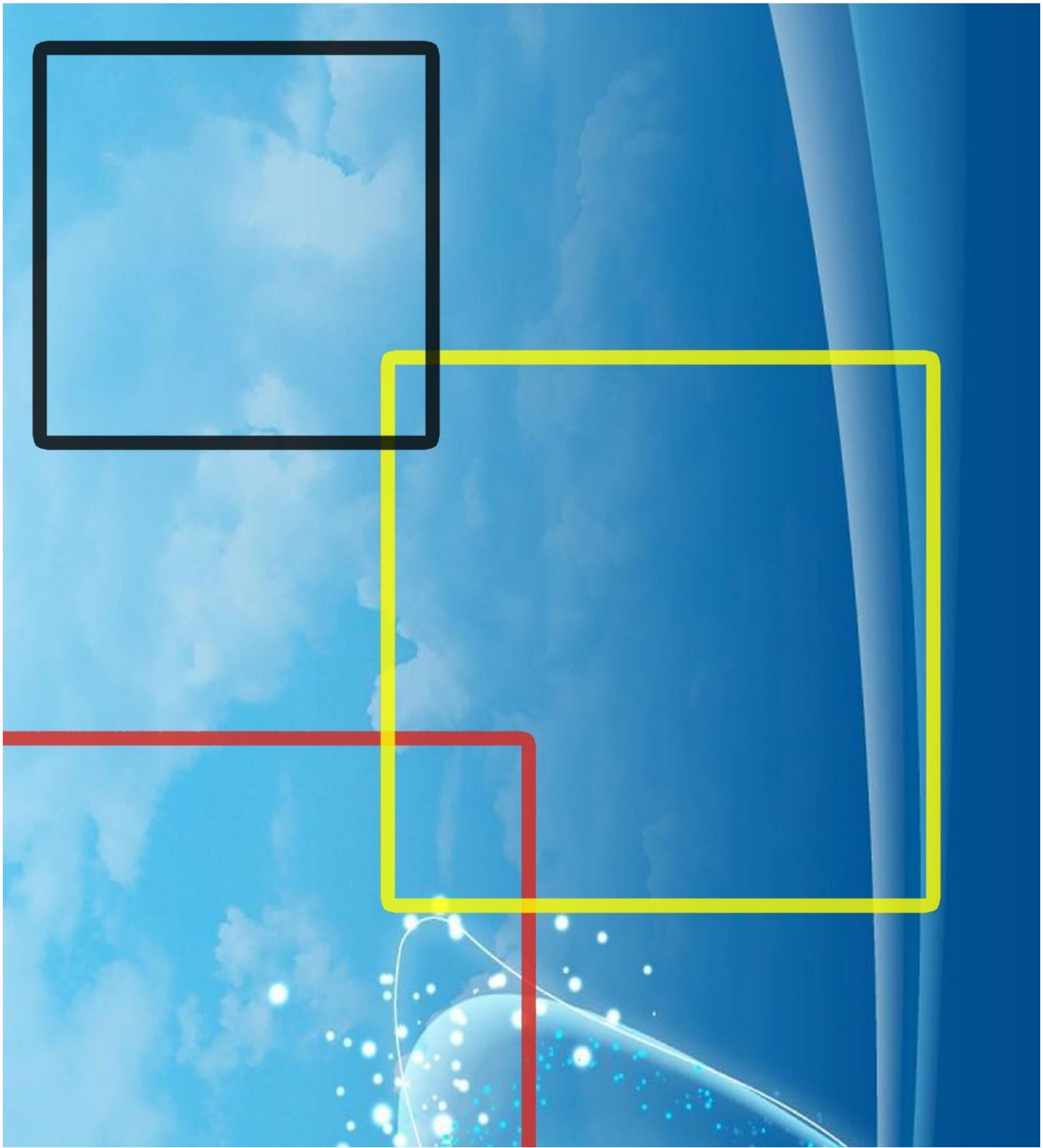
VAN VETERINARY JOURNAL

Year: 2024 / Volume: 35 / Issue: 3

ISSN : 2149-3359
e-ISSN : 2149-8644



November - 2024

VAN VETERINARY JOURNAL

This journal previously published as: **Yüzüncü Yıl Üniversitesi Veteriner Fakültesi Dergisi**

ISSN: 2149-3359 E-ISSN: 2149-8644

This journal is published three times a year. Journal Tittle Abbreviation: Van Vet J
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2024	35	3

This journal indexed / abstracted in Asos Index, CAB Abstracts, EBSCOhost, Google Scholar, Index Copernicus, Sobiad, TR Dizin and Turkiye Atıf Dizini

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Cite this article as: **Terim Kapakin KA, Bolat İ, Manavoğlu Kirman E et al. (2024).** Histopathological and Immunohistochemical Investigation on Effects of Boric Acid Used in Treatment of Rats with Knee Osteoartritis on Kidney Tissues. *Van Vet J*, 35 (3), 145-151. DOI: https://doi.org/10.36483/vanvetj.1477410

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

Histopathological and Immunohistochemical Investigation on Effects of Boric Acid Used in Treatment of Rats with Knee Osteoartritis on Kidney Tissues

Kübra Asena TERİM KAPAKİN[1] İsmail BOLAT[1,*] Esra MANAVOĞLU KİRMAN[1] Gülşah GÜNDOĞDU[2] Köksal GÜNDOĞDU[3] Fatma DEMİRKAYA MİLOĞLU[4] Şeymanur YILMAZ TAŞÇI[4]

[1] *Atatürk University, Faculty of Veterinary Medicine, Department of Pathology, Erzurum, Türkiye*
[2] *Pamukkale University, Faculty of Medicine, Department of Physiology, Denizli, Türkiye*
[3] *Denizli State Hospital Orthopedics and Traumatology, Denizli, Türkiye*
[4] *Atatürk University, Faculty of Pharmacy, Department of Analytical Chemistry, Erzurum, Türkiye*

Received: 02.05.2024 Accepted: 31.10.2024

ABSTRACT Osteoarthritis (OA) is a disease that often occurs in the knee joints and is characterized by disruption of cartilage homeostasis. Due to the systemic inflammation it creates, it affects not only the joint area, but also many tissues and organs. In this study, the damage caused by systemic inflammatory reactions due to OA in kidney tissue and the protective effect of boric acid were investigated. Wistar albino rats were used in the study. An experimental knee OA model was created by intraarticular injection of monosodium iodoacetate (MIA) in rats. It was formed from 4 groups as Control, OA, OA + 4 mg Boric Acid, OA + 10 mg Boric Acid to work. At the end of the study, kidney tissues were taken from the rats and TNF-α, IL-1β, NOS2 and MMP13 analyzes were performed by histopathological and immunohistochemical methods. Histopathological examinations revealed severe degenerative and necrotic changes in tubular epithelial cells in the OA groups, and these changes decreased in the boric acid-administered group depending on the dose. In immunohistochemical analyzes, it was determined that systemic inflammatory reactions occurring in OA application decreased in a dose-dependent manner with boric acid application. In conclusion; It was determined that kidney tissues were damaged due to systemic inflammatory reactions in rats with OA and boric acid had a protective effect against this damage.

Keywords: *Boric acid, Histopathology, Kidney, Immunohistochemistry, Osteoarthritis.*

ÖZ

Diz Osteoartritli Sıçanların Tedavisinde Kullanılan Borik Asitin Böbrek Dokuları Üzerindeki Etkilerinin Histopatolojik ve İmmünohistokimyasal Olarak Araştırılması

Osteoartrit (OA) sıklıkla diz eklemlerinde ortaya çıkan ve kıkırdak homeostazının bozulması ile karakterize bir hastalıktır. Yarattığı sistemik enflamasyon nedeniyle sadece eklem bölgesini değil, birçok doku ve organı da etkilemektedir. Bu çalışmada, OA'ya bağlı sistemik enflamatuar reaksiyonların böbrek dokusunda oluşturduğu hasar ve borik asidin koruyucu etkisi araştırılmıştır. Çalışmada Wistar albino sıçanlar kullanıldı. Sıçanlarda monosodyum iyodoasetatın (MIA) intraartiküler enjeksiyonu ile deneysel diz OA modeli oluşturuldu. Çalışma; Kontrol, OA, OA + 4 mg Borik Asit, OA + 10 mg Borik Asit olmak üzere 4 gruptan oluşturuldu. Çalışma sonunda sıçanlardan böbrek dokuları alınarak histopatolojik ve immünohistokimyasal yöntemlerle TNF-α, IL-1β, NOS2 ve MMP13 analizleri yapıldı. Histopatolojik incelemelerde OA gruplarında tübüler epitel hücrelerinde şiddetli dejeneratif ve nekrotik değişiklikler görülmüş, bu değişiklikler borik asit uygulanan grupta doza bağlı olarak azalmıştır. İmmünohistokimyasal analizlerde ise OA uygulamasında ortaya çıkan sistemik enflamatuar reaksiyonların borik asit uygulaması ile doza bağımlı bir şekilde azaldığı tespit edildi. Sonuç olarak; OA'li sıçanlarda sistemik enflamatuar reaksiyonlar nedeniyle böbrek dokularının hasar gördüğü ve borik asidin bu hasara karşı koruyucu etkisi olduğu tespit edildi.

Anahtar Kelimeler: *Borik asit, Böbrek, Histopatoloji, İmmünohistokimya, Osteoartrit.*

✉ *Corresponding author: ismail.bolat@atauni.edu.tr

INTRODUCTION

Osteoarthritis (OA) is a joint disease that occurs with the disruption of the balance between tissue destruction and regeneration, resulting in degeneration of cartilage homeostasis. It is known that this type of degeneration around the connective tissue is caused by many various factors, including mechanical stress and biochemical changes (Allen et al. 2022). The articular cartilage covers the ends of the femur and tibia, acting as a cushion and absorbing the physical impact of the joint area. When exposed to mechanical and chemical stress, it wears out and causes severely impaired functional change (Ni et al. 2022).

The pathophysiology of OA includes local inflammation induced mainly by proinflammatory cytokines such as interleukin 1β (IL1-β) and tumor necrosis factor α (TNF-α), and expression of inducible nitric oxide synthase (iNOS) and up regulation of matrix metalloproteinases (MMPs) leads (Goldring and Goldring 2004). For this reason, it is known that degenerative changes develop in many tissues and organs in the body in OA (Selig et al. 2022).

Clinical manifestations of osteoarthritis limit daily activities. Later, it may lead to other disorders such as sleep disturbance, depression, disability and deterioration in quality of life (Allen et al. 2022). Regeneration is not possible in cartilage tissue and this is almost non-existent in the elderly. OA treatment focuses on partial relief of symptoms and pain caused by inflammation, without a definitive solution. The most commonly used drug groups in OA treatment arenon-steroid anti-inflammatory drug (NSAIDs). Analgesic and anti-inflammatory drugs alleviate the clinical symptoms of OA. However, it should not be forgotten that NSAIDs also have different side effects (Selig et al. 2022).

In a worldwide study; While the number was 247.51 million in 1990, it increased to 527.81 million in 2019, an increase of 113.25% (Long et al. 2022).As the estimates of the WHO, 25% of people over the age of 65 experience pain and loss of function due to osteoarthritis. Every age group is affected by this disease, but this number increases at the age of 50 for men and over 40 for women (Bodur et al. 2011). According to the data in Türkiye, it has been reported that the rate of OA patients over the age of 50 is 14.8%, 22.5% in women and 8% in men (Bilge et al. 2018).

Monosodium iodoacetate (MIA) is known to experimentally induce OA symptoms in animals (Fonsi et al. 2019). It contributes to the formation of OA by disrupting energy and cellular metabolism in joints and chondrocytes (Chien et al. 2016). With MIA, inflammatory reactions increase in cartilage tissue and initiate pain by causing significant damage to nerve cells (Kuyinu et al. 2016).

Boron (B) is not found in its basic form in nature and is bound to oxygen, where 98.4% is boric acid and 1.6% is borate (Yildirim et al. 2022). Boric acid (H_3BO_3) dissolved form of boron and is the most common form of this form. Boron is mostly found in tissues and fluids as boric acid. Boric acid is excreted from the kidneys very quickly after being taken into the body. It is kept in the feces with brain, bone, kidney, testes and liver tissue, muscle, prostate, adrenals and body fluids such as plasma, semen, milk, saliva until it is excreted (Dilmani et al. 2023).

It is known that boric acid has a key feature in the body, especially in hormonal balance and mineralisation reactions (Estevez-Fregoso et al. 2023).

In addition, it has been shown that it shows serious protective activity in DNA in cells thanks to its antioxidant activity, especially in oxidative stress cases (Kar et al. 2020).

It has also been shown to have effective anti-inflammatory, anticoagulant, hypolipidemic, antiosteoporotic, and antineoplastic effects in animals both in vitro and in vivo (Haveric et al. 2020). When we look at the field of health, boric acid is used as a boron compound in brain tumors, wound and burn treatments, ointments, mouthwashes, eye drops and lens solutions as an antiseptic (Kuru and Yarat 2017).

Boron is involved in the metabolism of vitamin D and calcium. It has been reported that it is effective in magnesium and calcium metabolism, strengthens the bone framework and reduces pain in arthritis (Söğüt and Acar 2020). Studies have shown that boron has a positive effect on progesterone, estrogen, testosterone, thyroid hormone, steroid and insulin levels (Desordi et al. 2017). Among the tasks of boron at the cellular level; to assist cell membrane functions, to support metabolic activities and the wall structure in organisms with cell walls (Güneş et al. 2017).

Boron increases the level of glutathione in cells and subsequently prevents both oxidative stress and oxidative stress-induced oxidative damage. Boric acid is known to induce inflammatory response by triggering the expression of TNF-α as well as angiogenesis (Perez-Rodriguez et al. 2017).

OA is a disease that has recently become common among people and causes lesions in many different organs, including joints, with the symptoms it causes. Although there are different treatment modalities for the disease, a direct solution is still not available. The aim of this study was to investigate the damage caused by systemic inflammatory reactions in the kidney tissue due to OA and the protective effect of boric acid against this damage.

MATERIAL AND METHODS

Ethics committee approval of this study was obtained from Atatürk University Animal Experiments Local Ethics Committee (Ethical Report: 2023/04).

Wistar albino male rats, weighing 250-300 g, 12-16 weeks old, taken from Atatürk University Experimental Animals Laboratory, were fasted the night before for the experiment, but were given access to water. By applying ketamine (30mg/kg) and 2% xylazine (6mg/kg) intraperitoneally to the rats, 3mg monosodium iodoacetate (MIA) was dissolved in 0.9% NaCl under general anesthesia and injected. Of the rats intra articularly of 50µL at once and knee OA model was created with the application (Gundogdu et al. 2024). 28 rats were divided into 4 groups. The power analysis program was used to determine the number of animals in the group (Figure 1, G-Power 3.1.9.7. program). It was determined that at least 7 rats in each group and at least 28 rats in total were needed to obtain 95% working power (Type II error, β) with 0.05 error (Type I, α). Data from a previous study were used for this analysis (Bolat et al. 2023).

- *Group 1 (Control):* Rats were not treated.
- *Group 2 (OA):* Only knee OA model was created in rats.
- *Group 3 (OA + 4 mg Boric Acid):* Rats were administered oral 4 mg/kg boric acid 4 boric acid orally for 3 weeks after OA formation (Bolat et al. 2023).

- *Group 4 (OA + 10 mg Boric Acid):* Rats were administered oral 10 mg/kg boric acid 4 boric acid orally for 3 weeks after OA formation (Bolat et al. 2023).

After the administrations, the weights of all rats were measured and the rats were given a combination of 30mg/kg ketamine hydrochloride (Ketalar, Parke-Davis, İstanbul, Türkiye) and 6 mg/kg 2% xylazine hydrochlorideintraperitoneally it was sacrificed by decapitation under general anesthesia and was take kidney.

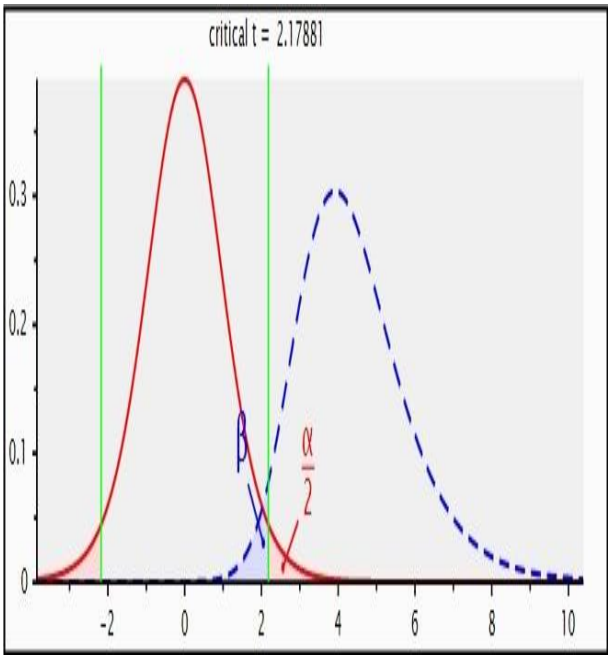

Figure 1: Power analysis graph obtained as a result of the power analysis applied to obtain a statistically significant difference of 14 scores between the groups in Knee Osteoarthritis scoring (Standard deviation±0.5).

