	Erkek	2	Al	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
8	Dişi	3	Al	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
9	Dişi	2	Al	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
10	Erkek	7	Al	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
11	Dişi	8	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
12	Dişi	11	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
13	Erkek	2	Al	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
14	Dişi	18	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
15	Dişi	2	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
16	Dişi	11	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
17	Dişi	12	Al	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
18	Dişi	1	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
19	Dişi	1	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif
20	Dişi	7	Doru	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif	Negatif

EPG: Gram dışkıdaki parazit yumurtası sayısı (number of eggs per gram of feces).

Enfekte oldukları belirlenen 20 ata, ivermektin ve praziquantel etken maddeleri sırasıyla 0,2 mg/kg ve 1,5 mg/kg dozlarında, bu maddelerin kombinasyonunu içeren ticari preparat ile oral yol ile uygulanmasından sonraki hem 14. hem de 28. günlerde, atların hiçbirinde *Strongylus* spp. yumurtalarına rastlanmadı. Böylece çalışmanın yapıldığı çiftliklerde her iki antelmentik maddenin, *Strongylus* spp. enfeksiyonlarına karşı yüksek derecede etki gösterdiği belirlendi (Tablo 2). *Strongylus* spp. etkenleri yönünden yapılan Real-Time PCR analizi sonuçlarının, hem antelmentik ilaç tedavisi öncesinde (Tablo 1), hem de sonrasında (Tablo 2), konvansiyonel tekniklerdeki sonuçlar ile bire bir uyumlu olduğu belirlendi.

Tartışma ve Sonuç

Yarış amaçlı yetiştirilen atlarda görülen paraziter enfeksiyonlar, atların gelişim ve performanslarını olumsuz yönde etkilemektedir (Seyoum ve ark., 2017). Dünyada ve Türkiye'de yapılan araştırmalara göre; atlarda sıklıkla *Strongylus* spp. türü helmint enfeksiyonlarının görüldüğü ve bu etkenlerin atlarda görülen sindirim sistemi helmintleri arasında en önemli tür olduğu bildirilmiştir (Çırak, 2003; Matthews, 2010).

Strongylidae etkenleri ile enfekte olduğu belirlenen on adet at çiftliğinde, çiftliklerin 7'sinde Benzimidazol dirençli Cyathostomin populasyonlarının tespit edildiği bildirilmiştir (Çırak ve ark., 2004). Strongylidae tip yumurta olduğunu tespit ettikleri 89 at üzerinde febendazolün etkisi araştırılmış, febendazolün, altı ahırın üçünde etkisiz, birinde şüpheli olarak etkisiz ve ikisinde de etkili olduğu belirlenmiş ve fenbendazole dirençli Strongylidae (Cyathostominae) etkenlerinin Litvanya'da yaygın olduğunun doğrulandığı bildirilmiştir (Dauparaitė ve ark., 2022).

Atlarda farklı yetiştirme biçimlerinde, helmintlerin yayılışı ve çeşitliliği üzerine etkisini değerlendirmek amacıyla yapılan bir çalışmada, 50 attan dışkı örnekleri alınarak, Strongylidae türlerinin pozitifliği bakımından incelenmiş ve örneklerin %52'sinde Strongylidae yumurtalarının tespit edildiği bildirilmiştir. Strongylidae yumurtaları pozitif tespit edilen örneklerin %40'ında *Cyathostomum* spp., %10'unda *Poteriostomum* spp., %8'inde *Strongylus vulgaris*, %8'inde *Strongylus equinus*, %4'ünde *Strongylus edentatus* etkenlerinin miks enfeksiyon şeklinde görüldüğü tespit edilmiştir. Aynı ortamlarda bir arada yetiştirilen atlarda Strongylidae ve *Parascaris equorum* yumurtalarına sırasıyla %100 ve %24 oranlarında belirlendiği bildirilmiştir. Bakım besleme koşulları açısından yetiştirme farklılıkları bulunan hayvan gruplarındaki helmint enfeksiyonlarında, gerek yayılış, gerekse enfeksiyon yoğunlukları açısından ciddi oranlarda farklılıklar tespit edildiği bildirilmekte olup, atlarda toplu yetiştirmelerin helmint enfeksiyonlarının yayılışı açısından daha riskli olduğu ve mücadelenin dikkatli yapılması gerektiği vurgulanmıştır (Aypak, 2013).

Strongylidae ve *Parascaris equorum* etkenleri ile enfekte atlarda, ivermektin ve pirantel etken maddelerine

karşı oluşabilecek antelmentik direncin belirlenmesi amacıyla Estonya'da yapılan bir çalışmada, 3 yaşın altındaki 41 yarış atından alınan dışkı örnekleri incelenmiş ve EPG sayısı 200'den fazla olan 32 attan alınan örnekler çalışma materyalini oluşturmuştur. Strongylidae tipi yumurta belirledikleri 32 ata, pirantel, *Parascaris equorum* pozitif tespit ettikleri atlara ise ivermektin uygulaması yapılmış, antelmentiklerin etkinliği EPG ve FECRT analizleri ile belirlenmiştir. *Parascaris equorum* ile enfekte oldukları belirlenen atların %50'sinde ivermektine, Strongylidae ile enfekte oldukları belirlenen atların %27'sinde pirantele karşı bir antelmentik direncin geliştiği bildirilmiştir. Araştırmacılar bu verilerin, pirantel etken maddesine karşı bildirilen ilk antelmentik direnç verileri olduğunu bildirmişlerdir (Lassen ve Peltola, 2015). Mısır'da işgücü amacıyla kullanılan, Strongylidae ve *P. equorum* etkenleri ile enfekte atlarda, ML veya benzimidazol direncinin belirlenmesi amacıyla yapılan bir çalışmada, 644 attan dışkı örneği alınmış ve EPG≥50 olarak tespit edilen 146 örnek analiz edilmiştir. Çalışmaya alınan atlar, ivermektin (n = 33), doramektin (n = 33) veya fenbendazol (n = 30) ile tedavi edilmiş, ardından FECRT analizlerini uygulanmıştır. Tedaviden sonra Strongylidae ve *P. equorum* enfeksiyonu prevalansının düşük düzeyde olduğu belirlenmiş ve Strongylidae etkenleri ile enfekte olan örneklerde, ML veya benzimidazol direncinin tespit edilmediği bildirilmiştir (Salem ve ark., 2021). Sunulan çalışmada *Strongylus* spp. enfeksiyonlarına karşı ivermektin ve praziquantel etken maddelerinde bir antelmentik direnç durumu tespit edilmemiştir. *Strongylus* spp. enfeksiyonlarda ivermektin etken maddesine karşı antelmentik direnç oluşmaması bakımından Salem ve ark. (2021) tarafından yapılan çalışma sonuçları ile benzerlik göstermekte olup, Lassen ve Pelştola (2015) tarafından yapılan çalışma ile farklılık göstermektedir. Bunun sebebinin, çalışmalardaki farklı etkenler ve bu etkenlere karşı farklı antelmentik maddelerin kullanılması ile ilişkili olabileceği düşünülmektedir.

Tayların *Parascaris* spp. enfeksiyonlarında ivermektinin etkinliğini incelemek için Polonya'da yapılan bir çalışmada, EPG≥50 yumurta tespit edilen 7 haradaki toplam 225 tay çalışmaya dahil edilmiş ve enfekte oldukları belirlenen taylara oral yol ile ivermektin tedavisi yapılmıştır. Tedaviden önce ve iki hafta sonra EPG yumurta sayıları FECR analizleri ile kaydedilmiştir. İvermektin tedavisini takiben, tayların %28,4'ünde *Parascaris* spp. yumurtalarının görüldüğü, ortalama tahmini FECR değerinin %44 ile %97 arasında değiştiği ve ortalama ilaç etkinliğinin %49,3 oranında olduğu bildirilmiştir. FECR değerlerinin yaşlı taylarda gençlere göre, erkek taylarda ise dişilere göre daha belirgin olduğu belirtilmiştir. Araştırmacılar çalışmalarının Polonya'daki taylarda *Parascaris* spp. etkenlerine karşı ivermektinin etkinliğinin azaldığını gösteren ilk çalışma olduğunu belirtmişlerdir (Studzińska ve ark., 2020). Sunulan çalışmada *Strongylus* spp. enfeksiyonlarında kullanılan ivermektin ve praziquantel etken maddelerine karşı bir antelmentik direnç durumu belirlenmemiştir. Polonya'da yapılan çalışmada ivermektinin etkinliğinin azaldığı bildirilmiştir (Studzińska ve ark., 2020). Çalışma sonuçlarındaki bu farklılığın, ivermektin etken maddesinin Polonya'da ülkemizden daha yoğun ve kontrolsüz bir şekilde kullanılmasından kaynaklanabileceği düşünülmektedir.

Polonya'da yapılan bir çalışmada, Strongylidae etkenleri ile enfekte atlarda ivermektinin etkinliğinin araştırılması için yapılan bir çalışmada, farklı damızlık at çiftliklerinden, 173 dışkı örneği incelenmiştir. Strongylidae etkenleri ile enfekte olduğunu belirledikleri atlara, ivermektin uygulamışlar, uygulamadan sonraki 14. gün EPG ve FECRT analizleri yapmışlardır. İlaç uygulamasından sonraki 14. günde yaptıkları incelemeler neticesinde etken maddenin %99,9 oranında parazitler üzerinde antelmentik etki gösterdiği ve ivermektine karşı bir antelmentik direncin gelişmediğini bildirmişlerdir (Zak ve ark., 2017). Strongylidae etkenlerinin, atlarda ivermektine karşı gelişmesi muhtemel antelmentik direncin araştırılması amacıyla Litvanya'da yapılan bir çalışmada, araştırmacılar 25 at çiftliğinden aldıkları 707 dışkı örneğinden 659'unun *Strongylus* spp. etkenleri yönünden pozitif olduğunu belirlemişlerdir. Enfekte oldukları belirlenen atlara ivermektin uygulandığı ilk gün ve uygulamadan sonraki 14. günde EPG ve FECRT analizleri yapmışlar, analizler neticesinde *Strongylus* spp. enfeksiyonlarında kullandıkları ivermektin etken maddesine karşı bir antelmentik direncin gelişmediğini belirlemişlerdir (Keidane ve ark., 2018). Atlarda, *Strongylus* spp. enfestasyonlarında, ivermektin ve praziquantel etken maddelerine karşı oluşabilecek antelmentik direncin belirlenmesi amacıyla yapılan bir çalışmada, etken yönünden pozitif olduğu tespit edilen atlara ivermektin ve praziquantel uygulaması yapılmış, tedavide 0. gün ve sonrası 21. günde yaptıkları EPG ve FECRT analizleri yaparak, bu maddelere karşı bir antelmentik direncin şekillenmediğini bildirmişlerdir (Marley ve ark., 2004). Sunulan çalışmada, *Strongylus* spp. enfeksiyonlarında kullanılan ivermektin ve praziquantel etken maddelerine karşı bir antelmentik direnç durumu gözlenmemiştir. Sunulan çalışma bu yönüyle Zak ve ark., (2017), Keidane ve ark., (2018), ve Marley ve ark., (2004) tarafından yapılan çalışmalardaki analiz sonuçları ile benzer sonuçlar vermektedir.

Afyonkarahisar'da tek tırnaklı hayvanlardaki helmint enfeksiyonlarının belirlenmesi amacıyla yapılan bir çalışmada, toplam 104 tek tırnaklı hayvandan (70 eşek, 34 at) taze dışkı örneği alınarak, flotasyon, sedimentasyon ve Baermann Wetzel teknikleriyle incelenmiş, 44 eşeğin (%62.86), 24 atın (%70.59) Strongylidae ve *Parascaris equorum* etkenlerinden en az biri ile enfekte olduğu bildirilmiştir. Enfekte atlarda Strongylid tip ve *Parascaris equorum* yumurtalarına sırasıyla %100 ve %33.33, enfekte eşeklerde ise Strongylid tip ve *Parascaris equorum* yumurtalarına sırasıyla %95.45 ve %4.54 oranlarında tespit edildiği bildirilmiştir. Araştırmacılar, Afyonkarahisar yöresindeki tektırnaklılarda önemli oranda helmint enfeksiyonları bulunduğunu ve etkili bir parazit kontrolünün yapılması gerektiğini vurgulamışlardır (Kozan ve Güzel, 2015). Sunulan çalışmada, at yetiştiriciliğinde ciddi problemlere sebep olmasından dolayı, *Strongylus* spp. enfeksiyonlarında, ivermektin ve praziquantelin tedavi edici etkileri ve bu maddelere karşı oluşması muhtemel antelmentik direnç durumu araştırıldı.

Yurtdışında yapılan çeşitli nekropsi çalışmalarında, *S. vulgaris* %22.5-70, *S. edentatus* %22.5-45, *S. equinus* %3-15 ve *Cyathostominae* spp. etkenleri %27-100 oranlarında bildirilmiştir (Barbosa ve ark., 2001; Bucknell ve ark., 1995; Collobert-Laugier ve ark., 2002; Dunsmore ve Jue, 1985; Pereira ve Vianna, 2006). Türkiye'de yapılan bir nekropsi çalışmasında Strongylidae erginlerine %100 oranında rastlanırken (Burgu ve ark., 1995) dışkı muayenelerinde Strongylidae yumurtalarına %30.4-100 arasında değişen oranlarda rastlanmıştır (Aydenizöz, 2003; Bakırcı ve ark., 2004; Çırak ve ark., 2004; Gül ve ark., 2003; Karaca ve ark., 2005; Pişkin ve ark., 1999; Umur ve Açıcı, 2009; Uslu ve Güçlü, 2007). Sunulan çalışmada farklı işletmelerden İngiliz safkan 60 yarış atından dışkı örnekleri alınmış ve örneklerin %33,3'ünde *Strongylus* spp. etkenleri pozitif olarak belirlenmiştir. *Strongylus* spp. etkenlerinin birçok ülkede atlarda yaygın olarak görülmesi bakımından sonuçlar benzerlik göstermektedir.

Sonuç olarak, çalışmamızın sonuçları ile antelmentik direnç problemine rastlanmamıştır, fakat atlarda meydana gelebilecek bir antelmentik direncin, önemli bir at yetiştiriciliği ve at sağlığı sorunu olması muhtemeldir. Araştırmalar, atlarda endoparazitlerin şu anda halihazırda mevcut olan ticari olarak satışı yapılan ilaç müstahzarlarına karşı direnç gelişebileceğini vurgulamaktadır. Bu nedenle atlara antelmentik ilaç uygulayan kişilerin eğitilmesi, antelmentik ilaç uygulamalarının kontrollü bir şekilde yönetilmesi, antelmentik direnç gelişimine karşı alınabilecek önemli tedbirlerdendir. Antelmentik ilaç uygulamalarının, hayvanlarda kontrollü şekilde yapılmaması durumunda, antelmentik direncin hayvanlarda oluşabileceği, hayvan sağlığı ve dolaylı olarak insan sağlığını da olumsuz yönde etkileyebileceği, ekonomik ve verim kayıplarının oluşabileceği unutulmamalıdır.

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Yazar Katkıları

Fikir/Kavram: HD, AY, MS
Tasarım: HD, AY, Bİ
Denetleme/Danışmanlık: AY, HD
Veri Toplama ve/veya İşleme: AY, HD, MAA, MS
Analiz ve/veya Yorum: HD, AY, MS, Bİ
Kaynak Taraması: HD, AY, Bİ, MD, MB
Makalenin Yazımı: HD, AY, Bİ
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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Evaluation of Serum Amino Acid and Carnitine Profile in Dogs with Transmissible Venereal Tumor

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Abstract: The presented study aimed to reveal the changes in serum amino acid and carnitine profiles in dogs with transmissible venereal tumor (TVT). The study material comprised 40 female dogs ranging in age from 3 to 5 years. The dogs were divided into two groups based on genital organ examinations. Group 1 (n=20) consisted of healthy dogs, while Group 2 (n=20) consisted of TVT-positive dogs. Blood samples were taken from dogs in both study groups, and serum was obtained. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for the determination of carnitine and amino acid profiles. The obtained data were compared using an independent samples t-test. The serum amino acid profiles of Lysine, Aspartic Acid, Tyrosine, Asparagine, Alanine, Arginine, Citrulline, Glutamic Acid, Glycine, Methylglutaryl, Phenylalanine, and Ornithine were found to be lower in the TVT group (P<0.05). The serum carnitine profiles of C0 (Free carnitine), C2 (Acetylcarnitine), C4 (Butyrylcarnitine), C4-DC (Methylmalonylcarnitine), C5 (Isovalerylcarnitine), C5:1 (Tiglylcarnitine), C5-OH (3-Hydroxyisovalerylcarnitine), C5-DC (Glutarylcarnitine), C6 (Hexanoylcarnitine), C6-DC (Adipoylcarnitine), C8 (Octanoylcarnitine), C8:1 (Octenoylcarnitine), C8-DC (Suberoylcarnitine), C10 (Decanoylcarnitine), C10:1 (Decenoylcarnitine), C12 (Dodecanoylcarnitine), C14 (Myristoylcarnitine), C14:1 (Myristoylcarnitine), C14:2 (Tetradecadienoylcarnitine), C18:1 (Oleoylcarnitine), C18:2 (Linoleylcarnitine), and C18:1-OH (Hydroxyoleylcarnitine) were found to be lower in the TVT group (P<0.01). The profiles of C3 (Propionylcarnitine) and C16 (Palmitoylcarnitine) were found to be higher in the TVT group (P<0.01). As a result, it was concluded that the significant changes in amino acid and carnitine values could be used as biomarkers for diagnosing TVT in dogs.

Keywords: *Amino acid, Carnitine, Dog, Transmissible venereal tumor.*

Transmissible Venereal Tümörlü Köpeklerde Serum Amino Asit ve Karnitin Profilinin Değerlendirilmesi

Özet: Sunulan çalışmada transmissible venereal tümörlü (TVT) köpeklerde serum amino asit ve karnitin profilindeki değişimleri ortaya koymak amaçlandı. Çalışma materyalini, 3-5 yaşları arasında değişen 40 adet dişi köpek oluşturdu. Köpekler genital organ muayenelerine göre iki gruba ayrıldı. Grup 1 (n=20) sağlıklı köpeklerden, Grup 2 (n=20) ise TVT pozitif köpeklerden oluştu. Her iki çalışma grubundaki köpeklerden kan örnekleri alınarak serum elde edildi. Karnitin ve amino asit profilinin belirlenmesinde, sıvı kromatografi-kütle spektrometresi (LC-MS/MS) kullanıldı. Elde edilen veriler bağımsız gruplar t-test ile karşılaştırıldı. Serum amino asitlerden Lizin, Aspartik Asit, Tirozin, Asparajin, Alanin, Arginin, Sitrülin, Glutamik Asit, Glisin, Metilglutaril, Fenilalanin ve Ornitin profilleri TVT grubunda daha düşük olduğu belirlendi (P<0.05). Serum karnitinlerden C0 (Serbest karnitin), C2 (Asetilkarnitin), C4 (Bütriylkarnitin), C4-DC (Metilmalonilkarnitin), C5 (İzovalerylkarnitin), C5:1 (Tiglikarnitin), C5-OH (3-Hidroksiizovalerylkarnitin), C5-DC (Glutarylkarnitin), C6 (Heksanoilkarnitin), C6-DC (Adipoilkarnitin), C8 (Oktanoilkarnitin), C8:1 (Oktenoylkarnitin), C8-DC (Suberoilkarnitin), C10 (Dekanoilkarnitin), C10:1 (Dekenoylkarnitin), C12 (Dodekanoilkarnitin), C14 (Miristoilkarnitin), C14:1 (Miristoilkarnitin), C14:2 (Tetradekadienoylkarnitin), C18:1 (Oleoylkarnitin), C18:2 (Linoleylkarnitin) ve C18:1-OH (Hidroksi-oleylkarnitin) profillerinin TVT grubunda daha düşük olduğu belirlendi (P<0.01). C3 (Propiyonilkarnitin) ve C16 (Palmitoilkarnitin) profilleri ise TVT grubunda daha yüksek tespit edildi (P<0.01). Sonuç olarak amino asit ve karnitin değerlerindeki belirgin değişimler bu iki profilin köpeklerde TVT teşhisinde biyobelirteç olarak kullanılabileceği kanısına varıldı.

Anahtar Kelimeler: *Amino asit, Karnitin, Köpek, Transmissible venereal tümör.*

Introduction

Transmissible venereal tumor (TVT), also referred to as sticker tumor, venereal granuloma, or infectious sarcoma, is defined as a contagious and benign reticuloendothelial tumor that primarily affects the external and sometimes internal genital regions of dogs (Tella et al., 2004). The tumor is usually spread through mating (Mukaratirwa and Gruys, 2003). It can also spread to non-genital areas such as the eyes, nose, and oral cavity through social behaviours like sniffing and licking (Abedin, 2020). In TVT, which typically appears cauliflower-like, ranging from crisp to reddish-brown in color, clinical signs such as pain, bleeding, and serosanguinous discharge from the external genital area are observed (Mac-Ewen, 2001). This tumor continues to be a significant problem in countries where mating is not controlled (Das and Das, 2000).

Amino acids have significant functions in numerous metabolic pathways, serving as both substrates and regulators. Assessing the concentrations of free amino acids in bodily fluids and specific tissues offers valuable insights into the biochemical and nutritional status linked with different diseases (Tochikubo and Ando, 2010). Traditionally, tumor metabolism has predominantly centered around carbon metabolism, particularly glycolysis and the tricarboxylic acid cycle. However, recent research has illuminated the significance of amino acids in cancer metabolism. Although glucose is a recognized energy source for tumor growth, amino acids also play a significant role as fuels that support cancer development (Lieu et al., 2020). Amino acids can serve as alternative fuel sources for cells (Green et al., 2016). All mammalian cells employ cellular metabolism to produce essential biomolecules for energy generation and to maintain homeostasis (Hanahan et al., 2011). Amino acids in tumor cells help meet these requirements by assisting in protein synthesis, energy and nucleotide generation, redox balance maintenance, and epigenetic modification (Pavlova et al., 2016).

Carnitine is a derivative of amino acids consisting of various forms, including free carnitine in its endogenous form and short, medium, and long-chain acylcarnitines (Wolf et al., 2013). Carnitine serves two primary functions: facilitating the transportation of long-chain fatty acids into the mitochondrial matrix for β-oxidation, generating cellular energy, and regulating the high intramitochondrial acyl-coenzyme A (CoA)/CoA ratio, thereby reducing the inhibition of many intramitochondrial enzymes involved in glucose and amino acid breakdown (Sandikci et al., 1999). Additionally, it possesses numerous metabolic roles, including stimulating hematopoiesis, blocking collagen-induced platelet aggregation, and averting programmed cell death in immune cells (Wolf et al., 2013). There is evidence showing that carnitine-mediated fatty acid oxidation in the pathogenesis of tumor development may contribute to the production of adenosine triphosphate (ATP), which could play a critical role in tumor progression (Carracedo et al., 2013). One strategy to meet the heightened energy demand of malignant cell proliferation is by engaging in glycolytic activity (Schmidt et al., 2010). Another approach is to acquire energy through fatty acid oxidation (FAO) from nearby adipose tissue, lipoproteins, and phospholipids (Carracedo et al., 2013).

The objective of this study is to explore the correlation between carnitine and amino acid levels in TVT, aiming to contribute novel perspectives to the existing literature and to ascertain its potential utility as a diagnostic biomarker for the disease.

Materials and Methods

Animal Selection: The study was conducted on 40 female mixed-breed dogs brought from Şanlıurfa Metropolitan Municipality Animal Shelter to Harran University Faculty of Veterinary Medicine Animal Hospital for treatment purposes. The study was carried out from February to May. The study utilized animals selected through a random sampling method, all subjected to identical feeding and management conditions. These animals were between 3 to 5 years of age and had an average weight of 26.73±5.12 kg. Dogs diagnosed with pregnancy, systemic disease, or undergoing chemotherapy for TVT treatment were not included in the study based on routine hematological, biochemical, urine analysis, and ultrasonographic examinations. The dogs were segregated into two groups following genital organ examinations. Group 1 (n=20) consisted of healthy dogs, while Group 2 (n=20) consisted of TVT-positive dogs. Those with swelling in the genital area, conformational abnormalities, excessive licking of the region, unusual odor, and evident cauliflower-like masses were considered positive for TVT. A vaginal smear sample was taken for a definitive diagnosis. A vaginal swab was rotated around its own axis within the vaginal mucosa, without contacting the clitoral fossa and external urethral orifice, at a 45-degree angle dorsally from between the labia, to ensure the collection of an adequate number of cells. The swab sample was immediately used to prepare two smear slides on a slide and stained using the Giemsa staining method without delay. The diagnosis of TVT was confirmed by the detection of intracytoplasmic vacuoles and numerous mitotic figures in the samples examined under a light microscope.

Laboratory Analyses: Blood samples were collected from dogs in both experimental groups via the cephalic vein using a 20G sterile syringe and transferred to 10 mL gel vacutainer tubes. Subsequently, the samples underwent centrifugation at 3000 rpm for 15 minutes to obtain serum. The determination of carnitine and amino acid profiles was performed using the LC-MS/MS device (Shimadzu, Japan) employing the method utilized by Tammo et al. (2021).

Statistical Analysis: Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS 26.0). Data normality was assessed analytically (Kolmogorov-Smirnov/Shapiro-Wilk tests). Descriptive statistics for variables that followed a normal distribution were presented as mean ± standard error of the mean (SEM). Group comparisons were made utilizing the Independent Samples

t-test, given the normal distribution of the data. P<0.05 was defined as statistically significant.

Results

The mean serum amino acid and carnitine values for the study groups are given in Tables 1 and 2, respectively. The profiles of serum amino acids including Lysine, Aspartic Acid, Tyrosine, Asparagine, Alanine, Arginine, Citrulline, and Glutamic Acid (P<0.001); Glycine (P<0.01); and Methylglutaryl, Phenylalanine, and Ornithine (P<0.05) were observed to be lower in the TVT group. The profiles of Valine and Methionine were observed to be not significantly different between the TVT and healthy groups (P>0.05). The profiles of serum carnitines including C0, C2, C4, C4-DC, C5, C5:1, C5-OH, C5-DC, C6, C6-DC, C8, C8:1, C8-DC, C10, C10:1, C14, C14:1, C18:1, C18:2, and C18:1-OH (P<0.001); C12 (Dodecanoylcarnitine) and C14:2 (Tetradecadienoyl-carnitine) (P<0.01) were observed to be lower in the TVT group. The profiles of C3 and C16 were observed to be higher in the TVT group (P<0.01). The profiles of C10-DC (Sebacoylcarnitine), C16:1 (Palmitoleylcarnitine), and C18 (Stearoylcarnitine) were observed to be not significantly different between the TVT and healthy groups (P>0.05).

Table 1. The mean serum amino acid values for the study groups.

Amino Acid Profile (µmol/L)	TVT Positive Group $\bar{X} \pm SEM$	Control Group $\bar{X} \pm SEM$	P value
Methy Glutaryl	0.0215±0.002	0.0280±0.002	**0.043**
Valine	356.13±20.58	388.29±4.21	0.253
Lysine	229.73±11.63	363.41±21.32	**0.000**
Methionine	62.40±3.98	67.27±6.52	0.355
Phenylalanine	62.26±3.97	75.53±1.48	**0.024**
Aspartic Acid	0.0580±0.006	0.1250±0.011	**0.000**
Tyrosine	51.39±2.82	83.97±4.66	**0.000**
Asparagine	50.62±3.81	82.46±6.06	**0.000**
Alanine	806.005±94.66	1413.36±57.46	**0.000**
Arginine	399.77±18.53	575.20±22.48	**0.000**
Citrulline	77.02±3.86	131.83±7.62	**0.000**
Glycine	267.24±5.09	375.55±21.33	**0.001**
Ornithine	30.09±1.95	36.46±1.83	**0.017**
Glutamic Acid	364.81±7.32	590.56±33.50	**0.000**

SEM: Standard error of the mean.

Discussion

Metabolomic analysis of carnitine and amino acids provides promising opportunities to elucidate complex metabolic changes associated with tumors and accelerate the identification of novel tumor biomarkers. Metabolomic profiling can aid in the identification of cancer biomarkers and provide clues for early cancer diagnosis. Amino acids and acylcarnitines, which play critical roles in cell physiology as essential metabolites and metabolic modulators, are potential biomarkers for tumor diagnosis (Aboud and Weiss, 2013). The presented study is the first to elucidate the changes in amino acid and acylcarnitine profiles in dogs with TVT disease, providing crucial data contributing to the understanding of these alterations.

Amino acids, the fundamental building blocks of proteins, have crucial functions in mammalian metabolism, cellular growth, genetic expression, and inflammatory reactions. For tumor cells to proliferate, protein (nitrogen supply) and amino acids (support nucleotide biosynthesis) are required. Alterations in amino acid concentrations can markedly impact the tumor microenvironment and immune response, highlighting modified amino acid profiles in individuals with cancer. It has been reported that there are significant differences in plasma amino acid profiles between early and late-stage cancers in both cancerous and healthy individuals (Ward and Thompson, 2012). Kubota et al. (1992) suggested that patients with different types of cancer exhibit a specific amino acid profile characterized by decreased levels of methionine, lysine, glycine, citrulline, aspartate, arginine, alanine, and phenylalanine, and increased levels of tyrosine, valine, and ornithine. To be more precise, Kubota et al. (1992) reported that plasma amino acid levels, especially alanine and arginine, are frequently elevated in breast cancer. Miyagi et al. (2011) demonstrated lower plasma concentrations of glutamine, citrulline, and arginine in patients with early-stage breast cancer compared to healthy individuals. Vissers et al. (2005) found decreased plasma levels of arginine and tryptophan amino acids in patients with breast cancer at different stages. In addition to findings in breast cancer, Lai et al. (2005) reported that plasma amino acids are often suppressed in gastrointestinal cancers, but no noticeable trend was observed in other types of cancer. Miyagi et al. (2011) also indicated decreased levels of amino acids in patients with colorectal and gastric cancer. Turkoglu et al. (2016) demonstrated increased plasma concentrations of glutamine and glycine, and decreased concentrations of phenylalanine and tryptophan in human ovarian cancer. Alanine and valine have increased in some cases and decreased in others (Turkoglu et al., 2016). In patients with endometrial cancer, plasma levels of phenylalanine, tryptophan, and valine decreased in accordance with age (Ihata et al., 2014). The amino acid profile in different tumor types is believed to stem from

Table 2. The mean serum carnitine values for the study groups.

Carnitine Profile	TVT Positive Group	Control Group	P value
	$\bar{X} \pm SEM$	$\bar{X} \pm SEM$	
C0 (Free carnitine)	84.13±1.38	98.54± 0.41	**0.000**
C2 (Acetylcarnitine)	8.01±0.19	9.71±0.13	**0.000**
C3 (Propionylcarnitine)	1.00±0.12	0.77±0.24	**0.000**
C4 (Butyrylcarnitine)	0.150±0.039	0.159±0.002	**0.000**
C4DC (Methylmalonylcarnitine)	0.031±0.0005	0.044±0.0007	**0.000**
C5 (Isovalerylcarnitine)	0.445±0.011	0.568±0.006	**0.000**
C5:1 (Tiglylcarnitine)	0.079±0.001	0.112±0.002	**0.000**
C5-OH (3-Hydroxyisovalerylcarnitine)	0.104±0.003	0.126±0.002	**0.000**
C5-DC (Glutarylcarnitine)	0.0513±0.002	0.0727±0.001	**0.000**
C6 (Hexanoylcarnitine)	0.0264±0.001	0.0564± 0.001	**0.000**
C6-DC (Adipoylcarnitine)	0.0366±0.001	0.0602±0.001	**0.000**
C8 (Octanoylcarnitine)	0.0524±0.004	0.0859±0.001	**0.000**
C8:1 (Octenoylcarnitine)	0.0246±0.001	0.0497±0.001	**0.000**
C8-DC (Suberoylcarnitine)	0.0315±0.002	0.0796±0.001	**0.000**
C10 (Decanoylcarnitine)	0.2014±0.011	0.3242±0.002	**0.000**
C10:1 (Decenoylcarnitine)	0.231± 0.003	0.2715±0.011	**0.000**
C10-DC (Sebacoylcarnitine)	0.0342±0.001	0.0377±0.002	0.134
C12 (Dodecanoylcarnitine)	0.0917±0.018	0.1447±0.000	**0.001**
C14 (Myristoylcarnitine)	0.1327±0.05	0.1960±0.000	**0.000**
C14:1 (Myristoleylcarnitine)	0.1197±0.004	0.2364±0.000	**0.000**
C14:2 (Tetradecadienoylcarnitine)	0.1000±0.004	0.1241±0.002	**0.001**
C16 (Palmitoylcarnitine)	0.3901±0.010	0.3404±0.003	**0.001**
C16:1 (Palmitoleylcarnitine)	0.0983±0.010	0.0910±0.002	0.289
C18 (Stearoylcarnitine)	0.3876±0.012	0.3723±0.002	1.000
C18:1 (Oleylcarnitine)	0.2509±0.012	0.4120±0.004	**0.000**
C18:2 (Linoleylcarnitine)	0.0624±0.002	0.1234±0.002	**0.000**
C18:1-OH (Hydroxyoleylcarnitine)	0.0178±0.001	0.0328±0.000	**0.000**

SEM: Standard error of the mean.

factors such as the tumor type, size, metabolism, and its impact on organ function where it resides. In a few studies conducted in veterinary medicine, it has been demonstrated that tyrosine levels decrease in dogs with melanoma, while phenylalanine and glutamic acid levels increase in dogs with lymphoma. Additionally, decreased levels of methionine, asparagine, glycine, and alanine have been reported in dogs with lymphoma (Kawabe et al., 2015). In dogs with mammary tumors, it has been reported that levels of methionine, asparagine, alanine, and citrulline decrease (Azuma et al., 2012). In the study presented, similar to other different tumors in the literature, it was observed that the serum amino acid levels significantly decreased in TVT-positive dogs. The reason for this is that rapidly dividing tumor cells utilize basic biosynthetic components (such as lipids, amino acids, nucleic acids, etc.) through aerobic glycolysis to meet their energy demands and obtain additional ATP (Hans et al., 2009). Additionally, disease, infection, and anticancer treatments can alter the utilization, digestion, absorption or of amino acids, leading to increased endogenous protein breakdown and thus changes in amino acid profiles. In cancer, this situation can be more profound due to the metabolic demands of the tumor (Jonker et al., 2012).

Acylcarnitines, which are derivatives of carnitine with acyl groups attached, have a significant function in transporting fatty acids for β-oxidation in both mitochondrial and peroxisomal compartments. Changes in their levels provide insights into disruptions in fatty acid oxidation, amino acid metabolism, and glycolysis, indicating the advancement and progression of cancer (Houten et al., 2020; McCann et al., 2021). Acylcarnitine levels are recognized as important biomarkers in identifying metabolic changes caused by various diseases, including congenital metabolic disorders, depression, metabolic disorders, Alzheimer's disease, diabetes, cardiovascular diseases, and certain types of cancer (Dambrova et al., 2022). Assessing acylcarnitine levels opens up new possibilities for diagnosing and prognosticating cancer. It has been reported that in patients with colorectal tumors, tumor cells accumulate long-chain acylcarnitines differently from normal tissues (Shen et al., 2021). In patients with esophageal squamous cell carcinoma, medium-chain acylcarnitine levels were found to be lower compared to the healthy control group (Xu et al., 2013). In solid tumors, serum levels of medium-chain acylcarnitines are generally low, while serum levels of long-chain acylcarnitines are generally high. There are a few studies reporting the use of acylcarnitines as diagnostic biomarkers

for certain cancers in humans (Lu et al., 2016; Niziol et al., 2018; Zhao et al., 2021). In advanced lung and breast cancer patients, there have been reports of decreased serum acylcarnitine concentrations and increased renal clearance of acylcarnitines (Sachan and Datson, 1987). Significant alterations in levels of short, medium, and long-chain acylcarnitines have been documented in patients diagnosed with hepatocellular carcinoma (Lu et al., 2016). Similar results have been reported in the profiles of acylcarnitines profiles of gliomas (Bogusiewicz et al., 2021). In the diagnosis of breast cancer, C12, C14, and C14:2 acylcarnitines have been recognized as potential biomarkers (Kozar et al., 2021), whereas a correlation has been observed between plasma levels of C2 acylcarnitine and the risk of breast cancer (His et al., 2019). In gastric cancer, the increase of C6DC, C16OH, C6, and C0 acylcarnitines has been reported as potential diagnostic biomarkers (Li et al., 2022). Moreover, it has been found that levels of C3, C4, C5, C14, and C16 acylcarnitines are significantly increased in patients with lung cancer (Ni et al., 2016). The increase in some acylcarnitine levels and the decrease in others observed in TVT-positive dogs in the presented study are consistent with changes in acylcarnitine levels occurring in different tumors, as reported in the literature. These changes are thought to be due to increased metabolic activity associated with TVT. Given that serum carnitine profiles are recognized to change with metabolic status, higher acylcarnitine clearance and excretion of non-acidic acylcarnitine in cancer patients may reflect an increase in metabolic state (Dodson, 1989). Decreased acylcarnitine concentrations may be attributed to increased lipid utilization, decreased production, and increased excretion of acidic acylcarnitines, or a combination of these factors (Dodson, 1989; Sachan and Datson, 1987).

