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RESEARCH ARTICLES

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RESEARCH ARTICLE

Effect of Pine Cone Vinegar on The Survival of *Listeria monocytogenes and Salmonella typhimurium* and Some Physico-Chemical Properties in Raw Beef

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ABSTRACT

Pine cones from pine (Pinus) trees contain various bioactive compounds with antibacterial and antioxidant activity. However, the use of pine cone products as functional foods or food additives is limited and there is a paucity of research in the literature. The objective of this study was the evaluation of pine cone vinegar as a marinade against *Listeria monocytogenes* and *Salmonella typhimurium* in raw beef. For this purpose, raw beef was marinated with pine cone vinegar at three different levels (25%, 50% and 75%) and three different marination times (2, 6 and 24 hours) and *L. monocytogenes, S. typhimurium* counts, pH and colour values (L*, a*, b*) were determined. Concentration*time interaction was significant for pH and the lowest pH value was found at 75%*24 hours interaction. Although the marinating process had a significant effect on the colour values of the meat, the concentration*time interaction was not significant for the a* value ($p>0.05$). The number of *S. typhimurium* in pine cone vinegar decreased by 3.7 log10 in 2 hours and by 5.26 log10 in 6 hours ($p<0.05$). The number of *L. monocytogenes* decreased by 4.17 log10 in 2 hours and 5.29 log10 in 6 hours ($p<0.05$). The number of *S. typhimurium* and *L. monocytogenes* decreased significantly in marinated beef samples, with the greatest decrease being observed at 24 hours for all concentrations. After 24 hours of marination, *Salmonella* counts decreased by 1.69, 1.68 and 1.73 log10, and *L. monocytogenes* counts decreased by 3.6, 3.43 and 3.13 log10, respectively. The findings of this study indicate that pine cone vinegar can serve as an effective decontaminant for meat and meat products, making it a valuable component in marinades.

Keywords: Beef, *Listeria monocytogenes*, Marinating, Pine cone, *Salmonella typhimurium*

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Çam Kozalağı Sirkesinin Çiğ Sığır Etinde *Listeria monocytogenes* ve *Salmonella typhimurium*'un Hayatta Kalması ve Bazı Fiziko-Kimyasal Özellikler Üzerine Etkisi

ÖZ

Çam (Pinus) ağacı türlerinden elde edilen çam kozalakları antibakteriyel ve antioksidan aktiviteye sahip çeşitli biyoaktif maddelere sahiptir. Ancak çam kozalağından elde edilen ürünlerin fonksiyonel gıda veya gıda katkı maddesi olarak kullanımı sınırlıdır ve literatürde bu konuda çok fazla araştırma bulunmamaktadır. Bu çalışmanın amacı da çam kozalağı sirkesinin çiğ sığır etlerinde *Listeria monocytogenes* ve *Salmonella typhimurium* karşı bir marinat olarak değerlendirmektir. Bu amaçla çam kozalağı sirkesi ile üç farklı konsantrasyon (%25, %50 ve %75) ve 3 farklı marinasyon süresininde (2, 6 ve 24 saat) çiğ sığır eti marine edilip, *L. monocytogenes*, *S. typhimurium* sayıları, pH ve renk değerleri (L*, a*, b*) belirlendi. pH için konsantrasyon*zaman etkileşimi önemli olduğu, en düşük pH değeri %75*24 saat etkileşiminde sapandı. Marinasyon işleminin etin renk değerleri üzerinde önemli etkileri olmasına rağmen, a* değeri için konsantrasyon*zaman etkileşimi önemli olmadığı belirlendi ($p>0,05$). Çam kozalağı sirkesinde S. typhimurium sayısının 2 saatte 3,7 $\log_{10}$, 6 saatte ise 5,26 $\log_{10}$ azaldığı tespit edildi ($p<0,05$). *L. monocytogenes* sayısının ise 2 saatte 4,17 $\log_{10}$, 6 saatte ise 5,29 $\log_{10}$ azaldığı tespit edildi ($p<0,05$). Marine edilmiş sığır eti örneklerinde *S. typhimurium* ve *L. monocytogenes* sayısının önemli düzeyde azaldığı en çok azalma tüm konsantrasyonlarda 24 saatte tespit edildi. 24 saat marinasyon sonrası *Salmonella* sayısı sırasıyla 1,69, 1,68 ve 1,73 $\log_{10}$ azaldığı, *L. monocytogenes* sayısı ise sırasıyla 3,6, 3,43 ve 3,13 $\log_{10}$ azaldığı saptandı. Bu çalışmanın sonuçları, çam kozalağı sirkesinin et ve et ürünlerinin dekontaminasyonu için kullanılabileceği ve marinatların önemli bir bileşeni olabileceğini ortaya koymuştur.

Anahtar Kelimeler: Çam kozalağı, *Listeria monocytogenes*, Marinasyon, *Salmonella typhimurium*, Sığır eti

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INTRODUCTION

Meat and meat products play a crucial role due to their nutrient content, high pH and water activity, which create an environment conducive to microbial growth (Aminzare et al. 2016). According to the Zoonoses Report published by EFSA and ECDC (2022), three of the most common zoonotic agents in humans are *Salmonella* spp, Shiga toxin-producing *Escherichia coli* and *L monocytogenes*. These pathogens are responsible for a significant proportion of foodborne diseases associated with meat consumption. Therefore, substances such as antimicrobials must be used to reduce the risk and protect human health (Kaur et al. 2023).

Marinating is a crucial step before consuming meat, serving both sensory quality and safety purposes. Not only does marination enhance tenderness and flavor, but it also protects against microbial and chemical spoilage, making meat safer for consumption. One popular method is immersion marinating, where pieces of meat are soaked in a liquid containing various ingredients such as acidic marinade plant extracts and spices (Lopes et al. 2022). Recently, there has been a growing demand for natural preservatives in response to consumer preferences for organic additives and green-labeled foods. Vinegar, rich in bioactive compounds, has emerged as an excellent candidate for meat marinades. Studies have reported that vinegar-based acidic marinades improve the flavour and safety of meat products (Park et al. 2014; Karam et al. 2020; Şengün et al. 2021; Fencioğlu et al. 2022). As a fermented product, vinegar has a long history of use as an acidulant, sauce, and flavoring in the food industry (Sengun et al. 2020; Pashazadeh et al. 2021).

Vinegar is produced not only from commonly used fruits like grapes and apples but also from various grains and other fruits. Fruit vinegars in particular are rich in organic acids and bioactive compounds (Luzón-Quintana et al. 2021; Özen et al. 2020). These bioactive compounds in vinegar have high antibacterial and antioxidant properties (Budak et al. 2014).

Pine cones obtained from various pine (Pinus) tree species contain bioactive substances such as flavonoids, polysaccharides, and phenolic compounds. These compounds exhibit anti-tumor, anti-inflammatory, antioxidant activities and antibacterial (Wang and Hong 2016; Yi et al. 2017). In traditional medicine, pine cones are considered non-toxic and have been used for purposes such as moisturizing the lungs, relieving coughs, and reducing fever (Zhang et al. 2010). However, the use of pine cone products as functional foods or food additives remains limited, especially regarding the effectiveness of pine cone vinegar in meat marinades. While various acidic marinades have been studied extensively, the use of pine cone vinegar as a marinade has not been widely explored. Considering that marination significantly affects the microstructure and properties of meat such as microbiological composition and chemical structure, a comprehensive approach is required to overcome the current knowledge gaps in this field.

This study was designed to investigate the efficacy of pine cone vinegar as a marinade against *L. monocytogenes* and *S. typhimurium* in raw beef. In this study, the effect of marinating with pine cone vinegar on the microbiological and physicochemical properties of raw beef was elucidated.

MATERIALS and METHODS

Musculus longissimus dorsi samples were obtained from local butchers in Şanlıurfa on the day of the experiment. The meat samples were immediately brought to the laboratory under cold chain conditions on the day of the experiment and the experiments were carried out as soon as possible. During all these processes, the meat samples were kept at 4±1 °C. Organic pine cone vinegar (Karşı köyden®) was purchased from a local market in Şanlıurfa. This vinegar is made from pine cones grown from the *Pinus brutia* pine tree species, water and salt and fermented for at least one year.

Preparation of inoculum

S. typhimurium (NCTC 74, 12416 and ATCC 14028) and *L. monocytogenes* (N 7144, RSKK 474 and 476) reference strains were used for inoculum preparation. These strains were incubated in tryptic soya broth (TSB) overnight at 37 °C to obtain fresh cultures. After incubation, the liquid cultures were centrifuged at 4200 x g for 10 min and the supernatant discarded. The resulting pellets were washed twice with sterile 0.1% peptone water (PW) (Merck, Darmstadt, Germany) and collected in a tube. The final volume was adjusted to 10 mL with 0.1% sterile PW (Merck, Darmstadt, Germany) and diluted decimally with PW. An inoculum of approximately 6.0 log10/mL was used for survival testing of pathogens in pine cone vinegar and meat samples.

Pathogens survival experiment in Pine cone vinegar

To assess the antibacterial effect of pine cone vinegar, approximately 6.0 log10/mL of *S. typhimurium* and *L. monocytogenes* were added to the vinegar (at 100% concentration). The pathogen counts were determined immediately after inoculation and at 2, 6, and 24 hours of incubation at 4 °C. The experiment was conducted in triplicate.

Preparation of the groups

To prepare the meat samples, they were first cut into small pieces (50±5 g) using a sterile lancet. Next, 500 µL of a diluted bacterial inoculum cocktail was spread

onto the meat samples using a cell spreader. After inoculation, the bacteria were allowed to adhere to the meat for 30 minutes at 4±1 °C. The samples were then randomly divided into ten groups: a control group (no treatment and 0. hours), three different pine cone vinegar concentrations (25%, 50%, and 75%), and three different marinating times (2, 6, and 24 hours). The marination process was carried out using the immersion method. While determining the concentrations of pine cone vinegar, the meat marinated with different concentrations was cooked and sensory analysis (colour, smell, taste, appearance, general appreciation) was performed as a preliminary study before starting the study. In the study, the ratios that did not adversely affect the sensory quality of the meat were prioritised. Pine cone vinegar was diluted with sterile distilled water to achieve the desired concentrations. The ratio of marinade to meat sample was 2:1 (100 mL marinade to 50 g tenderloin), and marination was conducted in sterile glass jars at 4±1 °C.

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 (İncili et al. 2021).

pH analyses

The pH values of the pine cone vinegar and meat samples were measured using a pH meter (HI 11310, Hanna Instruments, USA). The pH of the pine cone vinegar was measured by dipping the probe directly into the vinegar. When measuring the pH of meat samples, 10 g of meat samples were homogenized in 90 mL of distilled water, and pH measurements were made using a digital pH meter (İncili et al. 2021).

Colour analysis

The color characteristics of the meat samples were measured using a Chroma Meter (model CR-5, Konica Minolta, Osaka, Japan) set to a standard observer angle of 10° and illuminator type D-65. The parameters assessed included Hunter L* (whiteness/darkness), a* (redness/greenness), and b* (yellowness/blueness) (Aydemir et al. 2024).

Statistical analyses

The microbial counts, pH, L*, a*, and b* values of the samples underwent statistical analysis. Microbiological data were logarithmically transformed for statistical purposes. We employed a general linear model (GLM) for the analysis. In the GLM procedure, we treated pine cone vinegar concentrations (25%, 50%, and 75%) and marination times (2, 6, and 24 hours) as fixed effects, while replications were assigned as random effects. Multiple comparisons were conducted using the Tukey test ($p<0.05$). Throughout this study, all data were obtained from three independent replicates, and the results are presented as mean ± standard error of the mean. We performed all statistical analyses using IBM SPSS Version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULT

pH value

The pH of the pine cone vinegar used in the study was 3.8±0.06 and the pH of the meat was measured at 5.8±0.08. The average pH values of the meat samples are given in Figure 1. Marinating time and pine cone vinegar concentration did not cause a statistical difference between the groups in terms of pH. The pH value of the tenderloin samples decreased as the marinating concentration and time increased.

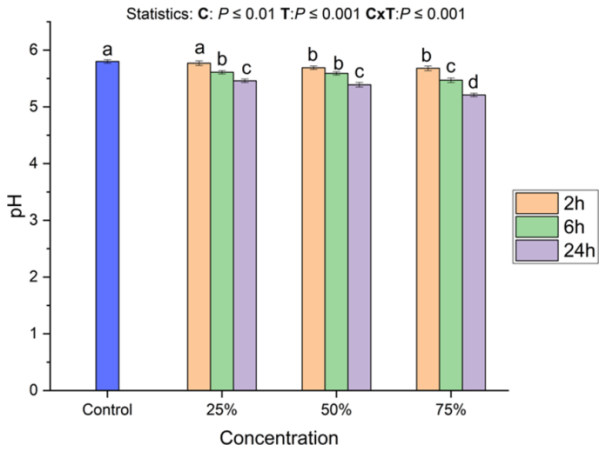

Figure 1: The mean pH values of the raw beef during storage period at 4 °C (Mean value±SE).[a-d] : Different superscripts indicates the statistical significance among the groups ($p<0.05$). *C:* Concentration of pine cone vinegar; *T:* Marination time; *C* × *T:* Interaction between concentration and time.

Colour value

Figure 2 shows that pine cone vinegar significantly changed the colour characteristics (L*, a* and b*) of the meat samples ($p<0.05$). The marinating process significantly decreased the a* value. However, increasing the vinegar concentration and extending the time to 24 hours slightly increased the L* and b* values compared to the control group.

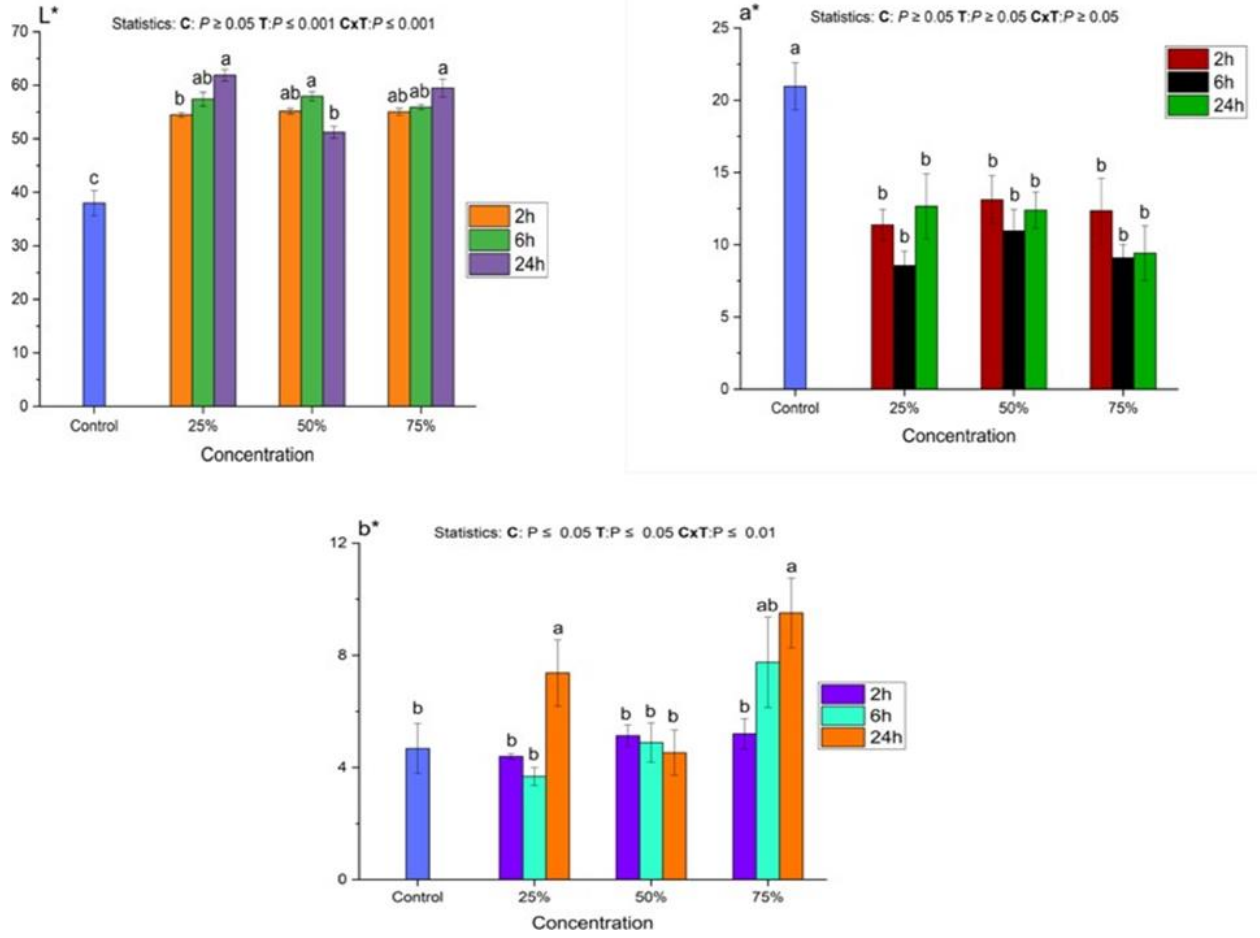

Figure 2: Average L*, a*, and b* values of the non-marinated and marinated beef samples at 4 °C. (Mean value±SE).[a-c] **:** Different superscripts indicates the statistical significance among the groups ($p<0.05$). ***C:*** Concentration of pine cone vinegar; ***T:*** Marination time; ***C × T:*** Interaction between concentration and time.

Salmonella typhimurium

The number of *S. typhimurium* in pine cone vinegar decreased by 3.7 log10 and 5.26 log10 after 2 and 6 hours, respectively ($p<0.05$). Although the number of *S. typhimurium* continued to decrease, the difference between 6 h and 24 h was not significant ($p>0.05$). Rapid reduction of *S. typhimurium* occurred in the pine cone vinegar, while a lower rate of decrease was observed in the marinated beef samples (Table 1). Compared to the control group, the number of *S. typhimurium* was significantly reduced in the marinated beef samples (Table 1). Specifically, *Salmonella* counts were reduced by 1.69, 1.68, and 1.73 log10 after 24 hours of marination at concentrations of 25%, 50%, and 75%, respectively. Although the time interaction was significant for *Salmonella* counts, the concentration*time interaction was not significant.

Listeria monocytogenes

The number of *L. monocytogenes* in pine cone vinegar decreased by 4.17 log10 after 2 hours and by 5.29 log10 after 6 hours ($p<0.05$). Although the number of *L. monocytogenes* continued to decrease, the difference between the 6-hour and 24-hour time points was not significant ($p>0.05$). Rapid reduction of *L. monocytogenes* occurred in the pine cone vinegar, while a lower rate of reduction was observed in the marinated beef samples (Table 1). Compared to the control group, the number of *L. monocytogenes* was significantly reduced in the marinated beef samples (Table 1). The greatest reduction was observed at 24 hours for all concentrations. Specifically, at 25%, 50%, and 75% concentrations, the number of *L. monocytogenes* decreased by 3.6, 3.43, and 3.13 log10, respectively, after 24 hours of marination. Both time

interaction and concentration*time interaction were significant for *L. monocytogenes* counts ($p<0.05$).

Table 1. Average counts for *S. typhimurium* and *L. monocytogenes* in non- marinated and marinated with pine cone vinegar beef at 4 °C (Mean log10 CFU/g±SE).

Concentration	Time	*L. monocytogenes*	*S. typhimurium*
Control	0h	5.66±0.28[a]	5.50±0.39[a]
25%	**2h**	5.14±0.06[a]	4.60±0.04[ab]
	6h	3.51±0.18[b]	4.23±0.15[b]
	24h	2.03±0.03[c]	3.81±0.11[b]
50%	**2h**	5.27±0.04[a]	4.16±0.53[ab]
	6h	3.53±0.06[b]	4.10±0.13[ab]
	24h	2.23±0.23[c]	3.82±0.04[b]
75%	**2h**	4.70±0.02[a]	4.22±0.17[ab]
	6h	3.38±0.08[b]	4.15±0.20[ab]
	24h	2.53±0.23[c]	3.77±0.24[b]
Statistics	**C**	p≥ 0.05	p≥0.05
	T	p≤ 0.01	p≤ 0.05
	CxT	p≤0.05	p≥ 0.05

[a-c]: Different superscripts indicates the statistical significance among the groups ($p<0.05$). ***C:*** Concentration of pine cone vinegar; ***T:*** Marination time; ***C × T:*** Interaction between concentration and time.

DISCUSSION

Effect of marination on physicochemical quality

Effect of marination on pH value

As marination concentration and time increased, the pH of the tenderloin samples decreased, with the lowest pH recorded at the 75% concentration*24 h interaction (Figure 1). The low pH of pine cone vinegar (3.8) is believed to influence the pH decrease of meat samples after marination. In fact, İncili et al (2023) reported a significant decrease in pH values when increasing the concentration of marinade used in meat samples. The decrease in pH due to prolonged marinating can be explained by the entry of more marinade into the meat and consequently the decrease in pH. As a matter of fact, Aydemir et al. (2024a) associated the decrease in the pH of beef tenderloin samples with the absorption of more marinade into the meat due to the prolongation of the marinating process. Consistent with our findings, other researchers also reported changes in pH in meat samples marinated with acidic marinades (İncili et al. 2023; Karatepe et al. 2023; Aydemir et al. 2024a; Aydemir et al. 2024b). A decrease in pH in meat samples is a desirable situation. This is because pH values below the isoelectric point (~5.3) of muscle have a direct effect on the physicochemical and textural properties of meat samples. In particular, a pH below the isoelectric point causes more marinade to enter the myofibrils. This causes the muscle fibres to swell and more water to be retained in the myofibrils. This softens the meat and improves its texture (Siroli et al. 2020; Karatepe et al. 2023). Lowering the pH also improves the microbiological quality of the meat. This is because low pH limits the survival of bacteria.

Effect of marination on color properties

Changes in colour are expected with increasing marination time. The colour of the meat surface is caused by the distribution of light reflected from the meat, as well as the selective absorption of myoglobin caused by major constituents such as muscle fibres and proteins and is also reported to be influenced by the amount of free liquid (Purslow et al. 2020). The absorption of marinade increases as the marinating time increases. In this case, as explained above, the pH of the meat decreases and colour changes occur in the meat samples. Indeed, de Avila Souza et al (2022) reported that myoglobin, which is responsible for the colour appearance of muscle, undergoes denaturation in acidic marinades, with myoglobin being converted to metmyoglobin under acidic conditions. In particular, the decrease in the a* value

of meat is more closely related to this situation. It was found that increasing the vinegar concentration and extending the marinating time to 24 hours resulted in a slight increase in L* values compared to the control group. The results of the study confirm the inverse relationship between pH and L*. Acid marinades reduce the water holding capacity of meat (Mazaheri Kalahrodi et al. 2021). As the water holding capacity of the meat decreases, the meat appears pale, soft and exudative (PSE). A high L* value has previously been reported to characterise pale meat (Barbut et al. 2008). Significant colour changes in meat samples marinated with acidic marinades have also been reported by other researchers (Gargi and Sengun 2021; İncili et al. 2021; Karatepe et al. 2023; Aydemir et al. 2024a; Aydemir et al. 2024b).

Effects of marination against *S. typhimurium, L. monocytogenes*

Vinegar is a special product that shows antimicrobial and antioxidant activity thanks to the bioactive compounds it contains (Bakır et al. 2017). Properties of organic acids in vinegar show antimicrobial effect by disrupting the integrity of the cell wall and membrane of bacteria (Lytou et al. 2019). Therefore, acidic marinades have been used to ensure and protect the microbial safety of meat and meat products. When the results of this study are examined, it is seen that pine cone vinegar caused a significant decrease in the number of pathogenic bacteria (Figure 1, Table 1). This reduction may be attributed to the low pH of pine cones (3.8) and their rich organic acids and bioactive compounds. It has been reported that pine cones are rich in bioactive compounds and have antimicrobial activity (Kim et al. 2019).

In the literature, there are studies investigating the antimicrobial effects of different vinegars against different microorganisms (Kelebek et al. 2017; Öztürk et al. 2015; Sengun et al. 2020; Karatepe et al. 2023). However, despite the widespread use of vinegar in meat marination, very limited research has been conducted on the effect of vinegar on pathogens on meat or meat products. To the best of our knowledge, the effect of pine cones on pathogenic bacteria in meat has not been investigated. Şengün et al (2021) Blackberry, rosehip reported that marinating beef with pomegranate and grape vinegar reduced the number of *S. typhimurium*, *L. monocytogenes* in meat samples by 1.420-1.913 log CFU/g and the highest reduction was found in rosehip vinegar marinade. Karatepe et al. (2023) reported that hawthorn vinegar marinating beef reduced the number of *S. typhimurium* in meat samples from 4.95 log CFU/g to 1.13 log CFU/g (100% concentration*24 *h)*, and *L. monocytogenes* from 4.98 log CFU/g to 1.39 log CFU/g (100% concentration*24 h). In addition, when meat products (chicken, lean boneless beef, etc.) were marinated with different acidic marinades (Rheum ribes L. juice, cucumber juice, V-made acidic pickles), different reduction moments (ranging from 0.54 to 3.3 log 10 CFU/g) in the number of these pathogens were reported (İncili et al. 2020; Şengün et al. 2020; İncili et al. 2021). The differences between the results of this study and the results of other studies can be explained by the type of meat used, the composition of the marinade used, the organic acid content and ratio of the marinade, the concentration of acidifying agent in the marinade, the time of keeping the meat in the marinade and the acid resistance of the pathogenic strains used in the studies.

The numbers of *S. typhimurium* and *L. monocytogenes* in pine cone vinegar decreased significantly after 2 h and 6 h, but not after 24 h (Figure 3). This can be explained by the fact that the bacteria develop resistance to the organic acids in the pine cone vinegar after a certain period of time and adapt to the pH of the pine cone. Indeed, Wang et al. (2019) environmental conditions such as exposure to acids can damage bacteria. However, bacteria that are not fatally injured can enter the stationary phase and/or regain the ability to regrow. It has also been mentioned that bacteria may show high adaptability to acidic environments (Chung et al. 2018). Similar to our findings, İncili et al. (2020) reported that the number of *S. typhimurium* bacteria in the homemade marinade they prepared decreased significantly until the 6th hour, but did not decrease at the 24th hour and was similar to the number of bacteria at the 6th hour. The situation was slightly different for the beef samples marinated in pine cone vinegar. The numbers of *S. typhimurium* and *L. monocytogenes* decreased most after 24 hours (Figure 3). This can be explained by the fact that the longer the time, the more the marinade is absorbed into the meat, the more organic acids enter the meat and the pH of the meat decreases. Thus, pine cone vinegar has a greater antibacterial effect on meat samples after 24 hours. In fact, Karatepe et al. (2023) emphasised that the longer the marinating time, the more the marinade is absorbed and the more organic acids are released into the meat. They also reported that marinating meat for 24 hours was the most effective treatment against bacteria.

The viability of *S. typhimurium* and *L. monocytogenes* in pine cone vinegar was found to be the same and decreased to a similar extent. However, *L. monocytogenes* was found to be more inactivated than *S. typhimurium* when beef was marinated in pine cone vinegar. This may be explained by the fact that bioactive compounds in pine cones are more effective against gram positive bacteria. This may also be explained by the better adhesion of Salmonella bacteria to meat compared to *Listeria b*acteria, thus providing protection from antimicrobial agents. In a study by Ilhak et al. (2018), explained that the reason why *Salmonella* was less inactivated in marinated chicken pieces was due to the protection of the

bacteria from the effects of antimicrobials due to the tight attachment of the bacteria to the meat.

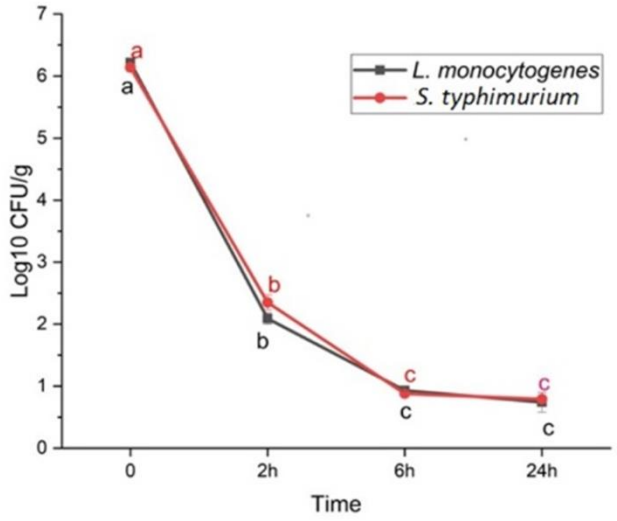

Figure 3. Survival of *S. typhimurium* and *L. monocytogenes* in pine cone vinegar at 4 °C for 24 hours (log10 CFU/g±SE).[a-c] : The mean values with different letters among the sampling hour are significantly different ($p<0.05$).

CONCLUSION

In conclusion, the pine cone vinegar marinade used in this study was found to have antibacterial activity against *S. typimurium* and *L. monocytogenes*. Pine cone vinegar, which has been used in folk medicine for many years against some diseases, may also have the potential to transform foods into functional foods. It can be suggested that pine cone vinegar marinade can be used to reduce microbial risks especially in red meat and its products. The results of this study may provide useful information for further studies investigating the effects of marinating meat and meat products with natural ingredients on the survival of foodborne pathogens and for all food handlers. Further studies are needed to evaluate the potential effects of pine cone vinegar on extending shelf life, improving chemical quality and sensory properties in different food matrices.

Conflict of interest: The authors have no conflicts of interest to report.

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RESEARCH ARTICLE

A Retrospective Evaluation of Surgical Diseases in Domestic and Wild Avian Species: 436 Cases (2017-2023)

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ABSTRACT

This retrospective study aims to evaluate the surgical diseases diagnosed in domestic and wild avian species presented to the Surgical Clinic of the Siirt University Animal Health Application and Research Hospital. In the study, data from 436 wild and domestic avian patients, including 196 budgerigars (*Melopsittacus undulatus*), 62 pigeons (*Columbalivia domestica*), 50 chickens (*Gallus gallus domesticus*), and other species, were analyzed. The distribution of cases was categorized by year, species, diagnosed diseases, disease etiologies, disease localizations, and treatment options. In examined avian patients, the most commonly encountered surgical condition was soft tissue trauma (42.20%), followed by wounds (23.16%), fractures (22.70%), congenital deformities (6.65%), and ocular diseases (5.27%). Among the etiologies of fractures, unknown causes and various traumas were identified. The diseases encountered in examined avian patients were treated using medical, surgical, and conservative methods. The data obtained from the study highlight the prevalence and diversity of surgical diseases in avian species and underscore the importance of specific treatment approaches for different avian species. The research is expected to contribute to studies related to surgical diseases that may be encountered in different avian species.
Keywords: Avian surgery, Fractures, Retrospective study, Soft tissue trauma

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Evcil ve Yabani Kanatlı Hayvanlarda Görülen Cerrahi Hastalıkların Retrospektif Değerlendirmesi: 436 Olgu (2017-2023)

ÖZ

Bu retrospektif çalışma, Siirt Üniversitesi Hayvan Sağlığı Uygulama ve Araştırma Hastanesi Cerrahi Kliniğine getirilen evcil ve yabani kanatlı türlerinde teşhis edilen cerrahi hastalıkların değerlendirilmesini amaçlamaktadır. Çalışmada 196 muhabbet kuşu (*Melopsittacus undulatus*), 62 güvercin (*Columbalivia domestica*), 50 tavuk (*Gallus gallus domesticus*) ve diğer türler dahil olmak üzere 436 yabani ve evcil kanatlı hastanın verileri incelendi. Vakaların yıllara, türlere, teşhis edilen hastalıklara, hastalık etiyolojilerine, hastalık lokalizasyonlarına ve tedavi seçeneklerine göre dağılımı kategorize edildi. Muayene edilen kanatlı hayvanlarda en sık rastlanan cerrahi hastalık yumuşak doku travması (%42,20) olurken, bu hastalığı sırasıyla yaralar (%23,16), kırıklar (%22,70), doğuştan deformiteler (%6,65) ve göz hastalıkları (%5,27) izledi. Kırık etiyolojileri arasında bilinmeyen nedenler ve çeşitli travmaların yer aldığı görüldü. Muayenesi yapılan kanatlı hayvanlarda karşılaşılan hastalıklar medikal, operatif ve konservatif olarak tedavi edildi. Çalışmadan elde edilen verilerin kanatlı hayvan türlerinde cerrahi hastalıkların prevalansını ve çeşitliliğini vurgulamakta ve farklı kanatlı türleri için özel tedavi yaklaşımlarının önemini ortaya koymaktadır. Araştırmanın, farklı kanatlı türlerinde karşılaşılabilecek cerrahi hastalıklarla ilgili çalışmalara katkı sunacağı düşünülmektedir.
Anahtar Kelimeler: Kanatlı cerrahisi, Kırıklar, Retrospektif çalışma, Yumuşak doku travması

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GİRİŞ

Yabani kanatlıların doğal yaşamdaki dengenin sağlıklı bir şekilde devam edebilmesi için oldukça önemli canlılar olduğu vurgulanmaktadır (Kibar ve Bumin 2006; Aslan ve ark. 2009; Altıntaş ve Akın 2024). Dünya genelinde 9700'e yakın kanatlı türü olduğu bilinmektedir (Tully ve ark. 2000). Evcil hayvanlar arasında kanatlılar evdeki yaşama hemen uyum sağlamaları, renk çeşitlilikleri, eğlenceli halleri ve uzun süre yaşayabilmeleri sebepleri ile günümüzde çok fazla tercih edilmektedirler (Davis 1996).

Yabani kanatlıların hayatları birçok faktör sebebi ile tehlikeyle karşı karşıyadır. Yaşam alanlarının tahrip edilmesi, çeşitli kazalar, diğer hayvanların saldırıları, farklı ilaç ve kimyasallar, zor iklim koşulları, bilinçsiz avlanmalar bu etkenlerden bazılarıdır (Joseph 1998; Aslan ve ark. 2009; Kibar ve Bumin 2006). Diğer hayvanlarda olduğu gibi kanatlılarda da ortopedik hastalıklar, yumuşak doku travmaları, göz hastalıkları gibi cerrahi hastalıklar görülmektedir (Altıntaş ve Akın 2024). Diğer hayvanlarda özellikle yara ve kırık sağaltımları sırasında uygulanan yöntemlerin kanatlı hayvanlarda da uygulanabileceği belirtilmektedir. Aslan ve ark. (2009) kanatlı hayvanlarda tespit ettikleri yaraların tedavisinde %0.5 povidon iode, basitrasin+neomisin (Thiocilline®, Abdi İbrahim İlaç Sanayi ve Ticaret A.Ş, İstanbul/Türkiye) ve *Centella asiatica* ekstresi (Madecassol®, Bayer Türk Kimya San. Ltd. Şti., İstanbul/Türkiye) karışımı pomad kullandıklarını belirtmişlerdir. Kırık tedavisinde ise fikzasyon amacıyla intramedullar pin ve destekli bandaj uygulamalarından yararlandıklarını bildirmişlerdir. Çalışmada 16 kuşun başarılı bir şekilde tedavie dildiği ancak 4 kuşun ise yapılan tedavilere cevap vermediği belirtilmiştir. Altıntaş ve Akın (2024) çalışmalarında yara olgularında etakridin laktat monohidrat (Rivanol®, İstanbul İlaç, İstanbul/Türkiye), hipokloröz asit (Crystalin®, NHP, İzmir/Türkiye)] ve bununla birlikte geniş spektrumlu antibiyotik [enrofloksasin (Baytril®, Bayer Türk Kimya San. Ltd. Şti., İstanbul/Türkiye)] uyguladıklarını bildirmişlerdir. Kırık olgularında ise pencereli yaş bandaj veya intramedullar pin (steinmann veya kirshner) yöntemleri ile tedavi uyguladıklarını belirtmişlerdir.

Kanatlı hayvanlarda karşılaşılan cerrahi olguların sağaltımında diğer hayvan türlerinde uygulanan yöntemler kullanılsa da anatomik farklılıklar nedeniyle bu uygulamaların çeşitli boyuttaki kanatlılara göre modifiye edilip uygun hale getirilmesi gerekmektedir. (Degernes 1994; Aslan ve ark. 2009). Bu doğrultuda veteriner cerrahi alanında tespit edilen farklı vakaları incelemenin, kanatlı hayvanlarda karşılaşılabilecek durumları değerlendirmek, tedavi seçeneklerini belirlemek ve ileride yapılacak çalışmaları planlamak için oldukça önemli olduğu vurgulanmaktadır (Altan ve ark. 2013; İşler ve ark. 2015; Altuğ ve ark. 2017)

Kanatlı hayvanlarda yapılan retrospektif çalışmalar incelendiğinde veteriner cerrahi alanındaki değerlendirmelerin sınırlı kaldığı dikkat çekmektedir (Aslan ve ark. 2009). Bu amaçla bu çalışmada 2017-2024 yılları arasında çeşitli nedenler ile Siirt Üniversitesi Hayvan Sağlığı Uygulama ve Araştırma Hastanesi Cerrahi Kliniği'ne getirilen kanatlı hayvanlarda tespit edilen çeşitli olguların retrospektif değerlendirilmesi amaçlandı.

MATERYAL ve METOT

Çalışmanın materyalini 2017-2023 yılları arasında Siirt Üniversitesi Hayvan Sağlığı Uygulama ve Araştırma Hastanesi Cerrahi Kliniği'ne getirilen farklı türde 436 kanatlı hayvan oluşturdu. Çalışmada yıllara göre gelen hasta sayısı, türleri ve hastalık tanıları, hastalık etiyolojileri, hastalık lokalizasyonları ve sağaltım seçenekleri kategorize edildi. Tanı koyma süreçlerinde; klinik muayene, radyolojik muayene (FDR Smart X, Fujifilm, Japonya), oftalmoskobik muayeneden (Ri-scope L, Riester, Almanya) yararlanıldı.

BULGULAR

Çalışmada incelenen 436 vakanın 24 (%5,50)'ünün 2017 yılında, 16 (%3,66)'sının 2018 yılında, 53 (%12,15)'ünün 2019 yılında, 58 (%13,30)'inin 2020 yılında, 116 (%26,60)'sının 2021 yılında, 130 (%29,81)'unun 2022 yılında ve 39 (%8,94)'unun ise 2023 yılında kliniğimize getirildiği belirlendi (Tablo 1).

Çalışma materyalini oluşturan 436 adet kanatlı hayvanın 196'sı (%44,95) muhabbet kuşu (*Melopsittacus undulatus*), 62'si (%14,22) güvercin (*Columbalivia domestica*) 50'si (%11,46) tavuk (*Gallus gallus domesticus*), 47'si (%10,72) papağan (*Psittaciformes* sp.), 13'ü (%2,98) keklik (*Alectoris chukar*), 11'i (%2,52) serçe (*Passerida* sp.), 10'u (%2,29) karga (*Corvus splendens*), 8'i (%1,83) şahin (*Buteo* sp.), 6'sı (%1,37) baykuş (*Strigiformes* sp.), 6'sı (%1,37) kumru (*Streptopelia decaocto*), 5'i (%1,14) atmaca (*Accipiter* sp.), 5'i (%1,14) ördek (*Anatinae* sp.), 5'i (%1,14) kartal (*Haliaeetus leucocephalus*), 4'ü (%0,91) hindi (*Meleagris gallopava*), 2'si (%0,45) ibibik kuşu (*Upupa epops*), 1'i (%0,22) bıldırcın (*Coturnix coturnix*), 1'i (%0,22) doğan (Falco), 1'i (%0,22) akbaba (Aegypiu), 1'i (%0,22) arı kuşu (Merops), 1'i (%0,22) kaz (Anserinae), 1'i (%0,22) kanarya (*Serinus canaria*) olarak kaydedildi (Tablo 1). Çalışmaya dahil edilen 436 kanatlının 115 (%26,37)'i erkek ve 138 (%31,65)'i dişi olarak belirlenirken 183 (%41,97)'ünün cinsiyeti belirlenemedi

Tablo 1. Çalışmada incelenen vakaların tür bazında yıllara göre dağılımı.

Table 1. Distribution of cases by species over years in the study.

Tür/Yıl	2017	2018	2019	2020	2021	2022	2023	Toplam
Muhabbet kuşu	5	6	28	36	57	54	10	196
Güvercin	3	5	5	3	18	18	10	62
Tavuk	5	1	11	8	12	10	3	50
Papağan	7	2	6	2	12	12	6	47
Keklik	0	2	1	4	2	2	2	13
Serçe	2	0	0	1	2	4	2	11
Karga	0	0	0	0	2	8	0	10
Şahin	1	0	0	0	0	4	3	8
Baykuş	0	0	0	0	0	6	0	6
Kumru	0	0	0	0	2	4	0	6
Atmaca	0	0	0	1	2	1	1	5
Ördek	0	0	1	2	1	1	0	5
Kartal	0	0	0	0	3	1	1	5
Hindi	0	0	1	1	2	0	0	4
İbibik kuşu	1	0	0	0	1	0	0	2
Bıldırcın	0	0	0	0	0	1	0	1
Doğan	0	0	0	0	0	1	0	1
Akbaba	0	0	0	0	0	1	0	1
Arı kuşu	0	0	0	0	0	1	0	1
Kaz	0	0	0	0	0	1	0	1
Kanarya	0	0	0	0	0	0	1	1
Toplam	24	16	53	58	116	130	39	436

Kanatlı hayvanların 184'ünde (%42,20) yumuşak doku travması, 101'inde (%23,16) yara, 99'unda (%22,70) kırık, 29'unda (%6,65) kongenital deformite, 23'ünde (%5,27) ise göz hastalıkları tespit edildi (Tablo 2). Kırık olgularının etiyolojileri incelendiğinde ise 43'ünün nedenin bilinmediği, 18'inin üzerine basma, 21'inin bir yere sıkışma, 1'inin diğer hayvanların saldırması, 17'sinin vurma/çarpma sonucu meydana geldiği tespit edildi. Teşhis edilen kırık olgularının anatomik lokalizasyonları tablo 3'te gösterildi. Olguların 21'i operatif olarak (intra medullar pin osteosentez), 78'i ise konservatif (bandaj) yöntemlerle tedavi edildi.

Tablo 2. Kuş hastalıklarının türler arasında dağılımı

Table 2. Avian diseases among avian species.

Tür	Yumuşak Doku Travması	Yara	Kırık	Kongenital Deformite	Göz Hastalıkları	Toplam
Muhabbet kuşu	100	30	50	11	5	**196**
Güvercin	20	21	15	0	6	**62**
Tavuk	20	14	6	7	3	**50**
Papağan	16	13	5	7	6	**47**
Keklik	5	1	6	0	1	**13**
Karga	4	2	1	3	0	**10**
Şahin	2	0	5	1	0	**8**
Baykuş	2	1	3	0	0	**6**
Serçe	4	6	1	0	0	**11**
Kumru	2	2	2	0	0	**6**
Atmaca	3	2	0	0	0	**5**
Ördek	2	2	0	0	1	**5**
Kartal	0	2	3	0	0	**5**
Hindi	2	0	1	0	1	**4**
İbibik kuşu	0	2	0	0	0	
Bıldırcın	0	0	1	0	0	**1**
Doğan	0	1	0	0	0	**1**
Akbaba	1	0	0	0	0	**1**
Arı kuşu	0	1	0	0	0	**1**
Kaz	0	1	0	0	0	**1**
Kanarya	1	0	0	0	0	**1**
TOPLAM	**184**	**101**	**99**	**29**	**23**	**436**

Tablo 3. Kırık olgularının anatomik lokalizasyonları.

Table 3. Anatomical localization of fracture cases.

Kırık (n=99)	**Humerus**	**Antebrachium**	**Metacarpus**	**Femur**	**Tibia**	**Metatarsus**
	16	15	3	16	46	3

Yumuşak doku travmalarının etiyolojileri incelendiğinde 82'inin sebebi bilinmezken, 31 tanesinin vurma çarpma, 33'ünün kafes tellerine takılma, 38'inin kapıya sıkışma sonucu meydana geldiği belirlendi. Olguların 72'si ön, 112'si ise arka ekstremitede tespit edildi. Vakaların tamamında medikal tedavi (lokal analjezikler, miyorelaksanlar ve vitaminler) önerildi.

Yara olgularının etiyolojileri incelendiğinde 54'ünün sebebi bilinmezken, 25 tanesinin hayvan saldırısı, 13'ünün sivri bir cisim batması ve 9'unun ise yanma sonucu meydana geldiği görüldü. Yara lokalizasyonları tablo 4'te gösterildi. Vakaların tamamında yara bölgesi uygun antiseptikler ile temizlenerek gerekli medikal tedavi uygulandı.

Olguların 29'unda kongenital deformite tespit edildi. Kongenital deformitelerin 4'ünün ön, 25'inin ise arka ektsremitede olduğu belirlendi. Deformitelerin 18'i valgus, 11'i varus olarak tespit edildi. Deformite tespit edilen hastalarda semptomatik tedaviler uygulandı.

Olguların 23'ünde göz hastalığı tespit edildi. Bunların 1'i korneada yara, 22'si ise konjunktivitis olarak değerlendirildi. Korneal yara olgusunda palpebral dikiş ve lokal olarak antibiyotikli göz damlası (Tobrased %0,3, Bilim İlaç, İstanbul/Türkiye)] uygulandı. Konjunktivitis olgularına ise lokal olarak antibiyotik+kortizon içeren göz damlası [tobramisin ve deksametazon (TobraDex %0,3/%0,1, Novartis, İstanbul/Türkiye)] uygulandı.

Tablo 4. Yara olgularının anatomik lokalizasyonu.

Table 4. Anatomical localization of wound cases.

Yara (n=101)	**Krenium**	**Kursak**	**Toraks**	**Ayak Tabanı**	**Arka Ekstremite**	**Ön Ekstremite**	**Falanks**
	18	23	18	8	17	14	3

TARTIŞMA

Cerrahi kliniklerine getirilen kanatlı hayvan sayısı araştırma süresi, coğrafi bölge farklılıkları, insanların duyarlılığı gibi faktörlere bağlı olarak değişkenlik göstermektedir. Bununla birlikte hastaların türleri ile ırklarının, hastalık insidansının ve hastalıkların lokalizasyonlarının da çeşitlilik gösterebileceği belirtilmektedir (Aslan ve ark. 2009; Molina-Lopez ve ark. 2013; İşler ve ark. 2015). Siirt bölgesinde yapılan bu çalışmada hasta türleri, sayıları, hastalık insidansları değerlendirildiğinde 5 yılda 436 hastanın kliniğe getirildiği belirlendi. Çalışmadaki tür çeşitliliği, sayıları ve tespit edilen hastalıkların diğer çalışmalardan (Fix ve Barrows 1990; Kibar ve Bumin 2006; Molina-Lopez ve ark. 2013) farklı olması literatür bilgileri desteklemektedir.

Konuyla ilgili daha önce yapılan çalışmalar incelendiğinde son yıllarda vaka sayılarının artış gösterdiği vurgulanmaktadır (Sarıerler ve Kılıç 2003; Akın ve ark. 2015; Altıntaş ve Akın 2024). Bu çalışmada olguların yıllık dağılımı incelendiğinde 2017 yılında 24, 2018 yılında 16, 2019 yılında 53, 2020 yılında 58, 2021 yılında 116, 2022 yılında 130 ve 2023 yılında 39 kanatlı hastanın kliniğimize getirildiği belirlendi. Bu verilerde 2018 yılından itibaren 2023 yılına kadar hasta sayısında artışı görülmektedir. Bu durum çalışmalarda belirtildiği şekilde kanatlı hayvanlara geçmiş yıllara göre daha çok ilgi duyulduğu ve duyarlılığın arttığı kanısını desteklemektedir. Çalışmada 2023 yılında hasta sayısındaki azalma söz konusu dönemde hastanede gerçekleştirilen tadilat sonucu hasta kabul edilemeyişi ile ilgili olarak ortaya çıkan bir durumdur.

Benzer çalışmalarda kliniklere getirilen yabani kanatlı sayılarının evde beslenen kanatlılara oranla daha az olduğu bildirilmektedir (Sarıerler ve Kılıç 2003; Aslan ve ark. 2009; Akın ve ark. 2015; Altıntaş ve Akın 2024). Benzer şekilde sunulan çalışmada da yabani kanatlı sayısının evcil kanatlı sayısına göre düşük olduğu (yabani kanatlı=18, evcil kanatlı=418) belirlenmiştir. Bu durumun, evcil hayvan olarak farklı sebeplerden (uyumları, renkleri, eğlenceli halleri ve uzun yaşam süreleri) dolayı kuşların daha fazla tercih edilmesi ile ilgili olduğu düşünülmektedir.

Kanatlı hayvanların cinsiyetlerinin belirlenmesinin her zaman kolay olmadığı belirtilmektedir (Helmer ve ark.

2005; Cerit ve Avanus 2007). Yapılan çalışmalarda genellikle anemnez bilgilerine göre cinsiyetin belirlendiği vurgulanmaktadır (Altıntaş ve Akın 2024). Molina-Lopez ve ark. (2013) 7553 vakayı içeren çalışmada olguların 3695'inin cinsiyetinin bilinmediğini belirtirken, 1.363'ünün dişi, 1.163'ünün erkek olduğunu belirlemişlerdir. Altıntaş ve Akın (2024) çalışmalarında 34 vakanın erkek, 18'inin dişi ve 74'ünün cinsiyetinin tespit edilemediğini belirtmişlerdir. Çalışmaya dahil edilen 436 kanatlının 115'i erkek, 138'i dişi olarak belirlenirken, 183'ünün cinsiyeti belirlenemedi. Diğer çalışmalarla paralel olarak sunulan bu çalışmada da olguların çoğunda cinsiyet tespit edilemedi.

Kanatlı hayvanlarda teşhis edilen hastalıkların değerlendirilmesiyle ilgili yapılan çalışmalarda kırık olgularına daha fazla rastlanıldığı bilgisi verilmektedir (Kibar ve Bumin 2006; Aslan ve ark. 2009; Korkmaz ve ark. 2014; Akın ve ark. 2015; Altıntaş ve Akın. 2024). İşler ve ark. (2015) yaptıkları çalışmada yabani kanatlılarda en sık görülen hastalıkları sınıflandırdıklarında sırasıyla ortopedik olgular (%87,17), genel cerrahi hastalıkları (%21,79), sinir lezyonlarının (%3,84) belirlendiği, evcil kanatlılarda ise sırasıyla genel cerrahi hastalıkları (%50), ortopedik olgular (%29,41) ve göz hastalıklarının (%14,70) tespit edildiğini bildirmişlerdir. Bu çalışmada ise 436 olguda tespit edilen hastalıklardan ilk sırada yumuşak doku travması (n=184) gelirken, bunu sırasıyla yara (n=101), ekstremite kırıkları (n=99), kongenital deformite (n=29) ve göz hastalıklarının (n=23) izlediği görülmektedir. Bu sonuçlarda yumuşak doku travmalarının da ortpedik hastalıklar içinde olduğu düşünüldüğünde, ortopedik hastalık olgularının fazla olması ile literatür bilgiler ile paralellik göstermektedir.

Kanatlı hayvanlarda kırık vakalarının lokalizasyonlarına bakıldığında; Kibar ve Bumin (2006) yabani kuşlar ile ilgili yaptıkları çalışmada kırık olgusunun %80'ninin kanatlarda, %20'sinin ise pelvik ekstremitelerde lokalize olduğunu belirlemişlerdir. Akın ve ark. (2015)'nın 57 kırık olgusu tespit ettikleri çalışmalarında kırık lokalizasyonun en sık humerus (17 olgu) bölgesinde olduğu görülmektedir. Altıntaş ve Akın (2024) yaptıkları çalışmada evcil kanatlılarda en fazla tibiotarsal kırıklar ile karşılaştıklarını belirtirken bu durumu, kuşların ayaklarını daha fazla kullanmaları ile açıklanmaktadır. Bu çalışmada da vakaların büyük bir bölümünü evcil kanatlılar oluşturmakta ve en fazla kırık olgusunun tibiatarsus bölgesinde olduğu tespit edilmiştir. Bu durum Altıntaş ve Akın (2024)'nın teorilerini desteklemektedir.

İşler ve ark. (2015) yaptıkları çalışmada hastaların %57,08'ine medikal sağaltım, %42,92'sine operatif sağaltım uygulandığı bildirilmiştir. Kliniğimize getirilen hastalarının sağaltım uygulamaları incelendiğinde medikal sağaltım %95,18 oranında, operatif sağaltım ise %4,81 oranında uygulanmıştır. Operasyon oranımızın düşük olması karşılaştığımız ekstremite kırıklarının genellikle muhabbet kuşları ve güvercinler gibi küçük boyutta kanatlılarda meydana gelmesine bağlanabileceği kanısına varılmıştır.

SONUÇ

Siirt ili Güneydoğu Anadolu bölgesinde yer almakla beraber coğrafi yapısı ve sahip olduğu tabiat parkı ile birçok yabani kanatlı kuş türüne ev sahipliği yapmaktadır. Bu durum da bölge halkının duyarlılığı artıkça ve Doğa Koruma ve Milli Parklar il Şube Müdürlüğü ile yapılan iş birliği protokolleri ile kliniğimize getirilen yabani kanatlı sayısının artacağını ve daha fazla ihtiyaç sahibi yabani kanatlının tedavilerini gerçekleştirebileceğimizi göstermektedir. Benzer çalışmalarda olduğu gibi kanatlı hayvanlarda genellikle ortopedik hastalıklar ve yara olguları ile daha sık karşılaşıldığı görülmektedir. Bu hastalıklarda ilk yardım uygulanmasının ve kısa sürede kliniğe getirilmesinin tedavide başarı oranını arttıracağı bilinmektedir. Bu kapsamda bölge halkının duyarlılığın ve iş birliği protokollerinin düzenlenmesinin oldukça yararlı olacağı düşünülmektedir. Veteriner cerrahi açısından değerlendirildiğinde ise diğer hayvan türlerinde görülen hastalıkların kanatlı hayvanlarda da belirlenmesinin yanı sıra tedavi seçeneklerinde türe özgü kararlar alınması kaçınılmazdır. Sunulan çalışma verilerinin ve ileride yapılacak daha kapsamlı araştırmaların kanatlı hayvanlarda karşılaşacak olguların teşhis ve etkin tedavi seçeneklerine katkı sağlayacağı kanaatine varılmıştır.

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

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RESEARCH ARTICLE

Investigation of the Effects of Rutin on Sodium Valproate-Induced Lung Damage in Rats

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ABSTRACT

Sodium valproate (SVP) is a drug widely used in epilepsy, migraine, and bipolar disorders. In addition to its therapeutic properties, it has toxic effects on many organs in high doses and prolonged intake. Rutin flavonoid derivative is a natural antioxidant and has been successfully used in many toxications. In the present study, it was aimed to investigate the effects of rutin on SVP-induced lung injury. In the study, 35 Sprague Dawley rats were divided into five equal groups as control, routine, SVP, SVP+Rutin 50 and SVP+Rutin 100 and oral administration was continued for 14 days. At the end of the study, lung tissue was obtained and oxidative stress (MDA, GSH, SOD, CAT, GPx, Nrf-2, HO-1), endoplasmic reticulum stress (ATF-6, PERK), inflammation (NF-κB, TNF-α), apoptosis (Bax, Bcl-2, Caspase-3) and autophagy (Beclin-1) parameters were analyzed. The data obtained showed that SVP weakened the defense system by decreasing antioxidant enzyme activities and increased lipid peroxidation, inflammation, apoptosis, and autophagy, leading to increased cell damage. It was determined that SVP and rutin 50 and rutin 100 doses strengthened the antioxidant defense system, suppressed lipid peroxidation, endoplasmic reticulum stress, inflammation, apoptosis, and autophagy, and were effective in protecting the cell from damage. As a result, it was determined that rutin use was beneficial against SVP-induced lung injury.

Key Words: Lung damage, Rat, Rutin, Sodium valproate

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Ratlarda Sodyum Valproat Kaynaklı Akciğer Hasarı Üzerine Rutin'in Etkilerinin Araştırılması

ÖZ

Sodium valproat (SVP), başta epilepsi olmak üzere migren ve bipolar bozukluklarda yaygın kullanılan bir ilaçtır. Tedavi edici özelliği yanı sıra yüksek doz ve uzun süre alımlarda çoğu organda toksik etki göstermektedir. Rutin flavanoit türevi doğal bir antioksidandır ve birçok toksikasyonda başarı ile kullanılmıştır. Sunulan çalışmada SVP kaynaklı akciğer hasarı üzerine rutin' in etkilerinin araştırılması amaçlanmıştır. Çalışmada 35 Sprague Dawley rat kontrol, Rutin, SVP, SVP+Rutin 50 ve SVP+Rutin 100 olmak üzere beş eşit gruba ayrılarak 14 gün oral yolla uygulamalar devam etmiştir. Çalışma sonunda akciğer dokusu alınarak oksidatif stres (MDA, GSH, SOD, CAT, GPx, Nrf-2, HO-1), endoplazmik retikulum stresi (ATF-6, PERK), inflamasyon (NF-κB, TNF-α), apoptoz (Bax, Bcl-2, Caspase-3) ve otofaji (Beclin-1) parametreleri incelenmiştir. Elde edilen veriler SVP' nin antioksidan enzim aktivitelerini azaltarak savunma sistemini zayıflattığını, lipid peroksidasyonu, inflamasyonu, apoptozisi ve otofajiyi artırarak hücrede hasarın artmasına neden olduğu göstermiştir. SVP ile birlikte rutin 50 ve rutin 100 dozlarının antioksidan savunma sistemini güçlendirdiği, lipid peroksidasyonunu, endoplasmik retikulum stresini, inflamasyonu, apoptoz ve otofajiyi baskılayarak hücreyi hasardan korumada etkili olduğu tespit edildi. Sonuç olarak SVP kaynaklı akciğer hasarına karşı rutin kullanımının faydalı olduğu belirlendi.

Anahtar Kelimeler: Akciğer hasarı, Rat, Rutin, Sodyum valproat

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INTRODUCTION

Epilepsy is one of the most common neurological disorders. Intermittent, exaggerated, atypical episodes of cerebral activity lead to clinical features ranging from debilitating seizures, cerebral processing abnormalities, and mental health problems to the life-threatening clinical condition epilepticus syndrome (SE) (Al-Rafiah and Mehdar 2021). Sodium Valproate (SVP) is a histone deacetylase inhibitor that has been used for over 30 years to treat epilepsy, which affects 70 million people worldwide (Zhou et al. 2020; Korıglu et al. 2021). The mechanism of action of SVP is known to be inhibition of γ-aminobutyric acid (GABA) metabolism and interruption of GABA reuptake into nerve endings (Adewole et al. 2023). Therefore, SVP is used in the treatment of many psychiatric disorders such as migraine, bipolar disorder, and schizophrenia as well as epilepsy (Kandemir et al. 2022). Although SVP, a popular drug due to its therapeutic benefits and low cost, is well tolerated in the body, it has been reported to cause renal, hepatic, and pulmonary toxicity with cardiovascular, neurologic, and gastrointestinal side effects, and SVP use has been reported to cause widespread alveolar bleeding, especially in humans (Nanau and Neuman 2013; Öztay et al. 2020). Therefore, the reliability of SVP has been questioned by clinicians who initially supported it (Chaudhary et al. 2015). Although there are many reasons underlying the toxic effect of SVP, induction of oxidative stress and inflammation are among the most important ones. Therefore, research on natural active ingredients that regulate these mechanisms against SVP toxicity continues intensively (Akaras et al. 2023).

Foods contain certain compounds with effective antioxidant properties, such as flavonoids. These compounds are known as dietary antioxidants because of their beneficial properties. More than 4000 flavonoids, including rutin, have been evaluated as dietary antioxidants (Genç et al. 2019). Rutin is a flavonoid glycoside found predominantly in citrus fruits such as grapefruit, orange, lemon, spinach, onion, apple, buckwheat seeds, and tea. A potent scavenger of superoxide radicals, rutin's pharmacological properties include anti-inflammatory, antioxidant, antiallergic, and anticarcinogenic effects (Küçükler et al. 2021; Gür and Kandemir 2023).

The present study aimed to investigate the effects of rutin on SVP-induced lung injury with some biochemical parameters and examine the possible mechanisms of injury and the effect of rutin on these mechanisms.

MATERIALS and METHODS

Drug and Chemicals

SVP (Depakin, Sanofi, Turkey), Rutin Hydrate (≥ 94%, Sigma (R5143), USA), and other chemicals were of analytical purity and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental Animals, Ethics Committee Aprproval and Experimental Design

In this study, 35 Sprague Dawley male rats from Ataturk University Animal Experimentation Center were used. The rats were kept in clean cages at a constant temperature of 24-25^{0}C and a 12-hour dark-light cycle. Water and feed were provided *ad-libitum*. The ethics committee approval of the study was obtained from Ataturk University Animal Experiments Local Ethics Committee with the meeting number 2023/14 and decision number 212 on 25.12.2023.

Five different groups of seven rats each were formed in the experiment. Rutin and SVP doses were determined with reference to previous studies (Kandemir et al. 2022; Akaras et al., 2023).

Group 1 (Control): Rats were given oral saline every day for 14 days.

Group 2 (Rutin): Rats received 100 mg/kg dose of rutin orally for 14 days.

Group 3 (SVP): Rats received SVP at a dose of 500 mg/kg orally for 14 days.

Group 4 (SVP+Rutin 50 mg/kg): Rats were orally administered SVP at a dose of 500 mg/kg for 14 days and 50 mg/kg of rutin was orally administered 30 minutes later.

Group 5 (SVP+Rutin 100 mg/kg): Rats were given SVP at a dose of 500 mg/kg orally for 14 days and 30 minutes later, 100 mg/kg of rutin was given orally.

24 hours after the last rutin treatment, rats were decapitated under mild sevoflurane anesthesia, lung tissues were removed and stored at -80 °C until biochemical analysis.

Analyzes of Oxidative Stress Parameters

Lung tissue was ground with liquid nitrogen (Tissue Lyser II, Qiagen) and homogenized in 1.15% potassium chloride buffer at a ratio of 1:10 (weight/volume). A portion of the homogenate was centrifuged at 10,000 rpm for 20 minutes at 4°C and the supernatant obtained was used to measure glutathione peroxidase (GPx) activity and glutathione (GSH) level. The remaining homogenate was centrifuged at 3500 rpm for 15 minutes and the supernatants were used for catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) analysis. GPx activity was measured according to Matkovics (1988), CAT activity according to Aebi (1984), SOD activity according to Sun et al. (1988), MDA level according to Placer et al. (1966) and GSH level according to Sedlak and Lindsay (1968). Total protein in the homogenate was determined according to the method of Lowry et al. (1951).