Histopathological Examination

Tissues containing 10% buffered formaldehyde and kept for 48-72 hours. After routine tissue follow-up, the tissues were blocked with paraffin and 4 µm thick sections were taken from each block with a microtome device (Leica RM 2255). Afterwards, hematoxylin-eosin staining was performed and histopathological evaluations were made under the light microscope. Necessary areas were imaged with light microscope (OLYMPUS). Kidney tissues were examined histopathologically for mononuclear cell infiltration, hyperemia, and degeneration and necrosis in epithelial cells. Histopathological lesions were evaluated as no (-), mild (+), moderate (++) and severe (+++) (Terim et al. 2013).

Immunohistochemical Staining Method

Primary antibodies were used, (tumor necrosis factor α (TNF-α) Cat No: sc-52746 Dilution ratio: 1/100 US, interleukin 1β (IL1-β) Cat No:sc-52012 Dilution ratio: 1/100 US, Nnitric oxide synthase 2 (NOS2) Cat No: sc-7271 Dilution ratio: 1/100 US, matrix metalloproteinases 13 (MMP) Cat No: sc-101564 Dilution ratio: 1/100 US). Following the IHC kit protocol (ab64264), 3-3' Diaminobenzidine (DAB) chromegene was dropped onto the tissues. Necessary areas were imaged with light microscope (OLYMPUS). According to the immunopositivity status, it was scored as absent (-), mild (+), moderate (++), intense (+++) positivity (Kapakin et al. 2012).

Statistical Analysis

GraphPad Prism 8.0.2 program was used for statistical analysis in histopathological and immunohistochemical examinations and the data were evaluated with $p<0.05$ considered significant. Kolmogorov Smirnov test was applied to determine the normality of distributions in groups.

G*Power analysis was used to determine the number of animals in the group.The non-parametric Kruskal-Wallis test was used to detect group interaction, and the Mann Whitney U test was used to determine differences between groups (Bolat et al. 2023).

RESULTS

Histopathological Findings

*Group 1:*The kidneys were normal (Figure 2A).

Group 2: Severe degeneration and necrosis of renal tubular epithelial cells, severe mononuclear cell infiltration in the intertubular region and severe hyperemia in the veins were detected (Figure 2).

Group 3: Severe degeneration and mild necrosis in renal tubule epithelial cells, moderate mononuclear cell infiltration in the intertubular region and severe hyperemia in the veins were detected (Figure 2).

Group 4: Mild degeneration of renal tubular epithelial cells, mononuclear cell infiltration in the intertubular region and moderate hyperemia in the veins were detected (Figure2). A statistically significant difference ($p<0.05$) was found when compared with group 2. Histopathological findings were showed at Figure 3 and Table 1.

Table 1: Histopathological findings and scoring in kidney tissues (n=7).

	Group 1	Group 2	Group 3	Group 4
Degeneration of epithelium	-	+++	+++	+
Necrosis in epithelium	-	+++	+	-
Mononuclear cell infiltration	-	+++	++	-
Hyperemia in the veins	-	+++	+++	++

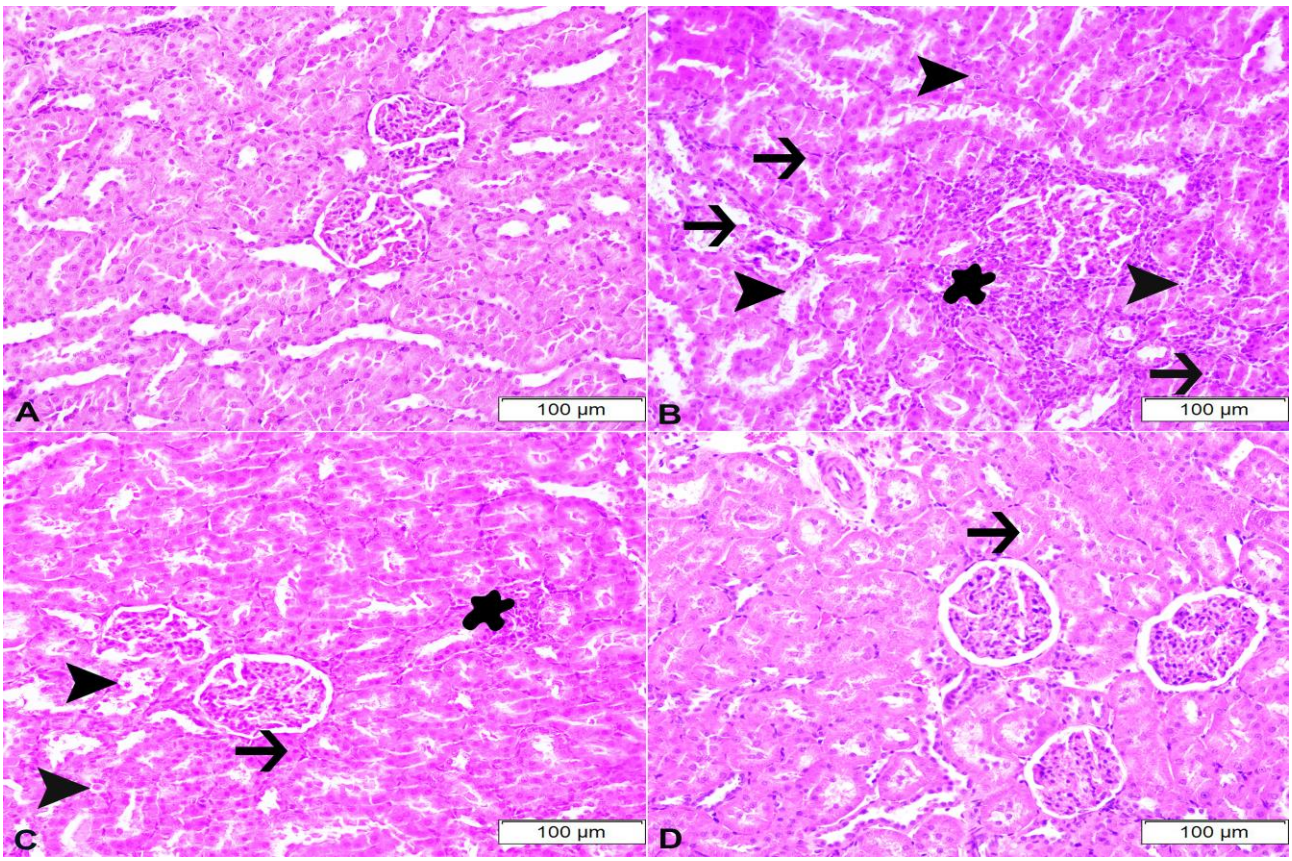

Figure 2: Kidney tissue, Group 1 (A), Group 2 (B), Group 3 (C), and Group 4 (D), renal tubule epithelial degeneration (arrows), necrosis (arrowheads) and inflammation (asteric), hematoxylin-eosin (H&E), Bar: 100µm.

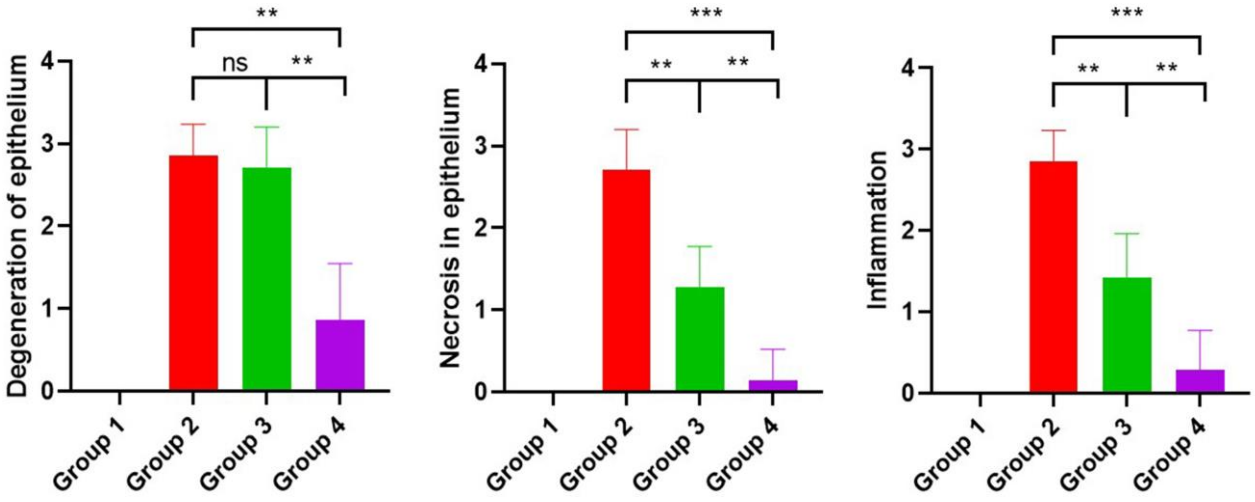

Figure 3: Scoring of histopathological findings in kidney tissue and statistical analysis findings. Degeneration in tubule epithelial cells (Group 2 vs 3 ns p=0.1865, Group 2 vs 4 ** p=0.0012, Group 3 vs 4 ** p=0.0017); Necrosis in tubule epithelial cells (Group 2 vs 3 ** p=0.0035, Group 2 vs 4 *** p=0.0006, Group 3 vs 4 ** p=0.0035); mononuclear cell infiltration (Group 2 vs 3 ** p=0.0023, Group 2 vs 4 *** p=0.0006, Group 3 vs 4 ** p=0.0087). (n=7). (ns: no significance).

Immunohistochemical Findings

Group 1: No TNF-α, IL1-β, MMP13 and NOS2 expression was observed in the kidneys (Figure 4).

Group 2: Severe TNF-α and IL1-β expressions were detected in glomeruli and inflammatory cells, and severe intracytoplasmic MMP13 and NOS2 expressions were seen in renal tubular epithelial cells (Figure 4).

Group 3: Mild TNF-α and moderate IL1-β expressions were detected in glomeruli and inflammatory cells, and moderate MMP13 and NOS2 expressions were identifed in renal tubular epithelial cells (Figure 4).

Group 4: Negative TNF-α and mild IL1-β expressions were detected in glomeruli and inflammatory cells, and mild MMP13 and NOS2 expressions were observed in renal tubular epithelial cells (Figure 4). A statistically significant difference ($p<0.05$) was found when immunohistochemical parameters were compared with Group 2. Immunohistochemical findings are presented in Figure 5 and Table 2.

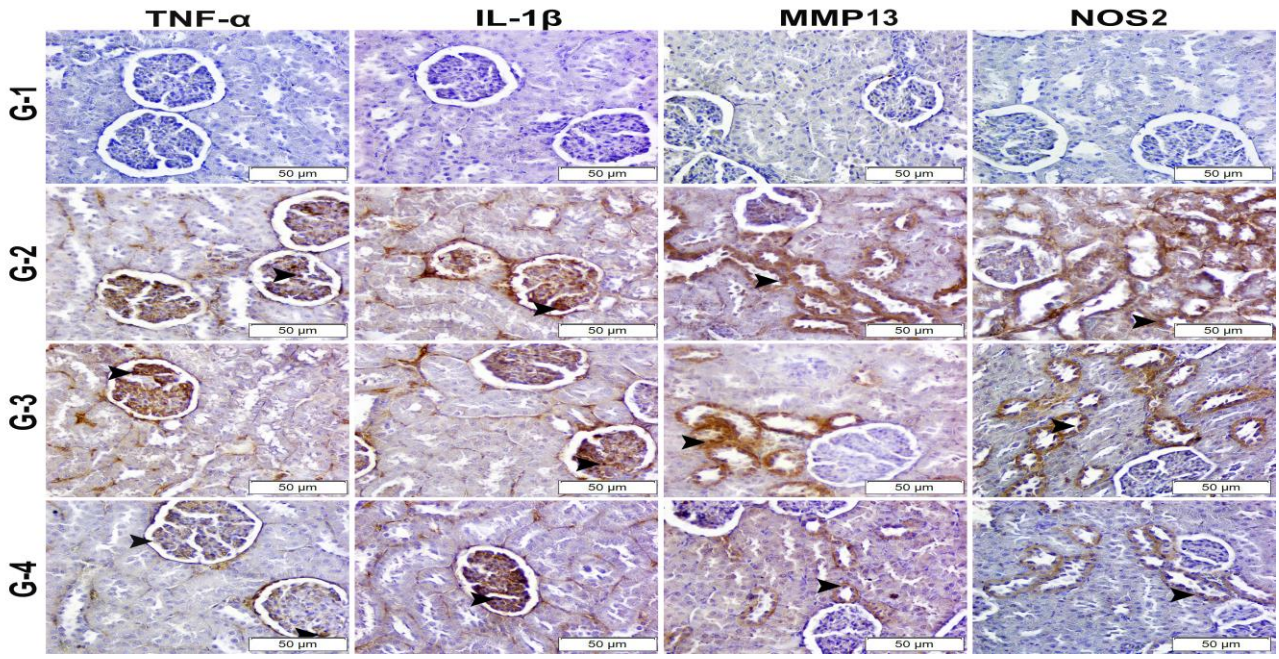

Figure 4: Kidney tissue, TNF-α and IL1-β expressions in glomeruli (arrowhead), MMP13 and NOS2 expressions in tubular epithelial cells (arrowhead), Immunoperoxidase-P (IHC-P), Bar: 50 µm.

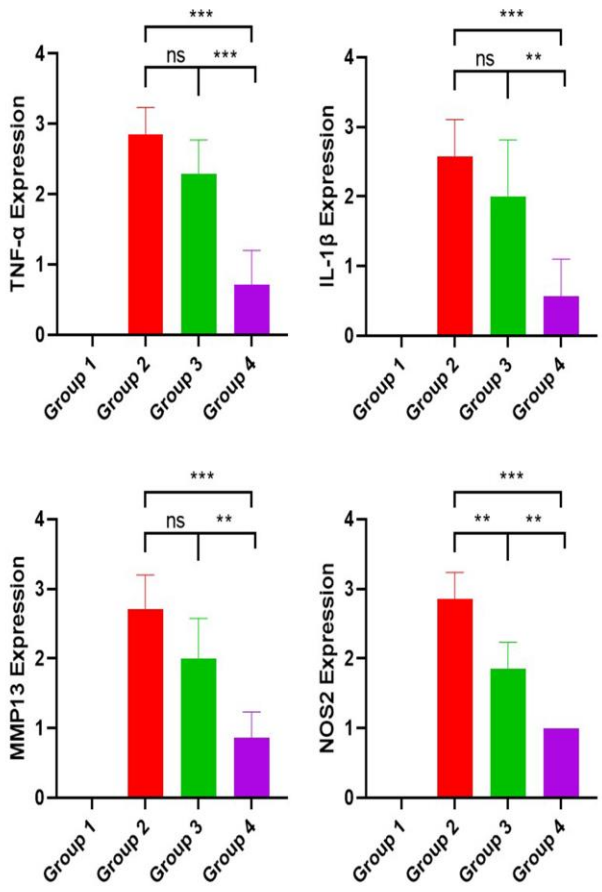

Figure 5: Immunohistochemical analysis results and statistical analysis data in kidney tissue.

TNF-α expression (Group 2 vs 3 ns p=0.1026, Group 2 vs 4 *** p=0.0006, Group 3 vs 4 *** p=0.0006); IL1-β expression (Group 2 vs 3 ns p=0.2739, Group 2 vs 4 *** p=0.0006, Group 3 vs 4 ** p=0.0087); MMP13 expression (Group 2 vs 3 ns p=0.0781, Group 2 vs 4 *** p=0.0006, Group 3 vs 4 ** p=0.0041); NOS2 expression (Group 2 vs 3 ** p=0.0041, Group 2 vs 4 *** p=0.0006, Group 3 vs 4 ** p=0.0047). (n=7). (ns: no significance).

Table 2: Scoring of immunohistochemical findings in kidney tissues (n=7).

	Group 1	Group 2	Group 3	Group 4
TNF-α	-	+++	++	+
IL-1β	-	+++	++	+
MMP13	-	+++	++	+
NOS2	-	+++	++	+

DISCUSSION AND CONCLUSION

Boron is a trace substance that needs oxygen and is widely found in nature. Boron is most abundant in nature in the form of boric acid and then borax. After BA is enter the body, it is absorbed very quickly in the gastrointestinal (GI) tract and enters the circulation. It takes an active role in different physiological and biochemical reactions by showing many protective activities in the body (Pawa and Ali 2006; Khaliq et al. 2018). Boric acid is used in the body, especially in steroid hormonal mechanisms, bone development and cellular mechanisms (King et al. 2015). Boric acid is widely found in vegetables and fruits, in nature especially in nuts, and people have easy access to it (Zhao and Wen et al. 2018).