Conclusion

In conclusion, amino acid and carnitine profiles have shown significant changes in dogs with TVT. The significant decreases in amino acid values and the significant decreases and increases in carnitine profiles have led to the notion that these two parameters could be utilized as biomarkers for TVT diagnosis. In further studies, utilizing a larger number of animals and specifically determining which parameters to measure, it has been inferred that a clearer diagnosis of the disease can be achieved.

Ethical Approval

This study was approved by the Harran University Animal Experiments Local Ethics Committee (21.12.2023, 2023/008 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Survival of Major Food Pathogens in Natural Zeolite (Clinoptilolite) at Different Ratios and in Chicken Wings After Dipping

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Abstract: The aim of this study was to determine the viability of *Salmonella* Typhimurium and *Listeria monocytogenes* in solutions prepared with readily available natural zeolite and in chicken wings decontaminated with these solutions. To determine the effect of zeolite on pathogen viability, solutions of different concentrations (5%, 10%, 25%) were prepared and contaminated. Their numbers were then determined at different times (2, 6, and 24 hours) during storage at 4 °C. To determine the effect of zeolite on the viability of pathogens in chicken wings, contaminated chicken wings were immersed in zeolite solutions prepared at three different concentrations (5%, 10%, 25%) for two different times (1.5 min, 3 min) and their numbers were determined. According to the results of this study, the number of *S.* Typhimurium decreased by approximately 2.5 $\log_{10}$, and the number of *L. monocytogenes* decreased by approximately 1.4 $\log_{10}$ in zeolite solutions. The number of pathogens was significantly reduced in decontaminated chicken wings ($P \leq 0.05$). In addition, increasing the concentration of zeolite and changing the time had a significant effect on the number of *S.* Typhimurium ($P \leq 0.05$). In conclusion, zeolite was found to be antimicrobial against *S.* Typhimurium and *L. monocytogenes* and has the potential to be used in the decontamination of poultry meat. It is envisaged that zeolite may be a natural alternative to ensure food safety in the near future. To this end, zeolite should be extensively investigated in other potential food applications.

Keywords: *Chicken wings, Clinoptilolite, Listeria monocytogenes, Salmonella Typhimurium, Zeolite.*

Farklı Oranlardaki Doğal Zeolitde (Klinoptilolit) ve Daldırma Sonrası Tavuk Kanatlarda Önemli Gıda Patojenlerin Yaşam Kabiliyetleri

Özet: Bu çalışmanın amacı kolaylıkla temin edilebilen doğal zeolit ile hazırlanmış solüsyonlarda ve bu solüsyonlar ile dekontamine edilmiş tavuk kanatlarında *Salmonella* Typhimurium ve *Listeria monocytogenes'in* yaşam kabiliyetlerini belirlemektir. Zeolitin, patojenlerin yaşam kabiliyeti üzerine etkisini belirlemek için farklı konsantrasyonlarda (%5, %10, %25) solüsyonlar hazırlanıp kontamine edildi. Daha sonra 4 °C'de muhafaza boyunca farklı sürelerde (2., 6., 24. saat) sayıları belirlendi. Zeolitin, tavuk kanatlarında patojenlerin yaşam kabiliyetine etkisinin belirlemesinde ise kontamine edilmiş tavuk kanatları üç farklı konsantrasyonda (%5, %10, %25) hazırlanan zeolit solüsyonlarına iki farklı sürede (1,5 ve 3 dk) daldırma işlemi yapılıp sayısı belirlendi. Bu çalışmanın sonuçlarına göre, zeolit solüsyonlarında *S.* Typhimurium sayısı yaklaşık 2,5 $\log_{10}$ azaldığını, *L. monocytogenes* sayısında ise yaklaşık 1,4 $\log_{10}$ azaldığı saptandı. Dekontamine edilmiş tavuk kanatlarında patojenlerin sayısı önemli ölçüde azaldığı saptandı ($P \leq 0,05$). Ayrıca zeolit konsantrasyonunun arttırılması ve sürenin değişimi *S.* Typhimurium sayısı üzerinde önemli etkisinin olduğu tespit edildi ($P \leq 0,05$). Sonuç olarak zeolitin *S.* Typhimurium ve *L. monocytogenes'*e karşı antimikrobiyal etkisinin olduğu ve kanatlı etlerinin dekontaminasyonunda kullanım potansiyeli olduğu ortaya konuldu. Yakın gelecekte gıda güvenliğinin sağlanması için zeolit doğal bir alternatif olabileceği öngürülmektedir. Bunun için zeolitin diğer potansiyel gıda uygulamaları içinde kapsamlı bir şekilde araştırılması gerekmektedir.

Anahtar Kelimeler: *Klinoptilolit, Listeria monocytogenes, Salmonella Typhimurium, Tavuk kanat, Zeolit.*

Introduction

Poultry meat has a very important place in human nutrition due to its protein content, which is rich in essential amino acids, B-complex vitamins and unsaturated fatty acids, as well as low fat and cholesterol content (Güngören et al., 2023; Keykhosravy et al., 2020; Mehdizadeh and Langroodi, 2019). However, contaminated poultry meat can deteriorate rapidly due to the creation of a favourable environment for microbial growth, such as water activity and high pH (Silva et al., 2018). Therefore, the presence of possible pathogens and spoilage microorganisms in these products causes health problems and economic losses in the poultry industry (İncili et al., 2020). Poultry meat and meat products appear to be responsible for a significant proportion of foodborne illness caused by these pathogens, and the use of antimicrobials is therefore important to reduce the risk of these pathogens and protect human health. Many methods have been tried to improve the microbial and chemical quality of poultry meat and meat products. A wide range of decontamination methods are available for chemical decontamination in the meat and meat products industry (Özbay and Sarıçoban, 2014). However, as these products still pose a risk in terms of shelf life and public health, interest in natural preservatives and additives has recently increased (Aydemir and Arslan, 2023).

Zeolites, commonly found in nature, are microporous crystalline aluminosilicates consisting of AlO_4 and SiO_4 tetrahedral units and are used in various industries (Huwei et al., 2021). Zeolites are edible, biocompatible and most likely non-toxic substances, but they have several special properties, such as molecular sieve structure, ionic exchangeability and water absorbency. These properties allow them to be used in various applications in different fields. (Hecht et al., 2011; Papaioannou et al., 2002).

Zeolites (cinoptilolite) have been used as additives in food and feed for several years due to their ability to adsorb toxins produced by moulds and parasites, fungal mycotoxins, ammonium ions and heavy metals (Deshmukh et al., 2023; Singh and Kumar, 2023; Tzia and Zorpas, 2012; Villa et al., 2022). In addition, the use of zeolites in food packaging materials can prevent spoilage reactions by selectively absorbing oxygen, thereby increasing the shelf life of the product (Kombaya-Touckia-Linin et al., 2019; Lu et al., 2017). They can also be used for odour absorption in the packaging of food products that are sensitive to odours and generate bad odours, or absorb odours from the environment (Sharma et al., 2023). Another important feature of zeolites, their antimicrobial effect that prevents the growth of harmful microorganisms, has been studied by various researchers (Janićijević et al., 2020; Prabhu and Devaraju, 2018; Sánchez et al., 2017; Soysal et al., 2015; Tunç and Duman, 2011). This demonstrates the potential of zeolites as antimicrobial agents.

The potential food applications of zeolites are that they are classified as generally recognised as safe (GRAS) substances by the United States Food and Drug Administration (USFDA, 2015) and the European Food Safety Authority (EFSA, 2011). These substances, which have been approved by organisations, have been widely researched for medical applications or environmental remediation, but have been less studied and researched for food applications. (Eroğlu et al., 2017 ; Lopes et al., 2021). Therefore, it was concluded that more research on zeolites is needed in various fields related to food science and technology.

The aim of this study was to determine the viability of *Salmonella* Typhimurium and *Listeria monocytogenes* in solutions prepared with readily available natural zeolite and in chicken wings decontaminated with these solutions.

Materials and Methods

Ethics Committee: This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees.

Preparation of inoculum: For the preparation of the inoculum, reference strains of *Salmonella* Typhimurium (NCTC 74, 12416, and ATCC 14028) and *Listeria monocytogenes* (N 7144, RSKK 474, and 476) were employed. The method used by İncili et al. (2020) was applied. An inoculum of approximately 6.0 $\log_{10}$/mL was used for the survival assay of pathogens in zeolite and for chicken wing samples.

Zeolite supply and preparation: Zeolite (Nanokar, Türkiye) was supplied by purchase. Zeolite was prepared at 5%, 10% and 25% concentrations. Sterile distilled water was used to prepare different zeolite concentrations and mixed until the zeolite dissolved.

Preparation of chicken wings: On the day of the experiment, fresh chicken wings were purchased from a local market in Şanlıurfa and brought to the laboratory in the cold chain, and the experiments were performed as soon as possible. To all samples, 0.5 mL of diluted bacterial cocktail was added by spreading it on all surfaces of the samples and allowed to adhere for 15 minutes at room temperature. The samples were then randomly selected and divided into ten groups: control (no treatment), three different zeolite concentrations (5%, 10% and 25%) and two different immersion times (1, 5 and 3 min). The groups were marinated by the immersion method. To obtain the concentrations of zeolite used in the study, the zeolite was diluted with sterile distilled water and shaken well to mix it. The ratio of immersion liquid to meat sample was 2:1 and immersion procedures were performed in sterile glass jars.

Pathogens survival experiment in zeolite: To evaluate the antibacterial effect of zeolite, approximately 6.0 $\log_{10}$/mL *Salmonella* Typhimurium and *Listeria monocytogenes* were added to three different zeolite concentrations (5%, 10% and 25%). The number of pathogens was determined immediately after inoculation and after 2, 6 and 24 hours of incubation at 4 °C. The experiment was performed in triplicate.

Microbiological analyses: Each marinated meat sample (25 ± 1 g) was collected under aseptic conditions and transferred to sterile sampling bags. Next, 225 mL of 0.1%

peptone water (PW) was added to the sampling bags, and the mixture was homogenized using a stomacher (BagMixer Interscience, France) for 3 minutes. For the detection of *L. monocytogenes*, Oxford agar (Biokar, France) was used, while xylose-lysine-deoxycholate agar (XLD agar) (Biokar, France) was employed for *S.* Typhimurium. The XLD and Oxford plates were incubated at 37±1 °C for 24 hours, and the number of colonies with specific morphology was recorded.

pH analyses: The pH of the chicken wing samples was measured using a pH meter (HI 11310, Hanna Instruments, USA). The fluid (rinse fluid) remaining in the sample bags after microbiological analysis of the chicken wing samples was used for pH analyses.

Statistical analyses: Microbial counts and pH values of the samples were subjected to statistical analysis. Microbiological data were logarithmically transformed for statistical analysis. The general linear model (GLM) was used for statistical analysis. In the GLM procedure, zeolite concentrations (5%, 10% and 25%) and immersion times (1, 5 and 3 min) were considered as fixed effects and replications as random effects. Multiple comparisons were made using the Tukey test (P≤0.05). In this study, all data were obtained from three independent replicates and results are presented as mean ± standard error of the mean.

Results

pH value: The average pH values of chicken wings samples at 4 °C are depicted in Table 1. There was no difference between the groups in terms of pH (P≥ 0.05).

Table 1. pH values of chicken wings (Mean ± SE).

Concentration	Time	pH
Control		6.67±0.28
5%	1.5 min.	6.63±0.01
	3 min.	6.44±0.06
10%	1.5 min.	6.61±0.07
	3 min.	6.53±0.07
25%	1.5 min.	6.53±0.12
	3 min.	6.58±0.02
Statistics	C	$P \geq 0.05$
	T	$P \geq 0.05$
	CxT	$P \geq 0.05$

C: Concentration, **T:**Time

Pathogens survival experiment at zeolite concentrations: The number of *S.* Typhimurium decreased by approximately 2.5 $\log_{10}$ (Figure 1), while the number of *L. monocytogenes* decreased by approximately 1.4 $\log_{10}$ (Figure 2). Although increasing the zeolite concentration had an insignificant effect on reducing the number of bacteria (P ≥ 0.05), time had a significant effect on the number of *S.* Typhimurium (P ≤ 0.05).

Survival of pathogens in chicken wings after zeolite decontamination: Compared to the control group, the number of *S.* Typhimurium was significantly reduced in the samples of chicken wings immersed in zeolite (Figure 3). The highest decrease was observed at 10% and 25% concentrations after 3 min. *S.* Typhimurium counts decreased by 0.86, 1.20 and 1.28 $\log_{10}$ after 3 min. decontamination at 5%, 10% and 25% concentrations, respectively. Concentration time interaction was not significant for *S.* Typhimurium counts. Compared to the control group, the number of *L. monocytogenes* was significantly reduced in the samples of chicken wings immersed in zeolite (Figure 4). *L. monocytogenes* counts decreased by 1.31, 1.46 and 1.59 $\log_{10}$ after 3 min. decontamination at 5%, 10% and 25% concentrations, respectively. Concentration time interaction was not significant for *L. monocytogenes* counts.

Discussion

The unique structural properties of zeolite provide them with adsorptive, ion exchange and molecular sieving properties (Dikić, 2021). Due to their existing properties, they can have an antimicrobial effect. In the present study, zeolite was found to significantly reduce the number of *S.* Typhimurium and *L. monocytogenes* in both the survival experiment and chicken wing meat (P≤0.05). There is previous evidence that zeolite can be used as an antimicrobial agent (Uchida et al., 1992; Mallek, 2012; Pajnik et al., 2020). Although zeolite has been used as an antimicrobial agent, to the best of our knowledge, no research on the decontamination of pathogens in chicken meat has been found (Villa et al., 2022). Mallek et al. (2012) reported that the addition of zeolite (0.5 or 1% a/a) to chicken diets resulted in a significant (P≤0.05) reduction in total culturable microbial levels and also resulted in improved organoleptic quality of meat.

The results of the present study showed that zeolite exhibited antimicrobial activity against Gram-positive (*L. monocytogenes*) and Gram-negative (*S.* Typhimurium) bacteria. The high ion exchange capacity of zeolite enhanced this antimicrobial property. It has been recommended that zeolite should be combined with ions such as zinc oxide (ZnO) ions, and silver (Ag +) ions for a higher antimicrobial effect (Dutta and Wang, 2019; Wang et al., 2019). In particular, Ag + doped zeolite systems have been reported to exhibit broad-spectrum antimicrobial activity against both Gram-negative (*Escherichia coli* and *S.* Typhimurium) and positive (*L. monocytogenes*, Staphylococcus aureus) bacteria (Janićijević et al., 2020; Sánchez et al., 2017).

It is known that S. Typhimurium can grow optimally at a pH range of 6.5-7.5 and can survive between pH 4.5 and 9.0, while L. monocytogenes is highly resistant to low temperature and pH (İncili et al., 2021). The pH values of the zeolite concentrations used in the study are at values where bacteria can survive (Table 1). Therefore, we believe that pH has no effect on the decrease in bacterial numbers. Its believe that the main reduction in bacterial numbers is due to the high ion exchange and absorption properties of the zeolite This is because it has been reported that clinoptilolite can adsorb bacteria and thus cause a decrease in bacterial numbers (Prasai et al., 2017). It is emphasised that the rough

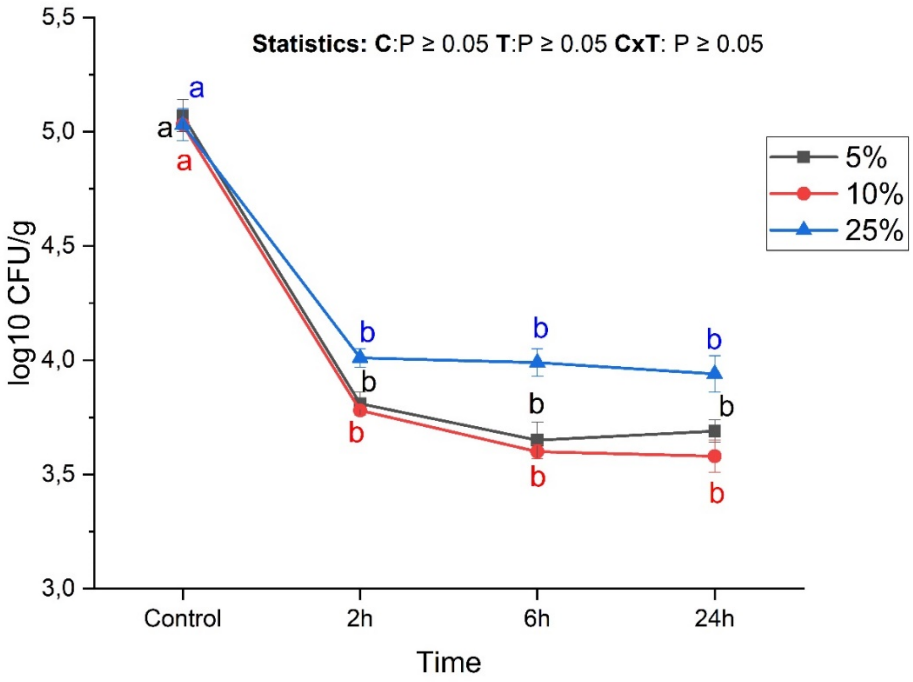

Figure 1. Survival of *Listeria monocytogenes* in zeolite at 4 °C for 24 hours (log10 CFU/g±SE). [a-b]: The mean values with different letters among the sampling hour are significantly different (P≤0.05). **C:** Concentration, **T:** Time.

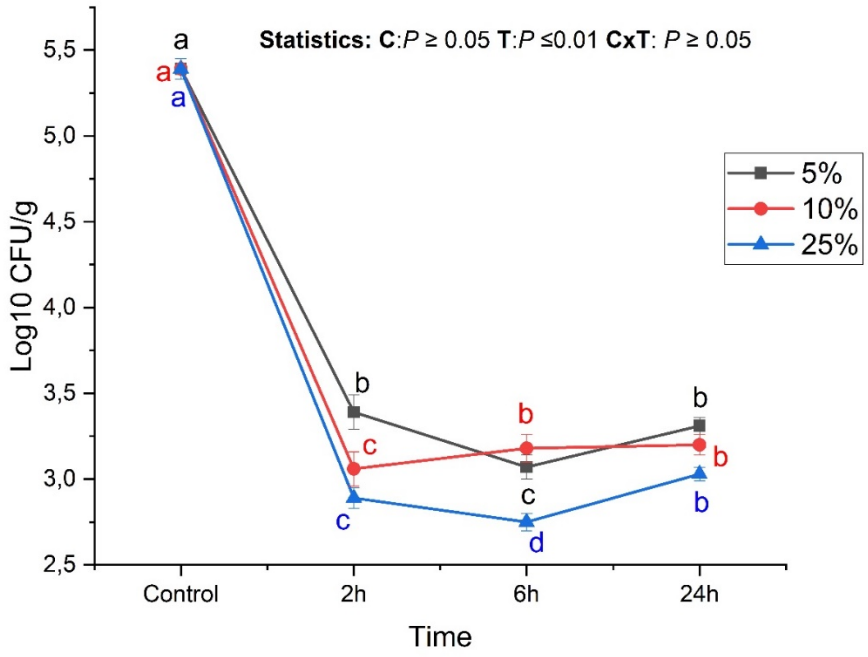

Figure 2. Survival of *Salmonella* Typhimurium in zeolite at 4 °C for 24 hours (log10 CFU/g±SE). [a-d]: The mean values with different letters among the sampling hour are significantly different (P≤0.05). **C:** Concentration, **T:**Time.

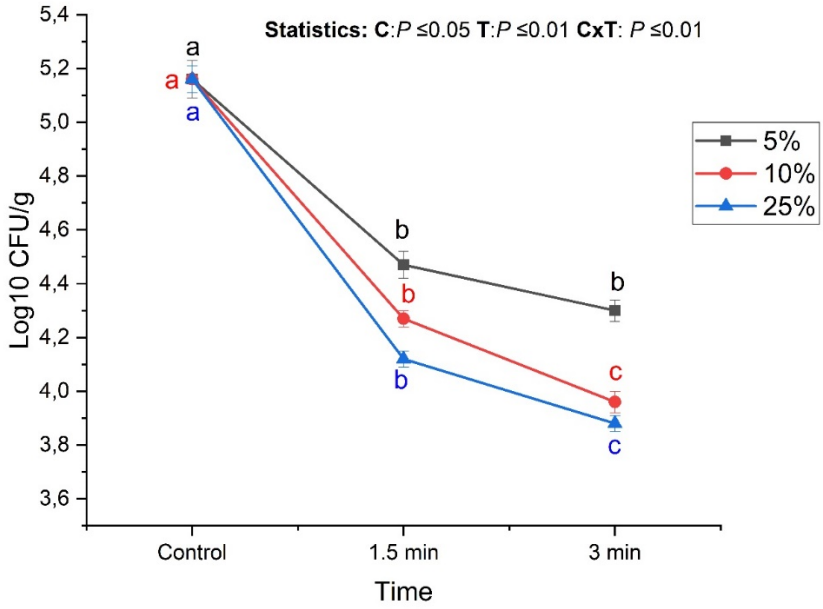

Figure 3. Mean *Salmonella* Typhimurium counts (log10 CFU/g±SE) in chicken wings at different zeolite concentrations at different times. a-c: The mean values with different letters among the sampling hour are significantly different (P≤0.05). **C:** Concentration, **T:**Time.

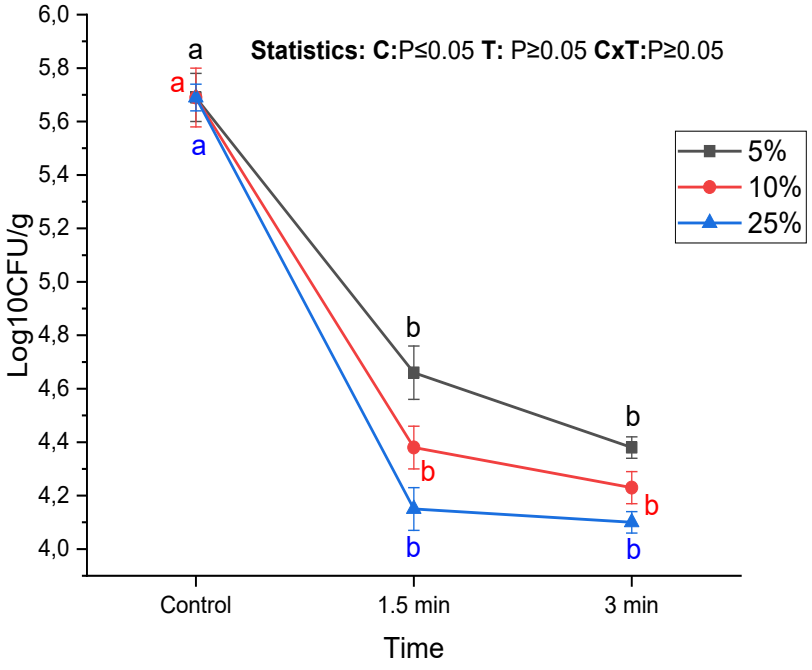

Figure 4. Mean *Listeria monocytogenes* counts (log10 CFU/g±SE) in chicken wings at different zeolite concentrations at different times. a-c: The mean values with different letters among the sampling hour are significantly different (P≤0.05). **C:** Concentration, **T:**Time.

surfaces of clinoptilolite particles provide a better microenvironment for the adsorption of bacteria (Hrenovic et al., 2005). An in vitro study by Wu et al. (2013) reported that *Escherichia coli* and *S.* Typhimurium were adsorbed by clinoptilolite (Wu et al., 2013). In addition, we believe that the different levels of *S.* Typhimurium and *L. monocytogenes* bacteria in zeolite solutions are due to the different effects of zeolite on gram-negative and gram-positive bacteria and,

most importantly, the different adsorption properties of zeolite for each bacterium. In fact, clinoptilolite has been reported to adsorb selectively on bacterial species (Prasai et al., 2017).

In zeolite solutions, the number of *S.* Typhimurium decreased by 2.5 $\log_{10}$ (Figure 1) and the number of *L. monocytogenes* decreased by approximately 1.4 $\log_{10}$ (Figure 2). For chicken wing decontamination, the numbers of *S.* Typhimurium and *L. monocytogenes* decreased by 1.28 (Figure 3) and 1.59 $\log_{10}$ (Figure 4), respectively. The greater reduction in *S.* Typhimurium in the zeolite solution can be explained by the fact that bacteria firmly attached to the wing meat can be protected from the effects of antibacterial agents (İncili et al., 2020). The opposite situation for *L. monocytogenes* can be explained by the fact that the bacteria cannot fully adhere to the wing meat after contamination and also that the bacteria enter the stationary phase in the zeolite solution.

In the zeolite solution, the number of *S.* Typhimurium decreased significantly after 2 and 6 hours, but not after 24 hours (Figure 2), while the number of *L. monocytogenes* decreased significantly after 2 hours, but not after 24 hours. This may be explained by the fact that bacteria develop resistance to the antimicrobial mechanisms of zeolite after a certain period of time. Indeed, Wang et al. (2019) showed that environmental conditions such as exposure to acids can harm bacteria. However, bacteria that are not fatally injured can enter the stationary phase and/or regain the ability to regrow. Bacteria have also been found to be highly adaptable to environmental conditions over time (Chung et al., 2018). In chicken wings decontaminated with zeolite solution, *S*. Typhimurium continued to decrease at 10% and 25% concentrations with time (Figure 3). However, the interaction of concentration and time was not effective for *L. monocytogenes* (Figure 4). This may be explained by the slowing of growth of *L. monocytogenes* against antimicrobial mechanisms and entry into stationary phase (İncili et al., 2020).

As a result, zeolite was shown to be antimicrobial against *S.* Typhimurium and *L. monocytogenes* and has a potential for use in the decontamination of poultry meat. Due to the high ion exchange properties of zeolite, modified zeolites can have very different, perhaps more potent, effects on pathogens. Therefore, zeolite can be combined with modification for stronger bactericidal effects. It is envisaged that zeolite may be a natural alternative for food safety in the near future. To this end, zeolite should be extensively investigated in other potential food applications

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Similarity Rate

We declare that the similarity rate of the article is 12% as stated in the report uploaded to the system.

Explanation

Some data from this study was presented as an abstract at the 10th Veterinary Food Hygiene Congress on 25-27 April 2024.

Author Contributions

Motivation / Concept: MEA, MNG, ES
Design: MEA
Control/Supervision: MEA
Data Collection and / or Processing: MEA, MNG
Analysis and / or Interpretation: MEA, MNG
Literature Review: MEA, ES
Writing the Article: MEA, ES
Critical Review: MEA

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

A new protocol for the induction of chronic mastitis with intramammary infusion of lipopolysaccharide (LPS) in Balb/c mice

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Abstract: Mastitis is inflammation of the mammary tissue and is commonly observed in farm animals. The problem causes severe financial losses in the dairy industry in terms of veterinary costs, milk disposal, and treatment expenses. Bacteria are the main actors in the etiology and cause acute and chronic inflammatory changes in the mammary tissue. Acute inflammatory changes are easily recognized clinically, and treatment is initiated immediately, but subacute inflammation progresses insidiously and leads to chronic inflammation with irreversible fibrotic changes. Standardized experimental models for the induction of acute mastitis in laboratory animals are available. Usually, infusion of bacteria or some bacterial structural components into mammary tissue is easily applied for this purpose. However, there are few studies on the induction of chronic mastitis with fibrotic changes, and the applications are relatively complex. In this study, LPS was infused through the teat duct three times on days 0, 5, and 10 to induce chronic mastitis in mice. Tissues were sampled on days 1, 6, and 15 to evaluate histopathological changes. While severe neutrophil infiltrates, a component of acute inflammation, were observed on day 1, lymphocyte infiltrates increased on day 6, consistent with subacute inflammation. On day 15, lesions representing chronic mastitis, such as fibrosis and lymphocyte infiltration, were observed. A model similar to the lesions in chronic mastitis of dairy cattle was successfully and easily established by LPS infusion in mice.

Keywords: *Balb/c, Chronic mastitis model, Lipopolysaccharide, Mammary gland, Masson's Trichrome.*

Balb/c farelerinde meme içi lipopolisakkarid uygulaması sonucu yeni bir kronik mastitis modelin indüklenmesi

Özet: Mastitis, meme dokusunun iltihaplanmasıdır ve dünya genelinde çiftlik hayvanlarında yaygın olarak görülmektedir. Bu sorun süt endüstrisinde veteriner masrafları, sütün imhası ve tedavi giderleri açısından ciddi mali kayıplara neden olmaktadır. Bakteriler etiyolojinin ana aktörleridir ve meme dokusunda akut ve kronik inflamatuar değişikliklere neden olurlar. Akut enflamatuar değişiklikler klinik olarak kolayca tanınır ve tedavi hemen başlatılır, ancak subakut inflamasyon sinsice ilerler ve geri dönüşü olmayan fibrotik değişikliklerle kronik inflamasyona yol açar. Laboratuvar hayvanlarında akut mastitis indüksiyonu için standartlaştırılmış deneysel modeller mevcuttur ve genellikle bakterilerin veya bazı bakteriyel yapısal bileşenlerin meme dokusuna infüzyonu bu amaçla kolayca uygulanmaktadır. Bununla birlikte, fibrotik değişikliklerle birlikte kronik mastitis indüksiyonu üzerine az sayıda çalışma vardır ve uygulamalar nispeten karmaşıktır. Sunulan çalışmada, farelerde kronik mastitisi indüklemek için LPS 0, 5 ve 10. günlerde üç kez meme kanalından infüze edilmiştir. Histopatolojik değişiklikleri değerlendirmek için 1, 6 ve 15. günlerde dokulardan örnek alınmıştır. Akut inflamasyonun bir bileşeni olan şiddetli nötrofil infiltratları 1. günde gözlenirken, lenfosit infiltratları subakut inflamasyonla uyumlu olarak 6. günde artmıştır. 15. günde, fibrozis ve lenfosit infiltrasyonu gibi kronik mastitisi temsil eden lezyonlar gözlenmiştir. Süt sığırlarının kronik mastitisindeki lezyonlara benzer bir model, farelerde LPS infüzyonu ile başarılı ve kolay bir şekilde oluşturulmuştur.

Anahtar Kelimeler: *Balb/c, Kronik mastitis modeli, Lipopolisakkarit, Meme bezi, Masson Trikrom.*

Introduction

Mastitis, the inflammation of udder tissue, is one of the most critical health issues of dairy cattle and causes serious economic losses. According to recent research, the average cost of an affected animal to the farm is about 444 USA dollars (Rollin et al., 2015). Infectious agents severely demolish mammary architecture and host inflammatory responses in acute and chronic mastitis (Ingman & Glynn, 2014; Zhao et al., 2015). Experimental induction of mastitis in dairy cattle may not be a good option for many reasons, including rearing conditions, contamination of teats with fecal bacteria after infusion of lipopolysaccharide (LPS), or live bacteria (Brouilette et al., 2023; Cheng & Han, 2020). Understanding the mechanism of mastitis is important not only for new treatment strategies but also for animal welfare (Cobirka et al., 2020). In this context, experimental animal models are strongly required to understand the mechanisms of tissue damage by various etiologic agents. So far, acute mastitis has been successfully induced by intramammary administration of various live bacteria such as *Escherichia coli*, *Staphylococcus aureus,* and *Candida krusei* or LPS in laboratory animals (Ingman et al., 2015). CD1 and Balb/c mice are very popular in studying mammary biology, function, and inflammation (Camperio et al., 2017). These models in CD1 and Balb/c breeds are primarily for the evaluation of early changes in the inflammatory process (Camperio et al., 2017). However, most infections in dairy cattle are subclinical, and damage to the udder tissue is often incurable when animals are suspected or diagnosed with mastitis (Cobirka et al., 2020; Lai et al., 2017). Ineffective treatments and prolonged inflammatory processes result in irreversible loss of parenchymal units and tissue fibrosis in mammary tissue (Kan et al., 2022). Fibrosis can be defined as the accumulation of interstitial cells and their unique extracellular matrix (ECM) proteins in tissues (Wynn, 2008; Wynn & Ramalingam, 2012). Mouse models utilized for the understanding of bovine chronic fibrosing mastitis are insufficient. In 1979, experimental chronic mastitis in mice was modeled by the intramammary inoculation of endotoxin 6 hours before *Staphylococcus aureus* administration (Anderson, 1979). Tuchscherr et al. (2005) have induced chronic mastitis in mice by the intramammary administration of particular strains of *Staphylococcus aureus* capable of producing polysaccharide wall components. However, these models have potential biological hazardous risks; *Staphylococcus aureus* has the capability to infect humans and other laboratory animals. If researchers aim to induce chronic fibrosing mastitis without the aforementioned risks, the submitted model herein can be a useful alternative. Establishing a fibrotic mastitis model in mice enables the investigation of specific genes, mediators, and immune cells enrolling in fibrosis formation. Development strategies of anti-fibrotic therapies can be easily possible. Therefore, we aimed to introduce a new model for generating mammary fibrosis in Balb/c mice.

Materials and Methods

The Animal Care and Use Committee of Bursa Uludag University, under the National Institute of Health Guide for the Care and Use of Laboratory Animals, approved all experimental procedures (Approval number: 2019-07/04).

Preparation of LPS: One mg of LPS from *Escherichia coli* O111:B4 (L4391 Sigma Aldrich, St. Louis, MO, United States) was dissolved in 5 ml of sterile phosphate-buffered saline (PBS) (P4417, Sigma Aldrich, St. Louis, MO, United States). Syringe-type filters (CLS431224, Sigma) were used to sterilize PBS (Barham et al., 2012).

Animals: Female Balb/c mice were obtained from the Experimental Animals Breeding and Research Center of Bursa Uludag University. They were housed at five mice per cage at temperatures of 20–22°C with 60-70% humidity in a controlled room set to a 12-h light/ dark cycle and had access to standard mice chow (Korkuteli, ANTALYA) and water ad libitum. Animals were mated at eight weeks of age, and lactating mice were used in experiments. After parturition, pups were allowed to suck their mothers for 8-10 days so that the inoculations could be given more easily. Pups were removed from dams 2-hour before intramammary injections and kept with nursing mice. Previous studies were based on determining the optimum dose of LPS to reduce the number of mice in preliminary experiments. The groups were first divided into 3 (control, PBS, LPS) according to the type of substance given. The number of animals in each group was nine, and a total of 27 animals were used. Three animals from each group were euthanized on day 1, three on day 6, three on day 6, and three on day 15, and the samples were examined.

Intramammary Infusion of LPS: All experimental procedures in mice were applied under anesthesia with sevoflurane (Sevorane liquid 250 mL, Abbvie, North Chicago IL, United States) using a portable anesthesia device (AMS Minor 612, Turkey). LPS solution (50 µl, 0.2 ug/µl) was infused directly into the right fourth mammary glands via the teat canal at days 0, 5, and 10. A 30-gauge blunt end needle (BD – Ultra Fine 0.5 mL insulin syringe, Becton, Dickinson, and Company, Franklin Lakes, NJ, United States) was utilized for all injections. An equal volume of sterile PBS solution was given into the right fourth mammary glands of mice in the vehicle group (Barham et al., 2012). Mice in the control group did not receive any infusion, three mice were euthanized at 1, 6, and 15 days for monitoring of healthy/or involuting mammary glands. To confirm the induction of mastitis following LPS injection, an animal was euthanized 24 hours after LPS infusion, tissue samples were taken and evaluated histopathologically.

Histopathologic examination: Mammary tissue samples were fixed in 4% paraformaldehyde solution for 24 hours, cut into small pieces, and transferred to tissue cassettes, dehydrated in ascending series of ethanol, cleared

in xylene, embedded in paraffin, cut into 4-µm serial sections with a microtome, and sections were placed into Poly-L-Lysine coated slides. Sections were stained with Hematoxylin-Eosin (H&E), and inflammatory changes were evaluated under the light microscope (CX41, Olympus Corporation, Shinjuku City, Tokyo, Japan).

Masson's Trichrome Staining: All slides were stained with Masson's Trichrome using a commercially available kit (Bio-Optica, Milano, Italy) according to the manufacturer's instructions to evaluate fibrotic changes.

Results

Histopathological findings:

On day 1;

Three animals from each experimental group (nine animals in total) were euthanized for day 1 evaluations. No inflammatory changes were detected in the control and PBS-vehicle groups microscopically (Fig. 1 A-B).