RT-PCR analysis

To analyze the relative mRNA transcript levels of the genes whose primer sequences are given in Table 1 in lung tissues, total RNA was first isolated from the tissues with QIAzol Lysis Reagent (79306; Qiagen). Then, cDNAs were synthesized from total RNAs with iScript cDNA Synthesis Kit (Bio-Rad). In the last step, cDNAs were reacted with primers of the relevant genes and iTaq Universal SYBR Green Supermix (BIORAD) in Rotor-Gene Q (Qiagen). After the reaction was completed, genes were normalized to β-actin using the 2-deltadeltaCT method (Livak and Schmittgen 2001).

Table 1: Primer sequences

Gene	Sequences (5'-3')	Length (bp)	Accession No
Nrf2	F: TTTGTAGATGACCATGAGTCGC	161	NM_031789.2
	R: TCCTGCCAAACTTGCTCCAT		
HO-1	F: ATGTCCCAGGATTTGTCCGA	144	NM_012580.2
	R: ATGGTACAAGGAGGCCATCA		
ATF-6	F: TCAACTCAGCACGTTCCTGA	130	NM_001107196.1
	R: GACCAGTGACAGGCTTCTCT		
PERK	F: GATGCCGAGAATCATGGGAA	198	NM_031599.2
	R: AGATTCGAGAAGGGACTCCA		
NF-κB	F: AGTCCCGCCCCTTCTAAAAC	106	NM_001276711.1
	R: CAATGGCCTCTGTGTAGCCC		
TNF-α	F: CTCGAGTGACAAGCCCGTAG	139	NM_012675.3
	R: ATCTGCTGGTACCACCAGTT		
Caspase-3	F: ACTGGAATGTCAGCTCGCAA	270	NM_012922.2
	R: GCAGTAGTCGCCTCTGAAGA		
Bcl-2	F: GACTTTGCAGAGATGTCCAG	214	NM_016993.2
	R: TCAGGTACTCAGTCATCCAC		
Bax	F: TTTCATCCAGGATCGAGCAG	154	NM_017059.2
	R: AATCATCCTCTGCAGCTCCA		
Beclin-1	F: TCTCGTCAAGGCGTCACTTC	198	NM_053739.2
	R: CCATTCTTTAGGCCCCGACG		
β-Actin	F: CAGCCTTCCTTCTTGGGTATG	360	NM_031144.3
	R: AGCTCAGTAACAGTCCGCCT		

One-way analysis of variance (ANOVA) and Tukey post hoc test (version 20.0; SPSS, Chicago, IL) were used to determine the differences between the groups and their significance levels. $p<0.05$ was considered a significant difference. All values were expressed as mean±standard derivation of the mean (SD).

RESULTS

Oxidative Stress

When lung tissue MDA levels were examined (Figure 1A), it was found that there was no difference between the control group and the rutin group ($p>0.05$), SVP administration increased the MDA level approximately 2-fold compared to the control and rutin groups ($p<0.001$), rutin 50 dose administered with SVP was not effective in reducing the MDA level, while rutin 100 dose successfully reduced the MDA level ($p<0.05$). In addition, GSH level (Figure 1B) and SOD (Figure 1C), CAT (Figure 1D) and GPx (Figure 1E) activities did not differ between the control and rutin groups ($p>0.05$), SVP administration caused a significant decrease in these antioxidant enzymes ($p<0.001$), and doses of rutin 50 and rutin 100 administered together with SVP caused a gradual and dose-dependent increase in antioxidant activities ($p<0.05$) and protected the lung tissue from oxidative damage.

When Nuclear released factor-2 (Nrf-2, Figure 2A) and Heme oxygenase-1 (HO-1, Figure 2B) mRNA expression levels, which strengthen the defense system by increasing the expression of antioxidant enzymes, were examined, it was determined that there was no difference between the control and rutin groups ($p>0.05$), SVP administration caused a decrease in Nrf-2 and HO-1 mRNA expression levels ($p<0.001$), while rutin 50 and 100 doses gradually increased these levels ($p<0.005$).

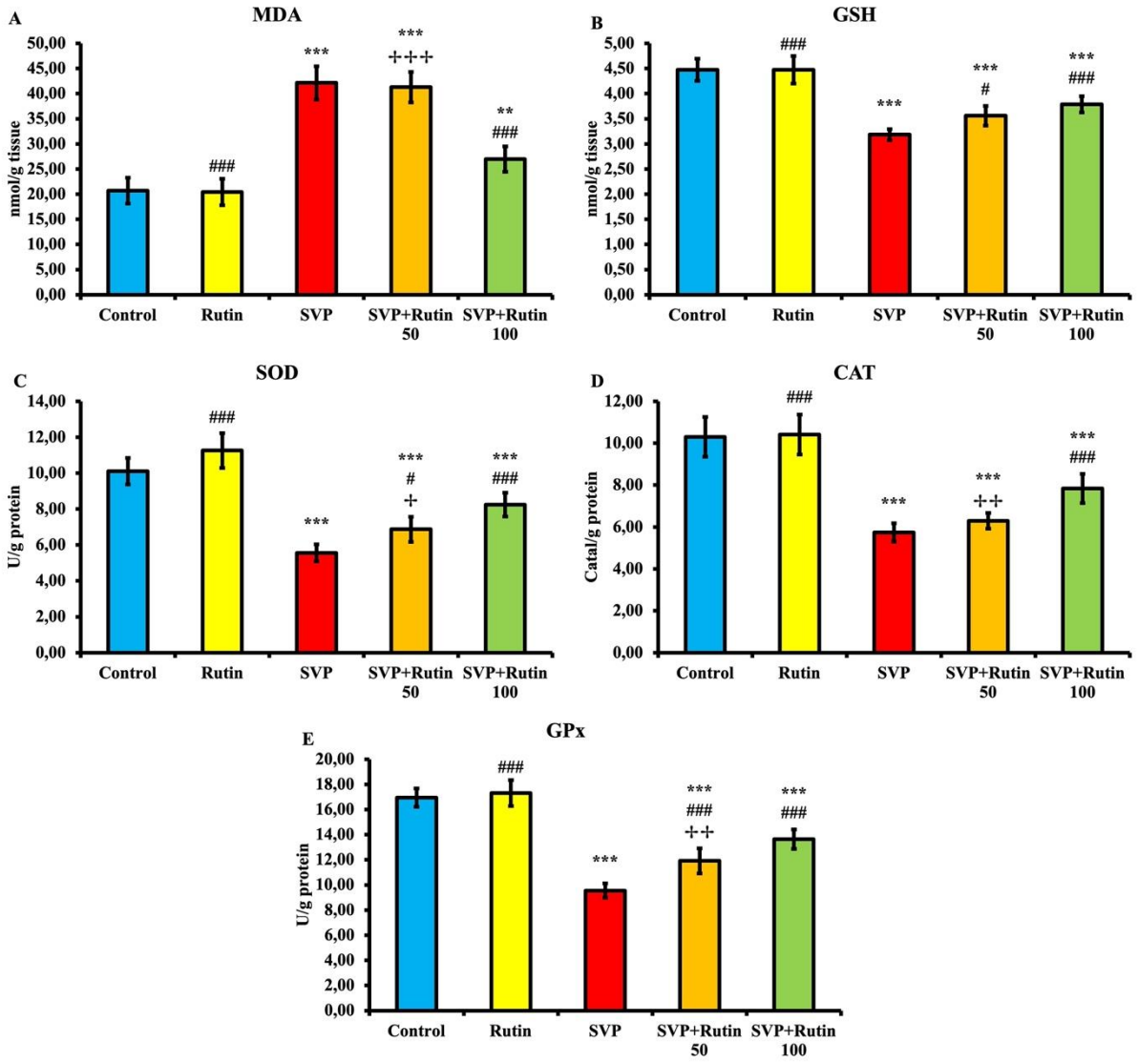

Figure 1: Lung tissue MDA (A) and GSH (B) levels and SOD (C), CAT (D) and GPx (E) activities after SVP and Rutin applications to rats. Statistical significance; Control and others: * p<0.05, ** p<0.01, *** p<0.001, SVP and orhers: # P < 0.05, ## p<0.01, ### p<0.001, SVP + Rutin 50 ve SVP + Rutin 100: ✢ P < 0.05, ✢✢ p<0.01, ✢✢✢ p<0.001.

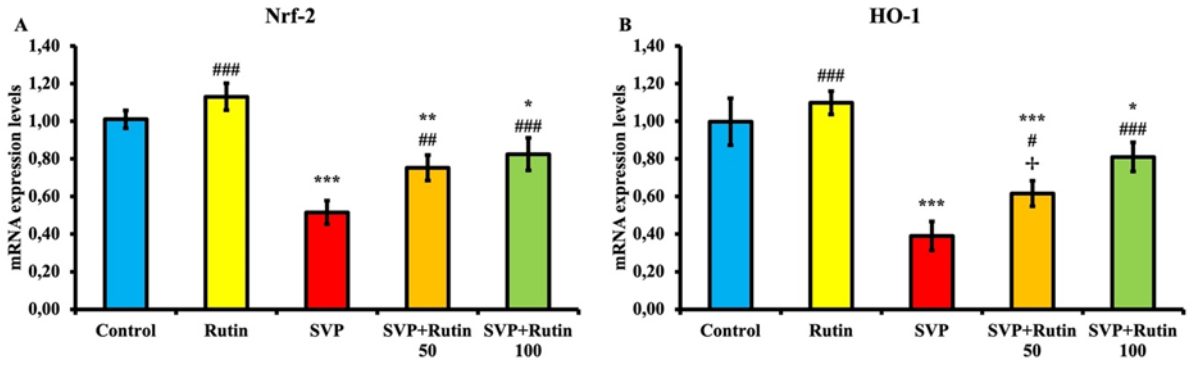

Figure 2: Lung tissue Nrf-2 (A) and HO-1 (B) mRNA expression levels after SVP and Rutin applications to rats. Statistical significance; Control and others: * p<0.05, ** p<0.01, *** p<0.001, SVP and others: # P < 0.05, ## p<0.01, ### p<0.001, SVP + Rutin 50 ve SVP + Rutin 100: ✢ P < 0.05, ✢✢ p<0.01, ✢✢✢ p<0.001.

Endoplasmic Reticulum Stress

When the activities of Transcription activating factor 6 (ATF-6, Figure 3A) and Protein kinase-like endoplasmic reticulum kinase mRNA expression levels (PERK, Figure 3B), which are among the endoplasmic reticulum stress parameters, were examined, it was found that there was no difference between the control and rutin groups in terms of ATF-6 and PERK mRNA expression levels ($p<0.05$), SVP treatment caused an increase in both parameters compared to control and rutin groups ($p<0.001$), rutin 50 and rutin 100 doses were effective in reducing endoplasmic reticulum stress by affecting ATF-6 and PERK mRNA expression levels ($p<0.05$).

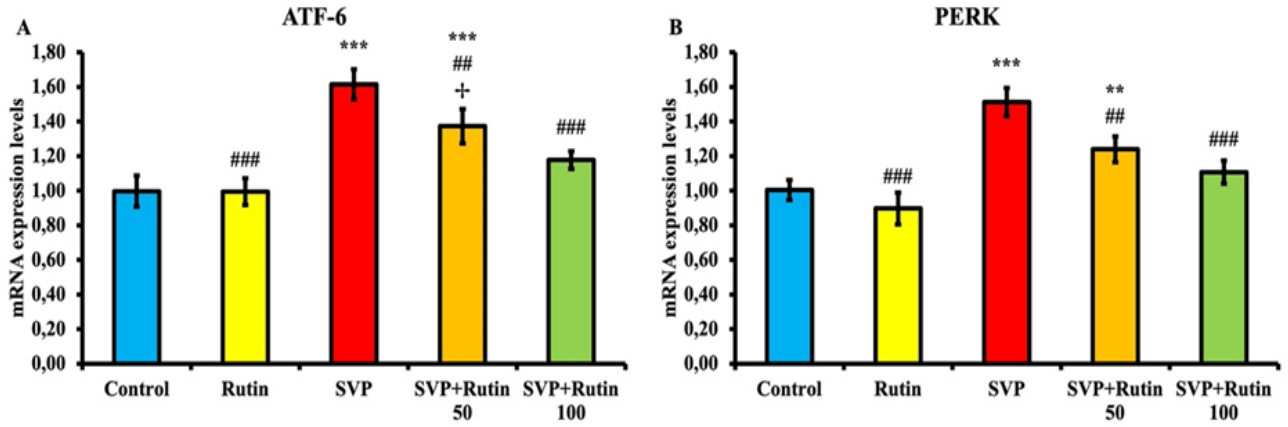

Figure 3: Lung tissue ATF-6 (A) and PERK (B) mRNA expression levels after SVP and Rutin applications to rats. Statistical significance; Control and others: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, SVP and others: # $P < 0.05$, ## $p<0.01$, ### $p<0.001$, SVP + Rutin 50 ve SVP + Rutin 100: + $P < 0.05$, ++ $p<0.01$, +++ $p<0.001$.

Inflammation

When Nuclear factor kappa B (NF-κB, Figure 4A) and Tumor necrosis factor alpha (TNF-α, Figure 4B) mRNA expression levels, which are among the most important markers of inflammation, were examined, SVP administration significantly increased NF-κB and TNF-α mRNA expression levels compared to control and rutin groups ($p<0.001$), while rutin 50 and rutin 100 doses given together with SVP suppressed inflammation by gradually and dose-dependently decreasing NF-κB and TNF-α levels ($p<0.05$).

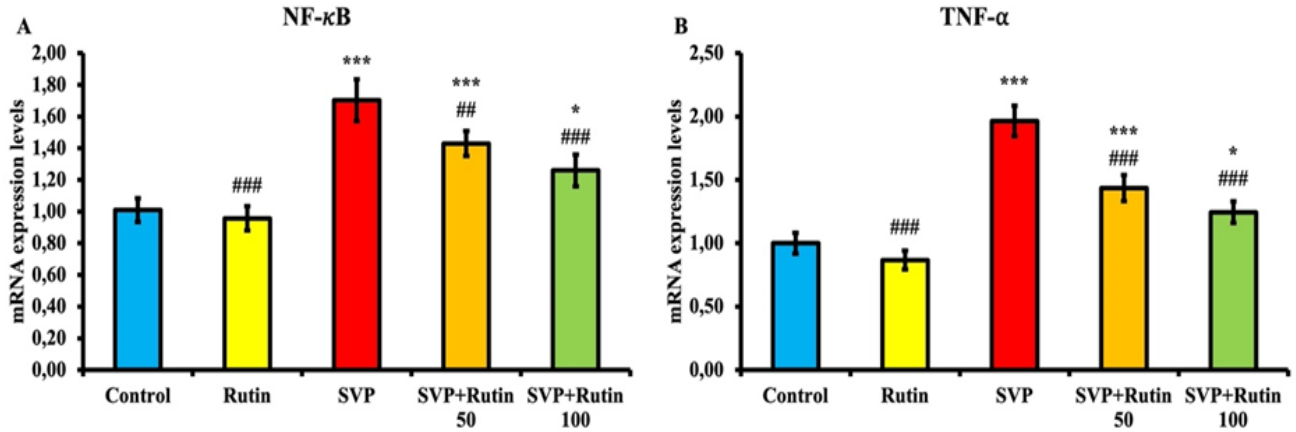

Figure 4: Lung tissue NF-κB (A) ve TNF-α (B) mRNA expression levels after SVP and Rutin applications to rats. Statistical significance; Control and others: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, SVP and others: # $P < 0.05$, ## $p<0.01$, ### $p<0.001$, SVP + Rutin 50 ve SVP + Rutin 100: + $P < 0.05$, ++ $p<0.01$, +++ $p<0.001$.

Apoptosis

The levels of Bcl-2-related x protein (Bax, Figure 5A), B-cell lymphoma gene-2 (Bcl-2, Figure 5B), and Cysteine aspartate-specific protease-3 mRNA expression levels (Caspase-3, Figure 5A), which are important markers of apoptosis, were analyzed. It was determined that proapoptotic marker Bax and apoptotic Caspase-3 mRNA expression levels were significantly increased with SVP treatment compared to control and rutin groups ($p<0.001$), while antiapoptotic marker Bcl-2 level was decreased with SVP treatment ($p<0.001$) and apoptosis was accelerated in the cells. Rutin 50 and 100 doses administered together with SVP were found to be successful ($p<0.05$) in suppressing apoptosis by decreasing Bax and caspase-3 and increasing Bcl-2 mRNA expression levels.

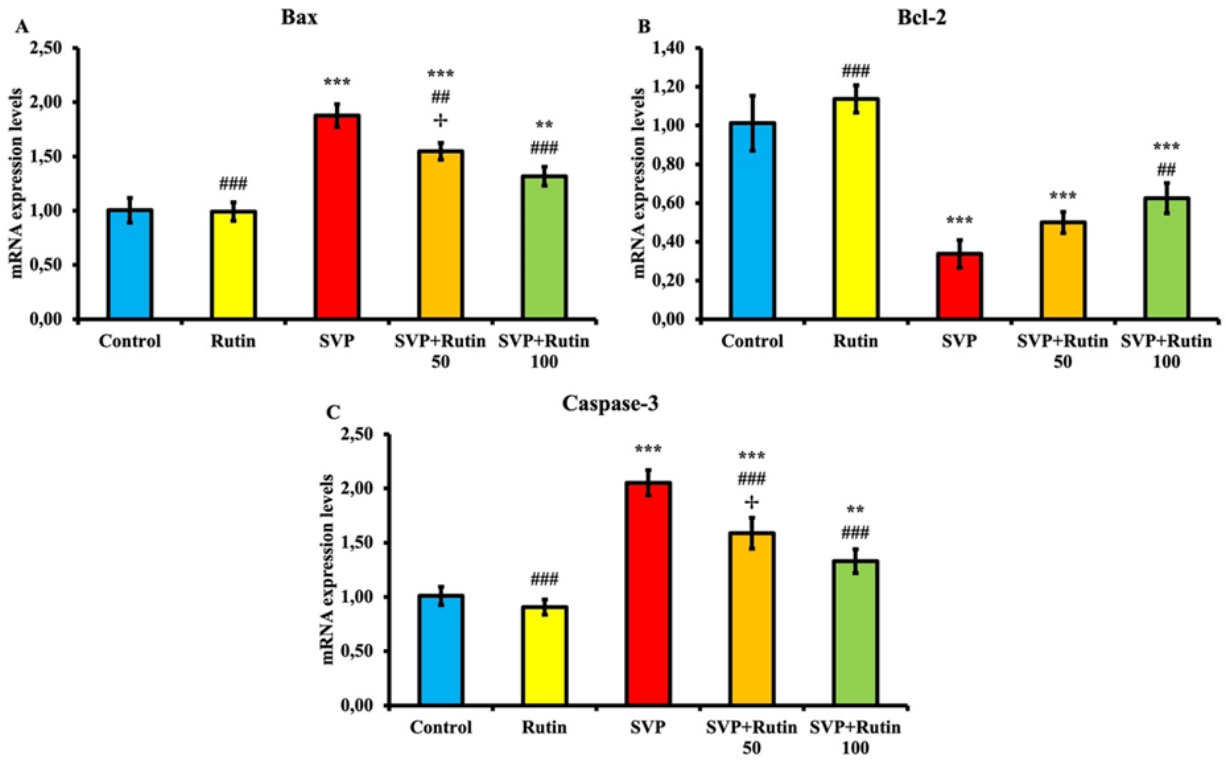

Figure 5: Lung tissue Bax (A), Bcl-2 (B) and Caspase-3 (C) mRNA expression levels after SVP and Rutin applications to rats. Statistical significance; Control and others * p<0.05, ** p<0.01, *** p<0.001, SVP and others: # P < 0.05, ## p<0.01, ### p<0.001, SVP + Rutin 50 ve SVP + Rutin 100: ✢ P < 0.05, ✢✢ p<0.01, ✢✢✢ p<0.001.

Autophagy

When Beclin-1 level, the most significant autophagy marker, was examined (Figure 6), it was found that there was no difference between the control and rutin groups (p>0.05), SVP administration caused an increase in lung tissue Beclin-1 mRNA expression levels (p<0.001), and both rutin 50 and 100 doses administered together with SVP were effective in suppressing autophagy by decreasing Beclin-1 mRNA expression levels (p<0.05).

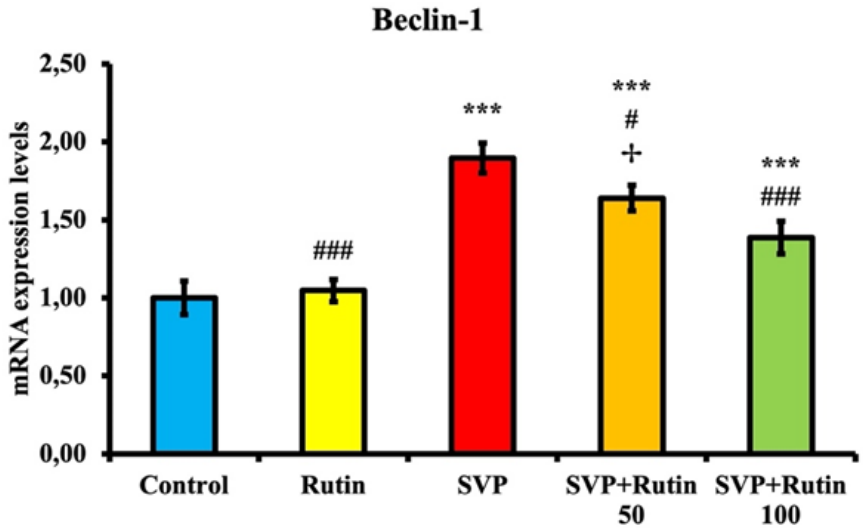

Figure 6: Lung tissue Beclin-1 mRNA expression levels after SVP and Rutin applications to rats. Statistical significance; Control and others:: * p<0.05, ** p<0.01, *** p<0.001, SVP and others: # P < 0.05, ## p<0.01, ### p<0.001, SVP + Rutin 50 ve SVP + Rutin 100: ✢ P < 0.05, ✢✢ p<0.01, ✢✢✢ p<0.001.

DISCUSSION

SVP is a well-known anti-epileptic drug and is also used to control convulsions, bipolar disorders and migraines (Gheena et al. 2022). In addition to its therapeutic properties, it has been reported to cause various organ damages including lung toxicity in long-term and high dose intake (Öztay et al.2020). Therefore, in the present study, the effects of rutin on sodium valproate-induced lung injury were investigated.

One of the main mechanisms of SVP toxicity is oxidative stress (Akarsu et al. 2023). Increased reaktive oxygene species (ROS) results in oxidative stress, which has a direct effect on acute and chronic lung injury. Increased ROS levels can directly cause tissue damage and promote inflammatory responses through the regulation of various pro-inflammatory mediators in the lungs (Akcılar et al. 2015a). It has been reported that there is excessive intracellular stress due to the process of peroxidation of membrane lipids and accumulation of free radicals, decreased enzymatic and non-enzymatic antioxidants, and increased MDA levels, and this is associated with metabolites derived from SVP. It was also reported that the decrease in intracellular antioxidants may be due to the overuse of antioxidants to neutralize the metabolites of SVP (Gheena et al. 2022). The organism has developed various ways to protect itself from the negative effects of oxidative attacks (Keleş et al. 2014; Ekinci-Akdemir et al. 2019). These mechanisms include various antioxidant enzymes such as SOD, CAT, GPx and non-enzymatic antioxidants such as GSH (Aydın et al. 2009; Kandemir et al. 2018; Kuzu et al. 2021; Kandemir et al. 2021). In the present study, it was determined that MDA levels in lung tissue of SVP-treated rats increased, SOD, CAT, and GPx activities and GSH levels decreased and oxidative stress developed in the cell. In addition, it was determined that rutin 50 and 100 doses administered with SVP decreased MDA levels and increased SOD, CAT, and GPx activities and GSH levels, strengthening the antioxidant system and protecting the cell from damage caused by oxidative stress. In studies conducted on the subject and targeting mechanisms in different organs, it was determined that SVP disrupted the cell membrane structure by increasing MDA levels and caused a decrease in antioxidant levels, and it was reported that rutin application was effective in reducing oxidative stress (Akaras et al. 2023a; Akarsu et al. 2023a, Kandemir et al. 2020).

The Nrf-2-antioxidant response element (ARE) system is stimulated by oxidative stress and plays an important role in tissue regeneration by regulating the expression of antioxidant and anti-inflammatory proteins in living organisms (Kocak et al. 2016). One of the target genes of Nrf-2 is HO-1. HO-1 oxidatively cleaves heme into biliverdin and carbon monoxide, thus eliminating the pro-oxidant effects of heme (Semiş et al. 2022). Activation of the Nrf-2 signaling pathway can effectively prevent oxidative stress-induced damage and contribute to tissue healing (Şimşek et al. 2023a; Tuncer et al. 2023a). In the present study, it was determined that SVP application caused a decrease in Nrf-2 and HO-1 mRNA experssion levels similar to antioxidant enzyme activities, and rutin application stimulated the Nrf-2 signaling pathway increased HO-1 mRNA experssion levels and decreased oxidative stress-induced damage. Nrf-2 and HO-1 have been examined as target parameters in many studies, it was reported that they were suppressed by different chemical agents, antioxidant enzyme activities decreased and the cell entered into oxidative stress effect, while flavonoids were found to increase antioxidant enzyme activities by stimulating Nrf-2 and HO-1 gene expression (Şimşek et al. 2023b; Gür et al. 2023; Çomaklı et al. 2023; Kankılıç et al. 2024a).

One of the mechanisms triggered by oxidative stress is endoplasmic reticulum (ER) stress (Semiş et al. 2021). The endoplasmic reticulum is an organelle involved in protein synthesis, folding and maturation, post-translational mechanisms, and calcium homeostasis (Ileriturk et al. 2023). Physiological and environmental factors contribute to the accumulation of unfolded and misfolded proteins in the ER lumen, as well as Ca imbalance. As a result, cells develop an unfolded protein response (UPR). As the stress persists, the UPR continues to elongate, ER stress develops and the cell undergoes apoptosis (Akaras et al. 2023b). It was reported by Chen et al. (2000) that SVP triggers ER stress by increasing oxidative stress. In the present study, SVP administration was found to increase ATF-6 and PERK mRNA experssion levels in lung tissue and thus ER stress. Rutin administration together with SVP was found to be effective in decreasing ATF-6 and PERK mRNA experssion levels and suppressing ER stress. It has been reported in different studies that rutin is effective in reducing ER stress and this effect is primarily achieved by reducing oxidative stress (Kandemir et al. 2022; Gür and Kandemir 2023).

Oxidative stress is also a factor in inflammatory damage (Şimşek et al. 2023c; Aksu et al. 2018). It is known that inflammation, another mechanism in the progression of lung injury, plays an important role and oxidative stress is one of the triggers of the inflammatory process in lung tissue. Inflammatory cytokines and chemokines are released by lung cells in association with oxidative stress. NF-κB is one of the main regulatory transcription factors regulating inflammatory responses and regulates proinflammatory cytokines such as TNF-α (Yeşildağ et al. 2022). TNF-α initiates and regulates the cytokine cascade in the cellular immune response process (Akarsu et al. 2023b). Different studies have shown that SVP administration accelerates inflammation by increasing NF-κB and TNF-α levels (Abu-Risha et al. 2024; Akaras et al. 2023a, Akarsu et al. 2023a). In the

present study, it was determined that SVP administration increased lung tissue NF-ϰB and TNF-α levels, and both doses of rutin administered together with SVP were effective and suppressed inflammation. It has also been demonstrated in different studies that rutin suppresses inflammation by decreasing NF-ϰB and TNF-α levels and shows anti-inflammatory properties (Küçükler et al. 2021; Tuncer et al. 2023b).

ROS are largely produced in mitochondria and have a significant impact on the development of apoptosis (Şimşek and Akaras, 2023). Apoptosis is a programmed cell death process that plays a role in the elimination of damaged cells under normal conditions (Akcılar et al. 2015b). In healthy tissues, apoptosis plays an important role in removing damaged or dangerous cells from the body (Kankılıç et al. 2024b). However, failure to control apoptosis causes damage in various tissues. Bcl-2 and caspase family proteins have an important role in the regulation of apoptosis. It is well known that oxidative stress mediates apoptosis by interacting with Bcl-2 and caspase family proteins (Yıldız et al. 2022). Caspases are inactive in cells, and when a caspase is activated, it initiates a cascade to activate other pro-caspases (Şimşek at al. 2016). The Bcl-2 family consists of pro-apoptotic and anti-apoptotic proteins that determine cell survival decisions. Bax in this family triggers apoptosis, while Bcl-2 is responsible for inhibiting apoptosis. The balance between these proteins determines the fate of cells in the apoptotic pathway. When an increase in the Bax/Bcl-2 ratio occurs, caspase 9 is activated. Activated Caspase-9 increases caspase 3 expression and apoptosis is triggered (Tabeshpour et al. 2020; Ekinci-Akdemir et al. 2022). It has been shown in many studies that SVP accelerates apoptosis by increasing Bax and caspase-3 levels and decreasing Bcl-2 levels in different organs, while rutin administration is effective in reducing apoptosis, especially decreasing the Bax/Bcl-2 ratio (Akaras et al. 2023a; Akarsu et al. 2023; Kandemir et al. 2022). In the present study, it was determined that Bax and Caspase-3 mRNA expression levels increased, Bcl-2 levels decreased and apoptosis accelerated in the SVP-treated group. Rutin administration together with AVP was found to be effective in reducing apoptosis at both doses.

Autophagy is a highly conserved physiological process that involves the recycling of proteins and molecules in the cytoplasm within autophagolysosomes (Kanlılıç et al. 2024c). When autophagy mediated by oxidative stress occurs at high levels, it triggers cell death and causes loss of function in tissues (Gür et al. 2022). One of the important markers in autophagy is Beclin-1 (Kanlılıç et al. 2024c). Beclin-1 is an essential protein involved in many important biological processes such as immunity, development, and tumor suppression (Tuncer et al. 2023c). In the present study, it was determined that there was a significant increase in Beclin-1 mRNA expression levels in the lung tissue of rats administered SVP and SVP-triggered autophagy. It was determined that rutin given together with SVP was effective at both doses and suppressed autophagy by decreasing the Beclin-1 mRNA expression levels. The anti-autophagic properties of rutin have been demonstrated in studies conducted in different tissues (Akaras et al. 2023a; Akarsu et al. 2023a).

CONCLUSION

When the data obtained in the study were evaluated, it was determined that SVP administration to rats caused lung tissue damage by increasing oxidative stress, ER stress, inflammation, apoptosis and autophagy, Rutin administration reduced this damage by showing antioxidant, anti-inflammatory, anti-apoptotic, and anti-autophagic effects, and as a result, the use of rutin in SVP-induced lung damage was beneficial.

Conflict of interest: The authors have no conflicts of interest to report.

Author' s Contributions: ÖK and CG contributed to the experimental design, biochemical analysis. ÖK drafted and wrote the manuscript. ÖK and CG reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Ataturk University Reserch Animals Application Center. This research was approved by The Ethics Committee of the Ataturk University (meeting number 2023/14, dated 25.12.2023 and decision number 212)

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RESEARCH ARTICLE

Stereological Investigation of the Effects of *Hypericum Perforatum* L. Plant on Skin Tissue in Second Degree Burns in Rats

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ABSTRACT

The aim of this study was to examine stereologically the effect of *Hypericum perforatum* L. oil on skin injuries resulting from burns. Experimental groups were selected 18 healthy male rats with an average weight of 250-300 grams as material. The rats were divided into 3 groups: control, burn, and burn+treatment, with 6 in each group. In the control group, second-degree burns were created and the rats were perfused. Control group rats were kept in formaldehyde for a week. Burns were applied to the burn, and burn+treatment groups with 100ºC water. 4gr/kg of Hypericum perforatum L. oil was given via gavage to the burn+treatment group only for 21 days. The burn group was not intervened. At the end of the process, rats in both the burn and burn+treatment groups were perfused. The skin of the animals, which were kept in formaldehyde for a week, and where second-degree burns occurred were dissected. After tissue tracking and tissue embedding procedures for all animals in the group, 5µm thick sections were taken to obtain 8-10 sections at a ratio of 1/75. Volume values were calculated using stereological methods from the sections photographed with an x4 objective. Calculations were made using the Shtereom I program in accordance with the Cavalieri's Principle. Non-parametric tests and Kruskal Wallis test were used in statistical evaluations. When the results were analyzed, it was observed that the administration of *Hypericum Perforatum* L. oil by gavage had an effect on the healing process of second degree burn wounds.
Key Words: Burn Wound, *Hypericum perforatum* L., Rat, Stereology, Volume.

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ÖZ

Sıçanlarda İkinci Derece Yanıklarda *Hypericum Perforatum* L. Bitkisinin Deri Dokusu Üzerine Etkilerinin Stereolojik Araştırılması

Yapılan bu çalışmada yanık sonucu oluşan deri yaralanmalarında *Hypericum perforatum* L. yağının etkisinin stereolojik olarak incelenmesi amaçlandı. Materyal olarak ortalama 250-300 gram ağırlığında sağlıklı 18 adet erkek rat seçilerek deney grupları oluşturuldu. Ratlar her grupta 6 adet olmak üzere kontrol, yanık ve yanık +tedavi olmak üzere 3 gruba ayrıldı. Kontrol grubunda ikinci derece yanık oluşturularak ratlar perfüze edildi. Bir hafta süreyle kontrol grubu ratlar formaldehitte bekletildi. Yanık ve yanık+tedavi gruplarına 100ºC'lik su ile yanık oluşturuldu. Sadece yanık+tedavi grubuna 4gr/kg *Hypericum perforatum* L. yağı gavaj yoluyla 21 gün süreyle verildi. Yanık grubuna müdahale edilmedi. Süreç sonunda hem yanık hem de yanık+tedavi grubundaki ratlar perfüze edildi. Bir hafta süreyle formaldehitte tutulan hayvanların ikinci derece yanık oluşturulan bölge derileri diseke edildi. Tüm gruptaki hayvanlar için doku takibi ve doku gömü işlemlerinden sonra 1/75 oranında 8-10 kesit elde edilecek şekilde ve 5µm kalınlığında kesitler alındı. 4'lük objektifle fotoğraflanan kesitlerden stereolojik yöntemlerle hacim değerleri hesaplandı. Hesaplamalar Cavalieri Prensibi'ne uygun olarak Shtereom I programı kullanılarak yapıldı. İstatistiksel değerlendirmelerde non-parametrik testler ve Kruskal Wallis testi kullanıldı. Sonuçlar analiz edildiğinde *Hypericum Perforatum* L. yağının gavaj yoluyla verilmesinin ikinci derece yanık yarasının iyileşme sürecinde etki ettiği gözlemlendi.
Anahtar Kelimeler: Hacim, *Hypericum perforatum* L., Rat, Stereoloji, Yanık Yarası

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INTRODUCTION

The skin consists of three layers, which from the outermost to the deepest are: epidermis, dermis, and hypodermis. It constitutes 15% of the total body mass in adult individuals. The areas where the epidermis layer is thickest are the soles of the feet and the palms of the hands. The thinnest is the epidermis of the eyelids. The dorsal region is an area where the epidermis is thick. Additionally, the epidermis is thick in the hip and belly areas (Öztürk 1999; Olcer and Gonul 2002; Aksoy 2013).

The burn is tissue damage that occurs as a result of exposure of part or all of the body to various conditions such as heat, radiation, chemicals or electricity. Infection may develop in the early stages of the burn. It can protect the integrity of skin and subcutaneous tissue in burns up to 40°C. At temperatures of 45ºC and above, the integrity of the skin and subcutaneous tissues may be disrupted due to irreversible denature of cell proteins. Destruction of tissues is defined as burn. The degree of denaturation is proportional to the contact surface area, temperature, and exposure time of the material causing the damage (Değerli 2006; Kaymaz 2018).

Burn degrees are classified depending on the cause, depth and width of the injury. In burn cases, treatment begins by first determining the depth of the burn area and the percentage of tissue. In burn injuries, the length of time it takes for injuries to heal is related to the size of the wound (Hettiaratchy and Papini 2004). Additionally, the age of the patient is also a factor in the recovery period. Especially in the elderly, delays may occur in the inflammation phase of wound healing due to the slowing down of body metabolism. Disruptions in the degranulation of lymphocytes and macrophages are the most important factor. It has been reported that the decrease in the synthesis of collagen and the decrease in tissue epithelialization, which are among the delaying factors in wound healing and are related to the mechanical resistance of the tissue (Witte and Barbul 1997). Second and 3rd degree burn injuries and inhalation burns are the most difficult burn injuries to heal. (Theoret 2005). Immunological balance and wound healing phases are negatively affected by sepsis, shock or inflammation that may occur in the body (Nursal et al. 1999).

Plants of the genus *Hypericum*, also known as St. John's Wort, Hypericaceae, occurs naturally on almost all continent in the world except Antarctica. 469 species of this genus have been identified. These plants can be found in the form of shrubs, trees or herbs. They are found in different habitats, such as high mountains in tropical or temperate regions, avoiding hot, salty, and extremely dry regions (Crockett and Robson 2011). *Hypericum perforatum* L. has been known as a plant with medicinal properties for centuries. It is known that oil-containing *Hypericum perforatum* L. preparations are used externally in the treatment of minor burns, wounds and inflammatory conditions. Its internal use has been preferred in the treatment of depressive and anxiety diseases (Greeson et al. 2001).

The term stereology is derived from the Greek word "stereos", which means three-dimensional object (Akalan and Demirkan 2013). It can be defined as the branch of science that allows interpretation of the actual three-dimensional properties of the object by using data obtained from two-dimensional sections (Mayhew and Gundersen 1996). In other words, stereology is reported as a method that uses two-dimensional cross-sections of an object to obtain quantitative information about its geometric structure (Cruz-Orive 1993).

The aim of this study is to reveal the effects of *Hypericum perforatum* oil applied by gavage method on the skin of rats with second-degree burns, on skin healing, stereologically by volumetric value calculations. Thus, scientific contributions will be made to the fields of anatomy, histology, surgery, and stereology.

MATERIALS and METHODS

In order to conduct this study, the decision dated 25.11.2021 and numbered 2021/11-14 was obtained from Van Yuzuncu Yil University Animal Experiments Local Ethics Committee. This study was supported by Yuzuncu Yil University Scientific Research Projects Coordination Unit with the project number TYL-2022-10179. In this study, 18 healthy male albino rats with an average weight of 250-300 grams, obtained from Van Yuzuncu Yil University Experimental Medicine Application and Research Center. Animals were housed in individual cages that did not allow contact with each other, under standard conditions (22±1 ºC, 12-hour light/dark cycle) with unlimited water consumption and daily feed supply.

The study was conducted with 18 rats distributed in a control burn group, a burn group and a burn+treatment group, 6 animals in each group. In the control burn rats the damaged tissue was removed immediately after burning without any treatment. After the burn tissue boundaries were determined, it was extirpated with the help of a scalpel and forceps. On the first day of the study, animals in all groups were anesthetized intraperitoneally with a combination of 50 mg/kg ketamine and 15 mg/kg xylazine. The back region was preferred as the area where the burn wound would be created. Thus, the animal was prevented from scratching and licking the burn area (Kahkeshani

et al. 2013). The rats were placed in the position lying on their abdominal regions. The hair on the back areas was removed with the help of a razor as much as the area to be burned. The water was heated to 100°C.

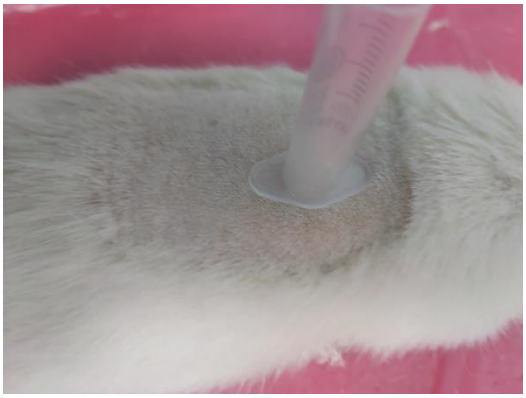

Figure 1. Stage of formation of burn wounds

From the first day of the study, 5mg/kg carprofen was administered subcutaneously to the burn+treatment and burn groups for analgesia for 7 days. Since the effect duration is 24 hours, the application was made every morning at 08.00. Rats in the burn+treatment group were given 1 ml of *Hypericum perforatum* L. oil by gavage through a tube placed in the esophagus every morning at 08:05 for 21 days.

The control group was sacrificed after the burn wound was created. For the sacrifice process, the rats were placed under general anesthesia. General anesthesia was achieved by intraperitoneal administration of 50 mg/kg ketalar and 15 mg/kg xylazine. After anesthesia rats were perfused. After perfusion, the rats were placed in 10% buffered formaldehyde and fixed. One week later, the skin areas where the burn wound was created were dissected. Tissue processing was applied to the dissected tissues from back region. Then, the tissues were blocked with paraffin. Sections were taken from the obtained blocks serially and parallelly, with a

Two independent areas with a diameter of 0.4 cm on the back of the rats were contacted with 100°C water during 20 seconds (Figure 1).

thickness of 5 µm, using sequential random sampling and the 1/75 stepping method. An average of 8-10 sections were obtained for each animal. The sections were stained with hematoxylin-eosin dye (Figure 2) The sections were examined by Olympus U-TV0, 5XC-3 (Tokyo, Japan). The resulting preparations were photographed under a microscope with a x4 objective The volume values of the burn wound areas were calculated using the Shtereom I program. This software uses stereological methods in a computer environment ensures its implementation. The program simultaneously records video of the microscope image. It is sent to the computer with the help of a camera and allows measurements to be made on it. Calculations can also be made on photographs transferred to the computer environment. Cavalieri's Principle was adhered to in the calculations by this program. After 21 days, the same procedures were repeated in the burn and burn+treatment groups. In order to strengthen the visual side of the work, shots with x10 and x40 objective were also obtained and included in the study.

Statistical Analysis

Four separate groups were created: "control and burn group", "control and burn+treatment group", "burn and burn+treatment group", "control, burn and burn+treatment group", and the Npar test was performed using Kruskal-Wallis and Mann-Whitney U test was applied. Statistical analyzes were completed in the study using SPSS version 24.0. The values were given in Table 1 , Table 2, Table 3, Table 4, Table 5, Table 6, Table 7.

Table 1. Non-parametric test results of burn, and burn+treatment groups.

Descriptive Statistics					
	Group Size	**Average**	**Standard Deviation**	**Minimum**	**Maximum**
Burn+Treatment	12	6328170833.500	2772592260.44741	3989541667.00	12805375000.00
Group	12	1.60	.516	1	2

Table 2. Mann-Whitney U test results of burn, and burn+treatment groups.

Rank				
	Group	**Group Size**	**Average Rank**	**Ranking Total**

Burn+Treatment	**Burn**	6	8.50	34.00
	Treatment	6	3.50	21.00
	Total	12		

Test Statistics	
	Burn+Treatment
Mann-Whitney U	.000
Wilcoxon W	21.000
Z-Score	-2.558
Approximate Significance (Bid-End)	.011

a. Grouping Variable: Groups1, b. Uncorrected for equations

Table 3. Non-parametric test results of control, and burn groups.

Descriptive Statistics					
	Group Size	**Average**	**Standard Deviation**	**Minimum**	**Maximum**
Control-Burn	12	7500750000.100	2044602671.469	5991916667.00	12805375000.00
Groups	12	1.40	.516	1	2

Table 4. Mann-Whitney U test results of control, and burn groups.

Rank				
	Groups	**Group Size**	**Average Rank**	**Ranking Total**
Control-Burn	**Control**	6	4.50	27.00
	Burn	6	7.00	28.00
	Total	12		

Test Statistics	
	Control-Burn
Mann-Whitney U	6.000
Wilcoxon W	27.000

Z-Score	-1.279
Approximate Significance (Bid-End)	.201
Definitive Analysis [2*(1-Undirected Analysis)]	.257b

a. Grouping Variable: Groups1, b. Uncorrected for equations

Table 5. Mann-Whitney U test results of control, and burn+treatment groups.

Rank				
	Groups 1	**Group Size**	**Average Size**	**Rank Total**
Control-Treatment	**Control**	6	9.50	57.00
	Treatment	6	3.50	21.00
	Total	12		

Test Statistics	
	Control-Treatment
Mann-Whitney U	.000
Wilcoxon W	21.000
Z-Score	-2.882
Approximate Significance (Bid-End)	.004
Definitive Analysis [2*(1-Undirected Analysis)]	.002b

a. Grouping Variable: Groups1, b. Uncorrected for equations

Table 6. Non-parametric test results of control, burn, and burn+treatment groups.

Descriptive Statistics					
	Group Size	**Average**	**Standart Deviation**	**Minimum**	**Maximum**
Anova	18	6424989583.4375	2163080871.10712	3989541667.00	12805375000.00
Groups 3	18	2.00	.894	1	3

Table 7. Kruskal-Wallis test results of control, burn, and burn+treatment groups.

Rank			
	Groups 3	**Group Size**	**Average Rank**
Anova	**Control**	6	10.50
	Burn	6	13.00
	Treatment	6	3.50
	Total	18	

Test Statistics A B	
	Anova
Chi-Square	11.250
Degrees of Freedom	2
Asymptotic Significance	.004
Asymptotıc Sıgnıficance	

A. Kruskal Wallis Test, B. Grouping Variable: Groups 3

RESULTS

Regional total volume values on the sections of control, burn, burn+treatment groups were obtained in cm^3 using stereological measurement methods. The values were given in Table 8.

According to Table 8 the burn area volume values in the control group were examined, it was observed that the highest volume value belonged to R1 with 0.3520 cm^3, while the lowest volume value was determined to be in R3 with 0.3050 cm^3. The mean burn area volume value in the control group was determined as 0.3168 cm^3. Additionally, according to Table 8 the highest CE value was found to be 0.041 in R3 and R4. The lowest CE value was observed at R1 as 0.038. The mean CE value of the control group was calculated to be 0.040. When the point numbers of the control group were examined, it was determined that the highest value was 704 and belonged to R1. It was determined that the lowest number of points belonged to R3 and R4 with 610. The mean number of points was calculated to be 633 (Table 8).

According to Table 8, it was observed that the highest volume value belonged to R1 with a value of 0.0644 cm^3, while the lowest volume value was determined in R4 with a value of 0.0358 cm^3. In addition, the average volume value of the burned area in the burn group was determined as 0.0503 cm^3. Considering Table 9 in terms of CE values, it was determined that the highest CE value belonged to R4 with 0.041, while the lowest CE value belonged to R1 with 0.034. The average CE value was found to be 0.036. When evaluated in terms of point number values, it was seen that the highest point number value of the burn area was 1052 and was detected in R1. It was also stated that the lowest point number value was 586 and was calculated in R4 (Table 8). The average number of points was found to be 822. When the calculations in Table 8 were evaluated in general, it was determined that the highest volume value belonged to R1, while the highest point number value was also calculated in R1. In addition to these values, the lowest CE value was also detected in R1. The fact that the volume and number of points values in R1 are parallel to each other and the CE value is low indicate a positive situation in terms of the reliability of the study. Because the number of points placed on the tissue whose volume will be determined and the volume value should be in an inverse proportional relationship with the CE value. In addition, when the CE and dot number values are examined in terms of

other rats, it is seen that among these three values, the dot number and volume values increase and the CE values decrease as these values increase, or vice versa. In other words, when the number of points and volume values decrease individually, the CE value also increases.

According to the values in Table 8, it was observed that the highest volume value belonged to R4 with 0.0317 cm^3, while the highest dot number value was determined to be 519 in R4. The average volume value was determined as 0.0272 cm^3. It was noted that the average CE value was 0.048 and the average number of points was calculated as 446. The highest CE value was detected in R2 as 0.056. In addition, the lowest volume value was determined to belong to R2 with 0.0214 cm^3, and in parallel with this, the lowest number of points was calculated in R2 with a figure of 351. It was determined that the lowest CE value belonged to R4 and R6, with 0.044. What is striking in Table 8 is that the volume and number of points values individually increased and decreased in parallel with each other. At the same time, it was determined that CE values decreased or increased inversely proportional to this increase or decrease. In Table 8, the average volume value was determined as 0.0272 cm^3. It was noted that the average CE value was 0.048 and the number of points was calculated as 446.

When the control, burn+treatment and burn groups were evaluated in terms of the average volume values of the dermis burn area, it was determined that the average volume value in the burn+treatment group decreased by approximately 45.92% compared to the burn group. This value was found by taking the difference between the average volume value of the burn group and the average volume value of the burn+treatment group and calculating that the percentage of the burn group was equal to this difference.

The coefficient of error (EC), obtained as a result of dividing the standard deviation to the arithmetic mean, was calculated separately for the burn area volume values of the control, burn and burn+treatment groups (Table 9). The results showed that this value was below 5%. This shows that the study is accurate both in terms of determining the number of animals and the number of sections to be taken, and also that it is reliable (Unal et al. 2002).

Table 8. Volume, CE and point number values of the burned skin area in the control, burn, burn+treatment groups

Burn Area Volumes (cm^3)/CE/Noise			
	VOLUME	**CE**	**NOISE**
	Control/Burn/Burn+treatment	**Control/Burn/Burn+treatment**	**Control/Burn/Burn+treatment**
R1	0.3520/0.0644/0.0292	0.038/0.034/0.047	704/1052/478
R2	0.3180/0.0466/0.0214	0.040/0.037/0.056	636/761/351
R3	0.3050/0.0477/0.0251	0.041/0.037/0.047	610/780/410
R4	0.3050/0.0358/0.0317	0.041/0.041/0.044	610/586/519
R5	0.3125/0.0576/0.0252	0.040/0.035/0.049	625/945/413
R6	0.3085/0.0497/0.0311	0.040/0.036/0.044	617/810/509
Average Value	0.3168/0.0503/0.0272	0.040/0.036/0.048	633/822/446

R: Rat, CE: Coefficient of Error, Noise: Number of points.

Table 9. Average values of coefficient of error (CE) of the volumes of the burn dermis regions of the groups.

Burn Area Average Values of Coefficient of Error		
Control	**Burn**	**Burn+Treatment**
0.040	0.036	0.048

DISCUSSION

Today, a wide variety of agents are used in wound treatment. Topical products widely used in burn wounds all over the world and in our country contain antimicrobial, antibiotic, and antiseptic substances.

Recently, the use of plant extract creams that increase epithelial formation and have antioxidant properties has been increasing rapidly (Unal 2006; Mehrabani et al. 2015; Afshar 2016).

In a study conducted by Soykan (2020), it was aimed to compare the changes in the wound area after creating a burn wound in rats and treating it with amniotic fluid, myrrh tree extract, and silver sulfadiazine, using pathological analysis and autocad. As a result of the data obtained, it was revealed that myrrh tree extract supports wound healing. Although there are similarities in this thesis study conducted by Soykan (2020), there are differences in terms of rat breed. In addition, in the study conducted by Soykan (2020), a total of four burn wounds were created in two different regions, while in this study, two separate burn wounds were created only in the back region for redundancy. Choosing the back area is to ensure that the animal can not reach that area and to prevent irritation. In this study, which was conducted to heal the burn wound, no application was made to heal the wound on its own in the group where the burn was created and left to heal on its own. Both studies are similar in terms of grouping in the study. However, the effects of different extracts were investigated in both studies used and the studies differ in this respect. In addition, in the study conducted by Soykan (2020), area calculation was made with the autocad program. However, in this thesis study, volume calculations were made, the results of which were closer to reality, and the Cavalieri's Principle was used by using the Shtereom I program. The part where the two studies overlap is that plants that are predicted to affect the healing process of burn wounds were used. According to the data obtained, the effect of myrrh tree on healing was calculated as 22%, while the effect of *Hypericum perforatum* L. oil on healing was measured as 45%. Kiyan et al. (2015) aimed to investigate the treatment of *Hypericum perforatum* L. (St. John's Wort) in thermal burns and compare it with silver sulfadiazine treatment. For this purpose, thirty-five Wistar-albino type rats were randomly selected and divided into five groups, with seven rats in each group. A second-degree thermal burn was created on the back of the rats by exposing them to 100 °C boiling water for 10 seconds in an area of 4x4 cm. All groups were irrigated with 50 cc saline solution for three minutes. No treatment was applied to the control group. Only irrigation was applied to the burn control group. Silver sulfadiazine was applied twice daily to the topical silver sulfadiazine group. *Hypericum perforatum* was applied to the topical *Hypericum perforatum* group four times a day, every six hours. Another topical substance used in the preparation of *Hypericum perforatum* was applied to the treatment group with ajan-gel, four times a day, every six hours. Tissues taken by biopsy were cut into 5µm-thick sections on a microtome, examined under a light microscope, and stained with hematoxylin-eosin in accordance with standard procedures. Biopsies taken from the groups were evaluated under a light microscope. Results were determined using the Verhofstad Histopathological Scoring system. The results obtained from this scoring were compared. It was then scored by taking the arithmetic average of the scores given. In addition, skin wound healing and epidermis thickness, inflammatory cell infiltration (neutrophils, monocytes, and macrophages), degenerative and total hair follicle number were also included in the histopathological evaluation. As a result, in the *Hypericum perforatum* treatment group, the collagen discoloration was localized in the lower part of the epidermal layer and did not progress to the depth of the dermis in the *Hypericum perforatum* treatment group compared to the other groups. It was determined that the group was protected according to the control group and was structurally closest to the control group. Epidermal thickness and number of vessels in the *Hypericum perforatum* group were found to be statistically significantly higher than the other groups ($p<0.05$). The number of degenerative hair follicles in the *Hypericum perforatum* group was found to be significantly less than the other groups ($p<0.05$). It was also determined that the total number of hair follicles increased significantly at the twenty-fourth hour ($p<0.05$). Kiyan et al. (2015) differs from our study in that *Hypericum perforatum* was applied topically and was compared with different substances. In this study, application was made using the gavage method. Instead, the volumetric effect of *Hypericum perforatum* oil on burn wound healing was examined. Kiyan et al. (2015) the experimental period is limited to 24 hours. In our study, the application period of both studies differs as the application was carried out for 21 days. Kiyan et al. (2015) is based on histological evaluations. However, this study is based on determining the healing rate of the tissue volumetrically using stereological methods.

In a study conducted by Çelikkol (2015), the extract obtained by soaking the dried flowers and leaves of the plant *Hypericum perforatum* L. in olive oil caused experimental wounds in rats. *Hypericum perforatum* plant, was applied topically to the right one of these defects. Any application was made to the defects on the left. The groups were sacrificed on the fourth, seventh, fourteenth, and twenty-first days. At the end of the experiment, the effect of St. John's wort oil on wound healing was determined. According to the results of histopathological findings and immunohistochemical evaluations although a significant increase was observed in parameters affecting wound healing such as Fibroblast Growth Factor (FBF), Vascular

Endothelial Growth Factor (VEBF), and Epidermal Growth Factor (EBF), no significant difference was found in the degree of epithelialization, polymorphonuclear leucocytes (PMNLs) infiltration and the amount of macrophages. The effect of St. John's wort oil on the healing of the wound surface area was found to be significant on the fourth and seventh days in soft tissue defects compared to the non-applied groups. The study conducted by Çelikkol (2015) is similar to our study in terms of using animals of the same breed. Additionally, in the study conducted by Çelikkol (2015), the last time the study was terminated was the same as the last time the study was terminated. However, Çelikkol (2015) also determined groups to terminate the study on the 4th, 7th, and 14th days. However, this study was terminated on the 21st day. Additionally, both studies differ in terms of the way the extract is applied. Çelikkol (2015) found topical application of the extract appropriate in his study. In fact, this situation distances the study from systemic side effects. But it can prolong the duration of effect. In this study, the gavage method was used to achieve the most effective results in the shortest time. Both studies differ in this respect. In the aforementioned study, histopathological and immunohistochemical results were obtained. However, in this study, the results were evaluated through stereology and it was aimed to obtain realistic results. The fact that macrophage counting was performed makes Çelikkol's (2015) study valuable. Cell count was not preferred in this study, and it was thought that cell count could be evaluated by including it in the study in future studies.

The purpose of the study on burns by Kotsiou and Tesseromatis (2020) was to investigate whether *Hypericum perforatum* plant extract is effective in the treatment of burn wounds. In the study, 32 male Wistar rats were used and these rats were divided into 4 different groups. While burn wounds in the first group were treated with *Hypericum perforatum* plant extract, animals in the second group were treated with nitrofurazone cream. In the third group, only a burn wound was created and no treatment was applied. In the fourth group, a burn wound was created and treated with placebo cream. In histological evaluation, staining was done with haematoxylin and eosin. It was found that the collagen coloration of the *Hypericum perforatum* treatment group was localized in the lower part of the epidermal layer and did not go deeper into the dermis compared to other groups, and the epidermis, hair follicles and sebaceous glands were preserved. It was determined that the epidermal thickness and number of vessels in the group applied Nitrofurazone Ointment USP (0.2%) every 12 hours and the *Hypericum perforatum* group were significantly higher than the other groups ($p<0.05$). The number of degenerated hair follicles in the *Hypericum perforatum* applied group was found to be significantly lower than the other groups ($p<0.05$). It was determined that the total number of hair follicles increased significantly compared to the control group ($p<0.05$) and this number did not differ ($p>0.05$). The results showed that *Hypericum perforatum* plant extract had similar effects to nitrofurazone cream in healing burn wounds. The burn wounds of rats treated with both *Hypericum perforatum* plant extract and nitrofurazone cream healed faster than those in the placebo group. There was no statistical difference in wound healing times between groups treated with *Hypericum perforatum* plant extract and nitrofurazone cream. Both studies are similar in that the study is related to animal selection, material used, and wound healing. The use of *Hypericum perforatum* L. oil makes both studies similar. However, it differs in that Hypericin cream is applied four times a day. Additionally, in this study, the last time the study was terminated differs from the time our study was completed. In this study, topical application of the extract and application of Hypericin as a cream were found to be appropriate. In this study, the gavage method was used to achieve the most effective results in the shortest time. Both studies differ in this respect. Kotsiou and Tesseromatis (2020) reached histopathological and immunohistochemical results in their study. However, in this study, the results were evaluated through stereology and it was aimed to obtain realistic results. The prediction of metalloproteinases and the examination of plasma MMP-8 and -9 make the study of Kotsiou and Tesseromatis (2020) valuable. ELISA was not preferred in this study, which led to the idea that this method could also be taken into consideration in future studies.

In a study conducted by Seyhan (2020); rats were divided into 3 equal groups of 8 animals each. These are Group A as the control group, Group B as the curcumin treatment group, and Group C as the *Hypericum perforatum* application group. A second-degree deep burn wound was created using a 2x2 cm diameter iron heating plate heated in boiling water for 5 minutes and placed on the skin by applying pressure for 20 seconds. Treatment was started 24 hours after the burn injury. Curcumin oil (2 cc) was applied to Group B and *Hypericum perforatum* oil was applied topically to Group C once a day for 20 days. Group A was considered as the control group and no drug treatment was applied. At the end of the experiment (day 21), all rats were sacrificed with an overdose of anesthetic and the burnt surface areas were removed for histopathological examinations. Tissues were embedded in paraffin wax and sections were cut at 5 µm thickness and stained with hematoxylin and eosin. At the end of the study, histological parameters, epithelialization, granulation tissue formation, inflammation and angiogenesis were evaluated in the

biopsy samples taken from the wound. Histological scores were made from 20 random fields per section from each sample at ×40 magnification. In Seyhan's (2020) study, second degree burn wounds were created with hot metal plates. In this study, boiled water at 100 ºC was preferred to create a second degree burn wound. There are method differences in both studies in terms of creating a burn wound. In addition, in the study conducted by Seyhan (2020), the last time the study was terminated is the same as the last time the study was terminated. The two studies are not similar in terms of the way the extract is applied. Seyhan (2020) found topical application of the extract appropriate in his study. In fact, this distances the study from systemic side effects. But it can prolong the duration of effect. In this study, the gavage method was used to achieve the most effective results in the shortest time. Both studies differ in this respect. In the aforementioned study, histological results were obtained. However, in this study, the results were evaluated through stereology and it was aimed to obtain realistic results.

CONCLUSION

Volumetric data have been obtained showing that the animals in which *Hypericum perforatum* oil was used supported faster recovery than the animals in which it was not used. As a result of this study, it was determined that there was a 45.92% difference in volume value between the treated group and the group without any treatment. We are of the opinion that this study may also lead to other studies investigating different histopathological parameters such as inflammatory cell infiltration (neutrophils, monocytes, and macrophages), degenerative and total hair follicle number, to be carried out at different durations and different burn degrees, and can be considered as a reference study.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: GÇ and AŞC contributed to the project idea, design and execution of the study. GÇ and AŞC contributed to the acquisition of data. GÇ analysed the data. GÇ and AŞC drafted and wrote the manuscript. GÇ reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Van Yuzuncu Yil University Research Animals Application Center. This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine, Van Yuzuncu Yil University (YUHADYEK, Ref No: 2021/11-14, Date: 25.11.2021).

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Explanation: This study was summarized from master's thesis of entitled "Stereological Investigation of the Effects of *Hypericum Perforatum* L. Plant on Skin Tissue in Second Degree Burns in Rats".

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RESEARCH ARTICLE

Protective Effect of Chlorogenic Acid against Testicular Damage Induced by Glyphosate Isopropilamine in Rats

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ABSTRACT

The aim of this subchronic toxicity study was to determine the prophylactic effect of chlorogenic acid (CGA) on the histopathologic-histologic changes and oxidative stress induced by Glyphosate isopropylamine (GLF-ISO) in testicular tissues. A sum of 42 male Wistar rats were divided into six equal groups, each containing 7 rats. For pretreatment, rats were given CGA at doses of 12.5, 25 and 50 mg/kg (po) and GLF-ISO at 787.85 mg/kg (po) for 7 weeks. GLF-ISO significantly increased malondialdehyde levels while decreasing SOD and CAT activities and GSH levels in testicular tissues. On the contrary, these parameters were improved in CGA-treated groups. Furthermore, CGA ameliorated the histopathological and histological changes in testicular tissues induced by GLF-ISO in a dose-dependent manner. The results indicate that testicular damage caused by GLF-ISO can potentially be prevented or managed by CGA.