There is not any study has been found examining the damage caused by the application of osteoarthritis in the kidney tissues. However, there are studies investigating the effects of boric acid against these damages in some studies that have created kidney damage with various substances (Malfait et al. 2016; Güney et al. 2022).

In a study where ethanol caused kidney damage, it was reported that boric acid prevented this damage in both biochemical and histopathological evaluations (Güney et al. 2022). Similarly, histopathologically and immunohistochemically, it has been determined that boric acid has a dose-dependent protective effect on kidney

tissue against the damage that occurs in kidney tissues with OA application.

MMPs induced by IL-1β, IL-6 and TNF-α secreted in the body as a result of OA; They increase cartilage destruction in tissues by activating inflammatory cytokines and chemokines (Malfait et al. 2016).Subsequently, nitric oxide (NO) and inducible nitric oxide synthase (iNOS), which are responsible for cartilage and bone degradation, induce the release of IL-1β in the body and systemic inflammatory reactions occur, causing damage to many tissues and organs (Mongkhon et al. 2014). Boric acid also plays a role in the body response by functioning in the hormonal and cellular mechanism in the body (Sogut et al. 2018). In addition, while BA plays a role in enysmatic activity in the body, it also increases the glutathione level in cells. In addition to its antiapoptotic activity at the cellular level, BA also reduces oxidative damage by suppressing ROS (Ince et al. 2018).

In recent studies; It has been reported that BA suppresses inflammatory reactions via the NF-kB pathway (Durick et al. 2005). Again, it was determined that BA application has an anti-inflammatory effect against inflammation caused by phytohemagglutinin (PHA-P) application (Armstrong et al. 2001). In the experimental study conducted in rats with OA; IL-1β, TNF-α, NOS2 and MMP13 expression levels in kidney tissues were increased by immunohistochemical methods as a result of systemic inflammatory reactions. However, it has been shown that BA significantly protects the kidney tissue against these inflammatory reactions in a dose-dependent manner, and new information has been added to the literature.

As a result, it was demonstrated for the first time that the systemic inflammatory response developed in rats with experimental osteoarthritis caused by MIA caused damage to the kidney tissues and that boric acid applied at different doses had a protective effect against such damage. For this reason, it was concluded that BA application may also be effective in other tissues and organs against the systemic inflammatory response that occurs with OA.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: İB, KATK
Supervision / Consultancy: İB, KATK, EMK
Data Collection and / or Processing: İB, KATK, EMK, ŞTT
Analysis and / or Interpretation: İB, GG, KG, SYT
Writing the Article: İB, KATK, EMK, GG
Critical Review: İB, KATK, FDM

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Van Vet J, 2024, 35 (3) 152-159

Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Eshagi Joganlo O, Keskin O (2024).** Isolation of Lytic *Pseudomonas aeruginosa* Phages and Investigation of Their Lytic Potency. *Van Vet J*, 35 (3), 152-159. DOI: https://doi.org/10.36483/vanvetj.1485075

ISSN: 2149-3359 **Araştırma Makalesi** e-ISSN: 2149-8644

Litik *Pseudomonas aeruginosa* Faj İzolasyonu ve Litik Etki Potansiyelinin Araştırılması

Omid ESHAGİ JOGANLO[1] Oktay KESKİN[1,*]

[1] *Harran Üniversitesi, Veteriner Fakültesi, Mikrobiyoloji Anabilim Dalı, 63200, Şanlıurfa, Türkiye*

Geliş Tarihi: 16.05.2024 Kabul Tarihi: 15.08.2024

ÖZ

Pseudomanas aeruginosa çevrede yaygın olarak bulunur. Genellikle çoklu antibiyotik dirençli ve sahip olduğu virülans faktörleri nedeniyle insan ve hayvanlarda tedavisi zor enfeksiyonlara neden olan önemli bir fırsatçı patojendir. Çoklu antibiyotik direncine sahip bakterilerin neden olduğu enfeksiyonların tedavisinde fajlar önem kazanmıştır. Bu çalışmada, *P. aeruginosa* için litik etkiye sahip faj/fajların izolasyonu ve bu fajların farklı *P. aeruginosa* izolatları için litik etkilerinin belirlenmesi amaçlandı. Bu amaçla kanalizasyon örnekleri, gübre örnekleri gibi fajların bulunabileceği kaynaklardan 3 farklı faj izolasyonu yapıldı ve bunların farklı *P. aeruginosa* izolatlarına litik etkileri araştırıldı. Bunun için Harran Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı Kültür Koleksiyonunda bulunan çeşitli klinik örneklerden ya da çevresel örneklerden izole edilmiş 40 *P. aeruginosa* izolatı kullanıldı. Bu konak bakterilerin çoklu antibiyotik direncine sahip oldukları belirlendi. Konak bakteriler penisilin, tylosin, oksitetrasiklin ve eritromisin için %100, ampilisin için %90, streptomisin ve amoksisilin/klavulonik asit için %87.5, sefkuinom için %85, doksisklin için %77.5, azithromisin için %1.5 ve gentamisin için %12.5 oranında dirençli olarak saptanırken, izolatların tamamı enrofloksasine duyarlı bulundu. Çalışmada izole edilerek kodlanan 3 fajdan PAFO fajı 25 (%62.5), PAFA fajı 35 (%87.5) ve PAFS fajı ise 30 izolat (%75) üzerinde lizis oluşturuken, her üç fajın da litik etki gösterdiği 10 izolat (%25) belirlendi. Sonuç olarak, *P. aeruginosa*'ya karşı litik bakteriyofajlar, özellikle antibiyotik direnci ve enfeksiyon kontrolü gibi zorluklarla başa çıkma potansiyeline sahip, spesifik ve etkili bir tedavi seçeneği olabilir. Bu nedenle çalışmada izole edilen fajların detaylı karakterizasyonlarının yapılması, tedavi ya da çevresel dekontaminasyon uygulamaları için ticari ürün haline dönüştürülme potansiyellerinin belirlenmesinin yararlı olacağı kanısına varıldı.

Anahtar Kelimeler: *Çoklu antibiyotik direnci, Litik bakteriyofaj, Pseudomonas aeruginosa, Tek Sağlık.*

ABSTRACT

Isolation of Lytic *Pseudomonas aeruginosa* Phages and Investigation of Their Lytic Potency

Pseudomanas aeruginosa is commonly found in the environment. It is an important opportunistic pathogen that causes difficult-to-treat infections in humans and animals due to its multiple antibiotic resistance and virulence factors. Treatment with phages has gained importance due to antibiotic resistance. In this study, it was aimed to isolate phage(s) with lytic effect for *P. aeruginosa* and to determine the lytic effects of these phages on different *P. aeruginosa* isolates. For this purpose, 3 different phages were isolated from sources where phages are likely to be found such as sewage samples, manure samples and the lytic effects of these phages on different *P. aeruginosa* isolates were investigated. For this purpose, 40 *P. aeruginosa* isolated from various clinical or environmental samples in the Culture Collection of Harran University, Faculty of Veterinary Medicine, Department of Microbiology were used. These host bacteria were considered to have multidrug resistance. The host bacteria were 100% resistant to penicillin, tylosin, oxytetracycline and erythromycin, 90% resistant to ampicillin, 87.5% resistant to streptomycin and amoxicillin/clavulonic acid, 85% resistant to cefquinom, 77.5% resistant to doxycycline, 1.5% resistant to azithromycin and 12.5% resistant to gentamicin, while all isolates were sensitive to enrofloxacin. Among the 3 phages isolated and coded in the study, PAFO phage caused lysis on 25 isolates (62.5%), PAFA phage caused lysis on 35 isolates (87.5%) and PAFS phage caused lysis on 30 isolates (75%), while 10 isolates (25%) in which all three phages showed lytic effect were determined. In conclusion, lytic bacteriophages against *P. aeruginosa* may be a specific and effective therapeutic option with the potential to tackle challenges such as antibiotic resistance and infection control. Therefore, it would be useful to further characterize the phages obtained in this study and determine their potential for commercialization for therapeutic or environmental contamination applications.

Keywords: *Lytic bacteriophage, Multidrug resistance, One Health, Pseudomonas aeruginosa.*

✉ *Corresponding author: okeskin@harran.edu.tr

GİRİŞ

Pseudomonas cinsi bakterilerin çoğu doğada toprak ve sularda yoğun olarak bulunmaktadır. *Pseudomonas* cinsinde 200'den fazla tür olmakla birlikte bunlardan bazıları saprofitik olup, bir kısmı da insan, hayvan ya da bitki patojeni olarak bilinmektedir (UKHSA 2024).

P. aeruginosa sağlıklı hayvanların dışkısında ve derisinde hayatta kalabilir ve fırsatçı karakteri nedeniyle hayvanın bağışıklık sistemini baskılayan veya normal florayı bozan tüm predispozan faktörler enfeksiyona neden olabilmektedir. Bu etken birçok hayvan türünde sporadik enfeksiyonlara neden olur ve fırsatçı patojen olarak sığır, koyun, keçi, domuz, köpek, kedi ve sürüngenlerde çeşitli hastalıklara neden olmaktadır. Etken, süt ineklerinde ekonomik kayıplara neden olan mastitis, solunum ve intestinal sistemde oluşan sistemik enfeksiyonlara, atlarda endometrit gibi genital sistem hastalıklarına, koyunlarda yeşil yün hastalığına, köpeklerde otitis ve idrar yolu enfeksiyonlarına yol açarken, vizon veya tilki gibi kürklü hayvanlarda ise hemorajik pnömoni gibi hastalıkların oluşumunda rol oynar (Schauer ve ark. 2021).

İnsanlarda hastane enfeksiyonlarına neden olan mikroorganizmalar arasında, büyük bir tehdit oluşturan çoklu ilaç direncine sahip *P. aeruginosa* öne çıkmaktadır (Harada ve ark. 2018). *P. aeuroginosa*, yoğun bakım ünitelerine kabul edilen hastalarda şiddetli alt solunum yolu enfeksiyonları ile ilişkili en baskın tür ve ventilatörle ilişkili pnömoni ile ilişkili ikinci en yaygın patojen olarak kabul edilmektedir (Sievert ve ark. 2013). *P. aeruginosa*, alt ve üst solunum yolu enfeksiyonunda en yaygın bakteriyel patojenlerden biridir, ayrıca kistik fibrozis (KF) hastalarında özellikle önemlidir (Fong ve ark. 2017). *P. aeruginosa* zamanla mevcut antibiyotiklere karşı giderek daha dirençli hale gelmiş ve hastane kaynaklı enfeksiyonlardan sorumlu ESKAPE olarak bilinen en tehlikeli altı (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* ve *Enterobacter* species) patojenden biri olarak kabul edilmektedir (Santajit ve Indrawattana 2016). Son zamanlarda hastane ortamlarında kolay yayılım sağladığı ve dirençli suşların giderek arttığı gözlenmekte ve en sık neden olduğu enfeksiyonlar arasında ise pnömoni, deri ve yumuşak doku enfeksiyonları, cerrahi müdahaleler sonucu oluşan osteomiyelit ve septik artrit, gastrointestinal sistem enfeksiyonları, göz ve kulak enfeksiyonları ile üriner sistem enfeksiyonları yer almaktadır (Kerr ve Snelling 2009).

Klinik uygulamadaki en büyük zorluklardan biri de çoklu ilaç direncine sahip izolatlardır. Bunlar, *P. aeruginosa*'nın da ciddi enfeksiyonlar oluşturması sebebiyle yer aldığı ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* ve *Enterobacter* türleri) olarak bilinen öncelikli patojenlerdir. Çoklu ilaç direnci, küresel halk sağlığına yönelik en büyük üç tehdit arasındadır ve genellikle aşırı ilaç kullanımı veya antimikrobiyallerin reçetesiz ya da uygunsuz kullanımından kaynaklanmaktadır (Sievert ve ark. 2013).

P. aeruginosa izolatlarında pek çok antibiyotiğe karşı direnç geliştiği görülmektedir. Bu nedenle antibiyotiklerin etkisiz kaldığı vakalarda bir tedavi seçeneği olarak bakterileri parçalayan viruslar olan baktariyofajların kullanılmasına dair çalışmalar hızla artmaktadır. Bu çalışmada, *P. aeruginosa* için litik etkiye sahip faj/fajların izolasyonu ve bu fajların farklı *P. aeruginosa* izolatları için litik etkilerinin belirlenmesi amaçlanmıştır.

MATERYAL VE METOT

Bu araştırma faaliyetinin etik kurul denetimine tabi olmadığı Harran Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'nun 08/11/2021 tarih ve 2021/008-04 nolu kararında belirtilmiştir.

Bakteri Suşları

Bu çalışmada Harran Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı Kültür Koleksiyonu'nda bulunan ve 31'i (10 mastitli süt, 6 Otitis externa ve 15 apse) hayvanlardaki klinik vakalardan, 6'sı sığır gübresinden, 3'ü de atık sulardan izole edilen toplam 40 *Pseudomonas aeruginosa* izolatı ve pozitif kontrol olarak *P. aeruginosa* ATCC 9027, negatif kontrol olarak da *Staphylococcus aureus* ATCC 29213 referans suşu kullanıldı.

Besiyerleri

Çalışmada kültür koleksiyonunda saklanan konak bakterilerin canlandırılması ve üretilmesi için Tryptic Soy Agar (TSA) ve Triptik Soy Broth (TSB), Faj izolasyonu için yapılan ekimlerde ise Luria Bertani Broth, (LB broth) ve Luria Bertani Agar (LBA), Kirby-Bauer disk difüzyon testi için Müller Hinton agar kullanıldı (HiMedia, Hindistan).

Faj İzolasyonunda Kullanılan Materyaller

Bu amaçla etkenin bulunabileceği ortamlar olarak değerlendirilen çiftliklerden toprak, atık sular, gübre örnekleri, lağım suları gibi çevresel örnekler 50 ml steril kaplara alınarak kullanıldı.

P. aeruginosa Suşlarının Pasajı ve Bakteriyel DNA İzolasyonu

Derin dondurucuda saklanan izolatları canlandırmak için Tryptic Soy Agar (TSA) besiyerine ekim yapıldı ve 24 saat 37 °C inkübe edildi. Besiyerinde üreyen koloniler Gram boyama ile boyanarak saflık yönünden kontrol edildi. Daha sonra DNA ekstraksiyonu için 1.5 ml'lik ependorfta 250µl distile su içinde bir öze dolusu kültür süspanse edildi. Hücrelerin parçalanması ve DNA'nın açığa çıkması için, örnekler 10 dakika kaynatıldı ve ardından 14.000 devirde 10 dakika santrifüj edildi. Supernatant, bakteri DNA kaynağı olarak kullanıldı.

Polimeraz Zincir Reaksiyonu (PCR)

P. aeruginosa izolatlarının doğrulanması Spilker ve ark. (2004) tarafından bildirilen primerler ve protokol kullanılarak gerçekleştirildi (Tablo 1). PCR reaksiyonu 25 µl total hacimde ve 16.65 µl steril distile su, 3 µl $MgCl^{+2}$, 2.5 µl PCR Buffer, herbir primerden 0.4 µl (100 pmol/µl stok), 0.2 µl dNTP miks (10 mM), 0.25 µl Taq polimeraz, 2 µl bakteriyel DNA olacak şekilde hazırlandı. Karışıma 95 °C'de 2 dakika başlangıç denatürasyonunu takiben, toplam 40 döngü olacak şekilde 94 °C'de 20 s denatürasyon, 58 °C'de 20 s primer bağlanması, 72 °C'de 40 s zincir uzama aşaması ve 72 °C 1 dk son zincir uzama aşaması uygulandı.

PCR Ürünlerinin Görüntülenmesi

Elde edilen PCR ürünlerinin görüntülenmesi için 10 µl DNA 2 µl 6× yükleme solüsyonu ile karıştırılarak 5 µg/ml etidyum bromid içeren %1.5 agaroz jele yüklenerek elektroforez gerçekleştirildi. Marker olarak 100 bp DNA Ladder (Genaid, İngiltere) kullanıldı. *P. aeruginosa* ATCC 9027 pozitif kontrol ve *Staphylococcus aureus* ATCC 29213 referans suşlarının DNA'ları negatif kontrol olarak kullanıldı.

Tablo 1: *P. aeruginosa*'nın moleküler olarak doğrulanması için kullanılan primerler (Spilker ve ark. 2004).

Table 1: Primers used for molecular confirmation of *P. aeruginosa* (Spilker et al. 2004).

Primer	Sequence (5'-3')	Location	Amplikon büyüklüğü size (bp)
PA-SS-F	GGGGGATCTTCGGACCTCA	189-206	956
PA-SS-R	TCCTTAGAGTGCCCACCCG	1124-1144	

Antibiyotik Duyarlılıklarının Belirlenmesi

Antimikrobiyal duyarlılık testi, Klinik ve Laboratuvar Standartları Enstitüsü (CLSI) kriterlerine (CLSI 2022) uygun olarak disk difüzyon yöntemiyle yapıldı. Fajlar için konak olarak kullanılacak 40 adet *P. aeruginosa* izolatının antibiyotik duyarlılıklarını belirlemek için penisilin G (P, 10 µg), streptomisin (S, 10 µg), azitromisin (AZM, 15 µg), amoksisilin/klavulonik asit (AMC, 20/10 µg), doksisiklin (DO, 30 µg), tilosin (TY, 15 µg), oksitetrasiklin (T, 30 µg), sefkuinom (CEQ, 30 µg), eritromisin (E, 15 µg), ampisilin (AM, 10 µg)), enrofloksasin (ENR, 5 µg) ve gentamisin (CN, 10 µg) içeren ticari (Oxoid, İngiltere) diskler kullanıldı.