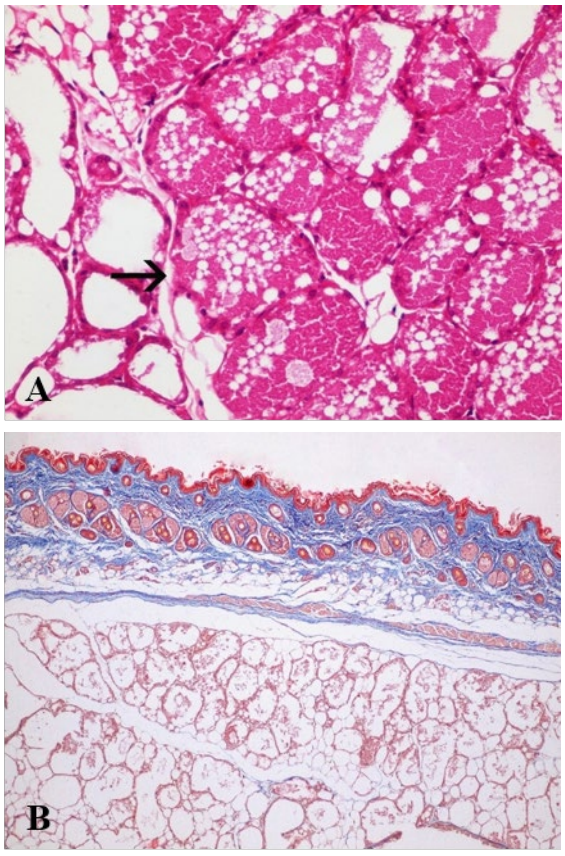

Figure 1. A) Healthy mice mammary tissue, no inflammatory changes were seen, and mammary tissue is active, alveolar lumens are filled with milk, H&E staining, control group x200 magnification, **black arrow:** mammary tubules. **B)** Healthy mice mammary tissue, no fibrous tissue around the alveoli, Masson's Trichrome staining, control group, x40 magnification.

Mammary tissue was active, and alveolar lumens were filled with milk in the control and PBS groups. Severe acute inflammatory changes represented by hyperemic blood vessels, neutrophil leucocyte infiltration within lumens of alveoli, and secretory tubules were observed along with degenerated-exfoliated secretory and tubular epithelium. Reactive mammary lymphadenopathy was evident in animals euthanized after the day of the first LPS injection. In addition to inflammatory changes, most alveoli continued synthesizing milk after a single LPS infusion (Fig. 2).

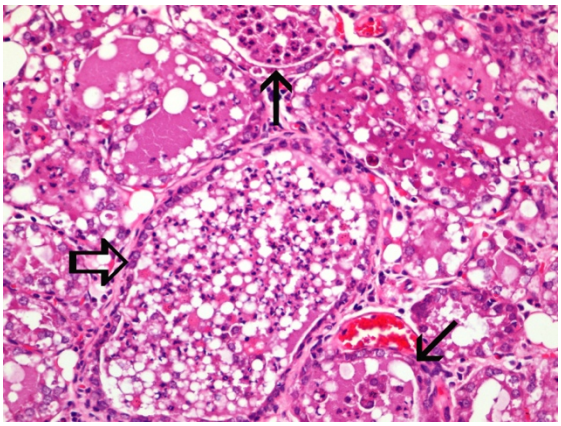

Figure 2. Acute mastitis on day 1, mice mammary tissue, severe neutrophils infiltration in tubules lumen **(transparent arrows)** and alveoli lumen **(black arrows)**, H&E staining, LPS group, x200 magnification.

On day 6;

Three animals from each experimental group (nine animals in total) were euthanized for day 6 evaluations. There were no inflammatory changes, and most alveolar lumens were filled with milk in the mammary tissues of the control and PBS groups. In some areas, signs of involution were characterized by the narrowing of alveoli and duct lumens and the absence of milk synthesis. Involuted mammary tissues were embedded in the increased amount of mammary adipose tissue. Acute inflammatory reaction altered mild to moderate subacute inflammatory response characterized by mononuclear cell infiltrations around alveoli and tubules; neutrophils were also observed in lumens of some secretory units (Fig. 3-A). Lymphoid follicular hyperplasia was seen in mammary lymph nodes. Mild to moderate ECM accumulation around the alveoli and ducts was initiated (Fig. 3-B).

On day 15;

Three animals from each experimental group (nine animals in total) were euthanized for day 15 evaluations. No signs of inflammation and whole mammary alveoli were involuted in control and PBS groups. Milk synthesis was not observed in any secretory unit; alveoli and ducts were isolated as islands in adipose tissue. In the LPS treatment group, mammary alveoli and ducts were heavily surrounded by mononuclear cells, including lymphocytes, macrophages, and plasma cells. Severe perialveolar and periductal fibrosis were observed in chronically inflamed mammary tissue. Mammary lymph nodes were greatly enlarged due to severe, diffuse follicular hyperplasia (Fig. 4-A). Masson's trichrome staining visualized the existence and accumulation of fibrotic tissue in mammary tissues (Fig. 4-B).

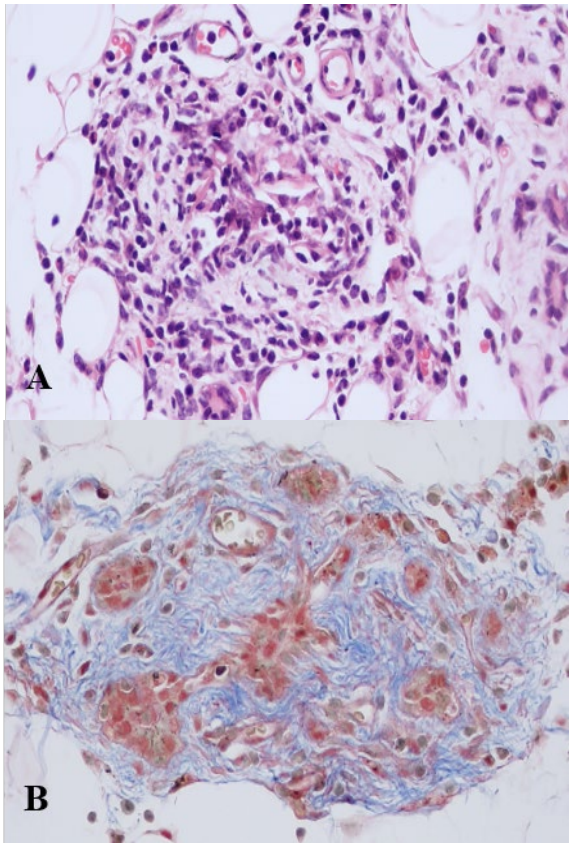

Figure 3. Mice mammary tissue on day 6 **A)** Around the tubules and alveoli lumens characterized by mononuclear and polymorphonuclear cell infiltration and increased fibrosis, H&E staining, LPS group, x200 magnification **B)** Increase connective tissue around the tubule and alveoli, Masson's Trichrome staining, LPS group, x200 magnification.

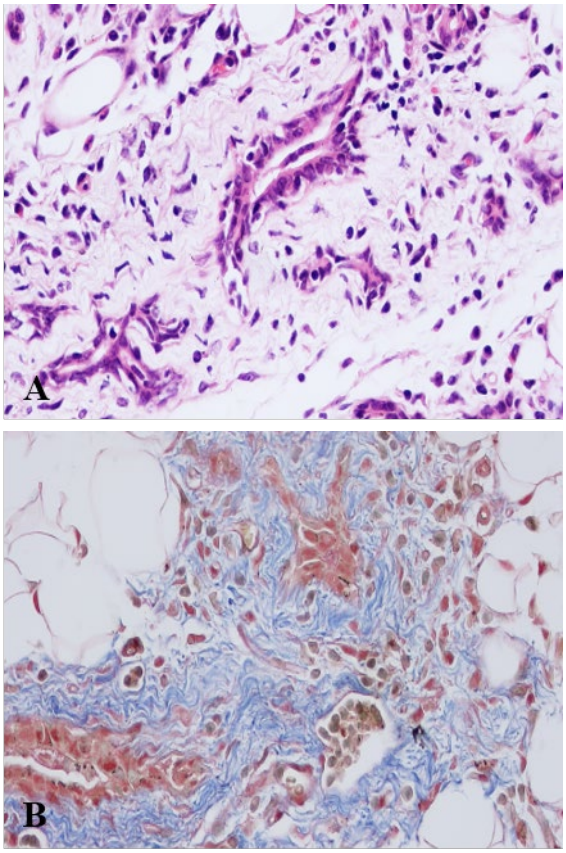

Figure 4. Mice mammary tissue on day 15; **A)** Severe perialveolar and periductal fibrosis and mononuclear infiltration in the perialveolar and periductal area in LPS group, H&E staining, x200 magnification, **B)** Severe fibrosis around the tubule and alveoli in LPS group Masson's Trichrome staining, LPS group, x200 magnification

Discussion

In this study, we have established a new model for fibrotic changes in mammary tissue after LPS infusion in Balb/c mice. Mastitis resulting in fibrosis is a common problem in dairy cattle, but its mechanism is poorly understood, and studies questioning pathogenesis are extremely limited. Mouse mastitis models are of utmost importance in understanding bovine udder health and disease when the costs originating from including cattle in experiments and their rearing conditions are considered. Alternative and inexpensive mouse models are applicable in almost all standard laboratory animal breeding facilities. In this context, several mouse mastitis models have been established using various inflammatory stimuli. The administration of live bacteria or bacterial cell wall fragments such as LPS via intramammary injection is a useful and well-established experimental approach in the induction of mastitis in laboratory animals. Most of these mouse models mimic acute inflammatory changes in mammary tissue. Even though acute mastitis is essential in cattle, most cases are subclinical insidious infections accompanied by tissue fibrosis resulting in early culling of affected animals. New, simple, and safe experimental models are needed since the data on the induction of chronic fibrosing mastitis in laboratory animals is very limited. Working with live bacteria may cause biological hazards to researchers and laboratory animals in the same facility. Additionally, culture, storage, and administration of bacteria in mice are difficult parts of experimental models. Different mouse mastitis models can be good alternatives for avoiding such disadvantages.

The number of experimental studies for initiating chronic mastitis in mice is limited. Tuchscherr et al. (2005) have reported that the administration of Staphylococcus aureus strains capable of producing capsular polysaccharides via the intramammary route elicited chronic mastitis in mice. In their model, severe tissue damage, polymorphonuclear, and mononuclear cell infiltrates in the mammary tissues of mice on the 4th, 8th, and 12th days following the administration of bacterial strains have been demonstrated. However, they did not mention changes compatible with increased connective tissue accumulation (fibrosis) in the mammary sample. In our study, major histopathological inflammatory changes were found to be similar to those of Tuchscherr et al. Hence, we successfully demonstrated the induction of fibrosis together with severe inflammatory changes in mammary tissue. Moreover, similar histopathological findings were noticed in tissue samples from slaughtered cattle selected due to chronic fibrotic mastitis (Özguden-Akkoc et al., 2023).

A recent study reported that single-dose subcutaneous injection of *S. aureus* suspension induced mammary tissue fibrosis in SPF Balb/c mice (Bi et al., 2020). However, such an administration route may cause injection site reactions in the dermis and subcutaneous tissues, leading to dermatitis, cellulitis, and abscesses. Further, this experimental manipulation increases the risk of lowering the bacterial burden at the injection site. Biohazard and zoonotic potential for humans should be kept in mind. In the present study, the infusion of LPS solution directly into teat canals allowed it to be diffused through the duct system and alveoli. Thus, an inflammatory reaction selectively involved the mammary tissue without affecting the adjacent tissues.

Conflict of Interest

The authors stated that they did not have any real, potential, or perceived conflict of interest.

Ethical Approval

This study was approved by the Bursa Uludag University Animal Experiments Local Ethics Committee (2019/07-04 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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Critical Review: OY, AA

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Morphometric Investigation of Sexual Dimorphism and Homotypic Variations of Ossicula Auditus in Morkaraman Sheep

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Abstract: In this study, it was aimed to determine the morphometric characteristics of the bones forming the ossicula auditus of Morkaraman sheep and to reveal the differences between sexes and sides. For this purpose, 24 (12 females/12 males) craniums of Morkaraman sheep were used in the study. After the craniums were macerated, the ossicula auditus in the cavum tympani were dissected out and morphometric data were determined by taking linear measurements. Sexually dimorphic structures and homotypic variations of the obtained morphometric data were statistically calculated. It was observed that the bones forming the ossicula auditis were malleus, incus and stapes, respectively; when the morphometric data obtained were analyzed, it was determined that there were statistically dimorphic differences between sexes in the parameters of malleus lenght, width of the caput mallei and length of the manubrium mallei (P<0.001), Lenght of the caput mallei (P<0.01) in the left malleus ossicle, lenght of the crus breve (P<0.001) in the right incus ossicle, Length of the crus longum and length of the corpus incudis (P<0.01), length of the crus breve and width of the corpus incudis (P<0.001) in the left incus ossicle. Homotypic variation was detected in the length of the caput mallei (P<0.01), length of the malleus and lenght of the manubrium mallei (P<0.001) parameters in malleus in male sheep, and width of the corpus incudis (P<0.001) parameter in incus in female sheep. At the same time, no symmetrical difference was found in other parameters (P>0.05). In the correlation analysis of the morphometric parameters of the ossicles, weak, moderate and strong correlation was observed in the positive direction, while only weak correlation was observed in the negative direction. As a result, it is thought to contribute to taxonomic and experimental studies by determining the morphometric parameters of the ossicula auditis bones of Morkaraman sheep.
Keywords: *Dimorphizm, Homotypic variation, Morphometry, Ossicula auditus.*

Morkaraman Koyunlarında Ossicula Auditus'un Eşeysel Dimorfizminin ve Homotipik Varyasyonlarının Morfometrik Olarak İncelenmesi

Özet: Bu çalışmada, Morkaraman koyunlarında ossicula auditus'u oluşturan kemiklerin morfometrik özelliklerinin belirlenmesi, cinsiyetler ve yönler arasındaki farklılıkların ortaya konulması amaçlanmıştır. Bu amaçla çalışmada 24 adet (12 dişi/12 erkek) Morkaraman koyunu kafatası kullanıldı. Cranium'lar maserasyona tabi tutulduktan sonra cavum tympani'deki ossicula auditus'lar diseke edildi ve doğrusal ölçümler alınarak morfometrik veriler belirlendi. Elde edilen morfometrik verilerin cinsiyete göre dimorfik yapıları ve homotipik varyasyonları istatistiksel olarak hesaplanmıştır. Ossicula auditus'u oluşturan kemiklerin sırasıyla malleus, incus ve stapes olduğu görülmüş, elde edilen morfometrik veriler analiz edildiğinde sol malleus kemikçiğinde,caput mallei uzunluğu (P<0.01) ile malleus uzunluğu, caput mallei genişliği ve manubrium mallei uzunluğu (P<0.001) parametrelerinde cinsiyetler arasında istatistiksel olarak dimorfik farklılıklar olduğu tespit edildi Sağ incus kemikçiğinde crus breve uzunluğu, LSC (P<0.001), sol incus kemikçiğinde ise crus longum uzunluğu ve corpus incudis uzunluğu (P<0.01), crus breve uzunluğu ve corpus incudis (P<0.001) parametrelerinde cinsiyetler arasında istatistiksel olarak dimorfik farklılıklar olduğu belirlendi. Erkek koyunlarda malleusta caput mallei uzunluğu (P<0.01), malleus uzunluğu ve manubrium malleiuzunluğu (P<0.001), dişi koyunlarda ise incusta corpus incudis uzunluğu (P<0.001) parametrelerinde homotipik varyasyon tespit edilirken, diğer parametrelerde simetrik farklılık bulunmadı (P>0.05). Kemikçiklerin morfometrik parametrelerinin korelasyon analizinde pozitif yönde zayıf, orta ve güçlü korelasyon gözlenirken, negatif yönde sadece zayıf korelasyon gözlenmiştir. Sonuç olarak, Morkaraman koyunlarının ossicula auditis kemiklerinin morfometrik parametrelerinin belirlenmesinin taksonomik ve deneysel çalışmalara katkı sağlayacağı düşünülmektedir.
Anahtar Kelimeler: *Dimorfizm, Homotipik varyasyon, Morfometri, Ossicula auditus.*

Introduction

In Turkey, which is geographically divided into seven regions, many sheep breeds with different morphological and physiological characteristics can adapt to the region due to climate and geographical differences. Morkaraman sheep is a fat-tailed indigenous breed that constitutes 20% of the sheep population in Turkey. Its breeding is carried out in Turkey's North Eastern and South Eastern Anatolia regions, mostly in Eastern Anatolia (Akmaz et al., 2021; Eyduran et al., 2008).

Apart from phenotypic characteristics, the skeletal system is often used in taxonomic classification. The most frequently used part of the skeletal system is the cranium bones (Kaymakçı, 2010; Soysal et al., 2003; Yaprak et al., 2023). Discrimination based on cranium morphology is difficult due to the many intraspecific polymorphisms and intraspecific diversity seen among sheep breeds (Bärmann et al., 2013). Therefore, craniometric or geometric morphometric methods are preferred (Bernal, 2007). Morphology and morphometry reflect the contribution of phenotype and genotype in the development of species (Wehausen and Ramey, 2000).

Ossicula auditus; located dorsal to the cavum tympani in the pars petrosa of the os temporale. Between the membrane tympani and the fenestra vestibuli (in the middle ear) are located the malleus, incus, and stapes, respectively. In young animals, there may also be a separate ossicle called os lenticulare between the incus and stapes. This ossicle fuses with the incus at a later age to form the processus (proc.) lenticulare. Ossicula auditus transmits sound vibrations in the membrana tympani from the auris media to the auris interna (König and Liebich, 2022). At the same time, the bones that make up the ossicula auditus are separated from each other by the m. tensor tympani and m. stapedius muscles, balancing the high vocal pressure (Reece, 2012).

Since the sheep ear model is close to the human ear model, it is preferred in experimental audiological studies and hearing aid trials (Cordero et al., 2011; Péus et al., 2020). In our study research, it was aimed to reveal the homotypic variations and sexual dimorphism of the ossicula audits of Morkaraman sheep by morphometric analysis.

Materials and Methods

The total number of samples was calculated as 10 for each pairwise comparison and 20 in total, with an effect size of 1.4, type 1 error of 0.05 and power of 80%. A total of 24 (12 female/12 male) Morkaraman sheep heads were used, considering the bone loss that may occur during the removal of the ossicula auditus. The materials used were selected in such a way that there was no statistical difference between the sexes in terms of body weight. All materials were found to be adult during dental examination.

After maceration of the fresh craniums, pars petrosa ossis temporalis and pars tympanica ossis temporalis were separated from the head for dissection of the ossicula auditus. The ossicula auditus located in the cavum tympani was dissected through the meatus acusticus externus. The ossicula auditus were visualized with a stereo-microscope (Nikon- SMZ-2T) and the measurements given in Table 1 and Figure 1 were taken to reveal the differences between the sexes and between the right and left ossicles. The measurement points are shown in Figure 1 (Demiraslan et al., 2015; Gürbüz et al., 2019; Kurtul et al., 2003).

Table 1. Measurement points of ossicula auditus.

Ossicula auditus	Measurement Parameters
Malleus	Malleus length (LM)
	Width of the caput mallei (WHM)
	Lenght of caput mallei (LHM)
	Length of the manubrium mallei (LhM)
Incus	Incus lenght (LI)
	Crus breve length (LSC)
	Crus longum lenght (LLC)
	Corpus incus lenght (HBI)
	Corpus incus width (WBI)
Stapes	Stapes lenght (LS)
	Caput stapedis width (WHS)
	Basis stapedis width (WBS)
	Crus rostrale lenght (LRC)
	Crus caudale lenght (LCC)
	Foramen intercrurale widht (WIF)
	Foramen intercrurale lenght (LIF)

Statistical analysis: The conformity of the measurement data obtained from the bones to normal distribution was evaluated by the Shapiro-Wilk test and Histogram graph, homogeneity of variances was assessed by Levene's test, and linearity was evaluated by scatter plot. Differences between measurement levels were analyzed using One-Way Analysis of Variance, and the relationships between variables were analyzed using Pearson product-moment and Spearman rank correlation coefficients. Correlation coefficients were evaluated as 0-0.19 no relationship, 0.20-0.39 weak, 0.40-0.69 moderate, 0.70-0.89 strong and 0.90-1.00 very strong relationship (Alpar, 2018). Analyses were performed using the Jamovi v2.3 package program. The significance level was determined as P<0.05.

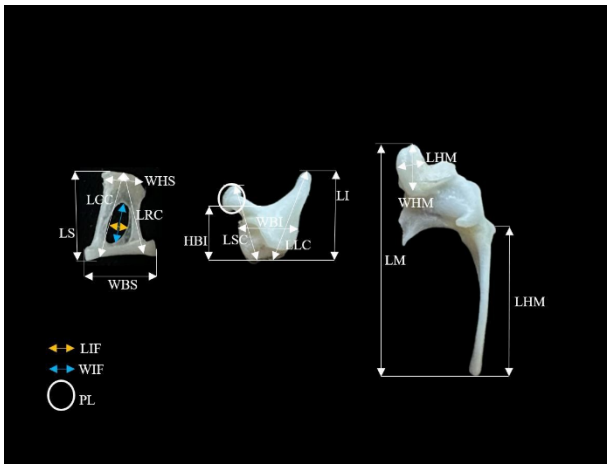

Figure 1. Measurements of the auditory ossicles. Abbreviations-see text (Table 1)

Results

It was observed that the bones forming the ossicula auditis in Morkaraman sheep were malleus, incus and stapes respectively and the os lenticulare formed the proc. lenticulare on the crus longum of the incus (Figure 1).

The morphometric data of malleus, incus and stapes and statistical analysis of these data in terms of gender and direction are given in Tables 2, 3, and 4. In the malleus measurement parameters (Table 2), there were statistically significant differences between the sexes for the left malleus ossicle, LH, WHM and LhM at P<0.001 and LHM at P<0.01, whereas there was no dimorphic difference between the sexes for the right malleus ossicle (P>0.05). When Table 3 was examined, it was determined that there was a significant difference between the sexes only in LSC P<0.001 in the right incus ossicle. Other parameters did not show statistically dimorphic variation (P>0.05). In the left incus ossicle, LLC and HBI were significantly different at P<0.01, LSC and WBI were significantly different at P<0.001. In the stapes ossicle, there was no statistically dimorphic difference between genders (P>0.05) (Table 4).

Table 2. Descriptive statistics of malleus bone measurements (mm).

Incus		Parameters ($\bar{X} \pm S_{\bar{X}}$)			
Gender	**Ear**	**LH (n=42)**	**WHM (n=44)**	**LHM (n=46)**	**LhM (n=43)**
Male	Right	10.01±0.16 ^{a}	3.51±0.06 ^{b}	2.27±0.08 ^{a}	6.93±0.23 ^{a}
	Left	9.09±0.20 ^{b}	3.38±0.08 ^{b}	2.02±0.07 ^{b}	5.99±0.13 ^{b}
Female	Right	10.42±0.10 ^{a}	3.64±0.06 ^{ab}	2.38±0.03 ^{a}	6.95±0.13 ^{a}
	Left	10.30±0.19 ^{a}	3.87±0.10 ^{a}	2.20±0.04 ^{a}	6.94±0.25 ^{a}
Total		3.60±0.05	9.95±0.12	2.97±0.03	2.21±0.03
P value		**<0.01**	**<0.001**	**<0.001**	**<0.01**

X: Arithmetic mean; SX: Standard error; ab: Statistical difference within the same column; n: Sample size.

Table 3. Descriptive statistics of incus bone measurements (mm).

Incus		Measurements ($\bar{X} \pm S_{\bar{X}}$)				
Gender	**Ear**	**LI (n=44)**	**LLC (n=44)**	**LSC (n=44)**	**HBI (n=44)**	**WBI (n=44)**
Male	Right	3.39±0.08 ^{b}	3.51±0.06 ^{b}	2.83±0.05 ^{b}	2.15±0.06 ^{b}	2.52±0.05 ^{b}
	Left	3.50±0.13 ^{ab}	3.38±0.08 ^{b}	2.80±0.03 ^{b}	2.18±0.03 ^{b}	2.42±0.04 ^{b}
Female	Right	3.66±0.06 ^{ab}	3.64±0.06 ^{ab}	3.05±0.04 ^{a}	2.30±0.04 ^{ab}	2.55±0.04 ^{b}
	Left	3.80±0.05 ^{a}	3.87±0.10 ^{a}	3.15±0.05 ^{a}	2.49±0.11 ^{a}	2.76±0.04 ^{a}
Total		3.60±0.05	3.61±0.05	2.97±0.03	2.29±0.04	2.57±0.03
P value		**<0.01**	**<0.01**	**<0.001**	**<0.01**	**<0.001**

X: Arithmetic mean; SX: Standard error; ab: Statistical difference within the same column; n: Sample size.

Table 4. Descriptive statistics of stapes bone measurements (mm).

Stapes		Measurements ($\bar{X} \pm S_{\bar{X}}$) (n=40)						
Gender	**Ear**	**LS**	**LLC**	**LRC**	**WHS**	**WBS**	**LIF**	**WIF**
Male	Right	3.46±0.12	3.78±0.10	3.82±0.13	1.91±0.06	3.38±0.07	1.43±0.08	0.92±0.07
	Left	3.40±0.04	3.73±0.06	3.73±0.09	1.83±0.07	3.42±0.09	1.46±0.06	1.03±0.06
Female	Right	3.11±0.32	3.75±0.10	3.66±0.16	1.86±0.07	3.36±0.04	1.56±0.11	0.97±0.11
	Left	3.38±0.08	3.73±0.09	3.70±0.10	1.86±0.06	3.38±0.05	1.54±0.08	1.05±0.05
Total		3.34±0.09	3.75±0.04	3.73±0.06	1.87±0.03	3.38±0.03	1.50±0.04	0.99±0.04
P value		0.524	0.969	0.795	0.836	0.959	0.650	0.636

X: Arithmetic mean; SX: Standard error; ab: Statistical difference within the same column; n: Sample size.

Examining homotypic variations of male and female animals, it was determined that the malleus ossicle of male sheep had significantly different LHM (P<0.01), LH, and Lhm (P<0.001) values (Table 2). In the incus ossicle, it was determined that there was a statistically significant difference in the WBI parameter only in females with a value of P<0.001, while the other parameters did not show homotypic variation (P>0.05). Male animals' incus ossicles showed no homotypic variance in any of the parameters that were looked at (P<0.05) (Table 3). It was determined that the stapes ossicle did not show statistically homotypic variation in all parameters in male and female animals (P>0.05) (Tablo 4).

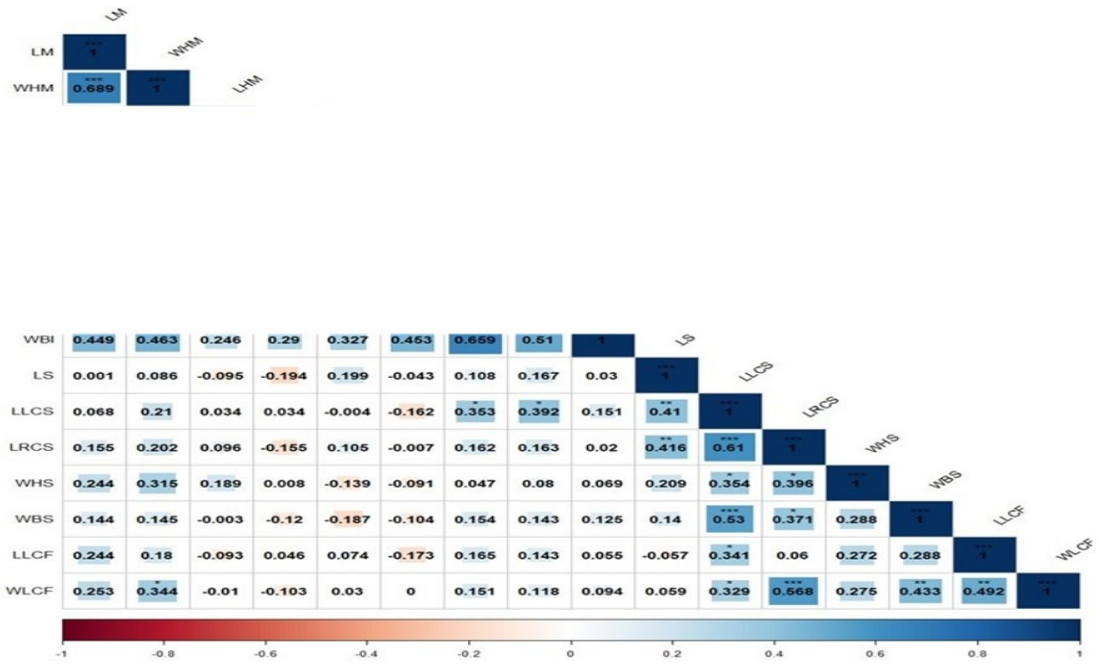

Figure 2. Correlation analysis of morphometric parameters.

The correlation analysis of the morphometric parameters of the malleus, incus and stapes forming the ossicula auditus is shown in Figure 2. The results of the correlation study showed that the ossicular parameters had weak, moderate, and strong positive relationships with one another, but only weak negative correlations.

Discussion and Conclusion

Bone morphology and morphometry have an important place in taxonomic classification and zooarchaeological studies, especially in species with high intraspecific polymorphism such as sheep (Demiraslan et al., 2024; Demircioğlu et al., 2021). In recent years, there have been morphological and morphometric studies on ossicula auditus in different animal species (Besoluk et al., 2019; Dalga & Aslan, 2019; Demiraslan et al., 2015; Gürbüz et al., 2019; Gürbüz and Demiraslan, 2023; Hadžiomerović et al., 2023; Kurtul et al., 2003; Martonos et al., 2021; Stoyanov, 2020). In this study, morphometric analysis of the ossicula auditus of Morkaraman sheep was performed and homotypic variations and sexual dimorphism were investigated.

In experimental ear studies, sheep ear model has been reported as the closest animal model to humans. In particular, it was determined that the anatomical and histological structures of the sheep middle ear were significantly similar to the human ear (Cordero et al., 2011; Lavinsky et al., 1999; Seibel et al., 2006). In this study, it was observed that the ossicula auditus of Morkaraman sheep were located as malleus, incus, and stapes, respectively from outside to inside in accordance with the literature, and os lenticulare was located on incus as proc. lenticulare (Demiraslan et al., 2015; Eyduran et al., 2008; Gürbüz et al., 2019; König and Liebich, 2009; Péus et al., 2020).

The left malleus (LH, WHM and LhM, LHM), right incus (LSC), and left incus (LLC, HBI, LSC, WBI) bones showed sexual dimorphism in the statistical comparison of the ossicula auditus between genders. No statistically significant difference was observed between genders in the other parameters. Gürbüz et al. (2016), reported that there was a statistical difference between the incus length and corpus incudis in Malakan horses between genders. In Merkep (Equus Asinus) (Demiraslan et al., 2015), it was reported that the morphometric parameters of the bones forming the ossicula auditus did not show statistical dimorphism between genders.

When the homotypic variations of the bones forming the ossicula auditus were examined in the study, statistical variations were observed in the LHM, LH and Lhm parameters of the malleus ossicle of male sheep, in the WBI parameter of the female incus ossicle, while no inter-directional variation was observed in any parameter of the stapes bone. In studies conducted on different animals, male Hemşin sheep (Dalga and Aslan, 2019), Malakan horses (Gürbüz et al., 2016), and wolves (Canis lupus) (Gürbüz et al., 2019), it has been reported that there is no homotypic variation between ossicula auditus bones. However, it was reported that there was statistically homotypic variation between the ossicula auditus bones in the parameters of incus length, crus breve length, stapes length, basis stapedis width, and crus anterior length in merkep *(Equus asinus)* (Demiraslan et al., 2015).

In this study, the morphometric parameters of ossicula auditus of Morkaraman sheep were determined and the differences of ossicles between sexes and directions were determined. The ossicles showing differences in gender and direction were analyzed one by one and the differences and similarities were revealed. In addition, correlation analysis of the data was performed and positive and negative correlations between the data were determined. It is thought that these data will be useful in the taxonomy of species and experimental studies.

Conflict of Interest

The authors stated that they did not have any real, potential, or perceived conflict of interest.

Ethical Approval

This study is not subject to ethical permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees". Approval document was received from Bingöl University HADYEK with E-85680299-020-169617 dated 08.08.2024.

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We declare that the similarity rate of the article is %5 as stated in the report uploaded to the system.

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Data Collection and/or Processing: FAK, AK
Analysis and / or Interpretation: FAK, AK
Literature Review: FAK, AK
Writing the Article: FAK, AK
Critical Review: FAK, AK

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Koyun ve Kuzuların Deri Apselerinden *Corynebacterium pseudotuberculosis*'in Teşhisi

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Özet: Koyun yetiştiriciliğinin yoğun olarak yapıldığı bir il olan Balıkesir'de, özellikle kırkım sonrası koyunlarda *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) kaynaklı enfeksiyonlara rastlanabilmektedir. Bu çalışma, Balıkesir ilinde koyunlarda meydana gelen lenfadenitis vakalarında *C. pseudotuberculosis* varlığını araştırmak amacıyla yapıldı. Svap materyallerinin ekimleri sonucunda üreyen kolonilerden (n=4) yapılan makroskobik, mikroskobik ve biyokimyasal testler sonucunda üreme görülen tüm materyallerden *C. pseudotuberculosis* identifiye edildi. Antibiyotik duyarlılıklarının belirlendiği disk difüzyon testinde tüm izolatlar tilmikosin, siprofloksasin, tetrasiklin ve klindamisine duyarlı bulundu. Koyunlara uygulanan apse ve tilmikosin ile uzun etkili amoksisilin sağaltımıyla apselerin iyileştiği görüldü. Sonuç olarak, bu çalışma, Balıkesir ilinde koyunların lenf düğümleri apselerinde *C. pseudotuberculosis*'in varlığı ve teşhisi hakkında bilgi sağlamıştır. Bu bulgular, Balıkesir ilinde *C. pseudotuberculosis* enfeksiyonunun epidemiyolojik açıdan varlığı, yayılması ve kontrol ve korunması ile hastalığa ait ekonomik kayıplarının azaltılmasına yönelik önlemlerin alınmasına yardımcı olabileceği düşünüldü.

Anahtar Kelimeler: *Apse, Corynebacterium pseudotuberculosis, Deri, Koyun, Kuzu.*

Diagnosis of *Corynebacterium pseudotuberculosis* from Skin Abscesses of Sheep and Lambs

Abstract: In Balıkesir province, where sheep farming is commonly practiced, infections caused by *Corynebacterium pseudotuberculosis* (*C. pseudotuberculosis*) are frequently observed, particularly in sheep after shearing. This study was conducted to investigate the presence of *C. pseudotuberculosis* in cases of lymphadenitis in sheep in Balıkesir province. Following the cultivation of swab samples (n:4), all materials from which colonies grew were identified as *C. pseudotuberculosis* based on macroscopic, microscopic, and biochemical tests. In the disk diffusion test determining antibiotic susceptibility, all isolated strains were sensitive to tilmicosin, ciprofloxacin, tetracycline, and clindamycin. It was observed that the abscesses in the sheep healed with the treatment of abscesses and tilmicosin along with long-acting amoxicillin. In conclusion, this study provided information about the presence and diagnosis of *C. pseudotuberculosis* in the lymph nodes of sheep in Balıkesir province. These findings were thought to help understanding the epidemiological presence, spread, and control of *C. pseudotuberculosis* infections in Balıkesir province, and in taking measures to reduce the economic losses associated with the disease.

Keywords: *Abscess, Corynebacterium pseudotuberculosis, Lamb, Sheep, Skin.*

Giriş

Corynebacterium pseudotuberculosis, Corynebacteriacae familyasında, Gram pozitif, çomak ve pleomorfik olarak X,V,Y şeklinde 2'li, 3'lü, 4'lü formlarda mikroskopta görülebilen, fakültatif anaerob üreme özelliğine sahip, mezofilik, sporsuz, hareketsiz, intaselüler bir bakteridir (Guerrero ve ark., 2018; Magdy Selim ve ark., 2022).

C. pseudotuberculosis, koyunlar, keçiler, atlar, sığırlar ve bazen insanlar üzerinde çeşitli kronik hastalıklara yol açan bir patojendir. Ancak, koyunlar ve keçiler bu organizmaya en duyarlı olan hayvanlardır ve bu bakterinin neden olduğu hastalık caseous lymphadenitis (CLA) olarak bilinir. *C. pseudotuberculosis ovis* serotipi koyunlarda kazeöz lenfadenitis hastalığına neden olur (Bernardes ve ark., 2021; do Nascimento Sousa ve ark., 2024; Torky ve ark., 2023).

Kazeöz lenfadenitis (KLA) hastalığına neden olan *C. pseudotuberculosis*, dünya genelinde rapor edilmiştir ve çeşitli ülkelerde ticari sürülerde önemli sorunlara yol açmaktadır (Akgül ve ark., 2018; Araujo ve ark., 2020). Hastalık, koyunlardan elde edilen yünün miktar ve kalitesinde azalma, süt ve et verimi düşmesine neden olarak ekonomik kayıplara yol açar. Enfekte karkasların apselerinden dolayı karkas kalitesinde düşüş ve süt veriminde azalma görülür. Enfeksiyon kaynakları, enfekte hayvanların lenf düğümlerinden yayılan irin ve enfekte materyallerdir. Hastalık, enfekte hayvanlardan ve/veya diğer kontamine materyallerden diğer sürülere bulaşır. Bakteri genellikle deri yaraları, burun boşluğu veya ağız yoluyla vücuda girer. Girişten sonra, bakteriler genellikle yerel lenf düğümlerine yerleşir ve bakteriyel toksinler ve bakteriyel invazyon nedeniyle iltihaplanma ve abse oluşur (Akgül ve ark., 2018; Bernardes ve ark., 2021; Meng ve ark., 2023; Oreiby ve ark., 2013).