Keywords: Glyphosate isopropilamine, Chlorogenic acid, Oxidative stress, Testicular damage

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Sıçanlarda Glifosat İzopropilamin ile Oluşturulan Testiküler Hasara Karşı Klorojenik Asidin Koruyucu Etkisi

ÖZ

Bu subkronik toksisite çalışmasının amacı, testis dokularında Glifosat izopropilamin (GLF-ISO) tarafından indüklenen histopatolojik-histolojik değişiklikler ve oksidatif stres üzerinde klorojenik asidin (CGA) profilaktik etkisini belirlemektir. Toplam 42 erkek Wistar sıçan, her biri 7 sıçan içeren altı eşit gruba ayrılmıştır. Ön muamele için sıçanlara 7 hafta boyunca 12.5, 25 ve 50 mg/kg (po) dozlarında CGA ve 787.85 mg/kg (po) dozunda GLF-ISO verilmiştir. GLF-ISO testis dokularında malondialdehit seviyelerini önemli ölçüde artırırken SOD ve CAT aktivitelerini ve GSH seviyelerini azaltmıştır. Aksine, bu parametreler CGA ile tedavi edilen gruplarda iyileşmiştir. Ayrıca, CGA, GLF-ISO tarafından indüklenen testis dokularındaki histopatolojik ve histolojik değişiklikleri doza bağlı bir şekilde iyileştirmiştir. Sonuçlar, GLF-ISO'nun neden olduğu testis hasarının CGA ile potansiyel olarak önlenebileceğini veya yönetilebileceğini göstermektedir.

Anahtar Kelimeler: Glyphosate İzopropilamin, Klorojenik asit, Oksidatif stres, Testiküler hasar

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INTRODUCTION

Glyphosate isopropylamine (GLF-ISO) is one of the most commonly used formulations of glyphosate, a broad-spectrum herbicide widely employed in agriculture, forestry, industrial weed control, lawns, gardens, and aquatic environments (Hanson 2024, NPIC 2024). Glyphosate itself is an acid molecule and is typically formulated as various salts to facilitate packaging and handling (Wikipedia 2024). Among these salts, the isopropylamine salt is most frequently utilized in commercial herbicidal products, including those produced by Monsanto (NPIC 2024, Wikipedia 2024). This formulation is known for its very low toxicity to rats, with an acute oral LD50 greater than 5000 mg/kg, indicating minimal risk of acute poisoning under normal usage conditions (Turkmen and Dogan 2020). Given its widespread use and low acute toxicity, GLF-ISO remains a critical tool in modern agricultural practices, although its long-term health impacts continue to be a subject of scientific investigation. A recent study (Avdatek et al. 2023) found that long-term exposure to GLF-ISO led to increased oxidative stress, decreased spermatologic parameters and decreased testosterone levels in rat testicular tissue. Previous studies have found that GLF-ISO causes adverse effects on the male reproductive system. Studies have suggested that glyphosate can induce oxidative stress and disrupt endocrine functions, leading to testicular damage. Research indicates that exposure to glyphosate increases the levels of oxidative stress markers and affects sperm integrity, which are critical indicators of reproductive health (Clair et al. 2012, Dai et al. 2016).
Reactive oxygen species (ROS) are vital in many areas of male reproductive function, including spermatozoa's ability to fertilize. However, an increase in ROS formation, caused by internal and external stimuli, can lead to oxidative stress, altering the structure and function of phospholipids and proteins. ROS assault DNA in the nucleus, fragmenting it and activating apoptosis, hence changing gene and protein expression (Juárez-Rojas et al. 2022). Research using animal models to prevent and restore male germinal tissue and function, especially against the oxidative stress effects of herbicides containing glyphosate, has focused on antioxidant compound as a possible technique for preserving male fertility (Avdatek et al. 2023, Avdatek et al. 2018, Hashim et al. 2022, Spadella et al. 2024). According to a recent study, chlorogenic acid (CGA) has the potential to be an important candidate to mitigate GLF-ISO-induced damage in various organs, including the testis and prostate (Jia et al. 2024).
Chlorogenic acids (CGAs) are a family of polyphenolic compounds predominantly found in coffee beans, particularly in green coffee extracts. They have been recognized for their various health benefits, which include antioxidant and anti-inflammatory activities (Tajik et al. 2017). In various in vitro and in vivo studies, CGAs have shown protective effects against oxidative stress, which is a key mechanism involved in many pathological conditions, including testicular damage. CGAs help mitigate oxidative damage by neutralizing free radicals and enhancing the activity of antioxidant enzymes like glutathione peroxidase (Uz et al. 2002).
However, there are no studies on the role of CGA in GLF-ISO-induced testicular damage. In this study, the protective effect of CGA on testicular damage induced by GLF-ISO via gavage in rats was investigated for the first time.

MATERIAL and METHODS

Chemicals

Knock-out, a glyphosate formulation marketed in Turkey by Hektas, was utilized in this investigation. 48% isopropylamine salt is present in the liquid-water soluble formulation of this active ingredient as inert substances and excipients. The selection of this glyphosate-based herbicide was based on the fact that it is one of the most popular herbicides for controlling weeds in Turkey, where it has been shown that eradication is challenging for formulations with high glyphosate levels. CGA (Cat no. C3878, purity ≥ 95%) was purchased from Sigma (Sigma–Aldrich, Shanghai, China).

Ethical statement, Animals and Experimental design

This study's Animal Experiments Local Ethics Committee permission was obtained (49533702-241). Forty-two male Wistar Albino rats with an average age of 2-3 months and weighing 180-200 g were obtained from Afyon Kocatepe University Experimental Animal Research and Application Center. Following one week of adaptation, randomized into six groups (n = 7), the animals were maintained in a controlled environment (22 °C, 12 h light-dark cycle) with free food and water.

Control group: Rats were given 0.5 mL distilled water solution (as chlorogenic acid solvent) per-orally (p.o.) for 49 days.
CGA50: Rats were given CGA (50 mg/kg) dissolved in 0.5 mL distilled water via p.o. route for 49 days.
GLF-ISO group: Rats were given GLF-ISO (LD_{50}/10, 787.85 mg/kg) dissolved in 0.5 mL distilled water via p.o. route for 49 days.
CGA12.5+GLF-ISO: CGA (12.5 mg/kg) was administered via p.o. one hour before GLF-ISO (800 mg/kg) administration and continued for 49 days.
CGA25+GLF-ISO: CGA (25 mg/kg) was administered via p.o. one hour before GLF-ISO (800 mg/kg) administration and continued for 49 days.

CGA50+GLF-ISO: CGA (50 mg/kg) was administered via p.o. one hour before GLF-ISO (800 mg/kg) administration and continued for 49 days.
The dose of GLF-ISO given to the animals was determined using the study by Turkmen and Dogan (2020), and the dose of CGA was determined using the study by Qi et al. (2011).

Sample Collection and Homogenate Preparation
One day after the last drug administration, rats were sacrificed under mild sevoflurane anesthesia. Testicular tissues of sacrificed rats were collected. Right testes were fixed in 10% neutral buffered formalin for histopathologic examination, while the left testes were stored in deep freezer at -20 for tissue biochemical analysis. Testes were taken out of the freezer at -20°C and put straight into the glass tubes to cool. After that, nine times as much phosphate-buffered saline (PBS; pH 7.4) was added to the testes to dilute them. Testes were minced in a glass and homogenized for three minutes in cold physiological saline on ice using a Teflon–glass homogenizer in preparation for the biochemical analyses (Türk et al. 2011).

Measurements of the oxidant-Antioxidant Balance
The method outlined by Ohkawa et al. in (1979) was utilized to quantify malondialdehyde (MDA), while the method reported by Beutler et al. (1963) was employed to assess the concentration of GSH in the tissue homogenates. The techniques outlined by Sun et al. (1988) and Aebi (1984) were utilized to assess the activity of the SOD and CAT antioxidant enzymes in the tissue samples. The researchers utilized the colorimetric technique outlined by Lowry et al. (1951) to quantify the protein concentration in the tissue. The spectrophotometric measurements were conducted using a Shimadzu 1601 UV–VIS spectrophotometer from Tokyo, Japan.

Histopathological evaluations
Testicular specimens from euthanized rats were fixed in 10% neutral buffered formalin for 24 hours, then kept in 80% ethyl alcohol overnight and paraffin blocks were prepared after routine procedures. The 5 micron thick sections taken from the paraffin blocks were stained with the Hematoxylin Eosin method (Luna 1968) and examined under a light microscope and the lesions were recorded. The measurements were performed under X20 objective. Testis lesions were graded semi-quantitatively according to the classifications of Gibson-Corley (2013) as -: no lesion, +: mild, ++: moderate, and +++: severe.

Histomorphometry
Paraffin sections were stained using Periodic Acid Schiff Reagent (PAS) staining method (Culling et al. 1985) to measure the seminiferous tubule diameters (STDs) and seminiferous epithelial heights (SEHs). Four sections were used from each rat. The rat spermatogenesis cycle comprises 14 stages (Hess 1990, Leblond and Clermont 1952); we observed stages VII−VIII of spermatogenesis. Ten round or nearly round stage VII−VIII tubules were selected randomly for each section (Ahbab et al. 2017). The STDs and SEHs were measured in four sections (approximately 40 STDs and SEHs/animal) using an image analysis program (Leica Q-Win Standard, Q-Win Plus 3.5 software, Leica Cambridge Ltd., Cambridge, UK). The measurements were taken at four different regions of each seminiferous tubule in the section.

Statistical evaluation
The SPSS (Version 22.0) statistical program was used for statistical analysis. Data were presented as mean ± standard deviation. In order to evaluate the data, a normality test was applied first. One-way analysis of variance (ANOVA) and the Duncan test for pairwise comparisons were used to determine the differences between groups for data showing normal values. The stage VII−VIII STDs and SEHs were analyzed using Kruskal-Wallis one-way analysis of variance and a post hoc multiple comparison test, the Mann-Whitney U test with Bonferroni correction. In all analyses, a p value less than 0.05 was considered statistically significant.

RESULTS

Effects of CGA on GLF-ISO-induced lipid peroxidation and antioxidant status
The effects of CGA, GLF-ISO, and their combination on testis oxidative stress and antioxidant parameters are presented in Table 1. GLF-ISO significantly increased the levels of MDA in the testis compared to the controls ($p<0.001$). The MDA levels reduced dose-dependently in the testis of rats, which respectively received 12.5, 25 and 50 mg/kg of CGA ($p<0.001$). Also, the oxidant indice did not significantly change due to CGA administration, compared to the control group ($p>0.05$).
Compared to the controls, GSH content, as well as SOD, and CAT activities, significantly reduced in the GLF-ISO group ($p<0.001$). On the other hand, CGA pretreatment at 12.5, 25 and 50 mg/kg for 49 consecutive days caused a dose-dependent increase in the GSH content, besides SOD and CAT activities, compared to the GLF-ISO group ($p<0.001$). Moreover, oxidative stress parameters did not change in normal rats after CGA administration, compared to the controls ($p>0.05$).

Table 1. Effects of glyphosate-isopropilamine(GLF-ISO; 787.85 mg/kg, 10% of the LD_{50}) and three different doses of chlorogenic acid (CGA; 12.5, 25, and 50 mg/kg) on levels of malondialdehyde (MDA) and glutathione (GSH) and activities of superoxide dismutase (SOD) and catalase (CAT) in testis tissue of rats homogenates

Groups	MDA (nmol/g tissue)	GSH (nmol/g tissue)	SOD (U/µg protein)	CAT (k/µg protein)
Control	0.99±0.35^{c}	7.35±0.72^{a}	2.12±0.56^{a}	4.15±0.98^{a}
Chloro50	0.91±0.28^{c}	7.66±0.69^{a}	2.28±0.59^{a}	4.04±0.91^{a}
GLF-ISO	7.26±2.10^{a}	3.54±0.36^{c}	0.89±0.25^{c}	0.94±0.21^{d}
Chloro12.5+GLF-ISO	4.72±2.00^{b}	6.01±0.58^{b}	1.02±0.33^{bc}	1.04±0.41^{d}
Chloro25+GLF-ISO	1.99±0.73^{c}	6.50±0.63^{ab}	1.70±0.48^{b}	1.62±0.35^{c}
Chloro50+GLF-ISO	1.56±0.91^{c}	7.12±0.76^{a}	2.05±0.54^{a}	2.84±0.67^{c}

Note: Mean ± standard deviation; $n = 7$; Values with different letters (a, b, c, d) in the same column are statistically significant ($p < 0.05$)

Effects of CGA on GLF-ISO-Induced Histopathological Changes

Histopathological changes in the testicular tissues of the animals in the experimental groups were described in detail and shown in Figure 1 and Table 2. In the GLF-ISO group, irregular basement membranes, vacuolization and hyalinization in the interstitial area were observed in the seminiferous tubules (Figure 1-A3; Table 2). Few histopathologic changes were observed in GLF-ISO groups (Figure 1-A4-A5-A6; Table 2). Normal tissue was observed in the control group (Figure 1-A1; Table 2) and in the CGA50 group (Figure 1-A2; Table 2).

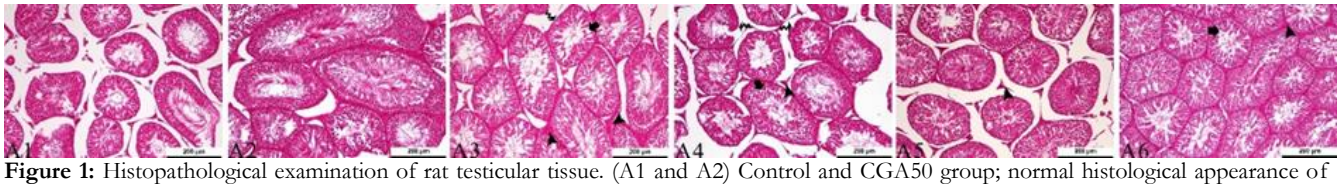

Figure 1: Histopathological examination of rat testicular tissue. (A1 and A2) Control and CGA50 group; normal histological appearance of testicular tissue. (A3) GLF-ISO group; Irregular basement membrane (curved arrow), Vacuolation in the seminiferous tubule (thick arrow) and Hyalinization in the interstitial area (arrowhead). (A4) Chloro12. 5+GLF-ISO group; Irregular basement membrane (curved arrow), Vacuolation in the seminiferous tubule (thick arrow) and Hyalinization in the interstitial area (arrowhead). (A5) Chloro25+GLF-ISO group; Hyalinization in the interstitial area (arrowhead). (A5) (A6) Chloro50+GLF-ISO group; Vacuolation in the seminiferous tubule (thick arrow) and Hyalinization in the interstitial area (arrowhead). All figures were stained with H&E. Original magnifications of 20x and 100 µm were used.

Tablo 2. Semi-quantitative histopathological scoring of testis tissue

Groups	Vacuolation in the seminiferous tubule	Hyalinization in the interstitial area	Irregular basement membrane in the seminiferous tubule
Control	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{c}
Chloro50	0.00±0.00^{b}	0,00±0.00^{b}	0.00±0.00^{c}
GLF	2.10±0.89^{a}	1.77±0.52^{a}	1.43±0.52^{a}
Chloro12.5+GLF-ISO	1.60±0.54^{a}	0,70±1.08^{a}	0.72±0.87^{b}
Chloro25+GLF-ISO	0.55±0.60^{b}	0,53±0.88^{a}	0.55±0.60^{bc}
Chloro50+GLF-ISO	0.36±0.57^{b}	0.18±0.45^{a}	0.36±0.57^{bc}
p	0.000	0.000	0.001

Note: Mean ± standard deviation; $n = 7$; Values with different letters (a, b, c) in the same column are statistically significant ($p < 0.05$)

Effects of CGA on GLF-ISO-Induced Histomorphometric Changes

Histomorphometric changes in the testicular tissues of the animals in the experimental groups were described in detail and shown in Table 3. In the GLF-ISO group, the STDs at stages VII−VIII were significantly reduced compared to the other groups ($p < 0.001$). The STDs in the CGA12.5+GLF-ISO group were significantly lower than the control group, but significantly higher than the GLF-ISO group. Moreover, there was no significant in the STDs between the CGA25+GLF-ISO and CGAKA50+GLF-ISO groups. The STDs in the CGA25+GLF-ISO and CGA50+GLF-ISO groups were significantly increased compared to the GLF-ISO group. There was no significant in the SEHs at stages VII−VIII between the control and GLF-ISO groups. On the other hand, the SEHs were similar between the CGA25+GLF-ISO and CGA50+GLF-ISO groups.

Table 3. Histomorphometric analysis of the testis in the experimental groups.

Group	n	STDs (µm) $(\bar{X} \pm S_{\bar{X}})$	SEHs (µm) $(\bar{X} \pm S_{\bar{X}})$
Control	7	199,12±0,80[c]	45,53±0,21[c]
CGA50	7	220,56±0,76[a]	55,72±0,22[ab]
GLF-ISO	7	179,99±0,68[e]	45,70±0,20[e]
CGA12.5+GLF-ISO	7	186,59±0,58[d]	50,52±0,14[d]
CGA25+GLF-ISO	7	198,49±0,70[bc]	51,53±0,10[c]
CGA50+GLF-ISO	7	202,72±0,8[b]	56,27±0,22[ac]
p		***	***

[a,b,c,d,e]Means within each grouping with different letter designations differ significantly.
CGA: Chlorogenic acid, GLF-ISO: Glyphosate isopropylamine, STDs: Seminiferous Tubule Diameters, SEH: Seminiferous Epithelium Heights, n: No of rats, $\bar{x}$: Mean, $S_{\bar{x}}$: Standard Error of Mean (SEM).
***: p<0.001.

DISCUSSION

Chronic exposure to glyphosate and glyphosate-based herbicides (GBHs) has been a subject of increasing scrutiny due to potential adverse effects on male reproductive health. Studies have demonstrated that exposure to glyphosate can affect various aspects of the testicular function and spermatogenesis (Liu et al. 2022, Owagboriaye et al. 2017). In this study, we tried to investigate the protective effect of different doses of CGA against testicular damage due to long-term exposure to GLF-ISO in terms of oxidative stress, histological and histopathological aspects.

Oxidative stress is the critical factor in the effect of glyphosate on testicular cells. Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify these reactive intermediates or repair the resulting damage. Several studies have highlighted the role of oxidative stress in male infertility and testicular dysfunction (Pavuluri et al. 2024). For instance, Sharma et al. (2013) noted that oxidative stress is a significant factor in male infertility, with high levels of ROS leading to lipid peroxidation and DNA damage in sperm cells. Moreover, glyphosate (GLF) exposure has been shown to significantly elevate levels of serum MDA and testicular ROS, while decreasing the activity of critical antioxidant enzymes such as CAT, and SOD (Bhardwaj et al. 2022). Such changes are indicative of oxidative damage and have been implicated in the disruption of testicular function. Recent research has highlighted the potential therapeutic role of antioxidants in ameliorating oxidative stress-induced testicular damage. For example, administration of antioxidants such as N-acetylcysteine (NAC) has been shown to reverse oxidative damage in GLF-treated testicular tissues by decreasing lipid peroxidation and enhancing the activity of antioxidant enzymes (Hashim et al. 2022). To counteract the oxidative damage induced by glyphosate, antioxidants have been suggested as potential mitigators. Studies have demonstrated the ameliorative effects of compounds like resveratrol and proanthocyanidin on testicular oxidative stress and DNA damage in rats exposed to GBHs (Avdatek et al. 2023, Avdatek et al. 2018). Similar to these studies, in our study, it was observed that MDA levels in testicular tissue were high, GSH levels and SOD and CAT activities were decreased in GLF-ISO-treated rats. The high MDA levels and low GSH levels, SOD and CAT activities in the GLF-ISO group may be related with the depletion of these enzymes due to the increase in oxidative stress. Compared to GLF-ISO group, MDA levels decreased dose-dependently in GLF-ISO groups treated with CGA and GSH levels, SOD and CAT activities increased significantly in testicular tissue. This suggests that CGA may have the ability to maintain and renew the activity of these enzymes.

Histopathologic and histologic analyses are crucial endpoints for assessing testicular toxicity, providing detailed information on structural and functional changes in the male reproductive system. In the context of glyphosate exposure, evaluation of testicular histopathology reveals important alterations that may affect spermatogenesis and overall testicular health. According to histopathologic examination and quantitative evaluation, GLF-ISO caused significant changes in the testis. Histopathologic and histologic analyses are crucial endpoints for assessing testicular toxicity, providing detailed information on structural and functional changes in the male reproductive system. In the context of glyphosate exposure, assessment of testicular histopathology reveals important changes that may affect spermatogenesis and overall testicular health. According to histopathologic examination and quantitative assessment, GLF-ISO caused significant changes in the testis. In the GLF-ISO group, irregular basement membrane and vacuolization formations in the testicular seminiferous tubule and hyalinization in the interstitial area were observed compared to the control and CGA50 groups. It was concluded that these histopathological disorders decreased in a dose-dependent manner with CGA administration. Avdatek et al. (2018) reported a decrease in sperm concentration and degeneration of Sertoli cells in the testis after administration of Knockdown 48 SL, a

commercial brand of GIS-ISO, to rats at a dose of 375 mg/kg for 8 weeks. In the same study, the authors concluded that resveratrol given as a protective agent ameliorated the histopathological changes caused by GIS-ISO. Nardi et al. (2017) showed that GLF treatment in rats decreased STDs. Similarly, we found that GLF-ISO administration reduced significantly STDs at stages VII−VIII. By contrast, the CGA12.5, CGA25, and CGA50+GLF-ISO treatments alleviated the adverse effects of GLF-ISO on STDs at stages VII−VIII of spermatogenesis. However, we found no difference in SEHs at stages VII−VIII among control and GLF-ISO groups. This could be due to the dose level and the method treatment of GLF-ISO.

CONCLUSION

In this study, CGA was shown to improve oxidative stress parameters and reduce histopathological damage in rats exposed to GLF-ISO-induced testicular damage in a dose-dependent manner, suggesting its potential as a protective agent against oxidative stress in reproductive organs. Further research is required to explore the long-term efficacy of these interventions and their applicability to human populations exposed to glyphosate.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: RT and YOB contributed to the project idea, design and execution of the study. OA, HHD, TT and OG contributed to the acquisition of data. RT, OA, HHD and TT analysed the data. RT and OG drafted and wrote the manuscript. YOB, OA, TT and OG reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Afyon Kocatepe University Reserch Animals Application Center. This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine, Afyon Kocatepe University (AKUHADYEK, Ref No: 49533702/241)

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RESEARCH ARTICLE

Investigation of cTn I, CK-MB, Myoglobin and D-Dimer Levels at Anemic Dogs Infected with *Ehrlichiosis*

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ABSTRACT

Ehrlichiosis is a vector-borne disease that affects humans and animals. Multiple tissues and organs are fascinating, and *Ehrlichiosis* can cause multiple organ failure. In one's way the known clinical findings and pathogenesis, it is not clear how *Ehrlichia* is effective and important on myocardial damage and disseminate intravascular coagulation (DIC) profile. Anemia is seen as one of the common clinical conditions in *Ehrlichiosis*. In this study, it was determined to state the existence of myocardial damage and thromboembolic condition at *Ehrlichiosis* with the severity of anemia. The animal material of this study consisted of 24 mono-infected, 9 co-infected, and 10 healthy animals. Additionally, animals were separated into mono, co-infected; with anemia profile non-anemic, mild, moderate, and severe anemic, and according to serologic and molecular results; acute infected, ınfected, and active ınfected. CK-MB, cTnI, and myoglobin levels were calculated in all groups to identify myocardial damage. Accordingly, D-Dimer concentrations were determined to set down the potential of the DIC profile. As a result of the data, D-Dimer levels significantly increased in mono, co-infected, mild, moderate, severe, and active infected animals ($p<0.05$). Significant statistical difference was seen in cTnI levels in mono, mild, moderate, and active infected groups ($p<0.05$). There were no significant statistical differences between CK-MB and myoglobin levels between study groups. As a result; It was observed that ischemia caused damage to the myocardium in the long view and composed DIC profile.

Key Words: CK-MB, cTn I, D-Dimer, *Ehrlichia*, Myoglobin

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Ehrlichiosis ile Enfekte Anemili Köpeklerde cTn I, CK-MB, Miyoglobin ve D-Dimer Seviyelerinin Belirlenmesi

ÖZ

Ehrlichiosis birçok doku ve organı etkileyen, çoklu organ yetmezliklerine neden olabilen bir hastalıklır. *Ehrlichia*nın yaygın damar içi pıhtılaşma (YDP) bozukluğu ve miyokardiyal hasar yönünden ne derece etkili ve önemli olduğu hala netlik kazanmamıştır. Anemi *Ehrlichiosis*'te yaygın görülen klinik tablolardan biridir. Yapılan bu çalışmada *Ehrlichiosis*'li hayvanlarda gelişen miyokardiyal hasar ve potansiyel YDP tablolarının anemi ile birlikte değerlendirilmesi amaçlandı. Çalışmanın hayvan materyali 10 sağlıklı, 24 mono-enfekte ve 9 ko-enfekte hayvandan oluşturuldu. Bunun yanında enfekte hayvanlar serolojik ve moleküler sonuçlarına göre aktif enfekte, enfekte ve akut enfekte; hastalık etkenlerine göre mono ve co enfekte, anemi durumlarına göre non anemik, hafif, orta ve şiddetli anemik olarak gruplandırıldı. Bu gruplarda cTnI, CK-MB ve miyoglobin seviyeleri, myokardiyal hasarın tespit edilmesi amacı ile ölçüldü. YDB profiline yatkınlığın belirlenmesi için D-Dimer konsantrasyonları saptandı. Elde edilen sonuçlar doğrultusunda D-Dimer konsantrasyonlarında anlamlı yükselmeler mono enfekte, co enfekte, hafif, orta ve şiddetli ile aktif enfekte gruplarında tespit edildi ($p<0.05$). Çıkan bu sonuçlar doğrultusunda, cTnI seviyelerinde mono enfekte, orta ve şiddetli anemili hayvanlar ile aktif enfekte gruplarında anlamlı istatistiksel farklar belirlendi ($p<0.05$). Diğer parametrelerde herhangi bir farklılığa rastlanmadı. Sonuç olarak *Ehrlichia* sonucu gelişen anemi doku ve organlarda iskemiye neden olarak, aneminin şiddeti ile uyumlu bir şekilde uzun süreli olgularda myokardiyal hasara sebep olduğu ve YDB profili geliştirdiği görüldü.

Anahtar kelimeler: CK-MB, cTn I, D-Dimer, *Ehrlichia*, Miyoglobin

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INTRODUCTION

Ehrlichiosis; is a zoonotic disease that can be transmitted by vectors. It affects both humans and animals. It has multiple clinical signs and when it is not treated, it could be severe and fatal (Paşa et al. 2017). Studies show that the disease can be seen at rates between 2.2% and 70% in different countries all over the World (Harrus et al. 2016). Fever, depression, lethargy, anorexia, loss of weight, secretion on eyes and nose, dyspnoea, lymphadenopathy, splenomegaly, edema at extremities and scrotum, disposition at bleeding, petechia on skin and mucosa, ecchymose and rarely epistaxis are most common clinical findings in Ehrlichiosis (Yağcı et al. 2010). As a laboratory result, thrombocytopenia, mild to moderate anemia (normocytic, normochromic, non-regenerative), and mild leukopenia can be monitored (Vismaya et al. 2020).

Several diseases can cause cardiac damage directly or indirectly. Cardiac myocardial damage can produce pro-inflammatory cytokines; like tumor necrosing factor alfa and interleukin 1, 6, 18. After damage to the myocardium, cardiac output can be formed and will end up with hypoperfusion at skeletal muscles. Anemia and vasculitis contrubite the heart damage with hypoperfusion (Gonzalez et al. 2022). This causes the activation of monocytes and produces the same cytokines. These cytokines affect myocardial functions negatively and spark off an increase in myocardial damage (Krejci et al. 2016). As a result of this damage to the myocardium; cardiac troponin I (cTn I), CK-MB, and Myoglobin can get into blood circulation from different tissues. By this purpose; cardiac troponin I, CK-MB, and Myoglobin can be used as diagnostic and prognostic marker of cardiac damage (Slack et al. 2005).

Hemostatic disorders are used as important markers for detecting the prognosis of diseases. Additionally, these disorders can cause bleeding problems (Zoia et al. 2022). disseminated intravascular coagulation profile (DIC) is defined as a syndrome in which thrombosis can be seen at capillary vessels and can progresses to secondary fibrinolysis (Stokol et al. 2022). This condition is not a primer defect. It develops because of reflection of the underlying primary factors (Bruchim et al. 2017). DIC is reported with Ehrlichiosis but the pathogenesis is not clearly explained (Dalugama and Gavarammana 2018). In the determination of DIC; It is reported that D-Dimer value, which is a cross-linked pure fibrin degradation product, can be used for diagnostic purposes (Machida et al. 2010; Sadosty et al. 2011).

Ehrlichiosis has different agents that can cause disease in dogs and mammalians. *Ehrlichia canis* is the causative agent of canine monocytic ehrlichiosis (CME). CME has different effects on several organs and makes multıple clinical signs. Similarly to other infectious diseases such as Babesiosis, Parvovirus, Chagas Disease, Leishmaniosis and Leptospirosis, Ehrlichiosis can cause myocarditis (Vitt et al. 2016). In addition to the reported effects of ehrlichiosis, its vital damage to the heart is not fully known. Evaluation of CME in terms of disseminated intravascular coagulation disorder (DIC) and thromboembolism risk, is considered important for both human and animal health (Evermann et al. 2012). In this study, we aimed to find if there is a relationship between the infected dogs with CME and the myocardial damage markers (cTn I, Myoglobin, CK-MB) and D-dimer levels, which are the markers of coagulation tendency.

MATERIAL METHODS

Animal Material and Study Groups

The study was ethically approved by the Animal Experiments Local Ethics Committee of Aydın Adnan Menderes University (Decision Number: 64583101/2016/60). The animal material of the study was organized with dogs that applied to Aydın Adnan Menderes University Veterinary Faculty Animal Hospital. 43 dogs of different ages, breeds and genders, without any previous heart disease, constituted the animal material of the study. The control group was created according to clinical, hematological, biochemical, serological and moleculer results. As a serologic method, immunochromatographic Rapid Diagnostic Test Kits (IDEXX, SNAP 4DX® plus, Ehrlichia Canis, A. phagocytophilum, Borrelia burgdorferi, Dirofilaria immitis; IDEXX, SNAP® Leishmania, Westbrook USA) were used and for molecular method we implemented real-time polymerase chain reaction (PCR). Presence of CME was searched with animals that had clinical findings suitable with Ehrlichiosis. The animals that had fever, depression, lethargy, anorexia, loss of weight, lymphadenopathy, splenomegaly, secretion on eyes and nose, dyspnoea, edema at extremities and scrotum, disposition at bleeding, petechia on skin and mucosa, ecchymose and epistaxis were subjected diagnostic methods for detecting Ehrlichia. The animals that had no clinical findings, normal hematologic and biochemical parameters, and negative results with serologic and molecular examinations constituted the control group. The Ehrlichia positive group was composed had positive results from one of the serological or molecular diagnostic methods.

Animals were separated into two groups; control (n=10) and CME positive (n=33). The infected group was dissociated from the mono (n=24) and co-infected (n=9) group. Co-infection status was evaluated according to the presence of A. phagocytophilum, Anaplasma platys, Borrelia burgdorferi, Dirofilaria immitis and Leishmaniosis.

These agents were searched with rapid tests. Ehrlichia mono-infected animals were divided into two groups according to their anemia status anemic (n=18) and non-anemic (n=6). Anemic animals were also grouped as mild (n=6), moderate (n=6) and severe (n=6). The severity of anemia was determined by the hematocrit value; mild: 30-37%, moderate: 20-29% and severe 13-20% (Turgut 2000). Both mono- and co-infected animals with CME were classified by serological and molecular testing results (Tanikawa et al. 2013; Barrantes-Gonzales et al. 2016). Serologically positive, PCR positive animals were classified as active infected, serologically positive, PCR negative animals were called infected, serologically negative, PCR positive animals were staged acute infected.

Sample Collection

For the collection of hematological data, 2.5 mL blood samples were taken into tubes with Ethylenediamine Tetraacetic Acid (EDTA), from vena cephalica antebrachium of animals. This sample was used for detecting whole blood, rapid test kits and PCR results. To determine cTn I, CK-MB, and myoglobin levels, 5 mL blood samples were collected in serum tubes (with silicone) from the same vena. Also, 2 mL blood samples were taken in tubes containing 3.2% sodium citrate for detecting D-Dimer levels.

Laboratory Analyses

Whole blood parameters were determined with an automatic hematology analyzer cell counter machine (Coulter-Abacus Junıor Vet, Hungary). Serum samples and plasma that was acquired from containing 3.2% sodium citrate tubes, were handled at fluorescence immunoassay rapid quantitative test machine (Finecare, Wondfo Biotech, China) to determine cTn I, CK-MB, Myoglobin and D-Dimer levels. Whole blood parameters, cTn I, CK-MB and D-Dimer levels were obtained immediately after blood samples were collected.

Serological Analyses

For serological diagnosis, blood samples, that taken into EDTA tubes, were processed with rapid diagnostic test kits. ELISA-based immunochromotographic rapid diagnostic test kits were used to detect CME mono and co-infection situation (IDEXX, SNAP 4DX® plus, Ehrlichia Canis, A. phagocytophilum, Borrelia burgdorferi, Dirofilaria immitis; IDEXX, SNAP® Leishmania, Westbrook USA). Test kits were applied within three hours after blood samples were taken.

Moleculer Analyses

Real-time PCR was used for molecular analysis. In PCR analysis DNA was prepared suitably to the protocol with 200 µL QiaGen® Genomic DNA Purification Kits (Qiagen Company, Germany). In this study, two Ehrlichia-specific PCR primers, that amplify 455 bp of the gene and are prepared based on the 16S rRNA gene sequence, were selected and used (Inokuma et al. 2004). The EDTA tube samples were used and stored at -20 °C until PCR applications were applied.

Statistical Analyses

In this study, in the analysis of values obtained for each group, the arithmetic mean ($\bar{x}$), standard deviation (ss), minimal-maximal values (Xmin-Xmax) and median values of the parameters were calculated, and whether the values normality distrubution condition was determined by Shapiro-Wilk test. For this purpose, the Kruskall Wallis and Mann Whitney U tests methods were applied to determine the difference between groups. Probability (p-value) < 0.05 was considered significant. SPSS 23 Statistics Packet Programme (IBM, Armonk, NY, USA) was used for statistical analyses.

RESULTS

Control group was constituted with normal hematologic parameters and negative serologic and molecular results (n=10). CME-positive animals had clinical complaints compatible with Ehrlichiosis. All infected dogs showed fever, edema at extremities, ecchymose, and epistaxis. The dogs were between two and eight years old. 13 dog was mixed breed. 5 dog's breed was Anatolian Shepherd Dog, 12 dog was Golden Retriever, and 3 dog was Pointer. All control group animals were constituted from mixed breed dogs. Statistical evaluation of the animals used in the study according to breed and age was not carried out because there were not enough numbers to form groups. CME positive animals (n=33) were classified according to the presence of *A. phagocytophilum, Borrelia burgdorferi, Dirofilaria immitis and Leishmaniosis,* as mono and co infected groups. All co-infected animals (n=9) had anaplasmosis and four had leishmaniosis. These agents' diagnoses were made with immunochromatographic rapid diagnostic test kits. Mono-infected CME positive animals were classified according to anemia situation. They were separated into 4 groups; non anemic (n=6), mild anemic (n=6), moderate anemic (n=6) and severe anemic (n=6). Mono-infected animals RBC, Hgb and HCT values and anemia classification status was shown in Table 1. Serological and molecular classification condition was shown in Table 2 and animals were staged as active infected (n=12), infected (n=15) and acute infected (n=6).

Table 1. Anemia classification results of CME mono-infected animals

No (Protocol No)	RBC (10^12/L)	Hgb (g/dL)	HCT (%)	Group
1 (10)	5.52	15.64	40.13	Non Anemic (n=6)
2 (15)	6.3	14.65	38.42	
3 (16)	5.62	18.22	37.12	
4 (17)	6.21	15.15	42.12	
5 (2)	6.01	12.11	35.12	
6 (8)	5.64	13.24	30.12	
7 (Fiona)	5.49	10.12	36.70	Mild Anemic (n=6)
8 (403)	5.26	10.12	35.50	
9 (14)	5.22	9.87	36.79	
10 (9)	5.4	8.65	36.5	
11 (3)	5.48	10.15	36.17	
12 (1)	5.46	8.64	30.41	
13 (SG 5)	3.49	10.98	22.66	Moderate Anemic (n=6)
14 (SG 1)	3.84	10.24	26.64	
15 (167)	4.71	10.25	28.33	
16 (12)	3.70	10.12	23.94	
17 (13)	3.61	6.24	24.90	
18 (7)	3.42	5.12	24.09	
19 (387)	3.46	10.12	19.90	Severe Anemic (n=6)
20 (508)	2.03	8.24	12.70	
21 (535)	2.53	6.12	17.22	
22 (SG 13)	4.70	3.15	5.56	
23 (SG 16)	1.76	2.24	12.62	
24 (SG 20)	2.94	2.25	19.41	

Table 2. Serological and molecular classification result

Group	Serological Result (Immunochramotographic Rapid Test)	Moleculer Result (Polymerase Chain Reaction)
Active Infected (n=12)	+	+
Infected (n=15)	+	-
Acute Infected (n=6)	-	+

In this study, control (n=10) and CME positive animals' (n=33); D-Dimer, cTn I, CK-MB, myoglobin levels were shown in Table 3. In the control groups, the plasma D-Dimer concentration was 0.49±0.51 mg-L and control group cTn I level 0.04±0.01 ng-mL. For D-Dimer levels, both co ($p=0.021$) and mono ($p=0.017$) infected groups had significantly different statistical value ($p<0.05$). Also, apart from non-anemic animals (n=6), D-Dimer levels of anemic animals in the study group were found to be significantly different ($p<0.05$) when compared to the control group. The p-values for mild (n=6), moderate (n=6), and severe (n=6) anemia groups were 0.012, 0.011, and 0.011, respectively. In serological and molecular classification; only active infected group ($p=0.01$) had significantly different statistical result ($p<0.05$). Mono infected groups cTn I value ($p=0.01$) were found meaningful statistical difference with control groups ($p<0.05$) but co-infected group had no statistical difference ($p>0.05$). Concurrently, moderate ($p=0.004$) and severe anemic ($p=0.042$) groups had significant statistical differences. In this study, it was determined that active infected group cTn I value ($p=0.042$) were statistically significantly different when compared with the control group ($p<0.05$). It was determined that there was no statistically significant ($p>0.05$) difference between CK-MB and myoglobin levels between the groups subjected to the classification groups.

Table 3. D-Dimer, cTnI, CK-MB and Myoglobin levels of control and study groups.

Group	Parameters											
	D-Dimer (mg-L)			cTnI (ng-mL)			CK-MB (ng-mL)			Myoglobin (ng-mL)		
	$\bar{x}\pm ss$ (min-max)	Median	p-value (Controlx Group)	$\bar{x}\pm ss$ (min-max)	Median	p-value (Controlx Group)	$\bar{x}\pm ss$ (min-max)	Median	p-value (Controlx Group)	$\bar{x}\pm ss$ (min-max)	Median	p-value (Controlx Group)
Control	0.49±0.51 (0.10-1.20)	0.10	-	0.04±0.01 (0.03-0.07)	0.05	-	0.65±0.94 (0.00-3.12)	0.43	-	10.90±5.39 (2.24-18.45)	10.72	-
Mono Infected	4.09±4.12 (0.00-10.00)	2.35	0.017 p<0.05*	0.63±0.82 (0.10-2.86)	0.18	0.000 p<0.05*	3.78±6.80 (0.30-33.36)	1.36	0.060 p>0.05	10.74±8.67 (2.00-41.85)	7.71	0.664 p>0.05
Co Infected	5.17±3.93 (0.00-10.00)	4.00	0.021 p<0.05*	0.56±0.83 (0.10-1.93)	0.10	0.077 p>0.05	2.14±2.48 (0.00-7.34)	2.29	0.060 p>0.05	8.22±3.83 (3.14-13.73)	7.57	0.664 p>0.05
Non Anemic	0.80±0.95 (0.10-2.60)	0.45	1.00 p>0.05	0.70±1.07 (0.10-2.81)	0.18	0.108 p>0.05	1.34±1.23 (0.30-3.45)	0.77	1.000 p>0.05	9.42±7.43 (2.00-19.70)	8.37	0.562 p>0.05
Mild Anemic	7.08±4.55 (0.30-10.00)	10.00	0.012 p<0.05*	0.60±1.11 (0.00-2.86)	0.17	0.144 p>0.05	3.01±3.36 (0.00-9.11)	2.25	0.929 p>0.05	12.26±6.81 (4.82-19.02)	12.57	0.562 p>0.05
Moderate Anemic	6.11±4.25 (2.00-10.00)	6.20	0.011 p<0.05*	0.83±0.60 (0.00-1.65)	0.76	0.004 p<0.05*	8.73±12.30 (0.98-33.36)	4.76	0.026 p<0.05*	14.35±13.51 (7.45-41.85)	9.20	0.562 p>0.05
Severe Anemic	5.71±3.70 (1.60-10.00)	4.95	0.011 p<0.05*	0.46±0.57 (0.10-1.38)	0.76	0.042 p<0.05*	1.90±2.91 (0.00-7.36)	0.30	1.000 p>0.05	6.92±4.86 (2.29-15.68)	6.63	0.562 p>0.05
Active Infected	4.99 ±4.31 (0.10-10.00)	5.30	0.011 p<0.05*	0.63±1.04 (0.10-1.10)	0.10	0.042 p<0.05*	2.19±2.45 (0.30-9.11)	1.84	1.000 p>0.05	10.65±6.88 (2.00-19.70)	7.06	0.562 p>0.05
Infected	4.43 ±4.09 (0.10-10.00)	2.30	0.762 p>0.05	0.68±0.72 (0.00-1.93)	0.27	0.681 p>0.05	2.53±2.61 (0.00-7.34)	1.25	0.753 p>0.05	8.26±4.41 (2.00-15.78	9.54	0.707 p>0.05
Acute Infected	3.08 ±3.67 (0.10-10.00)	2.60	0.762 p>0.05	0.43±0.48 (0.10-1.38)	0.27	0.681 p>0.05	7.71±12.85 (0.30-33.36)	2.53	0.753 p>0.05	13.35±13.98 (6.71-41.85)	7.71	0.707 p>0.05

***:** Statistically significant difference between control and group (p<0.05).

DISCUSSION

The World Health Organization (WHO) defines Ehrlichiosis as an important disease with zoonotic potential, which can be severe and fatal if left untreated. It has been reported that 50% of Ehrlichia infections can be seen in Asia, Africa, Europe and America continents throughout the world (Tsachev et al. 2006), and the prevalence of the disease can reach 67%, especially in regions with tropical climate characteristics (Karagenç et al. 2005).

In literature data, it has been reported that vectors can have more than one infection agent because of that co-infection is seen frequently in vectorial diseases (Solano-Gallego et al. 2011). In ehrlichiosis *Anaplasma, Leishmania, Borrelia, Bartonella, Rickettsia, Babesia,* and viral agents can be detected together. (Baneth et al. 2008; Shaw et al. 2009; Harrus et al. 2016). In this study, the presence of co-infection was detected in 9 dogs with Ehrlichiosis to the literature information mentioned above. In all of the co-ınfected animals, anaplasma was detected and leishmaniosis is found in 4 animals.

D-dimer is an important sign of fibrinolytic activation. it can increase trauma, surgery, infection, ınflammation, gestation, DIC, venous thrombosis, ischemic cardiomyopathy and thrombosis (Kobayashi et al. 2020). D-dimer is swayed from blood clots and attends blood circulation. When coagulation occurs due to many reasons such as infection; D-dimer analysis becomes a valuable marker for involuntary thrombosis (Sato et al. 2020). So D-dimer is indicated as an important marker when deep venous thrombosis and DIC occur (Han et al. 2022). In thıs study, the results of D-dimer levels are found compatible with works of literature. As a result of the analyses performed, it was determined that the D-dimer results of mono infected, co infected, mild, moderate, severe and active infected groups, were significantly different from the control group ($p<0.05$). It is recommended that more studies should be conducted on the subject of knowing how ehrlichiosis or co-infection agents predispose to DIC (Caldin et al. 2000). In the study conducted by Caldin et al. (2000), the sensitivity of D-Dimer was reported as 100% and the specificity as 97% in the development profile of DIC. It was reported by Paşa et al. (2017) that the D-Dimer level increased in infected dogs with ehrlichiosis (Paşa et al. 2017). In this study, it was seen that the findings on D-dimer were compatible with the studies of the researchers, and in line with the results obtained, it was concluded that the development potential of DIC and venous thromboembolism in Ehrlichia-infected animals were formed.

Infarctus and hypoxia consist of causing anemia Due to anemia, the increased metabolic heart requirement on the cardiovascular system and heart cannot be met (Portman et al. 1995). It has been reported that myocardial damage may develop as a result of anemia, which can occur in infections caused by Ehrlichia and other co-infected agents (Diniz 2008). One of the cardiac markers cTn I is associated with myocyte degeneration that acute heart damage (Diniz et al. 2007; Braunwald 2008). And it has been reported that myocyte degeneration usually consists of after severe ischemia (O'Brien et al. 2006; Braunwald 2008). It has been determined that myoglobin is released into the circulation due to insufficient tissue perfusion or cellular damage in trauma situations (Liu et al. 2019). Troponins and myoglobin, which are heart-derived proteins, can be used for the presence and grading of myocardial damage in the detection of possible myocardial damage (Schober et al. 2002; Oyama et al. 2004; Q'Brien et al. 2006; Langhorn and Willesen 2015). Sleeper et al. (2001) reported that healthy dogs' cTn I levels could be between <0.03 ng-ml and 0.07 ng-ml (mean and standard deviation 0.02 ± 0.01). In thıs study, it was seen that control group cTn I value was composable to works of literature. Among the study groups, cTn I levels of mono-infected animals, moderate and severe anemic group and active infected animals were found to be statistically significantly higher ($p<0.05$). This condition on cardiac troponin changes occurs due to hypoxia as described in people with anemia (Diniz et al. 2008). In humans, the half-life of cTnI is approximately 2 hours. However, a slow breakdown of contractile apparatus shows a considerably longer half-life (Langhorn and Willesen 2016). This situation is almost similar in dogs with humans. Because of this condition, cTn I is used as a sensitive biomarker in myocardial cell injuries (Burgener et al. 2006). In this study, we thouhgt that anemia in mono-infected animals can be associated with myocyte degeneration due to insufficient perfusion of the heart and the increase in cTn I levels accordingly. Similarly, the insignificance of cTn I change in animals with mild anemia supports the human studies mentioned above.

In this study, there is no difference between the groups about myoglobin ($p>0.05$). Holmgren ve Valberg (1992) was reported that assessment of clinical tables with myonecrosis follow the myoglobin in plasma was a suitable method for detecting cardiac damage (Holmgren and Valberg 1992). In another study, it was reported that the first myoglobin levels were significantly higher in the gradual measurements in animals with gastric dilatation-volvulus and trauma, and the plasma MYG levels decreased in repeated measurements at the 24th and 48th hours (Burgener et al. 2006). It is thought that the decrease in myoglobin level is since the half-life is stated as 9 minutes (Klocke et al. 1982). On the other hand, Mair et al (1992, 1994) reported that there is no way to detect an increase of plasma myoglobin concentration whether it originates from skeletal muscles or heart damage (Mair et al. 1992; Mair et al. 1994). In

insufficient oxygenation in muscles Myoglobin carries essential oxygen to muscles (Karagül et al. 2000). Considering this information, it is thought that the lack of significant myoglobin levels between the groups may be due to the differences in the clinical pathogenesis of Ehrlichia in terms of anemia profile and the inability to fully clarify the origin of myoglobin from the heart.

Creatine kinase has two different bases unıt; M (Muscle) and B (Brain). As a result of the interaction of these unıts with each other, creatine kinase has three isoenzymes; CK-MB, CK-MM and CK-BB (Lang 1981). Besides the point of place that ısoenzymes came from are different, their effects are not known in veterinary medicine (Aktas et al. 1994). The most well-known information about creatine kinase belongs to dogs in veterinary medicine (Slack et al. 2005). In the last decades, there has been some suspicion of using CK-MB as a marker at cardiac damage but ıt was reported that in dogs with left ventricular hypertrophy, CK-MM levels decrease by 50%, and CK-MB levels increase %10 (Ye et al. 2001). In septic foals there is an uprising for CK-MB levels, however, it has been reported that there is no difference in CK-MB levels in living or non-viable animals, so it cannot be used as an indicator in terms of prognosis (Slack et al. 2005). And also, when compared with the cardiac-specific troponins CK-MB has lower specificity than them (Silverman et al. 1974; Adams et al. 1993).

Significant increases in CK-MB levels can occur within 2-4 hours following the development of myocardial damage. It is reported that conventional CK-MB levels decreased to normal levels 12 hours after the symptoms of acute cardiac injury were controlled and it could be detected in only 27% of the animals included in the study (Puleo et al. 1987). In the same study, it was reported that CK-MB isomers (CK-MB1, CK-MB2) expressed 98% reliability (Puleo et al. 1987). In a different study, the half-life of CK-MB concentration in plasma was defined as 2 hours (Burgener et al. 2006). The limited availability of the CK isomer, CK-MB, in the heart causes its rapid degradation. This situation limits the use of this marker, in the detection of circulating heart damage due to the inadequacy of its release in damage cases (Burgener et al. 2006). We thought that the high reversibility capacity of CK-MB could explain the absence of a significant difference in CK-MB levels between the group's capacity of CK-MB.

CONCLUSION

In conclusion, it was determined that myocardial damage was formed due to ischemia in the heart in anemic dogs with Ehrlichiosis, and in moderate and severe anemic dogs CTnI concentrations increased. D-dimer levels also increased with CME. In the fields of medicine, It is thought that these markers can be used in the clinical follow-up of the prognosis and treatment of patients with Ehrlichiosis in the follow-up of DIC status and heart damage. To gain a comprehensive understanding of all stages of CME, we suggest conducting further studies on larger populations of dogs.

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

Authors' Contributions: YP and SP contributed to the article idea, design and execution the study. YP collected datas. YP and SP analyzed data. All outhors contributed to the critical revision of the manuscript and have read and approved the final version.

Ethical Approvel: The study was ethically approved by the Animal Experiments Local Ethics Committee of Aydın Adnan Menderes University (Decision Number: 64583101/2016/60).

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RESEARCH ARTICLE

Evaluation of Enteropathogens and Fecal pH Changes in Neonatal Calves with Diarrhea

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ABSTRACT

The aim of this study is to investigate fecal pH changes in diarrheal newborn calves infected with enteropathogens such as *Bovine rotavirus*, *Bovine Coronavirus*, *Cryptosporidium* spp, *Escherichia coli K99* and *Giardia lamblia*. fecal pH values of calves with identified pathogens were measured using nitrazine paper and digital pH meter, and it was revealed that there was a relationship between the detected factor and the age of the case. The animal material of the study consisted of 96 diarrheal calves aged between 1 and 28 days. Fecal samples were taken from calves with diarrhea. Rapid diagnostic kits were used to diagnose enteropathogens in the stool samples taken. When the pH analysis results of all cases were examined, fecal pH values of both single and co-infected cases were determined to be predominantly between 5-6 and 6.1-7. Fecal pH values of infected calves were determined to be maximum between 6.1-7. When the pH values of all cases were examined, it was determined that cases with 8-15 days of diarrhea were detected more frequently. It was determined that the fecal pH values of these calves with diarrhea varied between 6.1-7. In the analysis of fecal pH values in 12 samples in which only *E. coli* was detected, it was determined that the fecal pH value of 11 of these calves was measured between 5 and 6, and only 1 of them had a pH value above 6. On the other hand, it was determined that the fecal pH value was measured below 6 in 12 of the remaining 84 calves. It was observed that the fecal pH value was between 5-6 in the majority of calves in which E. coli was detected. It was determined that the fecal pH value was above 6 in 73 of a total of 96 calves. Additionally, pH was not detected above 7.1 in any of the cases infected with enteropathogens. The pH value of single and co-infected cases was predominantly determined between 6.1 and 7.

As a result, it was determined that the fecal pH values of cases infected with enterogens were not higher than 7, and the fecal pH values of enteropathogens were concentrated in the range of 6.1-7. For faster diagnosis, prevention, control and treatment of enteropathogens that cause diarrhea in newborn calves, attention can be taken in the diagnosis and treatment of the disease when fecal pH values are an easily detected parameter. It is thought that it will shed light on future studies regarding fecal pH.

Keywords: Calf, Diarrhea, Diagnosis, pH

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İshalli Yenidoğan Buzağılarda Enteropatojenler ile Dışkı pH Değişimlerinin Değerlendirilmesi

ÖZ

Bu çalışmanın amacı Sığır rotavirüsü, Sığır Coronavirüsü, *Cryptosporidium spp*, *Escherichia coli K99* ve *Giardia lamblia* gibi enteropatojenlerle enfekte olmuş ishalli yeni doğan buzağılarda dışkı pH değişikliklerinin araştırılmasıdır. Enteropatojen tespit edilen buzağıların dışkı pH değerleri nitrazin kâğıdı ve dijital pH metre kullanılarak ölçüldü ve tespit edilen faktör ile olgunun yaşı arasında bir ilişki olduğu ortaya çıktı. Araştırmanın hayvan materyalini yaşları 1 ile 28 gün arasında değişen 96 ishalli buzağı oluşturdu. İshalli buzağılardan dışkı örnekleri alındı. Alınan dışkı örneklerinde enteropatojenlerin tanısının konulması için hızlı tanı kitleri kullanıldı. Tüm vakaların pH analiz sonuçları incelendiğinde hem tek hem de ko-enfekte vakaların dışkı pH değerlerinin ağırlıklı olarak 5-6 ile 6,1-7 arasında olduğu belirlendi. Enfekte buzağıların dışkı pH değerlerinin maksimum 6,1-7 arasında olduğu belirlendi. Tüm vakaların pH değerleri incelendiğinde 8-15 gün süren ishal vakalarının daha sık tespit edildiği belirlendi. İshalli bu buzağıların dışkı pH değerlerinin 6,1-7 arasında değiştiği belirlendi. Sadece E. coli tespit edilen 12 örnekte dışkı pH değerleri analizinde, bu buzağılardan 11 tanesinin dışkı pH değerinin 5 ile 6 arasında ölçüldüğü, sadece 1 tanesinin pH değerinin 6'nın üzerinde olduğu belirlendi. Öte yandan geri kalan 84 buzağının 12'sinde dışkı pH değerinin 6'nın altında ölçüldüğü belirlendi. E. coli tespit edilen buzağıların büyük çoğunluğunda dışkı pH değerinin 5-6 arasında olduğu görüldü. Toplam 96 buzağının 73'ünde dışkı pH değerinin 6'nın üzerinde olduğu belirlendi. Ayrıca enteropatojenlerle enfekte olguların hiçbirinde pH 7,1'in üzerinde saptanmadı. Hem tek hem de ko-enfekte vakaların pH değeri ağırlıklı olarak 6,1 ile 7 arasında belirlendi.

Sonuç olarak enterojenlerle enfekte olguların dışkı pH değerlerinin 7'den yüksek olmadığı, enteropatojenlerin dışkı pH değerlerinin ise 6,1-7 aralığında yoğunlaştığı belirlendi. Yeni doğan buzağılarda ishale neden olan enteropatojenlerin daha hızlı teşhisi, önlenmesi, kontrolü ve tedavisi için dışkı pH değerlerinin kolay tespit edilen bir parametre olduğu için hastalığın teşhis ve tedavisinde dikkat edilebilir. Dışkı pH'ı ile ilgili ileride yapılacak çalışmalara ışık tutacağı düşünülmektedir.

Anahtar Kelimeler: Buzağı, İshal, pH, Tanı

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INTRODUCTION

Diarrhea is one of the most common causes of morbidity and mortality in young calves, especially in both dairy and beef calves younger than one month (Torche et al. 2020; Chen et al. 2022). In ruminants, they do not transfer immunoglobulin from the mother to the fetus during pregnancy. For this reason, calves are usually born hypogammaglobulinemic or agammaglobulinemic and receive the necessary immunoglobulins through colostrum and milk after birth (Yılmaz and Akgül 2014; Kozat, 2019). Calves start producing their their immunoglobulin on the 10th day after birth and reach normal plasma immunoglobulin levels when they are 60 days old. This may cause infectious diarrhea to be common in neonatal calves (Yılmaz and Akgül 2014; Çakıroğlu et al. 2010). Neonatal calf diarrhea (NCD) is one of the most common multifactorial diseases (Probo and Veronesi, 2022). Virus, bacteria, and protozoa infection, as well as immunological status and management factors (housing, feeding, and hygienic conditions) play an important role as determinants and predisposing factors, respectively. During the first 28 days of their lives, enteropathogens such as *Bovine rotavirus, Bovine coronavirus, Escherichia coli* (*E. coli*) and *Cryptosporidium* are common factors that cause diarrhea in neonatal calves, either alone or as mixed infections (Kozat and Voyvoda, 2006). Among these enteropathogens, it has been reported that *Bovine rotavirus* is the most common viral agent in neonatal calf diarrhea, along with *Bovine coronavirus* (Snodgrass et al., 1986; Ramig, 2004). Among bacterial factors, *E. coli* is an important enteropathogen in neonatal calves. Additionally, *Cryptosporidium* spp. The prevalence and zoonotic importance of parasitic agents such as Giardia are increasing day by day. In addition to viral, bacterial and parasitic factors, non-infectious factors such as unfavorable shelter conditions, animal breeders with low education levels, insufficient colostrum intake and neglect of umbilical cord care are also effective in neonatal calf diarrhea (Kozat, 2019). In Turkey, important studies are carried out to obtain healthy calves and protect them from diseases. In this context, it has been observed that research on the causes and factors that cause diarrhea, especially in calves, has increased rapidly in recent years (Al and Balıkçı, 2012; Kozat and Voyvoda, 2006)

Calves with diarrhea may show signs like loss of appetite, weight loss, and poop that is loose, bloody, and sometimes contains mucus. In severe cases, research shows that the poop can be very watery, bloody, and even have bits of lining from the intestines. Animals in this condition may also become thin, dehydrated, weak, and uninterested in their surroundings (Kozat and Voyvoda, 2006; Lorenz et al. 2011; Yang et al. 2017; Ganapathy et al. 2023). In calves with diarrhea, changes occur in biochemical and hematological parameters as well as clinical findings. Studies on newborn calves with diarrhea indicate that in cases of diarrhea, there is a decrease in sodium and chloride concentrations in the blood plasma and an increase in the potassium concentration in the blood plasma (Dincer et al. 2021; Ganapathy et al. 2023, Guzelbektes et al. 2007). Regardless of the cause, diarrhea can cause dehydration, electrolyte imbalance, metabolic acidosis and hypovolemia (Dincer et al. 2021; Ganapathy et al. 2023; Torche et al. 2020). Hypovolemia results in kidney failure and heart block due to hyperkalemia and septicemia due to secondary bacterial overgrowth in the small intestine. The kidney reduces urine production to compensate for the increased fluid losses caused by diarrhea (Shehta et al. 2022). Several studies (Kozat and Voyvoda, 2006; Saleh et al. 2022) found differences in these parameters compared to healthy calves. These differences include lower levels of total protein, albumin, and the albumin-globulin ratio, along with unchanged levels of beta globulin and potentially higher levels of alpha, gamma globulins, and total globulins. Other research (Subhash Malik et al. 2012) suggests that dehydrated calves with diarrhea also have higher red blood cell counts and white blood cell counts, indicating dehydration. Additionally, the severity of these changes depends on the type, duration, and intensity of the diarrhea (Nayak et al. 2019). While there are many studies on changing hematological and biochemical parameters in cases of diarrhea, there are few studies on changes in fecal pH value (Turgut and Ok 1998). In cases of diarrhea, enteropathogens disrupt the intestinal mucosal barrier system and cause damage to the intestinal epithelium (Kozat, 2023). It is thought that the pH of the stool will change as a result of the deterioration of the intestinal mucosal barrier system. Although the severity of clinical findings varies, changes in stool pH value are observed depending on enteropathogens. *Enterobacteriaceae* causes organic acid production, and the organic acids formed cause to decrease pH value of fecal. However, the same study stated that as a result of the damage to the gastrointestinal system during diarrhea, the normal flora changes and the beneficial bacteria decrease, the fecal pH value may increase, and the resulting alkaline environment may lead to the proliferation of harmful bacteria in human medicine (Osuka et al. 2012).

In the literature review, no research was found in the field of veterinary medicine regarding enteropathogens and changes in fecal pH in cases of diarrhea. This research will be to reveal the relationship between fecal pH and enteropathogens in cases of diarrhea. In this study, the objective of the present study was to evaluate fluctuations in fecal pH levels in diarrheic and normal fecal samples from neonatal calves, while also revealing whether there is a relationship between the factors that play a role in diarrhea and the fecal pH value.

MATERIAL and METHOD

Animals

Within the scope of this research, 96 newborn calves with diarrhea, 47 male and 49 female, were used as study material. During the clinical examination of the sick calves included in the research; the consistency, odor, content and color of the stool and the frequency of defecation were noted in detail.

Examination protocol of calves

Detailed systemic clinical examinations were executed on the diarrheal calves used in the study. During the examination, the consistency and content of the stool, the condition of the mucosa and the color of the conjunctiva were evaluated. In addition, clinical characteristics such as body temperature, skin elasticity, position of the eyeball in the orbit and sucking reflex of the calves were examined.

Taking stool samples and determining the causative agents

Stool samples were collected in stool containers or by swab or by swab following the technique. After the stool was collected, the BoviD-5 Ag rapid test kit (RC1302DD) originating from the Republic of Korea was used for agent analysis. Before the test, the box of the kit was carefully opened and materials such as test devices, assay diluent, and dropper were removed and checked for completeness. Then, a sample was taken from the diarrheal calf using a sterile fecal container and wearing gloves. The feces taken with the sampling apparatus were mixed with the assay diluent liquid and a homogeneous mixture was obtained by ensuring that the sample was completely dissolved. The sample taken from the resulting mixture with a dropper was carefully added to the sample section of the testing device and the process was completed. The test result was obtained by waiting 5-10 minutes for the sample to react in the test device.

pH determination from stool samples

Stool pH values were determined by dipping nitrazine paper into the stool samples taken into a sterile container and using a digital pH meter (AD11 Waterproof pH-TEMP Pocket Tester with replaceable electrode).

Statistical Analysis

The obtained data were used to determine the rate and frequency of occurrence of the factors for descriptive statistics. SPSS (version-21) statistical package program was used in the calculations.