Bakteriyofaj İzolasyonu

Çevresel örneklerden (lağım suları, gübre) alınan örnek süspansiyonlar 10.000×g devirde 10 dakika santrifüj edildikten sonra süpernatant 0,22 µm por çapına sahip filtreden süzülürek, başka steril bir tüpe akatarıldı. Bu filtratttan daha sonra 100 µl 2 mM $CaCl_2$ içeren 10 ml LB broth içine konularak ve üzerine log fazındaki konakçı bakterinin kültüründen 100 µl eklenerek karışım 50 rpm devirde 37 °C de 24-48 saat çalkalanarak inkübe edildi. Bu sürenin sonunda kültür 10.000×g devirde 10 dakika santrifüj edilerek üstteki süpernatant ayrı bir steril tüpe alınarak üzerine 0.5 ml kloroform eklendi ve daha sonra test bakterilerinin duyarlılığının belirlenmesinde (spot testinde) kullanıldı. Bu amaçla konak olarak kullanılan izolatların logaritmik fazdaki sıvı kültüründen TSA'a 100 µl yayma ekim metodu ile ekim yapılarak üzerine 10 µl faj filtratı damlatılarak 37 °C'de 24 saat inkübasyona bırakıldı. Litik faj aktivitesine sahip filtratlar 4 °C'de saklandı.

İzole Edilen Bakteriyofajların Çift Tabaka Agar Yöntemi ile Saflaştırılması

Elde edilen steril bakteriyofaj solüsyonunda bulunması muhtemel farklı fajların saflaştırılması amacı ile solüsyonun 10^{-10}'a kadar 10 katlı dilüsyonları gerçekleştirildi. Bu amaçla çift tabakalı agar yöntemi kullanıldı. Dilüsyon tüplerinden alınan 0,1 ml bakteriyofaj solusyonu, 100 µl log fazındaki konakçı bakteri süspansiyonu ile 4 ml yumuşak (%0.7) LBA (ortalama 48 °C'de bulunan) karıştırılarak alttaki LBA yüzeyinin üzerine eşit oranda döküldü. Steril pastör pipeti ile tek plak oluşumu görülen petrilerden bakteriyofaj plakları kesilerek TSB içerisine aktarıldı. Kesilen bakteriyofaj plakları sıvı besiyerinde karıştırılarak bakteriyofajların besiyerine geçmesi sağlandı. Elde edilen bakteriyofaj süspansiyonu yine on katlı olarak dilüye edilerek çift tabaka agar yöntemi uygulanarak 18 saat 37 °C'de inkübe edildi. Bu işlem üçkez tekrar edilerek saf bakteriyofaj eldesi yapıldı. Bu solusyon pürifiye olmuş saf bakteriyofaj stoku olarak -80 °C de kullanılıncaya kadar küçük taksimatlar halinde saklandı.

Bakteriyofajların Çoğaltılması

Litik olan ve saflaştırılan fajların çalışmada kullanılan 40 izolat üzerine olan litik etkilerini belirlemek için üç farklı faj çoğaltılarak titreleri yükseltildi. Bu amaçla konak logaritmik fazdaki sıvı kültüründen TSA'ya 100 µl ekilerek petrinin tüm yüzeyine yayıldı. Yüzey kuruduktan sonra steril faj filtratından 200 µl agar yüzeyine yayıldı. 37 °C'de 24 saat inkübasyonun ardından üzerinde litik alanlar bulunan petriler steril FTS ile toplanıp, aynı işlem en az 3 pasaj devam ederek fajların çoğaltılması sağlandı. Son olarak FTS içinde toplanan faj ve bakteri karışımları santrifüj edildikten sonra 0.22 µm filtreden geçirilerek elde edilen steril bakteriyofaj solüsyonlarının titreleri belirlendi.

Bakteriyofaj Titresinin Belirlenmesi

Sıvı kültürlerdeki bakteriyofaj titrelerinin belirlenmesi, çift katmanlı agar yöntemi ile belirlendi. İlk olarak bakteriyofaj suşları sterile saline–magnesium–gelatine buffer (SGM) solüsyonunda 10^{-1}'den 10^{-10} oranına kadar dilüe edildi. Sonra 100 µl faj dilüsyonu ve konak bakteri süspansiyonundan (~10^8 cfu/ml) 100 µl alındı ve 4 ml soft agar (%0.4) ile karıştırılarak LBA ($CaCl_2$ ilave edilmiş) içeren petriler üzerine döküldü. Besiyeri katılaştıktan sonra 37 °C'de gece boyunca inkübe edildi. Bir gecelik inkübasyonun ardından bakteriyofaj plak oluşturma birimi (PFU/mL) belirlendi.

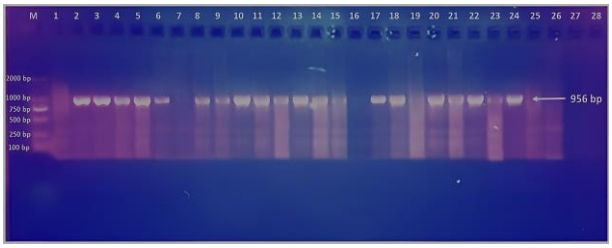
Bakteriyofajların Farklı İzolatlarda Litik Etkilerinin Belirlenmesi

İzole edilmiş bakteriyofajların litik aktivitesi, bakteriyofaj süspansiyonundan 10-20 µl "spot test" yöntemi kullanılarak değerlendirildi. Bakteriyofajların litik aktiviteleri, faj damlası uygulamasından sonra LB agar üzerinde oluşan plakların şeffaflığına göre belirlendi.

BULGULAR

PCR Bulguları

İncelenen izolatlardan PCR ile *P. aeruginosa* olduğu doğrulanan 40 izolat çalışmada kullanıldı (Şekil 1).



Şekil 1: PCR sonucu elde edilen bantların agaroz jel elektroforezde görüntüsü.

Figure 1: Agarose gel electrophoresis of PCR products.

M: DNA ladder, 1: Negatif Kontrol, 2: Pozitif Kontrol, 3-6, 8-15, 17, 18, 20-24 *P. aeruginosa*; 7, 16, 19, 25 ve 26 *Pseudomonas* spp. İzolatları.

İzolatların Antibiyotik Duyarlılıklarının Belirlenmesi

İzole edilen bakteriyofajların litik etkilerinin araştırılmasında konak bakteri olarak kullanılacak 40 adet *P. aeruginosa* izolatının antibiyotik duyarlılıkları (Tablo 2) ve duyarlılık/dirençlilik oranlar (Tablo 3) tablolarda verildi. On iki antibiyotiğe karşı duyarlılıkları test edilen konak bakterilerin tamamı 6-10 etken maddeye dirençli bulunduğu için konak bakteri olarak kullanılan izolatların tamamı (40/40) çoklu antibiyotik direncine sahip (MDR) izolatlar olarak değerlendirildi (Şekil 2).

Bakteriyofajların İzolasyonu

Bakteriyofaj izolasyon çalışmaları sonucunda spot testinde pozitif reaksiyon veren litik fajlar izole edildi. Elde edilen steril bakteriyofaj solüsyonunda bulunması muhtemel farklı fajların saflaştırılması amacı ile 10 katlı dilüsyonları gerçekleştirilen fajların 10^{-7} dilüsyonunda sayılabilecek faj plakları oluştu (Şekil 3). Buradan farklı görünümdeki plaklardan seçilerek saflaştırıldıktan sonra PAFA, PAFO ve PAFS olarak adlandırılan 3 farklı bakteriyofaj elde edildi (Şekil 4).

Bakteriyofajların Çoğaltılması ve Titrelerinin Belirlenmesi

Saflaştırılan her üç fajın da çoğaltıldığında 3×10^{10} PFU/ml aralığında faj titresine sahip olduğu belirlendi. Sulandırılan faj stoklarının her bir sulandırmasından yapılan ekimler sonucunda her bir agar tabakası üzerindeki plaklar sayılarak ve faj titresi hesaplandı.

Bakteriyofajların Farklı *Pseudomonas* Suşlarına Olan Litik Etkilerinin Belirlenmesi

P. aeruginosa olduğu doğrulanan toplam 40 adet izolat, elde edilen PAFA, PAFO ve PAFS kodlu bakteriyofajlarla test edilerek bu suşlara olan litik etkileri belirlenmiştir (Tablo 2; Şekil 5).

Elde edilen sonuçlara göre PAFO fajı 25 izolat (%62.5), PAFA fajı 35 izolat (% 87.5) ve PAFS fajı 30 izolat (%75) için litik etki oluştururken, her üç fajın da litik etki gösterdiği 10 izolat (%25) belirlendi.

Tablo 2: *P. aeruginosa* izolatlarının test edilen antibiyotiklere ve izole edilem bakteriyofajlara duyarlılıkları.

Table 2: Susceptibility of *P. aeruginosa* isolates to tested antibiotics and isolated bacteriophages.

Suş No	Antibiyotikler												Litik etki		
	P	S	AZM	AMC	DO	TY	T	CEQ	E	AM	ENR	CN	PAFO	PAFA	PAFS
1											S	S		+	+
2			S								S	S	+	+	+
3			S					S			S	S	+		+
4			S								S	S	+	+	
5		S	S		S					S	S			+	+
6			S								S	S	+	+	+
7		S			S					S	S	S	+	+	+
8			S	S	S						S	S		+	+
9			S								S	S		+	+
10			S								S	S	+	+	
11			S								S	S	+	+	+
12		S	S		S			S			S	S	+	+	
13		S			S			S			S			+	+
14		S	S								S	S		+	+
15			S	S	S			S			S	S	+	+	
16			S								S	S	+	+	
17			S								S	S	+	+	
18										S	S	S	+	+	+
19			S								S	S	+	+	
20		S	S					S		S	S			+	+
21			S								S	S	+		+
22		S	S								S	S		+	+
23			S								S	S		+	+
24			S								S	S	+	+	+
25			S					S			S	S	+	+	
26			S								S	S	+	+	+
27			S								S		+	+	
28			S	S	S						S	S		+	+
29			S								S	S	+		+
30											S	S	+		+
31			S								S	S		+	+
32			S								S	S	+	+	
33			S								S	S		+	+
34			S	S	S						S	S	+	+	+
35			S								S	S	+	+	+
36											S	S	+	+	+
37			S	S	S						S			+	+
38											S	S		+	+
39			S								S	S	+		+
40			S								S	S		+	+
Toplam	**0**	**5**	**33**	**5**	**9**	**0**	**0**	**6**	**0**	**4**	**40**	**35**			

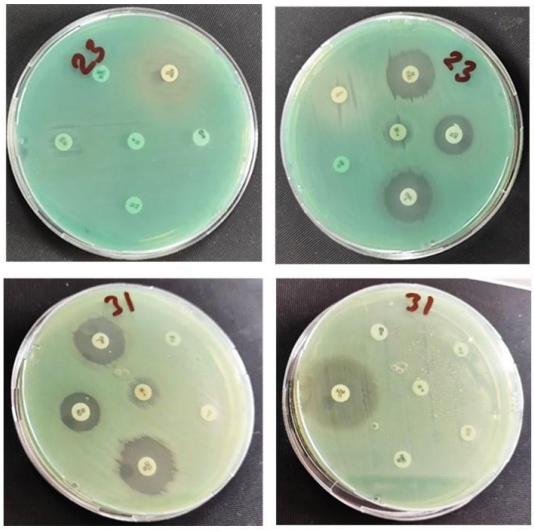
Antibiyotik duyarlılıkları açısından S: Duyarlı, Boşluk: Dirençli P: penisilin G, S: streptomisin, AZM: azitromisin, AMC: amoksisilin/klavulonik asit, DO: doksisiklin, TY: tilosin, T: oksitetrasiklin, CEQ: sefkuinom, E: eritromisin, AM: ampisilin, ENR: enrofloksasin, CN: gentamisin. Faj litik etkisi açısından + : Lizis var Boşluk: Lizis yok.

Tablo 3: *P. aeruginosa* izolatlarının disk difüzyon tekniği ile belirlenen duyarlılık/dirençlilik oranları.

Table 3: Susceptibility/resistance rates of *P. aeruginosa* isolates determined by disk diffusion technique.

Antibiyotik*	Duyarlı izolat n (%)	Dirençli izolat n (%)
P	0 (0)	40 (100)
S	5 (12.5)	35 (87.5)
AZM	33 (82.5)	7 (17.5)
AMC	5 (12.5)	35 (87.5)
DO	9 (22.5)	31 (77.5)
TY	0 (0)	40 (100)
T	0 (0)	40 (100)
CEQ	6 (15)	34 (85)
E	0 (0)	40 (100)
AM	4 (10)	36 (90)
ENR	40 (100)	0 (0)
CN	35 (87.5)	5 (12.5)

*P: penisilin G, S: streptomisin, AZM: azitromisin, AMC: amoksisilin/klavulonik asit, DO: doksisiklin, TY: tilosin, T: oksitetrasiklin, CEQ: sefkuinom, E: eritromisin, AM: ampisilin, ENR: enrofloksasin, CN: gentamisin.

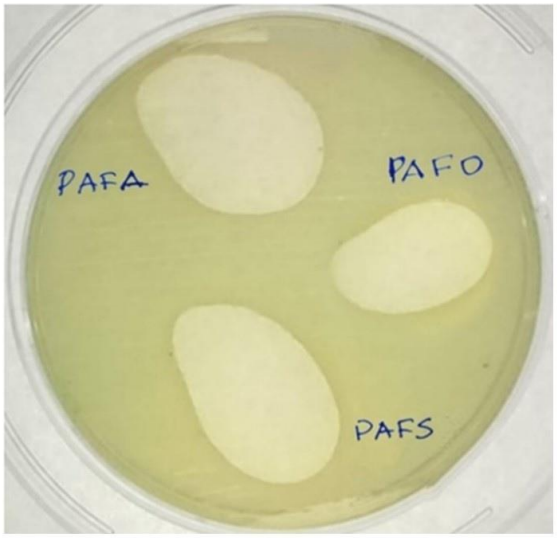


Şekil 2: Disk difüzyon tekniği ile yapılan antibiyogram testi sonuçları.

Figure 2: Antibiogram test results by disk diffusion technique.

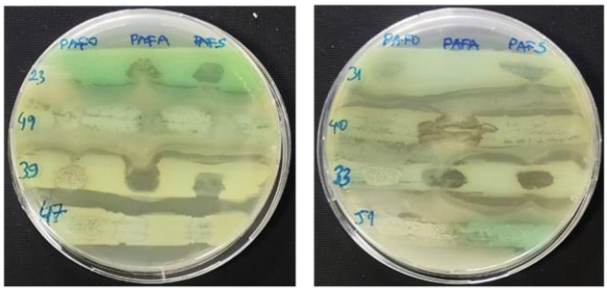
Şekil 3: Çift tabaka agar yöntemindeki faj plaklarının görünümü (PAFS).

Figure 3: View of phage plaques in the double layer agar method.



Şekil 4: İzole edilen üç fajın litik etkisi (Konak bakteri 29 numaralı *P. aeruginosa* izolatı).

Figure 4: Lytic effect of three isolated phages (Host bacterium *P. aeruginosa* isolate 29).



Şekil 5: Test edilen her üç fajın bazı konak *P. aeruginosa* izolatlarına litik etkileri.

Figure 5: Lytic effects of all three phages tested on some host *P. aeruginosa* isolates.

TARTIŞMA VE SONUÇ

P. aeruginosa farklı hayvan türlerinde çeşitli enfeksiyonlara neden fırsatçı bir patojendir. *P. aeruginosa* hayvanların deri ve sindirim sisteminde yaygın olarak bulunduğu için bağışıklık sisteminin baskılandığı durumlarda fırsatçı enfeksiyonlara neden olmaktadır.

P. aeruginosa'ya bağlı olarak köpeklerde otitis eksterna, sığırlarda mastitis ve abortus, korneal ülser, atlarda metritis, koyunlarda yapağı çürüklüğü ve kanatlı hayvanlarda embriyonal ölümleri ortaya çıkmaktadır (Arda ve ark. 1997).