Hastalık koyunlarda yüzeysel ve iç organlarda olmak üzere iki formda görülür. Yüzeysel form en sık görülen formdur.Parotid, submandibular, prescapular, prefemoral, popliteal lenf düğümleri gibi yüzeysel lenf düğümlerinde apse oluşumu ile karakterizedir. Baş ve boyun bölgesindeki lenf düğümlerinde şişlikler ve apse oluşumu gözlemlenebilir. Apselerin cilt altındaki sert şişliklerle karakterize edildiği görülür. Apseler genellikle yavaş yavaş gelişir ve zamanla olgunlaşır. Apselerin patlaması, açık yaralar bırakabilir (de Farias ve ark., 2018; Magdy Selim ve ark., 2022).

İç organ formu ise daha nadir bir formdur. İç organların ve derin lenf düğümlerinin etkilenmesi ile karakterizedir. Bu durumda genel sağlık durumu bozulabilir, iştahsızlık, zayıflık ve ağırlık kaybı görülebilir (Akgül ve ark., 2018; de Farias ve ark., 2018; Magdy Selim ve ark., 2022).

KLA'nın teşhisi, genellikle klinik bulgularla başlar ve laboratuvar testleriyle doğrulanır. Bakteriyel kültür ve PCR, kesin tanı koymada önemli teşhis yöntemleridir, ancak ELISA gibi serolojik testler ve görüntüleme yöntemleri de destekleyici olarak kullanılabilir (Akgül ve ark., 2018; Alves ve ark., 2020; Guerrero ve ark., 2018; Magdy Selim ve ark., 2022; Meng ve ark., 2023).

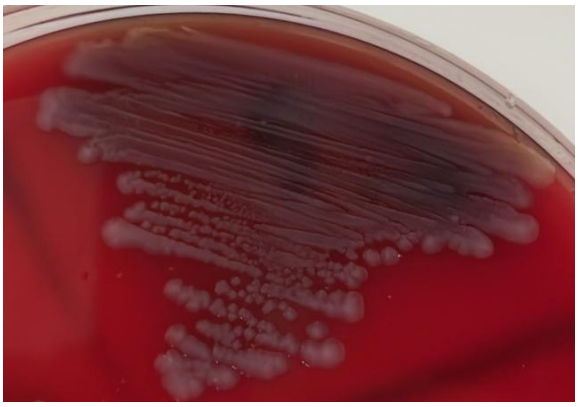
Kültür ve PCR metotları teşhis amaçlı olarak alınan apse içeriği veya etkilenen lenf düğümünden alınan materyaller ile yapılır. Koyun kanlı agarda *C. pseudotuberculosis* yuvarlak, beyaz, parlak ve kaygan koloniler şeklinde görünür ve hafif bir hemoliz alanı ile çevrilidir. Biyokimyasal olarak katalaz pozitif, üreaz pozitif, nitrat redüktaz negatif, indol negatif, H_2S negatif, glukoz, maltoz, mannoz, fruktoz ve glikojeni fermente etme özelliğindedir (Magdy Selim ve ark., 2022; Torky ve ark., 2023).

Serolojik testler, kan serumunda antikor tespiti temeline dayanır. Bu testler, enfekte hayvanları tespit etmek ve sürü taraması yapmak için kullanılır, ancak kesin bir teşhis için destekleyici bir rol oynar (Akgül ve ark., 2018).

Koyun yetiştiriciliğinin yoğun olarak yapıldığı bir il olan Balıkesir'de, özellikle kırkım sonrası koyunlarda *C. pseudotuberculosis*'in neden olduğu enfeksiyonlara rastlanabilmektedir. Bu çalışma, Balıkesir ilinde koyunlarda meydana gelen lenfadenitis vakalarında *C. pseudotuberculosis* varlığının ortaya konulması amacıyla yapıldı.

Materyal ve Metot

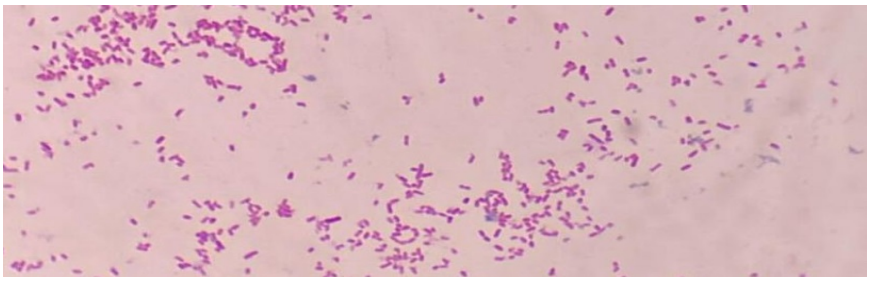
Materyal Alınan Hayvanlar: Bu çalışmada, entansif besi uygulanan, ad-libitum olarak kuzu fabrika yemi ile kaba yem olarak saman ile beslenen, toplam 5 aylık yaşta 70 kuzu, 1.5 yaşında 20 toklu, 1-4 yaş aralığında 20 damızlık koyun ve 10 koç ve yeni doğan yaklaşık 100 kuzu bulunan çiftlikte; kırkım sonrası kuzu ve koyunların skapula ve abdominal bölgelerinde oluşan apselerden (Şekil 1, Şekil 2) çiftlik veteriner hekiminin mikrobiyolojik teşhis amacıyla steril transport medium içeren svaplara aldığı ve soğuk zincirde laboratuvara gönderdiği 4 apse içeriği bakteriyolojik muayene için kullanıldı. Çalışmada kullanılan klinik materyal, mikrobiyolojik teşhis amaçlı çiftlik veteriner hekimi tarafından alınarak laboratuvara gönderildiğinden Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmelik'in 8.k-5 maddesine göre etik kurul iznine tabi değildir.



Şekil 1. *Corynebacterium pseudotuberculosis*'in %5 koyun kanlı agarda koloni yapısı.

İzolasyon ve İdentifikasyon: İzolasyon amacıyla %5 koyun kanlı agar (Merck, Almanya) ve Brain Hearth Infusion Broth (BHI; Oxoid, Birleşik Krallık) eş zamanlı olarak kullanıldı. Svap örnekleri %5 koyun kanlı agar ve BHI'ya ekimleri yapıldı

ve 37 °C'de 48 saat etüvde inkübe edildi (Torky ve ark., 2023). İnkubasyon sonrası üreyen kolonilerden identifikasyon amacıyla Gram boyama, koloni morfolojisi, hareket muayenesi, katalaz, üreaz, indol, nitrat redüksiyon testleri yapıldı ve TSI agarda (Merck, Almanya) biyokimyasal olarak değerlendirildi (Torky ve ark., 2023)



Şekil 2. %5 Koyun kanlı agarda üreyen *Corynebacterium pseudotuberculosis* etkeninin Gram boyama sonucu mikroskobik görüntüsü.

Antibiyogram Testi: *C. pseudotuberculosis* olarak identifiye edilen kolonilerden EUCAST prosedürüne göre McFarland 0.5 bulanıklık standardında inokulum hazırlandı ve Mueller-Hinton agar (Oxoid, Birleşik Krallık) kullanılarak disk difüzyon testi yapıldı (EUCAST, 2017; EUCAST, 2022).

Disk difüzyon testinde tilmikosin (15µg, Oxoid, Birleşik Krallık), klindamisin (2µg, Liofilchem, İtalya), siprofloksasin (5µg, Oxoid, Birleşik Krallık) ve tetrasiklin (30µg, Oxoid, Birleşik Krallık) kullanıldı (Torky, EUCAST). Disk difüzyon test sonuçları EUCAST (2017), EUCAST (2022) ve CLSI (2023) standardları ile tilmikosin etken maddesi için Liofilchem (2023) antibiyotik disk kriterlerine göre değerlendirildi (CLSI, 2023; EUCAST, 2017; EUCAST, 2022; Liofilchem, 2023; Torky ve ark., 2023;).

Bulgular

Ekimi yapılan 4 svap materyalinin tamamında (%100) üreme görüldü. Svapların ekimleri sonucunda üreyen kolonilerin Gram boyama, biyokimyasal testler ve agarda üreme morfolojileri değerlendirildi. Gram pozitif çomak, %5 koyun kanlı agarda yuvarlak beyazımsı parlak koloni, katalaz pozitif, üreaz pozitif, nitrat redüksiyon testi negatif, indol negatif, H_2S negatif, glikoz ve fruktoz fermentatif olan 4 bakteri *C. pseudotuberculosis* olarak identifiye edildi (Şekil 1, Şekil 2).

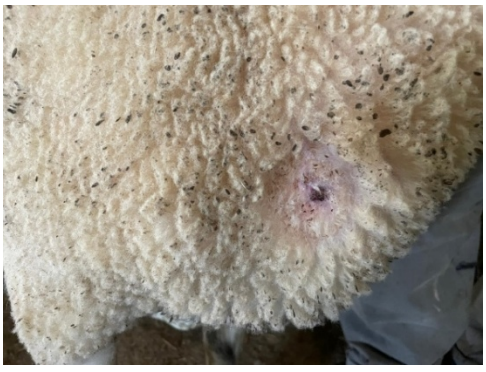
Antibiyotik duyarlılıklarının belirlendiği disk difüzyon testinde izole edilen 4 (%100) *C. pseudotuberculosis* suşu tilmikosin, siprofloksasin, tetrasiklin ve klindamisin'e duyarlı bulundu.

C. pseudotuberculosis çıkan materyallerin alındığı hayvanlar çiftlik veteriner hekimi tarafından apse tedavisi uygulandı ve antibiyogram testinde duyarlı olarak bulunan tilmikosin ve uzun etkili amoksisilin etken madde içeren antibiyotik ile sırasıyla tek doz ve bir gün arayla uygulanarak tedavi edildi. Bu tedavi sonucunda apselerde iyileşme görüldü (Şekil 3, Şekil 4).

Tartışma ve Sonuç

Balıkesir Valiliği'nin yayımladığı verilere göre Balıkesir ilinde 2022 yılında 1.272.236 baş koyun bulunmaktadır. Balıkesir ili hayvan yetiştiriciliği açısından ülkemizde büyük bir yere ve öneme sahiptir. Koyun yetiştiriciliği açısından bakıldığında gastronomik açıdan Balıkesir kuzusu olarak coğrafi işarete de sahiptir. Bu nedenle Balıkesir ilinde ekonomik olarak da katkı sağlayan önemli bir yetiştiricilik alanıdır.

Şekil 3. Tedavi sonrası iyileşme gösteren apsenin görünümü.



Şekil 4. Tedavi sonrası iyileşme gösteren apsenin görünümü.

C. pseudotuberculosis'in neden olduğu ve koyunlarda özellikle deride bulunan portantrelerden girerek meydana gelen kazeöz lenfadenitis enfeksiyonu, yetiştiricilikte süperfisyal ve viseral lenf yumrularında irinli ve granülomatöz lezyonlarla karakterize olan ve ekonomik kayıplara neden olan kronik bir enfeksiyondur (Akgül ve ark., 2018; Oreiby ve ark., 2013; Torky ve ark., 2023). Yung ve ark. (2015) hastalıkta en çok sırasıyla parotis, submandibular ve servikal lenf düğümlerinin etkilendiğini bildirmiştir. Bu çalışmada en çok abdominal ve skapular lenf düğümlerinde lezyon görüldü. Bu sonuç, Yung ve ark. (2015)'nın en çok

görülen lenf düğümlerinden farklı olarak diğer lenf düğümlerinde de enfeksiyonun görülebileceğini gösterdi.

Etkenin sebep olduğu hastalık dünyada ve ülkemizde serolojik, moleküler ve kültür yöntemleri ile yapılan çalışmalarda değişik prevalans oranları ile saptanmıştır. (Ekinci Yıldız ve İçen, 2022).

Aslan ve ark. (2016), Kayseri ilinde yaptıkları çalışmada ELISA yöntemiyle %36,4 prevalans bulduklarını bildirmişlerdir. Çetinkaya ve ark. (2002), Elazığ'da yaptıkları çalışmada prevalansın %3,5 olduğunu ve en çok preskapular lenf yumrusunun etkilendiğini bildirmişlerdir. Ekinci Yıldız ve İçen (2022), materyallerden *C. pseudotuberculosis* teşhisi için ELISA, PCR ve kültür yöntemlerini karşılaştırmışlar ve en fazla pozitiflik oranını PCR ile bulduklarını ve daha sonra sırasıyla ELISA ve kültür yönteminin geldiğini bildirmişlerdir. Bu çalışmada koyun ve kuzularda görülen lezyonlardan alınan 4 apse materyalinde etken saptandı. Toplam 90 koyun ve kuzuda %4,44 oranında *C. pseudotuberculosis* identifiye edildi. Bu sonuç Çetinkaya ve ark. (2002)'nın bildirdiği oran ile benzerdir.

Hastalığın tedavisinde antibiyotikler sıklıkla kullanılmaktadır. Yapılan çalışmalarda *C. pseudotuberculosis*'in farklı antibiyotiklere karşı duyarlı olduğu bildirilmiştir. Algammal (2016), izole ettikleri *C. pseudotuberculosis* suşlarının sırasıyla penisilin, eritromisin, siprofloksasin, amikasin, neomisin ve stretomisin'e duyarlı; metisilin ve novobiyosin'e orta duyarlı olduklarını bildirmişlerdir. Damaty ve ark. (2023), izole ettikleri elli dört *C. pseudotuberculosis* suşunun tamamının basitrasin ve florfenikole dirençli, norfloksasine ise duyarlı olduğunu bildirmişlerdir. Ayrıca penisilin, eritromisin ve cefadrine karşı yüksek direnç ile birlikte %7,4 oranında vankomisin direnci tespit etmişlerdir. Bu çalışmada sahada kullanılan antibiyotik etken maddeleri disk difüzyon testi ile değerlendirildi. Algammal (2016)'nın bildirdiği siprofiloksasin duyarlılığı bu çalışmada izole edilen suşların tamamında tespit edildi. Ayrıca timikosin, tetrasiklin ve klindamisin'e de izole edilen dört *C. pseudotuberculosis* suşu duyarlı bulundu. Bu antibiyotik etken maddelerin tedavide kullanılabileceği düşünüldü.

Hastalık deri yaraları, burun ve ağız boşluğu ile koyunların vücuduna girerek, lenf düğümlerinde apselere neden olmaktadır. Bu apseler deri yüzeyine açıldığında, enfekte içerik direk temas yoluyla bulaşma kaynağı olmakta ve sağlıklı hayvanları enfekte etmektedir Ayrıca akciğerde meydana gelen enfeksiyonda öksürme yoluyla da sağlıklı hayvanlar enfekte olabilmektedir (Oreiby ve ark., 2013; Umer ve ark., 2017).

Bu çalışmada kırkım sonrası abdominal ve scapular bölgede meydana gelen derideki portantrelerden koyunların vücuduna giren etken, bu bölgelerde bulunan lenf düğümlerinde apselere neden olarak lezyon meydana getirmiştir ve apselerin açılması sonucu sürüdeki sağlıklı hayvanları içerik kontaminasyonu sonucu enfekte edebileceği düşünüldü.

Bu çalışmada, kültür yöntemi ile tüm örneklerde *C. pseudotuberculosis* izole ve identifiye edildi.

Antibiyotik duyarlılıklarını belirlemek üzere gerçekleştirilen disk difüzyon testinde, tüm *C. pseudotuberculosis* suşlarının tilmikosin, siprofloksasin, tetrasiklin ve klindamisine duyarlı olduğu görüldü. Koyunlara uygulanan apse ve tilmikosin tedavisi ile uzun etkili amoksisilin sağaltımıyla apselerin iyileştiği görüldü.

Sonuç olarak, bu çalışma, Balıkesir ilinde koyunların lenf düğümleri apselerinde *C. pseudotuberculosis*'in varlığı ve teşhisi hakkında bilgi sağlamıştır. Bu bulguların, Balıkesir ilinde *C. pseudotuberculosis* enfeksiyonunun epidemiyolojik açıdan varlığı, yayılması, sağaltımı ile kontrol ve korunmasına yönelik önlemlerin alınmasına yardımcı olabileceği düşünüldü.

Çıkar çatışması

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

Etik izin

Bu çalışma çiftlik veteriner hekimi tarafından mikrobiyolojik teşhis amaçlı alınan ve mikrobiyolojik teşhis amacıyla laboratuvara gönderilen materyal ile yapılmıştır. Etik Kurul iznine tabi değildir. Ayrıca yazarlar Araştırma ve Yayın Etiğine uyulduğunu beyan etmişlerdir.

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Makalenin benzerlik oranının sisteme yüklenen raporda belirtildiği gibi % 3 olduğunu beyan ederiz.

Teşekkür

Yazar, Balıkesir Üniversitesi Kepsut MYO Veterinerlik Bölümü Laborant ve Veteriner Sağlık Programı öğrencileri Irmak Ergün, Meryem İpekoğlu, Bahadır Tilki ve Sefa Sayan'a laboratuvar hazırlıkları için teşekkür eder.

Yazar Katkıları

Fikir/Kavram: O.B
Tasarım: O.B
Denetleme/Danışmanlık: O.B
Veri Toplama ve/veya İşleme: O.B
Analiz ve/veya Yorum: O.B
Kaynak Taraması: O.B
Makalenin Yazımı: O.B
Eleştirel İnceleme: O.B

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Traumatic Diaphragmatic Hernia in Cats and Factors Affecting Survival (A Clinical Study with 24 Cats)

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Abstract: Diaphragm hernia is still a severe problem in cats exposed to trauma. This study aimed to contribute to clinical practice and colleagues by notifying clinical observations on traumatic diaphragm rupture in cats and factors effective on survival. In this study, 24 cats with traumatic diaphragm hernia were presented. Contrast radiography has provided great convenience in diagnosing suspicious cases. 20 out of 24 cats (83%) remained alive, but four died. The localization of the diaphragm of these cats in 3 cats was in the left half, which was relatively high (50%) in the left tears. In the defects on the right, the herniated organs were the liver, small intestine, and omentum, while the stomach, small intestine, and spleen in the defects on the left. As a result, survival in 83% is important, and this is a good prognosis for aperture hernia. Despite many negative stress factors, good operation management and postoperative maintenance can increase survival.

Keywords: *Cat, Diaphragmatic hernia, Survival rate, Trauma.*

Kedilerde Travmatik Diyafram Fıtığı ve Sağkalımı Etkileyen Faktörler (24 Kediyle Klinik Bir Çalışma)

Özet: Travmaya maruz kalan kedilerde diyafram fıtığı hala ciddi bir sorundur. Bu nedenle kedilerde travmatik diyafram yırtılması ve hayatta kalma üzerine etkili faktörler ile ilgili klinik gözlemler aktarılarak klinik pratiğe ve meslektaşlara katkı sağlanması önemlidir. Bu çalışmada travmatik diyafram fıtığı tanısı alan 24 kedi sunuldu. Kontrastlı radyografi şüpheli durumlarda tanı açısından büyük kolaylık sağladı. Çalışmada bulunan 24 kediden 20'si (%83) hayatta kaldı ancak 4'ü öldü. Bu kedilerin diyafram lokalizasyonu 3 kedide sol yarıda olup, sol yırtıkta bu oran oldukça yüksekti (%50). Sağdaki defektlerde fıtıklaşan organ karaciğer, ince bağırsak ve omentum, soldaki defektlerde ise mide, ince bağırsak ve dalaktı. Sonuç olarak %83 oranında hayatta kalma önemlidir ve bu da diyafram fıtığı için iyi bir prognozdur. Birçok olumsuz stres faktörüne rağmen iyi bir ameliyat yönetimi ve ameliyat sonrası bakım, hayatta kalma oranıyla birlikte artırılabilir.

Anahtar Kelimeler: *Diyafram fıtığı, Kedi, Sağkalım oranı, Travma.*

Introduction

The diaphragm is a membranous muscle section separating the abdomen and chest. Rupture or disruption of the integrity of this thin muscle structure is called diaphragmatic hernia. This condition may be congenital or acquired. In acquired cases, the etiology is often trauma. The source of trauma may be a fall from a height, a traffic accident or another animal attack (Ozer et al., 2007; Temiz, 2017).

In congenital diaphragmatic hernias, peritoneal-pericardial diaphragmatic hernia (PPDH) is more common, and PPDH is caused by defective development of the fetus. The overall prevalence of PPDH in domestic cat populations has been reported to be 0.062–0.59%. This condition should be considered separately from traumatic type diaphragmatic hernia or rupture. When a traumatic diaphragmatic tear/hernia occurs, organs such as the omentum, stomach, liver, and intestines can enter the chest cavity from the abdomen and cause compression of the lungs, preventing them from fully inflating and causing respiratory distress known as dyspnea. Additionally, irritation of the heart muscle often causes abnormal heart rhythms. Fluid can leak into the chest cavity, further complicating and worsening cardiopulmonary function (Margolis et al. 2018; Temiz, 2017).

The diaphragm consists of a central tendinous part and an outer muscular part. The muscle part is divided into three parts (pars lumbalis, pars costalis and pars sternalis), and the pars costalis is often ruptured. The diaphragm has three openings: the caval foramen, the esophageal cavity, and the aortic cavity. Three different types of diaphragmatic tears are commonly seen: circumferential (40%), radial (40%) and combination (20%) (Pereira et al., 2023). Clinical symptoms may vary depending on the location of the rupture and the herniated organs. The most common symptom is shortness of breath. In addition, cardiac arrhythmias and shock, enteric symptoms and vomiting may occur (Yaygıngül et al., 2019).

In patients with respiratory distress, management such as oxygen therapy, fluid therapy, and the use of inhaler treatments along with monitoring have a major role in the prognosis (Aulia et al., 2022; Deveci et al., 2022; Fucks de Souza et al., 2021).

This study aimed to inform clinical practice and our colleagues about the factors affecting clinical observations and survival rates in cats with traumatic diaphragmatic hernia.

Material and Methods

This study is not subject to HADYEK permission by Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

In this study, 24 cat cases diagnosed with traumatic diaphragmatic hernia among cats brought to Dicle University Veterinary Faculty Animal Hospital surgical clinics on different dates were investigated. Diaphragmatic hernias that were not accepted for surgery by their owners during the period of this study and were congenital were not included in the study. In addition to information such as age, gender, source or type of trauma, clinical findings, intraoperative findings, postoperative period findings, and complications were noted. A detailed clinical examination was performed for each cat (Table 1).

Table 1. General information about the cats operated on in the study.

	Gender	Cause of trauma	Age/month	Body weight/kg	breed
1	Male	Fall from height syndrome	12	4.8	Mix breed
2	Male	Fall from height syndrome	18	4.6	Persians
3	Male	Traffic accident	8	1.5	Mix breed
4	Female	Fall from height syndrome	15	4.7	Mix breed
5	Male	Fall from height syndrome	17	3.9	scottish fold
6	Female	Traffic accident	15	4.1	Mix breed
7	Male	Fall from height syndrome	19	4.3	scottish fold
8	Male	Fall from height syndrome	14	4.5	Mix breed
9	Male	Fall from height syndrome	17	3.8	Persians
10	Female	Traffic accident	23	4.8	Mix breed
11	Male	Fall from height syndrome	24	4.7	scottish fold
12	Female	Fall from height syndrome	29	4.8	Mix breed
13	Male	Traffic accident	72	3.9	Persians
14	Male	Fall from height syndrome	70	4.2	Mix breed
15	Male	Fall from height syndrome	68	4.6	Scotish fold
16	Female	Fall from height syndrome	60	3.6	Mix breed
17	Male	Fall from height syndrome	54	3.8	Siamese breed
18	Male	Fall from height syndrome	58	4.8	Siamese breed
19	Male	Traffic accident	62	3.6	Mix breed
20	Male	Traffic accident	36	3.9	scottish fold
21	Female	Fall from height syndrome	18	4.6	scottish fold
22	Male	Fall from height syndrome	68	6.7	Mix breed
23	Female	Fall from height syndrome	43	7.0	Mix breed
24	Female	Unknown	18	4.5	Mix breed

Since these patients were trauma patients, trauma management was taken into consideration. In the first evaluation, he was evaluated with plans A (air way), B (breathing), C (circulation), D (neurological disability). Thoracic trauma was evaluated until proven otherwise. After their general condition was stable, direct radiographs including the thorax and abdomen were examined (Ecotron EPX-F5000 (110 kv-100mA) Fujifilm) for definitive diagnosis in cats. In cases where the diagnosis was doubtful with plain radiography, it was evaluated with contrast radiography (with iohexol 40/50 mg/kg oral, Omnipaque™ Opakim-Istanbul) (Fig 1-3).

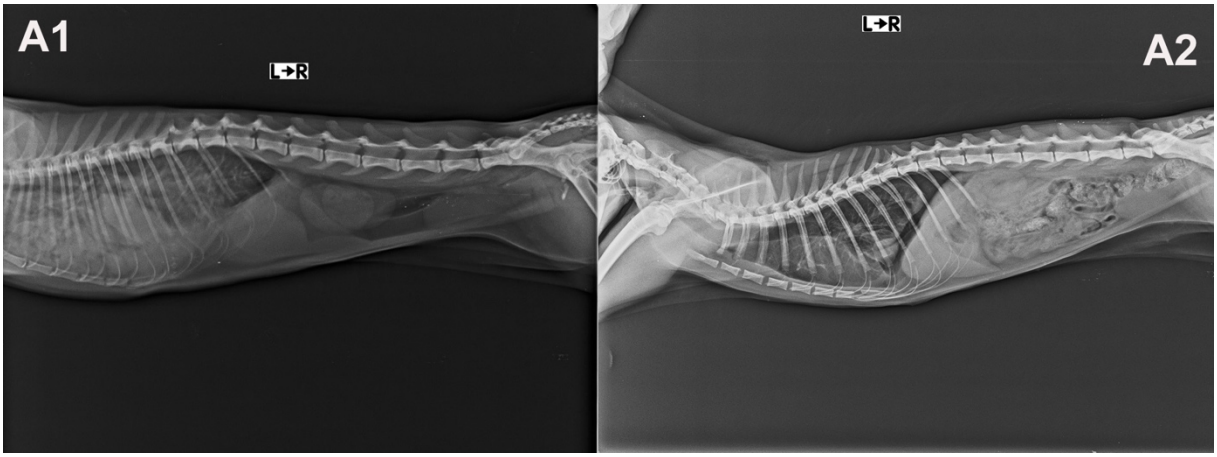

Figure 1. A1 Direct radiological appearance of intestinal loops in the thorax. **A2** Post-operative image of the case.

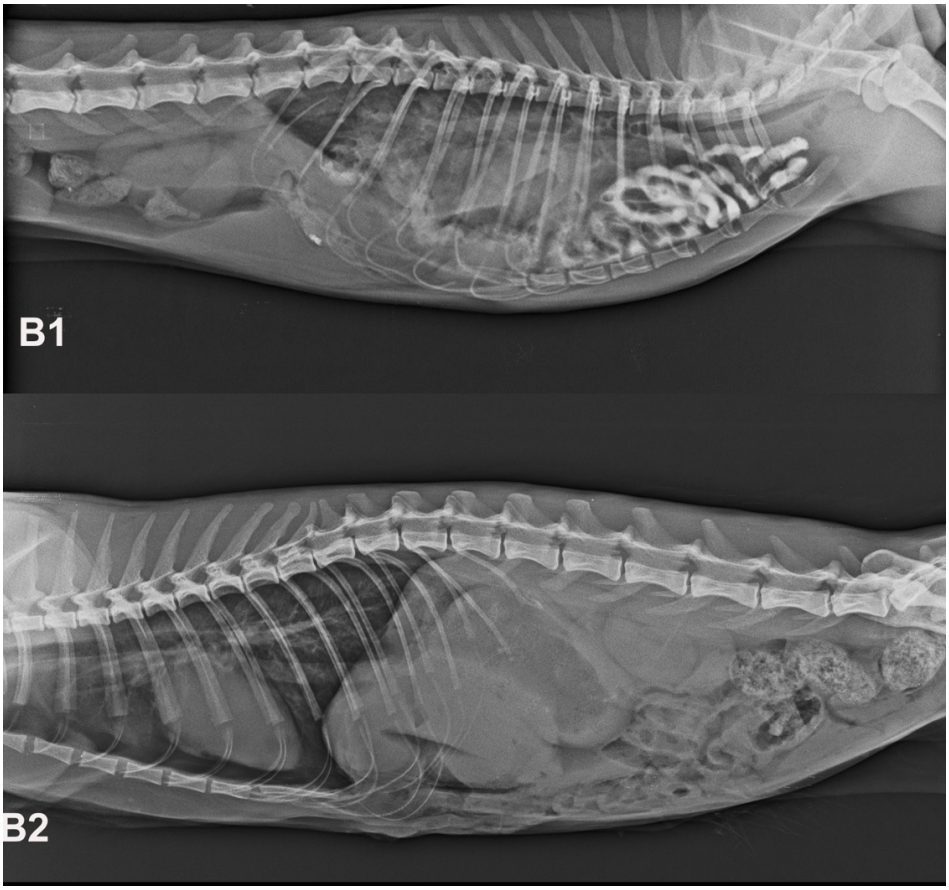

Figure 2. B1 View of the intestinal segment within the thorax using contrast radiography. **B2** Post-operative image of the case.

Venous catheterization was provided to the ramus dorsal of vena sephana parva, and then, during the clinical examination, venous blood was taken before anesthesia and at the 24th postoperative hour for hematological and biochemical analyses.

Even if the general condition of the patients was stable, their surgery was planned after 48 hours. For perioperative effect, cefazolin sodium (Cezol 250 mg, Deva, Türkiye, 20 mg/kg IM) was administered as an antibiotic before surgery, and meloxicam (Maxicam, Sanovel, Türkiye, 0.3 mg/kg SC)

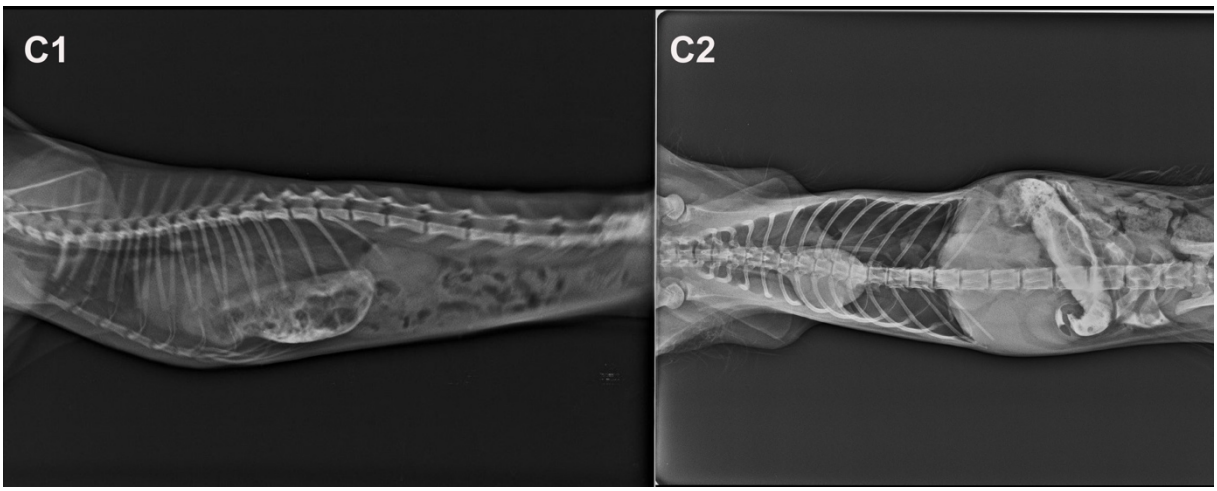

Figure 3. C1 View of the stomach within the thorax in a cat with a diaphragmatic hernia. **C2** View of thorax postoperatively.

was administered as an analgesic. In addition, preoperative oxygen was administered using a mask or in the intensive care cabin for 10 minutes. After injection xylazine HCL (Xylazin Bio, Interhas, Türkiye, 1 mg/kg/IV) and ketamine HCL (Ketasol, Interhas, Türkiye, 10 mg/kg/IV) was given for induction, endotracheal intubation was achieved (with No. 3-3.5 tube) and then anesthesia was maintained with sevoflurane in 100% O2. Mannitol and Lactated Ringer's solution (10 ml/kg/hour, IV) were given for perioperative and intraoperative fluid management.

In cats that were placed on their backs, the aseptically prepared operating area was covered with sterile drapes. The diaphragm was approached through a ventral median line laparotomy extending from the xiphoid cartilage to the umbilicus. The organs that passed into the chest cavity were transferred back to the abdominal region. This study categorized the localization of diaphragmatic hernia in two different ways. The first is defined by Deveci (Deveci et al., 2022), and the second is circumferential, radial, and combination, as defined by Bjorling (Bjorling and Sicard, 2004). The diaphragmatic defect was repaired with simple continuous or X suture techniques with non-absorbable thread (Prolene, Ethicon, USP 2/0-3/0). Negative pressure of the chest cavity was created before the final stitching. The operating area was then routinely closed. Anesthesia was terminated and extubated. Oxygenation continued in the intensive care cabin for half an hour. Additionally, the administration of cefazolin sodium (Cezol 250 mg, Deva, Türkiye, 20 mg/kg IM, once a day) was continued for 7 days postoperatively.

Results

The etiology of diaphragmatic hernia in all cases was traumatic. Among these, there were falls from height in 18 cases (75%), motor vehicle accidents in 5 cases (20%), and blunt trauma of unknown cause (a cat running away from home) in 1 case (4%). The clinical presentation times of the cases were between 1-7 days after the trauma. The age of the cats varied, and the average age ranged from 8 months to 72 months. 16 (66%) of the cats were male and 8 (33%) were female. Their average body weight was between 1.5-7 kg.

In the clinical examination of the study, all patients (100%) had tachypnea, respiratory distress, open mouth breathing, abdominal breathing and exercise intolerance. There were also cases of anorexia (15 cats, 62.5%) and vomiting (3 cats, 12.5%). While direct radiography was sufficient for diagnosis in 16 cases (66%), contrast radiography was performed in the other 8 (33%) suspicious cases. It was found remarkable that the diaphragmatic borders were not clear, and the heart silhouette disappeared in the radiographic examination. Additionally, abdominal organs were observed to be displaced. Intestinal segments within the thorax were more easily identified. In addition to diaphragmatic hernia in the cats in this study, radius-ulna fracture was detected in 1 cat, humeral fracture in 1 cat, cleft palate in 2 cats, and soft tissue trauma in 3 cats.

According to the scale of Deveci et al. (2022), the location of the diaphragmatic tear in this study was at 10 o'clock in 12 cases (50%), at 11 o'clock in 5 cases (20%), at 14 o'clock in 3 cases (12.5%), at 12 o'clock in 2 cases (8%), and along the length in 1 case (4%). According to the scale from Bjorling and Sicard (2004), the localization of the defect was determined as circumferential in 21 cases (87.5%), radial in 2 cases (8%), and a combination in 1 case (4%).

In this study, the herniated organs in the defects on the right side were the liver, small intestine, and omentum, while the stomach and small intestine herniated in the defects on the left side. The spleen was in the thorax in only one case (with a longitudinal defect).

A total of 3 (12.5%) cats died, 2 during the operation and 1 within two hours after the operation. During the operation, it was determined that both cats that died had lung lacerations and excessive secretions. In 2 of these cats, the diaphragm defect was on the left side. In other words, 2 of 4 cats (50%) with defects in the left half of the diaphragm (3 cats only left, 1 cat left + right half) died, which was found to be quite high. Another cat with a right diaphragmatic rupture died 24 hours after the operation. Despite all interventions (tocaricocentesis, IV support, oxygen support, cardiopulmonary resuscitation) in this cat with excessive secretion in the thoracic region, the cat could not be saved.

The mortality rate was calculated to be 8% in 2 cats where only the right half was affected.

In this study, no complications such as bleeding, organ rupture, or surgical site infection were encountered during the organ rejection operation. It was found remarkable that clinical findings such as difficulty in breathing, abdominal breathing and open mouth breathing improved within 24 hours after surgery.

Discussion and Conclusion

Diaphragmatic hernias, other than congenital ones, in cats develop due to traumatic causes and are among the critical life-threatening surgical cases in small animal practice (Borges et al., 2023). In this study, we aimed to provide information to clinical practice and our colleagues about the factors affecting the survival rate, as well as clinical observations in cats with traumatic diaphragmatic hernia.

Although it is more common especially in falls from height and traffic accidents, it can also occur in other types of trauma. A patient with trauma should be examined for cleft palate and diaphragm rupture (Borges et al., 2023; Fucks de Souza et al., 2021). In all cats included in the study, the etiology of diaphragmatic hernia was traumatic, primarily falling from heights and traffic accidents. The clinical presentation times of the cases were between 1 and 7 days after the trauma. Deveci et al. (2022) stated that traumatic diaphragmatic hernias that have been present for 15 days or more are chronic. Some authors emphasize that in order to reduce mortality in traumatic diaphragmatic hernia, surgery should not be performed within the first 24 hours following trauma and that surgery should be planned after the patient's physiological parameters are stable (Deveci et al., 2022; Mehrjerdi et al., 2022; Özer et al., 2007). Deveci et al. stated that due to advances in equipment and care conditions in the field of surgery, the patient can be operated on within the first 24 hours if appropriate conditions are provided in the preoperative, operative and postoperative process after stabilization. Therefore, diaphragmatic hernia may not be considered an emergency. However, the hemodynamic status of the patient should be stable after the trauma. In this study, all operative procedures were planned and performed after the patient's general condition was stable.