RESULTS

Clinicl findings

In the study, when we looked at the color, content and consistency of diarrhea, it was determined that they were yellow watery, yellow gruel-like, yellow-green watery, green watery, green gruel-like, grey-white, brown and bloody and mucous.

Biochemical Findings

According to the stool analysis results, enteropathogens were detected in 79 of 96 diarrheal cases, while no enteropathogens were detected in 17 cases. Of the 79 cases in which enteropathogens were detected, single enteropathogens were detected in sixty-one, double enteropathogens were detected in sixteen, and triple enteropathogens were detected in two (Table 1). Among the 61 calves were *Cryptosporidium* spp. in 25, *E. coli K99* in 12, *Bovine rotavirus* in 11, *Bovine coronavirus* in 7 and *Giardia lamblia* in 6. Additionally, 2 calves had *Cryptosporidium* spp.+*Giardia lamblia*, 8 cases *Cryptosporidium* spp. + *Bovine rotavirus*, 3 cases of *Bovine coronavirus*+*Bovine rotavirus*, 1 case of *Bovine coronavirus* + *Cryptosporidium* spp., 2 cases of *Bovine rotavirus*+*E. coli*, 1 case of *E. coli*+ *Cryptosporidium* spp.+*Bovine rotavirus,* 1 in *Bovine coronavirus*+*Bovine rotavirus* + *Cryptosporidium* spp. the factors were detected as co-infection. Of the 96 calves within the scope of the research, 27.08% had *Bovine rotavirus*, 12.5% had Bovine coronavirus, 15.62% had *E. coli*, 39.58% had *Cryptosporidium* spp. and in 8.33%, *Giardia lamblia* was detected alone or simultaneously with other enteropathogens.

Fecal pH results

Within the scope of the research, fecal pH values of 96 calves were measured. When the pH analysis results in all cases were examined, the pH values of both single and co-infected cases were determined in two groups as 5-6 and 6.1-7. The pH values of infected calves were detected in the density group between 6.1-7. When the pH values of all cases were examined, cases with 8-15 days of diarrhea were detected more frequently and the pH value of the calves was determined to be 6.1-7. In the analysis of fecal pH values in 12 samples in which only *E. coli* was detected, it was determined that the fecal pH value of 11 of these calves was measured between 5 and 6, and only 1 of them had a pH value above 6. On the other hand, it was determined that the fecal pH value was measured below 6 in 12 of the remaining 84 calves. It was determined that the fecal pH value was above 6 in 73 of a total of 96 calves (Table 1, Table 2). Additionally, pH of none of the cases infected with enteropathogens was detected above 7.1. The pH value of both single and co-infected cases was predominantly determined between 6.1 and 7. In this study, single enteropathogens were detected in 61 cases among 96 diarrheal cases. Fecal pH values of cases with single enteropathogens (n=61) were detected between 5-6 in 17 cases and 6.1-7 in 44 cases.

Table 1. Distribution of calf fecal pH value according to infectious factors

	Agent (s)	pH 5- to- 6	pH 6.1-to-7	pH 7.1 and above	Total
Single (n=61)	*Bovine rotavirus*	2	9	0	11
	Bovine coronavirus	0	7	0	7
	E. coli	11	1	0	12
	Giardia lamblia	2	4	0	6
	Cryptosporidium spp.	2	23	0	25
	Bovine coronavirus+ Bovine rotavirus	0	3	0	3
Double (n=16)	*Bovine rotavirus + Cryptosporidium* spp.	1	7	0	8
	Cryptosporidium spp.+ *Giardia lamblia*	1	1	0	2
	Cryptosporidium spp.+ *Bovine coronavirus*	0	1	0	1
	Bovine rotavirus+E.coli	2	0	0	2
	Bovine rotavirus+ Cryptosporidium spp. + *E.coli*	0	1	0	1
Triple (n=2)	*Bovine rotavirus + Bovine coronavirus + Cryptosporidium* spp..	0	1	0	1
Unknown (n=17)		2	14	1	17

Table 2. Distribution of pH value determined in stool samples according to age

pH Age (s)	pH between 5-6 (n=22)	pH between 6.1-7 (n=73)	pH 7.1 and above (n=1)	Total
1-7 days	17	18	0	35
8-15 days	4	41	1	46
16-28 days	1	14	0	15

DISCUSSION

It is a fact that calf diarrhea still causes economic losses in cattle farms, despite modern veterinary practices. In addition to diarrhea treatments, determining etiological factors and taking preventive measures is becoming increasingly important. As stated by Walker (1998), diarrhea in newborn calves with high morbidity and mortality constitutes a serious problem in the cattle industry. Worldwide, one of the main causes of calf deaths and financial losses to the cattle industry is losses due to diarrhea (Kozat, 2019, Von Buenau et al. 2005; Wudu et al. 2008). Precautions taken starting from the pregnancy period are of great importance to reduce the frequency of calf diarrhea (Kozat, 2019). It has been emphasized in many studies that taking the right precautions in cases of diarrhea, especially giving colostrum on time and sufficiently, plays a critical role in strengthening the immune system of calves (Göncü et al., 2013; Kozat, 2019). In addition to colostrum, improving the housing conditions of enterprises and ensuring hygiene can make significant contributions to the growth of calves in a healthier and more disease-resistant manner (Kozat and Voyvoda, 2006).

Diarrhea and changes in gut bacteria create a two-way street of health problems in dehydrated calves. This highlights the interconnectedness of the issues and emphasizes the importance of considering both the gut bacteria and blood tests for a complete understanding of the calf's health (Li et al. 2020). According to recent research, cases of diarrhea in calves in the first four weeks after birth have been linked to various pathogens. Enteropatogens such as *Bovine rotavirus, Coronavirus, E. coli, Cryptosporidium* and *Giardia* are stated as the most important causes of calf diarrhea (De la Fuente et al. 1998; Langoni et al. 2004; Yang et al. 2017). It is emphasized that there is no single reason for the occurrence of neonatal calf diarrhea, instead, more than one factor plays a role, and regular and accurate fluid-electrolyte treatment is as important as an effective chemotherapy treatment to reduce high mortality rates due to diarrhea (Cho and Yoon, 2014). Studies have shown that calves with diarrhea have a decrease in intestinal microbiota diversity, significant changes in the fecal microbial composition, and dysbiosis (Gomez et al., 2017). They reported that they detected a significant decrease in the alpha diversity of bacterial and fungal communities, especially in the intestine of calves (Jang et al. 2019; Liu et al. 2022). At the same time, diarrhea is characterized by a decrease in beneficial bacteria that produce short-chain fatty acids (SCFAs), which play a role in reducing the risk of diarrhea (Li et al. 2023). In this study, the fecal pH value of *bovine rotavirus*-infected calves with diarrhea was between 6.1 and 7 in 9 calves and between 5 and 6 in 2 calves. The maximum change in fecal pH values is between 6.1-7, which can be interpreted as being caused by disorders caused by the agent in the intestines.

When a calf gets infected with bovine coronavirus (BCoV), it first attacks the beginning of the small intestine and often spreads throughout the entire small and large intestines. BCoV mainly targets mature cells on the surface of tiny finger-like structures called villi, but it can also damage cells deeper in the gut lining (crypt cells). This damage takes longer to heal, making the illness last longer. Infected villi in the small intestine and cells in the large intestine get replaced by immature cells, and the ridges within the large intestine shrink (Cho and Yoon, 2014). In this research, the fecal pH value of 7 cases with *Bovine coronavirus* infection values was detected between 6.1-7.

Cryptosporidium species are zoonotic protozoans that cause gastrointestinal infections in various species. In particular, *Cryptosporidium parvum* is considered one of the main causes of calf diarrhea and is important as a potential zoonotic factor (Trotz-Williams et al. 2008; Chalmers et al. 2011). In studies conducted in other countries around the world, *Cryptosporidium* spp. different rates have been obtained regarding its prevalence. 47.9% in newborns in Spain (Castro-Hermida et al., 2002), 17.9% in France, 33.5% in Vietnam (Nguyen et al., 2007), 13% in Canada, 27.9% in England (Brook et al. 2008), 35% in America (Santın et al., 2004) and 11% in Sweden (Lefay et al. 2000; Björkman et al. 2003; McAllister et al. 2005). In this study, only *Cryptosporidium* infection was detected in 25 of 96 calves with neonatal diarrhea aged 1-28 days, while 38 (39.58%) were found to have other enteropatogens along with cryptosporidium infection. This shows that *Cryptosporidium* can be effective alone or in combination with other factors to cause diarrhea. *Escherichia coli* infections are considered one of the leading causes of calf diarrhea, which usually occurs within 2-10 days after birth, and can rarely be seen within the first 24 hours after birth (Kozat and Voyvoda, 2006). *Enterotoxigenic E. coli (ETEC)* strains from calves are mediated by adhesin antigens, mainly F5 (K99) and F41 fimbriae. The distal part of the small intestine is most suitable for ETEC colonization due to low pH (less than 6.5). It binds to the intestinal epithelium and proliferates in the enterocytes of the intestinal villi (Foster and Smith, 2009). *E. coli K99* rates vary around the world, for example 0.3% in Switzerland (Torsein et al. 2011), 2.6% in Germany (Bartels et al., 2010). In a study conducted in Spain, the mixed ratio of *E. coli K99* and *C. parvum* was found to be 27.8% (De la Fuente et al. 1998). In this study, it was determined that the fecal pH value in patients with *E. coli* infection was 6 and lower than 6 in 11 out of 12 calves.

Giardia lamblia is becoming increasingly important as it causes growth retardation in farm animals, reducing feed utilization and causing economic losses by causing diarrhea (O'Handley et al., 2003). In this study, Giardia lamblia infection was detected in 6 cases. The fecal pH of two of the 6 cases was found to be between 5-6, and the fecal pH of four calves was found to be between 6.1-7.

The pH value of a stool sample is usually measured using nitrazine paper. Normally, fecal pH varies between 7.0-7.5; However, lower than 5.5 indicates an acidic stool (Eherer and Fordtran, 1992). Higher or lower than normal fecal pH value is related to the severity of infection and can be used as an evaluation tool for the clinical course of diseases (Osuka et al. 2012). In the research conducted on fecal pH changes in diarrheal and healthy calves, the average pH after birth increased from the 1st week (pH 5.39), decreased in the 2nd week (6.02) and 3-4 weeks (6.30). In the same study, in the diarrhea group, week 1 showed a higher pH (6.18) and week 3-4. They reported that they detected lower pH (5.77) in diarrheal stools in weeks (Walker et al. 1998). In this study, the pH value of the feces was measured and it was determined that the pH value of 23 of 96 neonatal calves with diarrhea was between 5-6 and 72 of them was between 6.1-7. The highest fecal pH value was found to be 6.1-7 in all age groups. *E. coli* infection was detected in 11 of the calves whose fecal pH value was between 5-6. In addition, it was determined that the fecal pH value of 17 (48.57%) of 35 calves with diarrhea aged 1-7 days was between 5-6, and 18 (51.43%) was between 6.1-7; It was determined that the fecal pH value of 4 (8.69%) of 46 calves with diarrhea, aged 8-15 days, was between 5-6, and 41 (91.31%) were between 6.1-7.

These findings suggest that stool pH may be related to both *E. coli* infections and age. It has been stated that fecal pH value is related to the severity of infection and measuring fecal pH value can be used as an evaluation tool for the course of diseases (Osuka et al. 2012). In another study, in the comparison of fecal pH values in diarrheal and normal calves, it was reported that there were wide differences especially between Lactate and Succinate concentrations of both groups, but the average fecal pH value in healthy first week old calves was 5.39, 6.02 in 1-2 week old calves and 6.30 in 3-4 week old calves (Sato and Koiwa, 2008).

A fecal pH value of 5-6 suggests the presence of an acidic environment, potentially increasing the chances of pathogenic bacteria such as *E. coli* to proliferate. This situation was reported by Osuka et al (2012) that organic acids produced by Enterobacteriaceae may cause the fecal pH value to decrease. The findings on this subject suggest that fecal pH value can be used as an important parameter in evaluating gastrointestinal health status and diagnosing the disease agent. However, more studies are needed on the relationship between infectious factors and fecal pH values.

CONCLUSION

In summary, in this study, the pathogens *Bovine rotavirus*, *Bovine coronavirus*, *Cryptosporidium* spp., *E. coli K99* and *Giardia lamblia* were rapidly detected in calves with neonatal diarrhea. A difference was detected between the rates of occurrence of these factors alone and in mixed forms. Additionally, the fecal pH values of calves identified as the causative agent were measured. Considering that there was a relationship between the measured fecal pH values and the factors and that the factors were single and mixed in all calves, single and mixed factors were detected to be concentrated between fecal pH values of 6.1-7. It was concluded that there was a connection between fecal pH value changes and the factors. It is thought that this study will shed light on future research on fecal pH values.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: VÇ and SK contributed to the project idea, design and execution of the study. VÇ contributed to the acquisition of data. VÇ wrote the manuscript. SK reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: Approval for this study was obtained from the Animal Experiments Local Ethics Committee of the Van Yüzüncü Yıl University (Date: 01.12.2022, Number: 2022/12-05). "This study is not subject to the permission of Van YUHADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees". The data, information and documents presented in this article were obtained within the framework of academic and ethical rules."

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Explanation: We have not presented as a oral, poster, and abstract vs.

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RESEARCH ARTICLE

Development of a Scale to Assess Veterinary Medicine Students' Attitudes Toward Biochemistry Course

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ABSTRACT

Biochemistry stands as a pivotal subject in the curriculum of veterinary medicine education, warranting an assessment of veterinary students' attitudes towards this course. This study aims to develop a scale to gauge veterinary students' sentiments regarding biochemistry. The sample comprises 220 students enrolled in a veterinary medicine program. Initially, a preliminary test form with 49 items was created through a thorough review of relevant literature and expert consultation. A pre-test form was administered to 20 veterinary students to assess item clarity, grammar, and response duration, followed by necessary adjustments. Subsequently, the trial form was distributed to the 220 enrolled students. The data collected were analyzed, revealing a Kaiser-Meyer-Olkin (KMO) value of 0.794, indicating the adequacy of the scale. Exploratory factor analysis showcased factor loadings ranging from .582 to .812. Moreover, a reliability analysis demonstrated a Cronbach's Alpha coefficient of .761, surpassing the threshold of .70, signifying the scale's reliability. Thus, based on the findings, it can be concluded that the scale developed is both valid and reliable for assessing veterinary students' attitudes towards biochemistry.

Keywords: Attitude, Biochemistry, Scale development, Veterinary medicine

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Veteriner Hekimliği Öğrencilerinin Biyokimya Dersi Konusundaki Tutumlarını Değerlendirmek İçin Bir Ölçeğin Geliştirilmesi

ÖZ

Veteriner hekimlik eğitiminde, biyokimya temel bir ders olarak kabul edilir. Bu nedenle, veteriner hekimlik öğrencilerinin biyokimya dersine karşı davranışları ve duyarlılıkları, bu dersle ilgili tutumlarının ölçülmesini gerektirir. Bu araştırmanın amacı, veteriner hekimlik öğrencilerinin biyokimya dersine yönelik tutumunu ölçen bir ölçek geliştirmektir. Örneklemini, veteriner hekimlik fakültesinde eğitim gören 220 öğrenci oluşturdu. Araştırma kapsamında, öncelikle ilgili literatür tarandı, bir soru havuzu oluşturuldu ve 49 maddeden oluşan bir ön test formu uzman görüşü alınarak hazırlandı. Bu ön test formu, ölçek maddelerinin anlaşılabilirliği, dilbilgisi ve cevap süresi gibi faktörler açısından değerlendirilmek üzere 20 veteriner fakültesi öğrencisine uygulandı ve gerekli düzenlemeler yapılarak deneme formu veteriner fakültesindeki ve devam eden 220 öğrenciye uygulandı. Elde edilen veriler bilgisayar ortamına aktarılarak analiz edildi. Ölçeğin Kaiser-Meyer-Olkin (KMO) değeri .794 olarak belirlendi. Yapılan açıklayıcı faktör analizi sonucunda, ölçek maddelerinin faktör yüklerinin .582 ile .812 arasında değiştiği görüldü. Ayrıca, 16 maddeden ve 4 alt boyuttan oluşan bir ölçek elde edildi. Cronbach Alfa iç tutarlılık ve güvenirlik katsayısı .761 olarak bulundu. Bu değer, Cronbach Alfa katsayısı .70'in üzerinde olduğu için gelişitirilen ölçeğin güvenilir olduğunu göstermektedir.

Anahtar kelimeler: Biyokimya, Ölçek geliştirme, Tutum, Veteriner Hekimliği

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INTRODUCTION

Biochemistry is an interdisciplinary field where experts from various professions such as chemists, physicists, biologists, medical doctors, veterinarians, dentists, pharmacists, agronomists, etc., collaborate (Yeğin 1973). It is an essential subject in medical education, requiring students to understand organismal functions at macroscopic, microscopic, and especially submicroscopic (molecular) levels. However, transitioning between these organizational levels can be challenging for students (Basağa et al. 1994; Schönborn and Anderson 2006).

Awareness of how genetic and environmental factors influence the biochemical aspects of the human body is crucial for modern medical practice, as medical advancements parallel developments in biochemistry. Despite its importance, many medical students and practitioners perceive biochemistry as unnecessary, viewing it as burdensome with minimal relevance to daily medical practice (Afshar and Han 2014). Many students entering medical faculties have already received introductory biochemistry education in high school, leading to biases and a lack of appreciation for the subject. However, integrating prior knowledge with medical applications is indispensable in medical education (Bottomley and Denny 2011).

A study emphasizes the need for biochemistry education in aligning with medical problems encountered in students' future careers, advocating for student-centered and problem-based teaching methods to bridge the gap between theoretical knowledge and clinical practice (Moreland 1996). Clinical decisions in medical diagnosis, prognosis, and treatment heavily rely on objective assessments conducted in clinical biochemistry laboratories. Technological advancements have streamlined biochemical analyses, enhancing the laboratory's significance in healthcare (Sönmez 2013).

Educational institutions in healthcare tailor curricula and strategies to facilitate active learning, enabling students to apply acquired knowledge comprehensively in their professional lives (Surapaneni 2010; Nair et al. 2013). Attitude and motivation are crucial in science education, influencing student success. Attitude towards a subject is a complex sensory attribute that guides behavior and decision-making, affecting learning outcomes (Huyugüzel Çavaş and Çavaş 2014; Najdi 2013). Educational institutions continually update teaching strategies to adapt to evolving needs, emphasizing the assessment of students' attitudes towards subjects as a vital aspect of curriculum design (Pehlivan and Köseoğlu 2011). In medical education, understanding students' attitudes towards biochemistry is essential for educators to enhance student engagement and foster positive attitudes towards the subject (Kaya 2013).

This study aims to develop a measurement tool to assess the attitudes of veterinary medical students towards Biochemistry. Given the lack of such a tool in the literature, this research seeks to address this gap by developing a specific measurement instrument.

MATERIAL and METHOD

In this study, validity and reliability procedures were conducted to develop a scale measuring the attitudes of veterinary medical students towards the Biochemistry course. The scale was designed in accordance with the five-point Likert scale model (Köklü, 1995) and the general survey model has been employed in this study. To ensure validity, content validity was assessed through expert opinion, while construct validity was evaluated using exploratory factor analysis (EFA). For reliability, the internal consistency of the scale was measured using Cronbach's Alpha coefficient. Both methods were applied to confirm the robustness of the scale before use.

Sample group

The study group comprised a total of 220 veterinary medical students who took the Biochemistry course at Afyon Kocatepe University Faculty of Veterinary Medicine during the 2017-2018 academic year. Among these students, 73 were female, and 147 were male. Kline (1994) suggested that a group of 100 people would be sufficient for the study (Pearson and Mundform, 2010). Therefore, the size of the sample has been decided as sufficient.

Scale Development Process

Item Pool Formation Stage

During this stage, research related to scale development was conducted by the project team. Based on a literature review and data obtained from student interviews, a pool of potential items for the scale was created. The scale was decided to be a five-point Likert scale, with ratings ranging from "Strongly Agree" to "Strongly Disagree" (Table 7). It has been ensured that a balanced number of positive and negative items, resulting in a draft scale consisting of 49 items.

Expert Consultation and Pilot Testing Stage

The questions in the item pool were evaluated by a panel of experts consisting 3 person in Biochemistry and curriculum development and assessment. Based on the experts' feedback on the items, the draft scale was revised, resulting in a 49-item scale comprising five dimensions. In the pilot testing stage, the scale was administered to 20 randomly selected veterinary medical students at Afyon Kocatepe University Faculty of Veterinary Medicine. Following the pilot testing, the clarity, comprehensibility, and completion time of the scale were assessed, and necessary adjustments were made to finalize the draft scale.

Final Testing Stage

The developed 49-item five-point Likert scale was administered to a total of 220 veterinary medical students who had taken the Biochemistry course at Afyon Kocatepe University Faculty of Veterinary Medicine during the 2017-2018 academic year. Students were informed about the research purpose of the administration and the importance of providing sincere responses regarding their attitudes towards the Biochemistry course. Subsequently, data analysis for scale development commenced with a sample size of 220.

A crucial aspect of factor analysis is the adequacy of the sample size for analysis. Different scholars have expressed varying opinions regarding the required sample size for factor analysis. For instance, while Nunnally (1978) suggests that the sample size should be ten times the number of items for factor analysis, Kass and Tinsley (1979) report the necessity for the sample size to be 5 to 10 times the number of items if the sample size is below 300. Comrey and Lee (1992) evaluate the sample size as weak if below 100, appropriate if around 200, good if around 300, very good if around 400, and excellent if 1000 or more.

Data Analysis

Validity Analyses

Factor analysis was employed for the validity assessment of the measurement tool in the study. Factor analysis is a multivariate analysis technique that elucidates many intercorrelations, structurally grouping correlated items into relatively independent factors. Exploratory factor analysis is conducted when attempting to reveal new structures or functional definitions of concepts using factor loading values of items (Büyüköztürk 2005).

Additionally, the suitability of data for factor analysis is determined by the Kaiser-Meyer-Olkin (KMO) measure and Bartlett's Test of Sphericity. The KMO test assesses the adequacy of sampling, ranging from 0 to 1, while Bartlett's Sphericity Test indicates the possibility of factor analysis if the significance value is below 0.05. Thus, if the KMO measure is above 0.6 and Bartlett's Test is significant, it suggests the suitability of data for factor analysis (Büyüköztürk 2008).

Reliability Analyses

The reliability of a test or measurement tool is associated with how accurately it measures what it is intended to measure. Cronbach's Alpha coefficient was utilized to assess the reliability of Likert-type scales. Cronbach's Alpha coefficient provides information regarding the internal consistency/homogeneity of the adapted scale and its subscales.

Data analysis in the study was conducted using statistical software, with a significance level set at < 0.05 for all statistical analyses.

RESULT

In order to determine the grouping of items (factors) primarily for the validity study, factor analysis was conducted. During the factor analysis, KMO and Bartlett values were determined, followed by principal component analysis, and finally, varimax rotation procedures were performed.

Evaluation of Suitability for Factor Analysis of the Data

The KMO measure obtained in this study is 0.794, indicating a high value. Additionally, the Bartlett's test of sphericity yielded a significant chi-square value, affirming the appropriateness of the data for factor analysis. Based on the results of the preliminary analysis conducted to determine the suitability of the data for factor analysis, the KMO value was found to be 0.794, and the Bartlett's Test of Sphericity yielded a significant result ($p < 0.05$). The chi-square value was 3691.043 with 1176 degrees of freedom (Df) (Table 1).

Table 1. Results of KMO and Bartlett Test

Kaiser-Meyer-Olkin (KMO) coefficient		0.794
Bartlett Sphericity Test	Approx. Chi-Square	3691.043
	Degree of freedom	1176
	Significance	0.000

Determination of Factor Number and Identification of Factor Items

The analysis of the scale consisting of 49 items was initiated through factor analysis. According to the obtained data, it was observed that some items in the scale had low factor loadings while others loaded onto multiple factors. Items with factor loadings less than 0.40 and a difference of less than 0.10 between the two largest values were excluded from the scale, resulting in 33 items being removed through factor analysis. To determine the number of factors that could indicate the correlation among items, the Scree plot graph, eigenvalues, and variance percentages

were utilized (Çokluk et al. 2012). Based on the main breaking points on the Scree plot graph, the scale was restricted to four factors. After determining the number of factors in the scale, the distribution of items across factors was investigated. The remaining items formed 4 factors. As a result of the item removal process, the contributions of the first, second, third, and fourth sub-dimensions to the total variance after rotation were 16.225%, 14.046%, 13.818%, and 11.130%, respectively. Through factor analysis, it was observed that the total variance explained by the 4 factors obtained was 55.217%.

To determine which factors exhibit strong correlations with the items, a rotated component matrix was constructed, and the congruence and factor loading values of the items were examined. The table and Scree plot graph regarding eigenvalues and variance percentages are provided below (Figure 1 and Table 3).

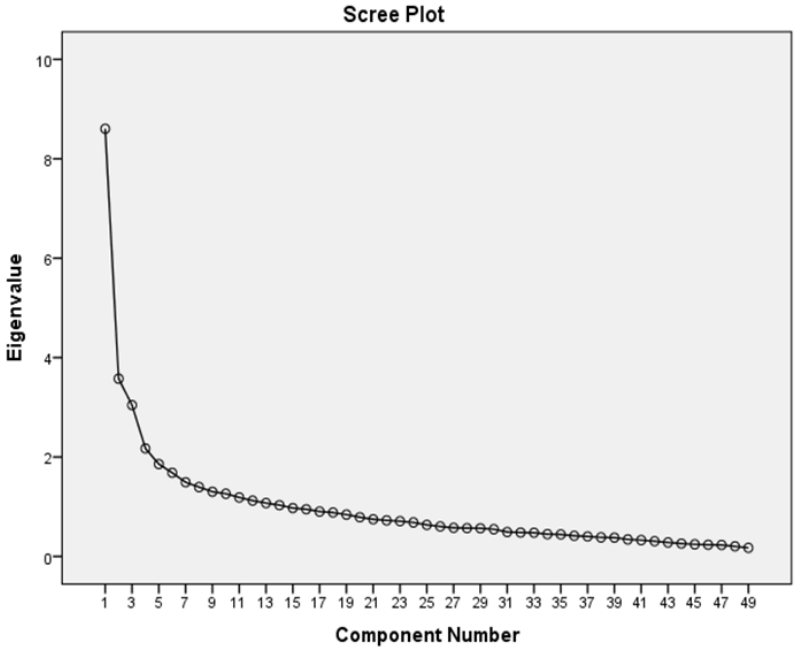

Figure 1: Line graph of attitude scale

In this study, the varimax (orthogonal rotation) method was employed for factor analysis. It is observed that the remaining items contribute to 55.217% of the total variance. Upon examining Table 2, it is noted that factor loadings range between .582 and .812. According to the analyses conducted, the first factor comprises items related to interest in the course, including items numbered 8, 14, 22, 9, and 13 (new item numbers: 1, 2, 3, 4, and 5), forming the interest subscale. The factor loadings of these 5 items range from .627 to .812, with item-total score correlations ranging from .328 to .632.

Additionally, the second factor pertains to expressions concerning laboratory work in the course, encompassing items numbered 43, 47, 26, and 37 (new item numbers: 6, 7, 8, and 9), forming the Application subscale. The factor loadings of these 4 items range from .582 to .807, with item-total score correlations ranging from .259 to .385.

Furthermore, the third factor addresses concerns related to the course, comprising items numbered 4, 5, 2, and 35 (new item numbers: 10, 11, 12, and 13), forming the Anxiety subscale. The factor loadings of these 4 items range from .623 to .809, with item-total score correlations ranging from .328 to .372.

Lastly, the fourth factor concerns the importance attributed to the course, consisting of items numbered 24, 15, and 16 (new item numbers: 14, 15, and 16), forming the Importance subscale. The factor loadings of these 3 items range from .618 to .786, with item-total score correlations ranging from .203 to .312.

As presented in Table 2, the contributions of the factors to the total variance are as follows: 16.225% for the first factor, 14.046% for the second factor, 13.817% for the third factor, and 11.130% for the fourth factor, totaling 55.217%. Acceptance of a variance explained between 40% and 60% is deemed sufficient in multifactorial designs (Çokluk et al. 2012).

Table 2. Rotated component matrix table of the attitude scale

Item Pool No	New Item No	F1	F2	F3	F4	Item Total Correlation	Common Factor Variance
M8	**1**	.812				.409	**.670**
M14	**2**	.717		.328		.448	**.635**
M22	**3**	.639				.366	**.447**
M9	**4**	.632	.321			.632	**.610**
M13	**5**	.627				.328	**.429**
M43	**6**		.807			.335	**.657**
M47	**7**		.733			.259	**.553**
M26	**8**		.693			.385	**.518**
M37	**9**		.582			.356	**.462**
M4	**10**			.809		.328	**.675**
M5	**11**		.464	.662		.344	**.501**
M2	**12**	.343	.370	.653		.372	**.606**
M35	**13**			.623		.344	**.434**
M24	**14**				.786	.203	**.630**
M15	**15**				.690	.312	**.537**
M6	**16**		.787		.618	.287	**.471**
Eigenvalue		3.685	1.939	1.752	1.458	**Total variance**	**55.217**
Explained Variance		**16.225**	**14.046**	**13.817**	**11.130**		

Reliability Findings for the Scale

Reliability analyses for the subscales and the overall scale, based on the final version comprising 16 items, are presented in Table 3. Upon examining Table 3, the Cronbach's alpha coefficient for the 1st factor is 0.757, for the 2nd factor it is 0.701, for the 3rd factor it is 0.674, and for the 4th factor it is 0.575. The total Cronbach's alpha value for the scale is 0.761.

Table 3. Number of items and Cronbach's alpha reliability coefficients for the attitude scale

Factors	Item Count	Cronbach's Alpha
1st Factor	5	0.757
2nd Factor	4	0.701
3rd Factor	4	0.674
4th Factor	3	0.575
Total	16	0.761

The environmental and correlation coefficients related to the factors in the attitude scale towards biochemistry course for veterinary medicine students are provided in Table 4. In Table 4, the second, third, and fourth factors show positive low-level significant correlations of .309, .304, and .181, respectively, with the total score, indicating a significantly reliable positive relationship of .752. The first, third, and fourth factors exhibit positive low-level significant correlations of .309, .129, and .156, respectively, with the total score, showing a significantly reliable positive relationship of .608. The first, second, and fourth factors demonstrate positive low-level significant correlations of .304, .129, and .218, respectively, with the total score, indicating a significantly reliable positive relationship of .659. The first, second, and third factors reveal positive low-level significant correlations of .181, .159, and .218,

respectively, with the total score, indicating a significantly reliable positive relationship of .536.

Table 4. Averages and correlation coefficients for sub-factors of the attitude scale

Factors	N	1st Factor	2nd Factor	3rd Factor	4th Factor	X	S.D.
1st Factor	220	-	.309	.304	.181	12.33	3.86
2nd Factor	220	.309	-	.129	.156	14.05	3.06
3rd Factor	220	.304	.129	-	.218	11.21	3.39
4th Factor	220	.181	.156	.218	-	8.60	2.57
Total	220	0.752	0.608	0.659	0.536	46.20	8.39

The arithmetic mean values for participants' responses to factors and the total score in the developed scale were 12.33, 14.05, 11.21, 8.60, and 46.20, respectively, with standard deviation values of 3.86, 3.06, 3.39, 2.57, and 8.39.

The top and bottom 27% group averages were determined based on the total score. Upper 27% (n = 59) and lower 27% (n = 59) groups were formed. A t-test was conducted to examine significant differences between the identified groups. The results regarding the discriminative capacity of the 16-item test are presented in Table 5. Based on the results presented in Table 5, it was found that all items were statistically significant at the 0.05 level between the upper and lower groups.

Table 5. Results of t-test for item means of scale items in lower and upper groups

Item Pool No	Groups	N	X	S.D.	t	p
M2	Upper Group	59	1.97	1.098	-6.620	.00
	Lower Group	59	3.34	1.154		
M4	Upper Group	59	2.10	1.140	-6.785	.00
	Lower Group	59	3.56	1.193		
M5	Upper Group	59	2.56	1.134	-6.159	.00
	Lower Group	59	3.80	1.047		
M6	Upper Group	59	2.31	1.193	-4.600	.00
	Lower Group	59	3.25	1.044		
M8	Upper Group	59	1.24	.506	-8.665	.00
	Lower Group	59	2.64	1.141		
M9	Upper Group	59	1.80	.761	-12.188	.00
	Lower Group	59	3.54	.795		
M13	Upper Group	59	2.02	1.042	-6.173	.00
	Lower Group	59	3.17	.985		
M14	Upper Group	59	1.78	.832	-9.919	.00
	Lower Group	59	3.39	.929		
M15	Upper Group	59	2.12	1.052	-5.838	.00
	Lower Group	59	3.37	1.272		
M22	Upper Group	59	1.61	.851	-7.135	.00
	Lower Group	59	3.02	1.252		
M24	Upper Group	59	2.49	1.165	-3.809	.00
	Lower Group	59	3.36	1.297		
M26	Upper Group	59	3.00	1.203	-6.020	.00
	Lower Group	59	4.12	.768		
M35	Upper Group	59	2.03	1.098	-6.613	.00
	Lower Group	59	3.31	.987		
M37	Upper Group	59	2.93	.980	-6.549	.00
	Lower Group	59	4.10	.959		
M43	Upper Group	59	2.97	1.231	-5.453	.00
	Lower Group	59	4.07	.944		
M47	Upper Group	59	2.90	1.199	-5.218	.00
	Lower Group	59	3.92	.896		

Naming of Factors

Upon evaluating the results in Table 6, it was observed that the items grouped under Factor 1 pertained to interest, those under Factor 2 related to application, those under Factor 3 reflected anxiety, and those under Factor 4 were indicative of the importance attributed to the course. Accordingly, these factors were named. The total score from the scale and its subscale are commented according to the highest and lowest point. Higher point shows a higher attitude and the lower point shows lower attitudes.

Table 6. Naming of factors

	ATTITUDE SCALE TOWARDS BIOCHEMISTRY COURSE AMONG VETERINARY MEDICINE STUDENTS	Strongly Agree	Agree	Undecided	Disagree	Strongly Disagree
INTEREST						
1	I am considering pursuing postgraduate education in the Department of Biochemistry in the future.	□	□	□	□	□
2	I believe I am more eager to study for the Biochemistry course compared to other courses.	□	□	□	□	□
3	I think the hours allocated for the Biochemistry course are insufficient.	□	□	□	□	□
4	I enjoy reading studies related to Biochemistry.	□	□	□	□	□
5	I feel upset when I cannot attend or will not be able to attend the Biochemistry course.	□	□	□	□	□
PRACTICAL CLASS						
6	I believe that experiments conducted in the laboratory during practical sessions are beneficial for Biochemistry.	□	□	□	□	□
7	I feel that I am doing an important job when conducting experiments in the laboratory on Biochemistry topics.	□	□	□	□	□
8	I think that the practical hours of the Biochemistry course increase the efficiency of the course.	□	□	□	□	□
9	I believe that the knowledge I have gained in clinical biochemistry facilitates my understanding of the procedures and reasons for diagnosis and treatment of diseases.	□	□	□	□	□
ANXIETY						
10	I think Biochemistry is one of the subjects I am very afraid of.	□	□	□	□	□
11	Attending Biochemistry class makes me nervous.	□	□	□	□	□
12	Biochemistry class doesn't scare me.	□	□	□	□	□
13	No matter how much I study, I fail in Biochemistry exams.	□	□	□	□	□
IMPORTANCE						
14	I don't believe that what I learn in Biochemistry class will be useful in my medical profession.	□	□	□	□	□
15	I cannot establish a connection between the theoretical knowledge I learned in Biochemistry class and treating patients in practice.	□	□	□	□	□
16	I think other courses in Veterinary Medicine education are more important than the Biochemistry course.	□	□	□	□	□

Table 7. Score range of the developed scale

Range	Option
1.00-1.80	Strongly Disagree
1.81-2.60	Disagree
2.61-3.40	Undecided
3.41-4.20	Agree
4.21-5.00	Strongly Agree

DISCUSSION

The significant value of the KMO measure and the Bartlett's test of sphericity indicates that the factor analysis can continue to refine the scale development. With a chi-square value of 3691.043 and Df of 1176, the data demonstrate suitability for exploratory factor analysis.

Upon examining the scree plot graph, where eigenvalues are depicted on the vertical axis and

factors on the horizontal axis, it is observed that after the fifth point, the steep decline in variance contribution diminishes. The downward trend from the first point illustrates the degree of contribution to variance with points, and each interval between two points represents a factor (Çokluk et al. 2012). Even after the fifth point, the contributions of components to variance decrease, and additional variances' contributions appear to be close to each other.

Upon reviewing the factor loadings in the developed scale, it can be observed that all items exhibit high factor loading values. The scale comprising four factors reflects the headings formed as a result of literature review during the scale development stages.

Bayram (2004) reported that a Cronbach's Alpha value above 0.70 is sufficient for reliability. The analysis revealed a significant and positive relationship between groups at a highly reliable level. Thus, it can be concluded that the developed scale exhibits a highly reliable level of reliability.

When examining the mean and correlation coefficients for the Subfactors of the Attitude Scale, it is evident that there is a significant correlation between the developed scale and the factors themselves, as well as with the total score. These findings serve as evidence of construct validity.

CONCLUSION

In this study, a 49-item measurement tool developed to assess the attitudes of veterinary faculty students towards the biochemistry course was initially prepared as a pilot form for content validity by consulting experts. The pilot form, comprising 49 items, was administered to 20 veterinary faculty students to assess comprehensibility, grammar, and adequacy of response time, and necessary adjustments were made thereafter. Subsequently, the pilot form of the scale was administered to 220 students currently attending the veterinary faculty. Factor analysis was performed for the data collected through the scales, resulting in 16 items collected under four factors in the scale. In addition, reliability coefficients (Cronbach's α) for the entire scale and sub-dimensions, discriminative validity analyses at the item level, and inter-factor correlation analyses were calculated. According to the eigenvalue criterion in the developed biochemistry course attitude scale, the total variance explained by the four significant factors is 55.217%. Following Varimax rotation analysis, the factor loadings of the items range between .582 and .812. The identified factors were named as "interest," "application," "anxiety," and "importance," respectively. The Cronbach's α coefficient for the entire scale was calculated as 0.761, indicating the overall consistency of the scale since Cronbach's α coefficients above 0.70 signify the scale's internal consistency. The statistically significant differences found for all items in the discriminative analysis across the entire scale ($p < .05$) demonstrate the discriminative nature of the item scores. The positive significant correlation observed among all factors and between all factors and the total score in the correlation analysis indicates that all factors in the scale share the same underlying structure. Based on all these validity and reliability procedures, it is concluded that this scale is a valid and reliable measurement tool that can be used to determine veterinary faculty students' attitudes towards the biochemistry course.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: AFF and İO contributed to the project idea, design and execution of the study. AFF, İO and GA contributed to the acquisition of data. AFF and İO analysed the data. AFF, İO, GA, and BD drafted and wrote the manuscript. AFF, İO, GA, and BD reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

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

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RESEARCH ARTICLE

Students and Veterinarians Views About Clinical Anatomy and Topographic Anatomy Courses in Veterinary Education

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ABSTRACT

In this study, it was aimed to evaluate the views of veterinarians and students about the topographic anatomy course and clinical anatomy course with the help of a survey. For this purpose, 4 separate surveys were created, 2 for the topographic anatomy course and 2 for the clinical anatomy course. For each course, one of these forms was used to the 3rd, 4th, and 5th grade students in Veterinary Medicine. Other survey forms were applied to veterinarians working in different regions in Turkey. As a result, 98% of the participants emphasized that anatomy knowledge was important for safe and adequate clinical practice. 59% of the students stated that the contents of the topographic anatomy course were not sufficient in this form, 92.3% stated that it should be explained with radiological images and, 96.2% explained with clinical sciences. Only 35.5% of veterinarians stated that the information they learned in the systematic anatomy course was sufficient for clinical practices. 92.1% of veterinarians stated that clinical anatomy courses should be present in the curriculum in recent years. According to the results, after taking systematic anatomy course, it was observed that it was a necessity to take the anatomy course associated with clinical sciences during the later periods.
Keywords: Anatomy, Course, Survey

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ÖZ

Veteriner Hekimliği Eğitiminde Klinik Anatomi ve Topografik Anatomi Derslerine İlişkin Öğrenci ve Veteriner Hekim Görüşleri

Bu çalışmada Veteriner hekimlerin ve öğrencilerin topografik anatomi dersi ve klinik anatomi dersi hakkındaki görüşlerinin anket yardımıyla değerlendirilmesi amaçlandı. Bu amaçla topografik anatomi dersi için 2, klinik anatomi dersi için 2 olmak üzere toplam 4 ayrı anket formu oluşturuldu. Veteriner Fakültesi 3., 4. ve 5. sınıf öğrencilerine her ders için bu formlardan biri uygulandı. Diğer anket formları ise Türkiye'nin farklı bölgelerinde görev yapan Veteriner hekimlere uygulandı. Sonuç olarak katılımcıların %98'i güvenli ve yeterli klinik uygulama için anatomi bilgisinin önemli olduğunu vurguladı. Öğrencilerin %59'u bu formda topografik anatomi ders içeriğinin yeterli olmadığını, %92,3'ü radyolojik görüntülerle, %96,2'si klinik bilimlerle zenginleştirilmesi gerektiğini belirtti. Veteriner hekimlerin sadece %35,5'i sistematik anatomi dersinde öğrendikleri bilgilerin klinik uygulamalar için yeterli olduğunu belirtti. Veteriner hekimlerin %92,1'i son yıllarda müfredatta klinik anatomi dersinin yer alması gerektiğini belirtti. Elde edilen sonuçlara göre zorunlu (sistematik) anatomi dersini alındıktan sonra daha sonraki dönemlerde klinik bilimlerle ilişkili anatomi dersinin verilmesinin bir gereklilik olduğu kanaatine varıldı.
Anahtar Kelimeler: Anatomi, Anket, Ders

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INTRODUCTION

According to the teaching methods, anatomy course is divided into subgroups such as systematic anatomy, clinical anatomy, radiological anatomy, and topographic anatomy. The systematic anatomy course examines the anatomical structure of domestic animals under the topics of locomotion, digestion, respiration, circulation, urogenital and nervous systems, and sensory organs (Demiraslan and Dayan 2021). Topographic anatomy course examines the anatomical formations regionally that make up the animal body. Here, the head, neck, chest, abdomen, dorsal areas, and front and hind limb areas are examined from superficial to profound (Dursun 2002). Radiological Anatomy is a form of topographic anatomy that has emerged with the development of technology and is increasingly important. Computed tomography, nuclear magnetic resonance imaging, positron emission tomography, and scintigraphy images, which are new methods used in the diagnosis of various diseases, are examined. These images are obtained by dividing the body from different angles into thin slices of a few millimeters (Gülekon 2017). Clinical anatomy is the branch of anatomy in which information covering systematic, topographic, and radiological anatomy is processed (Demiraslan and Dayan 2023).

Education is a process that includes mobility in its structure and therefore it changes from time to time. What form of education should be provided in veterinary faculties and which education models would be more effective are still being discussed. Efforts to improve anatomy education, which occupies an important place in veterinary medicine education, continue in Veterinary Faculties (Gültiken 2012; Gürbüz et al. 2013; Beresheim et al. 2024). Another important problem is that the boundaries of the course content in anatomy education can be drawn and what kind of anatomy education program is required can be defined by clinicians and students as well as anatomists (Phillips 1987). Anatomy education aims to prepare the student for clinical sciences and to integrate clinical sciences and education. For this reason, it is important how anatomy courses will be taught in undergraduate education (Akkoç et al. 2021).

Anatomy education is necessary to understand and solve clinical problems and diagnostic imaging methods. In recent years, radiological images and clinical anatomical problems have been frequently included in classical anatomy textbooks (König and Liebich 2004; Drake et al. 2005; Dyce et al. 2009; Demiraslan and Dayan 2023). Combining anatomy education with clinical information and radiological images is becoming increasingly important as it enables students to better understand anatomy and gives them an idea of what they may encounter in the future (Carmichael and Pawlina 2000).

The opinions of students and experts regarding education are taken into consideration. Therefore, survey-based information is considered important as a guide in shaping education (Ramalingaswami 1987). Laakkonen (2021) stated that, unlike medical education, there is little survey-based information on the use of art as a learning approach in veterinary anatomy. In this prospective, survey-based study, it was aimed to evaluate the opinions of students and veterinarians about the Topographic Anatomy and Clinical Anatomy course, which is given after receiving basic anatomy training, with the help of a survey.

MATERIALS and METHODS

Ethical Approval

The necessary permission is given to Burdur Mehmet Akif Ersoy University Ethics Committee of Non-Entrepreneurial Clinical Research (01.03/2023, The situation of Topographic Anatomy course in anatomy education: Student and Veterinarian opinions: Decision No: GO 2023/138. Student and Veterinarian opinions about Clinical Anatomy: Decision No: GO 2023/139).

Survey

In the study, a total of 4 separate surveys were created, 2 for the topographic anatomy course and 2 for the clinical anatomy course that is given after the basic anatomy course. For each course (Topographic anatomy and clinical anatomy), one of these forms was applied to 3rd, 4th and, 5th grade students (n = 160) studying at Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine. Other survey forms prepared separately for each course were applied to veterinarians (n=140) working in different regions of Turkey. The veterinarians who participated in the survey forms were randomly selected from among volunteers and those who were actively practicing medicine, and the students were randomly selected from among volunteers. The veterinarians stated that their field of work was 49.3% clinic/hospital, 24% public, 14.7% university, and 13.3% private sector. The questions in the survey forms were created by us using the literature (Arı and Şendemir 2003; Uygur et al. 2013; Gülekon 2017; Akkoç et al. 2021).

The first part of all survey forms included information about the definitions and course contents of systematic anatomy, topographic anatomy, radiological anatomy and, clinical anatomy. The survey forms were administered to the participants in 2023.

Survey for Topographic Anatomy Course

The survey prepared for students consisted of a total of 26 questions, including 1 demographic question, 1 open-ended question, 22 multiple-choice (agree,

undecided, disagree) questions and, 2 multiple-choice questions. The survey prepared for veterinarians consisted of a total of 24 questions, including 1 demographic question, 21 options (agree, undecided, disagree), 1 multiple-choice question and 1, open-ended question.

Survey for Clinical Anatomy Course

The survey prepared for the students consisted of a total of 17 questions, including 1 demographic question, 1 open-ended question, and 15 questions with options (agree, undecided, disagree). The survey prepared for veterinarians was composed of a total of 19 questions, including 1 demographic question, 17 options (agree, undecided, disagree) questions and, 1 open-ended question.

Collection of Data

All surveys prepared for clinical anatomy and topographic anatomy courses were administered to students and veterinarians via Google Forms. However, not all participants answered some of the questions in the survey.

Analysis of Data

Descriptive statistical values of the data were determined with the help of Google Forms.

RESULTS

As a result of the study, the opinions of veterinarians and students about the topographic anatomy and clinical anatomy courses given after the basic anatomy course were collected and each course was evaluated separately.

Results for Topographic Anatomy Course

98% of veterinarians (Vets) and 92% of students (Sts) supported the idea that anatomy is important for the clinic. 61% of Vets said, "I can make a radiologic evaluation with my knowledge of anatomy". Accordingly, the majority of participants approved that knowledge of anatomy is important for clinical sciences.

47% of Vets and 31% of Sts said, "The anatomical knowledge I have learned is sufficient for my specialization." However, it was noteworthy that 96.3% of vets stated that they felt the need to review their anatomy knowledge from time to time in their professional lives. Accordingly, it was determined that the anatomy knowledge learned during the undergraduate period was not sufficient for the majority of the participants.

The majority of participants stated that topographic anatomy (Vets: 72%, Sts: 48%), clinical anatomy (Vets: 85%, Sts: 59%) and radiological anatomy courses (Vets: 90%, Sts: 64%) should be taught compulsorily after taking the basic anatomy course. Among these courses, there was more interest in clinical anatomy and radiological anatomy courses.

Students agree with this "The abdomen and pelvis should be described in the most detail, followed by the chest and back". Accordingly, it was seen that it was necessary to teach abdomen and pelvis subjects in more detail.

Table 1 shows the opinions of veterinarians and students about the topographic anatomy course. It was concluded that the years in which the topographic anatomy course was given should be reconsidered and that the topographic anatomy course should be taught in the clinical years.

Results for Clinic Anatomy Course

35% of Vets and 56% of Sts stated that the information they learned in the systematic anatomy course was sufficient. 63% of Vets and 28% of Sts stated that another anatomy course should be given after the aystematic (basic) anatomy course. In this regard, it can be seen that the majority of students say that another anatomy course is not needed. This situation is normal due to concerns such as extra course load. Considering the experiences of Vets, it seems that after the systematic (basic) anatomy course, another anatomy course is needed in the future.

61% of Sts said that clinical anatomy should be taught instead of systematic anatomy. 38% of Vets stated that a topographic anatomy course should be taught instead of systematic anatomy, and 43% stated that a clinical anatomy course should be taught instead of topographic anatomy. Accordingly, the rate of choosing the clinical anatomy course was higher.

4% of Vets and 9% of Sts stated that they saw no difference between clinical anatomy and topographic anatomy. It was understood that the participants had sufficient knowledge about the contents of the courses. This was an important determination of the reliability of the answers. 18% of Vets and 28% of Sts stated that the information they learned in the topographic anatomy course was sufficient for clinical sciences. Accordingly, the majority of the participants did not find the content of the topographic anatomy course sufficient in terms of clinical anatomy. However, 88% of Vets and 75% of Sts stated that they would prefer the clinical anatomy course if it was an elective course.

Almost all of the participants (except for 2%) stated that practices of clinical anatomy should be performed on live animals. This was a significant rate (98%). This showed that it was necessary to provide anatomy education on live animals.

71% of Vets and 51% of Sts think that the clinical practices of a veterinarian who has not received clinical anatomy training are inadequate. However, most of the participants (Vets: 69%, Sts: 75%) said, "If I had a choice, I would deliver an adopted animal to a veterinarian who has taken a clinical anatomy course." Accordingly, it was seen that the clinical anatomy course was necessary in clinical practice.

However, a significant majority of the participants (Vets: 92%, Sts: 84%) stated that the clinical anatomy course should be included in the curriculum in the last semesters of education.

Sts stated that the subjects that should be explained in most detail in the topographic anatomy course should be the abdomen and pelvis regions (87.2%), chest and dorsal regions (62.8%), head-neck regions (44.9%) and leg regions (33.3%), respectively. However, it was stated that the best-learned subjects were the head and neck regions (48.7%), abdomen-pelvis regions (47.4%), chest-dorsal regions (46.2%) and leg regions (32.1%), respectively.

Table 2 shows the opinions of veterinarians and students about the clinical anatomy course. When all survey results were evaluated, it was determined that clinical anatomy and radiological anatomy courses are important courses for professional life and are expected to be in the teaching plan in the last semesters of education.

Table 1. Opinions of veterinarians (Vets) and students (Sts) about the topographic anatomy course given after taking the basic anatomy courses (-: not asked in the survey)

Survey	Vets (%)	Sts (%)
Anatomical knowledge (education) is important for safe and adequate medical (clinical) practice.	98.8	92.3
In my professional life, I need to review anatomy information from time to time.	96.3	-
I can make radiological evaluation with my knowledge of anatomy.	61.3	12.5
The anatomical information I have learned is sufficient for my expertise.	47.5	27.5
After taking the basic anatomy course, a Veterinarian who has not taken the topographic anatomy course can practice her/his profession.	37.5	51
Topographic anatomy courses should be given compulsory in the future after the basic anatomy course.	72.5	-
After the basic (systematic) anatomy course, I choose topographic anatomy courses as an elective course.	-	48.7
Clinical anatomy courses should be given compulsorily after the basic anatomy course.	85	-
After the basic anatomy course, I choose clinical anatomy courses as an elective course.	-	59
Radiological anatomy courses should be given compulsorily after the systematic anatomy course.	90	
After the basic anatomy course, I choose radiological anatomy courses as an elective course.	-	64.1
Topographic anatomy course should be taught in conjunction with clinical sciences.	92.5	96.2
As a result of the topographic anatomy training, I can reconcile clinical sciences.	-	51.3
Topographic anatomy course should be taught in conjunction with radiological images.	95	92.3
As a result of the topographic anatomy training, I can reconcile radiological images.	-	37.2
The information I learned in the Topographic Anatomy course is sufficient for my professional life.	-	30.8
Topographic anatomy course should be taught with maximum detail.	48.8	43.6
Topographic anatomy course should be given in the 3rd or 4th educational years (clinical years).	61.3	42.3

Table 2. Opinions of Veterinarians (Vets) and students (Sts) about the Clinical Anatomy course (-: not asked in the survey)

Survey	**Vets (%)**	**Sts (%)**
The knowledge I have learned in basic (systematic) anatomy is sufficient.	35.5	56.3
Another anatomy course is needed after basic (systematic) anatomy	63.2	28.1
Clinical Anatomy should be taught instead of basic (systematic) anatomy	38.2	60.9
Clinical Anatomy should be taught instead of topographic anatomy	43.4	-
The information I received in the topographic anatomy course is sufficient for clinical practices	18.4	28.1
There is no difference between clinical anatomy and topographic anatomy courses	3.9	9.4
If it were an elective course, I would definitely choose the clinical anatomy course.	88.2	75
The practices of clinical anatomy on live animals are required	98.7	-
The clinical practices of a veterinarian who has not taken a clinical anatomy course will be inadequate.	71.1	51.6
If I had a choice, I would hand over an adopted animal to a veterinarian who took a clinical anatomy course.	68.4	75
Clinical anatomy course should definitely be included in the curriculum in the last semesters of education.	92.1	84.1

DISCUSSION

Feedback to prove the quality and efficiency of education and to correct any deficiencies that may exist has a very important place. For this reason, the feedback method is frequently used in that it aims to obtain successful results in education by taking students' opinions and thoughts about education (Erpek et al. 2002). In our study, questions were asked to the participants including students and veterinarians about topographic and clinical anatomy courses and course contents. Thus, the need and necessity of topographic and clinical anatomy courses in anatomy education, which have an important place in veterinary education, were examined. Since there are a limited number of survey studies on veterinary anatomy courses in the literature, data from the study were compared with the survey results regarding anatomy education in Medical Faculties.

In the teaching plan of Veterinary Faculties, basic (systematic) anatomy course is included in the 1st and 2nd education years. In these years, the practice of anatomy is given using cadavers, models or, various simulations. In the 4th and 5th education years, students are taught clinical courses such as surgery, internal medicine, obstetrics and gynecology. To learn and apply clinical skills during these years, clinical practices of previously learned anatomy is needed. Literature about medical education shows that long-term retention of anatomical information is low in interns. Therefore, reteaching the anatomy course during clinical years was perceived as beneficial by students (Gülekon 2017). Congara et al. (2017) presented a survey about the benefit of reteaching anatomy during the clinical period of education in veterinary education. Congara et al.19 stated that the majority of the students agreed that anatomical knowledge was necessary for clinical education, and 71% of interns would prefer anatomy to be retaught if offered as a private course. In the study conducted, similar to the findings of Congara et al. (2017), the majority of the participants stated that the clinical anatomy course should be included in the teaching plan in recent years of education and stated that they would choose the clinical anatomy course if it were an elective course.

In a study conducted at the Faculty of Medicine (Arı and Şendemir 2003), students (52.3%) stated that the information they learned in anatomy education was insufficient for their clinical years and future. However, Ayademi and Ojeifo (1988) stated in their study that students had similar attitudes. Similarly, in the study, only 35% of veterinarians stated that the knowledge they learned in anatomy courses was sufficient for their future.

It is very important to integrate basic anatomy knowledge with clinical knowledge to learn and understand anatomy (Patel and Dauphinee 1984; Crisp 1989; Baciewicz et al. 1990). Because uncertainty in anatomical information affects the confidence and decision-making process of veterinarians working in the clinic (Wheble and Channon 1995). In this regard, teaching anatomy by associating it with a clinical case/case ensures that the subject is learned in the best way and improves the student's ability to do self-research. In this study, the majority of the participants stated that topographic anatomy courses should be taught in association with clinical and radiology anatomy.

Torres et al. (2016) organized a cross-sectional radiological anatomy course for medical students. While the success rate before the course was 56.2%, the success rate after the course was 75%. Again, after the course, 92% of the students stated that the course was more useful in learning anatomical and topographic structures, 90% stated that their anatomical knowledge improved, and 92% stated that this information would be useful to them in the future. Moscova et al. (2015) organized radiological anatomy (Radiography, ultrasonography, CT, and MRI) courses for 1st and 2nd-grade students. At the end of the course, 48-63% of the students stated that there was an increase in their understanding of the lessons, 72-77% stated that cross-sectional anatomy made it easier for them to learn anatomical information, and 76-80% stated that they could use this information in their future lives. Schober et al. (2014) in a survey conducted at the faculty of medicine, stated that 80% of the students stated that clinical and radiological information made it easier to understand anatomy courses. In the study, the expectations of students and vets were similar to the researchers found (Schober et al. 2014; Moscova et al. 2015; Torres et al. 2016;).

After training the basic anatomy at the faculty of medicine, a survey was conducted on the necessity of Radiological and Clinical anatomical information during the clinical years (Gülekon 2017). As a result of this study (Gülekon 2017) the majority of students stated that Clinical and radiological anatomy courses increased their motivation and helped them understand the anatomy better. Interns requested that these courses should be given as a refresher course during clinical periods. Uygur et al. (2013) stated in their study that anatomical information such as radiological anatomy, cross-sectional anatomy, and clinical anatomy should be added to the anatomy course content. In our study, it was determined that the expectations of the participants were similar to the literature. Regarding this, vets and students stated that after taking the basic anatomy course, the radiological anatomy (Vets: 90%, St: 64%) and clinical anatomy courses (Vets: 85%, St: 59%) should be taught during their clinical years.

There is a lack of guidance for educators regarding the level of anatomical knowledge required for a graduated veterinarian to be considered competent. Gummery et al. (2023) conducted a detailed study on veterinary anatomy learning outcomes to support curriculum development in this field. The study

results include eliminating unnecessary details and focusing more on the relevance of the competencies required for the new graduate. However, in the learning outcomes prepared separately according to systematical anatomy, it is emphasized that it is important for students to be able to define the functional properties of anatomical structures, their topographic relationships, their clinical significance, and the normal features of radiological images. Similarly, in the study, the majority of the participants stated that the current topographic anatomy course should not be taught in detail. However, it has been determined that it is necessary to add clinical and radiological anatomy courses to the Veterinary Anatomy curriculum in the clinical years after basic anatomy education.

CONCLUSION

As a result of the study, in the survey conducted about the content, need, and necessity of the topographic anatomy and the clinical anatomy courses given or recommended to be given after taking the basic anatomy course, it was determined that the following items were expected by the participants.

1. Arrangements regarding the years in which the topographic anatomy course is given should be made in such a way that it will be added to the education plan in the last periods of education (clinical years).
2. The content of the topographic anatomy course should be revised to include radiological anatomy and clinical sciences.
3. In the last periods of education (clinical years), the clinical and radiological anatomy courses must be included in the teaching plan.
4. Comparative anatomy training should be given to live animals.

We believe that the success of veterinarians to be trained will increase by enriching the contents of anatomy courses to be given in the following years after receiving basic anatomy training with imaging methods and clinical sciences.

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

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RESEARCH ARTICLE

Spatial Analysis of Honey Yield by Province Based on Registered Honey Production Data: Exploring Spatial Patterns

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ABSTRACT

Throughout history, bees and their products have gained ever-increasing importance. Honey, valued for its diverse uses, has driven the emergence of new industries and products, fueled by the growing demand for beekeeping as a sustainable source of income. Providing spatial information is very important to ensure the sustainability of products. Therefore, this study aimed to reveal the spatial distribution of honey yield outputs in Türkiye using exploratory spatial data analyses. Honey yield outputs in four time periods (2005–2010; 2011–2016; 2017–2022; 2005–2022) were tested by autocorrelation analysis, and Moran's I scatter plot was produced for each period. Standard Z statistics were found to be 4.1064, 3.1910, 2.1980 and 3.4427, respectively ($p<0.05$). Results showed that, it was observed that there are spatial associations and different spatial clusters in honey yield at the provincial level in Türkiye. It has been shown that honey yield in Türkiye tends to be partially clustered and production outputs tend to decrease in the east. This analysis implies several consequences for the sustainability of bee-based food production, including the potential for spillover effects from hot spot regions and the need to prioritize resource allocation towards these areas.

Key Words: Beekeeping, Honey, Spatial Autocorrelation, Spatial Pattern, Türkiye

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Kayıtlı Bal Üretim Verilerine Dayalı İl Bazında Bal Veriminin Mekânsal Analizi: Mekânsal Kalıpların Keşfedilmesi

ÖZ

Tarih boyunca arılar ve ürünleri giderek artan bir önem kazanmıştır. Çeşitli kullanım alanları nedeniyle değer verilen bal, sürdürülebilir bir gelir kaynağı olarak arıcılığa yönelik artan taleple desteklenen yeni endüstrilerin ve ürünlerin ortaya çıkmasına neden olmuştur. Ürünlerin sürdürülebilirliğini sağlamak için mekânsal bilgi sağlamak çok önemlidir. Bu nedenle, bu çalışma açıklayıcı mekânsal veri analizleri kullanarak Türkiye'deki bal verimi çıktılarının mekânsal dağılımını ortaya koymayı amaçlamıştır. Dört zaman periyodundaki (2005–2010; 2011–2017; 2017–2022; 2005–2022) bal verimi çıktıları otokorelasyon analizi ile test edilmiş ve her periyot için Moran'ın I saçılım grafiği üretilmiştir. Standart Z istatistikleri sırasıyla 4,1064, 3,1910, 2,1980 ve 3,4427 olarak belirlenmiştir ($p<0.05$). Sonuçlar, Türkiye'de il düzeyinde bal veriminde mekânsal birliktelikler ve farklı mekânsal kümelenmeler olduğunu göstermiştir. Türkiye'de bal veriminin kısmen kümelenme eğiliminde olduğu ve doğuda üretim çıktılarının azalma eğiliminde olduğu gösterilmiştir. Bu analiz, arı bazlı gıda üretiminin sürdürülebilirliği açısından, sıcak nokta bölgelerinden yayılma etkileri potansiyeli ve kaynak tahsisinin bu alanlara önceliklendirilmesi ihtiyacı dahil olmak üzere çeşitli sonuçlara işaret etmektedir.