P. aeruginosa, birden fazla mekanizma yoluyla, genellikle aynı anda direnç oluşturma konusunda olağanüstü bir yeteneğe sahiptir ve bu da neredeyse mevcut tüm antibiyotiklere karşı dirençle sonuçlanmaktadır (Harada ve ark. 2012). Abbas ve ark. (2022) yaptıkları çalışmada 24 *P. aeruginosa* izolatının 22'sini (%91.66) MDR olarak belirlemişlerdir. Araştırıcılar bu 22 MDR izolatının 19'unda (%86.36) biyofilm üretimini pozitif olurak saptarken hepsinde biyofilm kodlayan gen (*psl*A) varlığını da göstermişlerdir. Pseudomonaslarda görülen çoklu antibiyotik direnci gerek insanlarda (Gül ve ark. 2004; Çufalı 2011; Deredjian ve ark. 2011; Gomes ve ark. 2011; Örüklü 2011; Öner ve ark. 2022) gerekse hayvanlarda (Aslantaş ve ark. 2022; Ünal 2005; Keskin ve ark. 2012; Şahin 2015; Zeyrek 2019; Eliasi ve ark. 2020; Abdou ve ark. 2021) pek çok araştırmacı tarafından rapor edilmiştir.

Gül ve ark (2004) klinik örneklerde elde edilen 71 *P. aeruginosa* izolatında seftazidime duyarlılığını araştırdıkları çalışmada disk difüzyon tekniği ile %42,3, E-test yöntemi ile de %50.7 oranında direnç tespit etmişlerdir. Çufalı (2011) klinik örneklerden elde edilen 128 *P. aeruginosa* izolatın 13 antibyotiğe karşı duyarllıklarını değerlendirdiği çalışmasında bu antibiyotiklere %10.1-95.9 arasına değişen dirençlilik saptamıştır. Deredjian ve ark. (2011) yapmış oldukları çalışmada hastane suşlarının %60'nın 3'ten 16'ya kadar antibiyotik olacak şekilde çoklu direnç fenotipi gösterdiini rapor etmişlerdir. Öner ve ark. (2022) Ocak 2017-Aralık 2021 tarihleri arasında beş yıllık süreçte *P. aeruginosa* izolatlarında saptanan direnç durumun değerlendirmişlerdir. Bu süreçte klinik örneklerden izole edilen 2876 *P. aeruginosa* izolatı incelenmiştir. İzolatlarda en düşük direnç oranları amikasine %3, gentamisine %6, en yüksek direnç oranları ise seftazidime %21 ve imipeneme %19 olarak belirlenmiş ve direnç durumunda yıllara göre anlamlı bir farklılık olduğunu bildirmişlerdir.

Ünal (2005) farklı kaynaklarda elde edilen *P. aeruginosa* izolatlarının tamamını (%100) enrofloksasin ve gentamisine duyarlı, karbenisiline orta derecede duyarlı bulurken ampisilin, aztronam, piperasilin, sefatoksim, tetrasiklin, eritromisin, streptomisin, kanamisin ve trimeyhoprim/sülfamethaksosol içi %100 direnç saptamışlardır. Keskin ve ark. (2012) bir süt çiftliğinde buzağılarda görülen yüksek ölümler nedeniyle incelen örnekten izole edilen *P. aeruginosa* suşunda ampisilin, amoksisilin, amokisilin/klavulanik asit, sefoksitin, siprofloksasin, eritromisin, gentamisin, norfloksasin, oksasillin, penisilin, rifampin, streptomisin, tetrasiklin, trimetoprim-sulfametoksazol ve vankomisine direnç belirlerken sadece imipenem duyarlı bulunmuştur. Şahin (2015) klinik mastisli inek sütlerinden elde edilen *P. aeruginosa* izolatları için danofloksasin, linkomisin/neomisin, sefaperazon ve kolistin sülfat için duyarlılık oranı %100 olarak bildirirken pensilin/novobiosin, amoksisilin/klavulonik asit, kanamsinin/sefaloksin ve eritromisine %100 direnç belirlemişler. Araştırıcılar florfenikol için %29, trimethoprim/sülfamethaksosol için %29 ve oksitetrasiklin için de %71 oranında direnç belirlediklerini rapor etmişlerdir. Zeyrek (2019) köpeklerin burun boşluğundan izole edilen *P. aeruginosa* suşlarında amoksisilin/klavulonik asit, ampisilin/sulbaktam, imipenem, kloksasilin ve penisilin/novobiosin için %100 direnç oranı bildirirken kanamisin/sefaleksin %64 olarak bildirilmiştir. Araştırıcılar oksitetrasiklin için %55, enrofloksasin ve gentamisin içinse %100 duyarlılık belirlemişlerdir. Eliasi ve ark. (2020) Güney Afrika'da bir veteriner hastanesinden temin edilen *P. aeruginosa* izolatlarında 19 antibiyotik için disk difüzyon tekniği ile duyarlılıkları araştırmışlar ve linkomisin için %98, penisilin-G için %96, amoksisilin/klavulonik asit için %93, amoksisilin/ampisilin, karbenisilin ve tylosin için %92, linkomisin/spektinomisin, orbifloksasin ve sefalotin için %90, kloramfenikol ve kanamisin için %89, doksisklin için %87, piperasilin için %86, seftisidim için %77, enrofloksasin için %73, gentamisin için %18, amikasin için %16, tobramisin için %12 ve imipenem için %6 dirençlilik saptamışlardır. Abdou ve ark. (2021) ördeklerden izole edilen 20 adet *P. aeruginosa* suşunda eritromisin, oksitetrasiklin, ampicilin ve amoksisilin için %95, tylosin için %90, doksisiklin ve sefotaksim için %85, norfloksasin için %55, enrofloksasin için %50, siprofloksasin için %45 ve florfenikol için %20 oranında direnç saptamışlardır. Aslantaş ve ark. (2022) klinik mastitisli sığır sütlerinden izole 44 suşun disk difizyon tekniği ile gentamisin, tobramisin, amikasin, piperasilin/tazobaktam, aztreonam, meropenem, imipenem, siprofloksasin, sefipim, piperasilin ve seftazidim duyarlılıklarını araştırmışlardır. Çalışmacılar izolatların çoğunu (%72.7) incelenen tüm antimikrobiyallere duyarlı bulduklarını, 8 izolatı siprofloksasine, bir izolatı gentamisin ve siprofloksasine, bir izolatı meropenem ve imipeneme, bir izolatı meropenem, imipenem ve siprofloksasine, bir izolatın da gentamisin, tobramisin, amikasin, siprofloksasin ve seftazidime dirençli bulunduğunu, bu sonuçlara göre siproflaksasin için direnç oranının %25, karbepenemler için %4.5 olarak hesaplandığını rapor etmişlerdir. Verilen bu araştırmaların tamamında sonuçlar MDR olarak değerlendirilmiştir. Sunulan bu çalışmada da konak olarak kullanılan *P. aeruginosa* izolatlarının tamamı üç ve üçten fazla antiyotiğe dirençli olduğundan çoklu dirence (MDR) sahip oldukları değerlendirilmiş olup araştırıcıların sonuçları ile uyumludur. Bu çalışmada test edilen 12 antibiyotik için dirençlilik oranları penisilin, tylosin, oksitetrasiklin ve eritromisin için %100, ampilisin için %90, streptomisin ve amoksisilin/klavulonik asit için %87.5, sefkuinom için %85, doksisklin için %77.5, azithromisin için %1.5 ve gentamisin için %12.5 olarak saptanırken, izolatların tamamı enrofloksasine duyarlı bulunmuştur. Elde edilen bu direnç oranları araştırıcıların bulguları ile genel olarak benzer bulunsa da bazı antibiyotikler için bildirilen direnç oranlarıyla uyumsuzluğun farklı coğrafik alanlardan izole edilen etkenlerin test edilmesi ya da zamana bağlı olarak kazanılmış direnç oranlarının artması ile ilişkili olabileceği değerlendirilmektedir.

Antibiyotiklerin yaygın kullanımı ve antibiyotik direncinin sürekli artması nedeniyle bakteriyofajlar, *P. aeruginosa*'nın neden olduğu enfeksiyonların tedavisinde etkili alternatifler gibi görünmektedir (Pires ve ark. 2015; Litwin ve ark. 2021). Veteriner hekimlikte, evcil hayvanlarda deri enfeksiyonlarıyla savaşmak için bakteriyofaj tedavisine ilişkin çalışmalar sınırlıdır, ancak bakteriyofajların *P. aeruginosa*'nın antibiyotiğe dirençli enfeksiyonunun tedavisinde potansiyele sahip olduğu kanıtlanmıştır (Furusawa ve ark. 2016).

Günümüzde fajlar insan ve veteriner hekimliğinde yeniden kullanım alanına kavuşmakta ve fajlara dayalı yeni ilaçlar geliştirilmektedir. Her ikisi de bakteriyel hastalıklara karşı ana tedavi ajanları olduğundan genellikle antibiyotiklerle karşılaştırılırlar. Verimlilikleri, diğer farmasötiklerle

uyumluluğu, doğal kökenleri ve alerjiye veya bağımlılığa neden olduğunun bilinmemesi gibi çeşitli yönlerden ifade edilmektedir (Sulakvelidze ve ark. 2001).

Bazı klinik çalışmalar, hasta insan (Wright ve ark. 2009) ve köpeklerde (Hawkins ve ark. 2010) enfeksiyöz otitis tedavisinde bakteriyofajların kullanımına yönelik cesaret verici sonuçlar sağlamıştır. Diğer çalışmalar, hayvanlarda deneysel olarak oluşturulan sistemik enfeksiyonların tedavisinde, özellikle biyofilmlere karşı bakteriyofajların faydalarını bildirmiştir (Ferriol-Gonzáles ve Domingo-Calap 2020).

P. aeruginosa enfeksiyonlarını kontrol etmek için β-laktamlar (penisilinler, karbapenemler, sefalosporinler, monobaktamlar), aminoglikozitler ve fluorokinolonlar dahil olmak üzere geleneksel olarak kullanılan antibiyotiklere karşı oldukça fazla dirençli suşlar bulunmaktadır. Bu bulgular izole fajın etkinliğini ve faj terapisinde uygulamaya uygunluğunu desteklemektedir. Geniş konakçı aralığına sahip fajların etkili biyokontrol olarak kabul edildiği ve faj terapisinde terapötik uygulama için daha çok tercih edildiği iyi bilinmektedir (Fernández ve ark. 2019).

Faj tedavisi, yaygın olarak kullanılan antimikrobiyallere yanıt vermeyen hastalar için umut verici bir tedavi seçeneğidir. Belirli salgın suşları hedef alabilen yeni fajların izolasyonu, fajların klinik uygulaması için esastır. *P. aeruginosa*, hastane kaynaklı enfeksiyonların önde gelen nedenidir ve antibiyotiklere karşı güçlü bir doğal direnç gösterir. Bu nedenle faj tedavisi, hastane kaynaklı enfeksiyonların önde gelen nedeni olan ve antibiyotiklere karşı güçlü doğal direnç gösteren kronik *P. aeruginosa* enfeksiyonlarının tedavisinde geçerli bir alternatiftir (Berube ve ark. 2016).

Bir bakteriyofajın litik spektrumu, potansiyel uygulamalarını belirleyen en önemli biyolojik özelliklerden biri olarak kabul edilmektedir. Bir fajın litik spektrumu, enfekte edebileceği bakteri cinsleri, türleri ve suşlarının aralığı ile tanımlanmaktadır. Bu biyolojik özellik faj biyokontrol uygulamalarında kritik öneme sahiptir çünkü fajın belirli bakteri türlerini hedefleme yeteneğini belirler (Kutter 2009).

Sharma ve ark. (2021) atık sulardan *P. aeroginasa* için pekçok alanda uygulama potansiyeline sahip litik bakteriyofaj izole ettiklerini bildirmişlerdir. Ghaffar ve ark. (2023) atık sulardan *P. aeruginosa*'ya karşı izole ettikleri yüksek litik etkiye sahip bir fajla yaptıkları çalışmada fajın konakta *P. aeruginosa* kolonizasyonunu ve patogenezini azalttığını, bu fajın *P. aeruginosa*'nın neden olduğu enfeksiyonları sınırlamak için faj kokteyli terapisi olarak diğer litik fajlarla veya antibiyotiklerle kombine olarak uygulanabileceğini belirtmişlerdir. Elgawish ve ark (2023) yapmış oldukları çalışmada hastane atık sularından izole ettikleri 3 farklı fajın antibiyotik tedavisine alternatif olabilecek faj terapisi için potansiyeli olan litik etkiye sahip olduklarını belirtmişlerdir. Bu çalışmada litik etki gösteren 3 faj elde edildi 31'i klinik örneklerden (10 apse, 6 otitis externa, 15 mastitisli süt), 6'sı çevresel kaynaktan (gübre) ve 3'ü de atık suların izole edilmiş toplam 40 *P. aeruginosa* izolatı için litik etkileri araştırıcıların bulguları ile uyumlu olarak değerlendirildi. Sahada özellikle lokalize enfeksiyonların tedavisinde *P. aeruginosa* litik fajlarının başarıyla kullanıldığı yönünde pek çok araştırma bulunmaktadır (Hawkins ve ark., 2010; Santos ve ark., 2011; Khairnar ve ark., 2013; Fujiki ve ark., 2020; Grecu ve ark., 2023). Bu çalışmada elde edilen fajların litik etki oranları da klinik kullanım açısından umut verici olmakla beraber, tedavi amacıyla kullanım potansiyellerinin belirlenmesi için adsorbsiyon süresi, latent süre, çoğalma oranı, adsorbsiyon oranı gibi bakterifaj dinamiklerinin belirlenmesi gerekmektedir. Ayrıca bu fajların virülens ya da antibiyotik direnç genleri taşıyıp taşımadıklarının saptanması da kullanım potansiyellerini belirleyici olacaktır.

P. aeruginosa'nın zoonotik potansiyeli, bu bakterinin kontrolünde ve insan sağlığını etkileme riskini azaltmada, veteriner sağlık, halk sağlığı ve çevre sağlığı alanları arasında iş birliği yapılmasını gerektiren bir konu olarak değerlendirilmelidir. Tek Sağlık Konsepti yaklaşımı, bu tür karmaşık sorunlara bütünsel ve etkili çözümler geliştirmek adına önemli bir çerçeve sunar.

Sonuç olarak, *P. aeruginosa*'ya karşı litik bakteriyofajlar, özellikle antibiyotik direnci ve enfeksiyon kontrolü gibi zorluklarla başa çıkma potansiyeline sahip, spesifik ve etkili bir tedavi seçeneği sunabilir. Bundan sonraki çalışmalarda, çalışmada elde edilen fajların daha detaylı karakterizasyonlarının yapılması, tedavi amacıyla ya da çevresel dekontaminasyon uygulamaları için ticari ürün haline dönüştürülme potansiyellerinin belirlenmesinin gerektiği kanısına varılmıştır.

ÇIKAR ÇATIŞMASI

Yazarlar bu çalışma için herhangi bir çıkar çatışması olmadığını beyan ederler.

TEŞEKKÜR VE BİLGİLENDİRME

Bu araştırma Harran Üniversitesi Bilimsel Araştırma Projeleri Koordinatörlüğü tarafından 21283 proje numarası ile desteklenmiştir.

Bu çalışma Omid ESHAGİ JOGANLO isimli yazarın yüksek lisans tezinden özetlenmiştir.

YAZAR KATKILARI

Fikir/Kavram: OK
Denetleme/Danışmanlık: OK
Veri Toplama ve/veya İşleme: OEJ, OK
Analiz ve/veya Yorum: OEJ, OK
Makalenin Yazımı: OEJ, OK
Eleştirel İnceleme: OK

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Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Bozukluhan K, Merhan O, Akyüz E et al. (2024).** Determination of Haptoglobin, Serum Amyloid A, Some Other Acute Phase Proteins, and Biochemical Parameters in Cattle with Hydatid Cysts. *Van Vet J*, 35 (3), 160-163.
DOI: https://doi.org/10.36483/vanvetj.1488935

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

Determination of Haptoglobin, Serum Amyloid A, Some Other Acute Phase Proteins, and Biochemical Parameters in Cattle with Hydatid Cysts

Kadir BOZUKLUHAN[1,*] Oğuz MERHAN[2] Enes AKYÜZ[1] Şemistan KIZILTEPE[3] Uğur AYDIN[4] Tahir GEZER[1] Derya KOCAMAZ[5] Gürbüz GÖKÇE[1]

[1] *University of Kafkas, Department of Internal Medicine, Faculty of Veterinary Medicine, 36100, Kars, Türkiye*
[2] *University of Kafkas, Department of Biochemistry, Faculty of Veterinary Medicine, 36100, Kars, Türkiye*
[3] *University of Iğdır, Tuzluca Vocational High School, 76000, Iğdır, Türkiye*
[4] *University of Kafkas, Department of Surgery, Faculty of Veterinary Medicine, 36100, Kars, Türkiye*
[5] *University of Cukurova, Department of Biology, Faculty of Science and Arts, 01010, Adana, Türkiye*

Received: 23.05.2024 Accepted: 13.09.2024

ABSTRACT The aim of this study is to determine some acute phase proteins and biochemical parameter levels in cattle infected with the hydatid cysts. In the study, a total of 30, Brown Swiss cattle including 15 infected with the hydatid cysts in the study group and 15 in the control group were used. Haptoglobin, serum amyloid A (SAA), ceruloplasmin, interleukin (IL)-6, total protein, albumin, aspartate amino transferase (AST), gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP) activity, urea, creatinine, iron (Fe) levels were determined colorimetrically. When the animals infected with hydatid cysts and control group were compared, it was determined that haptoglobin, SAA, IL-6, AST, ALP, GGT activity, urea, creatinine levels and acute phase protein index values increased, while albumin levels decreased. In addition, it was determined that the ceruloplasmin and total protein levels increased, globulin, albumin/globulin and Fe levels decreased, but they were statistically insignificant. In conclusion, it was determined that there were significant changes in the biochemical parameters and increased haptoglobin and SAA synthesis in the animals infected with the hydatid cysts, and it is thought that these parameters may contribute to the pathogenesis and diagnosis of the disease.