Many different studies have emphasized good trauma management of traumatic diaphragmatic hernias. Oxygen support should be taken into consideration, especially in addition to taking the necessary interventions and precautions to keep the animal alive. In addition, if fluid therapy is to be performed, fluid selection and administration rate should be adjusted very well (Fucks de Souza et al., 2021; Subramaniam et al., 2020). As a matter of fact, in this study, first of all, it was tried to keep people alive and a good trauma management was carried out. Oxygen support was provided and surgeries were planned after the general condition of the patients was improved.

The main systems affected in patients with diaphragmatic hernia are the circulatory and respiratory systems. Therefore, clinical symptoms such as tachypnea, respiratory distress, and abdominal breathing are extremely prominent (Aulia et al., 2022; de Oliveira, 2020; Subramaniam et al., 2020). In addition, symptoms such as vomiting, difficulty breathing, and relaxation by holding the front of the body up may also be observed (Aulia et al., 2022). In our study, all patients had tachypnea, respiratory distress, open-mouth breathing, abdominal breathing, and exercise intolerance. In addition, cases of anorexia (15 cats, 62.5%) and vomiting (3 cats, 12.5%) were also encountered. In our study, radiology played an important role in diagnosing diaphragmatic rupture. In addition, intestinal segments in the thorax, where abdominal organs are displaced, were more easily identified.

When the localization of the defect was evaluated according to the Deveci et al. (2022) scale and the Bjorling and Sicard (2004) scale, the relationship between the diaphragm defect and the herniated organs is very clear according to the data in this study. On the other hand, it is questionable whether there is a relationship between diaphragm defect and survival. Although it is not certain, it can be said that the mortality rate is higher in left-sided hernias. This situation can be interpreted as follows; diaphragmatic hernias from the left side or along the length are always more serious. It can be explained by both the pressure on the organs and the change in venous pressure.

In defects on the right side of the diaphragm, the liver and small intestine herniated, while in defects on the left side, the stomach and small intestine were displaced (Cariou et al., 2009; Hyun, 2004; Mehrjerdi et al., 2022; Tsioli et al., 2020). In our study, the herniated organs in defects on the right side were the liver, small intestine, and omentum. However, the relationship between the defect or herniated organ and prognosis or survival is not fully known. In this sense, we think that each case should be decided according to its own clinical picture.

In summary, a total of 4 cats died in this study and all of the remaining 20 cats (83%) survived. Postoperative survival rate was reported as 82.2% by Beşaltı et al. (2011), 73.3% by Yaygıngül et al. (2019), 71% by Mehrjerdi et al. (2022), 83.3% in acute cases and 69.2% in chronic cases by Deveci et al. (2022). Özer et al. (2007) reported the mortality rate as 6.8% in their study. Our study results are parallel to the results obtained by Deveci et al. (2022) and Beşaltı et al. (2011). It has been reported that (Oliveira, 2020; Deveci et al., 2022; Ozer et al., 2007) deaths may occur during or even after surgery in cats with diaphragmatic hernia, but these deaths are not as high as expected. Additional measures must be taken to reduce the mortality rate further. Preoperative planning, intraoperative support and postoperative care conditions are also important. In order for the patient to be operated on, physiological parameters must be stable. In addition, preoperative oxygen support and postoperative oxygen support are very important. During the operative procedure, care should be taken to ensure that the lungs do not collapse and the negative pressure within the rib cage should be adjusted (Deveci et al., 2022; Mehrjerdi et al., 2022; Ozer et al., 2007).

It has been reported by different researchers that clinical symptoms such as difficulty breathing, abdominal

breathing, and open-mouth breathing improve very quickly after surgery (Besalti et al., 2011; Hyun, 2004; Minihan et al., 2004; Nikiphorou et al., 2016; Pereira et al., 2023). In our study, these symptoms also improved in the first 24 hours postoperatively.

Some authors who have assumed two weeks as chronic cases, pneumothorax, diseases that occur during the separation of the organs, characteristic lobe torsion, intestinal ganglion diseases increase mortality (Beşaltı et al., 2011; Minihan and Işık., 2004). During the operation, the spurting organs should be carefully placed back in their original place so that they are not seen and do not undergo torsion. Compression applied to the caudal vena cava will cause venous components (Souza et al., 2021). The lobes where the microorganism herniates should be removed separately and care should be taken not to undergo torsion. In all cases with this effect, the operation was performed by observing this situation and no complications were encountered. In the case that occurred in the postoperative period, rather than these components, the forces that can be obtained from the excessive secretion ability in the thorax.

Traumatic diaphragmatic hernia in cats, location, size, type of defect in the diaphragm and other accompanying disorders or pathologies, if any, affect the mortality rate. For this reason, the priority in trauma patients should be to keep them alive and especially breathing support is needed. It is clinically easy to suspect a diaphragmatic hernia and confirm the diagnosis. If necessary, support can be obtained from contrast radiography for this purpose. In case of a traumatic diaphragm rupture, it would be beneficial not to act in a hurry and wait for the first 24 hours for the operation. Once the general condition of the patient is stable, the operation can be performed by making the necessary planning.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

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Analysis or interpretation: SY, BEK, NS
Literature Search: EÇ, BEK, SA
Writing: BEK

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Effect of Acetylsalicylic Acid Treatment on Gait Score and Femur Osteometry in Broiler Chickens

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Abstract: The current research examined the effects of adding 0.03%, 0.06%, 0.1%, 0.3%, and 0.6% acetylsalicylic acid (ASA) to drinking water between 24 and 45 days on gait score and femur osteometry. The gait score average of the birds slaughtered on the 49th day was above 3, and no difference was found between the groups. It was determined that ASA treatment did not affect the osteometric and index values of the femur bones taken and examined after slaughter. In conclusion, it was concluded that ASA was not effective in preventing skeletal deformations caused by rapid growth in broilers.

Keywords: *Acetylsalicylic acid, Femur, Rapid growth, Welfare.*

Etlik Piliçlerde Asetilsalisilik Asit Uygulamasının Yürüme Skoru ve Femur Osteometrisi Üzerine Etkisi

Özet: Yapılan araştırmada 24 ve 45. günler arasında içme suyuna %0.03, %0.06, %0.1, %0.3 ve %0.6 asetilsalisilik asit (ASA) eklenmesinin yürüyüş skoru ve femur osteometrisi üzerine etkileri incelenmiştir. 49. günde kesilen tavukların yürüyüş skoru ortalaması 3'ün üzerinde olup gruplar arasında fark bulunmamıştır. Kesim sonrası alınarak incelenen femur kemiklerinin osteometrik değerlerini ve indeks değerlerini etkilemediği belirlenmiştir. Sonuç olarak ASA'nın etlik piliçlerde hızlı büyümeye bağlı iskelet deformasyonlarını önlemede etkili olmadığı sonucuna varılmıştır.

Anahtar Kelimeler: *Asetilsalisilik asit, Femur, Hızlı büyüme, Refah.*

Introduction

Orthopaedic problems are considered an important concern for broiler production and welfare. Foot and leg problems cause lameness, which causes pain, decreased mobility, and difficulty acting on basic behaviours, such as accessing water resources in chickens. In conclusion, this results in reduced yield and economic losses (Bessei, 2006; de Jong et al., 2016; Gocsik et al., 2017; Yang et al., 2023). Fast growth is a critical aspect impacting musculoskeletal health in commercial broilers. Physiologically, skeletal development should be consistent with the organism's growth rate. The growth rate of broilers increases dramatically in the early stage, but the skeletal development rate is slower than weight gain. Furthermore, these phenomena make weaker skeletons relative to huge body weight, quickly leading to skeletal disorders in broilers, especially in the legs and feet (Duggan et al., 2015; Shim et al., 2012; Xu et al., 2022; Yan et al., 2019). Some reports demonstrate that between 14% and 50% of broilers exhibit moderate to severe foot problems in commercial flocks during the final stages of the growing period (de Jong et al., 2016; Yang et al., 2023). In addition, studies indicate that fast-growing broilers have decreased bone mineral density and a lower bone ash percentage than slow-growing broilers (Shim et al., 2012).

Acetylsalicylic acid (ASA) is a non-steroidal anti-inflammatory drug that affects numerous biological pathways. As it is known, the ASA's mechanism of action acts by inhibiting cyclo-oxygenase-1 (COX-1), cyclo-oxygenase-2 (COX-2), and anti-platelet aggregation by reducing cytokine production (Hida et al., 2023). ASA additionally supports osteogenesis and prevents osteoclastogenesis, which means it can potentially improve bone health. (Cao et al., 2015; Liu et al., 2015; Wada et al., 2013). Moreover, some studies suggest that ASA could regulate bone metabolism (Fang et al., 2018; Shi et al., 2008; Yamaza et al., 2008).

This study aimed to benefit from ASA's osteogenesis effects to help reduce orthopedic problems in fast-growing broiler chickens. This research examined the impact of different ASA dosages on the gait score and femur osteometry of chickens during their growth phase.

Materials and Methods

Housing, feeding protocol, and experimental design: 300 one-day-old Ross 308 male broiler chickens (Aviagen, Newbridge, UK) were obtained from a commercial farm and participated in a 49-day study. The chicks were selected at random into six groups: (C) Control (untreated); (ASA1) added 0.03% acetylsalicylic acid to drinking water; (ASA2) added 0.06% acetylsalicylic acid to drinking water; (ASA3) added 0.1% acetylsalicylic acid to drinking water; (ASA4) added 0.3% acetylsalicylic acid to drinking water; (ASA5) added 0.6% ASA to drinking water. Each group included five repetitions and ten birds over the study period. The diet was uniform for all six treatment groups. The broilers have full access to both feed and water. They followed a feeding protocol, starting with a starter diet for the first 24 days of their lives, then transitioning to a grower diet from day 24 to day 35, and then switching to a finisher diet from day 36 to day 49 (Table 1). Additionally, water-soluble ASA treatment was administered to the groups throughout the growth phase until the end of the finisher phase (24-45 days). The nutritional content and requirements of the subject were assessed according to NRC guidelines. The chicks were housed in a 1 m×1 m pen; the floor was covered with wood shavings in a mechanical ventilation room. Throughout the initial stage (first five days), the temperature within the feeding chamber was maintained at a uniform range of 32-34°C. Afterward, the temperature was systematically decreased by two °C weekly until it reached the final range of 23±1°C. The relative humidity was consistently maintained at 40 to 60 percent. For the initial three days, the broilers were exposed to nonstop illumination, which was then replaced by a routine of 23 hours of light and 1 hour of night-time. At the end of the experiment, 90 animals, three from each replication and 15 from each group reflecting the replication average, were selected and ethically slaughtered.

Gait score: Gait score (GS) assessment was performed before the chickens were slaughtered. The gait scores were determined by consensus between the two trained observers. Trained assessors have classified all broilers into six score levels (0–5) following the Welfare Quality Assessment Protocol (Welfare Quality®, 2009), shown in Table 2.

Femur osteometry: The surrounding tissues were removed after grouping the right femur bone samples obtained from slaughtered chickens. Then, samples were kept in hydrogen peroxide for 2 hours. Measurements were made after the bones were cleaned of fat and dried. Measurements were conducted following the data-collecting protocol established by A. von den Driesch (von den Driesch, 1976). Figure 1 presents the metrics, including abbreviations and definitions used in the dataset.

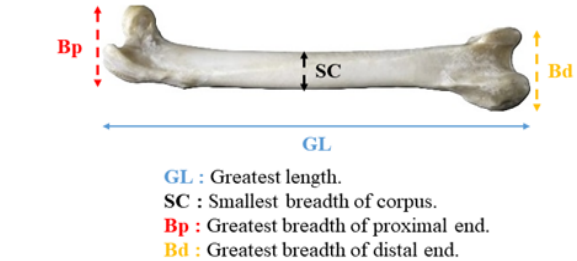

Figure 1. Abbreviations and definitions used in right femur osteometric measurements.

Statistical analysis: The ANOVA test was performed to compare the groups. For significant parameters, the Tukey HSD test was used for intra-group comparisons. For data with scoring, the Kruskall-Wallis H test and Mann-Whitney U

Table 1. Ingredients, chemical composition, and energy of the diets used during the grower period (24 to 35 d of age) and finisher period (36 to 49d of age).

Basal diet ingredients	Grower period (24-35)	Finisher period (36-49)
Corn	60.29	63.53
Soybean meal (44% crude protein)	28.34	24.20
wheat bran (Razmol)	4.00	5.00
Vegetable oil	4.21	4.15
Dicalcium Phosphate	1.33	1.33
Ground Llimestone	0.97	0.89
DL- Methionine	0.25	0.25
L- Lysine hydrochloride	0.16	0.20
L- Threonine	0.08	0.08
Salt	0.35	0.35
Vitamin and mineral supplements*	0.20	0.20
Nutritional Composition, (%)**		
Dry matter	90.00	89.90
Crude protein	19.50	18.00
Ether extract	6.07	6.08
Ash	5.51	5.25
Crude fiber	2.73	2.72
Starch	41.61	43.65
Calcium	0.78	0.74
Available phosphorous	0.39	0.38
Sodium	0.17	0.17
Chlorine	0.25	0.25
Methionine+Cystine	0.90	0.85
Lysine	1.15	1.07
Threonine	0.80	0.73
Tryptophan	0.26	0.23
Linoleic acid	3.45	3.48
ME, kcal/kg**	3200	3225

*: Vitamin-mineral premix supplied per kg: Vitamin A, 12000 IU; Vitamin D3, 3000 IU; Vitamin E, 30 mg; Manganese, 80 mg; Iron, 60 mg; Zinc, 60 mg; Copper, 5 mg; Iodine, 1.5 mg; Cobalt, 0.3 mg; Selenium 0.15 mg.
**: Calculated.

Table 2. Practicing a gait score for broiler chickens

Gait score	Measurement for gait score assessment
0	Typical, smooth, and agile. The toes are flexed while lifted
1	There is a slight anomaly, but it cannot be identified.
2	It is an actual and identifiable abnormality, but it has limited effects on walking ability.
3	Apparent abnormality affects the ability to walk. The bird has imbalanced steps and squats within 15 seconds.
4	Severe abnormality, but still capable of stepping. The chicken takes over five seconds to rise when nudged and squats after a few steps.
5	Incapable of stepping

test were utilized. The SPSS 21 package tool was used for statistical analysis. The difference between group means was statistically significant (P<0.05).

Results

Gait score: The gait score is an indicator commonly utilized for the measuring lameness in broilers. In current research, broiler gait scores were not affected by whether ASA was applied or not (P>0.05). As stated in Figure 2, the average gait score of the broilers was between 3-4 in all groups.

Femur osteometry: Table 3 shows the impact of adding acetylsalicylic acid to drinking water at different doses on the osteometric properties of the right femur in broiler chickens. Both osteometric measurements and index values showed no difference between the groups (P>0.05).

Discussion and Conclusion

Previous research has suggested that a gait score of 3 or more is a painful situation linked to poor welfare and inactivity (Caplen et al., 2013; Nääs et al., 2009). Broiler gait score is highly correlated with body weight. The rapid growth of broilers can lead to the placement of abnormally high weights on relatively infancy bones, which could impact their gait score (Kittelsen et al., 2017; Yang et al., 2023). Researchers have reported that ASA treatment positively affects broilers' carcass weight and yield (Fathi et al., 2016; Tavakoli et al., 2022). Also, researchers have found that administering one-time painkillers may increase the walking ability of heavier and older chickens. Studies point to a slight improvement in gait score, particularly for birds severely affected by lameness (Almeida Paz et al., 2019; Caplen et al., 2013; Nääs et al., 2009). However, this study showed that long-time ASA treatment did not affect the gait score in birds. The absorption of ASA following oral administration is fast and complete. The absorption is directly proportional to the dose and, thus, follows first-order pharmacokinetics

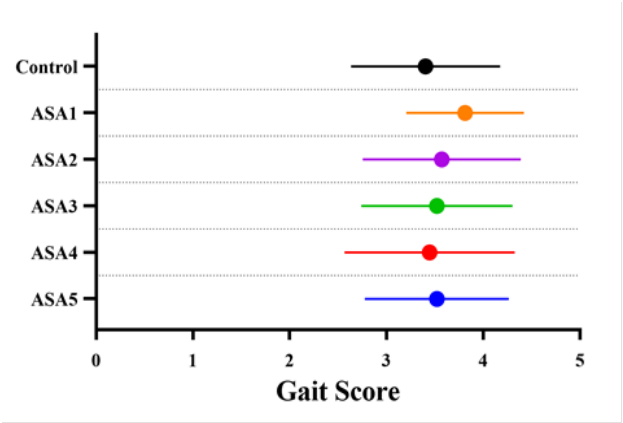

Figure 2. Effect of ASA treatment in drinking water on broiler Gait score. Control (without treatment); (ASA1) added 0.03% acetylsalicylic acid to water; (ASA2) added 0.06% acetylsalicylic acid to water; (ASA3) added 0.1% acetylsalicylic acid to water; (ASA4) added 0.3% acetylsalicylic acid to water; and (ASA5) added 0.6% ASA to water. The mean and standard error represent the values. P>0.05.

Table 3. The effect of acetylsalicylic acid added to drinking water at different doses on broiler chickens' osteometric properties of the right femur.

Osteometric Properties (mm)	Control	ASA Groups					P
		ASA1	ASA2	ASA3	ASA4	ASA5	
GL	114.89±1.40	114.16±1.39	114.77±0.78	115.60±0.55	115.37±0.53	115.48±0.81	NS
Bp	20.43±0.37	20.89±0.26	20.87±0.35	20.33±0.35	20.64±0.19	20.51±0.32	NS
SC	8.76±0.21	9.14±0.17	8.72±0.18	8.88±0.24	9.20±0.21	8.89±0.23	NS
Bd	24.08±0.43	24.21±0.47	23.94±0.33	23.81±0.44	24.30±0.31	23.36±0.36	NS
Bp*100/GL	17.78±0.28	18.13±0.23	18.19±0.26	17.60±0.32	17.90±0.17	17.78±0.30	NS
SC*100/GL	7.63±0.19	8.03±0.24	7.60±0.14	7.68±0.20	7.97±0.17	7.71±0.22	NS
Bd*100/GL	20.80±0.36	21.22±0.37	20.88±0.34	20.60±0.39	21.07±0.31	20.26±0.39	NS

Control (without treatment); (ASA1) added 0.03% acetylsalicylic acid to water; (ASA2) added 0.06% acetylsalicylic acid to water; (ASA3) added 0.1% acetylsalicylic acid to water; (ASA4) added 0.3% acetylsalicylic acid to water; and (ASA5) added 0.6% ASA to water. Data are shown as means ± standard error of the mean. Not Significant (NS): P>0.05, Greatest length (GL), Greatest breadth of the proximal end (Bp), Smallest breadth of the corpus (SC), Greatest breadth of the distal end (Bd).

(Stevens et al., 2019). The pharmaceutical effect of the drug can explain why long-term use of ASA did not affect lameness in fast-growing broilers in this study.

Over the last 60 years, the genetic selection of broilers has concentrated primarily on production qualities, including growth rate and feed efficiency. This has resulted in severe

welfare troubles for broilers, such as cardiovascular issues (sudden death syndrome, ascites, etc.) or musculoskeletal disorders (Contact dermatitis, Bacterial chondronecrosis with osteomyelitis, leg weakness, and lameness) (Hartcher and Lum, 2020). Restricting feeding due to chronic hunger and stress could not be an option (Decuypere et al., 2010). Genetic lines of broilers with slower growth rates exhibit better cardiovascular function, reduced mortality, and a decreased prevalence of musculoskeletal problems and bone deformities. Numerous studies argue for slower-growing strains, which do not present the same welfare problems as fast-growing commercial strains (Bessei, 2006; Hartcher and Lum, 2020; Wilhelmsson et al., 2019).

Rapidly growing broilers may want to avoid walking or exercising. Broilers spend about 76% of their time lying down, which increases with age. As they get closer to market age, broilers spend only 3.3% of their time walking (Weeks et al., 2000). Fast-growth broilers with limited circumstances for movement and exercises are particularly weak regarding osteoporosis, lower tibia and femur mineral density, bone mass, and bone-breaking strength. Also, rapid growth is associated with skeletal deformities such as tibial dyschondroplasia, which results in lameness. One study showed that (Derakhshanfar et al., 2013) dietary ASA administration at a dose of 400 mg/L for three weeks induced histopathological changes in the development of tibiotarsal bones in chickens. However, the authors put forward the thesis that the ASA application may be beneficial based on the indirect evidence they found. Despite using much higher doses of ASA in this study, the gait score remained above 3, and the femur index was similar among groups. Acetylsalicylic acid (ASA) is primarily effective as a non-steroidal anti-inflammatory drug. Many studies suggest that ASA possesses bone protective effects (Dadwal et al., 2020; de Souza Rendohl et al., 2021). ASA can promote osteoblast precursor stem cell survival and osteoblast differentiation (Cao et al., 2015). However, these effects provide limited benefits and still need to be proved. Our study showed no difference between the groups regarding osteometric measurements, index values, or gait scores.

While breeding is a competitive industry influenced by economic factors, it is also a sensitive domain due to its importance for animal welfare. Ensuring animal welfare is essential for a sustainable chicken meat industry. More research is required to avoid musculoskeletal welfare problems related to genetic selection. As a result, adding ASA to drinking water may not be a helpful solution for musculoskeletal problems. Therefore, using ASA to improve bone condition and walking ability may not be suitable.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study was approved by the Harran University Animal Experiments Local Ethics Committee (05.08.2021, 2021/01-05 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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Control/Supervision: ÜGŞ
Data Collection and / or Processing: GG, İD, YK, SA, BK
Analysis and / or Interpretation: GG, İD, YK, SA, BK
Literature Review: GG, İD
Writing the Article: GG, İD, ÜGŞ
Critical Review: ÜGŞ

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Exploring Shape Variance in Waterbirds' Pad Feet: A Geometric Morphometric Analysis

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Abstract: Waterbirds usually have webbed feet, which help them move easily through water. These pad feet fall into four main categories: palmate, semipalmate, totipalmate, and lobate. In this study, morphological diversity among the pad feet of different waterbird breeds such as the West Indian whistling duck (*Anas bahamensis*), mandarin duck (*Aix galericulata*), red-breasted goose (*Branta ruficollis*), wood duck (*Aix sponsa*), mute swan (*Cygnus olor*), greylag goose (*Anser anser*), mallard (*Anas platyrhynchos*), Pekin duck (*Anas platyrhynchos domesticus*), redhead duck (*Aythya americana*), Egyptian goose (*Alopochen aegyptiaca*), and pelican (*Pelecanus onocrotalus*) was examined by the geometric morphometric method. 2D images of 12 waterbirds' pad feet from different parts of Türkiye were analyzed from a dorsal view. In total thirteen landmarks were used. The analysis focused on principal component 1 and principal component 2 values. Principal component 1 shows slightly greater changes occurring on the lateral toes II and IV, as well as in the interdigital webbing below the average. Principal component 2 also reveals greater shape changes on the toes II and IV, which are more lateral. Geometric morphometric analysis proves valuable in identifying variations in the shape of the pad feet among various breeds of waterbirds, making it an effective tool for taxonomic purposes.

Keywords: *Anatomy, Avian, Difference, Evaluation, Foot, Form.*

Su Kuşlarının Ayak Şekil Varyasyonlarının İncelenmesi: Geometrik Morfometrik Analiz

Özet: Su kuşları genellikle suyun içinde kolayca hareket etmelerine yardımcı olan perdeli ayaklara sahiptir. Bu perdeli ayaklar dört ana kategoriye ayrılır: palmate, semipalmate, totipalmate ve lobate. Bu çalışmada, Batı Hint düdükçü ördeği (*Anas bahamensis*), mandarin ördeği (*Aix galericulata*), kızıl göğüslü kaz (*Branta ruficollis*), ağaç ördeği (*Aix sponsa*), mute kuğusu (Cygnus olor), boz kaz (*Anser anser*), yeşilbaş ördek (*Anas platyrhynchos*), Pekin ördeği (Anas platyrhynchos domesticus), kızılbaş ördek (*Aythya americana*), Mısır kazı (*Alopochen aegyptiaca*) ve pelikan (*Pelecanus onocrotalus*) gibi farklı su kuşu türlerinin perdeli ayaklarındaki morfolojik çeşitlilik geometrik morfometrik yöntem ile incelenmiştir. Türkiye'nin farklı bölgelerinden alınan 12 su kuşunun perdeli ayaklarının dorsal görünümden 2D görüntüleri analiz edilmiştir. Toplamda on üç belirleyici nokta kullanılmıştır. Analiz, temel bileşen 1 ve temel bileşen 2 değerlerine odaklanmıştır. Temel bileşen 1, yan parmaklar II ve IV ile ortalama altındaki parmak arası perdenin olduğu bölgede meydana gelen hafif değişiklikleri gösterirken, temel bileşen 2, daha yanlarda yer alan parmaklar II ve IV'teki şekil değişikliklerini ortaya koymaktadır. Geometrik morfometrik analiz, su kuşlarının perdeli ayaklarının şekil varyasyonlarını belirlemede değerli bir araç olduğunu ve bu analizlerin taksonomik amaçlar için etkili olduğunu kanıtlamaktadır.

Anahtar Kelimeler: *Anatomi, Ayak, Değerlendirme, Fark, Kanatlı, Şekil.*

Introduction

Waterbirds typically possess webbed feet, enabling them to easily propel themselves through water. These webbed feet are classified into four main types: palmate, semipalmate, totipalmate, and lobate. Among these, palmate feet are the most prevalent among waterbirds, characterized by the complete connection of the three front-facing toes (toes II, III, and IV) through webbing (Lovette et al., 2016; Raikow, 1985; Tokita et al., 2020).

Special feet known as pad feet are central to their ability to navigate water surfaces and wetland habitats. These remarkable anatomical structures distinguish water birds from their terrestrial counterparts, offering unique advantages for life in and around water (Birkhead et al., 2017). Pad feet, characterized by their flattened shape and webbed toes, serve as multifunctional tools for water birds, facilitating activities such as swimming, walking on mud or vegetation, and perching on floating objects (Koenig et al., 2016). The diversity of pad feet adaptations reflects the varied ecological niches birds water birds occupy, from the elegant swan gliding across serene lakes to the agile heron stalking its prey in marshy wetlands (Birkhead and Van Balen, 2008). These adaptations enable water birds to easily navigate wetland habitats, whether wading through shallow waters, paddling across lakes, or diving beneath the surface in search of prey (Proctor and Lynch, 1993).

Geometric morphometry (GM) is a shape analysis approach that relies on the examination of anatomical curves, points, and contours, utilizing data derived from two- or three-dimensional Cartesian coordinates (Aytek, 2017; Bookstein, 1997; Boz et al., 2023; Demircioğlu et al., 2021; Gündemir et al., 2020; Manuta et al., 2024; Szara et al., 2022). At its core, GM focuses on shape analysis, discerning subtle differences by tracking the displacement of biologically homologous landmarks (Bookstein, 1991; Zelditch et al., 2004), thereby explicitly defining “shape” in terms of proportions and relative arrangements of parts that remain consistent regardless of scaling, thereby providing a quantitative analysis (Rohlf et al., 1993). Principal Component Analysis (PCA) emerges as one of the most commonly employed methods for exploratory multivariate analysis. It serves to visualize the primary features of shape variation within a dataset and functions as an ordination method to unveil patterns in the relationships among observations (Klingenberg et al., 2000).

The geometric morphometric analyses conducted can elucidate how variations in the toe structures of water birds are correlated with food sources, hunting strategies, social structures, and other ecological factors. These findings contribute to a better understanding of the biological diversity and adaptation processes among water birds, providing deeper insights into their evolutionary processes and life strategies. In this way, by better understanding and managing the diversity and ecological adaptations among water birds, we can more effectively contribute to the sustainability of these species and their roles in ecosystems. However, the focus of this study is to examine how changes in finger shapes are associated with the evolutionary processes and life strategies of water birds.

In conclusion, numerous studies have highlighted the distinct genotypes of bird feet pads, showcasing variations in physiological, morphological, and behavioral traits compared to their ancestors (Höfling and Abourachid, 2021; Rico-Guevara et al., 2019; Tokita et al., 2020; Winkler and Leisler, 1985). In this study, we will examine the finger shapes of water birds such as the West Indian whistling duck (*Anas bahamensis*), mandarin duck (*Aix galericulata*), red-breasted goose (*Branta ruficollis*), wood duck (*Aix sponsa*), mute swan (*Cygnus olor*), greylag goose (*Anser anser*), mallard (*Anas platyrhynchos*), Pekin duck (*Anas platyrhynchos domesticus*), redhead duck (*Aythya americana*), Egyptian goose (*Alopochen aegyptiaca*), and pelican (*Pelecanus onocrotalus*) using geometric morphometric analysis (GMA). This article delves into the fascinating realm of pad feet in water birds across different breeds, employing landmark-based GMA to explore their diversity and functionality. Despite limitations in our dataset, the research underscores the efficacy of geometric morphometrics in revealing subtle shape differences in pad feet. With this method, we will investigate how the finger structures of water birds change in relation to their life strategies and ecological impacts. The investigation, focusing on waterbirds, adds to the broader discourse on avian morphology, highlighting the importance of geometric morphometrics in elucidating the complexities of pad feet diversity in Turkish waterbirds.

Material and Methods

Animals: A total of 12 birds’ feet were utilized in the study obtained by the Istanbul University-Cerrahpaşa Faculty of Veterinary Medicine, Department of Wildlife Diseases and Ecology. These include West Indian whistling duck (*Anas bahamensis*), mandarin duck (*Aix galericulata*), red-breasted goose (*Branta ruficollis*), wood duck (*Aix sponsa*), mute swan (*Cygnus olor*), greylag goose (*Anser anser*), mallard (*Anas platyrhynchos*), Pekin duck (*Anas platyrhynchos domesticus*), redhead duck (*Aythya americana*), Egyptian goose (*Alopochen aegyptiaca*), and pelican (*Pelecanus onocrotalus*). All animals showed no pathological lesions, and all individuals used for the study were adults. Clinical examinations were provided by specialists before the samples were collected.

Landmarks: Analyses of foot pad shape were performed on dorsal photographs of each bird. Photographs were taken from a distance of 25 centimeters. Subsequently, the images were digitized using the 'tps' extension with tpsUtil (version 1.74) (Rohlf, 2004). If necessary, the images were rotated to reduce accidental variation in landmark placement. For this study, a specific set of landmarks (LMs) was placed on the foot pad images using tpsDig version 2.29 (Rohlf, 1997). In total, 13 landmarks (LMs) were positioned along the dorsal view of the feet.

Geometric morphometrics: In the study, the results of the dorsal view of woterbird feet pads were subjected to geometric morphometric analyses, which were recorded separately. All analyses were conducted using MorphoJ software version 1.07a (Klingenberg, 2011). MorphoJ is a program package designed for geometric morphometric analysis of two- and three-dimensional landmark data (Klingenberg et al., 2002). After obtaining the Cartesian x, y coordinates for all landmarks, shape data was extracted using a full Procrustes fit (Dryden and Mardia, 1998; Rohlf and Slice, 1990). Subsequently, Generalized Procrustes Analysis (GPA) was applied to the imported landmark (LM) data before analysis to account for the shape variations in the foot pads. Principal Component Analysis (PCA) was then performed to explore the overall morphological variability of foot shape among breeds (Gündemir et al., 2020; Klingenberg and McIntyre, 1998; Klingenberg et al., 2002; Manuta et al., 2023). The data in the study were obtained from the TÜBİTAK 2209A project titled "Investigation of the Foot and Toe Shapes of Waterfowl Using Geometric Morphometric Analysis.

Results

Digitus II, III, and IV: The other three toes on the underside of the foot. These toes point forward and merge with the interdigital membrane. *Torus metatarsalis*: A swollen, cushion-like structure located at the back of the foot. This structure aids birds in maintaining balance on their feet. Interdigital membrane (*Tela interdigitalis*): Flap-like skin structures between the second, third, and fourth toes. These structures assist water birds in swimming and moving on water surfaces. The landmarks used in our study are shown in Figure 1, and the description of each landmark is provided below.

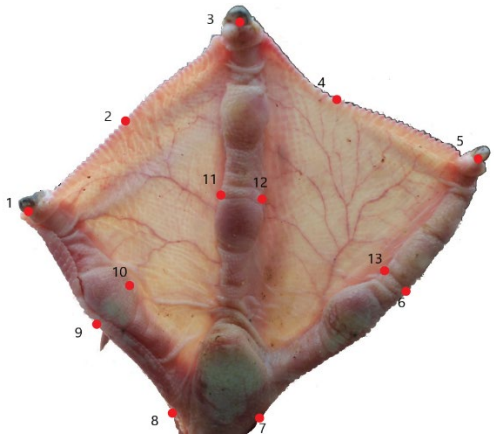

Figure 1. Landmarks used for the study of waterbirds pad feet in dorsal view. In total, 13 landmarks two-dimensional landmarks were used.

1. The point between the claw (*unguis*) and distal phalanx (*phalanx distalis*) of the second digit (*digitus secundus*).
2. The midpoint of the medial interdigital webbing (*tela interdigitalis medialis*).
3. The point between the claw (*unguis*) and distal phalanx (*phalanx distalis*) of the third digit (*digitus tertius*).
4. The midpoint of the lateral interdigital webbing (*tela interdigitalis lateralis*).
5. The point between the claw (*unguis*) and distal phalanx (*phalanx distalis*) of the fourth digit (*digitus quartus*).
6. The midpoint of the medial side of the fourth digit (*digitus quartus*).
7. The midpoint of the metatarsal tubercle (*torus metatarsalis*) is lateral.
8. The midpoint of the metatarsal tubercle (*torus metatarsalis*) medial.
9. The midpoint of the lateral side of the second digit (*digitus secundus*).
10. The midpoint of the medial side of the second digit (*digitus secundus*).
11. The midpoint of the lateral side of the third digit (*digitus tertius*).
12. The midpoint of the medial side of the third digit (*digitus tertius*).
13. The midpoint of the medial side of the fourth digit (*digitus quartus*).

Displays the shape changes associated with the first two principal components of the PCA with wire-frames for the extreme positive and negative values for each PC for the pooled sample (Figure 2). The shape variation between samples was analyzed by principal component analysis (PCA) using 13 landmarks in 2 dimensions in different feet pads (Table 1). The results of PCA using the landmark coordinates,

Table 1. Results of Principal Component Analysis (PCA).

Principal Component	Eigenvalues %	Variance %	Cumulative %
PC1	0,00406770	42,59	42,59
PC2	0,00188852	19,77	62,37
PC3	0,00118962	12,45	74,82
PC4	0,00098774	10,34	85,17
PC5	0,00049664	5,20	90,37

determined in the water bird feet, are shown in (Figure 2). Accordingly, the first principal component (PC1) explained 42,59% of the total shape variance, and the first four principal components (PC1 + PC2 + PC3) explained the rest of 74,82 %. The analysis focused on PC1 and PC2 values. Using wire-frame warp plots for visualization, we can observe the intricate structures and variations in thearrangements, webbing, and overall foot shape among waterbird species. PC1 shows slightly greater changes occurring on the lateral toes II and IV, as well as in the interdigital webbing below the average. PC2 reveals greater shape changes on the toes II and IV, which are more lateral. Additionally, a smaller metatarsal tubercle was observed.