Anahtar Kelimeler: Arıcılık, Bal, Mekânsal Otokorelasyon, Mekânsal Desen, Türkiye

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INTRODUCTION

The beekeeping industry in Türkiye plays a significant role in the country's economy, with exports representing a significant proportion of its overall value. The notable increase in beekeeping activities observed in recent years is indicative of this trend (Çevrimli and Sakarya 2018; Polat et al. 2023). The main product of beekeeping activities is honey. Honey has rich nutritional value and is a product that can be used in alternative medicine, cosmetics, and many other areas (Haydak 1970; Denisow and Denisow-Pietrzyk 2016). Bees are mysterious creatures that can offer us different products and services at every stage of life (Wratten et al. 2012; Sonmez Oskay et al. 2023). Beekeeping activities contribute to regional development in the economy across numerous developing nations through the production of items including honey, beeswax, royal jelly, pollen and bee venom which are crucial for agricultural pollination and human health (Lee et al. 2008; Wright et al. 2018; Sarı et al. 2020). There are many unexplored benefits offered by beekeeping, including therapeutic, apitherapy, tourism, gastronomy and support for ecological health (Şahingöz and İnci 2018; Bozkurt 2019; Onbaşlı et al. 2019; Akpınar and Bozkurt 2021; Tabatabei and Nisbet 2021). It has been reported in many studies that honey and its products reduce the inflammatory response of the COVID-19 epidemic, which has caused the deaths of many people recently, and have promising effects against the epidemic (Al Naggar et al. 2020; Berretta et al. 2020; Lima et al. 2020; Yang et al. 2020; Al Naggar et al. 2021). It has also been reported that the use of honey products in nutritional habits increased during the COVID-19 pandemic period (Doğan et al. 2021).

A large portion of indirect financial gain is generated by agriculture-related activities as honey bees are the primary pollinator for 33% of species of crops (Maris et al. 2008; Oldroyd and Nanork 2009). Türkiye has a lot of opportunities for beekeeping because of its diverse flora, favorable habitat conditions, and presence of colonies (Köseman et al. 2016). However, the Turkish beekeeping sector has not been able to benefit sufficiently from the abundant resources available. To ensure effective and long-lasting efficiency, oversight and analysis of beekeeping practices have become more crucial (Sarı et al. 2020).

There are many studies on the beekeeping industry in the literature. Sarı (2023) predicted future land use to determine the potential impacts of land use changes on beekeeping and to identify circumstances that the beekeeping industry may face in the future. Examining the effects of land structure on beekeeping is important. In another study, Sarı et al. (2020) created a conceptual model for beekeeping suitability assessment that not only improves beekeeping in Konya province but also can be applied to any region of the world. Beekeeping activities have been a subject researched at different times in different regions. Kumova and Korkmaz (2000) evaluated the place and importance of the Çukurova Region in Türkiye's Beekeeping. Similarly, another study Teoman and Yeni (2021) evaluated the formation of a cluster in Türkiye's Black Sea Region to develop a more effective market framework for the honey and beekeeping products sector. Beekeeping, which is always one of the most important agricultural activities that should be emphasized, has been the subject of various studies. Koday and Karadağ (2020) researched the regional distribution of beekeeping activities and honey production in Türkiye. One of the key parameters in beekeeping is the business aspect. Businesses play an integral role in the supply, diversity and efficiency of products. Kaya and Gürcan (2021) employed data envelopment analysis to investigate the activities of beekeeping enterprises from both technical and economic perspectives. One of the most significant issues in beekeeping is migratory beekeeping, as businesses engaged in this practice account for the majority of honey production in Türkiye. Akpınar and Bozkurt (2022) evaluated the current situation and problems of the beekeeping sector of immigrant beekeepers in Afyonkarahisar.

The selection of a particular analytical method needs to be determined by an evaluation of the data's features and previous information on the observations, as is the case with any analysis that uses statistics. Among these, Exploratory Spatial Data Analysis (ESDA), which is based on Geographic Information Systems (GIS) methods, is frequently used as a foundation for spatial analysis and has been described to be a successful way to quantify both global and local spatial autocorrelation (Anselin 1996; Anselin 2003). To better organize the beekeeping industry in the nation, promote the growth of the local agricultural economy, and provide superior amenities and services to meet nutritional requirements, agricultural policymakers and associated governments can benefit from analyzing the spatial patterns of beekeeping products. Making adequate spatial pattern management is crucial from the perspective of the local manager to identify the best places for the beekeeping sector. This study was conducted with the help of the ESDA method, to examine whether there is a spatial autocorrelation and to examine the spatial patterns of honey production by using the registered production amount and number of hives in 81 provinces.

MATERIAL and METHOD

Study Area

The data on outputs of honey at the province level in 2005-2022 were obtained from the Turkish Statistical Institute (TurkStat). Turkish provinces are smaller administrative and geographical units than regions. Geographically, Türkiye is separated into seven regions: Mediterranean, Eastern Anatolia, Aegean, Central Anatolia, Southeastern Anatolia, Black Sea and Marmara (Figure 1). This study focuses on Türkiye, with 81 provinces as sample analysis units.

Figure 1: Geographical Regions of Türkiye

Statistical Analysis

Honey production (tons) and hive data recorded in the database of the Turkish Statistical Institute were created in Excel format by province and honey yield was calculated by dividing the total amount of honey by the number of hives on a provincial basis. The Levene's test for homogeneity of variances and the Shapiro-Wilk test for normality were used as parametric test assumptions to analyze the data before statistical analysis. Each region's descriptive statistics were computed and displayed as "Mean ± SEM, Median, Minimum, Maximum." Kruskal-Wallis H Test was employed to evaluate whether there was a regional difference between the total honey yields between 2005 and 2022. Dunn-Bonferroni Test was used for post-hoc analysis. The statistical analysis was performed using SPSS 23.0 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp).

Spatial Statistical Analysis

The entire study period was split into three subperiods, 2005–2010, 2011–2016, and 2017–2022, and analyzed spatially. This study focused on the spatial clustering and spatial pattern of honey yield. Firstly, the distribution of calculated honey yield by provinces was mapped. In this study, the exploratory spatial data analysis (ESDA) is used to identify the presence of spatial dependency and heterogeneity in honey yield among provinces in Türkiye. ESDA is a set of methods for describing and visualizing spatial patterns of distribution, detecting clustering and outliers, revealing patterns of spatial relationships, and suggesting spatial structures (Anselin 1999).

Spatial Autocorrelation Analysis

Spatial autocorrelation measures are divided into two categories as global scales and local scales based on the scope or scale of the analysis. The Moran's I statistic, a well-established measure for investigating spatial autocorrelation and detecting global spatial clustering, was employed (Moran 1948; Moran 1950).

LISA Analysis

In this study, to evaluate local spatial relationships, identify local spatial autocorrelation, and assess the importance of hot spots and cold spots, the Local Indicator of Spatial Association (LISA) is used (Anselin 1996; Yang and Wong 2013; Bayir 2023).

To construct certain shape files (shp) using QGIS 3.18.3 software, we first integrated the data of the outputs of honey yield for each province into a vector map of Türkiye with administrative boundaries at the provincial level. GeoDa software is then used to perform ESDA analysis in the current analysis based

on certain shape files. Using a Monte Carlo permutation method, significance is tested (Anselin 2003). The LISA significance maps are then produced, containing data on the importance of the local spatial patterns. (1) High-High shows provinces with high honey yield are adjacent to provinces with high yield (positive spatial autocorrelation, is indicated in red); (2) Low-Low shows provinces with low yield that are adjacent to provinces with low yield (positive spatial autocorrelation, is indicated in dark blue); (3) Low-High shows provinces with low yield that are adjacent to provinces with high yield (negative spatial autocorrelation, is indicated in green); (4) High-Low indicates provinces with high yield that are adjacent to provinces with low yield (negative spatial autocorrelation, is indicated in yellow) and (5) "not significant" indicates provinces with no spatial autocorrelation.

RESULTS

Statistical analyzes were made according to registered TurkStat data. However, it should not be forgotten that most of the honey production in Türkiye is carried out by businesses engaged in migratory beekeeping. Honey yields were determined by proportioning the honey production amounts (kg) of each province to the number of colonies. Descriptive statistics of honey yields calculated for 18 years by region are given (Table 1). According to these results, a statistical difference was determined between regions in honey yield values ($p<0.01$). A comparative analysis of the 18-year honey yield of each province in the regions revealed that the Marmara Region yields the greatest quantity of honey, while the Southeastern Anatolia Region yields the least.

Table 1. Distribution of honey yield data reported between 2005–2022 by regions in Türkiye

Region	Number of provinces	n	Mean±SEM	Median (Min-Max)	P
Aegean	8	144	13.50 ± 0.32^{ad}	13.97 (4.02–23.57)	p< 0.01
Black Sea	18	324	11.94 ± 0.30^{b}	11.16 (2.97–31.34)	
Central Anatolia	13	234	11.76 ± 0.27^{ab}	11.63 (3.02–21.34)	
Eastern Anatolia	14	252	13.02 ± 0.32^{a}	12.68 (2.36–26.27)	
Marmara	11	198	13.57 ± 0.37^{ad}	12.94 (6.73–65.41)	
Mediterranean	8	144	13.31 ± 0.36^{a}	12.37 (5.87–25.60)	
Southeastern Anatolia	9	162	9.96 ± 0.42^{c}	8.70 (1.63–31.63)	

(^{a,b,c} letter values within a column with different superscripts differ significantly at $p< 0.01$. SEM: Standart Error of Mean, n: 18 x number of provinces in each region)

The study period was divided into three subperiods: (a) 2005–2010; (b) 2011–2016; and (c) 2017–2022. Each subperiod was then analysed in terms of its spatial distribution. In addition, the entire period from 2005 to 2022 was also analysed spatially. These areas were shaded with different colors according to the ratio of the total honey production of each province to the number of hives. Honey yield was evaluated according to five levels (Figure 2-A, B, C, D).

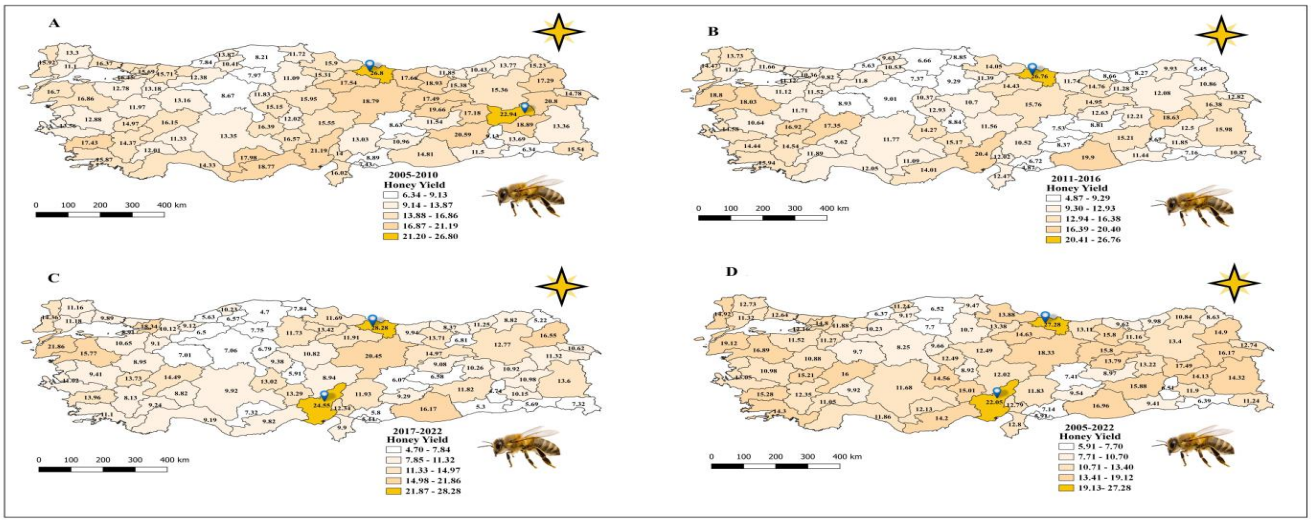

Figure 2: Distribution of honey yield in Türkiye for 2005-2010, 2011-2016, 2017-2022, and 2005-2022 (at province level, A, B, C, D)

When all periods were evaluated, the province with the highest productivity was Ordu, located in the Black Sea Region. Following Ordu, Muş province in Eastern Anatolia between 2005-2010 and Adana province in the Mediterranean Region between 2017-2022 were the provinces with the highest productivity. It was determined that the yield was higher in Eastern Anatolia in the first period and the yield decreased over time. Especially in the Black Sea, Central Anatolia and the Southeastern, some provinces with very low honey yield were observed (white color) (Figure 2-A, B, C, D).

Spatial Autocorrelation of Honey Yield

Moran's I measurements were made for the relevant periods to assess the association between the value of honey yield in each province and the value of honey yield in provinces adjacent and the Moran's I scatter plot of each period was produced (Figure 3-A, B, C, D). Moran's I values were all greater than 0, showing that honey yield had a positive spatial association and that exhibited spatial clustering. However, there was not a very high positive spatial autocorrelation (Table 2).

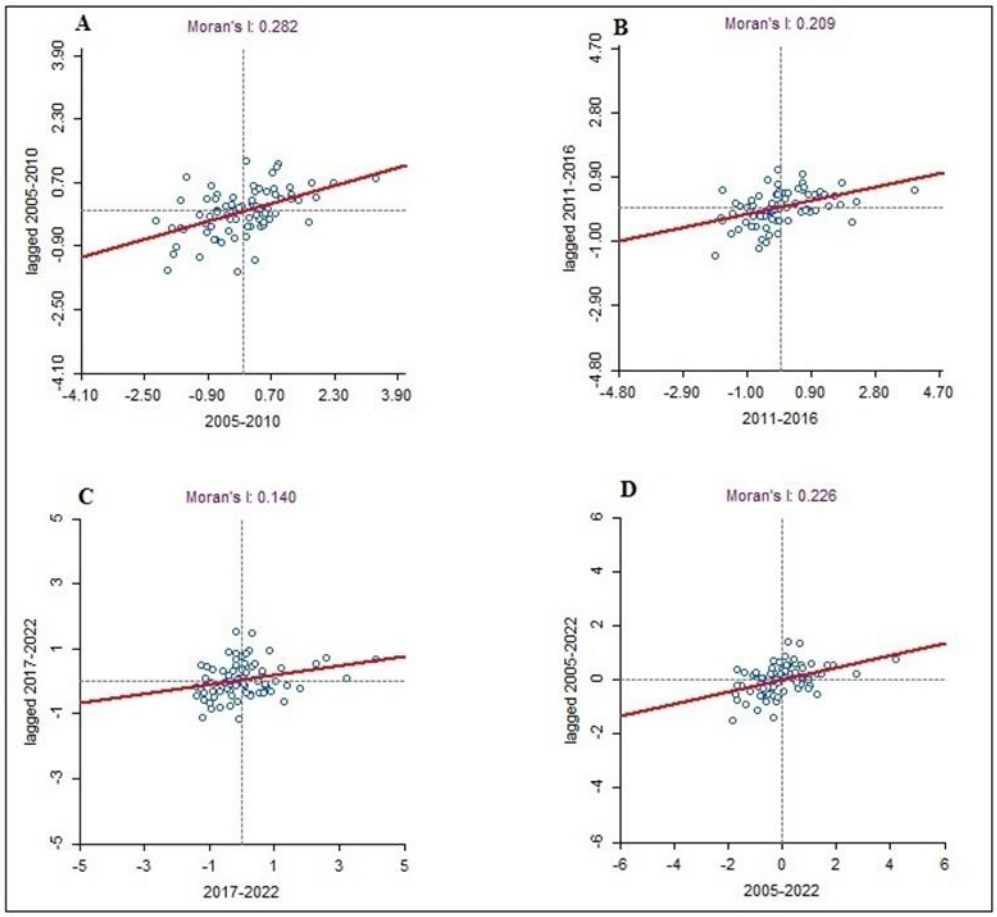

Figure 3: Moran's *I* scatter plot of the value of honey yield

Table 2. Spatial autocorrelation of different period considered for hotspot analysis of the honey yield using Moran's I statistics

Period	Moran's I	Z-score	*P*	Pattern
2005-2010	0.282	4.1064	0.002	clustered
2011-2016	0.209	3.1910	0.003	clustered
2017-2022	0.140	2.1980	0.021	clustered
2005-2022	0.226	3.4427	0.002	clustered

For 2005-2010, 2011-2016, 2017-2022 and 2005-2022, only 18.52%, 9.88%, 8.64% and 11.11% respectively, and a positive spatial association (containing categories High-High and Low-Low) was described significant provinces (95% confidence interval). For 2005-2010, 2011-2016, 2017-2022 and 2005-2022, only 28.40%, 18.52%, 19.75% and 22.22% respectively, and a positive spatial association (containing categories High-High and Low-Low) was described significant provinces (90% confidence interval) (Table 3).

Table 3. Number of cities in various LISA clusters

Sig. filter	2005-2010			2011-2016			2017-2022			2005-2022		
	HH	LL	Total %	HH	LL	Total %	HH	LL	Total %	HH	LL	Total %
5%	8	7	18.52	2	6	9.88	3	4	8.64	4	5	11.11
10%	10	13	28.4	5	10	18.52	7	9	19.75	7	11	22.22

HH: High-High cluster, LL: Low-Low cluster.

The maps showed that the production of honey clearly exhibits local clustering tendencies. Compared to local spatial outliers (High-Low or Low-High), there was more local clusters (High-High or Low-Low). When the honey yield was evaluated between 2005-2010, it was seen that there was two important local spatial clusters (eight High-High clusters). These High-High clusters were Ordu and Giresun in the Eastern Black Sea region, Tokat in the Middle Black Sea Region and Erzurum, Ağrı, Muş, Bingöl and Iğdır provinces in the Eastern Anatolia region. There were also significant local spatial outliers (one Low-High and one High-Low). When honey yield between 2011 and 2016 was evaluated, it was seen that there was important local spatial clusters (six Low-Low clusters). But, when evaluated between 2017-2022, it was seen that local spatial clustering has decreased. When the entire time period was evaluated, it was seen that there was High-High clusters in the provinces of Ordu, Samsun, Giresun and Tokat (Figure 4-A, B, C, D).

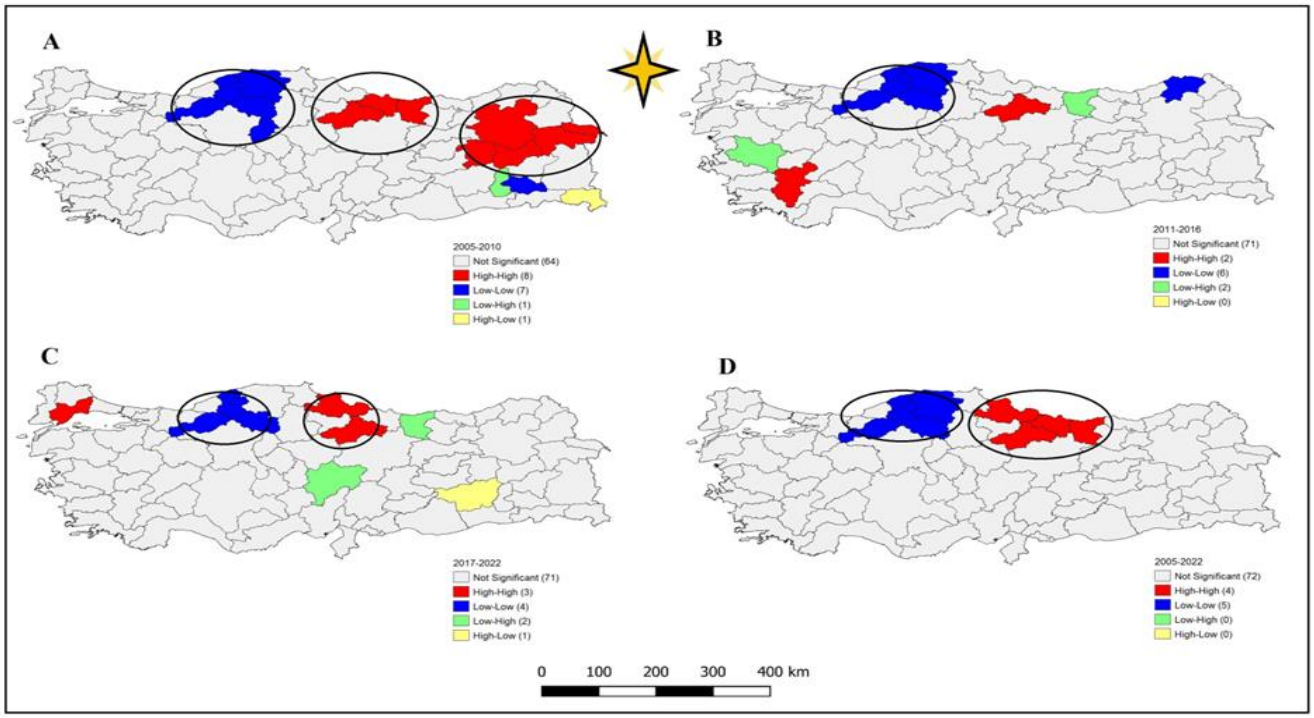

Figure 4: Spatial clustering and outliers of honey yield using LISA clustering for 2005-2010, 2011-2016, 2017-2022, and 2005-2022 (95% confidence interval, p-value=0.05, A, B, C, D).

When analyses were performed at a 90% confidence interval, clusters could be detected in different regions. For example, between 2017 and 2022, High-High clusters were identified in Edirne, Tekirdağ and Çanakkale provinces. According to, in the analyses made with both confidence intervals, it was determined that there were clusters in Eastern Anatolia at first and were not seen over time. Clusters were seen in Ordu province and its surroundings, which have the highest productivity, in every period. Results showed that different clusters can be detected by changing the parameters produced (Figure 5-A, B, C, D).

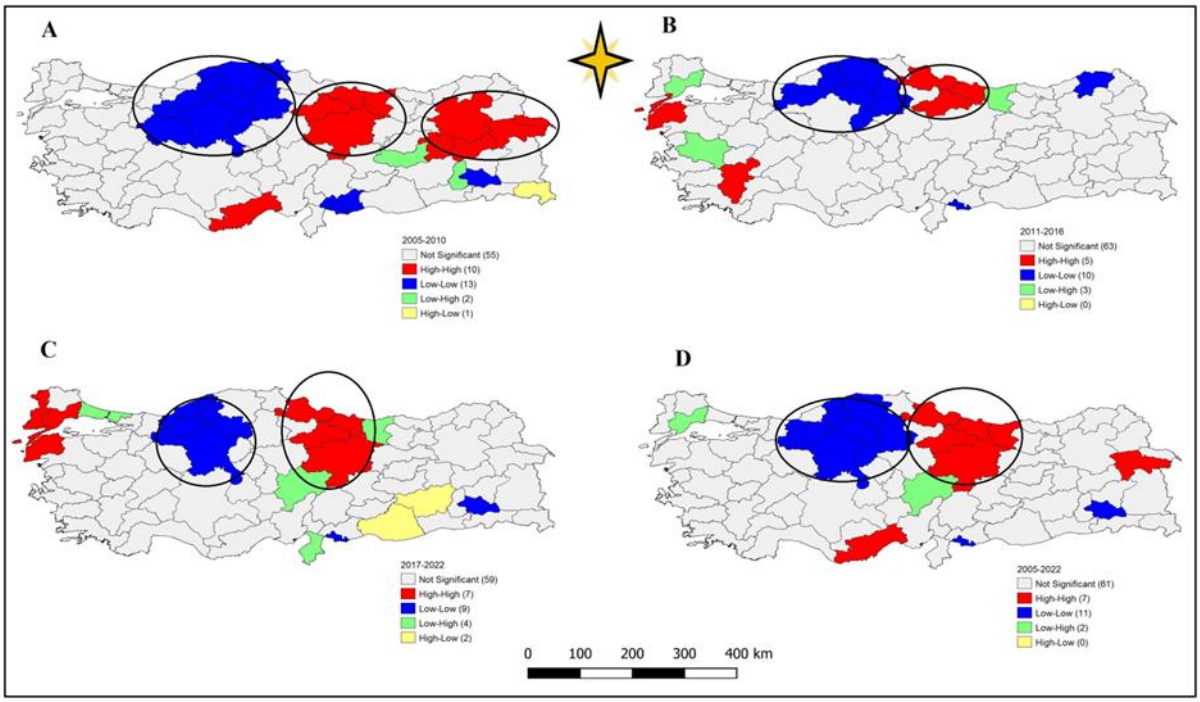

Figure 5: Spatial clustering and outliers of honey yield using LISA clustering for 2005-2010, 2011-2016, 2017-2022, and 2005-2022 (90% confidence interval, p-value=0.10, A, B, C, D)

DISCUSSION

Beekeeping is generally an animal husbandry activity carried out to produce honey. In addition, beekeeping activities are the branch of production most compatible with the economic and ecological cultivation model for every society. In the present study, the spatial distribution characteristics of honey yield were defined by using exploratory spatial analysis methodologies. As far as we know, the data regarding the calculated honey yield has not been examined so far with exploratory spatial analysis methods by creating a database based on GIS.

In this study, Marmara (13.57), Aegean (13.50) and Mediterranean (13.31) regions were the three regions with the highest productivity according to recorded data. Before the forest fires on the Aegean and Mediterranean coasts in 2021, the Eastern Black Sea Region (22.5%), the Mediterranean Region (19.2%) and the Aegean Region (13.4%) were the top three regions of Türkiye in honey production in 2020 (Burucu 2021). The necessity of migratory beekeeping for high production and profitability has been emphasized (Kekeçoğlu et al. 2014). It has been reported that the Aegean Region has an important place in honey production and the rate of migratory beekeeping enterprises is 82% (Özbilgin et al. 1999; Korkmaz et al. 2018). In Türkiye, beekeeping businesses that engage in migratory or permanent beekeeping activities differ from one another (Akpınar and Bozkurt 2022). In contrast to their stationary counterparts, migratory beekeepers use honey bee colonies with better hive capacities and superiority (Özbilgin et al. 1999, Cengiz and Dülger 2018; Akpınar and Bozkurt 2022). By moving hives along a prearranged path and timing it to correspond with the honey plant's blossoming times, migratory beekeepers can prolong the honey season (Korkmaz et al. 2018).

When provinces with similar honey yields tend to be next to one another, there is positive spatial autocorrelation; nevertheless, when provinces with high and low honey yields are next to one another, there is negative spatial autocorrelation. In this study, Moran I statistics for all periods examined showed that there was a spatial relationship, although it was not a very strong spatial relationship. In this context, ensuring interaction between provinces is of great importance in terms of increasing honey yield. According to local Moran I statistics and honey yield results calculated by taking into account registered data, it can be said that registered beekeepers in Ordu province make significant contributions to honey production. For this purpose, everyone should fulfill their duties in the most effective way in order to maintain the extremely strong beekeeping potential in Ordu province (Sıralı 2016). There is an issue that should not be ignored here. Mobile beekeeping, which we call migratory beekeeping, is practiced in many parts of our country and makes up a considerable portion of Türkiye's overall honey production (Akpınar and Bozkurt 2021). As a matter of fact, the majority of beekeepers in Ordu province take their bees to flower fields in Eastern provinces in the summer. For this reason, the total honey production and yield of this province are high due to high honey production per hive (Koday and Karadağ 2020). If the cooperation of beekeepers in this region

can be well ensured, significant increases in honey production can be achieved by increasing production not only in Ordu province but also in neighboring provinces. Because when provinces with similar honey yield tend to be side by side, High-High clusters occur.

High-High clusters were detected in Edirne, Tekirdağ, located in the Thrace region, and Çanakkale, which is adjacent to these provinces. Although colony productivity, colony strength and diligence are important factors in achieving high efficiency in beekeeping, the variety and abundance of nectar and pollen sources are also important (Sıralı 2002). In beekeeping, beehives are placed in agricultural areas to provide as many pollen and nectar sources as possible to the bees and to ensure the pollination of cultivated plants (Bozkurt 2019; Decourtye et al. 2019). Thus, identifying appropriate production areas and their capabilities will guarantee optimal utilization of plant resources and will have a direct impact on output and efficiency (Doğaroğlu and Genç 1994). Cultural plants important for beekeeping are grown in the Thrace Region. This makes the existing climatic and floral conditions conducive to beekeeping, and the northern parts of the region are considered some of the best places for the production of the highest quality honey in our country (Sıralı 1993; Sıralı 2002).

In recent years, honey and other hive products, long valued for their properties and high demand, have experienced a surging popularity both domestically and internationally in Türkiye. This rise, coupled with the growing recognition of beekeeping as a viable source of alternative income and the increasing importance of bees and their by-products, is fueling the development of new beekeeping-related goods and businesses (Ceyhan et al. 2017; Topal et al. 2021). To continue expanding the beekeeping sector in ways that benefit people, communities, and the environment, we must promote sustainable growth. Otherwise, poorly thought-out plans could be developed that would lead to incorrect procedures, such as introducing honeybees to regions where they are not suitable since increasing in one area can upset delicately balanced ecosystems (Sarı et al. 2020). Based on this idea, cooperation can be established with beekeepers in Ordu province, which has the highest production and High-High clustering, in order to follow sustainable honey supply chains for products in Türkiye. The rate of migratory beekeeping is high in Ordu province and the effect of migratory beekeeping on production is very important. According to the clustering results obtained, there was a High-High clustering in Erzurum, Ağrı, Muş, Bingöl and Iğdır provinces between 2005 and 2010, but no clustering could be determined in the following years. In fact, High-High clusters can be created again by providing more incentives in these provinces in the region, which has a significant potential. Because, the majority of beekeepers in Ordu province take their bees to flower fields in Erzurum, Kars, Ardahan, Ağrı, Iğdır, Muş and Bingöl provinces during the summer (Koday and Karadağ 2020). Thus, the use of outlets from these hotspots will inevitably contribute to the advancement of the immediate environment. Additionally, areas will be provided to raise honey bees suitable for the region.

Our research supports to some extent previous studies showing regional differences in the beekeeping industry. In addition to the fact that previous studies were generally limited in terms of the area examined, a spatial examination of honey yield was not carried out (Sarı et al. 2020; Teoman and Yeni 2021; Aşkan 2023; Polat et al. 2023; Sarı 2023). However, unlike previous studies, we detected clusters (High-High and Low-Low); For example, we observed that the clustering in the distribution of honey yield was more intense in Eastern Anatolia in the first period, but gradually decreased. We also observed clusters in Western Marmara. The findings clearly show the benefit of using honey yield output as a statistical indicator to identify hotspots at the province level. We also discovered that there are various spatial clustering structures (High-High and Low-Low) and clusters associated with honey yield. Therefore, these cluster maps from our study have significant consequences for future work mapping the supply and demand for honeybee byproducts, as well as for planning how to connect these hotspot provinces to their neighboring provinces through roadways and regional collaborations. Thus, more hotspot locations that are larger can be built to increase honey production and decrease uneven yield within regions. In addition, aggregating activities related to beekeeping (historical beekeeping activities, bee products, beehive air, bee museums, apitherapy, production activities) to a larger region can attract beekeepers and people who tend to earn money and increase the beekeeping industry and country income (Vilas-Boas 2018; Adanacıoğlu et al. 2019; Semkiw and Skubida 2021).

The distribution of bee populations can alter depending on both environmental factors like vegetation, temperature, altitude, and water supply as well as human factors like population density and product demand (Sarı et al. 2020; Sarı 2023). In our study, High-High clusters were observed in eastern Türkiye between 2005 and 2010, but the density decreased afterward. Consistent with the findings of our study, Koç et al. (2010) stated in their study that in their long-term trend analysis in the Eastern Anatolia region will be no improvement in honey and beeswax production in the long term. However, due to a variety of reasons, including climate, regional variation, and sunlight, the eastern regions contain an enormous variety of plant flora. Despite the region's hard winters, the spring and summer seasons may get rather warm because of the region's abundant sunshine. Beekeeping is a great opportunity for rural areas with rich plant flora, as it requires relatively little

investment and does not have a limited area, and provides an important economic opportunity (Ateş and Yaşar 2020). If beekeeping enterprises become more efficient, production will increase and the increasing demand will be met, and with the increase in production, the economic income of the producer will increase. In this way, it is believed that the tourism industry, particularly in rural regions, will grow and become stronger while also promoting rural growth and *Apis mellifera (API)* and gastronomic tourism (Aşkan 2023).

The protection of all ecosystems together with the most accurate and effective use of resources is the basis of sustainable agricultural production, which includes beekeeping as well as all areas related to food production (Bozkurt 2019). With the rise in global population, the honey bee's ability to produce, which provides vital items for the health and well-being of humans, has grown considerably more crucial. It is also important to note how future changes in the world may affect many of the environmental factors supporting the beekeeping industry (Decourtye et al. 2019; Mouillard-Lample et al. 2023). Utilizing geographic information techniques, various geographical analyses, and the combination of non-stationary spatial variables with environmental and socio-economic data will all help in such a situation. The emergence of spatial patterns in space and time can therefore be predicted with more accuracy by understanding the connections between the spatial distribution of honey yield and environmental processes.

Our investigation highlights a few gains. The initial and significant gain is the integration of new data for the analysis of honey yield distribution in Türkiye. Another significant gain relates to the scale of the study area in terms of space. By using provincial-level data, it is possible to reach more accurate and dependable conclusions with a broader perspective on honey yield on a national scale. The summary of honey yield 18-years period is an extra substantial gain in our study. Additionally, from the perspective of the nation's economic growth and development, these novel consequence maps of our study may offer more significant inputs for subsequent attempts to map the need and supply of bee-based products.

CONCLUSION

This study, for the first time, investigated the spatial relationships of honey yield at the provincial level in Türkiye in the periods 2005-2010, 2011-2016, 2017-2022 and 2005-2022, respectively. Honey production outputs in 81 provinces of Türkiye were used as an indicator to examine spatial correlations of honey yield. The point that needs to be taken into consideration in this study is to consider the extent to which beekeepers registered in the regions where the cluster is present engage in migratory beekeeping and to what extent they contribute to the production achieved. We can say that beekeepers in Ordu province, where High-High clustering has always been seen, contribute greatly to production, and people living in the Eastern Anatolia Region do not attach as much importance to beekeeping activities as before. As a result, we recommend increasing the competitiveness between provinces with high production in high potential regions and surrounding provinces, enabling better use of existing resources and establishing strong collaborations.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: TB, EA and Şİ conceived the study. EA collected data and designed it. TB performed the statistical and spatial analysis. EA and Şİ wrote the manuscript. All authors reviewed the manuscript.

Ethical approval: This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules. Data gathered from the official website of the Turkish Statistical Institute (TurkStat) regarding is public use data.

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RESEARCH ARTICLE

Pit1/Hinfi Polymorphism in Holstein Cattle in Afyonkarahisar Province

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ABSTRACT

Pit1 (Pituitary Specific Transcription Factor-1) gene has been shown to be resided on the centromeric region of chromosome 1 in cattle. This gene, also known as POUF1F has responsible for development of pituitary gland and hormone expression in mammals. Pit1 deficiency causes a dwarfism phenotype in mice, dogs and fish. Previous studies have demonstrated to associated with polymorphisms of the Pit1 gene and growth and reproductive performance and milk, meat yield traits. In the cattle, a HinfI polymorphism on Pit1 gene (c.1178G>A) have been reported that there is an association between milk and reproduction yield parameters. In this study, we aimed existence and distribution of Pit1/HinfI polymorphism in 81 head Holstein cattle in Afyonkarahisar province. In our study, we defined that there were 5 AA, 30 AG and 46 GG genotypes in our survey population. We also detected to frequency of A allele as 0.25 and the G allele as 0.75. We calculated to genetic index values such as PIC (0.3027418) and Heterozygosity (0.3742044) using RStudio package. Chi-square value was found 0.049 and was exhibited to survey population in Hardy-Weinberg equilibrium. The Pit1/HinfI polymorphism is a potential option for use in marker-assisted selection studies in light of these findings for Holstein cattle in Afyonkarahisar province.

Keywords: HinfI, Holstein sığır, PCR-RFLP, Pit1

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Afyonkarahisar'da Yetiştirilen Holstein Sığırlarda Pit1/Hinfi Polimorfizmi

ÖZ

Pit1 (Hipofize Özgü Transkripsiyon Faktörü-1) Sığırlarda 1.kromozomun sentromerik bölgesinde bulunan yaklaşık 129 aminoasitten oluşan bir protein kodlayan bir gendir. Memelilerde hipofiz bezinin gelişiminden ve hormon ekspresyonundan sorumlu olan Pit1 geni, POUF1F olarak da bilinmektedir. Pit1 eksikliğinde farelerde, köpeklerde, balıklarda cücelik fenotipi gözlenmektedir. Yapılan çalışmalar, Pit1 genindeki polimorfizmlerin sığırlarda, koyunlarda, domuzlarda, tavuklarda büyüme, üreme performansı, et ve süt verimi özellikleriyle ilişkili olduğunu göstermiştir. Sığırlarda Pit1/HinfI (c.1178G>A) polimorfizminin süt verimi ve döl verimi parametreleriyle ilişkili olduğu ifade edilmektedir. Bu çalışmada, Afyonkarahisar ilinde yetiştirilen 81 baş Holstein sığır Pit1/HinfI polimorfizminin varlığı ve dağılımının belirlenmesi amaçlanmıştır. PCR-RFLP tekniği kullanılarak hayvanların bu polimorfizm açısından genotiplendirilmiştir. Çalışma sonucunda 81 hayvandan 5 tanesinin AA, 30 tanesinin AG ve 46 tanesinin GG genotipinde olduğu bulunmuştur. G allelinin frekansı 0,75, A allelinin frekansı ise 0,25 olarak hesaplanmıştır. Genetik indeks değerlerinden PIC değeri 0,3027418 ve Heterozigotluk değeri 0,3742044 olarak hesaplanmıştır. χ^2 değeri 0,049 bulunmuş olup, popülasyonun Hardy-Weinberg dengesinde olduğu ortaya konulmuştur. Bu bulgular ışığında Pit1/HinfI polimorfizminin Afyonkarahisar ilinde yetiştirilen Holstein sığırlar için markör destekli seleksiyon çalışmalarında kullanılabilecek potansiyel bir aday olduğu düşünülmektedir.

Anahtar kelimeler: HinfI, Holstein sığır, PCR-RFLP, Pit1

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INTRODUCTION

Pituitary specific transcription factor -1 (Pit1) gene has been shown to be resided on the centromeric region of chromosome 1 in cattle. This gene encodes a protein of 129 amino acids and approximately 33 kDa in weight. (Moody et al. 1995; Selvaggi et al. 2011; Thuy et al. 2018). Pit1 gene is liable for the expression of hormones and the development of pituitary gland in mammals (Doosti et al. 2011). It has been shown that the expression of prolactin hormone in lactotroph cells (Supowit et al. 1992) and growth hormone in somatotroph cells (Tuggle and Trenkle, 1996) in anterior pituitary is controlled by Pit1 gene. The Pit1 protein, also called POUF1F, contains a POU domain for high affinity binding to target DNA (Ingraham vd., 1990, Hendriks-Stegeman vd., 2000). The Pit1/POUF1F protein binds to the promoter areas of the genes that encode growth hormone and prolactin hormone via its POU domain, resulting in the stimulation of transcription (Mangalam vd., 1989, Trokavicka vd., 2015).

Mutations on Pit1 gene may cause the inadequate expression of GH, PRL and TSH hormones in anterior pituitary (Cohen et al. 1996; Renaville et al. 1997). Dwarfism in mouse (Li et al. 1990, Flurkey et al. 2002), fish (Nica et al. 2004) and dogs (Lantinga-van Leeuwen et al. 1999) showed that Pit1 deficiency is responsible for growth hormone regulation.

Pit1 gene polymorphisms have been reported to show an association between growth and reproduction traits in cattle (Pytlewski et al. 2018), buffaloes (Zghair and Hassoni, 2021), sheep (Bai et al. 2016), swine (Piórkowska et al. 2015) and chickens (Nie et al. 2008). Pit1 gene polymorphisms are correlated to the first calving age, weaning, and the daily gain weight of calves in Limousine cattle (Pytlewski et al. 2022). These polymorphisms are reported to affect the milk yield and the components of milk, such as the amounts of lipids and proteins (De Mattos et al. 2004; Edriss et al. 2009, Zhou et al. 2016; Thuy et al. 2018).

Initially, Wollard et al. (1994) showed using by HinfI restriction enzyme that there was a transition mutation from G to A in 451 bp length region (c.1178G>A) on Pit1 gene. This polymorphism has been shown to be associated with reproductive parameters such as the first calving age, insemination numbers, calf and the cow weight during calving in Holstein cattle (Pytlewski et al. 2018). Pit1/HinfI polymoprhism was also associated with milk yield and milk composition in Holstein and Sahiwal cattle (Renaville vd., 1997; Hosseinzadeh et al. 2015a; Chauhan et al. 2015; Anggraeni et al. 2020). However, it has been reported that there is no relation between polymorphism and dairy traits in Simmental and Brown Swiss breeds (Aytekin and Boztepe, 2013; Sönmez and Ünal, 2023).

Previous studies have shown that Pit 1 gene polymorphisms are a potential marker for selection studies. In this study, we purposed to detect the existence and distribution of Pit1/HinfI polymorphism in 81 Holstein cattle in Afyonkarahisar province.

MATERIAL and METHODs

In this study, blood samples collected from Holstein cattle and laid up at -80 °C in the Medical Biology and Genetics laboratory in Afyon Kocatepe University-Faculty of Veterinary Medicine were used.

In this study, blood samples previously collected from Holstein cattle and laid up at -80 °C in the Medical Biology and Genetics Laboratory of Afyon Kocatepe University, Faculty of Veterinary Medicine were used. All procedures were approved by the local ethics committee (AKÜ-HADYEK-260-20) Afyon Kocatepe University.

DNA Isolation

DNA was isolated from blood samples using by spin-column method. Ten micro liter proteinase K, 200 µl blood sample and 200 µl Extraction buffer were added into 1.5 ml microcentrifuge tubes and vortexed for 10-15 seconds. Mixture was then incubated at 56 ℃ for 15 minutes. After the completion of incubation, 210 µl Binding Buffer was added into the lysate and transferred into the spin column and centrifuged at 8000 rpm for 1 minute. Aliquates of 650 µl Wash buffer I was added into the spin-column and centrifuged again at 8000 rpm for 1 minute. Collection tube was discarded and 500 µl Wash Buffer II was added into tube emptied tube and centrifuged at 8000 rpm for 1 minute. Collection tube was discarded and 250 µl Wash Buffer II was added into spin column and centrifuged at 14000 rpm for 3 minutes. After centrifugation, column was transferred to new 1.5 ml microcentrifuge tube and 100 µl TE (10 mMTris- 1mM EDTA, pH: 8.0) buffer was added into column and incubated at room temperature for 5 minutes. When incubation was completed, tubes centrifuged at 8000 rpm for 1 minute. Isolated DNA was stored at -80 ℃.

Polymerase Chain Reaction (PCR)

Primer sequences used was represented in Table 1. Primers optimal annealing temperature was determined as 56 ℃ by using Gradient PCR process. Primer pairs for target region at intron between 5 and 6 exons were used as described by Woollard et al (1994). Primer pairs were checked for hairpin and dimerization by using Primer3 programmed.

Table1. Primer sequences for Pit1 gene amplification

Primer	Sequence	Tm (Temperature Melting)
Forward	AAACCATCATCTCCCTTCTT	
5'→3'		56℃
Reverse 5'→3'	AATGTACAATGTGCCTTCTGAG	

PCR amplification was performed by using Dream Taq Polymerase kit 5U/ µl (Thermofisher Scientific-Litvania). 10 x PCR buffer 1 µl, 0.3 mM Forward primer, 0.3 mM Reverse Primer, 0.3 mM dNTP mix, 0.0625 µl (1.25 U) Dream Taq DNA polymerase, 1.5 µl DNA (~ 15 ng) was added into 0.2 ml PCR tubes. PCR conditions was shown in Table 2. When PCR process was completed, PCR products were run in %2 agarose gel electrophoresis at 90 Volt and viewed at UV imaging system (Vilber Lourmat BIO-VISION).

Table 2. PCR conditions

Component	Volume (µl)	Incubation Temperature
Nuclease Free Water	5.5	
Fast Digest Green Buffer	1	37℃-30 minute
HinfI Restriction Enzyme	0.5	80℃-15 minute (for inactivation of enzyme)
PCR product	8	
Total	15	

RFLP-Restriction Fragment Length Polymorphism

After completion of PCR, the PCR products were cut by using the HinfI restriction endonuclease for the detection of polymorphism.

Protocol for RFLP was shown in Table 3. HinfI restriction enzyme was used to detect the genotypes from PCR product. DNA fragments obtained from RFLP was run in 3% agarose gel electrophoresis at 90 Volt for 30 minutes and then viewed at UV imaging system.

Statistical Analysis

R-Studio package was used to allele and genotype frequencies with this PIC value and Heterozygosity value. We also calculated the genetic index value such as PIC value and Heterozygosity value, using RStudio Package. The population's Hardy-Weinberg equilibrium was defined using the chi-square (χ^2) test.

Table 3. Contents of RFLP reaction

Component	Volume (µl)	Incubation Temperature
Nuclease Free Water	5.5	
Fast Digest Green Buffer	1	
HinfI Restriction Enzyme	0.5	37℃-30 minute
PCR product	8	80℃-15 minute (for inactivation of enzyme)
Total	15	

RESULTS

In this study, the target region of Pit1 gene (between intron 5-exon6) was amplified by PCR.. RFLP analysis was performed for genotyping by HinfI enzyme. In RFLP, 451 bp length DNA fragment was cut using HinfI into 244 and 207 bp length two fragments (Figure 1). In the surveyed population (81 head Holstein cattle in Afyonkarahisar), we determined that there were 5 AA, 30 AG and 46 GG genotypes. Frequency of G allele appeared as 0.75, whereas A allele was 0.25.

We also determined the PIC value as 0.3027418 and the heterozygosity value as 0.3742044 using the R-Studio package (Table 4). If PIC value ranges from 0.25 to 0.50, this polymorphism can be used for marker as mid-level informativeness (Bostein et al. 1980; Selvaggi and Dario, 2011). Our PIC value, demonstrated that the Pit1/HinfI polymorphism could be a mid-level informative marker to use in selection studies.

The survey population was in Hardy-Weinberg equilibrium in terms of HinfI polymorphism in the target region of Pit1 gene, according to our results (P>0.05). As a result of the χ^2 test calculation based on the observed and expected genotype frequencies that was 0.049 and this value was below the 0.05 significance level (TD1:0.05= 3.841) with 1 degree of freedom in the χ^2 distribution table.

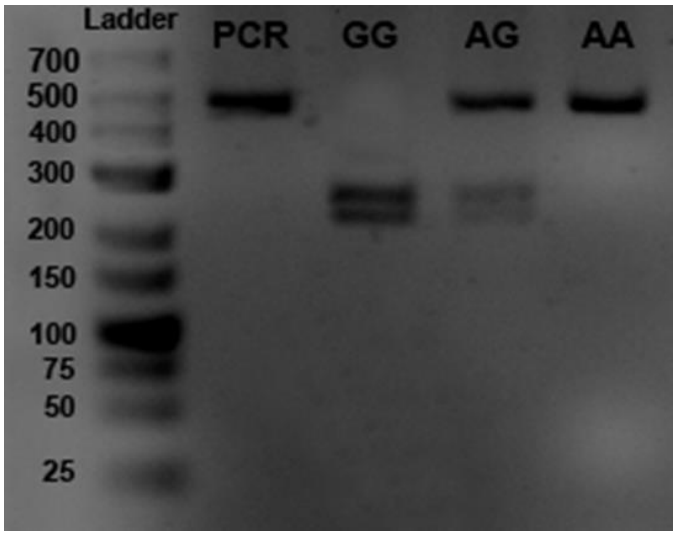

Figure 1: Results of RFLP. Only 451 bp in fragment in AA genotype, 244 and 207 bp fragments in GG genotype, all fragments in heterozygote AG.

Table 4. Genotypic and allelic frequencies and genetic index values

Frequency of Allele			Frequency of Genotype			χ^2
	Count	Proportion		Count	Proportion	
A	40	0.25	AA	5	0.06	
G	122	0.75	AG	30	0.37	
			GG	46	0.57	0.049

Genetic Indexes	
PIC value	0.3027418
Hu (Heterozygosity)	0.3742044

DISCUSSION

In 350 Holstein cattle, Bayram et al. (2017) found that the frequency of the G allele was 0.68 and the A allele was 0.32. According to Hosseinzadeh et al. (2015b), the frequency of the G allele was 0.74 and the frequency of the A allele was 0.26 in Holstein cattle in the HinfI restriction site. Misrianti et al (2010) they defined that the frequency of G allele was 0.75 and the A allele was 0.25 in Holstein cattle, exactly the same in our study. Genotype frequency was also AA 2%, AG 44%, and GG 53%. Besides, there is no detection of Pit1/HinfI polymorphism in 320 Indonesian buffalo. In the case of this polymorphism, the buffalo population was monomorphic (Ref). In 1024 Polish Holstein cattle, Pytlewski et al (2018) reported that the G allele frequency was 0.795 and the A allele frequency was 0.205. AA, AG, and GG genotypes frequencies also notified as 4.7 %, 31.5 % and 63.7 %, respectively. In 125 Holstein cattle, the frequency of the G allele was 0.784 and the frequency of the A allele was 0.216, according to Thuy et al (2018). The frequency of the GG, AG, and AA genotypes was determined to be 8%, 27.2%, and 64.8%, respectively. Dybus et al (2004) demonstrated frequency of G allele was 0.757 and A allele was 0.243 in 900 head of Holstein cattle in Poland. Frequency of AA, AG and GG genotypes also reported 5%, 38% and 56%, respectively, in the same study. Edriss et al (2009) reported to frequency of G allele was 0.744 and A allele was 0.256. Genotype frequencies were also reported as AA 3%, AG 45%, and GG 52%. All of these studies were conducted on Holstein cattle breeds. The results of all these studies were close to each other and the results of our studies were similar to the results of those studies.

In 288 Simmental cattle, Trokavicka et al. (2015) reported that the frequency of G allele was 0.774 and the A allele was 0.225. In Simmental cattle (n=67), the frequency of the G allele was 0.58 and the frequency of the A allele was 0.42 and the population under examination was in Hardy-Weinberg equilibrium according to Sönmez & Ünal (2023). Aytekin and Boztepe (2013) reported the frequency

of G allele was 0.626 in 301 Brown Swiss cattle, while the A allele was 0.374. Studies conducted in other dairy cattle breeds including Simmental and Brown Swiss showed that there were approximately similar results in Holstein breed. Ardıçlı et al. (2023) determined that the frequency of G allele was 0.7778 and the A allele was 0. 2222. Genetic index values such as the PIC value and heterozygosity were noted as 0.2859 and 0.3457, respectively.

In 2009, Zhang and colleagues examined in Chinese native breed Qinchuan and its various crossbreed (Pure Qinchuan-QQ n=67, Limusin x Qinchuan-LQ n=47, Angus x Qinchuan- AQ n=36 and Germany Yellow x Qinchuan-DQ n=42) for HinfI/Pit1 polymorphism. They noted the frequency of the G allele was 0.768, 0.819, 0.667, and 0.88, whereas the A allele was 0.232, 0.181, 0333, and 0.178, respectively, in QQ, LQ, AQ, and DQ cattle. The Pit1/HinfI polymorphism allele frequencies in Podolica cattle were reported by Selvaggi and Dario (2011) to be A = 0.3 and G = 0.7 and 14.42% AA, 31.73% AG, and 53.85% GG were the genotype frequencies. In addition, the PIC value was calculated as 0.332 which was in parallel with our results. According to Moravčiková et al (2013) frequency of G allele was 0.704 and A allele was 0.295 at 110 spotted regions in Slovak cattle and the population was Hardy-Weinberg equilibrium. The G allele frequency was detected as 0.659 and the A allele frequency was 0.341 in 296 Auliekol cattle of Kazakhstan. As Taipova et al (2020) reported that the studied population was in Hardy-Weinberg equilibrium. De Mattos et al (2004) investigated to the Pit1/HinfI polymorphism which was used for progeny testing Gyr bulls. The results of this investigation indicated that the frequency of G allele 0.95 and A allele was 0.05, whereas genotype frequencies were AG 10 % and GG 90%. AA genotype was not observed in this study. Hartati et al (2018) defined the allelic frequencies A and G alleles as 0.005 and 0.995 in 107 Indonesian native cattle breeds. Moreover, they found the genotype frequencies as AG 0.9% and 99.1% GG. Additionally, they showed that there was no AA genotype in the population and the population was in Hardy-Weinberg equilibrium. On the contrary to our findings, they determined to PIC value extremely low as 0.009. In this population, the genetic diversity concerning the targeted polymorphism is notably low. These two studies did not coincided to AA genotype and the ratio of heterozygosity was very low. Thus, frequency of A allele was extremely low. Zghairand and Hassoni (2021) determined that the frequency of G allele was 0.90 and the A allele was 0.10 in 27 buffaloes. They did not obtain any AA genotype in the population. This research showed that the A allele originating from only heterozygous individuals is present in the population. Therefore, this study reveals very important findings since it is the only study in which the A allele was detected in buffaloes. Gritsienko et al. (2020) reported that the frequency of the G allele was 0.69, 0.63 and 0.50. The A allele was also 0.31, 0.37 and 0.50 in Ukrainian native breeds including Ukrainian red (n = 32), Ukrainian black mottled (n = 32), and Ukranian red mottled (n = 28), respectively. It has been determined that G allele frequency is 0.71 and the A allele frequency is 0.29 in 69 Russian Holstein, (Pozovnikova et al (2020).

The frequency of A allele was found as 0.356 and the G allele was found 0.644 in 104 Anatolian Black (native Turkish cattle breed) by Sakar and Zülkadir (2022). Genotype frequencies also noted AA 9%, AG 51,9% and GG 38,5%. They identified to heterozygosity value (He) as 0.458. Aytekin and Bayraktar (2022) showed that the frequency of G allel was 0.74, 0.68, 0.90 and 0.77 in Anatolian Black, Holstein, Brown Swiss, and Simmental cattle, respectively. AA genotype was not detected in the population. The A allele was 0.26, 0.32, 0.1 and 0.23. This study demonstrated the minimum frequency of the A allele in Brown Swiss breed and also the maximum frequency in Holstein, as shown in other studies. Toğyar ve Özdemir (2023), investigated to the polymorphism in 70 Brown Swiss and 71 Simmetnal cattle. They determined frequency of G allele was 0.69 in Brown Swiss and 0.76 in Simmental cattle. The A allele frequency was 0.31 in Brown Swiss and 0.24 in Simmental cattle.

Khaizaran et al (2014) studied Pit1/HinfI polymorphism on 101 Palastinian Holstein, 18 crossbred and 25 native breed cattle. The frequencies of A allele were showed 0.31, 0.66 and 0.78 and the G allele were 0.68, 0.33 and 0.22, respectively in the mentioned breeds. It was observed that the frequency of the G allele was higher in the Holstein, which has been known to have high dairy characteristics in comparison to the Palestinian native and hybrid cattle breeds. Similarly, Doosti et al. (2011) investigated to the polymorphism in 224 Holstein cattle and 210 Iranian native cattle. In native cattle, they determined frequency of G allele was 0.25 and the A allele was 0.75. However, in the Holstein cattle, they defined frequency of G allele was 0.701 and the A allele was 0.298. These studies indicated that there is a high frequency of A allele in native cattle breeds than that of Holstein cattle breed.

CONCLUSION

In conclusion, Pit1 gene region is polymorphic in Holstein cattle breed in Afyonkarahisar. Wild type G allele is common while A allele is rare. Thus, Pit1/HinfI polymorphism is convenient to use for marker assisted selection study. Our study did not declare an association with any yield traits or phenotypic data. However, previous other studies were proved to Pit1/HinfI polymorphism affects on milk yield, milk composition, meat quality and reproductive traits.

Conflict of Interest: The author declares no conflict of interest.

Authors Contribution Rate: PGB: %35, ED: %35, ME: %15 CU: %15

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RESEARCH ARTICLE

Effect of Shilajit on Freezing Rooster Semen

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ABSTRACT

Reactive oxygen species (ROS) are created in excess during the cryopreservation process, which speeds up the rate of lipid peroxidation (LPO). This negatively impacts spermatozoa functions and reduces their capacity to fertilize. The spermatozoon plasma membrane consists of significant amounts of polyunsaturated fatty acids, which can be easily oxidized by ROS and produce harmful agents that are toxic to cells. The plasma membrane of rooster spermatozoa contains very small amounts of mitochondria, cytoplasm, and cytoplasmic antioxidants. Cryopreservation of rooster semen has been associated with adverse effects, including increased lipid peroxidation, structural damage in the mitochondria and acrosomal area, changes in the integrity and permeability of the spermatozoon plasma membrane, and severe damage to DNA. In the study, semen taken from 20 Plymouth Rock roosters were pooled to eliminate individual differences. By adding 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL and 25 µg/mL shilajit to Beltsville Poultry Semen Extender diluent, 5 experimental and 1 control groups were formed and frozen in 0.25 mL straws. After thawing in a water bath at 37oC, spermatologic parameters were analyzed with the CASA system. Viability evaluations were made with eosin – nigrosin stain and morphological evaluations were made with Hancock method. Sperm DNA integrity was examined with the COMET assay. As a result, it was concluded that the addition of 10, 15, 20 µg/mL shilajit to rooster semen extender improves semen quality parameters and DNA integrity of semen after cryopreservation.
Keywords: Cryopreservation, Rooster, Shilajit, Sperm

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ÖZ

Horoz Spermasının Dondurulmasında Shilajit'in Etkisi

Reaktif oksijen türleri (ROS), kriyoprezervasyon sürecinde aşırı miktarda oluşmakta ve lipid peroksidasyonu (LPO) hızını artırmaktadır. Bu durum, spermatozoa fonksiyonlarını olumsuz etkilemekte ve fertilizasyon yeteneğini azaltmaktadır. Spermatozoon plazma membranı, önemli miktarda çoklu doymamış yağ asidi içermekte ve ROS tarafından kolayca oksitlenebilmekte ve hücrelere toksik olan zararlı maddeler üretebilmektedirler. Horoz spermatozoon plazma membranı, çok az miktarda mitokondri, sitoplazma ve sitoplazmik antioksidan içermektedir. Horoz spermasının kriyoprezervasyonu, artmış lipid peroksidasyonu, mitokondri ve akrozomal bölgede yapısal hasar, spermatozoon plazma membranının bütünlüğü ve geçirgenliğinde değişiklikler ve DNA'da ciddi hasar gibi olumsuz etkilerle ilişkilendirilmiştir. Çalışmada, 20 Plymouth Rock ırkı horozdan alınan sperma bireysel farklılıkları ortadan kaldırmak için bir araya getirildi. Beltsville Poultry Semen Extender (BPSE) Sulandırıcısına 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL ve 25 µg/mL miktarlarda shilajit eklenerek 5 deney ve 1 kontrol grubu oluşturuldu ve 0.25 mL payetler içerisinde donduruldu. 37°C su banyosunda çözdürüldükten sonra spermatolojik parametreler CASA sistemi ile belirlendi. Spermatozoa canlılık oranı eosin – nigrosin boyama metodu ile, morfolojik değerlendirmeler ise Hancock yöntemi ile yapıldı. Spermatozoonn DNA bütünlüğü COMET analiz yöntemi ile değerlendirildi. Sonuç olarak, horoz sperma sulandırıcısına 10, 15, 20 µg/mL shilajit eklenmesinin, kriyoprezervasyon sonrası spermatolojik parametreleri ve DNA bütünlüğünü olumlu yönde etkilediği sonucuna varıldı.
Anahtar Kelimeler: Horoz, Kriyoprezervasyon, Shilajit, Sperma

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INTRODUCTION

With the discovery of the cryoprotective properties of glycerol, sperm cryopreservation has begun to be developed for many species. The first freezing of semen was carried out by Polge in 1951 using rooster semen (Polge, 1951). However, fertility rates with frozen poultry semen are highly variable but not reliable enough for commercial use or preservation of genetic material (Long, 2006).

The overproduction of reactive oxygen species (ROS) during the cryopreservation process accelerates lipid peroxidation (LPO), resulting in harmful effects on spermatozoa functions and a reduction in their fertilization ability. Tissues/cells contain highly oxidizable polyunsaturated fatty acids (PUFA) that are susceptible to lipid peroxidation (Chatterjee et al., 2001; Halliwell & Gutteridge, 2015).

The spermatozoon plasma membrane is rich PUFA, making it highly susceptible to oxidation by ROS, leading to the production of harmful agents that are toxic to cells. On the other hand, spermatozoa are exposed to physical and biochemical stress during the cryopreservation process, which could result in a decline in characteristics such spermatozoa metabolism, motility, plasma membrane integrity, and fertility (Wang et al., 1997).

Oxidative stress is a condition associated with an increased rate of cellular damage caused by oxygen and oxygen-derived oxidants, commonly known as ROS (Sikka et al., 1995). Oxidative stress accelerates a number of pathological conditions that affect fertilization ability (Joyce, 1987; Maneesh et al., 2005; Sharma & Agarwal, 1996).

The main factors hypothesized to potentially play a role in DNA-level damage during the freeze-thaw process are post-thawing osmotic stress and oxidative stress. Spermatozoon remains vulnerable to these stresses as it loses its cytoplasm with its enzymatic defenses (Alvarez & Storey, 1992; Bilodeau et al., 2000) ROS produced in this way can induce DNA damage (Twigg et al., 1998).

Rooster spermatozoa are marked by a relatively low content of cytoplasm, mitochondria, and cytoplasmic antioxidants, along with a high concentration of PUFA in their plasma membranes. These features make rooster spermatozoa especially vulnerable to damage during cryopreservation (Partyka et al., 2010). The cryopreservation of rooster semen has been associated with detrimental effects such as increased lipid peroxidation, structural damage to the mitochondria and acrosomal region, disruptions in plasma membrane integrity and permeability, and considerable DNA fragmentation. (Blesbois et al., 2005; Partyka et al., 2012; Partyka et al., 2010).

Shilajit, also known as shilajatu, mumie or mummiyo, is an exudation of varying consistency, pale brown to blackish-brown in color, found in rocks in many mountain ranges of the world, particularly the Himalayan mountain range in India (Kong et al., 1987; Srivastava et al., 1988).

The main physiological effect of shilajit has been found to be due to the presence of bioactive dibenzo alpha pyrons, together with humic and fulvic acids, which act as carrier molecules for the active ingredients (Ghosal, 1990).