Keywords: *Haptoglobin, Hydatid cysts, Cattle, Serum amyloid A.*

ÖZ

Hidatik Kistli Sığırlarda Haptoglobin, Serum Amiloid A ve Diğer Bazı Akut Faz Proteinleri ile Biyokimyasal Parametrelerin Belirlenmesi

Çalışmada amaç hidatik kist ile enfekte sığırlarda bazı akut faz proteinleri ve biyokimyasal parametre düzeylerinin belirlenmesidir. Çalışmada çalışma grubunda hidatik kist ile enfekte 15, kontrol grubunda 15 olmak üzere toplam 30 adet Montofon sığır kullanıldı. Haptoglobin, serum amiloid A (SAA), seruloplazmin, interlökin (IL)-6, total protein, albümin, aspartat amino transferaz (AST), gama glutamil transferaz (GGT) ve alkalin fosfataz (ALP) aktivitesi, üre, kreatinin, demir (Fe) düzeyleri kolorimetrik olarak belirlendi. Hidatik kist ile enfekte hayvanlar ile kontrol grubu karşılaştırıldığında, haptoglobin, SAA, IL-6, AST, ALP, GGT aktivitesi, üre, kreatinin düzeyleri ve akut faz protein indeksi değerlerinin arttığı, albümin düzeylerinin ise azaldığı belirlendi. Ayrıca seruloplazmin ve total protein düzeylerinin arttığı, globulin, albümin/globulin ve Fe düzeylerinin ise azaldığı ancak istatistiksel olarak anlamlı olmadığı belirlendi. Sonuç olarak hidatik kist ile enfekte hayvanlarda biyokimyasal parametrelerde önemli değişiklikler, haptoglobin ve SAA sentezinde artış olduğu belirlenmiş olup bu parametrelerin hastalığın patogenezine ve tanısına katkı sağlayabileceği düşünülmektedir.

Anahtar Kelimeler: *Haptoglobin, Hidatik kistler, Sığır, Serum amiloid A.*

✉ *Corresponding author: kbozukluhan@hotmail.com

INTRODUCTION

Hydatid cyst is a parasitic zoonotic disease-causing economic loss as a result of expulsion of cystic organs such as liver and lung above all including decrease in meat and milk yield, decrease in wool quality, and increase in infertility rate in farm animals (Balkaya and Şimşek 2010; Acıöz 2018). While the final host of the disease agent Echinococcus granulosus is domestic and wild carnivores such as cats, dogs, foxes and jackals; its intermediate hosts are ruminants, other mammals, and humans (Moro and Schantz 2009; Avcıoğlu 2013). Oncosphere ingested by the host with water and contaminated feed or food forms cysts of various volumes by blood to the liver, lymph lungs, spleen, heart or brain by being released in the intestine (Avcıoğlu 2013; Regassa 2019). The symptoms vary according to the organ where the cysts are located, the number and size of the cysts. The cysts placing in the liver cause jaundice, enlargement of the liver, and ascites and the cysts placing in the respiratory system cause cough, dyspnea, wheezing, and polypnea (Avcıoğlu 2013). Imaging techniques and serological tests are used in order to diagnose the disease in the intermediate host (Gökce et al. 2009; Regassa 2019).

The infectious and non-infectious factors such as inflammation, tissue damage, infection, and toxin cause acute phase response (APR) in the organism. The acute phase proteins (APP), which are nonspecific inflammation markers, are synthesized from the liver as a result of the APR (Abdulkhaleq et al. 2018; Iliev and Geoergiva 2018). Although blood concentration and its importance vary according to the animal species, haptoglobin and serum amyloid A (SAA) are the APPs that have diagnostic importance for cattle. Levels of the APPs are proportional to the extent of the tissue damage and the severity of the disease. In the studies, it revealed that the APPs may be used in differentiation between the bacterial and viral infections, in the differential diagnosis of clinical and subclinical diseases, in parasitic diseases, in the follow-up of treatment efficacy, and in determining the prognosis of sick animals (Tothova et al. 2014). Cytokines are important in the host immunity and their levels may vary depending on the parasite's genus, host type, organ in which it is located and metabolic products (Bayraktar et al. 2005; Abdulkhaleq et al. 2018). While interleukin (IL)-4, IL-6 and IL-10 secreted by T helper (Th)2 lymphocytes are associated with susceptibility to disease in hydatid cysts, IL-2 and interferon gamma secreted by Th1 cells are associated with protective immunity (Rigano et al. 2004). APR occurs depending on the cellular and humoral response in the tissues or organs where the hydatid cyst is located, and ultimately causes changes in APP synthesis in the liver. Therefore, our aim in this study is to determine some APPs (haptoglobin, SAA and ceruloplasmin) and some biochemical parameter levels in cattle infected with the hydatid cysts.

MATERIAL AND METHODS

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (Approval no: 2021/11).

Animals

In the study, a total of 30, 3-4 years old, Brown Swiss cattle including 15 infected with the hydatid cysts in the study group and 15 in the control group were used. The animals brought to the Department of Internal Medicine, Faculty of Veterinary Medicine, Kafkas University, were clinically examined and diagnosed with hydatid cyst. After slaughter, the diagnosis was confirmed. The control group consisted of clinically healthy animals with the same care and feeding conditions.

Samples

The blood samples from vena jugularis of the animals were taken into tubes without anticoagulant. The samples taken into the tubes without anticoagulant were centrifuged at 3000 rpm for 15 minutes. The serum samples were stored at -20 °C until the analysis would be made.

Determination of Acute Phase Proteins and Biochemical Parameters

Ceruloplasmin levels were determined spectrophotometric method, developed by Colombo and Ricterich (1964), while haptoglobin levels were determined by methods of Skinner et al. (1991). While the SAA and IL-6 level were determined by using the ELISA kit (Tridelta development limited, Ireland; BT Lab, China, respectively), aspartate amino transferase (AST), gamma glutamyl transferase (GGT), and alkaline phosphatase (ALP) activity, urea, creatinine, total protein and albumin levels were found colorimetrically by using the commercial test kit (Biolabo, France). The APP index=[Positive APP (g/dL)/Negative APP (g/dL)]x106 was calculated by using the APP data obtained from the analyzes (Martinez-Subiela and Ceron 2005).

Statistical Analysis

SPSS software program (Version 20, Chicago, Illinois, USA) was used to analyze the data. Since the groups were normally distributed as a result of the Kolmogorov-Smirnov normality test, the student-T test was used to compare the groups. In statistical evaluation, $p<0.05$ value was considered statistically significant.

RESULTS

In the anamnesis, it was learned that various antibiotics were administered to the animals for many times, but no results were obtained from the treatments applied. It was determined that there was atony in the rumen, dyspnea, cough, shallow and rapid respiration (32/min), and tachycardia (92/min) in the heart by the clinical examination. In addition, wheezing of the lung sounds and diffuse dullness in the lung percussion area were detected in the auscultation examination made. Many cysts showing well-circumscribed opacity were detected in the lungs on the radiographic examination made. When the animals infected with hydatid cysts and control group were compared; It was determined that haptoglobin, SAA, IL-6, AST, ALP, GGT activity, urea and creatinine levels ($p=0.001$) increased, while albumin levels ($p=0.021$) decreased. Also, it was determined that ceruloplasmin and total protein levels increased, globulin, albumin/globulin and iron (Fe) levels decreased, but they were statistically insignificant (Table 1). In addition, the haptoglobin/albumin ($p=0.001$) and SAA/albumin ($p=0.010$) index values calculated in the study were found to be higher than the control group (Table 2).

Table 1: Levels of acute phase protein and biochemical parameters in clinically healthy and cattle infected with hydatid cysts.

Parameters	Control ±SEM	Infected with hydatid cysts ±SEM	p
Haptoglobin (g/L)	0.091±0.004	0.155±0.015	0.001
Serum Amyloid A (µg/mL)	16.25±1.23	21.73±1.08	0.002
Ceruloplasmin (mg/dL)	13.56±1.11	17.85±1.82	0.540
Interleukin-6 (pg/mL)	61.94±6.39	165.68±7.08	0.001
Total Protein (mg/dL)	7.02±0.25	6.71±0.16	0.310
Albumin (mg/dL)	3.21±0.13	2.86±0.06	0.021
Globulin (mg/dL)	3.81±0.29	3.85±0.18	0.911
Albumin/Globulin rate	0.99±0.18	0.78±0.05	0.257
AST (U/L)	42.54±2.17	69.49±2.20	0.001
GGT (U/L)	27.41±1.21	49.79±2.84	0.001
ALP (U/L)	28.46±1.16	57.64±3.76	0.001
Urea (mmol/L)	7.24±0.21	9.77±0.54	0.001
Creatinine (µmol/L)	77.03±2.66	134.47±4.24	0.001
Iron (µg/dL)	101.72±3.13	92.66±5.00	0.136

AST: Aspartate Amino Transferase, GGT: Gamma Glutamyl Transferase, ALP: Alkaline Phosphatase

Table 2: Acute phase protein index values of hydatid cysts and healthy cattle.

Parameters	Control ±SEM	Infected with hydatid cysts ±SEM	p
Haptoglobin/ Albumin (g/dL)	2893.54±145.02	5409.61±484.87	0.001
Serum Amyloid A/Albumin (g/dL)	522.38±49.44	765.15±43.05	0.010

DISCUSSION AND CONCLUSION

Symptoms such as cough, dyspnea, wheezing and rapid breathing, tachycardia in cattle infected with hydatid cyst were determined as reported in the studies (Avcıoğlu 2013). The cytokines and other inflammatory mediators are secreted in the hydatid cysts disease depending on the cellular and humoral immune response in the organism because cysts fluid and germinative membrane have antigenic properties in the cysts formed in the intermediate host (Haniloo et al. 2008; Avcıoğlu 2013). It was reported in the studies conducted in the human medicine that the hydatid cysts cause an increase in the cytokine level (Rigano et al. 2004; Bayraktar et al. 2005). In veterinary medicine, a single study was conducted, and it was reported that IL-6 levels increased in cattle infected with hydatid cysts when the infected group was compared with the control group (Sevimli et al. 2015). In the study, it was determined that the IL-6 level increased in the cattle infected with the hydatid cysts. Probably, the reason for the increase may be due to the immunological response against the parasite. The increase in cytokine level depending on the cellular and humoral immune response in the organism causes the APP synthesis in the liver.

Haptoglobin, SAA, and ceruloplasmine are APPs with diagnostic importance for cattle. Haptoglobin, which is very low in the serum of healthy cattle, can increase up to 100 times following inflammation and infection (Pradeep 2014). Studies have shown that in many bacterial (Bozukluhan et al. 2018a; Bozukluhan et al. 2021; Kırbaş et al. 2021), viral (Bozukluhan et al. 2018b; Merhan et al. 2021), parasitic diseases (Bozukluhan et al. 2017; Merhan et al. 2017), and dystocia (Bayyit and Merhan 2020) the level of infection varies according to the type and prevalence. The APPs are nonspecific markers of the tissue damage, and the increase in the haptoglobin level reflects the severity of the infection (Eckersall and Bell 2010). Skinner et al. (1991) defined a serum haptoglobin level in the range of 0.2-0.4 g/L as mild, and between 1-2 g/L as severe infection. In the studies, it was reported that the haptoglobin, SAA and ceruloplasmin levels increased in the mixed-infected goats (Ulutaş et al. 2008), Dictyocaulus viviparus (Ganheim et al. 2004), Cryptosporidium parvum (Enemark et al. 2003), cattle infected with Babesia bigemina (Mohammadi et al. 2021), cattle with hypodermosis (Merhan et al. 2017) and calves infected with Toxocara vitulorum (Bozukluhan et al. 2017). Omidi et al. (2017) reported that there was no significant change in haptoglobin, SAA and albumin levels in a study they conducted in cattle with hydatid cysts. Also, Sevimli et al. (2015) in another study they conducted in cattle with hydatid cysts, they reported that there was an increase in SAA, but the haptoglobin level decreased. In the study, it was determined that haptoglobin and SAA levels increased in cattle infected with hydatid cyst compared to the control group. Skinner et al. (1991) reported that the haptoglobin level was approximately 0.2 g/L, indicating that the severity of the disease is mild. Probably, the reason for this increase may be the tissue destruction in the organs depending on the size of the cysts.

Albumin, which is a negative APP, has functions such as binding and transporting many organic and inorganic molecules (bilirubin, penicillin and calcium, etc.), acting as a source for amino acids, and maintaining the continuity of plasma pressure. It is synthesized by the liver. Its destruction occurs mostly in the kidneys. It was reported that its concentration decreases in liver diseases, anorexia, tissue damage and inflammatory conditions, kidney and intestinal diseases (Tennant and Center 2008). In the study, the albumin level decreased, and it was thought that this may be due to increased albumin catabolism because of inflammation and tissue damage due to infection, or dysfunction due to tissue damage in the liver.

The acute phase protein index is calculated using positive and negative APPs. It has been reported that the calculated index value varies according to the severity and process of the inflammation, and that these changes can be used in the diagnosis of diseases and in the follow-up of healing processes (Martinez-Subiela and Ceron 2005). The index value calculated in the study also increases in relation to the APP concentration, and it is thought that APPs and index values can be used to distinguish between sick and healthy cattle.

Biochemical changes in the blood are used in the diagnosis of many diseases. Studies have reported that parasitic infections cause significant changes in blood parameters and host biochemistry of animals (Ayaz et al. 2006; Irak et al. 2019). Parasitic infections cause damage to the liver tissue and cause changes in the parameters used in the evaluation of hepatic functions such as AST, ALP, GGT and total protein (Sahin and Akgul 2006; Tennant and Center 2008). AST and GGT activity are used to determine liver parenchymal damage, and ALP is used to determine cholestasis (Hoffmann and Solter 2008; Comba et al. 2017). Irak et al. (2019) reported that there was no statistical difference between the groups in terms of GGT, ALT, triglyceride and cholesterol levels in their study in sheep infected with hydatid cyst, but the difference was significant in terms of total protein, globulin and AST activity. In addition, Cinar et al. (2018) reported a significant increase in AST and ALP activity and a decrease in total protein level in another study they conducted in

sheep with hydatid cysts. It is thought that the reason for the increase in AST, ALP and GGT activities in the study may be due to the physiopathological changes in the liver.

Urea and creatinine, which are important parameters in the evaluation of renal functions, are affected by increased protein catabolism in case of infection, as well as the loss of perfusion in the kidneys due to systemic inflammation in infections, resulting in impaired nutrition and functions of the kidneys (Gokce and Woldehiwet 1999; Tennant and Center 2008; Aral 2015). In the study, the reason for the increase in serum urea and creatinine levels may be due to the increase in the rate of protein catabolism or the effect of kidney functions because of systemic inflammation due to the disease.