Discussion and Conclusion

Pad feet, the specialized structures found in water birds, possess unique physical traits specifically adapted for survival in aquatic habitats. With their graceful movements and effortless navigation of aquatic realms, water birds are marvels of avian adaptation (Birkhead, 2018). The aim of this study was to investigate differences in pad feet across

different waterbird breeds, such as the West Indian whistling duck (*Anas bahamensis*), mandarin duck (*Aix galericulata*),

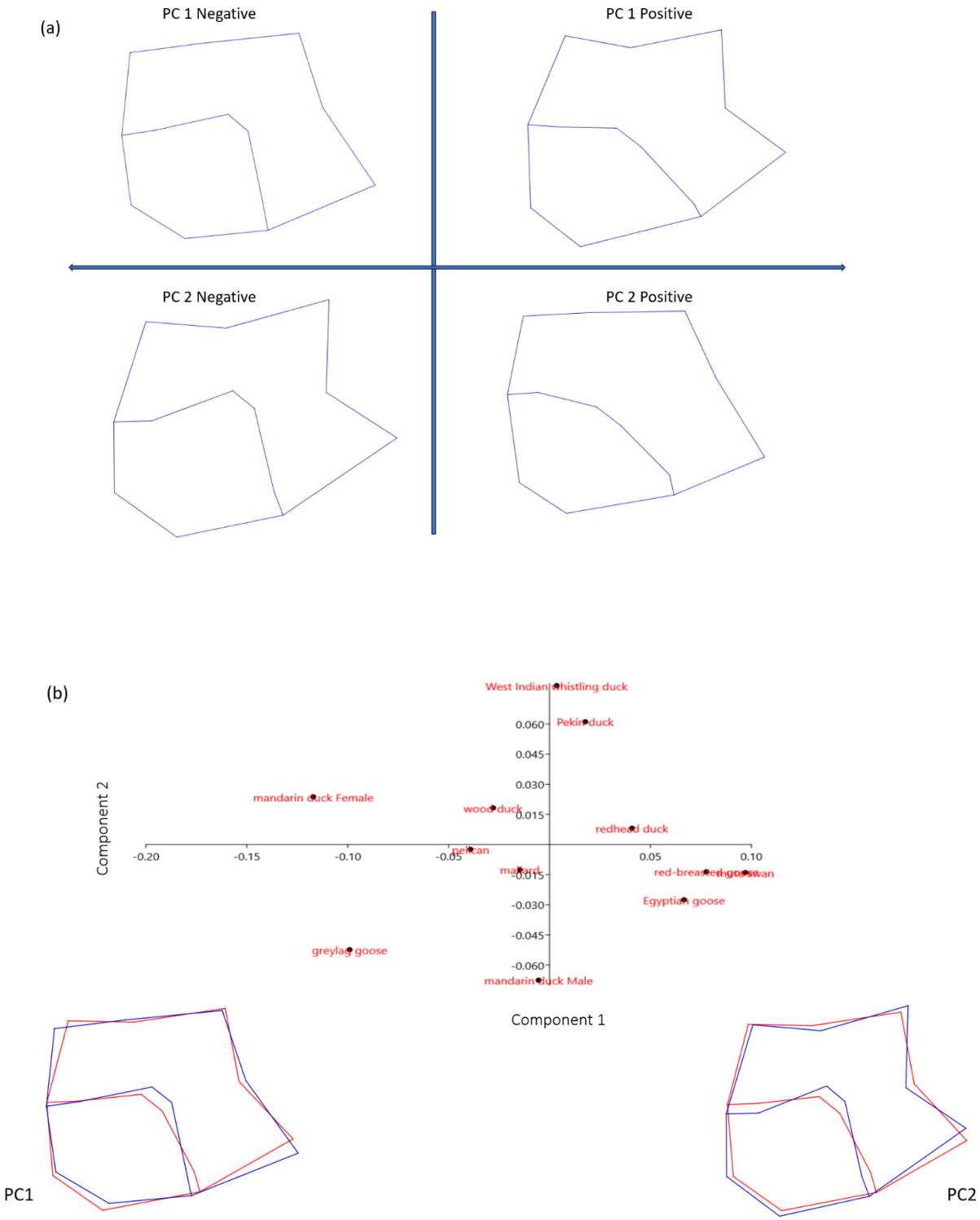

Figure 2. Scatter plot of PC1 and PC2 of the pad feet shape in dorsal view for interspecies. Wire-frames warp plots of shape changes depicting the positive and negative changes associated with PC1 (42,59%), PC2 (19,77%) of changes (top), and PCA showing variation among different breeds of water birds feet pads, as mapped by 13 landmarks (bottom). Blue outlines

represent the mean shape configuration, while the red outlines show the shape changes associated with the positive extremes of the PC axes.

red-breasted goose (*Branta ruficollis*), wood duck (*Aix sponsa*), mute swan (*Cygnus olor*), greylag goose (*Anser anser*), mallard (*Anas platyrhynchos*), Pekin duck (*Anas platyrhynchos domesticus*), redhead duck (*Aythya americana*), Egyptian goose (*Alopochen aegyptiaca*), and pelican (*Pelecanus onocrotalus*), using Principal Component Analysis (PCA) as a tool of geometric morphometric methods. Specifically, we examined scatter plots and wire-frame representations to elucidate potential variations. Our findings reveal notable distinctions among waterbird species, suggesting diverse morphology pad foot morphology adaptations.

Following geometric morphometry analysis based on landmarks, results showed a wide range of phenotypes in the shape of the pad feet of the specimens used in this study. Scatter plots generated from PCA provide visual representations of morphology pad foot morphology variation across different waterbird species. PCA allowed the extraction of principal components, with the first principal component (PC1) explaining 42.59%, while PC2 explained 19.77% of the total variation. Wire-frame representations further enhance our understanding by illustrating shape differences in pad feet morphology.

The observed variations in pad feet morphology among waterbird species are not superficial; they represent distinct adaptations tailored to each species' specific ecological niches and behaviors. These adaptations play a crucial role in waterbirds' survival and reproductive success by enabling them to effectively exploit their habitats and resources (Lin and Xu, 2017). For instance, species with elongated, webbed toes may exhibit enhanced swimming abilities, allowing them to efficiently navigate through water bodies in search of prey (Segesdi and Pecsics, 2022; Tokita et al., 2020). Conversely, species with shorter, more robust toes may excel in terrestrial locomotion, enabling them to forage on land or traverse different substrates with ease (Brown et al., 2002; Sargata-Vicens et al., 1992). Understanding these morphological adaptations provides valuable insights into waterbirds' evolutionary history and ecological diversification (Lin and Xu, 2017). By elucidating the functional significance of pad feet morphology, we gain a deeper appreciation for the remarkable diversity of avian adaptations and the complex interplay between form and function in the natural world.

The West Indian whistling duck is renowned for its strong, agile feet, which enable it to navigate through dense vegetation and shallow water bodies while foraging for aquatic vegetation, insects, and small invertebrates. Additionally, this species typically possesses moderately webbed feet with long, slender toes (Madge and Burn, 1988). Based on the results of our study, we observed the highest positive PC1 value compared to other specimens, indicating significant differences. Conversely, the mandarin duck male exhibited the lowest negative PC1 value. Mandarin ducks have well-developed webbed feet with pronounced webbing between the toes, extending almost to the tips. Unlike the West Indian whistling duck, mandarin ducks are less reliant on terrestrial locomotion and are primarily adapted for a semi-aquatic lifestyle, spending much of their time on or near water bodies (Kear, 2005). Consistent with research by Johnsgard (2010), our study's PCA results highlight distinct differences between our specimens.

Furthermore, pelican feet are characterized by long, webbed toes with reduced webbing between the front toes, allowing for greater maneuverability in water (Ogden et al., 1983; Tokita et al., 2020). Goose feet may vary in size and shape depending on the species, with moderate webbing between the toes (Kear, 2005; Livezey, 1986). Ducks exhibit varied foot morphology depending on their habitat and feeding behaviors; for instance, dabbling ducks have relatively small, webbed feet adapted for shallow water foraging and dabbling (Cherry and Morris, 2008; Sargata-Vicens et al., 1992). Swans possess large, powerful feet adapted for swimming and walking on land, characterized by long, slender toes with prominent webbing between them, providing strong propulsion in water (Gill, 2007; Johnsgard, 2010).

The ecological implications of pad feet morphology extend beyond individual species to influence community dynamics, ecosystem structure, and conservation strategies (Tokeshi, 2009). Waterbirds, acting as keystone species in aquatic ecosystems, play pivotal roles in nutrient cycling, habitat structure, and prey populations (Tokita et al., 2020). Pad foot morphology can significantly impact a species' foraging behavior, habitat preferences, and competitive interactions with other organisms. For instance, waterbirds with specialized adaptations for diving may exploit deeper water habitats, while those with more agile feet may dominate shallow wetlands. Moreover, changes in pad feet morphology in response to environmental pressures, such as habitat loss, pollution, and climate change, can serve as crucial indicators of ecosystem health and resilience. of monitoring of monitoring these morphological traits over time provides valuable insights into the impacts of anthropogenic disturbances on waterbird populations and their associated habitats (Serrano and Tella, 2018; Smith and Buhl, 2015).

Overall, of comparing pad feet morphology among different waterbird species reveals fascinating adaptations shaped by evolutionary processes and ecological pressures. Through detailed studies and analyses, we can unravel the complexities of avian morphology and gain a deeper understanding of the intricate relationships between form and function in the natural world.

In conclusion, based on these hypotheses, environmental factors may be the reason for the differences in pad feet among our breeds — West Indian whistling duck (*Anas bahamensis*), mandarin duck (*Aix galericulata*), red-breasted goose (*Branta ruficollis*), wood duck (*Aix sponsa*), mute swan (*Cygnus olor*), greylag goose (*Anser anser*), mallard (*Anas platyrhynchos*), Pekin duck (*Anas*

platyrhynchos domesticus), redhead duck (*Aythya americana*), Egyptian goose (*Alopochen aegyptiaca*), and pelican (*Pelecanus onocrotalus*). We believe our study will guide future researchers in employing methods such as biomechanics, comparative genomics, developmental genetics, and functional experiments to fully explain the observed evolutionary transitions in feet morphology and pad feet shape of waterbirds.

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The data in this study were obtained from the TÜBİTAK 2209a project titled "Examination of Foot and Toe Shapes of Waterbirds Using Geometric Morphometric Analysis.

Conflict of Interest

The authors stated that they did not have any real, potential, or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on the Working Principles and Procedures of Animal Experimentation Ethics Committees".

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

The effect of PGF2α injection in different times on the pregnancy rate in progesterone-based synchronization in Holstein cows

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Abstract: This study investigated the effect of Prostaglandin F2α (PGF2α) injection at different times on pregnancy rate (PR) in Holstein cows undergoing progesterone-based fixed-time artificial insemination (FTAI). Group 1 (G1, n=30): A progesterone releasing intravaginal device (PRID) was placed intravaginally with a gonadotropin-releasing hormone (GnRH) injection on day 0. Eight days later, PGF2α was injected. PRID was removed on the 9th day, and after 60 hours, FTAI was performed with a GnRH injection. Group 2 (G2, n=30): Unlike G1, PGF2α was injected twice, with an interval of 24 hours, 8 and 9 days after the intravaginal placement of PRID. Group 3 (G3, n=30): PGF2α was injected 9 days after the intravaginal placement of PRID. Pregnancy diagnoses were performed by transrectal ultrasonography 45 days after FTAI. Blood samples were taken on the 8th day (for Groups 1 and 2), the 9th day (for Groups 2 and 3, PRID removal day), and on the day of FTAI (for Groups 1, 2 and 3) and serum progesterone (P4) concentration was determined. The pregnancy rate (PR) was 36.67%, 53.33%, and 43.33% in Groups 1, 2, and 3, respectively (P=0.194). In cows with a body condition score (BCS) <3, repeated PGF2α injections within a 24-hour interval increased the PR (G2: 70%) compared to the other two groups (G1: 20%, G3: 25%) (P=0.045). P4 concentrations decreased after PGF2α injections (P<0.001). In cows experiencing luteolysis, a significant difference was found in PR between G1 and G3 (P=0.04). In conclusion, PGF2α injection at 24-hour intervals in P4-based FTAI protocols in cows quantitatively increased both pregnancy and luteolysis rates and may improve fertility.
Keywords: *Cow, Luteolysis, PGF2α, Pregnancy, Progesterone.*

Holstein ineklerinde progesteron bazlı senkronizasyonda farklı zamanlarda PGF2α enjeksiyonunun gebelik oranına etkisi

Özet: Bu çalışmada, progesteron bazlı sabit zamanlı suni tohumlama (FTAI) uygulanan Holştayn ineklerde farklı zamanlarda Prostaglandin F2α (PGF2α) enjeksiyonunun gebelik oranı üzerine etkisi araştırıldı. Grup 1 (G1, n=30): Progesteron salgılayan intravajinal cihaz (PRID) 0. günde vajinaya yerleştirildi ve gonadotropin salgılatıcı hormon (GnRH) enjeksiyonu yapıldı. Sekiz gün sonra PGF2α enjekte edildi. PRID, 9. günde çıkarıldı ve 60 saat sonra GnRH enjeksiyonu ile FTAI gerçekleştirildi. Grup 2 (G2, n=30): Grup 1'den farklı olarak PGF2α, sabit zamanlı suni tohumlama (FTAI) intravajinal yerleştirilmesinden 8 ve 9 gün sonra, aralarında 24 saatlik bir süre bulunarak iki kez enjekte edildi. Grup 3 (G3, n=30): PGF2α, PRID'in intravajinal yerleştirilmesinden 9 gün sonra enjekte edildi. Gebelik teşhisleri FTAI'den 45 gün sonra transrektal ultrasonografi ile yapıldı. Kan örnekleri, 8. gün (G1 ve G2 için), 9. gün (PRID çıkarılma günü) ve FTAI gününde alındı ve serum progesteron (P4) konsantrasyonu belirlendi. Gebelik oranları Grup 1, 2 ve 3'te sırasıyla %36.67, %53.33 ve %43.33 olarak bulundu (P=0.194). Vücut kondisyon skoru (BCS) <3 olan ineklerde, 24 saat arayla tekrarlanan PGF2α enjeksiyonları gebelik oranını (G2: %70) diğer iki grupla (G1: %20, G3: %25) karşılaştırıldığında artırdı (P=0.045). P4 konsantrasyonları PGF2α enjeksiyonlarından sonra azaldı (P<0.001). Luteolizis uygulanan ineklerde Grup 1 ve Grup 3 arasında gebelik oranlarında anlamlı fark bulundu (P=0.04). Sonuç olarak, ineklerde P4 bazlı FTAI protokollerinde 24 saatlik aralıklarla PGF2α enjeksiyonunun gebelik ve lüteoliz oranlarını nicel olarak artırdığı ve fertiliteyi iyileştirebileceği sonucuna varıldı.
Anahtar Kelimeler: *Gebelik, İnek, Luteolizis, PGF2α, Progesteron.*

Introduction

In the last 30 years, challenges have arisen in detecting estrus signs in cows, paralleling the increase in milk yield. Consequently, pregnancy rate (PR) on first insemination has declined to 40%. The accurate determination of estrus symptoms and timely artificial insemination (AI) play a crucial role in improving fertility. Timed AI protocols have been developed to alleviate difficulties in detecting estrus symptoms. These protocols are widely adopted in both dairy and beef cattle worldwide. Using highly productive bulls has led to increased profitability both genetically and economically. Furthermore, it allows for pre-determination and planning of calf production (Kaçar, 2019).

The goal of a successful estrus synchronization program in dairy cows aims to achieve high fertility using fixed-time artificial insemination (FTAI) without the need for estrus detection. Strategies for ovulation control involve manipulating the corpus luteum (CL) lifespan with prostaglandin F2α (PGF2α), inducing follicle development, synchronizing ovulation, or inhibiting estrus through progesterone (P4) treatment (Thatcher et al., 2006). Depending on the presence of the CL in different periods of the estrus cycle in cows, PGF2α acts at different rates 78% at 5-9th days, 87.9% at 10-13th days, and %100 at 14-19th days of the estrus cycle (Freitas et al., 2021). It was determined that cows with a preovulatory dominant follicle showed signs of estrus 72 hours after PGF2α injection when PGF2α injection was performed showed signs of estrus 72 h later (Martins et al., 2011). Optimizing the size of the dominant follicle is important for timed AI protocols and synchronization of ovulation (Wiltbank et al., 2014). Pregnancy rates were found to be similar between cows with estrous and cows with timed AI (Santos et al., 2010; Wiltbank et al., 2014). Timed synchronization protocols reduced the time between birth and first delivery and increased the proportion of cows conceiving after the voluntary waiting period (Kim et al., 2020). In this study, the aim was to investigate the effect of PGF2α injection at different times on PR in Holstein cows undergoing P4-based FTAI.

Materials and Methods

The research was approved by the Kafkas University Local Ethics Committee for Animal Experiments (HADYEK numbered 2019/129) and permission was obtained from the Ministry of Agriculture and Forestry of Türkiye (dated 19.06.2019 and numbered 64445328-020-E1820373). The experiments were performed at private corporations located in Denizli, Türkiye.

Animals: The study was carried out between January and May, in Holstein cows, housed in a semi-open barn, fed with mixed ration and had access to *ad libitum* water. In the study, 90 clinically healthy Holstein cows, aged between 2-6 years old, weighing 450-550 kg, with a body condition score (BCS) of 2.5-3.5 (according to a 5-point scale with 0.25-point increments), producing 14-25 liters of milk, and at least 60 days postpartum, were used. Internal and external antiparasitic treatments and vaccination of cows were performed at least 25 days prior to synchronization protocols. The cows were fed twice daily, and drinkable water was supplied *ad libitum* from fixed water drinkers. Cows were fed with alfalfa hay, barley straw, wheat straw, corn silage, sugar beet pulp, crushed barley, and concentrated feed (2750 metabolic energy; crude protein 21%, crude cellulose 10%, crude fat 4%, crude ash 8%, calcium 1%, phosphorus 0.6% and sodium 0.45%). Cows were then randomized into 3 groups:

Group 1 (G1, *n*=30): The cows in this group were administered a progesterone releasing intravaginal device (PRID) intravaginally on day 0 together with 2 mL injection of gonadotropin-releasing hormone (GnRH, 50 µg Gonadorelin diacetate tetrahydrate, Ovarelin®, Ceva, Türkiye). Eight days after the application, a 5 mL injection of PGF2α (5 mg/mL Dinoprost, Enzaprost-T®, Ceva, Türkiye) was administered. The PRID was removed on day 9, and 60 hours later, FTAI was performed together with a 2 mL injection of GnRH. **Group 2 (G2, *n*=30):** The cows in this group were administered a PRID intravaginally on day 0 together with 2 mL injection of GnRH, similar to G1. On days 8 and 9 after the application, a 5 mL injection of PGF2α was administered. The PRID was removed on day 9, and 60 hours later, a 2 mL injection of GnRH was made together with FTAI. **Group 3 (G3, *n=30*):** The cows in this group were administered a PRID intravaginally on day 0, together with a 2 mL injection of GnRH similar to G1 and G2. Nine days after the application, a 5 mL injection of PGF2α was administered, and the PRID was removed. Sixty hours after PRID was removed, a 2 mL injection of GnRH was made with FTAI. Holstein sperma (Medivet®, Türkiye) was used for AI. Sperma was defrosted in a hot water bath at 37°C for 30 seconds just before the AI, and then placed recto-vaginally to the cornu uteri on which the Graafian follicle was detected. Single AI was performed for all groups in the study. To eliminate application differences, all AI procedures were performed by the same veterinary physician. Pregnancies were determined by transrectal ultrasonographic examination (5-7.5 MHz linear probe, Draminski iScan®, Draminski, Poland) which was performed 45 days after the AI procedure (Fig. 1).

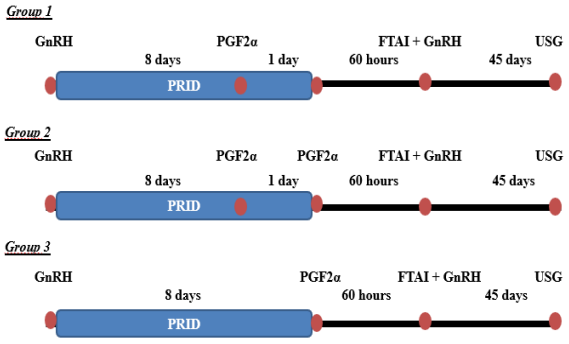

Figure 1. Procedures that were applied to all groups. GnRH: Gonadotrophin Releasing Hormone, PRID: Progesterone-releasing intravaginal device, PGF2α: ProstaglandinF2α, FTAI: Fixed-time artificial insemination, USG: Ultrasonography.

Analyzes of Blood Samples: On day 8 (Groups 1 and 2), day 9 (PRID removal days), and the day of AI, blood samples were collected from the *vena coccygea* into 8.5 ml vacutainer gel tubes (BD Vacutainer, BD, Türkiye). For the determination of P4 concentrations, blood samples were subjected to centrifugation at 1,300 g (NF400R®, Nüve,Türkiye) for 10 minutes to obtain serum samples. The serum samples were then stored at -18°C until the experiments.

Hormone analyzes were performed at the Department of Biochemistry of Kafkas of Science and Literature, Kars, Türkiye.

The P4 concentration in serum samples of cows obtained on different days was determined using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits (Catalogue no: 20112-1008, SunRed Biotechnology Company, Shanghai, China) according to the manufacturer's instructions. The assays were then analyzed with an ELISA reader (Epoch®, Biotek, USA). The assays were analyzed using an ELISA reader (Epoch®, Biotek, USA). The sensitivity of the kit was 0.029 ng/mL, with a measurement range of 0.05-40 ng/mL.

In our study, the changes in serum P4 on the 8th and 9th days of the experiment were determined. Additionally, serum P4 levels and active CL were determined on the day of AI, and the rate of luteolysis was determined. Cows with a serum P4 concentration of ≥1 ng/mL on day -3 and <1 ng/mL on day 0 are considered to have CL regression (Ribeiro et al., 2012). In this study, luteolysis of the CL P4 was accepted in cows with a serum P4 concentration of 1 ng/mL during the first PGF2α injection and <1 ng/mL at AI.

Statistical analysis: SPSS 18 (SPSS®, Chicago, IL, USA) package was used for the statistical analysis of the data obtained from the experiments. The PR was calculated using the Chi-square test. Age, milk yield, BCS, and P4 levels of the groups were evaluated using One-Way ANOVA, and multiple comparisons were made with the Tukey HSD test. A paired-t test was used for paired comparison in the intra-group analyzes, multiple dependent groups were evaluated with Repeated Measures ANOVA, and multiple comparisons were calculated using Bonferroni correction. The data in the study were presented as mean ± standard error of the mean (SEM). Values of P <0.05 were considered statistically significant in the evaluation of results.

Results

The age, BCS, and milk yields of the cows in the groups showed no significant difference (P>0.05). The average age of all groups was 4.38-4.5 years (P=0.894), BCS ranged between 2.90 and 2.95 (P=0.843), and milk yield ranged between 20.8 and 22.1 L (P=0.175).

Although PR did not differ statistically among the groups, the PR was found to be higher in G2 compared to the other groups. The PR of G2 was approximately 16% higher than in G1, and there was a trend towards statistical significance (Table 1).

Although the highest luteolysis rate was 70.0% (21/30) in G2, a significant difference (P=0.183) was not observed. However, there was a statistical trend towards luteolysis formation in G2 and G3 (P=0.067, Table 1).

For pregnancy rates in cows with luteolysis, there was no significant difference between G1 and G2 (P=0.309). Similarly, no significant difference in PR was determined between G2 and G3 (P=0.2). However, a significant difference was found between G1 and G3 (P=0.04, Table 1).

Investigations were conducted based on the BCS, and it was observed that PGF2α injections at 24-hour intervals did not significantly affect the PR in cows with BCS≥3 (P=0.931). However, in cows with BCS<3, it was determined that PGF2α applications significantly increased their PR (G2: 70%) (P<0.05) compared to the G1 (G1: 20%) (Table 2).

Progesterone concentrations in cows at PGF2α injections and at the time of AI are presented in Table 3. It was found that serum P4 levels did not significantly differ on different days between groups (P>0.05). However, it was determined that the concentration of P4 significantly decreased within groups after PGF2α injections (P<0.001).

Progesterone concentrations were 0.82 ± 0.05 ng/mL in G1, 0.84 ± 0.05 ng/mL in G2, and 0.75 ± 0.07 ng/mL in G3. There was no significant difference in P4 concentrations between groups (P=0.51; Table 4).

Table 1. Luteolysis rate, pregnancy rate and pregnancy rates with luteolysis of groups.

Groups	Luteolysis, % (n/total)	Pregnancy rate of cows in all groups, % (n/total)	Pregnancy rates of cows with luteolysis, % (n/total)
Group 1	60.0 (18/30)	36.67 (11/30)	61.1^{a} (11/18)
Group 2	70.0 (21/30)	53.33 (16/30)	76.2 (16/21)
Group 3	46.7 (14/30)	43.33 (13/30)	92.9^{b} (13/14)
P value	0.183	0.431	0.040

$^{a-b}$: Shows statistically significant differences between groups in the same line (P<0.05).

Table 2. Pregnancy rates (%) according to body condition score of cows.

Groups	BCS <3		BCS ≥3		*P* value
	%	n/Total	%	n/Total	
Group 1	20[a]	2/10	45	9/20	0.180
Group 2	70[b]	7/10	45	9/20	0.196
Group 3	25[ab]	2/8	50	11/22	0.222
P Value	0.045		0.931		-
Total	39.29	11/28	46.77	29/62	0.704

For BCS<3; *P* levels were determined as *P*=0.025 between Group 1 and Group 2, *P*=0.80 between Group 1 and Group 3, and *P*=0.058 between Group 2 and Group 3 (BCS=body condition score). a-b: Shows statistically significant differences between groups in the same line.

Table 3. Progesterone concentrations (ng/mL) between groups and different days (ng/mL).

Groups	8th day	9th day	FTAI	*P* value
Group 1	1.85±0.13[a]	-	1.12±0.09[b]	<0.001
Group 2	2.06±0.05[a]	1.73±0.22[a]	1.01±0.05[b]	<0.001
Group 3	-	2.01±0.14[a]	1.17±0.08[b]	<0.001
P value	0.372	0.268	0.292	-

[a-b]: Shows statistically significant differences between groups in the same line (*P*<0.001). FTAI: Fixed Time artificial insemination. The data in the table were presented as mean ± standard error of the mean (SEM).

Table 4. Progesterone concentration (ng/mL) in cows with luteolysis (ng/mL).

Groups	Mean	Standard error of the mean (SEM)
Group 1	0.82	0.05
Group 2	0.84	0.05
Group 3	0.75	0.07
P value	0.510	

Discussion

The absence of complete luteolysis in dairy cows with FTAI practices is reported to reduce fertility (Ribeiro et al., 2012; Giordona et al., 2013). In dairy cows, two PGF2α injections at 8-hour intervals have been suggested to be more effective than a single PGF2α injection in inducing luteolysis (Hölper et al., 2023). It was determined that PR increased as a result of FTAI with PGF2α injected at intervals of 7-8 hours in beef cattle (Alnimer et al., 2019). In Simmental cattle, on the 7th and 8th days of OvSynch protocol, 29.5% PR was obtained in the classical OvSynch group, while it was 36.5% in the OvSynch group where a double dose of PGF2α was injected. In the beef cows that received the five-day CoSynch protocol, fertility rates were reported as 48%, 51%, and 55%, respectively, following a single dose of PGF2α on day 5, a double dose on day 5, and two doses on the 5th day at 8-hour intervals (Giordona et al., 2012). In Simmental cows, when synchronized with the seven-day OvSynch+CIDR protocol, a 9% increase in PR was observed, and a positive statistical trend was detected as a result of a second injection of PGF2α 24 hours after the first PGF2α injection (Kaçar et al., 2018). It was reported that PGF2α injections did not increase the PR in the cows administered the PRID-Synch protocol for seven and five days, with an interval of 24 hours. However, performing a second injection of PGF2α reduced the percentage of cows whose luteal regression was not completed, and a tendency to increase PR was determined by timed AI (Santos et al., 2016). In contrast to these studies, a study with the OvSynch protocol found no significant difference in ovulation and PR after the second GnRH application in cows with or without an additional PGF2α treatment one day after (Brusveen et al., 2009). In the presented study, the rate of luteolysis in the groups was not different. It was determined that PGF2α applied twice at twenty-four-hour intervals did not increase the rate of luteolysis compared to the other groups. The inconsistency in luteolysis rates and pregnancy outcomes across studies could stem from several factors. For instance, differences in breed-specific responses to hormonal treatments may play a significant role, as breeds exhibit distinct reproductive physiology and sensitivity to PGF2α. Additionally, the physiological status of the cows, such as parity, lactation stage, or the functional status of the CL at the time of treatment, can significantly influence treatment outcomes. Study-specific conditions, including variations in protocol implementation, timing of injections, or environmental stressors, may also contribute to the observed differences (Nascimento et al., 2014). Therefore, further research is needed to elucidate these differences and to optimize synchronization protocols by considering breed-specific responses, physiological conditions, and environmental

influences. Such efforts could lead to a better understanding of how PGF2α injections can be utilized more effectively in improving luteolysis rates and overall reproductive performance.

To increase the regression of a newly created CL resulting from GnRH injection and optimize fertility, two doses of PGF2α administration were required. High P4 concentrations were found to suppress LH release and prevent ovulation when GnRH is administered (Lima et al., 2013). Increased circulating P4 concentrations around AI time create a suboptimal environment during the transport of sperm and ovum in the genital canal, resulting in decreased fertility (Brusveen et al., 2009). The circulating P4 levels above 0.5 ng/mL during timed AI were reported to reduce fertility by more than 50% (Souza et al., 2007). This was significantly effective in the regression of CL due to the large percentage of cows with high levels of P4 during the second GnRH treatment of the OvSynch protocol. Surprisingly, despite the significant increase in the percentage of cows with low P4 during the second GnRH (63.2% and 91.0%), it was observed that the pregnancy rate did not increase in the cows with high P4 concentrations at the second PGF2α treatment. It is possible that giving an additional dose of PGF2α earlier (approximately 12 hours following the normal PGF2α treatment) would lower the circulating P4 earlier and yield a positive effect on the PR (Brusveen et al., 2009). The ratio of cows with a low P4 concentration 48 hours after the first PGF2α treatment of the Ovsynch protocol was reported as 94% (Souza et al., 2007). In cows that received a second injection of PGF2α during the Ovsynch protocol, the proportion of cows with a P4 value lower than 0.4 ng/mL during the final GnRH administration was higher compared to the control group (95.6% and 84.6%, respectively). However, it has been demonstrated that the fertility of cows with complete luteolysis did not reflect that observation (Brusveen et al., 2009). In the present study, the number of cows with P4 levels <1 ng/mL during AI was higher, at 70%, in cows that received a PGF2α injection with a 24 hour-interval. However, this luteolysis rate was not reflected in the pregnancy rate. The lowest luteolysis rate was observed in cows that received PGF2α on the 9th day, at 46.7%. Interestingly, these cows were found to have a higher PR compared to other groups. According to these results, it may be suggested that luteolysis in this group was complete and sufficient, making the uterus more suitable for pregnancy. 24 hours after the first PGF2α application using the ReSynch protocol, a second injection of PGF2α has been shown to induce luteal regression fully and tends to increase the PR. However, it was found that a double dose of PGF2α injection administered at one time produced no effect (Barletta et al., 2018). On the 5th day of the sexual cycle, 41% of heifers responded to a single PGF2α treatment, whereas none of the lactating or non-lactating cows responded. On the seventh day, 88% of heifers and 90% of non-lactating cows responded to a single PGF2α treatment, whereas only 66% of lactating cows responded (Nascimento et al., 2014). Therefore, in this study, we found no differences in PR between the groups. However, PGF2α injections at 24-hour intervals showed a statistical trend towards increasing PR compared to the cows administered PGF2α on day 8 (G1). Despite the numerical difference in PR, the absence of a statistically significant difference can be attributed to the limited number of cows used in each group. This indicates that the sample size may be insufficient to detect significant differences. A larger sample size is needed in future studies to obtain more robust results and to understand better the effects of PGF2α injections on luteolysis and pregnancy rates. On the eighth day, PGF2α injection has been shown to reduce PR, suggesting that early and inadequate luteolysis may negatively affect fertility rates.

In Nelore breed cattle with progesterone-based AI, the pregnancy rate was determined to be 69.75% in cows with BCS≥2.75, whereas it was only 32.98% in cows with BCS <2.75. For protocols with FTAI applications, it was recommended that BCS should be at least 2.5 to ensure a positive energy balance in cows and heifers (Pereira et al., 2018). On the other hand, animals with higher BCS are expected to have higher levels of nutritional reserves, which can be observed from the distribution of body fat on the animal's body surface. The PR in cows with BCS≤2.5 was found to be lower at 28.7%, compared to the groups with BCSs of 2.75-3 (37%) and > 3.25 (40.9%) (Ribeiro et al., 2012). PGF2α injections at 24-hour intervals did not change the PR of the cows with BCS≥3 compared to other groups. Surprisingly, in cows with BCS<3, PGF2α injections at 24-hour intervals increased the PR compared to different groups. This finding has been interpreted as meaning that two PGF2α injections can provide complete luteolysis in cows with low BCS.

Conclusion

In conclusion, in P4-based FTAI applications, PGF2α injections with a 24-hour interval in increased PR in Holstein cows. In addition, the rate of luteolysis was higher in the cows in this group. In light of these findings, PGF2α injections at 24-hour intervals may be effective in improving fertility. However, these results should be further verified by additional research using larger sample sizes.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (19.09.2019, 2019/129 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Sequencing the Parasitic Nematode *Contracaecum* spp. in Edible Fish (*Planiliza Abu*)

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Abstract: *Contracaecum rudolphii* Hartwich,1964 (Nematoda: Ascaridoidea, Anisakidae) is a typical anisakid recorded globally. Consumption of undercooked seafood, raw or infected, contains the nematode larvae that cause human anisakidosis. Fish (*Planiliza abu*) specimens were obtained from a local market in Karbala, Iraq. This type of fish came from the Razzaza Lake. The prevalence of *Contracaecum* spp. parasites were done over eight months from June 2022 until January 2023, using ITS1, morphological and molecular analysis of the nematodes Contracaecum spp from fish. Of 395 fish, 124 (31.3%) had visceral infections caused by *Contracaecum* spp. larval type (L3). The morphological and genetic identification of *Contracaecum* spp. was validated in the parasitology laboratory of the Veterinary Medicine College at Kerbala University. The result showed that the infection rate in January 2023 was (46.6%) and increased while it was (20%) in September 2022. With the use of ITS1 gene, the molecular analysis for *Contracaecum spp.* was to investigate *Contracaecum* spp. and to confirm it. However, the nematode count, number of infected fish, and length were all substantially different at the P≤0.05. This study detected the isolate=(a1) at the locus=OP787071 and sequenced the parasites. The isolates were confirmed as *Contracaecum rudolphii*, isolate a1 internal transcribed spacer 1, partial sequence. In conclusion, molecular genotyping might be a useful technique for identifying the *Contracaecum* L3 larval species, life-cycle biology, transmission methods, and types of intermediate hosts.

Keywords: *Anisakidosis, Contracaecum rudolphii, Fish, ITS1, Zoonosis.*

Introduction

Anisakiasis is a parasitic infection caused by larval stages of nematodes from the Anisakidae family's genera *Anisakis*, *Pseudoterranova*, and *Contracaecum*. Fish infested by the *Contracaecum* spp. parasites can infect humans as accidental hosts by consuming raw or undercooked infective stage larvae (L3) (Aibinu et al., 2019). The cases of human larvae are incidentally taken by undercooked fish meat or eating raw, and they may lead to anisakidosis, causing fever, stomach pains, vomiting and diarrhea (Decruyenaere et al., 2022; Hirosawa et al., 2020; Shamsi and Butcher, 2011; Shibata et al., 2020; Younis et al., 2017). They are known to have extremely harmful effects on wildlife fish, birds, and marine mammals (Shamsi, 2019). Anisakidosis is a new illness that causes a variety of clinical symptoms in humans and is caused by members of the Anisakidae family (Golden et al., 2022). The detected anisakidae nematodes were in stage 3 (L3), and the mitochondrial gene rRNA was molecularly identified using PCR (Shamsi and Suthar 2016). Both strains (*C. rudolphii* A and B) were found in freshwaters of Crucian carp (*Carassius carassius*), while *C. rudolphii senso lato* was found in the Caspian round goby (*Neogobius melanostomus*) from the Baltic Sea. Only the temporarily strain designated *C. rudolphii* B was identified in Poland. Using (TS-1 and ITS-2) of the ribosomal DNA of nematode from *C. rudolphii* B may be the dominant type in both brackish and freshwater (Szostakowska and Fagerholm, 2007). Additionally, a new location has been added to the parasite species' geographical spread. *Prussian carp*, *Carassius gibelio* were caught in Karataş Lake in Burdur-Turkey. Only *C. rudolphii* was found in one sample (2.63%) (İnnal et al., 2020). However, *Contracaecum Rudolph* samples that were collected from cormorant populations in Italy and Europe revealed two sibling species, *C. rudolph* A, which was more prevalent in brackish water fish, and *C. rudolphii* B, *w*hich was found infecting only freshwater fish identified by sequence analysis of the mtDNA cox2, and ITS region of rDNA gene loci (Mattiucci et al., 2020).

To date, *Contracaecum rudolphii* complex currently has five recognized members, those being A, B, C, D, E, and F (D'amelio et al., 2012; Mattiucci et al., 2008; Mattiucci et al., 2020). While all infected fishes by *Contracaecum* larvae in north Iraq's Sulaimani Province represented exactly one species (*C. rudolphii* B) through testing the sequences of ITS1, ITS2, and COX2 **(**Abdullah et al., 2021a**)**. On the other hand, in the al-Sanaf marsh, southern Iraq, the ITS-1 regions of rDNA showed two distinct species; *C. septentrionale* and *C.microcephalus* (Mohammad and Hbaiel, 2019). In this study, molecular studies of Contracaecum larvae in *Planiliza abu* from Razzaza Lake in Kerbala were used.

Materials and Methods

Location: Karbala, also spelled Kerbala, is the administrative center of the Karbala Governorate in central Iraq, approximately (100 km) southwest of Baghdad. It is home to an estimated one million pilgrims who travel there annually. Razzaza or Razaza Lake, is located in western Iraq, west of Karbala (3241N, 4340E). It is Iraq's second-largest freshwater lake, and it used to be an important source of fish. The lake, which covers an area of 1810 square km and is located 40 meters above sea level, has a storage capacity of 26 billion cubic meters of water. Part of the water from Lake Habbaniyah is discharged into Razzaza Lake through a controlled exit route or channel from the Euphrates (Fig. 1).

Figure 1. Map of Iraq, Karbala province, and Razzaza Lake location.