Xiao et al. (2018) has been observed that fulvic acid has a positive effect on sperm morphology, reduces malondialdehyde level, helps to preserve the integrity of the spermatozoon membrane, preserves the integrity of the spermatozoon acrosome and causes an increase in sperm motility.

Sultan et al. (2021), on nili ravi buffaloes, found that after thawing 3% shilajit was added to the semen extender, spermatological parameters such as progressive motility, spermatozoa plasma membrane integrity, viability rate and DNA integrity increased and oxidative stress occurred during cryopreservation decreased.

One of the easiest techniques for identifying single- and double-strand breaks in spermatozoa is the comet test, also known as single-cell gel electrophoresis (McKelvey-Martin et al., 1997). The test's basic idea is that damaged DNA strands can be separated by an electric field while the strands' charge and size assist to make this occur. Following separation, single- and double-stranded broken DNA pieces move to the comet's tail, whereas intact DNA remains in the comet's head (Klaude et al., 1996). As a result, spermatozoa with high DNA strand breakage levels show an ascending comet look (Singh & E. Stephens, 1998) and a dense appearance (Hughes et al., 1996).

To improve the test's efficiency, further metrics such the comet tail moment, olive tail moment, and nucleus diameter were added. The comet test has been applied in numerous investigations, including the assessment of UV radiation, carcinogens, toxicants, and radiotherapy effects, to evaluate DNA damage in a variety of cell types (Singh et al., 1988).

The hypothesis of the study is that the combined effect of fulvic acid and many other components in the content of shilajitin can preserve DNA integrity in frozen rooster semen after thawing. The aim of this study was to examine the effects of five different doses of shilajit, which is an antioxidant, on spermatological parameters and spermatozoon DNA integrity (COMET) in the freezing of rooster semen.

MATERIALS and METHODS

The study was carried out in accordance with the guidelines of the Ethics Committee decision numbered 2020/10 of the Republic of Turkey Ministry of Agriculture and Forestry, Poultry Research Institute.

Roosters and Semen Collection

In our study, semen was collected from Plymouth Rock (n=20) breed roosters, aged 49 weeks, fed ad

libitum and treated with 16 hours of light and 8 hours of dark photoperiod in individual cages, by dorso-abdominal massage method (Tarif et al., 2013). Roosters with semen motility of 90% and above, which were taken and examined, were included in the study. After the preliminary examination, semen from 20 roosters were pooled and divided into 6 equal parts.

Sperm Prosessing and Cryopreservation

Beltsville poultry semen extender (BPSE) was used as diluent and divided into 6 equal parts by adding 5% glycerol as cryoprotectant. By adding various doses of shilajit (S) to the BPSE diluent, study groups were created given as Control (0 µg/mL), S5 (5 µg/mL), S10 (10 µg/mL), S15 (15 µg/mL), S20 (20 µg/mL) and S25 (25 µg) /mL) and semen was diluted. Diluted semen was cooled for 2 hours at 4oC in the refrigerator. After equilibration, the semen was drawn into 0.25 mL straws and sealed with polyvinyl alcohol. Straws were frozen at a distance of 4 cm from the liquid nitrogen surface in the cryobox for 7 minutes and stored in liquid nitrogen. After being thawed in a water bath for 30 seconds at 37 oC, straws were evaluated.

Sperm Motion Parameters

Sperm motility was performed using the CASA system (SCA, Sperm Class Analyzer, Version 6.5.0.91; Microptic, Barcelona, Spain) and a phase contrast microscope (Eclipse Ci-L, Nikon, Japan) with a heating plate. Thawed semen was transferred to Eppendorf tubes. 7 µL of semen was placed on a slide and examined by covering it with a coverslip. Motility (M, %), progressive motility (PM, %), mean path velocity from kinematic parameters (VAP, µm/s), linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), lateral head displacement width (ALH, µm), straight progression (STR, %), linearity (LIN, %) parameters were examined.

Sperm Morphology

Hancock stain was used to evaluate morphology (Schäfer & Holzmann, 2000). The percentages of head, mid-piece, tail, and total spermatozoa abnormalities were calculated. A droplet of semen was mixed with Hancock's stain and then applied to a slide. Phase-contrast microscopy (Eclipse Ci-L, Nikon, Japan) at an enlargement of 100 was used to analyze any anomalies in the sperm (n = 200/slide).

Sperm Viability

The eosin-nigrosin staining method was used to assess the vitality of spermatozoa (Ommati et al., 2013). After thoroughly mixing two droplets of nigrosin-eosin and semen, the mixture was spread out on a microscope slide and allowed to air dry before being examined at 400X magnification using a phase-contrast microscope (Eclipse Ci-L, Nikon, Japan). From each sample, the number of sperm was assessed to be 200; unstained sperm were classified as viable, whereas stained spermatozoa were classified as nonviable.

Comet Assay

Frozen rooster semen thawed at 37 °C for 30 seconds was transferred to eppendorf tubes. The semen in Eppendorf tubes were diluted 1:1 with phosphate buffer solution (PBS), which does not contain Ca+2 and Mg+2, and washed by centrifugation at +4oC for 10 minutes at 800 rpm and the supernatant was removed. The semen was reconstituted and centrifuged and the washing process was repeated. The supernatant was removed again and the spermatozoa were diluted 1:1 with PBS (Fraser & Strzezek, 2004; Nandre, 2007).

120 µL of 0.75% low-melting agarose (LMA) gel prepared in PBS was dropped on the sandblasted slides and smeared. After smearing, it was left to dry at room temperature and the first agarose layer was formed. 10 µL of semen diluted with PBS and 90 µL of 1% LMA Gel were mixed in an eppendorf tube at 37 °C. The entire prepared 100 µL mixture was spread on the first agarose layer and covered with a 24 x 60 mm coverslip and left on the ice pack until solidified. After solidification, the coverslips were carefully pulled and slide preparation was completed (Hughes et al., 1997; Singh et al., 2003).

Lysis solution is used to lyse the cell and nuclear membranes and to release the DNA helixes in agarose. After the spermatozoa were embedded in the agarose gel on the prepared slide, the slides were incubated at +4 oC for 1 hour in a copplin jar using Comet Assay Lysis Solution (R&D Systems, Comet Assay Lysis Solution, Catalog number: 4250-050-01) containing high concentrations of salt and detergent and 1% Triton X-100. After one hour of incubation, 1 mL of Dithioerithrol (DDT) was added to the prepared lysis solution and incubated at +4 oC for 1 hour. At the end of the incubation, 0.5 mL Proteinase K was added into the Copplin jar and incubated in an incubator at +37 oC overnight (Hughes et al., 1997; Singh et al., 2003).

Samples prepared by modifying Shanmugam et al. (2016), were incubated for 20 minutes in a freshly prepared and cooled electrophoresis buffer solution (600 mM NaOH ve 2 mM EDTA, pH 7,3) in an electrophoresis tank for the purpose of separating DNA strands before being carried out in electrophoresis. After the incubation of the spermatozoa embedded in the agarose layer was completed in the electrophoresis buffer solution, they were subjected to electrophoresis in the same buffer solution at 20 volts and 30 mA electrical field for 15 minutes.

After the electrophoresis of the prepared samples, the slides were washed with a freshly prepared Tris buffer solution (0.4 M Tris HCl, pH 7.5) to remove the electrophoresis solution from the samples, and neutralization of the samples was performed (Shanmugam et al., 2016).

After the neutralization process was completed, the DNAs were stained using a fluorescent dye, ethidium bromide (5 µg/mL). For this purpose, a drop of ethidium bromide was dripped onto the samples and

covered with a 24 x 60 mm coverslip and evaluated within 4 hours (Gliozzi et al., 2011).
Samples stained with Ethidium bromide were examined at 400X magnification using a fluorescent attachment phase contrast microscope (Olympus CX-31). 100 comet images from all groups were evaluated (TriTek Comet Score™ Freeware v1.5). All evaluation steps were performed in a dim light environment to avoid further DNA damage (Gundogan et al., 2010). Tail DNA (%), tail Length (μm), Comet Length (μm) and Olive Tail Moment parameters were recorded to define the DNA damage detected as a result of the evaluation.

Statistical analysis

The analyzes in the study were compared and evaluated according to the between-group and in-group analysis. While the one-way ANOVA method was used for in-group analyses, factorial trial design was used for intergroup comparisons. In addition, the differences between the means were investigated with the Duncan Multiple Comparison Test, and the interaction effects were carried out according to the Tukey Multiple Comparison Method. All statistical analyzes and evaluations were made according to the SAS (2009) statistical software. A difference that considered significant was $p<0.05$.

RESULTS

Sperm Motion Parameters

In the statistical evaluation made as a result of the examination of sperm motility and kinematic parameters; It was determined that the differences between the kinematic parameters of VAP, VSL and ALH were significant ($P<0.05$). While no significant difference was detected in motility and progressive motility parameters, the highest motility (77.40 ± 4.34), progressive motility (21.96 ± 1.50), kinematic parameters VAP (33.24 ± 1.04), VSL (18.58) Values ±0.75), VCL (61.09 ±3.28), ALH (1.94 ±0.06), STR (43.89 ±1.54) and LIN (24.45 ±1.60) It was detected in the S20 group (Table 1).

Sperm Morphology and Viability

In the statistical evaluation made as a result of the examination of sperm morphology and viability rate parameters; It was determined that the differences between head, middle part, tail and total parameters of abnormal spermatozoa were significant ($P<0.01$). While there was no significant difference in the ratio of dead/live spermatozoa, the highest survival rate (76.60 ± 3.90%) was found in the S20 group. Among the abnormal spermatozoa values, the lowest head abnormal value (3.50 ± 0.56) was found in the S15 group, the lowest middle part abnormal value (4.40 ± 0.73) was in the S5 group, the lowest tail abnormal value (4.40 ± 0.73) was in the S10 group, and the lowest total abnormal sperm value (26.30 ± 1.54) was in the S5 group (Table 2).

Comet Assay

In the statistical evaluation made as a result of the examination of spermatozoa DNA damage parameters; The differences between the DNA damage parameters Tail DNA ($P<0.0001$), Tail length ($P<0.0001$), Comet length ($P < 0.01$) and Olive tail moment ($P < 0.001$) were found to be significant. The lowest value between groups in Tail DNA value was detected in the S15 group (20.99 ± 0.49), while the highest value was detected in the S25 group (27.52 ± 0.58). The lowest value between the groups in the tail length value was detected in the S15 group (13.20 ± 0.77), while the highest value was detected in the S25 group (19.71 ± 1.00). The lowest value between the groups in olive tail moment value was detected in the S15 group (5.17 ± 0.09), while the highest value was detected in the S25 group (7.04 ± 0.41) (Table 3).

Table 1. Motility and kinematic parameters after thawing of rooster semen.

Parameters	SK	S5	S10	S15	S20	S25	P Value
	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
MOT(%)	73.41 ± 3.00	75.46 ± 3.47	73.33 ± 3.28	74.84 ± 3.61	77.40 ± 4.34	74.58 ± 3.89	> 0.05
PM (%)	17.74 ± 1.50	20.52 ± 1.14	18.56 ± 1.12	19.23 ± 0.99	21.96 ± 1.50	18.83 ± 1.54	> 0.05
VAP (μm/s)	27.89 ± 1.34^{ab}	31.19 ± 0.99^{ab}	29.14 ± 1.03^{ab}	30.48 ± 1.13^{ab}	33.24 ± 1.04^{a}	27.20 ± 3.87^{b}	< 0.05
VSL (μm/s)	15.60 ± 0.93^{b}	17.41 ± 0.68^{ab}	16.44 ± 0.73^{ab}	17.03 ± 0.75^{ab}	18.58 ± 0.75^{a}	16.58 ± 0.86^{ab}	< 0.05
VCL (μm/s)	54.49 ± 2.16	60.23 ± 1.65	57.46 ± 1.67	58.15 ± 1.65	61.09 ± 3.28	58.27 ± 2.40	> 0.05
ALH (μm)	1.65 ± 0.06^{b}	1.82 ± 0.04^{ab}	1.75 ± 0.04^{b}	1.77 ± 0.04^{ab}	1.94 ± 0.06^{a}	1.76 ± 0.06^{b}	< 0.05
STR (%)	43.50 ± 1.34	43.76 ± 0.77	42.83 ± 1.18	43.89 ± 1.21	43.89 ± 1.54	43.09 ± 1.61	> 0.05
LIN (%)	23.29 ± 1.31	23.76 ± 0.90	22.83 ± 0.95	24.33 ± 1.39	24.45 ± 1.60	22.88 ± 1.59	> 0.05

a, b: Values with different letters in each row are statistically significant.
MOT (%): Motility. PM (%): Progressive Motility, VAP (μm/s): average path velocity, VSL (μm/s): straight-line velocity, VCL (μm/s): curvilinear velocity, ALH (μm): amplitude of lateral head displacement, STR (%): straightness, LIN (%): linearity. SK: Control (0 μg/mL shilajit), S5: 5 μg/mL shilajit, S10: 10 μg/mL shilajit, S15: 15 μg/mL shilajit, S20: 20 μg/mL shilajit , S25: 25 μg/mL shilajit

Table 2. Statistical results of sperm morphology and viability rate after thawing.

Parameters		SK	S5	S10	S15	S20	S25	P Value
		$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
Sperm Viability (%)		72.70 ± 3.54	75.70 ± 3.97	73.40 ± 3.74	74.70 ± 3.16	76.60 ± 3.90	73.50 ± 3.56	> 0.05
Sperm Morphology (%)	**Head**	7.00 ± 0.80^{a}	4.40 ± 0.52^{b}	4.10 ± 0.40^{b}	3.50 ± 0.56^{b}	4.70 ± 0.57^{b}	4.30 ± 0.57^{b}	< 0.01
	Mid-piece	6.10 ± 0.99^{bc}	4.40 ± 0.73^{c}	8.70 ± 0.95^{a}	9.40 ± 0.76^{a}	7.50 ± 0.95^{ab}	7.60 ± 0.52^{ab}	< 0.01
	Tail	19.50 ± 1.62^{abc}	17.50 ± 1.15^{bc}	14.20 ± 1.09^{c}	17.90 ± 1.53^{bc}	22.40 ± 3.08^{ab}	23.80 ± 1.89^{a}	< 0.01
	Total	32.60 ± 2.14^{ab}	26.30 ± 1.54^{b}	27.00 ± 0.73^{b}	30.80 ± 1.81^{ab}	34.60 ± 3.57^{a}	35.70 ± 1.84^{a}	< 0.01

a, b, c: Values annotated with distinct letters within each row indicate statistically significant differences. SC: Control (0 μg/mL shilajit), S5: 5 μg/mL shilajit, S10: 10 μg/mL shilajit, S15: 15 μg/mL shilajit, S20: 20 μg/mL shilajit , S25: 25 μg/mL shilajit

Table 3: COMET results after post-thawing rooster sperm

Parameters	SK	S5	S10	S15	S20	S25	P Value
	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	$\overline{X} \pm S_{\overline{X}}$	
Tail DNA (%)	26.08 ± 0.55^{ab}	23.45 ± 1.06^{bc}	20.93 ± 1.59^{c}	20.99 ± 0.49^{c}	24.93 ± 0.46^{ab}	27.52 ± 0.58^{a}	< 0.0001
Tail length (μm)	15.87 ± 0.66^{b}	15.62 ± 1.07^{b}	13.66 ± 0.71^{b}	13.20 ± 0.77^{b}	15.57 ± 0.78^{b}	19.71 ± 1.00^{a}	< 0.0001
Comet length (μm)	45.86 ± 2.17^{b}	44.68 ± 1.58^{b}	44.23 ± 0.74^{b}	42.67 ± 0.38^{b}	47.33 ± 2.04^{b}	51.91 ± 1.82^{a}	< 0.01
Olive tail moment	6.74 ± 0.48^{ab}	5.71 ± 0.34^{bc}	5.00 ± 0.36^{c}	5.17 ± 0.09^{c}	6.48 ± 0.37^{ab}	7.04 ± 0.41^{a}	< 0.001

a, b, c: Values annotated with distinct letters within each row indicate statistically significant differences. SC: Control (0 μg/mL shilajit), S5: 5 μg/mL shilajit, S10: 10 μg/mL shilajit, S15: 15 μg/mL shilajit, S20: 20 μg/mL shilajit , S25: 25 μg/mL shilajit

DISCUSSION

The data in the studies in which the motility and progressive motility of rooster semen frozen by adding antioxidants to the semen were evaluated with the CASA system after thawing were examined. The values of the S20 group in our study were higher than the values in the study in which Safa et al. (2016) used nano-Selenium and Vitamin E as antioxidants, similar to the values in the study in which Najafi et al. (2020) used astaxanthin and lower than the values in the study in which Najafi et al. (2021) used alpha lipolic acid Interestingly, the differences observed between the findings of the mean values of motility and progressive motility findings determined by using the CASA system after thawing of frozen rooster semen with various doses of shilajit added to the BPSE extender obtained in the study and the findings reported in other studies are interestingly due to the reason that shilajit supplementation to thex rooster semen extender increases semen motility. It was concluded that the semiquinone-hydroquinone complex structure resulted in improvement in spermatozoa motility at all stages of cryopreservation, attributable to the radical scavenging effect of dibenzo-pyrones and fulvic acid.
Calculation of sperm kinematic parameters is based on the principle of functional performance of axonemes and membranes of spermatozoa, metric measurements of spermatozoon motility. The definitions of spermatozoa kinematic parameters are based on different measurements of the central positions of the two-dimensional spermatozoon head per unit time. One of the kinematic parameters, VAP (μm/s) value expresses the average velocity of the sperm head per unit time along the mean trajectory of the sperm. The data in the studies in which the kinematic parameters were evaluated with the CASA system after thawing of rooster semen frozen by adding antioxidants to the semen were examined. The VAP value in our study was observed to be higher than the VAP value of the resveratrol group in Rezaie et al. (2021) in parallel with the value of the astaxanthin group in Najafi et al. (2020) and in parallel with values of the crocin and naringenin groups in Mehdipour et al. (2020).
The VSL (μm/s) value, one of the kinematic parameters, expresses the average velocity of the spermatozoon head per unit time along a straight line from its initial position to its final position. The data in the studies in which the kinematic parameters were evaluated with the CASA system after the thawing of the frozen rooster semen by adding antioxidants to the semen were examined. The VSL value in our study was

observed to be lower than the VSL value of the querectin-loaded nano-structured lipid carrier group in A. Najafi et al. (2020), in parallel with the VSL values of the crocin and naringenin groups in Mehdipour et al. (2020) and higher than the VSL value of the resveratrol used groups in Rezaie et al. (2021).

The ALH (μm) value, one of the kinematic parameters, expresses the amplitude of variations of the spermatozoon head orbit relative to the average orbit. The data from the studies in which kinematic parameters of frozen rooster sperm were evaluated with CASA system at the end of thawing by adding antioxidants to the sperm were examined. The ALH (μm) value in our study was observed to be higher than the ALH values belonging to the nano selenium group used in Safa et al. (2016), lower than the ALH values belonging to the groups in which ellagic acid-loaded liposomes were used in Najafi et al. (2019), lower than the ALH values belonging to the querectin-loaded nanostructured lipid carrier group used in A. Najafi et al. (2020), lower than the ALH values belonging to the astaxanthin group used in Najafi et al. (2020), lower than the ALH (μm) values of crocin and naringenin group used in Mehdipour et al. (2020)(Mehdipour et al., 2020), lower than the ALH (μm) values of the resveratrol-used groups in Rezaie et al. (2021).

The fact that some of the values obtained as a result of examining the kinematic parameters made after thawing in frozen rooster semen with the CASA system show parallelism is a sign that our thesis study is in harmony with similar studies. The differences observed in the kinematic parameters after thawing may be due to the modifications made in the components of the semen extender, the differences in the percentage values of the cryoprotectant used, and the efficacy of the antioxidant substances, as well as the rooster breed. In addition, it is thought that these differences may be caused by differences between different CASA software used in Deciphering kinematic parameters and the image and measurement settings of the poultry module in the same software.

It has been reported that high VCL and ALH values and low LIN values in cattle are observed in hyperactive spermatozoa, and also in terms of fertility parameters, the VCL value in cattle should be higher than 70, and the ALH value should be higher than 7 (Kathiravan et al., 2011). As a result of literature researches conducted in terms of kinematic parameters in poultry spermatozoa, no such information has been found. For this reason, it is thought that further studies should be carried out in order to better interpret the kinematic parameters in poultry.

The data in the studies in which the viability rates of rooster semen frozen by adding antioxidants to the semen were evaluated after thawing with 2% eosin-nigrosin were examined. According to the results of the study in which A. Najafi et al. (2020), used querectin as an antioxidant, the values in our study were higher than the viability rate in the groups using querectin, querectin-loaded nanoliposomes and querectin-loaded nano-structured lipid carriers. On the other hand According to the results of the study in which Mehdipour et al. (2020), used crocin and naringenin as antioxidants, the vitality rate was higher than the groups using crocin and naringenin and according to the results of the study where Masoudi et al. (2020), used glutathione as an antioxidant it was observed that the vitality rate was lower.

The data in the studies on the ratios of abnormal spermatozoa with Hancock stain after thawing of rooster semen frozen by adding antioxidants to the semen were examined. The values we found in our study were observed to be lower than the rate of total abnormal spermatozoa after thawing, according to the results of the study in which Lotfi et al. (2017), used hyaluronic acid as an antioxidant. In addition, according to the results of the study in which Najafi et al. (2021) used alpha lipolic acid and alpha lipolic acid nanostructured lipid carrier as antioxidants, it was higher than the post-thawed groups and according to the results of the study, in which Siari et al. (2022) used querectin as an antioxidant, the abnormal spermatozoa rates were higher in the post-thawed group was observed.

The variations observed between the statistical findings of the mean values obtained through manipulations and those reported in other studies are attributed to differences in the effectiveness of antioxidants and the presence of compounds such as fulvic acids, humic acids, humins, fatty acids, triterpenes, selenium, phospholipids, resins, latex, gums, albumins, and selenium. It has been concluded that shilajit, which consists of approximately 80-85% humic substances, including triterpenes, sterols, and aromatics, has the potential to enhance cellular metabolism and improve cell viability.

When the findings obtained as a result of examining the DNA damage of rooster semen after thawing were examined, it was observed that the values of some researchers (Gliozzi et al., 2017; Gliozzi et al., 2011) were lower. Although it is thought that the reason why DNA damage parameters were found lower than other studies with limited literature information is the effectiveness of the antioxidant we used in our research, it was determined that DNA damage decreased in the S10 and S15 groups, increased DNA damage in the S25 group, and there was no difference in DNA damage between the control group and the control group. It has been concluded that fulvic acids, which are intensely found in shilajit used in our research, show antioxidant activity at the cellular level by neutralizing the effects of free radicals, and may reduce the amount of damage by protecting the cell nucleus and mitochondria. It is thought that factors such as the differences in the procedures applied in the COMET technique used in the evaluation of DNA damage in our study, minor modifications made in the technique, imaging, evaluation software and the person performing the analysis may also be effective in the formation of the differences.

CONCLUSION

As a result, in addition to the positive effects of the use of shilajit in freezing rooster sperm in terms of spermatological parameters and DNA damage, it was found that high doses negatively affect spermatological and DNA damage parameters. It has been concluded that the addition of 10, 15, 20 μg/mL shilajit to rooster sperm diluent improves sperm quality parameters such as movement parameters of sperm, viability and DNA integrity after cryopreservation. It is thought that the effect of shilajit on freezing by adding it to rooster semen should be supported by more comprehensive studies by adding in vivo parameters.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: MYN and MS contributed to the project idea, design and execution of the study. MYN contributed to the acquisition of data. MYN and MS analysed the data. MYN drafted and wrote the manuscript. MYN and MS reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Republic of Turkey Ministry of Agriculture and Forestry, Poultry Research Institute and Ondokuz Mayis University Faculty of Veterinary Medicine. This research was approved by The Ethics Committee of the Republic of Turkey Ministry of Agriculture and Forestry (Ref No: 2020/10, Tarih: 12/2020).

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RESEARCH ARTICLE

The Investigation of the Effect of Boron on Intestinal Incision Wound Healing in Rats

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ABSTRACT

In this study 250-300 g 38 adult male Wistar Albino rats were used, randomly divided into six groups as four groups that received boron (n=7/group) and two control groups (n=5/group). Etibor-48 (Borax pentahydrate) was diluted in saline and administered by gavage. The Boron 1 group received 10 mg.kg-1 boron for three days and the Boron 2 group received 10 mg.kg-1 for seven days, Boron 3 group received 30 mg.kg-1 for three days, and the Boron 4 group, received 30 mg.kg-1 for seven days before the start of the study. On day 0, colon incisional wound was performed for all rats under general anesthesia and sutured. Control groups took no drugs and were euthanized on the third and seventh postoperative days. Blood sampling were done via cardiac punction at necropsy to measure Interleukin-1, IL-6, TNF-α, MPO, MDA, NO, GSH, and AOA. The sutured bowel incision line was evaluated histopathologically. Inflammatory cell measurement results in Boron 1, 2, 3, and 4 groups, and Control 3- and 7-day groups were 2.93±0.41, 3.93±0.41, 3.61±0.55, 3.93±0.41, 1.26±0.41, and 1.43±0.51, respectively. There were statistical difference between the groups ($p<0.05$). AOA measurement results in the Boron 1, 2, 3, and 4 groups, and Control 3- and 7-day groups were 7.38±0.64, 8.27±0.57, 9.07±1.16, 9.06±0.86, 10.00±1.47, and 9.86±0.54 mmol.L-1, respectively. There were statistical difference between the Boron 1 and the Control 3- and 7-day groups ($p<0.05$). It is concluded that Borax pentahydrate solution 30 mg.kg-1 had a positive effect on intestinal incisional wound healing, contrary to the literature.
.**Keywords:** Antioxidant activity, Borax pentahydrate, intestinal incisional wound healing, rat

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Ratlarda Barsak Ensizyon Yarası İyileşmesi Üzerine Bor'un Etkisinin Araştırılması

ÖZ

Bu çalışmada 250-300 g, 38 erişkin erkek Wistar Albino rat kullanıldı, rastgele olarak Bor verilen dört grup (n=7/grup) ve iki kontrol grubu (n=5/grup) olmak üzere altı gruba ayrıldı. Etibor-48 (Boraks pentahidrat) serum fizyolojik içinde seyreltildi ve gavaj yoluyla uygulandı. Bor 1 grubuna üç gün 10 mg.kg-1 bor, Bor 2 grubuna yedi gün 10 mg.kg-1 bor, Bor 3 grubuna üç gün 30 mg.kg-1 bor ve Bor 4 gruba, çalışmanın başlamasından yedi gün önce 30 mg.kg-1 bor verildi. 0. günde tüm ratlara genel anestezi altında kolon anastomozu yapıldı. Ameliyat sonrası kontrol gruplarına hiçbir ilaç uygulanmadı ve postoperatif üçüncü ve yedinci gün ötenazi yapıldı. MPO, IL-1, IL-6, TNF-α, MDA, NO, GSH ve AOA'yı ölçmek için nekropsi sırasında tüm sıçanlardan kardiyak punksiyonla kan örneği alındı. Anastomoz bölgesi histopatolojik olarak değerlendirildi. Bor 1, 2, 3 ve 4 grupları ile Kontrol 3 ve 7 günlük gruplarda inflamatuar hücre ölçüm sonuçları sırasıyla 2,93±0,41, 3,93±0,41, 3,61±0,55, 3,93±0,41, 1,26±0,41 ve 1,43±. 0.51 olarak bulundu. Gruplar arasında istatistiksel farklar vardı ($p<0.05$). Bor 1, 2, 3 ve 4 grupları ile Kontrol 3 ve 7 günlük gruplarda AOA ölçüm sonuçları sırasıyla 7,38±0,64, 8,27±0,57, 9,07±1,16, 9,06±0,86, 10,00±1,47 ve 9,86±0,54 mmol/l idi. Bor 1 ve Kontrol 3- ve 7 günlük gruplar arasında istatistiksel fark vardı ($p<0.05$). 30 mg.kg-1 Boraks pentahidrat solüsyonunun bağırsak yara iyileşmesi üzerine literatürün aksine olumlu etkisi olduğu sonucuna varılmıştır.
Anahtar Kelimeler: Antioksidan aktivite, barsak ensizyonel yara iyileşmesi, Borax pentahydrate, sıçan.

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INTRODUCTION

Wound healing is mainly comparable in all tissues; however, it has some distinct features in the gastrointestinal system (Kiliçoğlu et al. 2005). In intestinal wounds, unlike skin wounds, smooth muscle cells also synthesize collagen together with fibroblasts, and the tensile force occurs much faster in the intestine (Yağci 2011). In the gastrointestinal tract, many different features exist that are not found in the skin, such as the presence of a large pool of microorganisms, the effect of serosa on suture line closure, and vascular nutrition specific to the gastrointestinal system. Although our knowledge about local and systemic factors affecting gastrointestinal anastomosis healing is increasing, anastomotic leak and separation are common serious problems with high mortality (Yağci 2011).

Boron is denoted by the symbol 'B', which is first in group 3A in the periodic table (Nielsen et al. 1987; Saritaş et al. 2019). It has an atomic number of 5, an atomic weight of 10.82, a specific gravity of 2.84, and a melting point of 2190±20°C. Boron is not found in a pure form in nature. Instead, it is found in the form of boron salts or silicates by combining with oxygen. There are nearly 200 boron compounds in nature, the primary ones being boric acid and borax (Moseman 1994; Demir 2005).

The present study aimed to examine the histopathological and biochemical effects of Borax pentahydrate on intestinal incisions wound healing in rats.

MATERIAL and METHODS

This research was initiated with the approval of Afyon Kocatepe University Animal Experiments Local Ethics Committee (AKUHADYEK), dated 24.05.2018 with Registry No. 64-17.
A total of 38 adult male rats weighing 250-300 g were used in the study. Rats were housed in standard cages with a 12/12 h light/dark cycle. Animals were allowed to drink water ad libitum and provided rat chow until two hours before the study started. Orally administered Etibor-48 (Borax pentahydrate) obtained from Eti Boron Mining Enterprises was used in the study.

Anesthesia Protocol

General anesthesia was provided using a combination of 13 mg.kg-1 xylazine hydrochloride (Rompun, 50 ml, 23.32 mg.ml-1, Bayer-Germany) and 87 mg.kg-1 ketamine hydrochloride (Alfamine 10%, Ata-Fen, İzmir, TÜRKİYE).

Surgical Procedure

For all rats, on day 0, under general anesthesia, after reaching the descending colon, a 2-3 cm longitudinal incision was made in the antimesenteric region, and a double layer of 6-0 Prolene non-absorbable polypropylene suture was applied (Korkmaz et al. 2015) and checked for leaks. The abdominal wall and skin were closed using established methods in all groups. Gentamicin 4 mg.kg-1 was administered parenterally for five days, and wound care was carried out until the end of the study.

Study Groups

The 38 rats used in the study were divided into six groups at random. Groups 1 through 4 were administered boron diluted in physiologic saline by oral gavage, while groups 5 and 6 served as untreated controls.
The Boron 1 (n=7) group received boron (10 mg.kg-1) for three days before the start of the study and continued until the third postoperative day, at which time the rats were euthanized.
The Boron 2 (n=7) group received boron (10 mg.kg-1) for seven days before the start of the study and continued until the seventh postoperative day, at which time the rats were euthanized.
The Boron 3 (n=7) group received boron (30 mg.kg-1) for three days before the start of the study and continued until the third postoperative day, at which time the rats were euthanized.
The Boron 4 (n=7) group received boron (30 mg.kg-1) for seven days before the start of the study and continued until the seventh postoperative day, at which time the rats were euthanized.
The Control 3-day group (n=5) and Control 7-day group (n=5) did not receive boron and were euthanized three and seven days postoperatively.
At the time of euthanasia on day 3 or 7, samples were taken from the suture line for histopathological examination, and cardiac blood samples were obtained for biochemical measurements.

Biochemical Measurements

Determination of IL-1, IL-6, TNF-α, and MPO Activity

The serum activity of IL-1 (Biont, Rat Interleukin 1 [IL-1] ELISA Kit Catalog No: YLA0153RA), IL-6 (Biont, Rat Interleukin 6 [IL-6] ELISA Kit Catalog No: YLA0031RA), TNF-α (Biont, Rat TNF-α ELISA Kit, Catalog No: YLA0118RA), and MPO (Biont, Rat Myeloperoxidase [MPO] ELISA Kit Catalog No: YLA0046RA) were determined using commercial ELISA kits.

Determination of Malondialdehyde (MDA) Level:

The MDA level was determined based on the double-boiling method, which was modified by Draper and Hadley (1990). During the first boiling, the bound MDA in the samples is liberated from the proteins, and the proteins are precipitated. In the second boiling, the absorbance of the colored complex formed by reacting with total MDA and thiobarbituric acid (TBA) is measured at 532 nm. The concentration of MDA is then calculated using the molar absorption coefficient.

Two test tubes, a control, and a sample were prepared. Trichloroacetic acid (TCA) 10% solution (2.5 ml) was placed in both tubes, after which 0.5 ml of sample was added to the sample tube and 0.5 ml of distilled water to the control tube. The tubes were sealed and kept in a boiling water bath for 15 minutes, then cooled under cold water and centrifuged at 3000 rpm for 10 minutes, after which 2 ml of the upper supernatant was transferred to another tube and 1 ml of 0.675% TBA solution added. The lids were tightly closed, and the tubes were placed in a boiling water bath again for 15 minutes, then cooled in cold water. The absorbance of the sample against the blank was measured at 532 nm in a spectrophotometer. Using the extinction coefficient of the MDA-TBA complex at 532 nm, the MDA value was determined in nmol.ml-1 for serum and nmol.mg-1 for tissue samples.

Determination of Antioxidant Activity (AOA)

The AOA was determined based upon the fact that Fe-EDTA complex standard solution reacts with hydrogen peroxide by the Fenton reaction, leading to formation of hydroxyl radicals. Reactive oxygen radicals degrade benzoate because of TBARS release. Antioxidants added to human fluid cause suppression of TBARS production. This reaction is measured calorimetrically, with suppression of color development detected as AOA (Koracevic et al. 2001).

Determination of Nitric Oxide (NO)

A modified method determined by nitrite + nitrate (NOx) levels, as reported by Miranda et al. (2001), was used to measure NO in tissue and serum samples.

Determination of Glutathione (GSH)

A glutathione assay kit (Cayman Chemical Company, item no.703002, USA) was used to determine the GSH level.

Histopathological Examination

Samples from animals that underwent necropsy were fixed in 10% neutral buffered formaldehyde solution. After 48 hours, they were trimmed and placed into cassettes, followed through a series of alcohol and xylene, then blocked in paraffin and cut to a thickness of 4-5 microns with a microtome and placed on slides. These sections were marked with hematoxylin-eosin and analyzed under a light microscope.

Statistical Analysis

The results were statistically evaluated by a one-way ANOVA test using the SPSS 16.0 statistical package program. The Duncan test was applied to results with statistical differences. Data were expressed as mean ± standard deviation. The level of significance was set as $p<0.05$.

RESULTS

The aim of this study was to investigate the effects of 10 mg.kg-1 and 30 mg.kg-1 orally given Boron on the healing of the intestinal incision line in the 3rd, 7th day and control groups, with histopathological and biochemical parameters.

In this study, histopathological examination results of tissue sections taken from the incision line in Boron 1, Boron 2, Boron 3, Boron 4, Control 3 and Control 7 groups, respectively; as inflammatory cell, fibroblastic activity, neovascularization and collagen level were given. Inflammatory cell measurement results in the groups, found as 2.93±0.41, 3.93±0.41, 3.61±0.55, 3.93±0.41, 1.26±0.41, 1.43±0.51, respectively. Fibroblastic activity values in the groups were recorded as 3.26±0.75, 3.43±0.82, 3.77±0.52, 3.77±0.52, 1.1±0.10, 1.43±0.52, respectively. Neovascularization measurement values found as 2.93±0.41, 3.77±0.52, 3.43±0.52, 3.93±0.41, 1.26±0.41, 1.43±0.52, respectively. Collagen measurement results were determined as 3.26±0.41, 3.43±0.52, 3.43±0.52, 4.10±0.00, 1.10±0.00, 1.10±0.00, respectively. Inflammatory cells, fibroblastic activity, neovascularization and collagen levels obtained as a result of histopathological examinations were found to be statistically significant when compared between all groups ($p<0.05$) (Table 1, Figure 1).

In this study, hemogram (WBC, LYM, MID, GRA, Hb, MCH, RBC, MCV, HCT, PLT) and biochemistry parameters in blood samples taken by intracardiac injection of Boron 1, Boron 2, Boron 3, Boron 4, Control 3 and Control 7 groups (MDA, IL-1, IL-6, TNF-α, MPO, AOA, GSH, NO) measurements were made. When the hemogram results were evaluated, WBC, LYM, MID, RBC, MCV, HCT and PLT levels were not statistically significant when compared between groups ($p>0.05$), while GRA, Hb and MCH parameters were statistically significant ($p<0.05$) (Table 2).

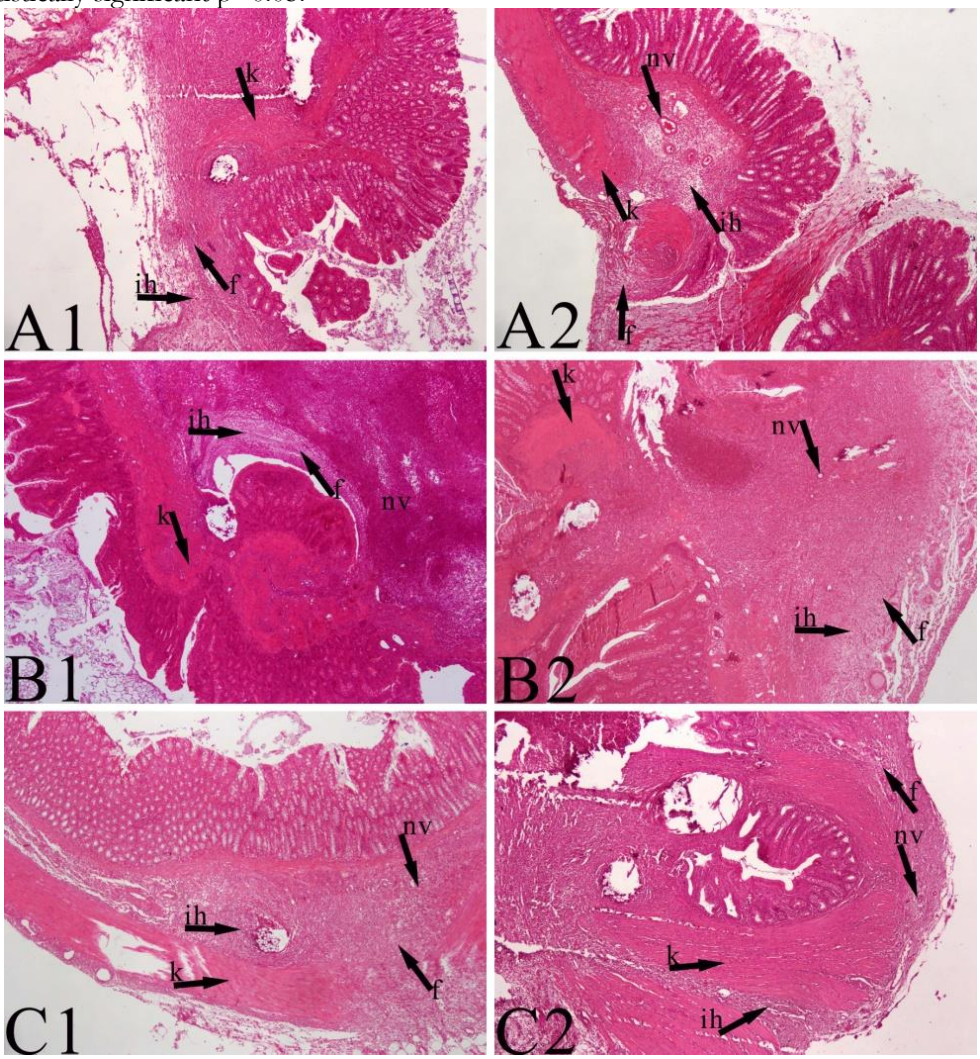
When all groups were compared as a result of biochemical measurements, no statistically significant difference was observed in GSH, TNF-α and IL-1 values ($p>0.05$) (Table 3).

When statistical comparison was made for all groups, it was found that between Boron 1 group and Boron 3, Boron 4, Control 3 and Control 7 groups, Boron 2 group and Boron 4, Control 3 and Control 7 groups, Boron 3 group and Control 7 groups difference was observed ($p<0.05$). According to IL-6 measurement results, the increase in Boron 1 group was found to be statistically significant in comparison with Boron 2, Boron 3, Boron 4, Control 3 and Control 7 groups ($p<0.05$). The increase in Boron 3 group is statistically significant in comparison to Boron 4, Control 3 and Control 7 groups ($p<0.05$) (Table 3).

Table 1: Histopathological results of inflammatory cells, fibroblastic activity, neovascularization, and collagen in groups.

	Histopathological results			
Groups	**Inflammatory cell**	**Fibroblastic activity**	**Neovascularization**	**Collagen**
Boron 1, 10 mg.kg-1 3 days	2.93±0.41[b]	3.26±0.75[a]	2.93±0.41[b]	3.26±0.41[b]
Boron 2, 10 mg.kg-1 7 days	3.93±0.41[a]	3.43±0.82[a]	3.77±0.52[b]	3.43±0.52[b]
Boron 3, 30 mg.kg-1 3 days	3.61±0.55[a]	3.77±0.52[a]	3.43±0.52[a]	3.43±0.52[b]
Boron 4, 30 mg.kg-1 7 days	3.93±0.41[a]	3.77±0.52[a]	3.93±0.41[a]	4.10±0.00[a]
Control 3 days	1.26±0.41[c]	1.11±0.10[b]	1.26±0.41[c]	1.10±0.00[c]
Control 7 days	1.43±0.51[c]	1.43±0.52[b]	1.43±0.52[c]	1.10±0.00[c]
P	**0.000**	**0.000**	**0.000**	**0.000**

[a, b, c:] Inflammatory cells, fibroblastic activity, neovascularization, and collagen values with different letters in the same column are statistically significant $p<0.05$.



A1: Control Day 3; A2: Control Day 7; B1: Boron 10 mg Day 3; B2: Boron 10 mg Day 7; C1: Boron 30 mg Day 3; C2: Boron 30 mg Day 7, nv: Neovascularization, f: Fibroblastic activity, ic: Inflammatory cell, c: Collagen.

Figure 1: Microscopic views of histopathological examination results in groups.

Table 2. Hemogram Results in Groups (Mean ±SD).

	Hemogram Results									
Groups	**WBC (10^9/L)**	**LYM (%)**	**MID (%)**	**GRA (%)**	**Hb (mg/dl)**	**MCH (pg)**	**RBC (10^{12}/L)**	**MCV (fl)**	**HCT (%)**	**PLT (10^9/L)**
Boron 1 10 mg.kg-1 (3 days)	6.24±1.67	47.27±9.41	9.00±1.95	43.72±9.10^{b}	15.15±1.12^{a}	18.85±0.88^{a}	8.06±0.75	45.77±1.89	36.81±2.58	617.71±93.85
Boron 2 10 mg.kg-1 (7 days)	8.46±5.92	52.27±22.07	11.08±3.45	36.64±21.34^{c}	14.11±1.44^{a}	19.30±1.20^{b}	7.35±1.07	47.28±1.85	34.66±4.37	818.85±168.84
Boron 3 30 mg.kg-1 (3 days)	5.76±4.08	51.90±6.22	11.55±2.51	36.54±6.47^{c}	15.41±0.46^{a}	18.01±0.63	8.57±0.45	45.41±1.96	38.87±1.30	524.85±212.64
Boron 4 30 mg.kg-1 (7 days)	6.12±1.74	42.20±14.54	10.95±6.66	46.84±12.88^{d}	13.84±1.76^{b}	18.11±0.96^{abc}	7.66±1.14	45.08±2.13	34.44±4.76	785.42±155.44
Control (3 days)	8.04±2.66	38.90±5.34	7.96±3.93	53.14±9.10^{e}	14.98±1.12^{b}	17.94±1.21	8.36±0.51	44.50±1.98	37.20±2.51	496.40±123.36
Control (7 days)	5.66±2.35	37.18±9.90	8.18±3.53	54.64±12.78^{a}	14.20±0.66^{b}	18.30±0.89^{abd}	7.79±0.59	45.08±2.13	35.02±1.54	855.40±81.62

WBC: Leukocytes (White Blood Cells), LYM: Lymphocyte, MID: Monocytes, GRA: Granulocyte, Hb: Hemoglobin, RBC: Red Blood Cells, PLT: Platelets, MCV: Mean Cell Volume, HCT: Hematocrit, MCH: Mean Corpuscular Hemoglobin

Values with different letters (a,b,c,d,e) between groups in the same column are statistically significant ($P < 0.05$).

Table 3. Biochemistry Analysis Results in Groups (Mean ±SD).

Biochemistry Analysis Results								
Groups	**MDA (mcmol/gH)**	**IL-1 (pg/ml)**	**IL-6 (ng/L)**	**TNF-α (ng/L)**	**MPO (ng/ml)**	**AOA (mmol/L)**	**GSH (nmol/gHb)**	**NO (mcmol/ml)**
Boron 1 10 mg.kg-1 (3 days)	5.03±0.11[a]	20.64±2.98	41.55±8.68[a]	47.12±8.17	22.72±5.61[a]	7.38±0.64[a]	2.41±0.32	12.56±0.80[a]
Boron 2 10 mg.kg-1 (7 days)	4.96±0.16[a]	19.52±1.70	25.34±5.30[b]	42.66±4.85	16.57±2.40[b]	8.27±0.57[ab]	2.25±0.27	10.52±1.20[ab]
Boron 3 30 mg.kg-1 (3 days)	4.37±0.44[ab]	21.41±2.10	31.43±3.01[abc]	43.46±4.93	19.98±1.43[ef]	9.07±1.16[ab]	2.20±0.37	11.33±0.74[ab]
Boron 4 30 mg.kg-1 (7 days)	3.81±0.61[ac]	21.24±1.76	19.62±3.61[abd]	45.36±10.29	15.51±1.66[c]	9.06±0.86[ab]	2.21±0.33	10.12±1.63[b]
Control (3 days)	4.03±0.80[ad]	22.67±1.43	25.54±4.99[afb]	39.82±6.31	16.11±2.86[d]	10.00±1.47[b]	2.11±0.06	10.12±1.55[b]
Control (7 days)	3.28±0.25[ae]	20.90±2.51	16.14±2.27[abe]	39.22±5.04	14.64±1.44[e]	9.86±0.54[b]	3.05±1.18	9.68±0.85[b]

Values with different letters (a, b, c, d, e) in the same column are statistically significant ($P<0.05$).

MDA: Malondialdehyde, IL-1: Interleukin-1, IL-6: Interleukin-6, TNF-α: Tumor Necrosis Factor-Alpha, MPO: Myeloperoxidase, IMA: Ischemic Modified

Albumin, AOA: Antioxidant Activity; GSH: Glutathione, NO: Nitric Oxide.

According to the statistical comparison of the MPO measurement results of all groups, the increase in Boron 1 group was statistically significant when compared with Boron 2, Boron 3, Boron 4, Control 3 and Control 7 groups ($p<0.05$). There is a statistical difference between Boron 3 group and Control 3 and Control 7 groups. The increase in boron 3 group was significant ($p<0.05$) (Table 3).

When the findings obtained through AOA measurements were compared between all groups, there was a difference between the Boron1 group and the Control 3 and Control 7-day groups ($p<0.05$). There was no difference between Boron 2, Boron 3, Boron 4 groups and any other groups ($p>0.05$) (Table 3).

When the findings obtained as a result of GSH measurements of all groups were compared statistically, the difference in Boron 1 group was statistically significant ($p<0.05$) when the increase in Boron 2, Boron 3, Boron 4, Control 3 and Control 7 groups was compared (Table 3).

DISCUSSION

Colon anastomoses are among the most common surgical procedures in humans and animals. In studies on wound healing and follow-up, anastomotic leakage is a frequent complication. High morbidity has led to an increase in the number of studies and research being conducted. The present study examined the histopathological and biochemical effects of orally administered boron on incisional wound healing of colon in rats.

Healing of wounds is a complicated process in which the tissue repairs itself (Stadelmann et al. 1998; Korkmaz et al. 2015). While the healing process of wounds is similar for different tissues, in the gastrointestinal tract, it has some characteristic properties such as tension time that develops much earlier than in the skin (Cronin et al. 1968; Korkmaz et al. 2015), and collagen is synthesized by smooth muscle cells in intestinal wounds (Graham et al. 1987; Korkmaz et al. 2015).

Intestinal wound healing involves inflammation, proliferation-fibroplasia, and maturation stages. Inflammation begins with vasodilation, secretion of vasoactive materials, increased vascular permeability, and neutrophil infiltration within three hours following vasoconstriction of the wound edges. Macrophages and fibroblasts transfer to the wound site, where macrophages control inflammation through cytokine release (Brasken 1991; Graham et al. 1992; Korkmaz et al. 2015).

There are both local and systemic aspects in the healing of intestinal wounds (Frostberg et al. 2014; Korkmaz et al. 2015). For the extracellular matrix significant factors include collagen fibers, fibroblasts, and immune cells which control wound strength during the early stages of postoperative healing process (Carrico et al. 1984; Frostberg et al. 2014; Korkmaz et al. 2015).

Tissue sections taken from the anastomosis line were evaluated histopathologically for inflammatory cells, fibroblastic activity, neovascularization, and collagen levels (Table 1). The number of inflammatory cells was high in the Boron 2, 3, and 4 groups, with statistical significance ($p<0.001$). Fibroblastic activity measurements from the anastomosis line found the Control 3- and 7-day groups to be statistically lower than in the Boron groups ($p<0.001$). When neovascularization findings obtained in all groups were compared, the highest level was found in the Boron 4 group. On the other hand, neovascularization in the Control 3- and 7-day groups was statistically lower than the Boron groups. The collagen measurement result of the Boron 4 group was statistically significant compared to the other Boron groups. On the other hand, collagen levels in the Control 3- and 7-day groups were statistically lower than all Boron groups.

It was noted that there was a significant increase in fibroblastic activity in the Boron 4 group compared to the Control groups and other Boron groups. It was determined that collagen formation increased 4-fold in the Boron 4 group compared to the Control 7-day group. Inflammatory cell formation was determined in the 7-day 10 mg and 30 mg Boron groups at a high rate. Histopathological evaluations revealed healing to be more uniform in the Boron 4 group, followed by the Boron 2 group; therefore, intestinal wound healing was clinically and histopathologically better in day-7 groups, in line with the literature.

Postoperative pain is one of the widespread issues in surgery. According to various reports, pain treatment is not sufficient in almost half of patients (Gottschalk and Smith 2001; Korkmaz et al. 2015). A multimodal approach to analgesia is necessary for the reduction of discomfort from different mechanisms. Opioids, local anesthetic agents, non-steroidal anti-inflammatory drugs (NSAIDs), paracetamol, and gabapentinoids are examples of drugs used to treat pain (Carstensen and Moller 2010; Wood 2010; Korkmaz et al. 2015).

Today, one of the most common complications in bowel operations is adhesions. Studies have reported that 12% to 17% of patients who undergo abdominal surgery for various reasons develop ileus due to serosal adhesions in the early or late postoperative period (Saribeyoğlu et al. 2008; Koç et al. 2013).

İnce et al. (2010) stated that boron and boric acid reduce liver GSH levels and boosts kidney GSH levels in rats. In our study, there were no statistical differences between any of the groups regarding GSH.

NO, MDA and MPO are substances produced by inflammatory cells or formed as by-products. NO is produced by the enzyme inducible nitric oxide synthetase (INOS) found in neutrophils. As a result of triggering the INOS enzyme in sepsis and inflammation, NO production increases (Faist et al.1996; Anup and Balasubramanian 2000; Koç et al. 2013). In the study by Koç et al. (2013) the NO level was significantly lower in an anastomotic group administered 4% icodextrin compared to a group without icodextrin use. In the present study, comparing the findings in NO levels in all groups, there was a statistically significant difference between the Boron 1 group and the Boron 4, Control 3-, and 7-day groups. There was no difference between the Boron 3 group and the other groups.

MDA is a by-product formed by the cells involved in the inflammatory response when oxygen radicals break down lipid-containing structures such as plasma and cell membranes. It is a parameter used to evaluate both tissue damage and the severity of inflammation (Singal et al. 1983; Kaul et al. 1993; Koç et al. 2013). In the study by Koç et al. (2013), MDA levels were significantly lower in the anastomosis group using 4% icodextrin. In our study, there was a significant difference ($p<0.05$) in the MDA level between the Boron 3 group and the Control 7-day group.

MPO is an enzyme used to create toxic agents that neutrophils use to break down the agents they phagocytize. It is used as an indicator of neutrophil infiltration in tissues. In the same study by Koç et al. (2013), MPO was significantly lower in the group in which 4% icodextrin was used. It has been reported that this low value indicated less adhesion formation due to a less severe inflammatory response. In the present study, when the MPO levels obtained in all groups were compared, there was a statistically significant difference between the Control 7-day and Boron 3 groups.

The biochemical role of boron is still not clear. Although the nutritional importance of boron in some pathological conditions such as arthritis and osteoporosis is not defined, it has been reported that boron increases optimal function throughout the life cycle (Naghii and Samman 1997; Wallace et al. 2002). Various studies have shown that boron is an important element for humans and animals and plays a relative role in macro mineral metabolism, endocrine functions (calcitonin, estrogen, insulin, thyroid), hormones, vitamin D metabolism, bone metabolism, and immune functions (Kabu and Akosman 2013; Kabu et al. 2015; Korkmaz et al. 2019).

A clinical study showed that 17-beta-estradiol and testosterone levels considerably increased in postmenopausal women who received a 3 mg.day-1 boron supplement for seven weeks. According to the same report, boron supplementation results in two-fold increase in testosterone coupling and a significant increase in calcium retention (Nielsen et al. 1987; Naghii et al. 2011). In another study, in males who received 10 mg of boron supplementation daily for four weeks, a significant increase in 17-beta-estradiol levels and increased plasma testosterone was reported (Nielsen 1994; Naghii and Samman 1997; Naghii et al. 2011). Wallace et al. argued that acute supplementation with 11.6 mg of boron as 102.6 mg of sodium tetraborate decahydrate with a meal resulted in a significant increase in plasma boron concentration in comparison to placebo in healthy middle-aged men (Wallace et al. 2002; Naghii et al. 2011). In general, researchers have stated that there was a 10-fold increase in plasma boron from fasting concentrations (Wallace et al. 2002; Naghii et al. 2011).

Naghii et al. (2011) stated that sex hormone-binding globulin showed significantly lower concentrations following boron consumption with no considerable difference, other than TNF-α which again showed low concentrations and six-hour boron supplementation had no significant effect on hormone concentrations.

In a study by Korkmaz et al. (2019) the fact that boron and hyaluronic acid increased antioxidant enzymes, SOD, and catalase levels in both blood and cartilage tissue showed that these two agents contribute to the antioxidant defense system. Also, the researchers reported that it was the first study on MDA, GSH, SOD, and catalase levels in both blood and articular cartilage tissue to evaluate the effect of boron administered intravenously to rats with an osteochondral defect. In this study, we determined that the AOA was significant.

Adhesion development in groups that underwent necropsy on the third and seventh postoperative days (Lange et al. 1995) was evaluated according to a 0-3+ scale. Adhesion development at the level of 0-1+ was observed in all groups; however, no adhesion formation was observed at the sutured incision line to the peritoneum and intra-abdominal organs.

It has been reported that a complex reaction called acute-phase inflammatory response begins immediately after surgical trauma (Pepys 1981), and the production of acute-phase proteins will increase immediately after surgical interventions (Roumen et al. 1992; Wilmore 1997). The substance that regulates the acute-phase protein response is IL-6 (Heinrich et al. 1990; Pullicino et al. 1990), and IL-6 secretion stimulates the secretion of other inflammatory cytokines such as TNF-α and IL-1 (Heinrich et al. 1990; Ertel et al. 1990; Yamamoto et al. 1993). In this study, however, there were no statistical differences in the results between the groups with regard to serum TNF-α values.

The increase in serum IL-6 level in the Boron 3 group was statistically significant compared to the Boron 4 and Control 3- and 7-day groups. Similarly, the increase in the Boron 1 group was statistically significant compared to all other groups. Comparing the findings obtained from all groups for IL-1, there were no differences between the groups ($P>0.05$) (Cruickshank et al. 1990). The acute-phase response reaches its peak value after trauma, and these mediators increase after surgical intervention (Conner et al. 1988; Nishimoto et al. 1989; Gruys 1994). Harada et al. (1997) found a statistically significant relationship between IL-6 and TNF-α levels. As such, an increase in the mentioned parameters was determined in the Boron 1 and Boron 3 groups. Based on these results, it was determined that the levels of inflammatory mediators decreased after the third day in parallel with the literature.

CONCLUSION

In conclusion, in this study, in which Borax pentahydrate solution was given orally by gavage, a 30 mg.kg-1 dose was determined to have positive effects on intestinal wound healing; however, more detailed studies are needed to expand upon these results.

Conflict of interest: The authors declareted that there are no conflicts of interest.

Authors' Contributions: Alarslan carried out the planning, experimental phase and writing of the study. Sarıtaş supervised the planning and writing stages of the study.

Ethical approval: This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (AKUHADYEK).

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RESEARCH ARTICLE

Some Fertility Traits of Ile de France Sheep and Preweaning Growth of Their Lambs in Intensive Conditions

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ABSTRACT

This study was conducted to examine some reproductive characteristics for Ile de France sheep and pre-weaning growth of their lambs in intensive conditions. In this research, 233 head two-year-old Ile de France sheep and 209 lambs born from them were used as animal material. Some fertility characteristics (pregnancy rate, infertility rate, abortion rate, stillbirth rate, birth rate, single birth rate, twin birth rate and litter size) of Ile de France sheep and pre-weaning growth characteristics of lambs (birth weight, live weight and daily live weight gain) were examined. Pregnancy rate, infertility rate, abortion rate, stillbirth and birth rate were determined 76.82, 23.18, 3.91, 1.68 and 72.53%, respectively in intensively reared Ile de France sheep. The litter size was calculated 1.24 in this study. It was found to the least squares means of birth weight, 30th day, 60th day, 90th day live weight and daily live weight gain of Ile de France lambs were 4.93, 10.01, 17.32, 27.80 kg and 212.20 grams, respectively. The effect of birth type was found to be significant ($p<0.05$; $p<0.001$) on the characteristics examined. As a result, it can be said that some fertility parameters of Ile de France sheep need to be improved and the birth weight of the lambs is at a good level. Considering the twinning rate of the ewes and the litter size, it was concluded that the reproductive performance of the herd may increase in the following production seasons.

Keywords: Birth weight, Fertility, Growth, Ile de France, Live weight, Sheep

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Entansif Koşullarda Yetiştirilen Ile De France Koyunlarda Bazı Döl Verimi Özellikleri ve Kuzularında Sütten Kesim Öncesi Büyüme

ÖZ

Bu çalışma entansif koşullarda yetiştirilen Ile de France koyunlarda bazı dölverimi özellikleri ile kuzularında sütten kesim öncesi büyümenin incelenmesi amacıyla yapılmıştır. Araştırmada hayvan materyali olarak iki yaşlı 233 baş Ile de France koyun ve bunlardan doğan 209 baş kuzu kullanılmıştır. Ile de France koyunların bazı dölverim özellikleri (gebelik oranı, kısırlık oranı, Abort oranı, ölü doğum oranı, doğum oranı, tek doğum oranı, ikiz doğum oranı ve doğuran koyun başına düşen kuzu sayısı) ile kuzularının sütten kesim öncesi büyüme özelliklerinden doğum ağırlığı, canlı ağırlık ve günlük canlı ağırlık artışı incelenmiştir. Entansif olarak yetiştirilen Ile de France koyunlarda gebelik oranı, kısırlık oranı, abort oranı, ölü doğum oranı ve doğum oranı sırasıyla; %76,82; 23,18; 3,91; 1,68 ve 72,53 olarak tespit edilmiştir. Bu çalışmada doğuran koyun başına düşen kuzu sayısı 1,24 olarak hesaplanmıştır. Ile de France kuzuların doğum ağırlığı, 30. gün, 60. gün, 90.gün canlı ağırlık ve günlük canlı ağırlık artışı genel ortalamaları sırasıyla; 4,93; 10,01; 17,32; 27,80 kg ve 212,20 gram olarak bulunmuştur. İncelenen özelliklere doğum tipinin etkisi önemli ($p<0,05$; $p<0.001$) bulunmuştur. Sonuç olarak Ile de France koyunların bazı dölverimi parametrelerinin iyileştirilmesi gerektiği, kuzularda doğum ağırlığının iyi seviyede olduğu söylenebilir. Ile de France koyunların ikizlik oranı ve doğuran koyun başına düşen kuzu sayısı dikkate alındığında sürü yönetiminin iyileştirilmesiyle dölverim performansının sonraki üretim sezonlarında yükselebileceği kanaatine varılmıştır.

Anahtar kelimeler: Büyüme, Canlı ağırlık, Doğum ağırlığı, Dölverimi, Ile de France, Koyun

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GİRİŞ

Koyunun MÖ 6000-8000' li yıllarda evciltildiği ve Muflon, Arkal ve Argali koyunlarının evcil koyunlara köken teşkil eden yabani koyunlar olduğu bildirilmektedir (Akçapınar ve Özbeyaz, 2021). Koyunlar diğer türlere nazaran meralardan daha iyi faydalanmaktadır. Türkiye coğrafi yapısı itibariyle koyun yetiştiriciliğine oldukça uygun bir ülkedir. Dünyada süt, et ve yapağı yönlü geliştirilmiş koyun ırkları bulunmaktadır. Türkiye koyun varlığının önemli bir kısmını kombine düşük verimli yerli ırklar oluşturmaktadır. Koyun yetiştiriciliğinde verimlerin artırılması için melezleme yapılmaktadır. Son yıllarda hem melezlemede kullanmak hem de saf olarak yetiştirilmek amacıyla bazı kültür ırkları Türkiye'ye getirilmektedir. Bu ırklardan Ile de France koyununun anavatanı Fransa olup etçi bir ırktır (Akçapınar 1994). İngiliz etçi koyun ırkı Leicester ile Rambouillet ve Merinos arasında yapılan melezleme ile elde edildiği ifade edilmektedir (Akçapınar 1994; Anonim 2024). Koyunların erken ve geç gelişen ırk olmasına göre puberta ve damızlıkta ilk kullanılma yaşı 6-10 ay ve 7-20 ay arasında değişmektedir (Akçapınar ve Özbeyaz, 2021). İngiliz etçi koyun ırkları gibi ırklar erken geliştikleri için 7-12 aylık yaşlarda damızlıkta kullanılabilmektedir (Akçapınar, 1994).