It was determined that there were significant changes in the biochemical parameters and increased haptoglobin and SAA synthesis in the animals infected with the hydatid cysts, and it is thought that these parameters may contribute to the pathogenesis and diagnosis of the disease.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: KB, OM
Supervision / Consultancy: GG
Data Collection and / or Processing: ŞK, UA, EA
Analysis and / or Interpretation: TG, DK
Writing the Article: KB, OM
Critical Review: KB, OM, GG

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Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Kızıltepe Ş, Ayvazoğlu C, Yaşar Ü et al. (2024).** Determination of Cardiac Troponin Levels in Dogs with Babesiosis. *Van Vet J*, 35 (3), 164-167. DOI: https://doi.org/10.36483/vanvetj.1403752

ISSN: 2149-3359 **Araştırma Makalesi** e-ISSN: 2149-8644

Babesiosis'li Köpeklerde Kardiyak Troponin Seviyelerinin Belirlenmesi

Şemistan KIZILTEPE[1,*] Cemalettin AYVAZOĞLU[2] Ümit YAŞAR[2] Zehra GÜL YAŞAR[2] Nilgün AYDIN[3] Gencay Taşkın TAŞÇI[3]

[1] *Iğdır Üniversitesi, Tuzluca Meslek Yüksekokulu, 76900, Iğdır, Türkiye*
[2] *Ardahan Üniversitesi, Nihat Delibalta Göle Meslek Yüksekokulu, 75700, Ardahan, Türkiye*
[3] *Kafkas Üniversitesi, Veteriner Fakültesi, Parazitoloji Anabilim Dalı, 36300, Kars, Türkiye*

Geliş Tarihi: 27.06.2024 Kabul Tarihi: 23.10.2024

ÖZ

Köpeklerde kardiyak biyobelirteçler, kardiyak hastalıkların erken teşhisi, prognozu veya tedavisinin izlenmesinde büyük öneme sahiptir. Yüksek hassasiyetli kardiyak troponinler (hs-cTn'ler), miyokardiyal hasarın hassas göstergeleri olarak kabul edilir. Bu çalışma *Babesia canis (B. canis)*'le doğal enfekte köpeklerde yüksek hassasiyetli kardiyak troponin I (hs-cTnI) ve T (hs-cTnT) seviyelerinin belirlenmesi amacıyla yapılmıştır. Çalışmanın materyalini; Iğdır Üniversitesi Hayvan Hastanesine getirilen ve Kafkas Üniversitesi Parazitoloji laboratuvarı tarafından PCR analizi ile *B. canis* teşhisi konulan 3-4 yaş aralığındaki 15 hasta köpek çalışma grubunu oluşturdu. Kontrol grubu için aynı yaş aralığında sağlıklı 10 adet köpek seçildi. Hasta ve sağlıklı olan köpeklerden *V. cephalica*'dan serum tüplerine (BD Vacutainer, BD, Franklin Lakes, NJ) 5'er mL kan alınarak serumları ayrıştırıldı. Bu serumlar -20 °C muhafaza edilerek 30 gün süre içerisinde analizleri yapılarak hs-cTnI ve hs-cTnT seviyeleri belirlendi. Çalışmamızda *B. canis*'li köpeklerin hs-cTnI ve hs-cTnT seviyelerinin kontrol grubuna göre önemli derecede yüksek olduğu belirlendi (sırasıyla; $p<0.01$; $p<0.05$). Sonuç olarak, babesiosisli köpeklerde hs-cTnT ve hs-cTnI seviyelerinde istatistiksel olarak anlamlı bir artış olduğu tespit edilmiştir. Serum hs-cTnI ve hs-cTnT seviyelerindeki küçük değişimlerin bile prognostik açıdan önemi olduğundan *B. canis*'in köpeklerde miyokardiyal hasara neden olduğu söylenebilir. Ancak her geçen gün kullanımı artan troponinlerin; prognoz takibinde kullanılabilirliği, miyokardiyal hasar teşhisli hastalara tedavi uygulamanın faydalı olup olmadığını ve bu hastalarda troponin konsantrasyonlarının iyileşme ile ilişkili olup olmadığını netleştirmek için gelecekteki araştırmalara ihtiyaç vardır.

Anahtar Kelimeler: *Babesiosis, Kardiyak troponin, Köpek.*

ABSTRACT

Determination of Cardiac Troponin Levels in Dogs with Babesiosis

Cardiac biomarkers in dogs are of great importance in the early diagnosis, prognosis, or monitoring of treatment for cardiac diseases. High-sensitivity cardiac troponins (hs-cTns) are considered sensitive indicators of myocardial damage. This study was conducted to determine the levels of high-sensitivity cardiac troponin I (hs-cTnI) and T (hs-cTnT) in dogs naturally infected with *Babesia canis* (*B. canis*). The study material consisted of 15 sick dogs aged 3-4 years, brought to the Iğdır University Animal Hospital and diagnosed with *B. canis* by PCR analysis at the Kafkas University Parasitology Laboratory. For the control group, 10 healthy dogs of the same age range were selected. From both sick and healthy dogs, 5 mL of blood was taken from the V. cephalica into serum tubes (BD Vacutainer, BD, Franklin Lakes, NJ), and the serum was separated. These sera were stored at -20 °C and analyzed within 30 days to determine hs-cTnI and hs-cTnT levels. In our study, it was determined that the hs-cTnI and hs-cTnT levels of dogs with *B. canis* were significantly higher than those of the control group ($p<0.01$; $p<0.05$, respectively). As a result, a statistically significant increase in hs-cTnT and hs-cTnI levels was detected in dogs with babesiosis. Since even small changes in serum hs-cTnI and hs-cTnT levels are prognostically important, it can be said that *B. canis* causes myocardial damage in dogs. However, future research is needed to clarify the usability of troponins, which are increasingly used, in prognosis monitoring, whether it is beneficial to apply treatment to patients diagnosed with myocardial damage, and whether troponin concentrations in these patients are related to recovery.

Keywords: *Babesiosis, Cardiac troponin, Dog.*

✉ *Corresponding author: semistan.kiziltepe@igdir.edu.tr

GİRİŞ

Küresel ısınma nedeniyle oluşan yağışlardaki düzensizlikler ve değişimler; keneler ve sivrisinekler gibi vektörlerin miktarını, yayılımlarını, yaşam sürelerini ve dirençlerini artırmaktadır. Bu durum, iklim değişikliğine bağlı olarak vektörlerle bulaşan zoonotik hastalıkların (VBZH) artacağı şeklinde yorumlanmaktadır (Koyuncu ve Akgün 2018). Bu nedenle VBZH; tüm dünyada halk sağlığı ve hayvan refahı açısından gerekli önlemler alınmadığı takdirde gün geçtikçe artan önemli bir sorun haline gelebilir.

Babesiosis; dünya genelinde yaygın olarak görülen ve keneler tarafından bulaştırılan VBZH hastalıklardandır (Kumar ve ark. 2023). Köpek babesiosisi, kene yoluyla yatay, köpek ısırığından kan transferi, kan nakli veya enfekte bir dişi köpekten yavru köpeklere plasenta boyunca dikey bulaşma ile nispeten yaygın bir hemoprotozoan enfeksiyondur (Karasová ve ark. 2022; Isik ve ark. 2023).

Köpeklerde babesiosise neden olan türler morfolojilerine göre büyük (*B. canis, B. vogeli, B. rossi*) ve küçük (*B. gibsoni, B. conradae, ve B. microti-like*) olmak üzere iki grup halinde sınıflandırılmıştır (Aysul ve ark. 2013; Weingart ve ark. 2023). Perakut, akut, kronik ve subklinik seyir gösteren köpek babesiosis'inin klinik belirtileri tür, vektör, ırk, yaş ve immün yanıt durumlarına göre değişebilmektedir (Rasoulzadeh ve ark. 2021). Etken genellikle klinik olarak, ateş, anoreksi, depresyon, hemoglobinüri, kusma, ikterus ve anemi gibi semptomlara sebep olabilmektedir (Erkılıç 2019; Kırmızıgül ve ark. 2020). Yapılan bir çalışmada babesiosis olgularında nadir de olsa kalp disfonksiyonlarının görüldüğü bildirilmiş ve oluşan bu kardiyak lezyonların muhtemel sebebi aşırı inflamatuar yanıt ve anemik hipoksinin gelişmesi gösterilmiştir (Lobetti ve ark. 2002).

Troponinler, kalp hastalıklarını teşhisinde ve prognozun takip edilmesinde faydalanılan bir polipeptitlerdir (Gavazza ve ark. 2021). Troponinler kalp myositlerinin yıkımlandığı durumlarda dolaşıma geçer. Troponinlerdeki hafif artışlar bile miyokardiyal hasarın bir göstergesidir (Carretón ve ark. 2017; AlSaad ve ark. 2020). Genellikle insan sağlığında kullanılan ve son dönemde veteriner hekimlik alanında da hızla kullanımı artan troponinler 3 alt birimden (I, T ve C) oluşmaktadır. Miyokard hasarı için cTn-I ve cTn-T spesifik olmasına rağmen cTn-I altın standart olarak kabul edilmiştir (Kırbaş ve ark. 2021). hs-cTnI ve hs-cTnT değerleri, cTn-I ve cTn-T değerlerine kıyasla daha yüksek hassasiyete sahiptir. Bu nedenle hs-cTnI ve hs-cTnT değerleri hastalıkların erken evrelerinde ortaya çıkabilecek hafif miyokard hasarlarının tespitine katkı sağlamaktadır (Klüser ve ark. 2019).

Yapılan bu çalışma ile *B. canis*'li köpeklerde hs-cTnI ve hs-cTnT seviyelerinin sağlıklılara göre değişimlerinin belirlenmesi amaçlanmıştır.

MATERYAL VE METOT

Çalışma, Kafkas Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu'nun 06.02.2024 tarih ve KAÜ-HADYEK/2024-021 onaylı izin alınarak etik kurul ilkelerine uygun yapılmıştır.

Çalışmanın materyali Iğdır Üniversitesi Hayvan Hastanesine getirilen ve Kafkas Üniversitesi Veteriner Fakültesi Parazitoloji Anabilim Dalı'nda PCR analizi ile *B. canis* teşhisi konulan 3-4 yaşında 15 erkek köpek ve kontrol grubu için de aynı yaş aralığında sağlıklı 10 erkek köpekten oluşturmuştur. Dişi köpekler östrojenin kalp koruyucu etkisi nedeniyle sonuçları değiştirebileceğinden (Kim-Schulze ve ark. 1996) çalışmaya dahil edilmemişlerdir. Çalışmaya dahil edilen hayvanlardan usulüne uygun olarak serum elde etmek amacıyla alınan kanlar 10 dakika 3000 devirde santrifüj edildi. Troponin konsantrasyonlarının -20 °C'de 3 ay stabil kaldığı bildirildiğinden (Langhorn ve Willesen 2016), serumlar -20ºC'de maksimum 30 gün süre içerisinde muhafaza edilerek testler yapıldı.

Babesia canis'le Enfekte Köpeklerin PCR ile Teşhisi

Hasta köpeklerde türün doğrulanması *B. canis*'e özgü primerler kullanılarak PCR ile yapıldı. PCR'de 746 bp bölgesini amplifiye eden Bab1 (5'-GTG AAC CTT ATC ACT TAA AGG-3') ve Bab3 (5'-CTA CAC AGA GCA CAC AGC C-3') primerleri kullanıldı. 25 µL'lik bir çözelti (8.5 µl nükleaz içermeyen su, 12.5µl ana karışım (Mytaq, Bioline), ileri (Bab1) ve ters (Bab3) primerler (20 pmol/µL), 1 µL ve 2 µL şablon DNA) PCR için bir termal döngüleyici (Biometra, Analytik Jena, ABD) kullanıldı. Her reaksiyon için pozitif ve negatif kontrol DNA örnekleri kullanıldı. PCR koşulları aşağıdaki gibiydi: 94 °C'de ilk 2 dakikalık denatürasyon; 35 tekrarlanan denatürasyon döngüsü (30 saniye boyunca 94 °C), tavlama (30 saniye boyunca 56 °C) ve uzatma (45 saniye boyunca 72 °C); ardından 72 °C'de 10 dakika uzatma yapıldı (Duarte ve ark. 2008; Ayvazoğlu ve ark. 2023).

Kardiak Troponinlerin Ölçümü

Hs-cTnI ve hs-cTnT düzeyleri, ticari test kiti (Canine High Sensitivity Cardiac Troponin I and T Kit, BT Lab, Çin) ile ELISA cihazında (Thermo Scientific Multiscan GO, TİP: 1510) ölçüldü. Sonuçlar üretici tarafından belirtildiği şekilde (ng/mL) değerlendirildi.

İstatistiksel Analiz

Elde edilen sonuçlar SPSS 20 paket programında normalite testi ile istatistiksel analizi yapıldı. Veriler normal dağılım gösterdiği için bağımsız t-testi yapıldı. Test sonucunda P değeri 0.05'ten küçük olan değerler istatistiksel olarak anlamlı kabul edildi ve değerler Ortalama±Standart Hata olarak verildi.

BULGULAR

Yapılan analizler neticesinde *B. canis* ile enfekte köpeklerde hs-cTnI ve hs-cTnT seviyelerinin sırasıyla 0.4265±0.101, 0.3158±0.104 ng/mL ve sağlıklı köpeklerde bu değerlerin sırasıyla 0.1112±0.008, 0.0789±0.019 ng/mL olduğu tespit edilmiştir (Tablo 1). Yapılan istatistiksel analizde *B. canis*'le enfekte köpeklerde hs-cTnI ve T seviyelerinin kontrol grubuna göre yüksek olduğu belirlendi ($p<0.01$; $p<0.05$).

Tablo 1: *Babesia canis* ile enfekte köpeklerde hs-cTnI ve hs-cTnT seviyeleri.

Parameters	Group	N	Mean±SE (Min-Max)	T/P
hs-cTnI (ng/mL)	Healthy	10	0.1112±0.008 (0.0709-0.1446)	T=10.454 P=0.008**
	B. canis	15	0.4265±0.101 (0.1145-1.5004)	
hs-cTnT (ng/mL)	Healthy	10	0.0789±0.019 (0,0177-0,1223)	T=5.284 P=0.04*
	B. canis	15	0.3158±0.104 (0.0277-1.5852)	

*: p<0.05, **: p<0.01, hs-cTnI: yüksek hassasiyetli kardiyak troponin I, hs-cTnT: yüksek hassasiyetli kardiyak troponin T, *B. Canis: Babesia canis,* Healthy: sağlıklı.

TARTIŞMA VE SONUÇ

Köpek babesiosisi, kene kaynaklı intraeritrositik *B. canis, B. rossi, B. gibsoni, B. vogeli, B. microti-like* ve *B. conradae* türlerinin neden olduğu bir enfeksiyondur (Ayoob ve ark. 2010; Aysul ve ark. 2013; Weingart ve ark. 2023). Babesiosis, eritrositlerin esnekliğini azaltarak geçişin yavaşlamasına ve kapiller dolumu artmasıyla eritrositin daha fazla hasar görmesine neden olur (Ayoob ve ark. 2010). Babesiosis enfeksiyonlarında hipoksemi, kırmızı biliyer sendrom, akut böbrek yetmezliği, pankreatit, rabdomiyoliz, yaygın damar içi pıhtılaşma, sistemik hipotansiyon, trombositopeni, hepatopati, merkezi sinir sistemi (MSS) disfonksiyonu, kardiyak disfonksiyon, kardiyojenik olmayan pulmoner ödem, hipoglisemi, hiperlaktatemi ve metabolik asidoz ortaya çıktığı bildirilmektedir (Jacobson ve Lobetti 1996; Welzl ve ark. 2001; de Gopegui ve ark. 2007). Ayrıca şiddetli klinik vakalarda histolojik kardiyak değişikliklerin oluştuğu da bildirilmiştir (Gow ve ark. 2011).

Yapılan çalışmalarda da hasta hayvanlarda oluşan kardiyak disfonksiyonlar genellikle EKG ile belirlenmiştir (Champion ve ark. 2013; Bartnicki ve ark. 2017; Reddy ve ark. 2022). Ancak yapılan farklı çalışmalarda, EKG anormallikleri ile histolojik değişiklikler veya miyokardiyal hasarın biyokimyasal kanıtı arasında bir ilişki olmadığı bildirilmektedir (Lobetti ve ark. 2002; Langhorn ve ark. 2014; Klüser ve ark. 2019). Bu kapsamda yapılan güncel çalışmalar, kardiyak biyobelirteçlerin daha spesifik sonuçlar verdiğini bildirilmişlerdir (Langhorn ve Willesen, 2016; Carretón ve ark. 2017; Kırbaş ve ark. 2021). Troponinler, perikardial efüzyon, kardiyak kontüzyon, aritmojenik sağ ventriküler kardiomiyopati, mitral kalp hastalığı, dilate kardiyomiyopati, kardiotoksisite gibi hastalıklarda prognostik ve tanısal olarak sıklıkla tercih edilen bir polipeptitlerdir (Schober ve ark. 2002; La Vecchio ve ark. 2009). Genellikle insan sağlığında kullanılan ve son dönemde veteriner hekimlik alanında da hızla kullanımı artan troponinler 3 alt birimden (I, T ve C) oluşmaktadır. Ancak miyokard hasarı için cTn-I ve cTn-T spesifik olup cTn-I altın standart olarak kabul edilmiştir (Serra ve ark. 2010; Kırbaş ve ark. 2021).

Yapılan bir çalışmada troponinlerin köpeklerde insana benzer bir salınım gerçekleştirdiği bildirilmiştir (Voss ve ark. 1995). Yapılan farklı çalışmalarda da sağlıklı köpeklerde cTn-I seviyesinin ≤0.17 ng/mL (Guglielmini ve ark. 2010; Polizopoulou ve ark. 2014; Winter ve ark. 2014) cTn-T seviyesinin ≤0.05 ng/mL (Shaw ve ark. 2004; Tarducci ve ark. 2004) olduğu bildirilmiştir. Farklı bir çalışmada ise sağlıklı köpeklerde hs-cTnI ve hs-cTnT seviyesi sırasıyla 0.141±0.023, 0.093±0.013 ng/mL olarak bildirilmiştir (Ayvazoğlu ve ark. 2022). Çalışmamızda da literatüre benzer olarak sağlıklı köpeklerde hs-cTnI seviyesi 0.1112±0.008 ng/mL, hs-cTnT seviyesi ise 0.0789±0.019 olarak belirlendi. Bu durum çalışmada kullanılan sağlıklı köpeklerde kalp hasarı olmadığını göstermektedir. Çalışmada kullanılan köpekler üzerinde de geçmişte klinik ve laboratuvar muayenelerinde herhangi bir hastalık bildirilmediğinden, kalp hastalığına sahip olmaları olası değildir. Bu nedenle çalışma sonuçları hem literatür bilgisine hem de yapılan klinik muayene ile uyumluydu.