Collection and Examination of Fish: The source of *Planiliza abu* is Razzaza Lake of Karbala, Iraq and samples were purchased from the local market of Karbala. A total (395) samples of fish that belong to one genus *Contracaecum* (Railliet and Henry, 1912) of the Anisakidae family were captured between June 2022 and January 2023. All of the fish were counted, measured, and weighed. Each specimen was traditionally dissected afterwards, and its anisakid larvae content was checked. Each specimen (viscera and flesh) was examined separately and put in Petri dishes. The length and weight were measured to give Prevalence (P), and mean intensity (mI) was calculated by (Shamsi and Suthar 2016). Following a visual inspection, the flesh and then the stereoscopic microscope were used to dissect the viscera, and the number of worms was determined for each sample (Shamsi et al., 2011; Yusni et al., 2022).

Morphological and Examination of *Contracaecum* Larvae: All of the isolated nematodes were examined morphologically. Individual fish larvae were mechanically removed, rinsed in saline solution for 30 minutes, and placed in 70% ethanol alcohol (Pons-Bordas et al., 2020). Lactophenol was used to clear the nematodes so that they could be morphologically evaluated. As suggested by the genus name, these worms' digestive system consists of two ceca that are situated in opposition to one another. The fronts of their bodies also feature an excretory orifice (Martínez et al., 2022), (Fig. 2,3,4, and 5). They should be regarded as the most important morphological traits for differentiating *Contracaecum spp.* from another parasitic anisakid because they endure the longest throughout all stages of growth (Shamsi, 2019).

Figure 2. Fish (*Planiliza abu*) infested by *Contracaecum rudolphii* (CR).

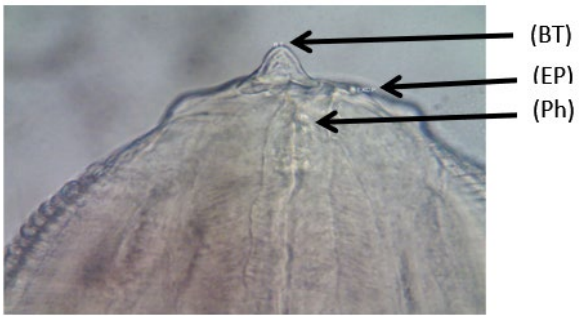

Figure 3. Anterior part of the third-stage larva. Boring tooth (BT), excretory pore (EP), and oesophagus (Ph) (Scale bar= 0.10 mm).

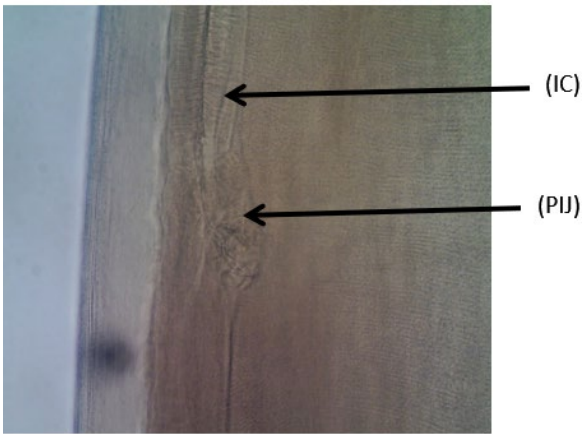

Figure 4. The pharynx-intestine junction area (PIJ), intestinal cecum (IC) (Scale bar = 0.10 mm).

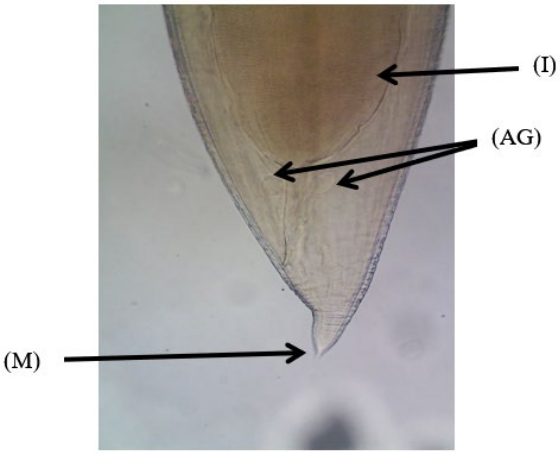

Figure 5. Posterior part of contracaecum: intestine (I), anal glands (AG), and mucron (M) (Scale-bar= 0.10 mm).

Statistical Analysis: The results data were analyzed by Chi-square. The SPSS statistical software (version 24) was used to analyze data. The Pearson correlation coefficient was analyzed between factors (Peck et al., 2015).

Molecular Analysis: A total of molecular were detected by the morphological examination used to detect eight third-stage larvae belonging to the *Contracaecum* genus of anisakid larvae. Then, eight *Contracaecum spp.* larvae were subjected to a molecular approach. DNA extraction from middle parts of *Contracaecum* larvae.

The molecular analysis was conducted on eight *Contracaecum* larvae. The larvae were selected randomly for each month. DNA extraction kit (Geneaid Biotech, Korea) was used. The total DNA was recovered from the center region of the larvae (Fig. 6). Amplifications focused on the ITS regions that were amplified using the primers NC5 (5'-GTA GGT GAACCT GCG GAA GGA TCA TT-3') and NC2 (5'-TTA GTTTCT TTT CCT CCG CT-3') (Zhu et al.,1998).

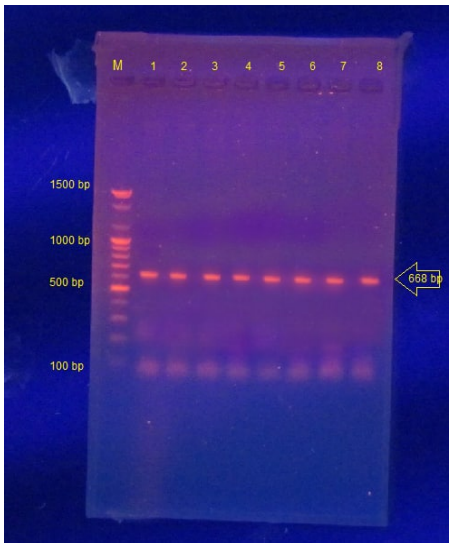

Figure 6. Electrophoresis of PCR product of *Contracaecum* of ITS1a gen for *Contracaecum* larvae at 668 bp according to DNA Ladder (100 -1500 bp), Lines: 1–8: positive all *Contracaecum* samples.

PCR conditions followed the protocol described by Pekmezci et al. (2014). Briefly, the Amplification of DNA fragments of interest from genomic DNA was performed using the polymerase chain reaction (PCR). The reaction volume was prepared as 25 µl which included 5 µl of a sample containing DNA, 1.5 µM forward primer, 1.5 µM of reverse primer, and double-distilled water to a final volume of 25 µl in the master mix kit (Promega, USA). The reaction was performed with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 seconds. (denaturation), 65 °C for 60 s (annealing) and 72 °C for 1 min (extension), with a final extension step at 72 °C for 7 min to ensure all amplification reactions had reached completion. The PCR products were analyzed by Safe-Red™ -agarose gel electrophoresis. PCR conditions were also used according to (D'amelio et al., 2007). UV transillumination was used and visualized on 1.5% agarose of the amplified rRNA products. The sequences of nucleotide acquired in the current study

were listed in GenBank under accession the number of OP787071.

To detect sequence similarities, NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) ran BLAST searches. For analysis, GenBank sequences of Contracaecum species were retrieved. The analysis included 28 nucleotide sequences, including one from Contracaecum Rudolph (OP787071). The similarity was determined using the Maximum Likelihood approach in the MEGA program (11.0.13). The search tree was built automatically using Neighbor-Join and the Maximum Composite Likelihood approach, and the topology with the greatest log-likelihood value was picked (Fig. 7).

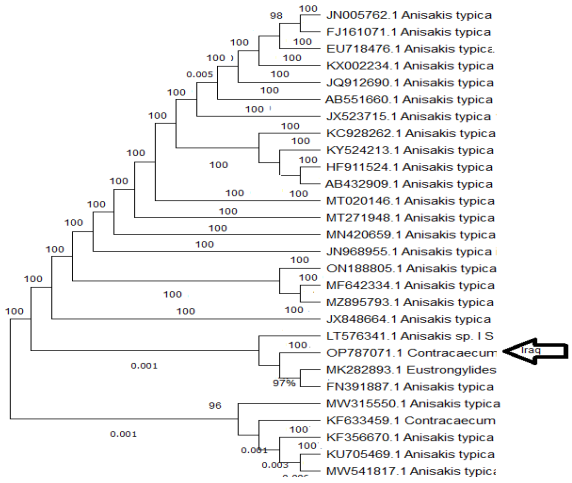

Figure 7. Phylogenetic analysis of ITS1 homologs from different Anskidae. Neighbor-joining reconstruction between the sequence of *Contracaecum rudolphii* obtained in this study and sequences of *Eustrongylides* and Anisakis typical species, recorded in GenBank and Phylogenetic analysis from the NCBI BLAST database. The MEGA (11.0.13) tool was used to compare the sequences to those performed independently for each gene fragment.

Ethical Approval: Ethics required are approved by the Ethical Committee of the University of Kerbala / College of Veterinary Medicine. Date: 23/02/2023, Ref: UOK.VET. PA.2022.05.

Results

The nematodes were examined and identified as Contracaecum third larval stage in the current study using a morphology consistency molecular genetic approach and phylogenetic analysis. Generally, all the nematodes have been *Contracaecum spp.* using light microscopic inspection depending on the special characteristics feature of this parasite (Figs. 2-7). This survey was completed during a period, from Jun 2022 to January 2023 to study nematode parasites of *Planiliza abu* of Razzaz Lake. Primer sequences of *C. rudolphii* isolate internal transcribed spacer 1(ITS1) (Table 1). The total prevalence of 395 fish was 124(31.3%). Infection rates were greatest in September and November and lowest from October to 2022 (Table 2). The relation among length, No. of nematode, and No. of infected fish have been signed at the level P≤0.05 according to (R) correlation (Table 3).

Discussion

The genus *Contracaecum spp.* includes more than 100 species, which are distributed globally from different hosts (Shamsi et al., 2009). Anisakiasis is widely distributed with their larvae recorded in various fish species from different countries, resulting *Anisakis* nematodes but it remains a neglected zoonotic disease (Aguilar-Marcelino et al., 2022; Shamsi and Barton, 2023). Because the zoonotic nematode Anisakidae family poses a risk to human health, it is crucial to identify fish (Buchmann and Mehrdana, 2016). Anisakidae is a family of nematode parasites, and one of the most significant fish-borne zoonoses in Europe is anisakidosis. It results from consuming the infectious larvae in their third stage causing the subacute abdomen and masquerading as an intraperitoneal malignancy (Dinas et al., 2024).

In this study, the nematodes were investigated and identified as Contracaecum third larval stage utilizing a morphological consistency molecular genetic technique. This survey was made from June 2022 to January 2023, and it studied the nematodes that parasitize the viscera the *P. abu* from Razzaza Lake. Generally, all the nematodes have been confirmed as *Contracaecum spp.* using morphological examination depending on the specific features of this parasitic nematode (Figs. 2-5). The total prevalence of 395 fish was 124 (31.3%) (Table 2). According to morphological examination, all of the fish were of the larval type Contracaecum. This result agrees with (Jawad et al., 2022), who confirmed nematodes in fish (*P. abu*) as *Contracaecum* spp. by Kerbala University's Veterinary Medicine College in the parasitology lab using morphological examination. The monthly infection in September and November had high infection rates, while in October, it had low infection rates of 32.0%, 31.5%, and 0.1%, respectively. In this study, the infection rates were the greatest in January 2023 (46.6%), but they were the lowest in September 2022 (20%), This may be climatic condition changes, founding or increased intermediate hosts and less fishering leading to favorable to increase infection (Table 2). Another study found 30% of *Contracaecum spp.* larvae in fish species from Lake Nasser, Egypt (Hamouda and Younis, 2022). In natural settings, the parasite despises the capacity to kill the intermediate host and prefers to finish the life cycle on the ultimate host. These factors may decrease the possibility of *Contracaecum* L3 larvae being transmitted to their ultimate host, resulting in a decreased total infection (Barson, 2004). In the current study, however, the gender length and infection of fish have not been significant within months (*P*>0.05). While with weight, none of the infected fish have been signed at the level P>0,05. The gender with length at level P≤0.01 and gender with weight at the level P≤0.05 according to Pearson correlation (Table 3). These characteristics were non-significant with host size, prevalence, infection severity, and body condition in *Clarias gariepinus* from Lake Chivero, Zimbabwe (Barson, 2004).

Table 1. Primer sequences of *C. rudolphii* isolate internal transcribed spacer 1(ITS1).

Primer	Sequence (5' – 3')	Amplicon size
ITS1(F)	GTA GGT GAACCT GCG GAA GGA TCA TT	
ITS1(R)	TTA GTTTCT TTT CCT CCG CT	**668 bp**

Table 2. Prevalence, length, gender and infection in the *Planiliza abu*.

Months of study	Length		Gender		Total no. of fish	No. of infected fish	%	No. of nematodes in fish
	infection	No infection	infected Male	Female infected				
Jun	10	9	2	13	58	15	25.8	48
2022 July	11	10	3	10	55	13	23.6	60
August	8	9	5	6	31	11	35.4	52
September	12	10	4	8	60	12	20	72
October	8.5	10.5	7	11	50	18	36	44
November	11	10.5	4	11	45	15	33.3	68
December	12	11.5	4	15	51	19	37.2	80
2023 January	13	12.5	5	16	45	21	46.6	76
					395	124	31.3	508

Table 3. Pearson correlation of infection in the *Planiliza abu*.

	Pearson correlation					
	Length	**Weight**	**Gender**	**No of infected fish**	**health fish**	**No of fish**
Length	1	0.789*	0.879**	0.686*	0.252	0.467
Weight		1	0.794*	0.66	0.143	0.475
Gender			1	0.515	0.209	0.509
No of infected fish				1	-0.177	-0.018
Health fish					1	0.899**
No of fish						1

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Currently, the overall mean intensity was 4 and 1-19 worms per fish degree of infection, which is consistent with the findings (Al-Zubaidy, 2009) and disagreement with (Barson, 2004) who discovered (1-7) worms per fish and mean intensity (2.2). This variation might be due to ecological ambient variables, location, sample size, and intermediate host characteristics. In some of the studies, a survey of parasites of *P. abu* of Razzaza Lake was conducted in Karbala, Iraq (Al-Saadi et al., 2010; Al-Zubaidy, 2009; Jawad et al., 2022) (25.9%, 0.8%; in 2019, 48.73% of 148 fish were caught, 65.08% of 277 in 2020, and 9.6% of 577 in 2021).

Iraq's southern al-Sanaf swamp Sequence compares between Contracaecum larvae and the ITS-1 sections of rDNA amplified using PCR and proved that *Nycticorax nycticorax* had two different species. The first *C. septentrionale* and second *C. microcephalum*, both initially identified in Iraq, entered into GenBank with the entry numbers of MK424799.1 and MK424795, respectively Mohammad and Hbaiel (2019).

In Sulaimani Province, Iraq, contra caecum has been identified in 13 different species of freshwater fish, one from each of the following families: Bagridae, Heteropneustidae, Mastacembelidae, Mugilidae, Siluridae, and Sisoridae. All *Contracaecum* larvae were determined by morphological and genomic (ITS1, ITS2, and COX2) analysis to be members of the same species (*C. rudolphii* B), with infection rates ranging from 0.92% to 19.35% (Abdullah et al., 2021a). ITS-1, ITS-2 and COX-2 showed that all infected fish species representing one species (*C. rudolphii* B), are gathered in five Cyprinid fish species in this location, and these findings were published at the same region utilizing modern study techniques (Abdullah et al., 2021b).

In this work, the phylogenetic connections between *C. rudolphii* and other genera, such as *Anisakis typica* and *Eustrongyloides spp.* were determined using maximum likelihood using the ITS-1 gene. MEGA (11.0.13) was used to create the phylogenetic tree. *Eustrongyloides spp.* (MK282893.1) and *Anisakis typica* (FN391887.1) were highly identical to *C. rudolphii* 1 with 97% similarity and formed a clade with them, (Table 1) (Figs. 6 and 7).

This study utilizes PCR and sequencing of eight Contracaecum larvae from 200 samples over eight months, comparable to another study from Shadegan Wetland, Iran. All of the discoveries were proven to be *Contracaecum* spp. based on the phylogenetic tree and genetic distance, which identified *A. pegreffii* and *C. rudolphii* as the species of all nematodes. Primers NC5-NC2 were used to amplify an ITS segment from the worms *Barbus grypus* and *Mesopotamichthys sharpeyi,* which were subsequently subjected to *Contracaecum speciosus* and *Anisakis* infections (Mohammadi et al., 2021).

The ITS ribosomal gene was identified using PCR, and the mitochondrial genes COX2 and rrnS were molecularly

characterized in Guerrero mullet fish (*Mugil curema*). *Contracaecum sp.* was discovered with a frequency of 283 (61.5%) of 460 nematodes in stage 3 (L3) (Martínez et al., 2022). In South Wales, Australia, the ITS-2 region of rDNA, found in the intestinal tissue of carp from Coonancoocabil Lagoon, was used to identify a unique *Contracaecum bancrofti* (type IV) (Shamsi et al., 2018). The sequences of the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of each morphospecies' nuclear ribosomal DNA are examined. The comparison of ITS-1 and ITS-2 sequencing data for individuals of *C. ogmorhini* sensu lato from pinnipeds with other species revealed that ITS-2 can be used for differentiation among Contracaecum species based on morphological data and was useful in confirming the taxonomic status of individual species in Australia (Shamsi et al., 2009).

In Ethiopia along Lake Tana Based on ITS1 analysis, two separate Contracaecum species were identified: *Contracaecum sp.* 1 and *Contracaecum sp.* 2, which shared 99% and 98% of their characteristics with *Contracaecum sp.* While *Contracaecum* sp. 1 and *Contracaecum* sp. 2 revealed 91% and 89% similarity with *Contracaecum multipapillatum* in their rrns, respectively (Kibet et al., 2021). This work highlights the need to integrate morphological and molecular techniques with (ITS1) to identify *Contracaecum rudolphii* larval stages, particularly those that occur in fish (*P. abu*).

Conclusion

In conclusion, the present study is the first molecular sequencing in fish (*P. Abu*), that has not been previously recorded from a local market in Razzaza Lake in Karbala, Iraq. The results have been confirmed that *C. rudolphii* depends on (ITS1) analysis. Further studies are needed to extend the knowledge of *Contracaecum* species distributed in a local market in Razzaza Lake in Karbala, Iraq. A good finding as a molecular genotyping might be a useful technique for identifying the Contracaecum L3 larval species., life-cycle biology. There is very little risk from zoonotic anisakids, such as *C. rudolphii*, in the area under study. Therefore, it is crucial to use genetic and molecular methods when learning about one species of fish, and should be expanded to other species. To reduce the risk of human infections, molecular searches for *Contracaecum* larvae in eaten seafood, particularly fish hosts, are required to support food safety.

Conflict of Interest

The authors declared no conflicts of interest regarding this manuscript's publication.

Ethical Approval

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Author Contributions

The authors are equall in Motivation, Concept, Design, Control/Supervision, Data Collection, Analysis, Literature Review, Writing the Article and Critical Review.

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Determination of urea, creatinine and urea/creatinine ratios in calves with diarrhoea

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Abstract: The objective of this study was to ascertain the levels of urea, creatinine (Crea) and the urea/creatinine ratio (UCR) in calves presenting with diarrhoea. The material of the study consisted of 20 calves with diarrhoea and 10 healthy calves. Once the aetiology of diarrhoeal calves had been determined, blood samples were taken, and urea, creatinine and UCR levels were determined. The findings of the study indicated that the levels of urea and UCR were statistically significantly elevated in calves with diarrhoea in comparison to the control group. However, the increase in creatinine level was statistically insignificant. The results of the study revealed that UCR is an important biomarker in the evaluation of renal failure in calves with diarrhoea.

Keywords: *Calf, creatinine, diarrhoea, urea, UCR.*

İshalli buzağılarda üre, kreatinin ve üre/kreatinin oranlarının belirlenmesi

Özet: Bu çalışmada ishalli buzağılarda üre, kreatin (Crea) ve üre/kreatinin oranının (UCR) belirlenmesi amaçlandı. Çalışmanın materyalini 20 ishalli buzağı ve 10 sağlıklı buzağı oluşturdu. İshalli buzağıların etiyolojileri belirlendikten sonra kan örnekleri alındı ve üre, kreatinin ve UCR düzeyleri belirlendi. Çalışmanın sonuçları ishalli buzağılarda kontrol grubuna göre istatistiksel olarak önemli derecede yüksek üre ve UCR düzeyi tespit edildi. Crea düzeyindeki artış ise istatistiksel olarak önemsizdi. Çalışmanın sonuçları ishalli buzağılarda böbrek yetmezliğinin değerlendirilmesinde UCR'nin önemli bir biyobelirteç olduğunu ortaya koymuştur.

Anahtar Kelimeler: *Buzağı, ishal, kreatinin, UCR.*

Introduction

Gastrointestinal disease affecting weaned calves, calf diarrhoea, is an important disease in veterinary medicine (Maier et al., 2022). The most frequently identified etiological agents in the aetiology of the disease include *Escherichia coli* (*E. coli*), *Giardia duodenalis*, coronavirus, *Cryptosporidium parvum* (*C. parvum*), and rotavirus (Balıkçı et al., 2024; Keleş et al., 2022; Mamak et al., 2023). Infectious agents are responsible for intestinal damage and a significant loss of body fluids within the intestine. This results in a rapid onset of dehydration in the calf (Kasa et al., 2020). Additionally, calf diarrhoea can result in dehydration, as well as electrolyte imbalance, metabolic acidosis, hypovolaemia and, over time, renal failure (Shehta et al., 2022).

Urea and creatinine (Crea) are commonly employed parameters in the assessment of renal failure in calf diarrhoea (Akyüz et al., 2022; Yanar et al., 2023). Nevertheless, the urea-creatinine ratio (UCR) has been a commonly employed metric in the field of human medicine in recent years (Statlender et al., 2024; Tonomura et al., 2023). The evidence suggests that UCR may serve as an effective marker for the assessment of renal failure. Furthermore, recent studies have indicated that UCR may serve as a prognostic indicator in humans (Brookes and Power 2022; van der Slikke et al., 2020). Nevertheless, the utilisation of UCR in veterinary medicine remains relatively constrained in comparison to its application in human medicine. In the literature review, there are very few studies evaluating UCR in calves with diarrhoea (Wiest and Klee, 1998).

In this context, the objective of this research was to evaluate the UCR level in calves with diarrhoea. The UCR level may serve as a suitable parameter in patients with renal failure resulting from dehydration, particularly in calves with diarrhoea, or in veterinary practice for the evaluation of direct renal failure.

Materials and Methods

Study design: The study population consisted of 20 calves (n=20) aged 1–10 days admitted to the Atatürk University Veterinary Faculty Animal Hospital large animal clinic with diarrhoea. A control group of 10 healthy calves (n=10) was also included. The study was approved by the Atatürk University Local Ethics Committee of Animal Experiments (Decision Number: 2024/18). To be eligible for inclusion in the study, calves with diarrhoea had to have received no prior treatment. Calves undergoing any form of treatment were excluded from the study. A faecal sample was initially obtained from diarrheal calves using a swab, and the aetiology of the diarrhoea was subsequently determined using a rapid test kit for five infectious agents, including *E. coli* (ETEC F5), *Giardia duodenalis*, coronavirus, *parvum* and rotavirus.

Blood sampling: Blood samples were collected from the *Vena jugularis* of calves presenting with diarrhoea and placed into serum tubes of approximately 10ml capacity. The serum samples were maintained at room temperature for 30 minutes and subsequently subjected to centrifugation at 3000 rpm for 10 minutes in a Beckman Coulter Allegra® X-30R centrifuge (USA) to obtain serum samples. Immediately following the determination of the serum samples, urea and crea measurements were performed using an auto analyser (Beckman Coulter® AU5800, USA). The urea-creatinine ratio was calculated using the following formula:

Urea/Creatinine ratio: The ratio of serum urea/serum crea was calculated.

Statistical Analysis: Before statistical analysis, the normality of the data distribution was tested using the Shapiro–Wilk test. Since the data obtained in this study did not have normal distribution, it was subjected to analysis using the Mann-Whitney *U* test, a non-parametric statistical test. Statistical analyses were performed using SPSS 27.0 software, with significance set at $P < 0.05$ to detect statistical differences between group.

Results

The results of the etiological analysis revealed that the most prevalent infectious agent was *E. coli*, with a prevalence rate of 60% (12/20). Furthermore, three calves exhibited signs of rotavirus-induced diarrhoea (3/20), while five calves displayed a rotavirus, coronavirus and *C. parvum* mixed infection (5/20).

The results of the biochemical analyses indicated a statistically significant ($P < 0.001$) elevation in urea levels (130.93 mg/dL [69.99-245.23 mg/dL]) in the calves with diarrhoea compared to the control group (29.02 mg/dL [0.98-45.20 mg/dL]) (Table 1). Furthermore, the concentration of crea in the serum of calves with diarrhoea (2.51 mg/dL [1.44-4.07 mg/dL]) was observed to be higher than that of the control group (2.3 mg/dL [0.09-2.97 mg/dL]). However, this elevation was not found to be statistically significant. Finally, the UCR was found to be significantly elevated ($P < .001$) in the diarrhoeic group (53.70 [31.88-125.12]) in comparison to the control group (14.22 [9.15-22.94]).

Discussion and Conclusion

The objective of this study was to ascertain the UCR level in the assessment of renal damage in calves with diarrhoea. In the study, *E. coli* was the most frequently identified agent in calves with diarrhoea. This finding is not aligned with the results of prevalence studies investigating the aetiological causes of calf diarrhoea in various provinces of Turkey (Balıkçı et al., 2023; Mamak et al., 2023). It is, however, no prevalence study was conducted in the region where the study was conducted. Moreover, it has been established that *E. coli*-induced calf diarrhoea represents a significant global health concern (Coşkun and Şahin 2023; Nguyen et al., 2011). In light of these findings, it is possible that the prevalence of calf diarrhoea in the Erzurum region may differ from the levels reported in the literature.

In the study, the serum urea level was found to be statistically significantly higher (P<.001) in calves with diarrhoea compared to the control group. These results were in line with previous reports (Eğlenti et al., 2020; Saleh et al., 2022). It is important to note, however, that there is also a study reporting non-statistically significant increases in urea levels due to diarrhoea (Torche et al., 2020). The discrepancies in clinical severity observed in the calves included in this study may be the underlying cause of these contradictory results. Dehydration and hypovolemia resulting from diarrhoea in calves (Kozat, 2021; Shehta et al., 2022) may lead to pre-renal azotemia (Molitoris, 2022), which could explain the observed increase in urea levels. Furthermore, the elevation in urine concentration resulting from dehydration may also be a contributing factor to the observed increase in urea levels (Thomas et al., 2008) as urea is an important factor in increasing urine concentration (Yang et al., 2005). In addition, previous studies have indicated that gastrointestinal bleeding may also be a contributing factor to elevated urea levels (Stellato et al., 1980; Tomizawa et al., 2015). In this study, the *E. coli* infection agent was detected in the majority of calves presenting with diarrhoea (60%), and no clinical signs indicative of gastrointestinal haemorrhage were observed in the calves. In light of these findings, it is reasonable to suggest that the elevated urea levels observed in calves with diarrhoea are due to dehydration and hypovolemia rather than gastrointestinal haemorrhage.

Table 1. Urea, crea and UCR levels of healthy and diarrhoeic calves.

Parameters	Control Medians (Range)	Diarrhoea Medians (Range)	*P* Value
Urea (mg/dL)	29.02 (0.98-45.20)[a]	130.93 (69.99-245.23)[b]	*P*<.001
Crea (mg/dL)	2.3 (0.09- 2.97)	2.51 (1.44-4.07)	*P*=0.448
UCR	14.22 (9.15-22.94)[a]	53.70 (31.88-125.12)[b]	*P*<.001

a, b The means shown in different lowercase letters between the groups (on the line) are statistically significant.

The crea values of the diarrhoeal calves were observed to be higher than those of the control group; however, this increase was not found to be statistically significant. This result was not consistent with previous reports (Akyüz and Kükürt, 2021; Makdam and Basbugan, 2020). This result of the study may be due to insufficient protein intake of the calves. Studies have shown that crea levels may increase when protein intake is insufficient (Valtonen et al., 1982). In this context, a partial increase in crea levels in calves may have occurred due to insufficient protein intake. Strikingly, however, the result of a recent study shows that there may be statistically significant or insignificant increases in crea levels depending on the etiological factors of calf diarrhoea. Furthermore, in this study, the increase in crea levels in calves infected with *E. coli* was statistically significant compared to the control group, whereas the increase in calves infected with rotavirus was not statistically significant (Tümer and Dincer, 2024). Although the majority of pathogens detected in calf diarrhoea in this study were *E.coli*, it is important to report the presence of calves with rotavirus infection. In this context, it can be argued that the CRE level in calf diarrhoea may vary depending on the aetiological agent.

The UCR level was found to be statistically significantly higher in the diarrheal group than in the control group. This finding was consistent with the results of previous human studies, which have demonstrated that UCR can be used as a prognostic marker in patients with renal failure (Brookes and Power, 2022; Tonomura et al., 2023). In the literature review, the number of studies on this subject in the field of veterinary medicine is relatively few (Wiest and Klee, 1998; Lobetti, 2012). However, the reason for the increased UCR level in human studies remains unclear. At this juncture, the potential mechanism may be attributed to a multitude of effects exerted by urea, including disruption of the intestinal epithelial barrier and alteration of the microbiome (Lau and Vaziri, 2017; Seki et al., 2019). In calves with diarrhoea, the impairment of the epithelial barrier and flora may have triggered a relative increase in urea level compared to creatinine level, leading to a statistical increase in UCR level. Nevertheless, further research is required to ascertain the impact of urea on calves with intestinal damage and to determine whether intestinal damage is a contributing factor to the elevation of serum urea levels in these patients. However, within the context of this study, it was established that UCR levels can be employed in the assessment of renal failure in calves with diarrhoea.

In conclusion, this study has demonstrated that UCR level, which has been frequently used in the evaluation of renal failure in human medicine in recent years, may act as an important marker for the evaluation of renal failure in calves with diarrhoea. Furthermore, it may be useful to evaluate UCR levels in patients who may cause secondary renal failure, especially in the field of veterinary medicine.

Conflict of Interest

The authors stated that they did not have a potential or perceived conflict of interest.

Ethical Approval

The study was approved by the Atatürk University Local Ethics Committee of Animal Experiments (Decision Number: 2024/18).

Similarity Rate

We declare that the similarity rate of the article is 15% as stated in the report uploaded to the system.

Author Contributions

Design: KEY
Control/Supervision: KEY, MSA
Data Collection and/or Processing: KEY, AÖ
Analysis and / or Interpretation: KEY, AÖ
Literature Review: KEY
Writing the Article: KEY
Critical Review: MSA

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Uterine prolapse observed during and immediately after parturition in three dogs: case report and literature review

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Abstract: Uterine prolapse (UPR) in bitches is a rare obstetric emergency. Generally, it occurs within 48 hours postpartum in bitches. Opposite this datum, in this case report three instances of UPR presented as two during parturition and one at 40 days postpartum. Besides one of them, which occurred during labor, was complicated by uterine rupture. Although no definitive etiology has been determined, it seems that the leading cause is weakness in pelvic connective tissues. Factors contributing to UPR include excessive relaxation of pelvic ligaments, difficult labor, and excessive straining. The extracellular matrix (ECM), composed mainly of collagen and elastin, is crucial in maintaining tissue integrity. Hormones like estrogen, progesterone, and relaxin significantly affect the ECM's structure during pregnancy and parturition, influencing pelvic elasticity and uterine strength. Besides, our cases showed that poor body condition, parity, age, and breed predisposition are also suspected as contributing factors. All the animals were treated by surgery. Three cases show mothers and/or puppies may survive with timely and appropriate surgery. Besides, we believe that ovariohysterectomy is the best treatment choice with both survivor and prevention effects. However, to clearly define both the primary factors and co-factors speculated by this report, further research is required to investigate.

Keywords: *Dog, Parturition, Uterine prolapse.*

Üç köpekte doğum sırasında ve hemen sonrasında görülen uterus prolapsusu: olgu sunumları ve literatür derlemesi

Özet: Köpeklerde uterus prolapsusu (UPR) nadir görülen bir obstetrik acil durumdur. Genellikle köpeklerde doğumdan sonraki 48 saat içinde meydana gelir. Bu veriye zıt olarak sunulan raporda ikisi doğum sırasında ve biri postpartum 40. günde şekillenen üç UPR olgusu bildirilmiştir. Doğum sırasında şekillenen olgulardan bir tanesi uterus rupturu ile komplike olarak gelişmiştir. Kesin bir etiyolojisi olmasa da başlıca neden pelvik bağ dokularının zayıflığı gibi görünmektedir. UPR'ye katkıda bulunan faktörler arasında pelvik bağların aşırı gevşemesi, güç doğum ve aşırı zorlanma yer alır. Esas olarak kolajen ve elastinden oluşan ekstraselüler matriks (ECM) doku bütünlüğünün korunmasında çok önemlidir. Östrojen, progesteron ve relaksin gibi hormonlar, gebelik ve doğum sırasında ECM'nin yapısını önemli ölçüde etkileyerek pelvik elastikiyetini ve uterus gücünü değiştirir. Ayrıca, olgularımız zayıf vücut kondisyonu, doğum sayısı, yaş ve cins yatkınlığının da predispoze faktörler arasında olabileceğinden şüphelendirmiştir. Tüm hayvanlar cerrahi olarak tedavi edilmiştir ve elle prolabe kitle reddenin küçük ırklarda işe yaramadığı ancak büyük ırklarda bir şans yarattığı belirlenmiştir. Üç vaka da anne ve/veya yavruların zamanında ve uygun cerrahi müdahale ile kurtarılabilmiştir. Hem sağ kalım hem de korunma açısından ovariohisterektominin en iyi tedavi seçeneği olduğu görüşüne de ulaşılmıştır. Ancak bu raporda öne sürülen hem birincil faktörleri hem de yardımcı faktörleri net bir şekilde tanımlayabilmek için daha fazla araştırma yapılması gerekmektedir.

Anahtar Kelimeler*: Doğum, Köpek, Uterus Prolapsusu.*

Introduction

Uterine prolapse (UPR) is an obstetrical emergency condition infrequently reported in dogs and cats (Nelissen, 2015). The urgency of these cases arises from the risk of contamination, obstruction of ovarian arteries and veins, rupture resulting in hemoperitoneum, peritonitis, dehydration, and hypothermia (Davidson, 2003). It is less common in dogs compared to cats (<0.003% vs 0.8%, respectively) (Davidson, 2003; Payan-Carreira et al., 2012). One or both uterine horns may prolapse through the dilated cervix and vulva (Nelissen, 2015) during the peripartum period (up to 48 hours postpartum) (Feldman and Nelson, 2004). There is no definitive cause of UPR but predisposing factors including excessive relaxation/stretching of the pelvic musculature, prolonged dystocia, inadequate obstetric maneuvers, oversized fetus, multiple pregnancies, uterine atony, excessive abdominal contractions, severe tenesmus, incomplete placental detachment (Binli et al., 2021; Özyurtlu and Kaya, 2005), and cystic endometrial hyperplasia (CEH) (Greiling et al., 2023). While it could be confused with vaginal prolapse and tumors (Nelissen, 2015), the diagnosis was made quickly with the medical history and visualization of a tubular mass protruding from the vulva.

In this report, three cases of UPR occurred without a history of obstetric maneuvers. Two cases (case 1 and case 2) were observed during parturition, and one of them was complicated by uterine rupture (UR) (case 1); the last one (case 3) occurred immediately postpartum and reoccurred despite being rejected several times.

Material and Methods

The animal material consisted of three dogs diagnosed with UPR in our clinic. General information on the cases is presented in Table 1.

Only non-experimental animals were used in this study. Internationally recognized high standards of individual veterinary clinical patient care were followed, and routine clinical treatment was performed. Therefore, ethical approval from a committee was not required.

Table 1. General information, examination and treatment findings of cases.

Signalements	Case 1	Case 2	Case 3
Breed	Kangal	Pekingese	French bulldog
Age (y)	4	4	1
BW (kg)	28	7	6.5
Parity	Multiparous	Multiparous	Primiparous
Prolapsed part	LUH	RUH	UB
Prolapsed time	8th h at parturition	24th h at parturition	İmmediately after parturition and continues to prolapse
Presenting to hospital	1 h later	6 h later	24 h later (after last occurrence)
Obstetric intervention	No	No	No
Previous treatment	No	No	Manually rep. x3
Physical examination	Slight dehydration	Slight dehydration	No abnormality
Inspection of mass	Bloody-green dis. PA Fetal structures Uterine rupture	Placental area. Ut. starting to dry Necrotization in PA	Bloody dis. Ut. starting to dry
Ultrasonography	Multiple fetuses Fetal viability	One fetus Fetal viability Invagination (Fig. 2B)	No fetuses No fetal structure Ut. diameter 1.5 cm
X Ray	>5 fetuses in abd.	1 fetus in abd.	No fetus in abd.
CBC	Slight anemia	In reference interval	Slight anemia
Treatment	Manually obstetrics Surgically rep. En-block OHE: – After 5 puppies were rescued with manually, 10 more puppies were rescued with surgery	Surgically rep. En-block OHE: – One puppy was rescued with surgery.	Surgically rep. Routine OHE
Complication	No	Cardiac arrest at surgery She rescued by CPR	No

BW: body weight, CBC: complete blood count, abd.: abdomen, LUH: left uterine horn, RUH: right uterine horn, UB: uterine body, Ut.: uterus, dis.: discharge, PA: placental areas, Rep: replacement, OHE: ovariohysterectomy, CPR: cardiopulmonary resuscitation.