Ile de france ırkında yapılan çalışmalarda gebelik oranı %85.00-94.98, doğum oranı %82,0 ve 90,80, ikiz doğum oranı %16,90, doğuran koyun başına düşen kuzu sayısı 1,17-1,65, farklı dönemlerde canlı ağırlıklara ilişkin genel ortalamalar doğum ağırlığı için 3,19-4,89 kg, 30.gün canlı ağırlık için 9,88-15,05 kg, 60.gün canlı ağırlık için 19,95 kg ve 90.gün canlı ağırlık için 29,98 kg olarak bildirilmektedir (Aktaş 1971; Ivanova ve Raicheva 2015; Atli 2017; Achkakanova ve Staykova 2019; Staykova ve Achkakanova 2019). Farklı düzeylerde Ile de France melezi kuzularda yapılan çalışmalarda doğum ağırlığı, 30., 60. ve 90. gün canlı ağırlık ortalamaları sırasıyla; 3,75-4,67; 9,16; 14,87; ve 22,31 kg olarak bulunmuştur (Aydoğan 1985; Gökdal ve ark. 2006; Cloete ve ark. 2007; Kandemir ve ark. 2013).

Bu çalışmanın amacı entansif koşullarda yetiştirilen Ile de France koyunlarda gebelik oranı, kısırlık oranı, abort oranı, ölü doğum oranı, doğum oranı, tek doğum oranı, ikiz doğum oranı ve doğuran koyun başına düşen kuzu sayısı ile kuzularında doğum ağırlığı, farklı dönemlerde canlı ağırlıklar ve günlük canlı ağırlık artışının incelenmesidir.

MATERYAL ve METOD

Bu çalışma Konya ilinde bulunan özel bir işletmedeki iki yaşlı 233 baş Ile de France koyun ve 209 baş kuzularında 2022-2023 yıllarında yürütülmüştür.

Koyun ve kuzular gezinti alanı bulunan kapalı ağılda entansif olarak yetiştirilmektedir. Tohumlamalar haziran ve ağustos ayları arasında beş baş koç ile serbest olarak yapılmıştır. Tohumlamadan önce herhangi bir özel uygulama yapılmamıştır. Doğumlar Kasım-Ocak ayları arasında gerçekleşmiştir. Tohumlama ve doğum sezonunda kayıt altına alınan koyunlarda gebelik oranı (gebe kalan koyun sayısı/koçaltı koyun sayısı), doğum oranı (doğuran koyun sayısı/koçaltı koyun sayısı), tek doğum oranı (tek doğuran koyun sayısı/ doğuran koyun sayısı), ikiz doğum oranı (ikiz doğuran koyun sayısı/ doğuran koyun sayısı), kısırlık oranı (gebe kalmayan koyun sayısı/koçaltı koyun sayısı), abort oranı (abort yapan koyun sayısı/gebe kalan koyun sayısı), ölü doğum oranı (ölü doğum yapan koyun sayısı/ gebe kalan koyun sayısı) ve doğuran koyun başına düşen kuzu sayısı (doğan kuzu sayısı/doğuran koyun sayısı) hesaplanmıştır. Kuzular doğumlarını takiben ilk 12 saat içerisinde tartılmış ve plastik küpe ile numaralandırılmıştır. İlk doğan kuzu 30 günlük yaşa ulaşmadan tartıma başlanarak 30 gün aralıklarla sütten kesime (yaklaşık 90.gün) kadar devam edilmiştir. Bu tartım verileri kullanılarak interpolasyon ile 30. ve 60. gün canlı ağırlık, ekstrapolasyon ile 90.gün canlı ağırlık değerleri hesaplanmıştır. Günlük canlı ağırlık artışı doğum ağırlığı ile son tartım arasında hesaplanmıştır. Kuzuların tartımında doğum ağırlığı için 10 grama hassas, diğer canlı ağırlıkların belirlenmesinde 50 grama hassas kantar kullanılmıştır. Koyun ve kuzuların beslenmesi işletmenin imkanları doğrultusunda rutin uyguladığı şekilde gerçekleştirilmiştir. Koyunlar %26,0 ham protein, %5,7 ham yağ, %3,7 ham selüloz, %19,0 ham kül ve %0,8 Sodyum içeriğine sahip konsantre yem, kuru yonca ve saman ile beslenmiştir. Kuzulara ise %16,0 ham protein, %3,4 ham yağ, %8,6 ham selüloz, %8,2 ham kül ve %0,25 Sodyum içeren kuzu yemi ve kuru yonca verilmiştir. Ayrıca koyunlar işletmenin rutin aşı programı kapsamında aşılanmıştır. İstatistik analizlerde incelenen özellikler için aşağıdaki modeller kullanılmıştır.

Doğum ağırlığı, 30. gün canlı ağırlık, 60.gün canlı ağırlık ve günlük canlı ağırlık artışı için;

$$Yijkl = \mu + Ci + DTj + DAk + eijkl$$

90. gün canlı ağırlık için;

$$Yijk = \mu + Ci + DTj + eijk$$

Bu modelde; Yijk: Gözlem değerini, μ: Beklenen ortalama değeri, Ci: cinsiyetin etkisini (i =erkek ve dişi), DTj: Doğum tipinin etkisini (j = tek, ikiz), DAk: Doğum ayının etkisini (k = Kasım, Aralık ve Ocak) ve eijkl: Rastgele hatayı ifade etmektedir. İstatistiki analizlerde SPSS for Windows programından yararlanılmıştır (Anonim 2009).

BULGULAR

İç Anadolu Bölgesi şartlarında özel bir işletmede entansif olarak yetiştirilen Ile de France koyunların bazı dölverim özelliklerine ait değerler Tablo 1' de verilmiştir. Bu koyunlarda gebelik oranı, kısırlık oranı, abort oranı, ölü doğum ve doğum oranı sırasıyla; %76,82; 23,18; 3,91; 1,68 ve 72,53 olarak tespit edilmiştir. Tek ve ikiz doğum oranı %76,33 ve 23,67'dir. Doğuran koyun başına düşen kuzu sayısı 1,24 olarak tespit edilmiştir.

Ile de France kuzularda sütten kesim öncesi büyüme özelliklerine ilişkin bulgular Tablo 2' de sunulmuştur. Doğum ağırlığı genel ortalaması 4,93 kg'dır. Doğum ağırlığına doğum tipi ve doğum ayının etkisi önemli ($p<0,001$) bulunmuştur. Cinsiyetin etkisi ise önemli değildir. Otuzuncu gün canlı ağırlık değeri ortalaması 10,01 kg olup doğum tipi ve doğum ayının etkisi önemli ($p<0,001$), cinsiyetin etkisi ise önemli değildir. Altmışıncı gün canlı ağırlık için hesaplanan genel ortalama 17,32 kg olmuştur. Bu parametreye sadece doğum tipinin etkisi önemli ($p<0,001$) bulunurken cinsiyet ve doğum ayının etkisi önemli olmamıştır. Doksanıncı gün canlı ağırlık genel ortalaması 27,80 kg'dır. Doksanıncı gün canlı ağırlığa sadece doğum tipinin etkisi önemli ($p<0,05$) olup cinsiyet ve doğum ayının etkisi önemli değildir. Günlük canlı ağırlık artışı genel ortalaması 212,20 gram olarak hesaplanmıştır. Bu özelliğe doğum tipinin etkisi önemli ($p<0,001$) bulunurken cinsiyet ve doğum ayının etkisi önemli olmamıştır.

Tablo 1. Ile de France koyunlarda bazı döl verim özellikleri
Table 1. Some fertility traits in Ile de France sheep

Özellik	**n**	**Oran (%)**
Koçaltı Koyun Sayısı	233	
Gebe Koyun Sayısı	179	76,82
Kısır Koyun Sayısı	54	23,18
Abort Yapan Koyun Sayısı	7	3,91
Ölü Doğum Yapan Koyun Sayısı	3	1,68
Doğuran Koyun Sayısı	169	72,53
Tek Doğuran Koyun Sayısı	129	76,33
İkiz Doğuran Koyun Sayısı	40	23,67
Doğuran Koyun Başına Düşen Kuzu Sayısı	1,24	

Tablo 2. Ile De France kuzularda sütten kesim öncesi canlı ağırlıklar ve günlük canlı ağırlık artışına ait ortalamalar ve standart hataları
Table 2. Preweaning live weights and daily weight gain means and standard errors in Ile De France lambs

Özellik		Doğum ağırlığı (kg)		30. gün canlı ağırlık (kg)		60. gün canlı ağırlık (kg)		90. gün canlı ağırlık (kg)		Günlük canlı ağırlık artışı (gram)	
		n	$\bar{X} \pm S_{\bar{x}}$	n	$\bar{X} \pm S_{\bar{x}}$	n	$\bar{X} \pm S_{\bar{x}}$	n	$\bar{X} \pm S_{\bar{x}}$	n	$\bar{X} \pm S_{\bar{x}}$
	μ	209	4,93±0,093	118	10,01±0,288	87	17,32±0,929	22	27,80±1,397	82	212,20±13,702
Cinsiyet											
	Erkek	103	4,97±0,097	55	10,07±0,394	47	17,31±1,033	12	28,77±1,94	45	214,64±15,278
	Dişi	106	4,88±0,093	63	9,95±0,313	40	17,34±1,019	10	26,83±2,079	37	209,76±15,162
Doğum tipi			***		***		***		*		***
	Tek	129	5,38±0,081 [a]	77	11,55±0,300 [a]	51	20,31±0,993 [a]	10	31,53±2,079	48	244,80±14,706 [a]
	İkiz	80	4,47±0,109 [b]	41	8,47±0,407 [b]	36	14,34±1,061 [b]	12	24,07±1,94	34	179,60±15,757 [b]
Doğum ayı			***		***						
	Kasım	93	5,47±0,090 [a]	82	11,17±0,239 [a]	82	18,16±0,453	-	-	77	236,24±6,892
	Aralık	27	4,99±0,172 [b]	24	9,30±0,458 [b]	5	16,49±1,801	-	-	5	188,16±26,507
	Ocak	89	4,32±0,093 [b]	12	9,57±0,657 [b]	-	-	-	-	-	-

*: P<0,05; ***:P<0,001.
[a,b]: Aynı sütunda her alt grupta farklı harf taşıyan ortalamalar arası farklılıklar önemlidir ($p<0,05$).

TARTIŞMA

Koyun yetiştiriciliğinde döl verimi ve kuzu verimi karlılık için önemli parametrelerdir. Bu çalışmada Entansif koşullarda yetiştirilen Ile de France koyunların gebelik oranı %76,82 olarak hesaplanmıştır. Bu değer saf Ile de France koyunlar için bildirilen (Aktaş 1971; Atli 2017; Achkakanova ve Staykova 2019) değerlerden (%85,00-94,98) düşük olmuştur. Ayrıca Karacabey ve Orta Anadolu Merinosu için bildirilen (İriş 2018; Behrem ve ark. 2022) gebelik oranı değerlerinden de düşüktür. Bu çalışmada Ile de France koyunların kısırlık oranı %23,18 olarak tespit edilmiştir. Kısırlık oranı, Aktaş (1971) ve Atli (2017) tarafından Ile de France koyunlarda yürütülen çalışmalarda bildirilen (%15,0 ve %7,7) değerlerden yüksektir. Entansif koşullarda yetiştirilen Ile de France koyunlarda yürütülen bu çalışmada abort ve ölü doğum oranı %3,91 ve %1,68 olarak tespit edilmiştir. Atli (2017) tarafından saf Ile de France koyunlarda yürütülen bir çalışmada abort oranı için bildirilen %1,7 değerine benzerdir. Doğum oranı, önemli dölverimi parametrelerinden biridir. Bu çalışmada Ile de France koyunlarda doğum oranı %72,53 olarak belirlenmiştir. Bu değer Aktaş (1971) ve Atli (2017) tarafından Ile de France koyunlarda yürütülen çalışmalarda bildirilen (%82,0 ve %90,80) değerlerden düşüktür. Koyun yetiştiriciliğinde ikizliğin artırılması kuzu veriminde artışa dolayısıyla gelirlerin artmasına katkı sağlamaktadır. Bu çalışmada ikiz doğum oranı %23,67 olarak hesaplanmıştır. Bu değer Atli (2017) tarafından Ile de France koyunlar için bildirilen %16,90 değerinden yüksektir. Doğuran koyun başına düşen kuzu sayısı 1,24 olup saf Ile de France koyunlar için bildirilen (Atli 2017; Achkakanova ve Staykova 2019; Staykova ve Achkakanova 2019) değer (1,17-1,65) aralığındadır. Kandemir ve ark (2013) tarafından Ile de France x Akkaraman melezi koyunlarda bildirilen 1,20 değerinden biraz yüksektir. Entansif koşullarda yetiştirilen Ile de France koyunlarda yürütülen bu araştırmada dölverimi özelliklerinden gebelik oranı ve doğum oranının literatür bildirimlerine göre düşük olması araştırmanın yürütüldüğü sürüdeki koyunların iki yaşında (ilk kuzulamayı bu işletmede yapması) ve koyunların uyum döneminde olmasından kaynaklanmış olabilir. Kıvırcık ve Lalahan (Kıvırcık x Akkaraman G_1) koyunlarında yürütülen bazı çalışmalarda da doğum oranı en düşük 2 yaşlı koyunlarda bildirilmiştir (Nageye 2020; Erol ve ark. 2017). Ayrıca araştırmalar arasındaki bakım, besleme ve iklim şartlarındaki farklılıklarda bu duruma neden olmuş olabilir. İkizlik ve doğuran koyun başına düşen kuzu sayıları dikkate alındığında ilerleyen üretim dönemlerinde sürüde üreme performansının yükselebileceği düşünülmektedir.

Büyümenin en önemli göstergelerinden olan farklı dönemlerdeki canlı ağırlık değerleri ilk damızlıkta kullanma zamanı ve et veriminin de temel parametreleridir. Bu çalışmada Ile de France kuzuların doğum ağırlığı ortalaması 4,93 kg tespit edilmiştir. Doğum ağırlığı değeri farklı araştırmalarda bildirilen saf (3,19-4,89 kg) ve farklı düzeylerde melez (3,75-4,67 kg) Ile de France kuzuların doğum ağırlığı aralığının üst sınırından biraz yüksek yüksek olmuştur (Aktaş 1971; Ivanova ve Raicheva 2015; Atli 2017; Achkakanova ve Staykova 2019; Aydoğan 1985; Gökdal ve ark. 2006; Cloete ve ark. 2007; Kandemir ve ark. 2013). Bu araştırmada doğum ağırlığına doğum tipi ve ayının etkisi istatistik olarak önemli bulunmuştur. Tek doğan kuzular ile kasım ayında doğanlar diğerlerinden daha yüksek doğum ağırlığına sahip olmuştur. Ile de France, Gökçeada, Hamdani, Akkaraman ve melezlerinde yürütülen çalışmalarda da doğum tipinin doğum ağırlığına etkisinin önemli olduğu bildirilmiştir (Odabaşıoğlu ve ark. 1995; Yılmaz ve ark. 2006; Ceyhan ve ark. 2013; Atli 2017). Bu araştırmada erkek kuzularda (4,97 kg) doğum ağırlığı dişilerden (4,88kg) yüksek olsa da aralarında farklılık istatistik olarak önemli bulunmamıştır. Bu araştırmanın bulgularına benzer şekilde Ile de France, Gökçeada, Hamdani, Akkaraman ve melezlerinde yürütülen farklı çalışmalarda da cinsiyetin doğum ağırlığına etkisinin önemli olmadığı bildirilmektedir (Odabaşıoğlu ve ark. 1995; Yılmaz ve ark. 2006; Ceyhan ve ark. 2013; Atli 2017). Ayrıca Çelikeloğlu ve ark. (2022) tarafından yürütülen bir çalışmada Ramlıç, Teksel ve F_1 melezi kuzularda doğum ayının doğum ağırlığına etkisinin önemli olduğu ifade edilmiştir. Araştırma bulguları ve literatür bildirişleri dikkate alınarak değerlendirildiğinde Ile de France kuzuların doğum ağırlığının iyi olduğu yaşama gücü ve adaptasyonda bu durumun önemli katkı sağlayacağı düşünülmektedir.

Bu çalışmada Ile de France kuzuların 30. gün canlı ağırlık ortalaması 10,01 kg olarak hesaplanmıştır. Bu değer farklı araştırmalarda (Ivanova ve Raicheva 2015; Atli 2017; Achkakanova ve Staykova 2019) saf Ile de France kuzular için bildirilen değer aralığında (9.88-15.05 kg) olup alt sınıra yakındır. Araştırmanın yürütüldüğü Ile de France kuzuların 60. ve 90. canlı ağırlık ortalamaları 17,32 ve 27,80 kg olarak tespit edilmiştir. Bu canlı ağırlık ortalamaları Atlı (2017) tarafından saf Ile de France kuzular için bildirilen değerlerden (19,95 kg ve 29,98 kg) düşük bulunmuştur. Ayrıca 30., 60. ve 90. gün canlı ağırlık ortalamaları Gökdal ve ark. (2006) tarafından Ile de France x Akkaraman melezi kuzularda yapılan çalışmada aynı dönemler için bildirdikleri canlı ağırlık ortalamalarından yüksektir. Bu durum genotip, bakım, besleme ve iklim şartlarındaki farklılıklardan kaynaklanmış olabilir. Bu çalışmada 30., 60. ve 90. gün canlı ağırlığa doğum tipinin etkisi önemli bulunmuş olup tek doğan kuzular ikizlerden bütün dönemlerde daha ağırdır. İncelenen özellikler bakımından genellikle erkekler dişilerden daha yüksek canlı ağırlığa sahip olmasına rağmen aralarındaki farklılık istatistik olarak önemli bulunmamıştır. Doğum ayının 30. gün

canlı ağırlığa etkisi istatistik olarak önemlidir. Doğum ağırlığına benzer şekilde kasım ayında doğan kuzular aralık ve ocak ayında doğanlara göre daha yüksek 30. gün canlı ağırlığa sahip olmuştur.

Ile de France kuzuların günlük canlı ağırlık artışı genel ortalaması 212,20 gramdır. Achkakanova ve Staykova (2019) tarafından saf Ile de France kuzuların 30-70. günler arasında bildirilen günlük canlı ağırlık artışı değerinden (228 gram) düşük olmuştur. Ayrıca Afyonkarahisar'da yürütülen çalışmalarda (Tekerli ve ark. 2022; Koçak ve ark. 2024) bildirilen Ramlıç (212 gram) ve Teksel x Pırlak melezi F_1 (212,52 gram) kuzulara benzer, Pırlak (203,98 ve 205,73 gram) kuzuların değerinden ise yüksek bulunmuştur. Araştırmalar arasındaki farklılıklar genotip, bakım, besleme ve günlük canlı ağırlık artışı hesaplama dönemlerinin değişik olmasından kaynaklanmış olabilir. Nitekim Tekerli ve ark. 2022 ile Koçak ve ark. 2024 tarafından yürütülen çalışmalarda günlük canlı ağırlık artışı, doğum-sütten kesim (yaklaşık 120 gün) arasında hesaplanmıştır. Ile de France kuzularda yürütülen bu çalışmadan farklı dönemdir. Günlük canlı ağırlık artışına etkisi incelenen faktörlerden sadece doğum tipinin etkisi önemli bulunmuştur. Tek doğan kuzular (244,80 gram) ikiz doğanlardan (179,60 gram) daha yüksek günlük canlı ağırlık artışına sahip olmuştur. Erkek kuzularda günlük canlı ağılık artışı ortalaması 214,64 gram iken dişilerde 209,76 gram olup aralarındaki farklılık istatistiki olarak önemli bulunmamıştır.

SONUÇ

Sonuç olarak Entansif koşullarda yetiştirilen Ile de France koyunlarda yürütülen bu çalışmada gebelik ve doğum oranının aynı ırk için bildirilen değerlerden düşük olduğu görülmektedir. Ile de France koyunların uyum dönemi, iki yaşında olması, ikizlik ve doğuran koyun başına düşen kuzu sayıları değerlendirildiğinde ilerleyen üretim dönemlerinde sürünün üreme performansının yükselebileceği düşünülmektedir. Ile de France kuzuların sütten kesim öncesi büyüme özellikleri incelendiğinde doğum ağırlığının iyi seviyede olduğu söylenebilir. Bu durumun kuzuların yaşama gücü ve adaptasyonuna katkı sağlayacağı değerlendirilmektir.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: SK and NY contributed to the study design and conduct of the study. Animal material preparation, data collection and statistical analysis were performed by NY and SK. The first draft of the manuscript was written by SK, and all the authors commented on previous versions of the manuscript.

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

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RESEARCH ARTICLE

Investigation of Anti-Müllerian Hormone Presence in Bitch Urine and Comparison of Anti-Müllerian Hormone Levels in Blood Serum and Urine

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ABSTRACT

The presented study aimed to investigate the presence of AMH in bitch urine and to determine whether there is a correlation between blood serum and urine AMH levels. Forty-two healthy crossbreed bitches brought to Kafkas University, Faculty of Veterinary Medicine, Animal Hospital, Department of Obstetrics and Gynecology with a request for ovariohysterectomy were included in the study. Blood samples were taken from the Vena cephalica antebrachii, and urine samples were collected using a urinary catheter of all bitches. After the blood and urine samples were centrifuged, AMH levels were determined using the ELISA method. The average AMH concentration was determined to be 4.56±0.53 ng/mL in urine and 7.75±1.19 ng/mL in blood serum. No significant correlation was found between blood and urine AMH levels (P>0.05). It was concluded that urine AMH levels were not related to blood serum AMH levels.

Keywords: Anti-Müllerian Hormone, Bitch, Blood serum, Urine

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Köpeklerde İdrarda Anti-Müllerian Hormon Varlığının Araştırılması ve Kan Serumu ve İdrarda Anti-Müllerian Hormon Düzeylerinin Karşılaştırılması

ÖZ

Sunulan çalışmada köpek idrarında AMH varlığının araştırılması ve kan serumu ile idrar AMH düzeyi arasında korelasyon olup olmadığının belirlenmesi amaçlanmıştır. Kafkas Üniversitesi, Veteriner Fakültesi Hayvan Hastanesi, Doğum ve Jinekoloji Kliniği'ne ovaryohisterektomi isteğiyle getirilen sağlıklı 42 melez ırk köpek çalışmaya dahil edildi. Tüm köpeklerin Vena cephalica antebrahii'den kan örnekleri alındı ve idrar kateteri kullanılarak idrar örnekleri toplandı. Kan ve idrar örnekleri santrifüj edildikten sonra AMH düzeyleri ELISA yöntemiyle belirlendi. Kan serumunda ortalama AMH düzeyi 7,75±1,19 ng/mL, idrarda ise 4,56±0,53 ng/mL bulundu. Kan ve idrar AMH düzeyi arasında anlamlı bir korelasyon saptanmadı (P>0,05). İdrar AMH düzeyinin kan serumundaki AMH düzeyi ile ilişkili olmadığı kanaatine varıldı.

Anahtar kelimeler: Anti-Müllerian hormon, İdrar, Kan serumu, Köpek.

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INTRODUCTION

Anti-Müllerian hormone (AMH), which is produced exclusively in ovarian tissue (Yağcı et al. 2016) and has a glycoprotein structure, belongs to the transforming growth factor-β (TGF-β) family (Bedenk et al. 2020). It limits the number of actively growing follicles by reducing the sensitivity of follicles with growth potential to FSH. Thus, it prevents premature exhaustion of primordial follicles (Akbarinejad et al. 2020). Since the oocyte reserve is preserved (Holst 2017), the reproductive life of the dog is extended (Akbarinejad et al. 2020). AMH levels are used both to investigate the ovarian reserve (Hollinshead et al. 2017; Yağcı et al. 2016) and to determine the presence or absence of the ovary in adult bitches with no information about their medical history (Axner and Holst 2015; Alm and Holst 2018; Yagci et al. 2016). It is also a reliable diagnostic tool for diagnosing ovarian remnant syndrome (Yilmaz et al. 2015). AMH is investigated either in ovarian granulosa cells (Karakaş Alkan et al. 2019) or blood (serum or plasma) samples (Kaya et al. 2024; Themmen et al. 2016). Since these methods are invasive, minimally invasive or non-invasive methods are being investigated as alternatives (Cai et al. 2023; Hallberg et al. 2024; Kaya et al. 2024; Pankhurst et al. 2016).

The excretion mechanism of AMH is not fully known (Griesinger et al. 2012). It is reported that only AMH that undergoes proteolytic degradation can be excreted in the urine (Pankhurst et al. 2016). It is stated that neuraminidases are effective in the destruction of AMH. It is thought that after exposure to the effect of neuraminidase, it is removed from the circulation by endocytosis in liver cells and destruction in hepatic lysozymes (Griesinger et al. 2012). In recent years, many studies have investigated the excretion pathway of AMH. In one of the studies investigating whether it is excreted via seminal plasma, the presence of AMH in the seminal fluid could not be detected (Hallberg et al. 2024), while AMH was detected in another study (Muhammed et al. 2018). There are a few studies investigating its excretion via urine. In one study, the presence of AMH in human urine was detected, and its level was determined quantitatively (Cai et al. 2023). However, another study reported that AMH in the urine of women with polycystic ovary syndrome was much lower than blood AMH levels and that urine AMH levels could not diagnose the disease (Ipandi 2024). No AMH was detected in urine samples from mice injected with recombinant human AMH, leading researchers to believe that AMH would not be excreted by the kidneys in mice (Pankhurst et al. 2016). In a study investigating the presence of AMH in the urine of cats, it was reported that AMH was present in urine, but its level did not fully reflect blood AMH levels. In this study, blood AMH levels were higher than 1 ng/mL in all non-neutered cats, whereas urine AMH levels were <1 ng/mL in 7 cats (Kaya et al. 2024). It is unknown whether the urinary excretion of AMH is species to the specific. The presence of AMH in urine has not been investigated in bitches. Therefore, in the present study, we investigated the presence of AMH in bitch urine and the correlation between blood AMH and urine AMH levels.

MATERIALS and METHODS

Animal Material

Forty-two crossbreed bitches brought to Kafkas University, Faculty of Veterinary Medicine, Animal Hospital, Obstetrics and Gynecology Clinic with a request for ovariohysterectomy were included in the study. These bitches, weighing an average of 11-31 kg (21.67±0.97) and aged 1-3 years. The study protocol was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK/2021-090).

Method of Study

Bitches that were found to be healthy as a result of clinical and ultrasonographic examinations were included in the study. Vaginal cytology was performed in these bitches to determine the period of the sexual cycle. Vaginal smear samples were taken from the dorsal wall of the vagina by gently rolling with the help of cotton swabs and rolling onto the slide. The samples were stained with Diff Quick (MGG Quick Stain, Chembio Laboratory Research, Türkiye). The period of the sexual cycle was determined according to the morphology of vaginal cell types in the microscopic examination (Olympus Cx23). Bitches in anoestrus and diestrus were included in the study. According to vaginal cytology results, 27 bitches were determined to be in anoestrus and 15 bitches were in diestrus. Bitches were classified as <15 kg, 15-24 kg, and ≥25 kg according to their body weight. When the bitches in the study were grouped according to body weight, it was determined that 7 bitches were small (9.5-14 kg), 24 bitches were medium (15-24 kg), and 11 bitches were large (25-41 kg).

Obtaining Blood Serum and AMH Analysis

Blood samples were taken from all bitches (Vena cephalica antebrahii). Urine samples were collected via a urinary catheter (Coloplast, EasiCath, Denmark). Urine and blood samples were centrifuged at 3000 rpm for 20 minutes, and the serum was separated. The serum was transferred to Eppendorf tubes and stored at -20°C until analysis. Analysis of serum and urine AMH levels was performed by the ELISA method using a commercial canine-specific kit (BT LAB, Bioassay Technology Zhejiang, China) according to

the manufacturer's recommendations. The measurement range was 0.2-60 ng/mL. Intra-assay and inter-assay coefficients of variation were <8% and <10%, respectively.

Statistical Analysis

Data analysis was performed using the IBM SPSS 26 statistical package program. All data were given as mean±standard error. Normality tests were performed using the Shapiro-Wilk test. Groups were compared according to sexual cycle period using the Mann-Whitney U test. Blood AMH levels in groups according to body weight were compared using the Kruskal-Wallis test. A Spearman correlation test was used to investigate whether there was a relationship between urine and blood AMH levels. P value <0.05 was considered statistically significant.

RESULTS

AMH levels in blood serum and urine were 7.75±1.19 ng/mL (1.29-40.55 ng/mL) and 4.56±0.53 ng/mL (1.20-20.30 ng/mL), respectively. There was no significant correlation between blood and urine AMH levels (P>0.05).

During the anoestrus period, the mean blood serum AMH level was determined as 8.91±1.83 ng/mL and in the urine as 4.56±0.78 ng/mL. During the diestrus period, the blood serum and urine AMH levels were determined as 5.87±0.83 ng/mL and 4.56±0.64 ng/mL, respectively. Blood AMH levels did not change statistically significantly according to the period of the sexual cycle.

Mean blood and urine AMH levels were determined as 7.53±2.18 ng/mL, 3.54±0.52 ng/mL in small breed, 5.38±0.61 ng/mL, 4.87±0.80 ng/mL in medium-sized, and 13.06±3.80 ng/mL, 3.54±0.52 ng/mL in large breed bitches, respectively. No statistically significant difference was found between the groups in blood AMH levels (P>0.05).

DISCUSSION

The anti-Müllerian hormone is used to detect females who have undergone surgical sterilization, to the presence of ovarian remnant syndrome, to diagnose granulosa cell tumors, and to determine ovarian reserve (Kaya et al. 2021). AMH is found in the bloodstream in 2 forms (proAMH and AMHN,C) (Pankhurst et al. 2016). ProAMH is a proprotein that is incapable of binding to AMH receptor II. Proproteins are converted to AMHN,C, which can bind to the receptor by undergoing proteolytic degradation via converting enzymes (such as subtilisin/kexin) (Pankhurst and McLennan 2016). While the proAMH form is more abundant in the ovary (follicular fluid and granulosa cells), the non-covalent complex AMHN,C is most abundant in the bloodstream (Pankhurst et al. 2016). Commercially available methods for determining AMH levels cannot distinguish between these two forms (Pankhurst and McLennan 2016). In recent years, the presence, levels, and clearance times of AMH in body fluids have been revealed in various studies. In a study investigating the level of AMH in intraperitoneal fluid, it was found that there was a similar level of AMH with blood serum and that there was a significant positive correlation between the AMH levels in the two fluids (Kostrzewa et al. 2020). Human recombinant AMH was administered to rats intraperitoneally and intratesticularly to investigate the changes in AMH levels in blood serum and testicular fluid. With intratesticular administration, AMH levels were detected in both serum and testicular fluid (Sriraman et al. 2001). While AMH in seminal plasma could not be detected in a study (Hallberg et al. 2024), AMH has been detected in urine in humans and cats (Cai et al. 2023; Kaya et al. 2024). This shows that AMH is present in some body fluids and its level can be detected. However, it is not found in all body fluids.

It is reported that the time it takes for AMH to be cleared from the body varies depending on the animal species. In a study conducted on rats by Anadol et al. (2016), the change in AMH levels in rats whose ovaries were removed was investigated. There was a statistically significant decrease even one day after the operation. AMH level decreased significantly until day 5 (0.52±0.13 ng/mL) and below the non-detectable level (0.31 ng/mL) on day 10 after the surgery. In another study, the AMH level, which was 5.8±1.5 ng/mL before ovariohysterectomy in anestrous dogs, decreased to 3.1±1.1 ng/mL on the first day following the operation and to 1.9±1.5 ng/mL on the fifth day. It decreased below 1 ng/mL on the 10th postoperative day (Anadol et al. 2020). In rats given human recombinant AMH, it was observed that the half-life was 8-12 hours, and then it was rapidly cleared from the circulation. Since the rat's own AMH did not react in primate-based MIS ELISA measurements, the rat's own AMH was not detected in the measurements, and it was reported that it did not affect the results (Sriraman et al. 2001). All these results are based on the rate of AMH loss after gonad removal. The most appropriate explanation is thought to be that this situation is due to differences in clearance between species or differences between recombinant and native AMH. It is reported that AMH in circulation is cleared from the body by proteolytic degradation and excretion in the urine (Pankhurst et al. 2016). The presence of AMH in urine was first determined in humans by Cai et al. (2023) and in cats by Kaya et al. (2024). No previous study was found in bitch urine. In the presented study, the presence of AMH in bitch urine was determined and presented quantitatively. When all these results were evaluated, it was seen that AMH was excreted via urine in bitches, but there was no correlation between blood and urine AMH levels.

CONCLUSION

Urinary AMH level in bitches was measured and assessed quantitatively for the first time. Urine AMH level is not consistent with blood AMH level.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: SK contributed to the project idea, design, and execution of the study. GK, MCD, and MAK contributed to the acquisition of data. SK evaluated vaginal cytology. İK analyzed the hormone levels. SK and CK analyzed the data. SK and CK drafted and wrote the manuscript. All authors have read and approved the finalized manuscript.

Ethical approval: The study protocol was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK/2021-090).

Explanation: We have presented as an oral at the Anadolu 11th International Conference on Applied Sciences (2022).

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RESEARCH ARTICLE

Distribution of Bacterial Agents and Diagnosed Diseases in Samples Sent to the Jockey Club of Türkiye İstanbul Equine Hospital Laboratory: A Retrospective Study (2015-2019)

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ABSTRACT

The categorization of diseases identified in Turkish race and breeding horses, as well as the bacterial agents derived from these equines, have not been thoroughly investigated. This study aimed to examine the bacterial agents in samples taken from sick horses brought to the covering stations and animal hospitals operating under the Jockey Club of Türkiye (TJK) between January 1, 2015, and December 31, 2019, and the retrospective distribution of diseases in the horses from which these samples were taken. It was determined that the most frequently sent sample to the TJK İstanbul Equine Hospital Laboratory was tracheal lavage fluid (47.3%) and that the samples were mostly sent in the spring (33.8%) and autumn (30.8%) seasons. It was determined that the most common respiratory system infections (49.37%), followed by genital system infections (29.11%) and gastrointestinal system infections (18.57%) were seen in the horses from which the samples were taken. *Escherichia coli* (15.6%) was the most commonly detected strain in all submitted samples. In respiratory system infections, the most identified pathogens were *Streptococcus equi* subsp. *zooepidemicus* (14.53%), followed by *Streptococcus dysgalactiae* subsp. *equisimilis* (11.11%) and *Streptococcus equi* subsp. *equi* (10.26%). As a result, it is expected that the findings obtained from this study will contribute to taking specific measures against diseases or bacterial agents with high prevalence in enterprises operating within the TJK and minimizing losses due to horse diseases.

Keywords: *Escherichia coli*, Horse, Respiratory system diseases, *Streptococcus equi* subsp. *zooepidemicus,* Tracheal lavage

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Türkiye Jokey Kulübü İstanbul At Hastanesi Laboratuvarına Gönderilen Numunelerdeki Bakteriyel Etkenlerin ve Teşhis Edilen Hastalıkların Dağılımı: Retrospektif Bir Çalışma (2015-2019)

ÖZ

Türkiye'de damızlık ve yarış atlarında teşhisi yapılmış hastalıkların ve bu atlardan elde edilen bakteriyel etkenlerin sınıflandırılmasına dair kapsamlı bir çalışma bulunmamaktadır. Bu çalışmada, 1 Ocak 2015-31 Aralık 2019 tarihleri arasında, Türkiye Jokey Kulübü (TJK) bünyesinde faaliyet gösteren aşım istasyonu ve hayvan hastanelerine getirilen hasta atlardan alınan numenelerdeki bakteriyel etkenlerin ve bu örneklerin alındığı atlardaki hastalıkların retrospektif olarak dağılımının araştırılması amaçlandı. TJK İstanbul At Hastanesi Laboratuvarı'na en çok gönderilen numunenin trakeal lavaj sıvısı (%47.3) olduğu ve numunelerinin en çok ilkbahar (%33.8) ve sonbahar (%30.8) mevsimlerinde gönderildiği tespit edildi. Gönderilen numunelerin alındığı atlarda en çok solunum sistemi enfeksiyonu (%49.37), daha sonra genital sistem enfeksiyonları (%29.11) ve gastrointestinal sistem enfeksiyonları (%18.57) görüldüğü belirlendi. Gönderilen bütün numunelerde en çok *Escherichia coli* (%15.6) tespit edildi. Solunum sistemi enfeksiyonlarında, en çok *Streptococcus equi* subsp. *zooepidemicus* (%14.53), daha sonra sırasıyla *Streptococcus dysgalactiae* subsp. *equisimilis* (%11.11) ve *Streptococcus equi* subsp. *equi* (%10.26) identifiye edildiği belirlendi. Sonuç olarak, bu çalışmadan elde edilen bulguların, TJK bünyesinde faaliyet gösteren işletmelerde prevalansı yüksek hastalık veya bakteriyel etkenlere karşı spesifik tedbirlerin alınmasına ve at hastalıklarına bağlı kayıpların minimalize edilmesine katkı sağlaması beklenmektedir.

Anahtar Kelimeler: At, *Escherichia coli, Streptococcus equi* subsp. *zooepidemicus*, Solunum sistemi hastalıkları, Trakeal lavaj

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INTRODUCTION

Respiratory diseases are a significant cause of morbidity and performance loss in horses of all ages, breeds, and disciplines (Morris and Seeherman 1991; Burrell et al. 1996). Infectious (Strangles, bacterial pneumonia, viral infections) and non-infectious diseases (asthma, laryngeal hemiplegia, recurrent airway obstruction [RAO], etc.) affecting both the upper and lower respiratory tracts are major respiratory system disorders in horses (Burrell et al. 1996; Ainsworth and Hackett 2004; Ekinci et al. 2024; Deniz et al. 2024). Bacterial pneumonia is a significant cause of morbidity and mortality in adult horses (Sweeney 1991). Secondary bacterial infections of the lower respiratory tract occur when opportunistic bacteria, typically part of the normal flora of the oral cavity or upper respiratory tract, gain access to the lower airways and disrupt natural respiratory defense mechanisms, leading to bronchopneumonia or pleuropneumonia (Burrell et al. 1996; Ainsworth and Hackett 2004). Respiratory disease has been detected in 40% to 42% of racehorses presented for poor performance evaluation (Martin et al. 2000).

In India, a study was conducted on 88 healthy and 53 horses with respiratory diseases to isolate and identify aerobic bacteria from the upper respiratory tract. Of the 321 isolates from both groups, 84.1% were identified as gram positive and 15.9% as gram negative bacteria. The most frequently isolated bacteria were *Streptococcus equi* subsp. *zooepidemicus* (17.44%), followed by *Micrococcus* spp. (9.96%), *Corynebacterium* spp. (9.65%), *Staphylococcus intermedius* (9.65%), *Staphylococcus aureus* (8.72%), *Bacillus* spp. (7.16%), *Streptococcus pneumoniae* (5.60%), *Staphylococcus chromogenes* (5.60%), *Streptococcus equisimilis* (5.29%), *Pseudomonas aeruginosa* (5.29%), *Rhodococcus equi* (3.73%), *Escherichia coli* (3.73%), *Klebsiella pneumoniae* (3.42%), *Proteus vulgaris* (3.42%), and *Streptococcus equi* subsp. *equi* (1.24%) (Mir et al. 2013). In a retrospective study analyzing antibiotic susceptibility test results of bacterial pathogens cultured from horses at the Zurich University Equine Clinic between 2012 and 2015, *Escherichia coli* (20%, 60/303) and *Staphylococcus aureus* (13%, 40/303) were commonly isolated. The highest rate of multidrug resistance was found in *Acinetobacter baumannii* isolates (96%, 23/24), followed by *Enterobacter cloacae* (86%, 24/28) and *Escherichia coli* (80%, 48/60) (van Spijk et al. 2016). In a retrospective study by Erol et al. (2012) investigating the tissue/organ distributions and antimicrobial susceptibilities of β-hemolytic streptococci in horses, a total of 2,497 β-hemolytic streptococci were isolated from 2,391 cases. Among these, *Streptococcus equi* subsp. *zooepidemicus* was the most frequently isolated species (72.0%). Other isolated species included *Streptococcus dysgalactiae* subsp. *equisimilis* (21.3%), *Streptococcus equi* subsp. *equi* (5.8%), and unidentified β-hemolytic streptococci (0.9%). *Escherichia coli* (*E. coli*) is a bacterium commonly found in the intestines of most mammals. It can be found almost everywhere in the environment due to its spread through feces, water, and soil, its ability to attach to plants, and its capacity to colonize plants (van Duijkeren et al. 2000). *Streptococcus equi* subsp. *equi*, *Streptococcus dysgalactiae* subsp. *equisimilis*, and *Streptococcus equi* subsp. *zooepidemicus* are the three main β-hemolytic *Streptococcus* species that cause serious and economically significant diseases in horses. *Streptococcus equi* subsp. *equi* is the causative agent of Strangles (also known as Lymphadenitis equorum), a highly contagious infection affecting the upper respiratory tract and specific to horses that is associated with their lymph nodes (Timoney 2004; Holden et al. 2009). *Streptococcus equi* subsp. *zooepidemicus* is considered a mucosal commensal in the oral cavity, pharynx, and respiratory tract of horses and acts as an opportunistic pathogen causing respiratory and urinary tract diseases (endometritis) in horses and has been reported as the most common bacterium causing placentitis in mares (Erol et al. 2012; Rasmussen et al. 2015; Díaz-Bertrana et al. 2021). The causative agent of Strangles, *Streptococcus equi* subsp. *equi*, is a primary bacterial pathogen of the upper respiratory tract and can cause mucosal invasion without predisposing factors (Libardoni et al. 2016; Jaramillo-Morales et al. 2023).

In Türkiye, there are limited studies on the classification of bacterial agents and diseases identified in breeding and racehorses (Yildirim et al. 2015; Çalışkan and Tel 2021; Diri et al. 2022; Baydar et al. 2023; Deniz et al. 2024). Indeed, the lack of classification of diseases observed in horses, which have very high monetary value, contributes to difficulties in tracking equine diseases in Türkiye, resulting in a scarcity of epidemiological data and inadequate hospital management. Additionally, the inability to document diseases leads to insufficient preventive measures against some significant illnesses and consequently results in economic losses. Moreover, taking preventive measures against prevalent diseases or bacterial agents is crucial for minimizing losses related to these agents or diseases and ensuring the effective and efficient use of resources in horse farms. This study aims to retrospectively investigate the distribution of bacterial agents identified from samples sent to the TJK İstanbul Equine Hospital laboratory between 2015 and 2019 and the diseases of the horses from which these samples were obtained.

MATERIALS and METHODS

Animal Material

The animal material for this study comprised resident and guest horses from the TJK (Jockey Club of

Türkiye) facilities, including animal hospitals and breeding stations, between January 1, 2015, and December 31, 2019.

Sample Collection

Fecal, nasal swab, abscess content, clitoral swab, uterine swab and tracheal lavage fluid samples taken from horses showing signs of disease to confirm the suspected diseases were sent to the laboratory (TJK İstanbul Equine Hospital Laboratory, Bakırköy, İstanbul, Türkiye) under appropriate storage conditions.

Bacteriologic analyses

Tracheal aspirate, nasal swab, endometrial swab, endometrial washing fluid and abscess content samples were cultured onto two 5% sheep blood agar (Laborlar, Türkiye) and mac conkey agar (Laborlar, Türkiye) for bacteriological examination. One blood agar was incubated in aerobic atmosphere and the other in microaerophilic atmosphere at 37 °C for 24-72 hours. After 72 hours, cultures with no growth were considered negative. In positive cultures, growths were checked for purity/presence of contaminant colonies. Cultures with 2< number of different types of growth were considered as contamination. Suspicious colonies in positive cultures were identified by conventional biochemical tests (Diagnostics i.n.c., Slovakia). After determining the morphological characteristics of the isolated colonies and the hemolysis characteristics of the colonies showing growth on blood agar, Gram staining (GBL, Türkiye) was performed on suspicious colonies to determine the Gram characteristics and morphology of the agents. Then catalase (GBL, Türkiye) and oxidase tests (Diagnostics i.n.c., Slovakia) were performed. Subsequently, a commercial kit (Diagnostics i.n.c., Slovakia) was used for conventional biochemical tests (Quinn et al. 1998). All bacteriological analyses were performed by a veterinarian specialized in microbiology and the results were recorded.

Data Collection

Data on the types of samples and identified bacterial agents sent to the TJK İstanbul Equine Hospital Laboratory between January 1, 2015, and December 31, 2019, were retrospectively obtained from laboratory records. Subsequently, information about the diseases of the horses from which these samples were collected, including age, breed, sex, and season, was retrieved from hospital and laboratory records.

Statistical Analysis

Statistical analyses were conducted using IBM-SPSS for Windows Release 25.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics were expressed as frequencies and percentages. The relationship between categorical variables was analyzed using the Chi-Square (χ^2) test (alternatively, Fisher's exact test). Graphs were generated using GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, CA, USA). A p-value of <0.05 was considered statistically significant.

RESULTS

In this retrospective study, samples sent to the TJK İstanbul Equine Hospital laboratory were identified as coming from the following facilities: İstanbul Equine Hospital, Silivri Breeding Station, İzmit Equine Hospital, Kartepe Equine Hospital, Bursa Equine Hospital, Karacabey Equine Hospital, İzmir Equine Hospital, Torbalı Breeding Station, Mahmudiye Equine Hospital, Ankara Equine Hospital, Elazig Equine Hospital, Diyarbakir Equine Hospital, Sanliurfa Equine Hospital, Sanliurfa Breeding Station, Adana Equine Hospital, and Seyhan Breeding Station. It was determined that 83.1% of the samples sent for bacterial culture were from Thoroughbred horses, while 16.9% were from Arabian horses. Among these horses, 56.5% were female and 43.5% were male, with an average age of 6.93±4.78 years.

It was determined that 81.4% of the samples were collected in 2019, 13.9% in 2018, 3.8% in 2016, 0.4% in 2015, and 0.4% in 2017. No statistically significant association was observed between the year category and the categories of identified bacterial agents (χ^2: 130.846, P= 0.997).

A total of 1,847 samples were sent to the İstanbul Equine Hospital Laboratory for bacteriological examination from horses showing signs of disease suspected of originating from specific systems or regions. Of these 1,847 samples, 237 (12.83%) were identified with one or more bacteria. Among the submitted samples, 47.3% were from tracheal lavage fluid, 28.3% from the uterus, 17.7% from the rectum, 1.69% from the clitoris, 1.27% from the kidneys and urethra, 0.84% from the air sacs, 0.84% from internal organs (liver, spleen), 0.42% from lung tissue, 0.42% from joints, 0.42% from the nasal cavity, 0.42% from the placenta, and 0.42% from synovial fluid (Figure 1).

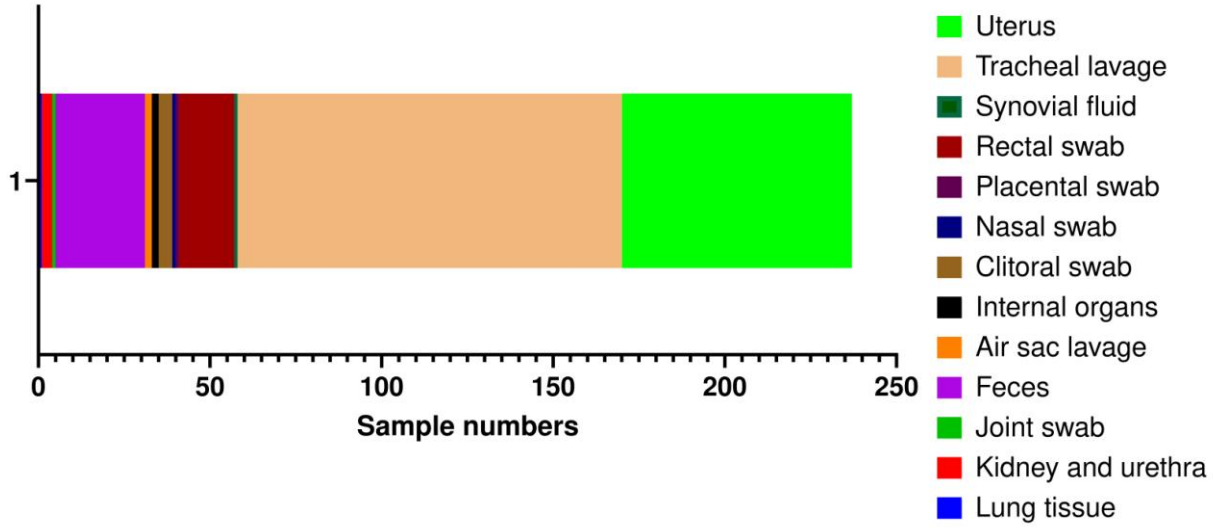

Figure 1. Distribution of samples sent to TJK İstanbul Equine Hospital Laboratory according to the regions where they were taken.

Samples sent to the TJK İstanbul Equine Hospital Laboratory were most frequently collected in March (15.6%), followed by September (12.7%), April (10.5%), June (10.1%), October (9.3%), November (8.9%), May (7.6%), January (6.3%), February (5.9%), August (5.9%), and December (0.4%). When examining the distribution of samples by season, the highest number were collected in spring (33.8%), followed by autumn (30.8%), summer (22.8%), and winter (12.7%) (Figure 2). However, no statistically significant association was observed between the seasonal categories of sample collection and the identified bacterial agents (χ^2: 159.990, P= 0.171).

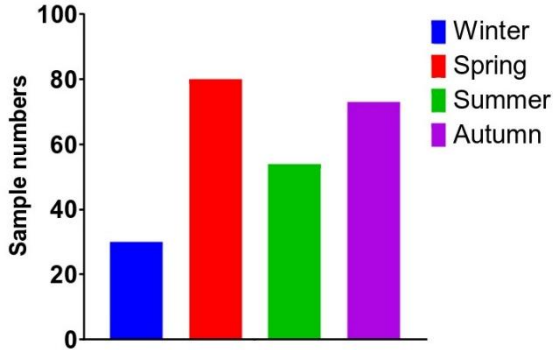

Figure 2. Distribution of samples sent to the TJK Istanbul Horse Hospital laboratory and from which bacterial agents were isolated, according to the seasons in which they were taken.

Among the horses from which samples were sent to the TJK İstanbul Equine Hospital Laboratory, the most frequently diagnosed condition was respiratory system infection (pneumonia) (49.37%, 117/237), followed by genital system infection (metritis, endometritis) (29.96%, 71/237), gastrointestinal system infections (enteritis, enterocolitis, colic) (18.57%, 44/237), urinary system infection (cystitis) (1.27%, 3/237), and arthritis (0.84%, 2/237) (Figure 3).

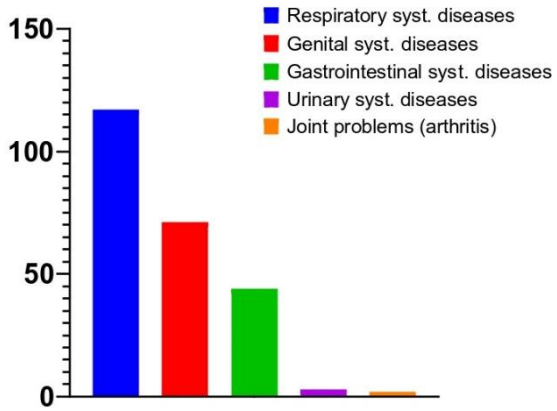

Figure 3. Distribution of diseases in horses from which samples were taken and bacterial agents were isolated, sent to the laboratory of TJK İstanbul Equine Hospital.

Among all the samples sent to the TJK İstanbul Equine Hospital Laboratory, *Escherichia coli* was the most frequently isolated bacterium (15.6%). The following bacteria were isolated in decreasing order: *Streptococcus equi* (11.0%), *S. zooepidemicus* (9.7%), *Streptococcus agalactiae* (7.6%), *Streptococcus dysgalactiae* subsp. *equisimilis* (7.6%), *Corynebacterium jeikeium* (5.9%), *Enterobacter cloacae* (4.6%), *Klebsiella pneumoniae* (4.6%), *Salmonella* spp. (2.5%), *Pseudomonas aeruginosa* (2.1%), *Aeromonas hydrophila* (1.7%), *Stenotrophomonas maltophilia* (1.7%), *Streptococcus equisimilis* (1.7%), *Yersinia pseudotuberculosis* (1.7%), *Pantoea agglomerans* (1.3%), *Proteus mirabilis* (1.3%), *Rhodococcus equi* (1.3%), *S. aureus* (1.3%), and *Sphingomonas paucimobilis* (1.3%) (Table 1).

In respiratory system infections, the most frequently isolated bacteria were *Streptococcus equi* subsp. *zooepidemicus* (14.53%, 17/117), followed by *Streptococcus dysgalactiae* subsp. *equisimilis* (11.11%, 13/117), *Streptococcus equi* subsp. *equi* (10.26%,

12/117), *Escherichia coli* (10.26%, 12/117), *Klebsiella pneumoniae* (9.40%, 11/117), and *Streptococcus agalactiae* (9.40%, 11/117) (Table 2).

In horses diagnosed with genital system infections (metritis/endometritis/placentitis, urovagina), the most frequently isolated bacteria were *Streptococcus equi* (18.06%, 13/72), followed by *Escherichia coli* (12.5%, 9/72), *Streptococcus agalactiae* (9.72%, 7/72), *Corynebacterium jeikeium* (8.33%, 6/72), *Streptococcus equi* subsp. *zooepidemicus* (8.33%, 6/72), and *Streptococcus dysgalactiae* subsp. *equisimilis* (6.94%, 5/72), among others.

In horses with gastrointestinal system infections, the most frequently isolated bacteria were *E. coli* (37.21%, 16/43), followed by *Corynebacterium jeikeium* (18.60%, 8/43), *Salmonella* spp. (13.95%, 6/43), and *Enterobacter cloacae* (11.63%, 5/43) (Table 3).

Table 1. Distribution of bacterial agents isolated from samples sent to TJK İstanbul Equine Hospital Laboratory

Bacterial agents	**%**	**n/total**
Escherichia coli	15.6	37/237
Streptococcus equi subsp. *equi*	11.0	26/237
Streptococcus equi subsp. *zooepidemicus*	9.7	23/237
Streptococcus agalactiae	7.6	18/237
Streptococcus dysgalactiae subsp. *equisimilis*	7.6	18/237
Corynebacterium jeikeium	5.9	14/237
Enterobacter cloacae	4.6	11/237
Klebsiella pneumoniae	4.6	11/237
Salmonella spp	2.5	6/237
Pseudomonas aeruginosa	2.1	5/237
Aeromonas hydrophila	1.7	4/237
Stenotrophomonas maltophilia	1.7	4/237
Streptococcus equisimilis	1.7	4/237
Yersinia pseudotüberculosis	1.7	4/237
Pantoea agglomerans	1.3	3/237
Proteus mirabilis	1.3	3/237
Rhodococcus equi	1.3	3/237
Staphylococcus aureus	1.3	3/237
Sphingomonas paucimobilis	1.3	3/237

Others; *Serratia rubidaea* (0.4%, 1/237), *Shigella* spp. (0.8%, 2/237), *Staphylococcus haemolyticus* (0.4%, 1/237), *Staphylococcus lugdunensis* (0.4%, 1/237), *Staphylococcus saprophyticus* (0.4%, 1/237), *Staphylococcus schleiferi* (0.4%, 1/237), *Staphylococcus simulans* (0.4%, 1/237), *Staphylococcus vitulinus* (0.4%, 1/237), *Streptococcus porcinus* (0.4%, 1/237), *Streptococcus sanguis* (0.4%, 1/237), *Streptococcus scheiferi* (0.4%, 1/237), *Vibrio metschnikovii* (0.4%, 1/237), *Streptococcus uberis* (0.4%, 1/237), *Micrococcus sedentarius* (0.8%, 2/237), *Gardneralla vaginalis* (0.4%, 1/237), *Enterococcus fecalis* (0.4%, 1/237), *Enterococcus faecium* (0.4%, 1/237), *Enterobacter* spp. (0.4%, 1/237), *Enterobacter sakazakii* (0.4%, 1/237), *Enterobacter gergoviae* (0.4%, 1/237), *Enterobacter cancerogenus* (0.4%, 1/237), *Edwardsiella hoshinae* (0.4%, 1/237), *Corynebacterium ulcerans* (0.4%, 1/237), *Corynebacterium* spp. (0.4%, 1/237), *Corynebacterium pseudotüberculosis* (0.4%, 1/237), *Corynebacterium bovis* (0.4%, 1/237), *Citrobacter freundii* (0.4%, 1/237), *Bacillus circulans* (0.4%, 1/237), *Aerococcus urinae* (0.4%, 1/237). Data were expressed as % (n/total).

Table 2. Distribution of bacterial agents isolated from respiratory system infections

Bacterial agents	%	n/total
Streptococcus equi subsp *zooepidemicus*	14.53	17/117
Streptococcus dysgalactiae subsp. *equisimilis*	11.11	13/117
Streptococcus equi subsp *equi*	11.11	13/117
Escherichia coli	10.26	12/117
Klebsiella pneumoniae	9.40	11/117
Streptococcus agalactiae	9.40	11/117
Enterobacter cloacae	4.27	5/117
Yersinia pseudotüberculosis	3.42	4/117
Aeromonas hydrophila	3.42	4/117
Stenotrophomonas maltophilia	2.56	3/117
Rhodococcus equi	2.56	3/117
Pseudomonas aeruginosa	2.56	3/117
Streptococcus equisimilis	1.71	2/117
Corynebacterium pseudotüberculosis	0.85	1/117
Corynebacterium bovis	0.85	1/117
Citrobacter freundii	0.85	1/117
Enterobacter cancerogenus	0.85	1/117
Enterobacter sakazakii	0.85	1/117
Enterococcus faecium	0.85	1/117
Micrococcus sedentarius	0.85	1/117
Pantoea agglomerans	0.85	1/117
Serratia rubidaea	0.85	1/117
Shigella spp.	0.85	1/117
Sphingomonas paucimobilis	0.85	1/117
Staphylococcus lugdunensis	0.85	1/117
Staphylococcus vitulinus	0.85	1/117
Streptococcus porcinus	0.85	1/117
Streptococcus scheiferi	0.85	1/117
Vibrio metschnikovii	0.85	1/117

Data were expressed as % (n/total).

Table 3. Distribution of bacterial agents isolated from horses diagnosed with gastrointestinal system infections.

Bacterial agents	%	n/total
Escherichia coli	37.21	16/43
Corynebacterium jeikeium	18.60	8/43
Salmonella spp.	13.95	6/43
Enterobacter cloacae	11.63	5/43
Enterobacter spp.	4.65	2/43
Pseudomonas aeruginosa	4.65	2/43
Bacillus circulans	2.33	1/43
Enterobacter gergoviae	2.33	1/43
Enterococcus faecium	2.33	1/43
Proteus mirabilis	2.33	1/43

Data were expressed as % (n/total).

DISCUSSION

This study reveals that among the samples sent to the TJK İstanbul Equine Hospital Laboratory for bacteriological analysis, tracheal lavage fluid was the most frequently collected type of sample (47.3%). The majority of samples were submitted in the spring (33.8%) and autumn (30.8%) seasons. The most commonly isolated bacterium from all samples was *Escherichia coli* (15.6%), followed by *Streptococcus equi* subsp. *equi* (11.0%) and *Streptococcus equi* subsp. *zooepidemicus* (9.7%). The horses from which the samples were collected were most commonly diagnosed with respiratory system diseases (49.37%), followed by genital system diseases (29.11%) and gastrointestinal system diseases (18.57%). The high incidence of respiratory system infections in the present study may be related to the fact that the majority of the horses from which the samples were taken were racehorses or had a racing history and that there was high horse mobility in some facilities. Respiratory system diseases are common in racehorses as in other young domestic animal species (Burrell et al., 1996). The start of the breeding season at TJK facilities in February and the subsequent transfer of horses from various sources may increase the incidence of respiratory infections. Another possible reason is that the immunity of foals born in spring (March, April, May) may be suppressed due to the stress caused by the separation of their mothers in the fall season. In addition, the increase in the pathogen load in the environment associated with the cooling of the weather and the onset of the rainy season in the fall may explain the increase in the number of respiratory infections.

Escherichia coli is a bacterium commonly found in the intestines of most mammals. It is widely distributed in the environment due to its presence in feces, its spread through water and soil, its ability to adhere to plants, and its potential to colonize plant surfaces (van Duijkeren et al., 2000). *E. coli* is a prevalent commensal organism in the intestines of horses (Feary et al., 2003). This bacterium is often cultured from the feces of both healthy and diarrheal horses (Maddox et al., 2011; Johns et al., 2012). In the current study, *E. coli* was the most frequently isolated bacterium from the samples, accounting for 15.6%. The primary concern regarding the presence of *E. coli* in horse feces relates to human health. This is because antimicrobial-resistant and multidrug-resistant strains of *E. coli* can be isolated from the feces of hospitalized horses, particularly those treated with oral antibiotics (Maddox et al., 2015).

In the current study, *E. coli* was the most frequently isolated bacterium in horses diagnosed with gastrointestinal system diseases (37.21%, 16/43), followed by *Corynebacterium jeikeium* (18.60%, 8/43) and *Salmonella* spp. (13.95%, 6/43). Enteritis, colitis, and/or enterocolitis are among the most common causes of disease and mortality in horses (Macías-Rioseco et al., 2020). Diagnosing the etiology of enteritis, colitis, and enterocolitis is challenging, and the cause remains undetermined in approximately 50% of cases (Uzal et al., 2015). Enteropathogens such as *Clostridium difficile*, *Clostridium perfringens*, *E. coli*, and *Salmonella* spp. have been associated with acute enteritis in horses (Browning et al., 1991; Mallicote et al., 2012). *Salmonella* spp., *Clostridium perfringens* type A NetF-positive, *C. perfringens* type C, *Clostridioides difficile*, *Clostridium piliforme*, *Paeniclostridium sordellii*, *Rhodococcus equi*, and *Neorickettsia risticii* are among the primary bacterial causes of enterocolitis in horses (Uzal et al., 2022). *Salmonella* spp. can be found in the intestines of clinically healthy horses. Stress and antibiotic therapy are considered major factors that predispose to clinical salmonellosis (Alinovi et al., 2003). Horses infected with *Salmonella* spp. may appear clinically healthy or may show mild to severe clinical signs. Factors contributing to this variability include host-related factors (e.g., stress, immune status, concurrent gastrointestinal diseases) and pathogen-related factors (e.g., serotype, infection dose). Horses with mild disease may exhibit slight fever, soft stools, and a temporary decrease in feed intake. Those with severe disease may show symptoms such as watery diarrhea, fever, toxemia, anorexia, and colic. Serious dehydration, electrolyte imbalances, acid-base disturbances, and protein-losing enteropathy may result from malabsorptive and hypersecretory diarrhea (Shaw & Stämpfli, 2018). *Corynebacterium jeikeium* has increasingly been identified in various clinical conditions, particularly in immunocompromised individuals, and is recognized as a significant nosocomial pathogen. *C. jeikeium* is one of the most frequently isolated medically significant corynebacterial species in intensive care unit patients. *C. jeikeium* is a non-motile, Gram-positive rod that appears as a cocobacillus. Other infections attributed to *C. jeikeium* include skin and wound infections, catheter-associated infections, enteritis, meningitis, osteomyelitis, peritonitis, pneumonia, and pyelonephritis (Denise, 2018).

Respiratory system diseases are common in racehorses, much like in other young companion animal species (Burrell et al., 1996). Respiratory infections can occur in horses of all ages, with affected horses typically exhibiting clinical signs such as exercise intolerance, coughing, nasal discharge, fever, dyspnea, tachypnea, general depression, and loss of appetite. Diagnostic techniques such as bronchoalveolar lavage, transtracheal wash, thoracic ultrasonography, or thoracic radiography are often used to confirm a suspected diagnosis (Ainsworth & Hackett, 2004). Respiratory tract diseases are particularly significant in horses, especially in young Thoroughbreds. Inflammation detected in the trachea and bronchi is referred to as inflammatory airway

disease, and it is more impactful and frequent compared to other respiratory symptoms in this population (Wood et al., 2005). Respiratory disorders are second in importance only to musculoskeletal disorders in limiting an equine athlete's performance (Hewson & Arroyo, 2015). In the current study, respiratory system infections were found to be the most prevalent among infections at facilities operating under TJK, accounting for 49.37%. A study conducted in Ethiopia similarly reported that cough and nasal discharge associated with respiratory infections were commonly observed in horses (Laing et al., 2021).

Streptococcus equi subsp. zooepidemicus is a part of the normal bacterial flora in horses and is responsible for a range of diseases including pneumonia, abortion, upper respiratory tract infections, wound infections, testicular infections, and neonatal infections (Newton et al. 2003; Lindahl et al. 2013). In the present study, it was found that the most commonly used sample type was tracheal lavage fluid (47.3%). Furthermore, *Streptococcus equi subsp. zooepidemicus* was the most frequently isolated pathogen in respiratory infections, accounting for 14.53% (17/117). Similarly, a retrospective study by Erol et al. (2012) comprehensively examined the tissue/organ distribution and antimicrobial susceptibility patterns of *β-hemolytic streptococci* in horses between January 1, 2000, and December 31, 2010. In this study, a total of 2,497 *β-hemolytic streptococci* were isolated from 2,391 cases, with *S. equi subsp. zooepidemicus* being the most frequently isolated type (72.0%). *S. zooepidemicus* is associated with *S. equi* subsp. *equi*, which is the causative agent of Strangles (germ, watering can disease). In the current study, *S. equi subsp. equi* (11.0%) and *S. equi subsp. zooepidemicus* (9.7%) were the most frequently isolated pathogens after *E. coli* (15.6%) among the samples sent to the TJK İstanbul Equine Hospital Laboratory. Jaramillo-Morales et al. (2022) reported that young horses with a recent history of transport have a higher likelihood of testing positive for *S. equi* in guttural pouch swabs, with prevalences of 13.5% for *S. equi subsp. equi* and 1.5% for *S. equi subsp. zooepidemicus*. In the USA, *S. equi* was found in 715 out of 9,409 horses with upper respiratory infections (7.6%) (Jaramillo-Morales et al. 2023). Another study reported a prevalence of *S. equi subsp. equi* in herds at 5.86% (Libardoni et al. 2016). Çalışkan and Tel (2021) examined a total of 60 samples, including 32 nasal swabs and 28 tracheal aspirates, for *S. equi* and *S. zooepidemicus*. They reported isolating *Streptococcus spp.* from 22 out of 60 samples (36%), of which 3 strains (19.1%) were *S. equi* and 19 strains (20.6%) were *S. zooepidemicus*. In another study conducted in Türkiye, *Streptococcus spp.* was isolated from 2 out of 133 samples (1.2%), with one being *S. equi subsp. zooepidemicus* and the other *S. pneumoniae* (Diri et al. 2022). Additionally, *S. equi subsp. zooepidemicus* was isolated from 6 out of 93 horses (6.5%) in another study (Acke et al. 2015).

In the present study, after *Streptococcus equi subsp. zooepidemicus* (14.53%), the most commonly isolated pathogens in respiratory system diseases were *Streptococcus dysgalactiae subsp. equisimilis* (11.11%, 13/117), *Streptococcus equi subsp. equi* (11.11%, 13/117), *Escherichia coli* (10.26%, 12/117), *Streptococcus agalactiae* (9.40%, 11/117), and *Klebsiella pneumoniae* (9.40%, 11/117). *S. equisimilis* has rarely been isolated from the placentas of aborted, stillborn, and premature foals (Hong et al. 1993). *Streptococcus dysgalactiae subsp. equisimilis* is commonly found as a commensal in skin and mucosal surfaces and is an opportunistic pathogen for various animal species, including humans, horses, dogs, and pigs (Timoney 2004; İncili et al. 2023). It has recently been isolated from horses with a history of respiratory disease or Strangles-like illness (Laus et al. 2007). *S. equisimilis* is the second most frequently identified bacterial agent in pneumonia cases in horses, following *S. equi subsp. zooepidemicus* (Erol et al. 2012). In one study, *Streptococcus dysgalactiae subsp. equisimilis* was detected in 29 out of 99 nasal swabs (29.3%) (Preziuso et al. 2010). Another study isolated *S. dysgalactiae subsp. equisimilis* from 22 out of 93 horses (23.7%) (Acke et al. 2015).

In this study, it was determined that the samples sent to the TJK İstanbul Equine Hospital Laboratory were most frequently collected in the spring (33.8%) and then in the fall (30.8%). These samples were primarily from horses with respiratory infections. Analyzing the monthly distribution of the samples, it was found that most samples were sent in March. This can be explained by the start of the breeding season at the TJK facilities in February and the subsequent transfer of horses from various sources, which could lead to the transmission of diseases to healthy horses. The prevalence of recurrent airway obstruction (RAO) in horses, which is associated with exposure to hay and straw, may increase due to horses being kept in stables during the winter months (Bracher et al. 1991). Inflammatory airway disease (IAD) tends to peak at the beginning of spring and then decrease throughout the year, with significant increases often observed during seasonal transitions (Wood et al. 2005). Kutasi et al. (2011) reported that the prevalence of recurrent airway obstruction (RAO) is higher in the spring and summer compared to the winter months. It was noted that many horses began to show symptoms during this period, or the clinical signs became more severe. Another study observed a trend of three main peaks for RAO and IAD throughout the year. These diseases were reported to peak in early spring, with a smaller peak in mid-summer and another peak at the end of summer (Kutasi et al. 2011; Couëtil and Ward 2003). The study indicated that the likelihood of RAO in horses is 1.6 times higher in winter and 1.5 times higher in spring compared to summer.

Bacterial species involved in infectious equine endometritis are generally residents of the mare's

normal microbiota. The most common bacterium triggering endometritis in mares is *Streptococcus equi* subsp. *zooepidemicus* (Purswell et al. 1989; Langoni et al. 1997; Benko et al. 2016). However, other microorganisms commonly seen in equine endometritis include *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas* spp., *Staphylococcus aureus*, *Corynebacterium* spp., *Bacillus* spp., and *Actinomyces* (Riddle et al. 2007; Leblanc et al. 2009; Davis et al. 2013). In this study, the most frequently isolated bacteria from mares diagnosed with genital system diseases (metritis, endometritis) were *Streptococcus equi* subsp. *equi* (18.06%, 13/72), followed by *E. coli* (12.5%, 9/72) and *Streptococcus agalactiae* (9.72%, 7/72). Postpartum metritis is typically associated with trauma during parturition the retention of fetal membranes within 10 days after birth (more commonly 2-4 days after birth) and contamination of the urogenital system (Morris et al. 2020). An increase of one unit in the endometrial edema score in early postpartum mares has been reported to increase the likelihood of diagnosing subclinical endometritis caused by *Streptococcus equi* subsp. *zooepidemicus* by 5.5 times (Rasmussen et al. 2015). Díaz-Bertrana et al. (2021) isolated *Staphylococcus* (25.1%), *Streptococcus* (18.2%), *Escherichia* (17.3%), and *Pseudomonas* (12.1%) from samples collected from 363 mares with a history of repeated infertility, positive endometrial cytology, and/or vaginal discharge.

Anaerobic cultures were only performed on samples obtained from abscess content. However, the fact that anaerobic and *Mycoplasma* spp. cultures were not conducted on the other samples is one of the limitations of this study.

CONCLUSION

In conclusion, the findings from this study can contribute to the implementation of specific measures against prevalent diseases or bacterial agents in animal hospitals, stud farms, boarding facilities, and breeding stations operated under the TJK. This, in turn, may help minimize losses related to equine diseases.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: MCT and GE contributed to the project idea, design and execution of the study. MCT contributed to the acquisition of data. MCT and GE analysed the data. MCT and GE drafted and wrote the manuscript. MCT and GE reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out in the Turkish Jockey Club, İstanbul Equine Hospital Laboratory. "This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k1). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules."

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RESEARCH ARTICLE

Determination of Hematological Indices in Dogs with Acute CDV Infection

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ABSTRACT

The objective of this study was to ascertain the hematological indices in canines that have been infected with the Canine Distemper Virus (CDV) in an acute phase. The study included ten dogs with acute CDV infection (n = 10) and ten healthy dogs (n = 10). The results of the study demonstrated a statistically significant elevation in the neutrophil-to-lymphocyte ratio (NLR) and monocyte-to-lymphocyte ratio (MLR) in dogs acutely infected with CDV compared to the control group. In conclusion, different hematological responses can occur during the acute phase of CDV infection, and NLR ratios have the potential to be a good inflammatory marker for monitoring the acute phase.

Keywords: Canine distemper virus, Dog, Hematological indices, Neutrophil to lymphocyte ratio

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Akut Kanin Distemper Virüs Enfeksiyonlu Köpeklerde Hematolojik İndekslerin Belirlenmesi

ÖZ

Bu çalışmanın amacı akut Canine Distemper Virus (CDV) ile enfekte köpeklerde hematolojik indislerin belirlenmesidir. Çalışmaya 10 akut CDV enfeksiyonlu (n = 10) ve 10 sağlıklı köpek (n = 10) dahil edilmiştir. Çalışmanın sonuçlarına göre akut CDV ile enfekte köpeklerde kontrol grubuna göre kıyasla istatistiksel olarak önemli derecede yüksek nötrofil-lenfosit oranı (NLR) ve monosit lenfosit oranı (MLR) tespit edilmiştir. Sonuç olarak CDV enfeksiyonunun akut döneminde farklı hematolojik yanıtların oluşabileceği ve NLR oranlarının akut dönemi izlemede iyi bir yangısal belirteç olarak rol oynayabilme potansiyeline sahip olduğu tespit edilmiştir.

Anahtar Kelimeler: Canine distemper virus, Hematolojik indisler, Köpek, Nötrofil-lenfosit oranı

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INTRODUCTION

Canine distemper virus (CDV) constitutes one of the most highly transmissible viral pathogens affecting domestic canine populations globally. Furthermore, it presents a substantial menace to the persistence of a diverse array of endangered wildlife species across the world (Martinez-Gutierrez & Ruiz-Saenz 2016). The clinical presentation can vary substantially, ranging from asymptomatic infection to a fulminant multisystemic disease process. This variability is influenced by a multitude of factors, including the infected species, age, immunological competence of the host, and the virulence of the specific CDV strain (Deem et al. 2000). During the acute phase of the disease, respiratory and digestive system infections manifest prominently, whereas the nervous system exhibits involvement in the advanced stages (Martella et al. 2008). Furthermore, CDV infection demonstrably induces a state of immunosuppression within the host organism (Dik et al. 2023). This phenomenon stands in stark contrast to the inflammatory response typically observed in canine infectious diseases, where an increase in cytokine and acute-phase protein synthesis is a hallmark feature (Paul et al. 2023). This exceptional situation underscores the critical need for the utilization of alternative inflammatory markers for a more comprehensive evaluation of the host response in CDV.

Within this context, hematological indices such as the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), monocyte-to-lymphocyte ratio (MLR), and eosinophil-to-lymphocyte ratio (ELR) have emerged as prominent tools for evaluating inflammation in recent human studies (Langley et al. 2021; Buonacera et al. 2022). Given the inflammatory response observed in CDV infection, these readily available hematological indices hold promise as potential markers for assessing inflammation in canine patients with CDV.

Noteworthy developments in recent veterinary medicine have been the exploration of hematological indices as inflammatory markers. While the NLR has emerged as a promising indicator for intestinal inflammation in dogs (Becher et al. 2021), its efficacy in evaluating respiratory tract infections appears less conclusive (Conway et al. 2021). Intriguingly, while research on the PLR in veterinary medicine remains less compared to the NLR, existing studies suggest its potential as an inflammatory marker in various canine infectious diseases. More specifically, research has documented PLR as a significant inflammatory marker in canine sepsis (Pierini et al. 2020), and critically ill dogs (Dourmashkin et al. 2023). Furthermore, the MLR and ELR have garnered increasing attention in recent human studies as potential markers for detecting inflammatory processes (Vakhshoori et al. 2023).

Notably, recent human studies emphasize the importance of evaluating these indices at different disease stages for a more comprehensive understanding of the immune response (Zhou et al. 2023; Tan et al. 2024). This emphasis on staged evaluation in human medicine resonates with the importance of considering disease stage when assessing hematologic indices in CDV infection. By evaluating these indicators throughout the disease process, it is possible to gain a more complete understanding of the immune response and potential inflammatory processes associated with CDV infection. Building upon the growing recognition of the value of staged evaluation of hematologic indices in human medicine, this study aimed to determine the specific profile of these indices in dogs acutely infected with CDV.

MATERIALS and METHODS

Animals

The study was approved by the Atatürk University Local Ethics Committee (Decision Number: 2024/19). A prospective, case-controlled observational study was performed in unvaccinated dogs with upper respiratory tract symptoms and/or diarrhea. The G*Power program, version 3.1, developed by Franz Faul at the University of Kiel, Germany, was used to determine the minimum sample size for each group based on NLR data (Thomas, 2014). The calculation was performed using information about NLR from a previous study conducted by Durán-Galea et al (2024) on dogs with leptospirosis, with the following parameters: effect size = 1.4032928, significance level = 0.05, and power = 0.90. The analysis determined that at least 10 dogs should be included in each group. Therefore, the study included 10 dogs (n = 10) of any breed or gender presenting with clinical signs suggestive of CDV infection, including diarrhea, lethargy, vomiting, sneezing, and ocular discharge. Dogs exhibiting neural signs suggestive of a chronic infection were excluded from the study. Moreover, dogs were unvaccinated for CDV and had no prior medical intervention. The initial stage involved a comprehensive patient evaluation, encompassing a thorough review of medical records, a detailed physical examination, and the analysis of fecal samples using flotation techniques. Screening for CDV infection in suspect dogs was performed using a rapid CDV antigen test kit (Anigen, Antigen-USA). Post-CPV screening, additional examinations for canine coronavirus (CCV) and parvovirus (CPV) were performed. Dogs diagnosed with co-infections (CCV or CPV) were excluded to maintain the study's focus on CDV.

Following the recruitment of the CDV-infected group, 10 control dogs (n = 10) were included to

ensure age-matched cohorts in the study. These control dogs underwent a comprehensive health evaluation, including a review of their medical history, a thorough physical examination, complete blood count analysis, and confirmation of their CDV-negative status.

Blood Sampling and Complete Blood Count Analysis

Prior to the commencement of treatment, blood samples were obtained via the use of 21-gauge needles, with some samples collected from the cephalic vein and the remainder obtained through jugular venipuncture and distributed into anticoagulant (EDTA) tubes (K2EDTA Vacuette, Shanghai, China). Whole blood counts were subsequently evaluated using an automated hematology analyzer (Abacus Junior Vet5®, Hungary). The NLR, PLR, MLR, and ELR were determined using the following formulas:

NLR = absolute counts of neutrophils/absolute counts of lymphocytes,

PLR = absolute counts of platelets/absolute counts of lymphocytes,

MLR = absolute counts of monocytes/absolute counts of lymphocytes,

ELR = absolute counts of eosinophils/absolute counts of lymphocytes.

Statistical Analysis

The Shapiro-Wilk normality test was employed to ascertain whether the data were normally distributed. As the results of the normality test indicated that the data obtained in this study did not exhibit normal distribution, a statistical analysis of the data was conducted using the Mann-Whitney U test, a non-parametric test suitable for comparing two groups (control and CDV) (Despande et al. 2018). Data are expressed as median (1st quartile-3rd quartile). SPSS 27.0 software was used to perform the Mann-Whitney U test, with significance set at $p < 0.05$ to identify statistically significant differences between the control and CDV groups.

RESULTS

The results of the study revealed that both the CDV-infected (n = 10; median age: 4 months, range: 3.0–6.0 months) and control groups (n = 10; median age: 4.5 months, range: 3.0–6.0 months) had similar median ages. Importantly, there was no statistically significant difference in the age distribution between the groups. The clinical signs observed in the canines included in the study were fever (6/10), dehydration (7/10), vomiting (2/10), diarrhea (7/10), cough (5/10), nasal discharge (9/10), and lacrimation (7/10).

Table 1. Comparisons of hematological analyses of canine distemper virus infected and control dogs.

Variables	Study Group (n=10)	Control Group (n=10)	P Value	Reference range (Khan et al., 2011; MSD Manual)
WBC (10^9/l)	16.29 (13.08-26.98)	7.61 (4.65-11.19)	<0.001	5.9-16.6
Lymph (10^9/l)	0.85 (0.23-2.21)	1.31 (0.52-3.60)	0.218	0.4-2.9
Mono (10^9/l)	0.87 (0.72-1.26)	0.55 (0.11-0.88)	0.005	0.1-1.4
Neut (10^9/l)	14.64 (11.04-24.99)	5.41 (3.11-9.00)	<0.001	2.9-12
Lymp (%)	5.2 (1.3-11.9)	14.18 (11.18-49.86)	<0.001	8-21
Mono (%)	5.45 (3.7-7.0)	6.12 (1.04-18.49)	0.436	1-9
Neut (%)	87.75 (81.1-92.8)	72.93 (44.32-84.83)	<0.001	51-84
Eos (%)	0.35 (0.2-2.0)	1.22 (0-4.47)	0.165	0-9
RBC (10^{12}/l)	5.73 (4.19-5.96)	7.39 (5.24-8.29)	<0.001	5.5-8.5
Hgb (g/dl)	9.75 (8-11.8)	16.5 (10.9-18.2)	<0.001	14.2-19.2
Hct (%)	33.48 (24.66-42.78)	50.16 (36.36-59.3)	<0.001	35-57
MCV (fl)	64.5 (53-73)	69 (68-75)	0.035	65-80
MCH (pg)	18.9 (15.9-20.4)	21.3 (19-24.5)	<0.001	12.2-25.4
MCHC (g/dl)	28.6 (26.90-33.60)	31.2 (28-36.3)	0.035	32-36
PLT (10^9/l)	459.5 (251-873)	389 (260-690)	0.218	211-621
MPV (fl)	10.9 (9.4-19.80)	7.95 (6.4-10.3)	<0.001	6.1-10.1

Eos; eosinophil, **Hct;** hematocrit, **Hgb;** hemoglobin concentration, **Lymph**; lymphocyte, **Neut**; neutrophil, **Mono**; monocyte, **MCV**; mean corpuscular volume, **MCH**; mean corpuscular hemoglobin volume, **MCHC;** mean corpuscular hemoglobin concentration, **MPV**; mean platelet volume, **PLT**; platelet, **RBC;** Red Blood Cell, **RDW**; red cell distribution, **WBC**; white blood cell.

The results of the hematological analysis are presented in Table 1. The findings of the hematological analyses indicated a statistically significant increase in white blood cell, monocyte, neutrophil count and percentage, and mean platelet volume, and a statistically significant decrease in lymphocyte percentage, red blood cell, hemoglobin, mean corpuscular hemoglobin concentrations, and hematocrit levels in CDV dogs in comparison to the control group. However, only neutrophil count, neutrophil percentage, and mean platelet volume were found to be not within the reference values.

Figure 1 presents the concentrations of NLR, PLR, MLR, and ELR ratios in the control and CDV groups. The NLR showed a significant increase ($p < 0.001$) in CDV-infected dogs (median: 16.84, interquartile range [Q1-Q3]: 8.53-30.30) compared to the control group (median: 5.42, interquartile range [Q1-Q3]: 3.04-6.06).

Similarly, MLR levels were significantly higher in CDV-infected dogs (median: 1.09, interquartile range [Q1-Q3]: 0.58-1.50, $p = 0.023$) compared to controls (median: 0.39, interquartile range [Q1-Q3]: 0.12-0.85). While PLR also tended to be higher in the CDV group (median: 616.11, interquartile range [Q1-Q3]: 311.92-856.76) compared to controls (median: 397.36, interquartile range [Q1-Q3]: 172.32-616.52), this increase was not statistically significant ($p = 0.247$).

In contrast to the other parameters, ELR levels in CDV-infected dogs (median: 0.07, interquartile range [Q1-Q3]: 0.05-0.17) tended to be lower compared to the control group (median: 0.09, interquartile range [Q1-Q3]: 0.03-0.16). However, this decrease was not statistically significant ($p = 0.631$).

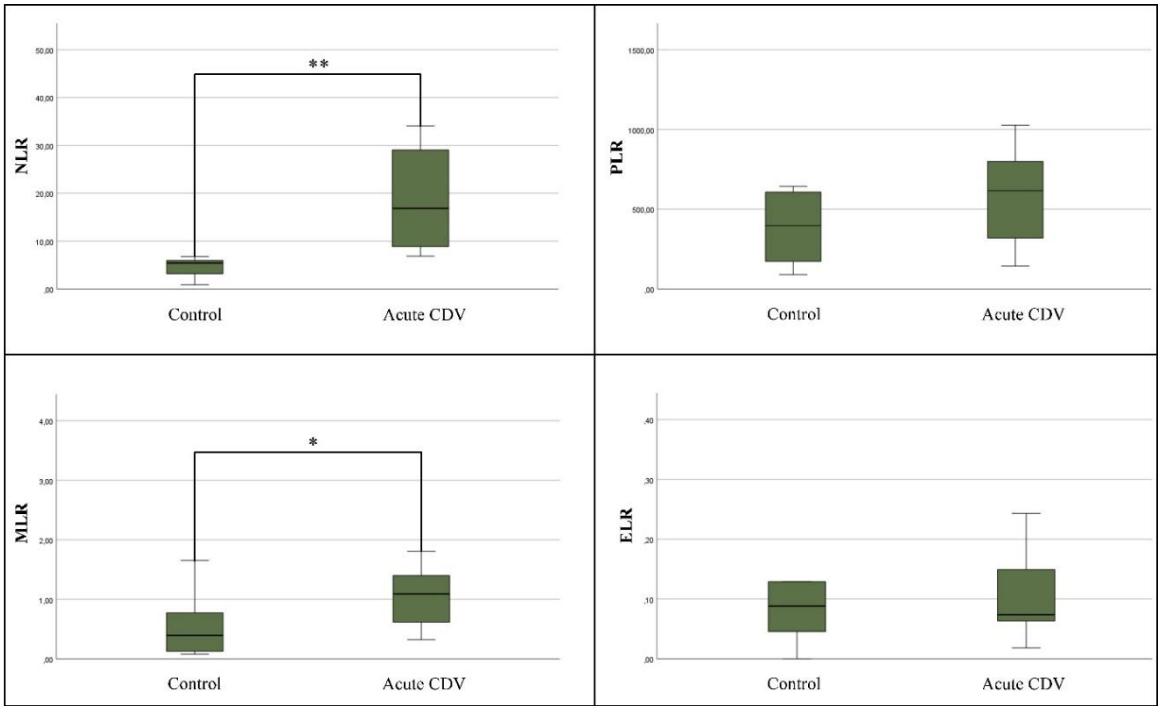

Figure 1. Comparisons of neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, and eosinophil-to-lymphocyte ratio in dogs acutely infected with CDV with ones in control group. (* = $p<0.05$, ** = $p< 0.01$).

DISCUSSION

A comparative analysis of hematological parameters was conducted in canines experiencing acute CDV infection. Results revealed a statistically significant elevation in the NLR among CDV-infected dogs (median: 16.84, interquartile range [Q1-Q3]: 8.53-30.30) compared to controls (median: 5.42, interquartile range [Q1-Q3]: 3.04-6.06). This finding aligns with previous research documenting increased NLR in chronic CDV cases (Pekmezci et al. 2022). However, the present study identified notably higher NLR values than those reported previously. This discrepancy can be attributed to pronounced neutrophilia, rather than solely lymphopenia, which has been associated with lymphoid apoptosis in acute CDV infection (Schobesberger et al. 2005). In contrast to the neutropenia typically observed in canine viral diseases (Gülersoy et al. 2022), the present study identified neutrophilia as a prominent feature of acute CDV infection. Considering the inverse relation between neutrophil count and disease severity (Goddard et al. 2008), an elevated NLR emerges as a potentially more sensitive inflammatory biomarker in acute CDV cases.

The results demonstrated an elevation in PLR levels in CDV-infected canines (median: 616.11, interquartile range [Q1-Q3]: 311.92-856.76) when compared to the control group (median: 397.36, interquartile range [Q1-Q3]: 172.32-616.52). However, this increase did not attain a statistically significant level. It is beneficial to highlight the increase in platelet count as the primary reason for the observed elevation in PLR levels, rather than lymphopenia. However, this finding is inconsistent with previous reports of thrombocytopenia in CDV infection (Jesus et al. 2021). In light of these findings, it can be postulated that the dogs were in the acute phase of the disease. Some studies in human medicine indicate that certain viruses can directly interact with platelets during the acute phase of infection, leading to their activation (Chaipan et al. 2016). Additionally, it has been demonstrated that cytokine release during the course of infection may result in platelet activation (Goeijenbier et al. 2012). In this context, although platelet activation is seen as a plausible cause in our results, it is important to note that these are only hypotheses and that the underlying cause should be determined with greater precision. Although no statistical difference was detected, this may be attributed to the small sample size. In future studies, it is strongly recommended that platelet levels in dogs acutely infected with CDV be investigated with a larger sample size.

A statistically significant elevation in the MLR was observed in CDV-infected dogs (median: 1.09, interquartile range [Q1-Q3]: 0.58-1.50, $p = 0.023$) compared to controls (median: 0.39, interquartile range [Q1-Q3]: 0.12-0.85). This finding is consistent with the established role of monocytes in orchestrating rapid pro-inflammatory and antiviral responses during acute viral infections (Nikitina et al., 2018). Notably, the signaling lymphocyte activation molecule (SLAM), a primary CDV receptor (Seki et al., 2003), can be rapidly upregulated on monocytes (Beineke et al. 2009), suggesting a potential mechanism for the observed increase in MLR. The enhanced monocyte activation, possibly mediated by SLAM engagement, may contribute to the amplified inflammatory response characteristic of acute CDV infection. These results suggest that MLR may serve as a valuable prognostic indicator of inflammation in dogs with acute CDV.

In contrast to other hematological parameters, a non-significant decrease in ELR was observed in CDV-infected dogs (median: 0.07, interquartile range [Q1-Q3]: 0.05-0.17) compared to healthy dogs (median: 0.09, interquartile range [Q1-Q3]: 0.03-0.16). This finding suggests a potential redistribution of eosinophils from the peripheral circulation to extravascular tissues. Corroborating this hypothesis, previous research has documented the presence of eosinophilic inclusion bodies within the bladder, stomach, kidneys, and lungs of CDV-infected canines (Headley & Graça 2000). The observed decrease in circulating eosinophils may reflect an early phase of this extravasation process, resulting in a non-significant difference in ELR.

The present study is subject to certain limitations. Firstly, due to financial constraints, molecular confirmation of CDV infection, such as PCR, was not possible. Secondly, the sample size was relatively small. However, it is important to note the low prevalence of CDV in the study region. Future research should prioritize the molecular diagnosis of CDV and employ a larger sample size to enhance the robustness of findings.

CONCLUSIONS

In conclusion, this study investigated the utility of hematological indices in monitoring inflammation during acute CDV infection. NLR and MLR may be important parameters to assess inflammation in this context. Given their cost-effectiveness, accessibility and widespread clinical application, NLR and MLR show promise as valuable tools for monitoring treatment response in dogs with acute CDV.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: KEY and ŞD contributed to the project idea, design and execution of the study. KEY, ŞD and MK contributed to the acquisition of data. KEY and ŞD analysed the data. KEY drafted and wrote the manuscript. KEY, ŞD and MK reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Atatürk University Animal Hospital. This research was approved by Atatürk University Local Ethics Committee (Decision Number: 2024/19)

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Kocatepe Veterinary Journal

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LETTER TO EDİTOR

Alcohol in Homeopathic Products May Cause Wrong *in vitro* and Local Effects

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In the research of newly discovered drugs and molecules, in vitro tests are widely used as an alternative or supplement to animal experiments. However, in tests conducted with water-insoluble substances, the content and density of the substances used as solvents are data that should not be neglected. In cases where solvents (alcohol, etc.) of the tested compound are used, it may cause false in vitro results in cytotoxicity tests. Previous in vitro tests have shown that alcohol has a cytotoxic effect on cancer cells on its own and that the rates are in the range of (0.4-2%). Homoeopathic products may contain different rates of alcohol (approximately 50-60%) during the preparation phase. These rates may decrease during the dilution phase. These drugs have been observed to have very successful results when used systemically. However, it has been seen in the literature that the same drugs (e.g. Thenarecron) have in vitro cell culture studies. It has been suggested in these studies that cytotoxic effects are observed and that they can be used as potential cancer drugs. It should not be forgotten that these effects may be due to the alcohol used as a solvent. Other in vitro studies have also shown that the mechanism of action of these drugs, which have autophagic effects on cancer cells, is caused by alcohol, not the active ingredients. The systemic effects of these and similar drugs are more valuable in terms of clinical aspects, and local or in vitro use may cause misinterpretations. In some in vitro studies, it has been observed that the alcohol used in the control group has similar effects on cancer cell lines as drugs with alcoholic extracts. Therefore, it is thought that the use of drugs containing solvents known to have cytotoxic effects (such as alcohol) in vitro may cause erroneous evaluations, that if their use is mandatory, one of the control groups must be solvent, and that parenteral (non-local) use on biological systems will be more beneficial.

As a result, it should be kept in mind that systemic use of homoeopathic products with alcoholic extracts gives more significant results and that in vitro effects such as autophagy and cytotoxicity may be due to their alcohol content.

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CASE REPORT

A Fish Trematode *Vitellibaculum* Identified in the Gastrointestinal Tract of the Little Gull (*Larus minutus*): The First Report of Trematode from Iran

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ABSTRACT

Vitellibaculum, which belong to the subfamily *Megasoleninae* within the family *Haploporidae,* are typically parasites of marine fish. This trematode has been reported from different regions of the world. The Little Gull (*Larus minutus*), a migratory bird common in the coastal regions of Iran, was the subject of this study. A dead bird found by the Environmental Protection Agency in Babolsar was referred to the laboratory of the Department of Parasitology of the Science and Research branch of the Islamic Azad University. After necropsy and sampling of the intestinal contents, a single trematode was identified. After staining and slide preparation, it was identified as *Vitellibaculum* and registered in the National Parasitology Museum of Tehran University.
Keywords: Iran, Little Gull, Trematode, *Vitellibaculum.*

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Küçük Martının (*Larus minutus*) Sindirim Sisteminde Tanımlanan Bir Balık Trematodu *Vitellibaculum*: İran'dan Trematodun İlk Raporu

ÖZ

Vitellibaculum, *Haploporidae* familyasının *Megasoleninae* alt familyasına ait bir deniz balığı paraziti olarak bilinmektedir ve dünyanın çeşitli bölgelerinden rapor edilmiştir. Bu çalışmada, İran'ın sahil bölgelerinde yaygın olarak bulunan göçmen bir kuş türü olan Küçük Martı (*Larus minutus*) incelenmiştir. Çalışma kapsamında, Babolsar'da Çevre Koruma Ajansı tarafından bulunan ölü bir kuş, İslam Azad Üniversitesi Bilim ve Araştırma Birimi Parazitoloji Laboratuvarı'na gönderilmiştir. Nekropsi ve bağırsak içeriği örneklemesi sonrasında bir trematod tespit edilmiştir. Boyama ve lam hazırlama işlemlerinin ardından, bu trematodun *Vitellibaculum* olduğu belirlenmiş ve Tahran Üniversitesi Ulusal Parazitoloji Müzesi'nde kayda geçirilmiştir
Anahtar Kelime: İran, Küçük Martı, Trematod, *Vitellibaculum*

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INTRODUCTION

The genus *Vitellibaculum* (Syn. *Allomegasolena* Siddiqi & Cable, 1960), first described by Montgomery in 1957, comprises trematodes that occur as helminth parasites in various marine fish species. These parasites are assigned to the family *Haploporidae*, in particular the subfamily *Megasoleninae* (Montgomery, 1957; Madhavi and Bray, 2018).

The subfamily *Megasoleninae* is characterized by the presence of extensive vitelline follicles and an external seminal vesicle. These follicles are widespread throughout the hindbody and converge either behind the testes or near the posterior end of the body, while the external seminal vesicle is elongated and relatively narrow. Some genera within this subfamily possess a single testis, while others have two testes arranged in tandem, occasionally oblique. Typically, the caeca extends to the posterior end of the body. The *Megasoleninae* includes five genera: *Megasolena, Hapladena*, *Vitellibaculum*, *Myodera*, and *Metamegasolena*. This subfamily is distributed worldwide and is commonly found in marine fishes (Pulis and Overstreet, 2013; Andres et al., 2018).

The *Vitellibaculum* has a long, narrow body. Its oral sucker is at the end, and the ventral sucker is large, without a stalk. It has two testes arranged in tandem and a hermaphroditic sac. Its external seminal vesicle is elongated, and its ovary is located before the testes. The life cycle of this species remains unclear, but some studies suggest the involvement of invertebrates as intermediate hosts (Jones et al., 2005).

The little gull *(Larus minutus*) is a migratory bird species found in Iran. These birds prefer to spend the winter in the coastal regions and have been reported on both the southern and northern coasts of the country. Their dispersion is particularly large along the Caspian Sea, encompassing the entire coastal areas of Gilan and Mazandaran provinces. Little gulls feed mainly on small marine organisms and tiny fish that inhabit the shallow waters of the sea (Scott and Adhami, 2006; Mansoori, 2009).

In this report, the trematode was accidentally discovered in the gastrointestinal canal of a little gull.

The Iranian seas, namely the Caspian Sea, the Persian Gulf, and the Oman Sea are home to a wide variety of fish species, and the fishing industry is an important sector in these regions. Consequently, various studies are being carried out in this sector concerning both the environment and the health of the fish. Samples are regularly taken from the fish caught and analyzed for food safety. Literature documents that these fish are infected with helminths and acanthocephalans (Ebrahim Zadeh Mosav et al, 2014; Tavakol et al., 2015). However, *Vitellibaculum* has not yet been reported in these fishes. This study is the first to report this trematode from the Middle East region, particularly from the Iranian basin.

MATERIAL and METHOD

In July 2018, the dead little gull was handed over by the Environmental Protection Agency of Mazandaran Province (Babolsar District) to the Faculty of Veterinary Medicine of the Islamic Azad University, Science and Research Branch, Tehran, for pathological examination.

Babolsar, located in the northern part of Iran, is one of the coastal cities of Mazandaran province. It is situated between the Caspian Sea and the Alborz Mountains, at 36°42'02"N, 52°39'00"E coordinates.

The autopsy of the deceased bird was performed, and samples were taken from different tissues to determine the possible cause of death. The gastrointestinal tract was separated for parasitological diagnosis. The entire canal was examined for the presence of parasites such as nematodes, cestodes, and trematodes. The intestinal contents were washed and passed through sieves with a diameter of 100 micrometers. A white trematode was observed in the residues in the sieves. After the initial microscopic examination confirmed that it was a trematode, aceto-alum-carmine staining was performed (Chandrawathani et al., 2019) and the trematode was fixed on a slide with antelan. The specimen was then forwarded to the National Parasitology Museum of Tehran University for further identification.

RESULTS

Microscopic examination of the trematodes revealed that the organism has a greatly elongated, narrow body. Body length and width are about 2.5 mm × 0.2 mm. It has a terminal oral sucker and a ventral sucker of equal size. The prepharynx is longer than the large pharynx, and the esophagus is also longer than the pharynx. In the hindbody's posterior half of the abdomen, there are two adjacent testes, one behind the other. The cylindrical, elongated external seminal vesicle is much longer than the hermaphrodite sac. The eggs are non-operculate, moderately numerous, and not filamentous. The vitellarium has numerous large follicles in lateral and sometimes medial fields that extend the entire length of the hindbody without fusing posteriorly (Jones et al., 2005). The specimen was diagnosed as *Vitellibaculum* (syn: *Allomegasolena*) and registered in the National Parasitology Museum of Tehran University.

Figure. 1: Overview of Vitellibaculum at 10X magnification, showcasing its elongated body, equal-sized terminal oral and ventral suckers, and notable reproductive structures.

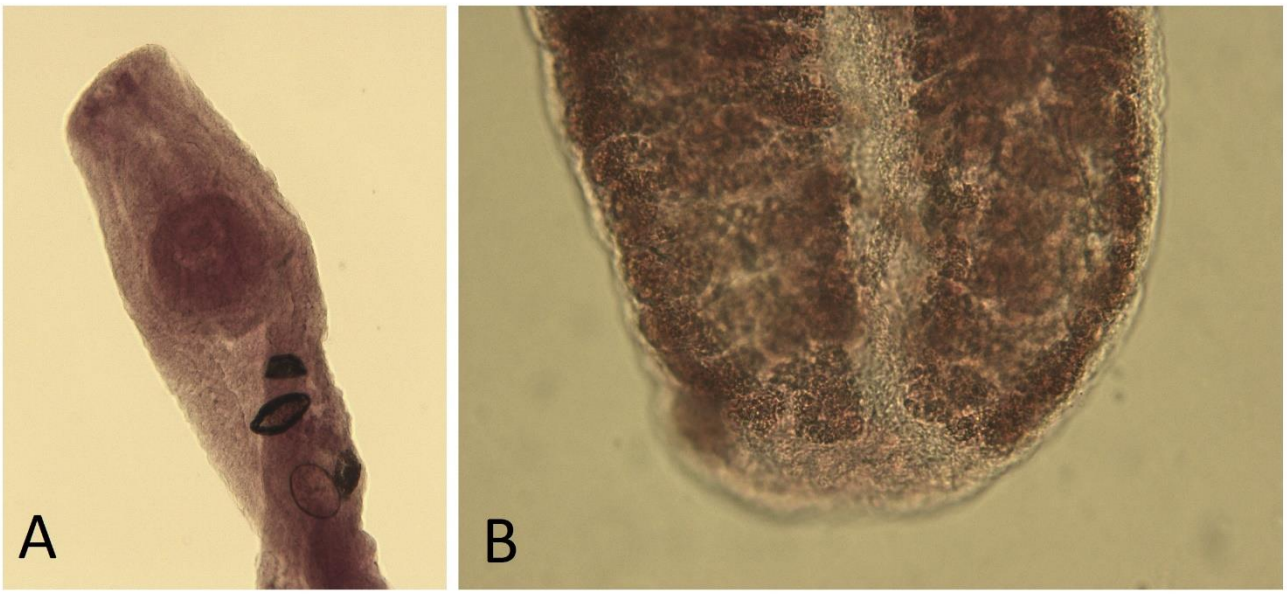

Figure. 2: A: Anterior end of *Vitellibaculum* at 10X magnification, highlighting the structure of the terminal oral sucker and ventral sucker; B: Posterior end of *Vitellibaculum* at 40X magnification, revealing the elongated external seminal vesicle.

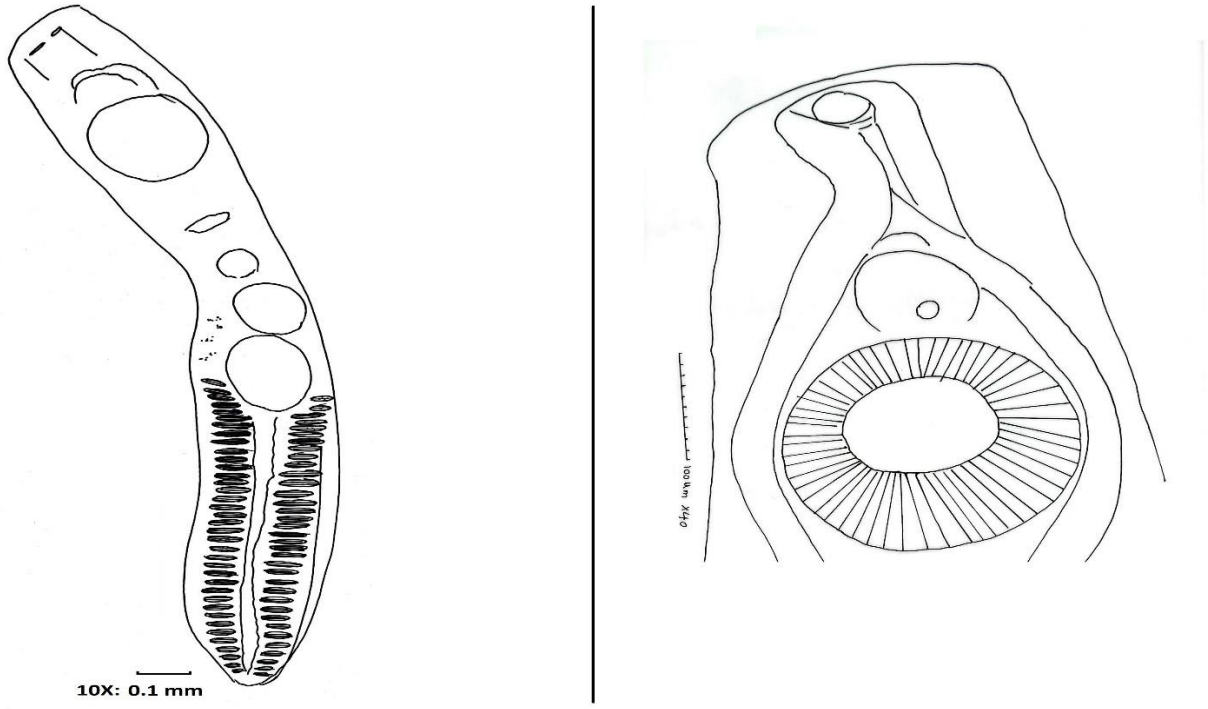

Figure.3: Ventral views of the oral and ventral suckers, along with non-operculate terminal eggs (Original)

DISCUSSION

Vitellibaculum is known to parasitize the intestines of marine fish. There is no documented evidence of lethal or pathological effects on fish yet. This trematode has been reported in various marine fish species. The life cycle of *Vitellibaculum* remains unclear, but there are two hypotheses: One states that it is a free-living cercariae stage, while the other proposes that invertebrates such as snails are used as intermediate hosts (Andres, 2014). This trematode has been reported from North and South America, particularly Brazil, Puerto Rico, and the USA, as well as from Libya in Africa (Montgomery, 1957; Overstreet, 1969; Fernandes and Kohn, 1984; Al-Bassel and Hussein, 2012; Roberts, 2021).

The taxonomic identification of *Vitellibaculum* remains complicated and controversial. Many trematodes in the family *Haploporidae* have only one testis, while members of the subfamily Megasoleninae, including genera such as *Megasolena, Hapladena, Vitellibaculum, Myodera,* and *Metamegasolena*, possess two testes. This characteristic suggests that *Vitellibaculum* and other *Megasoleninae* with two testes may not belong to the family *Haploporidae*. However, this hypothesis has yet to be confirmed by molecular identification methods (Andres, 2014). However, since the first identification of this trematode in 1957, there have been no reports of trematodes in fish from the seas of the Middle East. The little gull (*Larus minutus*) is found in many coastal areas of Iran. This bird is long and much smaller than the black-crowned cormorant. Its flight resembles that of a sea swallow. The behavior of this bird is similar to that of cormorants, and like the swallows, it hunts fish and insects on the water surface in flight. This bird is often found in coastal areas, estuaries, and on the edges of marshes. In Iran, it migrates in large numbers in winter to the low-lying areas along the coast of the Caspian Sea and is occasionally seen on the edges of marshes in Fars, Khuzestan, and on the coasts of the Persian Gulf. These birds migrate from south to north within Iran, and some of them spend the summer on the northern coast of the Caspian Sea and the winter on the southern coast. These birds are distributed along the entire southern coast of the Caspian Sea (Khaleghizadehi & Sehhatisabet, 2007; Khalilipour et al., 2007; Mansoori, 2009).

It seems that the little gull is not the usual host of *Vitellibaculum*, but it can feed on infected fish that carry the trematode. After consumption, the trematode could possibly survive in the gastrointestinal tract. However, no trematode eggs were detected in the fecal analysis of the cecum and large intestine, and only one trematode was found in the bird's intestine.

CONCLUSION

This study suggests that the trematode cannot maintain physiological activity in the bird's body. Nevertheless, the gastrointestinal secretions of the bird do not damage the trematode, indicating that the tegument structure of the *Vitellibaculum* is resistant to all physiological secretions and physical activities of the gastrointestinal tract of *Larus minutus*. This resistance means that although the metabolic functions of the trematode are inhibited, its structural integrity remains

intact, allowing it to survive passage through the bird's digestive system.

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Ethical approval: The current study does not require ethics approval.

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CASE REPORT

A Case of Type II Polydactyly and Flexor Tendon Contracture in a Calf

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ABSTRACT

Polydactyly is an abnormality defined by the presence of one or more extra digits. Polydactyly may occur alone or in association with other congenital defects. Polydactyly is easily diagnosed by clinical examination. X-rays are used to identify possible bony abnormalities. A 3 days old Montafon crossbred calf was brought to our hospital with the complaint of extra digit on the left forelimb and inability to extend the same leg. The patient could stand up with three legs but had difficulty in walking. As a follow-up of clinical and radiologic examinations, a diagnosis of unilateral forelimb Type II polydactyly and arqure was made. The decision was made to operate on the patient to prevent a loss of productivity in the future. The extra digit was removed and Z tenotomy was performed for flexor tendon contracture. In conclusion, we describe a case of type II polydactyly and flexor tendon contracture of the calf which was successfully corrected surgically.

Key Words: Calves, congenital anomaly, polydactyly, treatment

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Bir Buzağıda Tip II Polidaktili ve Fleksör Tendon Kontraktürü Olgusu

ÖZ

Polidaktili, bir veya daha fazla ekstra parmağın varlığı ile tanımlanan bir anormalliktir. Polidaktili tek başına veya diğer konjenital defektlerle birlikte görülebilir. Polidaktili klinik muayene ile kolayca teşhis edilir. Olası kemik anormalliklerini belirlemek için röntgen çekilir. 3 günlük montofon melezi bir buzağı sol ön bacakta ekstra parmak ve aynı bacağı uzatamama şikâyeti ile hastanemize getirildi. Hasta üç ayağı ile ayağa kalkabiliyor ancak yürümekte zorluk çekiyordu. Klinik ve radyolojik incelemeler sonucunda tek taraflı ön bacak Tip II polidaktili ve arkür tanısı konuldu. Gelecekte üretkenlik kaybını önlemek için hastanın ameliyat edilmesine karar verildi. Ekstra parmak alındı ve fleksör tendon kontraktürü için Z tenotomi uygulandı. Sonuç olarak, buzağıda cerrahi olarak başarıyla düzeltilen tip II polidaktili ve fleksör tendon kontraktürü olgusunu tanımlıyoruz.

Anahtar Kelimeler: Buzağılar, konjenital anomali, polidaktili, tedavi

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INTRODUCTION

Polydactyly is a defect defined by the formation of one or more additional digits (Mosbah et al., 2012; Rafee, 2016). This anomaly has been reported in humans and many animal species such as birds, cats, dogs, horses, camel, and cattle (Johnson et al., 1981; Barber, 1990; Bani-Ismail et al., 1999; Paryani, 2015; Hamelin et al., 2017; Woehler & Holzmann, 2020). Polydactyly may be unilateral or bilateral and occur in combination with other congenital defects (Leipold, et al., 1972; Barber, 1990; Gugjoo et al., 2013). Polydactyly is easily diagnosed by clinical examination. A full X-ray examination of the area is necessary to detect possible osseous deformities. In an animal with polydactyly, lameness increases with age. This leads to a decrease in the animal's productivity and quality of life, and therefore to economic loss (Uygur et al., 2022). It is less common in cattle, but when it does occur, it is more common on the forelimbs of cattle. Polydactyly in cattle can be divided into seven types, graded as types I to VII (Rafee, 2016).

Congenital contracted flexor tendon is a common defect in cattle and can be seen in many breeds. Causes of contracted flexor tendons include abnormal positions in utero, genetic predisposition, malnutrition and exposure to teratogens. Contracted tendons can appear in association with other congenital anomalies such as cleft palate, dwarfism and arthrogryposis (Anderson & St Jean, 1996; Genccelep et al., 2019). Congenital contracture of the flexor tendons is defined as a congenital malformation characterized by curvature of the extremities, multiple joint stiffness and muscle dysplasia. Splinting, bandage applications such as polyvinyl chloride (PVC) and fiberglass materials, and tenotomy are generally used in the treatment of this disorder (Kiliç & Tekin, 2021).

In this case, type II polydactyly and arqure were identified in a 3-day-old calf and the operative treatment performed to prevent economic loss in the future is described.

CASE PRESENTATION

A 3-day-old, 52 kg male Montafon Crossbreed calf was brought to the Department of Surgery of Aydın Adnan Menderes University, Faculty of Veterinary Medicine due to extra digit on the left forelimb. According to the anamnesis obtained from the owner, it was learned that the calf was born from the first pregnancy of a two-year-old heifer. Clinical examination revealed that the patient could stand up with three legs but had difficulty in walking. There was an extra metacarpal bone and nail in the left forelimb and the carpal joint could not be extended. The clinical signs included; respiratory rate (28/min), temperature (38,3 °C), and heart rate (120/min) were in the normal range. Haematology and the biochemical profile were in the normal range. Orthogonal radiographic examination of the extremity was performed. The clinical examination revealed unilateral polydactyly and arqure in the calf and the diagnosis was confirmed by radiological examination. (Figure 1).

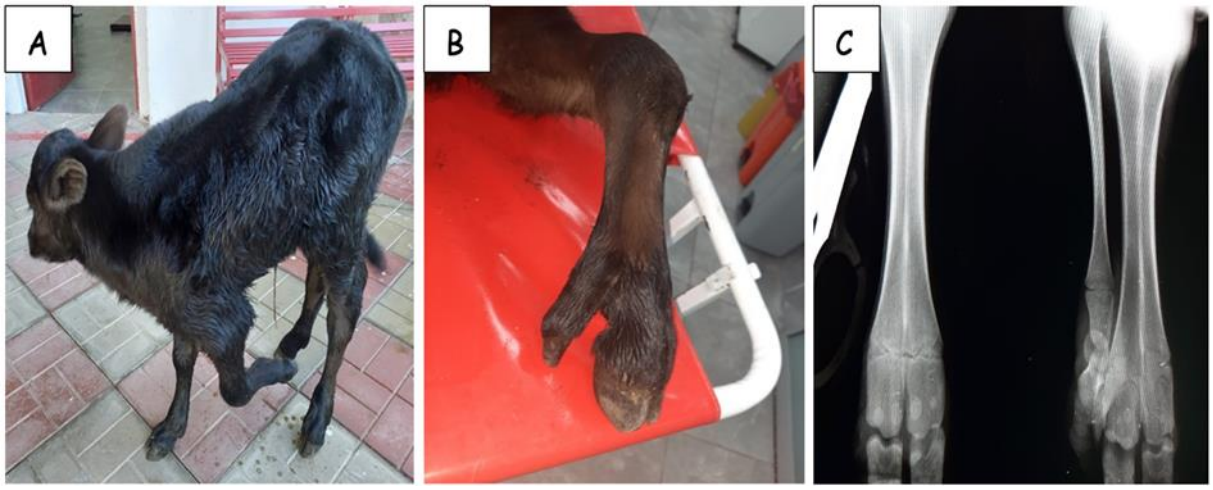

Figure 1: (A) Standing clinical view of the patient. (B) Lateral clinical view of the patient's right extremity. (C) Anterioposterior X-ray image of the patient's forelimbs.

The operation was decided in order to prevent possible productivity losses in the future. The calf was administrated Xylazine (Xylazinbio® 2%, Biovate) at a dose of 0.1 mg/kg (IM) for premedication and Ketamine (Ketasol® 10%, Richterpharma) at a dose of 2 mg/kg (IM) 10 minutes later for induction. The animal was intubated and the cuff was inflated. Anesthesia maintenance was performed with Isoflurane (Isoflurane USP100%, 100 ml, ABD) in 100% oxygen. Operation area was prepared aseptically

and covered sterile drapes. A proximal to distal skin incision was made on the medial aspect of the extra metacarpal bone. The subcutaneous connective tissues and surrounding structures were carefully dissected, with attention to vessel and muscles. The extra metacarpal bone was proximally osteotomized at its attachment site, and the claw was entirely removed (Figure 2).

Afterwards, a skin incision was made over the contracted tendon, which prevented the carpal joint from extending, and this tendon was extended by performing a Z tenetomy (Figure 3). The skin was routinely closed.

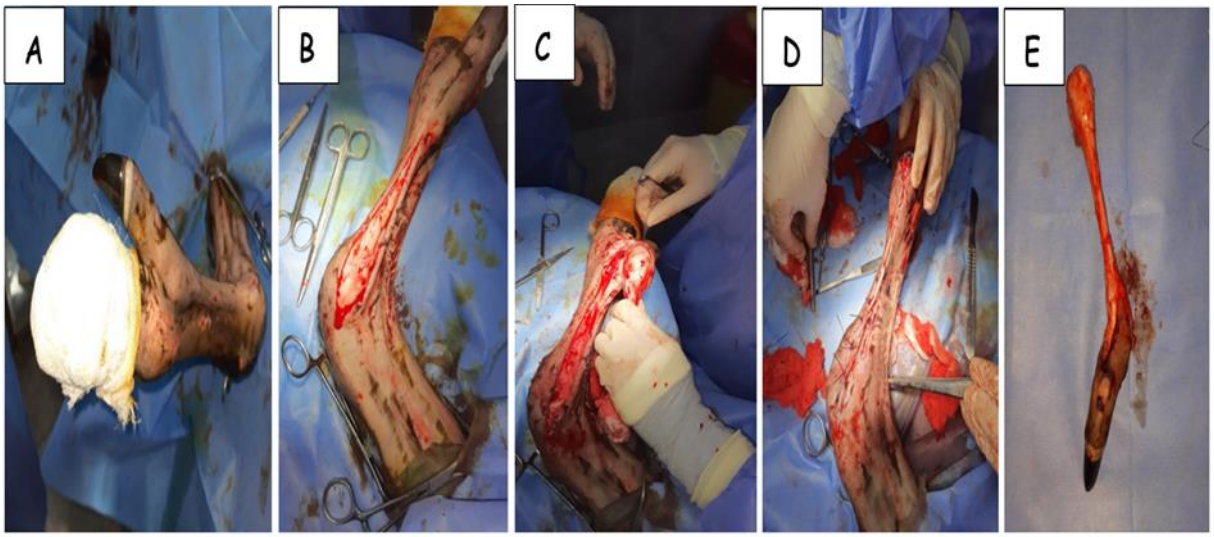

Figure 2: (A) Delimitation of the area to be operated on. (B) Skin incision over the extra bone. (C) View of the extra bone as it is removed. (D) Closure of the incision site after removal of the extra bone. (E) Removed extra bone.

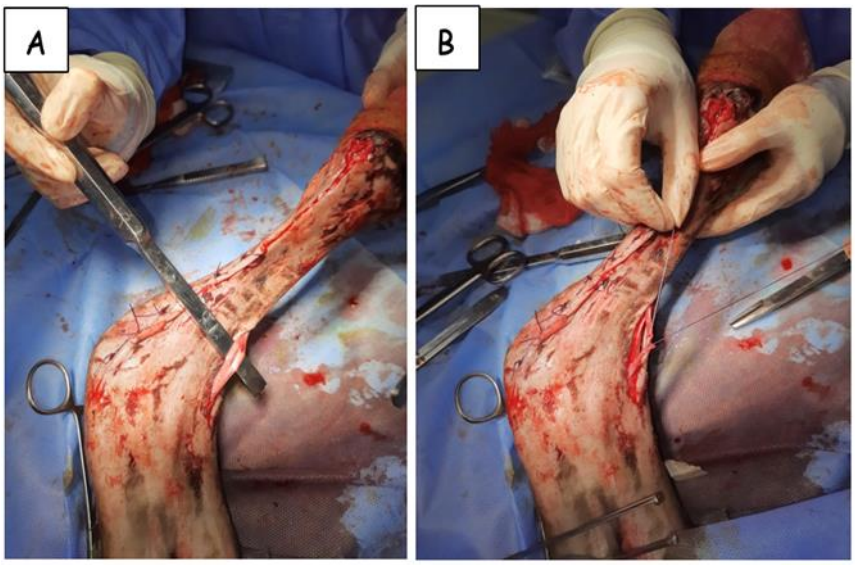

Figure 3: (A) The contracted tendon dissected from other tissues. (B) Z tenetomy of the tendon.

After the operation, a PVC (polyvinyl clorite) bandage was applied to the tenetomized extremity of this calf with carpal flexural deformity to provide more support.

Postoperatively, the calf was given Meloxicam (Bavet Meloxicam®, Turkey, 0.2 mg/kg, 5 days), Amoxicillin/Clavulanic Acid (Synulox®, 35 mg/ml Clavulanic Acid, 140 mg/ml Amoxicillin, Zoetis, 7 days) and orthogonal x-ray of the extremity was taken (Figure 4). Sutures were removed 14 days after the operation and the bandage was changed. One month later, the patient was able to use his leg comfortably but the tendon is a tissue that heals late, so the calf's forelimb was put in PVC bandage for 1 more week.

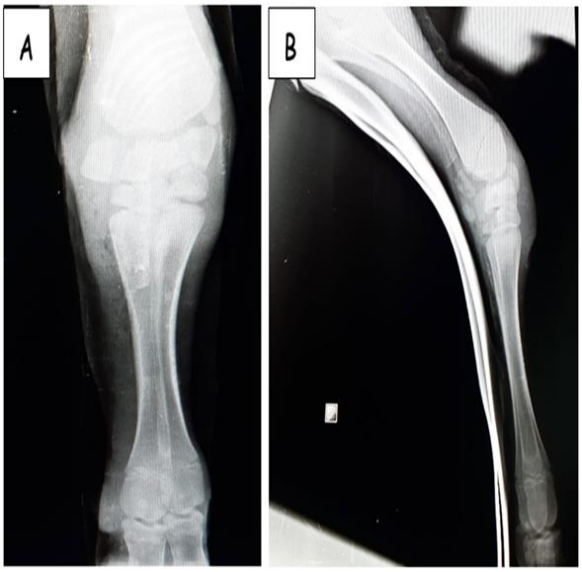

Figure 4: Postoperative (a) anterioposterior and (b) mediolateral X-ray image.

DISCUSSION

Anomalies in animals are caused by nutritional disorders, stress factors, genetic and environmental factors or a combination of these, vitamin deficiencies, errors in breeder selection, teratogens and lack of preference for artificial insemination (Newman et al., 1999; Vermunt et al., 2000; Uygur et al., 2022). Polydactyly is a congenital disorder and is increasingly common in cattle. There are seven types of polydactylism seen in cattle; Type I- bilateral polydactyly of both forelimbs with additional metacarpal bones or phalanges, Type II- unilateral polydactyly of the forelimbs or hindlimbs with additional metacarpal or metatarsal bones and phalanges, Type III- Additional digits in all four limbs, Type IV- Rarely seen, involves bilateral duplication of digits on the forelimb or hindlimb, Type V- Polydactyly, Type VI- Bilateral incomplete formation of metacarpal II and Type VII- Polydactyly with a complex of malformations. Polydactylism usually affects both forelimbs, but less frequently malformations of one or all four limbs are described (Mosbah et al., 2012).

Diagnosis is usually based on clinical examination. X-ray examinations are useful to assess the extent of bone abnormalities associated with the extra metacarpus. In this case, clinical and radiological examination was performed. As a result, Type II, i.e. unilateral additional metacarpals and phalanges in the left forelimb, as well as carpal flexural deformity are present.

According to some reports, polydactyly occurs alone or rarely in association with other developmental or inherited malformations such as tendon contracture (Crowe & Swerczek, 1985; Villagomez & Alonso, 1998). In this case, it was seen together with flexor tendon contracture.

In the treatment of polydactyly, surgical removal of the extra digits is recommended to prevent lameness, restore normal limb conformation and improve the cosmetic appearance of the limb (Bani-Ismail et al., 1999; Carstanjen et al., 2007). In this study, the excess finger was surgically removed and Z tenotomy was performed for flexor tendon contracture.

CONCLUSION

Polydactylism can be seen alone or with other congenital anomalies and is rarely seen in cattle. Our case was diagnosed with polydactyly and arqure of the forelimb based on clinical appearance and radiography. In conclusion, we describe a case of type II polydactyly and flexor tendon contracture of the calf which was successfully corrected surgically.

Conflict of interest: Authors declare that they have no financial interests or personal conflicts that may affect the study in this article.

Authors' Contributions: RY contributed Clinical case attention, Data collection, Literature review, Writing original draft, Writing review & editing of the study..ESA contributed clinical case attention, writing review & editing of the study. All authors have read and approved the finalized manuscript.

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

Explanation: This study has not been presented (as a oral, poster, abstract vs) anywhere before.

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