Case Reports: The history of the cases is given below.

In case 1, labor had started seven hours earlier, and one puppy was born alive. While being brought to the hospital due to excessive contractions and lack of parturition, the prolapse and simultaneous UR has been observed by the owner.

Figure 1. Clinical presentation of case 2 (A) and case 3 (B).

In case 2, labor had started 24 hours before she was brought to the clinic, and ended after the delivery of three live puppies, and the mass was seen with the resumption of abdominal contractions.

In case 3, the dog had delivered four live puppies about 40 days ago. A mass in a red color appeared immediately after last puppy had been delivered and disappeared shortly after. The mass seemed once again 20 days after the labor, and it had been rejected manually by the owner due to it did not disappear spontaneously; this process was repeated three more times, and on the fourth and final occasion, the owner presented the dog to the hospital within 24 hours (Figure 1).

In all cases, preoperative fluid (Lactated Ringer's, IV, 10 ml/kg/h, Polifleks®; Polifarma) and antibiotic (Ceftriaxone, IV, 25 mg/kg, Novosef®; Sanofi) treatment was started; the prolapsed mass was washed and cleaned with saline solution (İzoVİP, Polifleks®; Polifarma) and compressed with hyperosmotic fluid (%30 Dekstroz, Polifleks®; Polifarma) for about half an hour to reduce edema; and surgery was performed as the primary treatment method. Unlike other cases, in case 1, the puppies that could be saved manually were delivered in the preoperative process. While en-bloc ovariohysterectomy (OHE) was performed in cases 1 and 2 due to the live puppies, a routine OHE was performed in case 3 (Figure 2). Surgery was performed under general anesthesia in all cases. After induction with propofol (6 mg/kg, Propofol®; Fresenius), anesthesia was continued with isoflurane (Isoflurane USP®, Adeka) and 100% after intubation. Intraoperative analgesia was provided with a constant-rate infusion of a combination of 500 ml saline (İzoVİP, Polifleks®; Polifarma), 30 mg ketamine (Ketasol®, Richter pharma), and 150 mg lidocaine (Adokain®, Sanovel) at a rate of 10 ml/kg/hour. Surgeries were performed with a midline incision. After entering the abdominal cavity, while the prolapsed uterus was pushed through to the inside of the vagina by an assistant externally, it was pulled into the abdominal cavity by the operator, intussusception was corrected, the tissue was restored to its anatomical position, and OHE was performed. The abdominal wall was closed as routinely. Antibiotic treatments were continued for seven days, and stitches were removed ten days after the surgery. A neonatal team immediately intervened and cared about the puppies. The puppies were kept under supervision in an incubator because the Apgar score of all the puppies were <8.

Discussion

In this report, three cases of UPR are discussed; two of which occurred during labor -one of them complicated with uterine rupture- and one of which occurred constantly recurring within 40 days of postpartum.

In canines, uterine horns reside entirely within the abdominal cavity. The uterine body is in both the abdominal and pelvic cavities, and they tethered the dorsolateral body wall and the lateral wall of the pelvic cavity by suspensory ligaments, respectively (Nelissen, 2015). Despite the distinct anatomical positioning of the uterus in dogs compared to humans, UPR in dogs can be similar to pelvic organ prolapse (POP) in humans. Therefore, etiologically, parallels may be drawn with other POPs, such as vaginal prolapse, rectal prolapse, or perineal hernia.

In dogs, the pelvic region bears approximately 36-37% of the body weight and is characterized by a robust framework comprising bone, muscle, and ligaments. In females, the anatomical architecture and functions of the

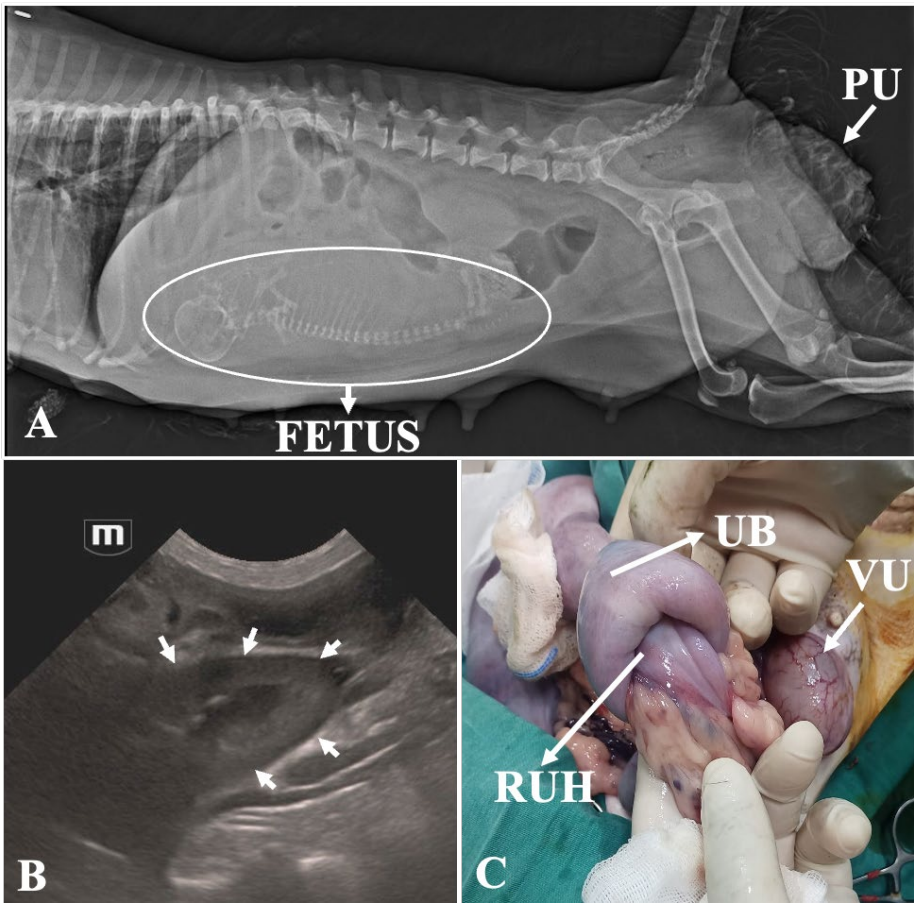

Figure 2. Images belong to case 2. A) The x-ray image of fetus in non-prolapsed uterine horns and uterine prolapse (UPR), B) Ultrasonographic appearance of uterine invagination (white arrows), C) Appearance of invaginated right uterine horn (RUH), uterine body (UB) and vesica urinary (VU) during operation.

pelvis, uterus, and vagina are designed to ensure the uninterrupted continuity of reproduction under optimal conditions. In this design, under the gonadal and non-gonadal effects of hormones such as estrogen (E2), progesterone (P4), and relaxin (RLN), all the reproductive organs must undergo significant anatomical, physiological and histological changes and adapt to pregnancy, labor, and peripartum period. These alterations encompass elongation of the suspensory ligaments to facilitate cranioventral expansion of the uterus concomitant with fetal growth, the capacity of the birth canal to withstand strong abdominal contractions during parturition, cervical dilation to accommodate the regress of fetuses propelled outward by contractions through the birth canal, edema formation in the vulva/vagina, and the physiological separation of the symphysis pelvis. These adaptive responses are examples of the requisite synchrony for successful reproduction.

Although the etiology of POP, such as UPR in humans and animals, remains unclear, there are common points, such as excessive relaxation/stretching of pelvic ligaments, dystocia, excessive abdominal contractions/straining. These factors can be considered macro-predisposing factors, but more recent studies have focused on the organization and abnormal structure of the connective tissue of the pelvic structure and uterosacral ligaments (Zhang et al., 2020). The macro-predisposing factors are shaped by tension, compression, and shear stresses that cause anatomical/physiological changes. They are referred to as mechanical forces at the cellular and subcellular levels. The complex structure and organization of the extracellular matrix (ECM) provides resistance to the mechanical load resulting from daily physiological activity, offers physical support to the cells, and determines the physical properties and behavior of the tissue. The ECM, whose main elements are proteoglycans and collagen, elastin, fibrous proteins, fibronectin, and laminin, organize to form rope-like that confers enormous tensile strength to tendons. Architecture also has the ability to degenerate and reorganize in response to physiological and pathophysiological processes in the same tissue (Karsdal et al., 2013). This dynamic structure is critical in processes such as pregnancy, labor, cervical ripening, and involution in the reproductive system. The most important component of ECM supporting the stability and plasticity of the pelvic floor is collagen synthesized by fibroblasts, with type I providing the mechanical tension, and type III providing the flexibility (Nallasamy et al., 2017). In these processes, weakening the supporting structures strength due to the alteration of the ECM architecture contributes to POP (Zhang et al., 2020), and in addition of gonadal activities, sex hormones also play a role with non-

gonadal or metabolic activities in organizing the macro-changes specific to pregnancy and parturition (Nallasamy et al., 2017). Here, E2 effects on muscle and connective tissue (Fede et al., 2019), and P4 may induces diabetes mellitus which is altering connective tissue function and structure by affecting collagen type I (Módena et al., 2016). Exposure of dogs to P4 during prolonged diestrus, even if not pregnant, may be effective in this regard (Johnston et al., 2001). Relaxin could induce relaxation of the pelvic ligaments and remodeling of the symphysis pelvis, which is associated with collagen degradation (Binli et al., 2023). The mechanism of ECM degradation is not clearly understood. However, this process depends on the combined activity of matrix metalloproteases (MMPs) and their regulation of the release, activation, or retention of growth factors, growth factor binding proteins, cell surface receptors, and cell-cell adhesion molecules. All collagens, especially collagen types I and III, can be degraded by MMPs such as MMP-2 and MMP-9 (Guler and Roovers, 2022). The balance between collagen synthesis and degradation is important for maintaining tissue integrity and tensile strength during continuous tissue remodeling, and disruption of this balance can lead to POP; for example, an increased type I/type III ratio has been found in prolapsed organs of women with POP (Sansilvestri-Morel et al., 2001). In addition, fibroblasts in prolapsed tissue have been found to produce more collagen, MMP-2 and MMP-9 activity increases, tissue inhibitors of metalloproteinases-1 (TIMP-1) activity decreases, and collagen turnover increases (Guler and Roovers, 2022). As this is a case report, we will not go into further molecular detail but will briefly mention the effects of sex hormones on MMP and collagen activity. Studies show that E2 increases connective tissue turnover in the pelvic floor, upregulates MMPs not all and causes an increase in ECM destruction by suppressing TIMPs, suppresses fibroblast proliferation, and increases MMP-2 activity (Fede et al., 2019; Kanca et al., 2011). Progesterone increases MMP-2 activity and decreases MMP-9 activity (Kanca et al., 2011) and, when combined with E2, causes a decrease in the active form of MMP-1 (Zong et al., 2007). Due to its collagenolytic effect, RLN alters ligament mechanics by releasing the MMP activators collagenase, causing induction of collagen type 1 in the symphysis pelvis, inducing MMP1 and MMP-3 expression while showing little effect on TIMPs (Kapila and Xie, 1998).

The role of sex hormones and repeated exposure to them in the etiology of POP is clear. Indeed, the likelihood of UPR in women increases with parity and age (Schulten et al., 2022). The fact that two of our cases (cases 1 and 2) were four years old and multiparous suggests that age and parity may be necessary in the etiology of UPR in dogs, causing repeated exposure to sex hormones and the pregnancy process. On the other hand, the fact that both our case 3 and the case in a previous report (Ağaoğlu et al., 2012) were young (2 years old) and primiparous rules out the importance of repeated exposure to sex hormones alone and even suggests that other factors. In fact, rectal prolapse in dogs often occurs in young animals (< 11 months of age) and is reported to be more common in males, in cases of severe tenesmus, and in only 6% of cases because of dystocia (Igna et al., 2021). These data, as stated in the previous literature (Binli et al., 2021), show that the effect of mechanical forces caused by excessive pushing during parturition increased abdominal tension due to tenesmus, inappropriate oxytocin treatment, or inappropriate obstetric maneuvers affecting the pelvic structures is also essential. In the reports we encountered, there were no attempts to assist parturition, such as oxytocin treatment or obstetric maneuvers. Therefore, we believe that hormonal factors and parturition play a role in the etiology of the cases presented here, not alone but combined with other factors: Body condition score (BCS) and genetic predisposition.

In case 1, the Kangal weighed 28 kg; in case 3, the French Bulldog weighed 6.5 kg. However, adult body weights should be 50-60 kg for the Kangal and 9-13 kg for the French Bulldog. On the other hand, Pekinese (case 2) weighed 7 kg, slightly above the required range of 3.2-6.4 kg. Overweight in women are known to have significant effects on uterine and vaginal prolapse (Myers et al., 2012). Weight loss and weight gain lead to lean mass changes, including adipose tissue and muscle. The ECM is directly linked to weight gain/loss, and adipose tissue plays a vital role in this process. The adipocyte can be expressed as a single fat droplet surrounded by a thick ECM, and the ECM around the adipocyte, which shrinks due to weight loss, is expected to adapt to changes in cell volume. This process can lead to tension and cellular stress. During long-term weight loss, a downregulation in genes that regulate the ECM in adipose tissue and changes in the expression levels of ECM components can be observed (Roumans et al., 2015). Based on this information, we believe that a poor BCS in cases 1 and 3 may have contributed to the formation of the case by negatively affecting the ECM structure.

Assessing age and breed predisposition is difficult because UPR cases are rare in dogs (Payan-Carreira et al., 2012). We agree with this opinion because the small number of cases/reports in dogs does not allow a comprehensive evaluation of UPR, such as a systematic review or meta-analysis. On the other hand, as two of our cases (case 2 and case 3) were small breeds and brachycephalic, we speculate that there may be a breed predisposition in the case. Although UPR has been reported in different breeds such as Pomeranian Spitz (Mashhadi et al., 2024), American Bully (Angrimani et al., 2020) and Great Dane (Sathiamoorhyt et al., 2013), we speculate that vaginal prolapse (Nelissen, 2015) and inguinal hernia (Binli et al., 2023) in dogs may be predisposed by age, small size and brachycephalic breed. This speculation was also supported by a report on mares and cows: Arabian mares (Boye et al., 2022) and beef cows (Peter, 2014) are more prone to UPR than other mares and dairy cows. On the other hand, it is clear that this speculation needs to be supported by genetic studies, and genetic studies in human medicine support our speculation. It has been found that women with a family history of POP are more likely to develop POP than those without. Twin studies have shown that genetic factors account for about 43% of the variation in susceptibility to POPs. Several genetic variants or polymorphisms that play a role in prolapse by affecting collagen synthesis and ECM remodeling have been

identified in women (Lim et al., 2014). We believe that this topic warrants further investigation in veterinary medicine.

Uterine rupture is an emergent and uncommon situation. Some of the predisposing factors that claim to UPR cases also available to uterine rupture. Although factors such as uterine torsion and trauma are the most important, conditions such as prolonged dystocia and pyometra, which make the uterine wall thin and fragile, also play an important role in etiology (Johnston et al., 2001). Although superfecundation and multiple pregnancies are common in dogs, a pregnancy with 16 puppies, as seen in case 1, can be considered unusual and may have resulted in rupture due to excessive stretching on the uterine wall. At the same time, the incidence of dystocia in dogs can be up to 100% in predisposed breeds. Uterine inertia is the most common cause of dystocia. While complete primary uterine inertia does not begin, incomplete primary uterine inertia begins and ends after a few fetuses have been delivered (Gendler et al., 2007). Factors involved include vaginal hyperplasia, vaginal or uterine prolapse, which usually results in a narrowing or obstruction of the birth canal (Gendler et al., 2007), and UPR is responsible for 0.6% of maternal dystocia (Feldman and Nelson, 2004). Case 1 was considered as an incomplete primary uterine inertia case because labor started, and a puppy was born. In this case, the uterus ruptured and prolapsed after continued excessive stretching, and the factors mentioned above, such as ECM architecture and BCS, also contributed. Unlike the Kangal, our other two cases belong to breeds predisposed to dystocia (Johnston et al., 2001). In case 2, the interruption of labor and the onset of labor after 24 hours of rest suggest incomplete primary uterine inertia in this case. It is known that pelvic disharmony and uterine inertia are common in breeds predisposed to dystocia (Johnston et al., 2001), and it is also known that uterine relaxation and uterine inertia provided by P4 may lead to UPR in women (Módena et al., 2016) and cows (Peter, 2014).

Treatment might be performed medically or surgically, and when deciding on the treatment method, factors such as the uterus condition, the animal's breeding value, and the owner's wishes are considered. We believe that even if the uterus is successfully replaced by medical treatment, there is a possibility of recurrence due to excessive stretching of the suspensory and pelvic ligaments, and this risk is even higher in a future pregnancy. It should also be considered that if pregnancy is not achieved, uterine pathologies such as pyometra may develop, as the prolapsed uterus is open to trauma, environmental conditions, and bacterial contamination. Uterine prolapse complicated with CEH suggests that the possibility of recurrence may be increased if pyometra develops. In a Great Dane with bilateral UPR, manual placement was reported to be successful with no short-term complications, but no information was provided on the long-term follow-up (Sathiamoorthy et al., 2013). In contrast to this case, the recurrence of the case after manual treatment in a Pomeranian Spitz (Mashhadi et al., 2024), and in case 3 presented here, leads us to question the validity of manual rejection in the treatment in dogs. The reason for the success of manual repositioning of UPR in cows is that the birth canal is sufficiently large and wide to allow manual treatment. In this case, Sathiamoorthy et al. (2013) attributed the procedure's success to the animal's pelvic floor size, which allowed for manual intervention. Both our case and Mashhadi et al. (2024) suggest that manual therapy is unsuccessful in small breeds.

On the other hand, there is the possibility of vascular congestion due to intussusception and consequent devitalization or ischemia-reperfusion injury (Zitkute et al., 2021). Intussusception is challenging to regulate as hemodynamics cannot be easily altered, and invasive procedures are required. Considering the above reasons, we agree with the publications reporting that the most appropriate treatment intervention in UPR is surgery (Greiling et al., 2023; Payan-Carreira et al., 2012), especially OHE which also prevents the transmission of a possible genetic predisposition.

Because UPR can lead to life-threatening conditions, precautions, and practices should be implemented for emergency intervention during the perioperative period. In case 2, although none of the above risks were present and the necessary perioperative precautions were taken, cardiac arrest occurred during the surgery. However, she was revived, there were no post-operative complications, and subsequent follow-up via telephone survey showed that she remained in good health. Considering that brachycephalic breeds such as Pekingese have a 1.57-fold increase in intra-anesthetic complication rate and a 4.33-fold increase in post-anesthetic complication rate than non-brachycephalic breeds (Gruenheid et al., 2018), we believe that the complication experienced was due to brachycephalic airway syndrome.

As a result, UPR in dogs is an obstetric condition that needs alertness because it is life-threatening, it is rarely seen, and it is impossible to take precautions (except elective OHE). On the other hand, mother and/or puppies can be survived with timely and appropriate surgical management. The etiology of UPR is not fully understood, but it is undoubtedly multifactorial in dogs. It seems that sex hormones, which cause significant changes in the ECM architecture of the pelvic floor and ligaments, are the main factors. However, we believe, and speculated here, that there are co-factors, including poor BCS, age, parity, and breed predisposition, that also contribute significantly to UPR etiology. However, to clearly define both the primary factors and co-factors speculated by this report, further research is required to investigate the effect of hormones, age, parity, and breed on ECM architecture of the pelvic floor and reproductive tract.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This work involved the use of non-experimental animal only. Established internationally recognized high standards

of individual veterinary clinical patient care were followed. Ethical approval from a committee was therefore not necessarily required.

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Critical Review: SSA, MF

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ YAYIN KURALLARI *

1- Harran Üniversitesi Veteriner Fakültesi Dergisi (Harran Üniv Vet Fak Derg), özellikle Veteriner Hekimliği bilim alanı ile ilgili olmak üzere insan ve hayvan sağlığını kapsayan Türkçe ve İngilizce olarak hazırlanmış orijinal klinik ve deneysel araştırmalar, olgu sunumları, derlemeler (çağrılı veya sorumlu yazara ait derleme konusu ile ilgili en az 3 araştırma makalesinin referans listesinde olması gereklidir), kısa bilimsel makale ve editöre mektuplar yayınlayan hakemli bir dergidir. Dergide İngilizce hazırlanmış makalelerin yayımlanmasına öncelik verilir. Dergi 6 ayda bir, yılda 2 sayı olarak yayınlanır. Yayınlanan makalelerden ücret alınmamaktadır.

2- Dergiye kabul edilen yayınlar başka bir yerde yayınlanmamış olmalıdır. Eş zamanlı olarak incelenmek üzere başka dergilere gönderilmiş olmamalıdır. Yayınlanan makalelerden doğacak her türlü hukuki ve cezai sorumluluk yazarlara aittir. Yazarlara yayın hakkı bedeli ödenmez. Gönderilen makaleler ve ekleri makale yayınlansın veya yayınlanmasın geri iade edilmez.

3- Daha önce kongrelerde tebliğ edilmiş ve özeti yayımlanmış çalışmalar, bu durum kapak sayfasında belirtilmek üzere kabul edilir. Bununla birlikte yayın, tezden üretilmiş ise ve destekleyen kuruluş var ise yayında belirtilmelidir.

4- Dergi Editörlüğüne ulaşan makale, dergi editörlüğünce ön değerlendirmeye tabi tutulur. Editörlük, ön değerlendirme sonucuna göre makaleyi reddetme veya hakem değerlendirmesine tabi tutmadan önce düzeltme isteme hakkına sahiptir.

5- Makaleler değerlendirme için en az iki hakeme gönderilir. Makale kabul sürecinde, iki hakemin görüşlerinin farklı olması durumunda editör, üçüncü bir hakemin veya danışma kurulunun görüşünü alarak karar verir.

6- Harran Üniversitesi Veteriner Fakültesi Dergisi, etik ilkelere saygı çerçevesinde, TÜBİTAK ULAKBİM tarafından Türkiye'de tüm üniversitelerin kullanımına açmış olduğu "ithenticate" intihal tespit programı aracılığıyla gönderilen tüm makale, olgu sunumu ve derlemelerin ön değerlendirmesinin yapılması ve sonuçların gönderilmesi gerekmektedir. Bu ön değerlendirme sonuçlarına göre, makale, olgu sunumu veya derlemelerin başka kaynaklarla benzerlik oranının **%15'i** (özet, abstract ve kaynaklar hariç) aşmaması gerekmektedir. "ithenticate" programı aracılığı ile yapılacak öndeğerlendirmede benzerlik oranının %15 değerini aşması durumunda yayımlanmak üzere dergimize gönderilen makale, olgu sunumu veya derlemeler değerlendirilmeye alınmayacaktır.

7- Gönderilen herhangi bir makalenin (tüm makale kategorileri için) referanslarının en az % 20'sinin son beş yılda yayınlanan referansları içermesi gerekir. Anonim kaynaklar asgari düzeyde tutulmalıdır.

8- Makale yayına kabul edildiği takdirde her türlü yayın hakkının devredildiğine dair beyanları kapsayan Telif Hakkı Devir Sözleşmesinin tüm yazarlar tarafından imzalanarak basımdan önce elektronik olarak dergi editörlüğüne gönderilmesi gerekmektedir. Telif Hakkı Devir Sözleşmesi gönderilmeyen makaleler yayımlamaya kabul edilmiş olsalar bile basılmazlar.

9- Harran Üniversitesi Veteriner Fakültesi Dergisi'ne gönderilecek makale, olgu sunumu, derleme vb. çalışmalar, https://dergipark.org.tr/tr/pub/huvfd adresinden gönderildiğinde değerlendirme sürecine alınmaktadır.

10- Harran Üniversitesi Veteriner Fakültesi Dergisi'ne gönderilecek makale, olgu sunumu, derleme vb. çalışmalar MS Word formatında, tüm fotoğraflar (resimler) en az 300 dpi çözünürlükte, TIFF veya JPEG formatında kaydedilmiş olmalıdır.

YAZIM KURALLARI

Yazılar, MS Word formatında, Times New Roman yazı tipinde, 12 punto, çift satır aralıklı ve her kenardan 2.5 cm boşluk bırakılarak hazırlanmalıdır. Makaleye satır numaraları (makalenin 2. sayfasından başlamak üzere sürekli olacak şekilde) eklenmelidir. Bu şekildeki yazılar, şekil ve tablolar dâhil olmak üzere orijinal bilimsel araştırmalar ve derlemelerde 15, kısa bilimsel makale ve olgu sunumlarında 5 sayfayı geçmemelidir.

Birimler ve ölçüler için Uluslararası Standart birimleri (SI-sistem) kullanılmalıdır.

Araştırma Makaleleri: Orijinal araştırma makaleleri aşağıdaki ana konu sıralamasına göre dizilmelidir: Başlık, Yazar adları (Sorumlu yazar (*) ile işaretlenmeli), Yazar adresleri, Yazar ORCID numaraları, Özet ve Anahtar kelimeler (3 - 6 kelime), İngilizce başlık, Abstract ve Keywords ile Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç, Teşekkür veya Bilgilendirme ile Kaynaklar. Her bir Tablo ve Şekil ayrı sayfalarda yer almalıdır.

YAZIM DÜZENİ

Özet: Orijinal araştırma makalelerinde 250, diğer makale türlerinde 200 kelimeyi geçmeyecek şekilde hazırlanmalıdır.

Anahtar Kelimeler: En fazla 6 tane olmak üzere her iki dildeki özetin altında alfabetik sırayla verilmelidir. Anahtar kelimeler, Türkiye Bilim Terimleri arasından seçilmelidir. Anahtar kelimelerin seçiminde Türkiye Bilim Terimleri internet adresinden (http://www.bilimterimleri.com) yararlanılmalıdır.

Giriş: Sonuçların anlaşılabilmesi ve yorumlanabilmesi için o konu ile ilgili yapılmış olan çalışmalar hakkında bilgilere yer verilmelidir. Giriş'te çalışmanın hipotezi belirtilmelidir. Çalışmanın amacı bu bölümün en sonunda açık olarak yazılmalıdır.

Materyal ve Metot: Bu bölümde deneysel çalışmalar diğer araştırmacılar tarafından tekrarlanabilecek yeterlilikteki detayı ile verilmelidir. Uluslararası indeksli dergilerde yayınlanmış bir makalede açıklanan bir teknik kullanıldığında, metodun çok kısa açıklanması ve ilgili orijinal makaleye atıf yapılması gereklidir. Makalede etik kurul izini ve/veya yasal/özel izin alınmasının gerekip gerekmediği bu bölümde belirtilmelidir. Materyal olarak hayvan kullanılan orijinal araştırma makalelerinde (klinik, deneysel, saha çalışmaları vb.); etik kurul onayı alınmış olmalıdır. Etik kurul onay/izin belgesinin "alındığı etik kurulun ismini, sayısını ve tarihini" içeren açıklayıcı bilgiler materyal ve metot bölümüne yazılmalıdır. Yayın kurulu etik kurul onay belgesini isteme hakkına sahiptir.

Bulgular: Araştırma bulguları açık ve anlaşılabilir şekilde verilmelidir. Bulgular, gerektiğinde tablo ve şekillerle desteklenmeli ve kısa olarak sunulmalıdır.

Tartışma ve Sonuç: Bulgular gereksiz ayrıntıya girmeden literatürler ışığında tartışılmalı ve bulguların önemi vurgulanmalıdır. Sonuç ya da öneri cümlesi ile bitirilmelidir.

Teşekkür: Çalışma veya makaleye kişisel katkı ve parasal destek burada belirtilmelidir.

Derleme: Derginin yayın alanlarındaki konularda yenilikleri içeren, güncel kaynaklardan yararlanılarak hazırlanmış makaleler olup, yazarların konu ile doğrudan ilişkili en az 3 adet çalışmalarının olması ve bunların derleme içinde kullanılması durumunda yayınlanmak üzere kabul edilebilecektir. Sorumlu yazar, derlemesini gönderirken konu ile ilgili makalelerinin de künye bilgilerini dergi editörlüğüne göndermelidir (makale künyeleri, makale metninin en son sayfasında sunulmalıdır). Harran Üniversitesi Veteriner Fakültesi Dergisi'nde değerlendirmeye alınan ve yayınlanan derlemeler **çağrılı derleme**lerden oluşmaktadır. Derlemelerde; Özet, Giriş, Sonuç ve Kaynaklar bölümleri bulunmalıdır.

Olgu Sunumu: Yazarların, karşılaştıkları yeni veya ender gözlemlenen olguların ele alındığı, bilimsel değere sahip bilgileri içeren eserlerdir. En fazla 15 kaynak kullanılmalı ve bu kaynakların güncel olmasına özen gösterilmelidir. Olgu sunumları; Özet, Giriş, Olgu tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalıdır.

Kısa Bilimsel Makale: Kısa bilimsel makalelerde dar kapsamlı olarak ele alınmış, yeni bilgi ve bulgular sunulmalıdır. Araştırma makalesi formatında hazırlanmalı ve en fazla 5 sayfa olmalıdır. En fazla 2 tablo veya şekil içermelidir.

Kaynaklar

Metin içinde atıf yapılırken;

1. Yazar veya yazarların soyadından sonra parantez içinde kaynağın yayın yılı belirtilmelidir; Adams (1998) tarafından; Wilkie ve Whittaker (1997) tarafından; Doyle ve ark. (2007) tarafından….
2. Cümlenin sonunda atıf yapıldığında ise yazar ismi ve yayın yılı parantez içinde belirtilmelidir; ... bildirilmiştir (Adams, 1998); .... bildirilmiştir (Wilkie ve Whittaker, 1997); ..... bildirilmiştir (Doyle ve ark., 2007).
3. Birden çok kaynağa atıf yapılması durumunda önce alfabetik sonra kronolojik sıralama yapılmalıdır;
   …. bildirilmiştir (Adams, 1998; Adams, 2008; Doyle ve ark., 2007; Wilkie ve Whittaker, 2006).
4. Aynı yazarın aynı yıl yayınları söz konusu ise her biri "a" harfinden başlayarak küçük harflerle işaretlenmelidir;
   …. (Adams, 2005a; Adams, 2005b;…).

Kaynak listesi aşağıdaki şekilde hazırlanmalıdır:

1. **Kaynak listesi yazar soyadına göre alfabetik olarak sıralanmalıdır.**
2. **Kaynaklarda yer alacak dergi adları ISI web of Science'a göre kısaltılmalı ve italik yazılmalıdır.**
3. **Kaynakların yazın şekli aşağıdaki şekilde olmalıdır.**

Makale; Sullivan JC, Sasser JM, Pollock JS, 2007: Sexual dimorphism in oxidant status in spontaneously hypertensive rats. *Am J Phsiol Integr Comp Physiol,* 292 (1), 64-68.

Kitap; Cadenas E, Packer L, 2001: Handbook of Antioxidants. 2nd ed., Marcel Dekker Inc., New York, USA.

Kitaptan bir bölüm: Bahk J, Marth EH (1990). Listeriosis and Listeria monocytogenes In: Foodborne Diseases, Cliver DO (Ed), 248-256, Academic Press, San Diego. **Web sayfası:** Anonim (1) http://www.emea.europa.eu, Erişim tarihi; 01.04.2010.

Tez: Er A, 2009: Makrolid grubu antibiyotiklerin endotoksemide sitokin düzeylerine etkisi. Doktora tezi, SÜ Sağlık Bilimleri Enstitüsü, Konya.

Bilimsel toplantıda sunulan bildiri: Allen WR, Wilsher S, Morris L, Crowhurst JS, Hillyer MH, Neal HN, 2006: Re-establishment of oviducal patency and fertility in infertile mares. In: Proceedings of the Ninth International Symposium on Equine Reproduction, Kerkrade, Holland, pp. 27-28.

Tablo ve Şekiller: Her bir tablo ve şekil ayrı sayfalara yerleştirilmelidir. Kullanım sırasına göre numaralandırılmalı, kısa başlıklarla ifade edilmeli ve metin içinde tablo numarası verilerek atıfta bulunulmalıdır. Tablo başlıkları makalenin yazım dilinde tablonun üst bölümüne yazılmalıdır. Tabloda kullanılan kısaltmalar ve gerekli açıklamalar tablo altında verilmelidir. Şekil başlıkları makalenin yazım dilinde şeklin alt bölümüne yazılmalıdır.

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Manuscripts should be prepared in MS Word format, Times New Roman font, with 12 font size, double line spacing and 2.5 cm space on each side. Line numbers (continually starting from page 2 of the article) should be added to the article. Articles of this type should not exceed 15 pages in original scientific research and reviews, including figures and tables, and 5 pages in short scientific articles and case reports.

International Standard Units (SI-system) should be used for units and dimensions.

Research Articles: Original research articles should be arranged in the order of the following main topics: Title, Author names (must be marked with the responsible author (*)), Author addresses, Author ORCID numbers, Abstract and Keywords (3 - 6 words), English title, Abstract and Introduction to Keywords, Material and Method, Results, Discussion and Conclusion, Thanks or Information and References. Each Table and Figure should be on separate pages.

STYLE AND FORMAT

Abstract: It should be prepared not to exceed 250 words in original research articles and 200 words in other types of articles.

Keywords: It should be given in alphabetical order below the summary in both languages, maximum 6. Keywords should be selected from Turkey Science Terms. Turkey Science Terms in the selection of keywords from the internet address (http://www.bilimterimleri.com) should be utilized.

Introduction: In order for the results to be understood and interpreted, information about the studies done on that subject should be included. In the introduction, the hypothesis of the study should be specified. The purpose of the study should be clearly written at the end of this section.

Material and Method: Experimental studies should be given in this section with sufficient detail that can be repeated by other researchers. When using a technique described in an article published in international indexed journals, it is necessary to describe the method very briefly and to cite the relevant original article. In the article, it should be stated in this section whether the ethical committee permission and / or legal / special permission should be obtained. In original research articles using animals as materials (clinical, experimental, field studies, etc.); ethics committee approval must have been obtained. Explanatory information including the name, number and date of the ethics committee's ethics committee approval / permit document should be written in the material and method section. The editorial board has the right to request the ethics committee approval document.

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Discussion and Conclusion: Findings should be discussed in the light of the literature before going into unnecessary detail and the importance of the findings should be emphasized. It should be finished with a conclusion or suggestion sentence.

Acknowledgment: Personal contribution and monetary support to the study or article should be stated here.

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While citing in the text;

1. The publication year of the reference should be specified in parentheses after the surname of the author or authors; By Adams (1998); By Wilkie and Whittaker (1997); Doyle et al. (2007) by….

2. When cited at the end of the sentence, the name of the author and the year of publication must be indicated in parentheses; ... have been reported (Adams, 1998); .... has been reported (Wilkie and Whittaker, 1997); ..... has been reported (Doyle et al., 2007).

3. In case of reference to more than one reference, first alphabetical and chronological order should be done;

.... reported (Adams, 1998; Adams, 2008; Doyle et al., 2007; Wilkie & Whittaker, 2006).

4. If the same author has publications in the same year, each should be marked in lowercase letters, starting with the letter "a";

.... (Adams, 2005a; Adams, 2005b;…).

The list of references should be prepared as follows:

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2. The names of the journals in the references should be shortened according to the ISI web of Science and should be written in italics.

3. Type of references should be as follows.

Journal article; Sullivan JC, Sasser JM, Pollock JS, 2007: Sexual dimorphism in oxidant status in spontaneously hypertensive rats. Am J Phsiol Integr Comp Physiol, 292 (1), 64-68.

Book; Cadenas E, Packer L, 2001: Handbook of Antioxidants. 2nd ed., Marcel Dekker Inc., New York, USA.

Chapter in a book: Bahk J, Marth EH (1990). Listeriosis and Listeria monocytogenes In: Foodborne Diseases, Cliver DO (Ed), 248-256, Academic Press, San Diego. Web page: Anonymous (1) http://www.emea.europa.eu, Access date; 01.04.2010.

Thesis: Er A, 2009: Effect of macrolide antibiotics on cytokine levels in endotoxemia. PhD thesis, SU Health Sciences Institute, Konya.

Paper presented at the scientific meeting: Allen WR, Wilsher S, Morris L, Crowhurst JS, Hillyer MH, Neal HN, 2006: Re-establishment of oviducal patency and fertility in infertile mares. In: Proceedings of the Ninth International Symposium on Equine Reproduction, Kerkrade, Holland, pp. 27-28.

Tables and Figures: Each table and figure should be placed on separate pages. It should be numbered according to the order of use, expressed in short titles, and should be cited by giving the table number in the text. Table titles should be written in the writing language of the article in the upper part of the table. Abbreviations and necessary explanations used in the table should be given under the table. Figure titles should be written at the bottom of the figure in the writing language of the article.

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