Yapılan çalışmalarda; kardiyomiyopati, mitral kapak dejenerasyonu, subaortik stenoz, babesiosis ve gastrik dilatasyon-volvulus gibi miyokard hasara sebep hastalıklarda troponin seviyelerinin yükseldiği bildirilmiştir (Hemdon ve ark. 2002; Lobetti ve ark. 2002; Schober ve ark. 2002; Dukes-McEwan ve ark. 2022). Kalp hasarı şekillendiğinde kanda cTn-I seviyesi 6-12 saat ve cTn-T seviyesi 2-5 saat içinde en yüksek seviyelere ulaştığı raporlanmıştır (Chow ve ark., 2017; Hellings ve ark. 2020). Babesiosisli köpeklerde yapılan bir çalışmada 50 köpekten 19 tanesinde cTn-I seviyesinin yükseldiği ve babesiosis'den ölen 2 köpekte cTn-I seviyesinin çok yüksek olduğu bildirilmiştir (Bartnicki ve ark. 2017). *D. immitis*'li köpeklerde yapılan bir çalışmada da hs-cTnI seviyesinin 0.234 ng/mL olduğu bildirilmiştir (Ayvazoğlu ve ark. 2022).

Çalışmamızda *B. canis* ile enfekte köpeklerde hs-cTnI seviyesinin 0.4265±0.101ng/mL olduğu tespit edildi. Bu durum literatürlerle benzer olarak *Babesia* spp. ile enfekte olan hayvanlarda kardiyak hasarın meydana geldiğini (Winter ve ark. 2014) ve bu klinik durumun belirteci olarak istatistiksel olarak anlamlı artış gösteren hs-cTnI serum seviyelerinin uygun bir belirteç olacağını düşündürmektedir (Langhorn ve ark. 2014).

Kronik mitral kapakçık hasarı olan köpeklerde cTn-T seviyesinin 0.024 ng/mL olduğu bildirilmiştir (Bakirel ve Gunes 2009). Farklı bir çalışmada da hs-cTnT seviyesinin *D. immitis*'li köpeklerde 0.164±0.035 ng/mL olarak bildirilmiştir (Ayvazoğlu ve ark. 2022). Çalışmamızda da *B. canis* ile enfekte köpeklerde hs-cTnT seviyesinin 0.3158±0.104 ng/mL olduğu tespit edildi. Bu durum literatürde bahsedildiği üzere kardiyak hasara neden olan enfeksiyonlarda klinik karakterizasyonu ve serum hs-cTnT konsantrasyon değişimlerinin bir belirteç olabileceğini düşündürdü.

Bazı hastalıklarda serum hs-cTnT konsantrasyon değişimlerinin kardiyak hasar belirteci olarak önemi belirtilmiştir (Bakirel ve Gunes 2009; Langhorn ve ark. 2014) Ancak; cTn-T seviyesi kalp kasının yanı sıra iskelet kasında minimal düzeyde salgılandığı rapor edildiğinden (Naseri ve ark. 2020) cTn-I seviyesinin kardiyak hasarında daha net sonuçlar vermektedir. Çalışmamızda da *B. canis* ile enfekte köpeklerde istatiksel olarak sağlıklılara göre hs-cTnT değişimlerinin ($p<0.05$) hassasiyetinin hs-cTnI kadar iyi ($p<0.01$) olmadığı tespit edilmiştir. Bu durum, kardiyak hasarlarda hs-cTnI analizinin hs-cTnT oranla daha duyarlı ve üstün bir test olduğunu düşündürmektedir (Lobetti ve ark. 2002).

Sonuç olarak, babesiosisli köpeklerde hs-cTnT ve hs-cTnI seviyelerinde istatistiksel olarak anlamlı bir artış olduğu tespit edilmiştir. Serum cTnI ve cTnT seviyelerindeki küçük değişimlerin bile prognostik açıdan önemi olduğundan (Langhorn ve ark. 2013; Carretón ve ark. 2017) *B. canis*'in köpeklerde miyokardiyal hasara neden olduğu söylenebilir. Ancak her geçen gün kullanımı artan troponinlerin; prognoz takibinde kullanılabilirliğinin, miyokardiyal hasar teşhisli hastalara tedavi uygulamanın faydalı olup olmadığının ve bu hastalarda troponin konsantrasyonlarının iyileşme ile ilişkili olup olmadığının netleştirilmesi için gelecekteki araştırmalara ihtiyaç vardır.

ÇIKAR ÇATIŞMASI

Yazarlar bu çalışma için herhangi bir çıkar çatışması olmadığını beyan ederler.

YAZAR KATKILARI

Fikir/Kavram: ŞK, CA
Denetleme/Danışmanlık: GTT
Veri Toplama ve/veya İşleme: ŞK, ÜY
Analiz ve/veya Yorum: ZGY, NA
Makalenin Yazımı: ŞK

Eleştirel İnceleme: GTT

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Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Coşkun N, Timurkan MÖ, Aydın H, Yılmaz V (2024).** Investigation of the Prevalence of Rotavirus Infection in Calves using Polyacrylamide Gel Electrophoresis. *Van Vet J*, 35 (3), 168-172. DOI: https://doi.org/10.36483/vanvetj.1513086

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

Investigation of the Prevalence of Rotavirus Infection in Calves using Polyacrylamide Gel Electrophoresis

Nüvit COŞKUN[1, *] Mehmet Özkan TİMURKAN[2] Hakan AYDIN[2] Volkan YILMAZ[1]

[1] *Kafkas University, Faculty of Veterinary Medicine, Department of Virology, 36100, Kars, Türkiye*
[2] *Atatürk University, Faculty of Veterinary Medicine, Department of Virology, 25700, Erzurum, Türkiye*

Received: 09.07.2024 Accepted: 13.11.2024

ABSTRACT Bovine rotaviruses cause loss of calves and cause great financial losses to breeders. Bovine rotaviruses, which are classified in the *Reovirales* order, *Sedoreoviridae* family and *Rotavirus* genus, are mostly classified as G and P genotypes according to VP7 and VP4 gene regions. In addition, 10 different species (group A-J) have been identified according to genetic and antigenic properties of another major antigen, VP6. Group A rotaviruses are the most common cause of diarrhea in calves, while group B and C infections are also known. For the protection of calves, rotavirus screening should be performed on a herd basis and the infection status of cattle should be revealed. For this purpose, stool samples of 100 calves with diarrhea symptoms in the inventory of Ataturk University, Faculty of Veterinary Medicine, Department of Virology were used. Polyacrylamide gel electrophoresis (PAGE), which allows the examination of segments of the genome, was used to check for the presence of the virus. Nucleic acid extraction was performed on the stool samples before electrophoresis and then extracts were loaded into the prepared polyacrylamide gel and run. The samples were stained with silver nitrate stain, segment patterns were determined, and the presence of rotavirus was analyzed. While 27 of the analyzed samples were positive, 5 samples were suspicious, and 68 samples were negative. The segment pattern of the positive samples was compatible with group A and all of them were classified in this group. Although they were in the same group, it was determined that the positive samples had 3 different electrophoretypes. As a result, it was determined that rotaviruses still have an important role in the etiology of calf diarrhea. Besides, the detected rotaviruses showed variation, although they were in group A, and breeders in the region should pay attention to control and hygiene measures.

Keywords: *Cattle, Diagnosis, Polyacrylamide gel electrophoresis, Rotavirus infections.*

ÖZ

Buzağıların Rotavirus Enfeksiyonunun Prevalansının Poliakrilamid Jel Elektroforez Yöntemi ile Araştırılması

Sığır rotavirus enfeksiyonları yenidoğanların ölümlerine neden olduğundan yetiştiriciler için önemli ekonomik kayıplara sebep olmaktadır. *Reovirales* takımı, *Sedoreoviridae* ailesi ve *Rotavirus* genusu içerisinde sınıflandırılan sığır rotavirusları en çok VP7 ve VP4 gen bölgelerine göre G ve P genotipleri olarak sınıflandırılmaktadır. Bunun haricinde diğer bir major antijen olan VP6 genetik ve antijenik özellikleri temel alınarak 10 farklı tür (grup A-J) belirlenmiştir. Grup A rotaviruslar sığırlarda en fazla ishale neden olurken grup B ve C enfeksiyonları da bilinmektedir. Buzağıların korunması için sürü bazında rotavirus taraması yapılmalı ve sığırların enfeksiyon durumu ortaya konulmalıdır. Bu amaçla Atatürk Üniversitesi Veteriner Fakültesi Viroloji Anabilim Dalı envanterinde bulunan ishal semptomu olan 100 adet buzağıya ait gaita örneği kullanıldı. Virus varlığını tespit etmek için grup ayrımına imkân veren poliakrilamid jel elektroforezi (PAGE) kullanıldı. Elektroforez öncesi gaita örneklerine nükleik asit ekstraksiyonu işlemi yapıldı ve sonrasında hazırlanan poliakrilamid jele yüklenerek yürütüldü. Yürütülen örnekler gümüş nitrat boyanarak segment paternleri belirlenerek rotavirus varlığına bakıldı. İncelenen örneklerin 27'sinde pozitiflik saptanırken 5 örnek şüpheli ve 68 örnek negatif olarak belirlendi. Pozitif bulunan örneklerin segment paterni grup A ile uyumlu bulundu ve tümünün bu grup içerisinde sınıflandığı görüldü. Her ne kadar aynı grup içerisinde yer alsa da tespit edilen pozitif örneklerin 3 farklı elektroforetipe sahip olduğu tespit edildi. Sonuç olarak rotavirusların buzağı ishalleri etiyolojisinde halen önemli rolü olduğu, tespit edilen rotavirusların grup A içerisinde yer almakla beraber varyasyon gösterdiği ve bölgede yetiştiricilerin kontrol ve hijyen önlemlerine dikkat etmesi gerektiği tespit edilmiştir.

Anahtar Kelimeler: *Poliakrilamid jel elektroforezi, Rotavirüs enfeksiyonları, Sığır, Tanı.*

✉ *Corresponding author: nuvitcoskun@gmail.com

INTRODUCTION

Neonatal calf diarrhea is one of the main problems in cattle breeding because of the serious economic losses. One of the main viral agents contributing to early-stage calf loss by diarrhea is bovine rotaviruses. Bovine rotaviruses are classified under the family *Sedoviridae*, genus *Rotavirus* (Karayel-Hacıoglu et al. 2022; Aksoy and Azkur 2023). Genetic material of the bovine rotavirus is a double-stranded RNA that has 11 segments and encodes 6 structural and 6 non-structural proteins (Aksoy and Azkur 2023; Ates and Yesilbag 2023). Besides having a zoonotic potential, rotaviruses have a high degree of reassortment by exchanging the segment with other species in the same genus. This leads to the widening of the host spectrum and interspecies transmission of rotaviruses (Karayel-Hacıoglu et al. 2022).

Rotaviruses are mostly classified with a binary nomination namely G and P depending on VP7 and VP4 respectively. There is an extended genotype classification Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx based on genes VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 to compare full genomes by rotavirus classification working group (RCWG. 2024). According to this classification, 42 G, 58 P, 32 I, 28 R, 24 C, 24 M, 39 A, 28 N, 28 T, 32 E, and 28 H genotypes have been described (RCWG. 2024). Moreover, based on the genetic and antigenic of the full genome and VP6 rotaviruses are classified into ten groups (or species, designated from A to J) (Banyai et al. 2017). Calf diarrhea is mostly caused by bovine rotaviruses which belong to group A, but groups B and C are also reported (Karayel-Hacıoglu et al. 2022; Ates and Yesilbag 2023).

Bovine rotaviruses are common causative agents of calf diarrhea which have been previously serologically and molecularly reported from our country and region from herds with diarrhea (Alkan et al. 2010; Alkan et al. 2015; Karayel et al. 2017; Bulut et al. 2020; Karayel-Hacıoglu et al. 2022; Aksoy and Azkur 2023; Ates and Yesilbag 2023).

As the disease can lead to a significant potential loss to cattle husbandry, diagnosis of the virus bears quite importance. Virus can be diagnosed by molecular methods, ELISA kits and rapid diagnostic tests. Diagnosis with reverse transcription polymerase chain reaction (RT-PCR) is the preferred method of detailed studies because subsequent phylogenetical analyses provide a larger perspective in examining mutations and segment changes (WHO 2009; Bulut et al. 2020). However, this method can be challenging because of the need to analyse multiple gene regions, mutations and the presence of novel strains, PCR inhibitors present in stool or other technical issues. An initial diagnosis is beneficial to make sure virus is present in the sample to work on RT-PCR optimizations. Polyacrylamide gel electrophoresis (PAGE) is an optimal method to visualize the segments of the virus. Thus, after PAGE is performed presence of the virus nucleic acid is known in the sample, so further molecular studies can be performed (WHO 2009). Also, molecular study of single segment can be an option when segments are separately migrated removing the segment and using gel extraction.

This study aims to investigate rotavirus presence, determine rotavirus groups according to segment patterns, and observe different variations in segment migration patterns of rotaviruses in calves with diarrhea.

MATERIAL AND METHODS

Material

Material of the study consisted of 100 stool samples of calves of different ages. Material is collected within Erzurum province. This study has been approved by Ataturk University Veterinary Faculty Local Ethics Committee with number 2024/04.

Polyacrylamide Gel Electrophoresis

Stools were first processed for dsRNA nucleic acid extraction. Gels were cast and run using Mini-Protean (Bio-Rad, California ABD) system. Gels were run vertically using the vertical tank. Gel staining was done using silver nitrate to visualize dsRNA segments. All processes were conducted as instructed by WHO Rotavirus Manual (WHO 2009).

RESULTS

Rotavirus genome was detected in 27 samples, no segment was observed in 68 of the samples and result of 5 samples could not be determined due to heavy smear in corresponding well of the samples. Thus, 5 samples were regarded as suspicious. Sample gels including positive and negative samples are presented in Figure 1 and Figure 2. All positive samples had a pattern compatible to rotavirus group A pattern. Although all positive samples were of group A, there were minor differences in migration pattern. Three different patterns were distinguishable among positive samples. Thus, these electropherotypes were designated type1, type 2, type 3 as shown in Figure 3.

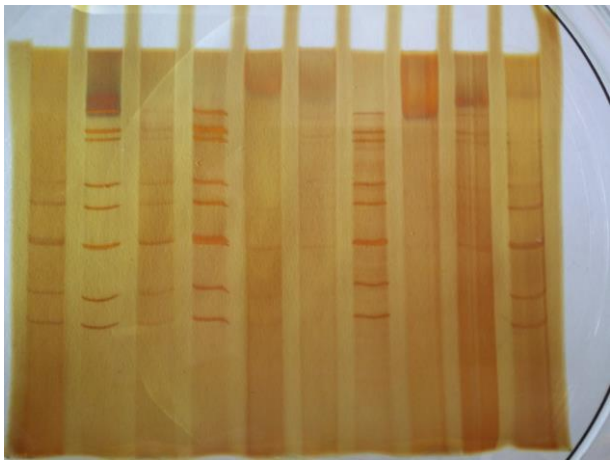

Figure 1: Sample PAGE gel image from various positive and negative cases. Image is visualised with normal camera in direct light. Positive samples have varying degree of visible segment patterns. Only well 8 (counted from left) does not have visible segments and considered negative. Some segments are not as apparent due to lack of contrast (Wells 5, 6 and 9). This is caused by decreased amount of nucleic acid in samples.

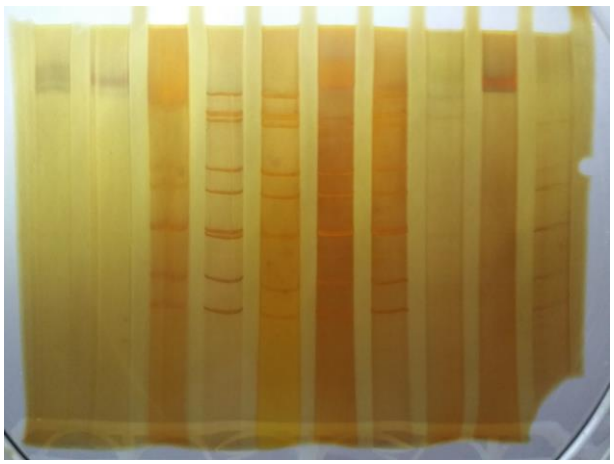

Figure 2: Sample PAGE gel image from various positive and negative cases. Image is visualised with normal camera in direct light. Positive samples have varying degree of visible segment patterns. Wells 1, 2, 8, 9 (counted from left) does not have visible segments and considered negative. Some segments are not as apparent due to lack of contrast (Wells 3 and 10).

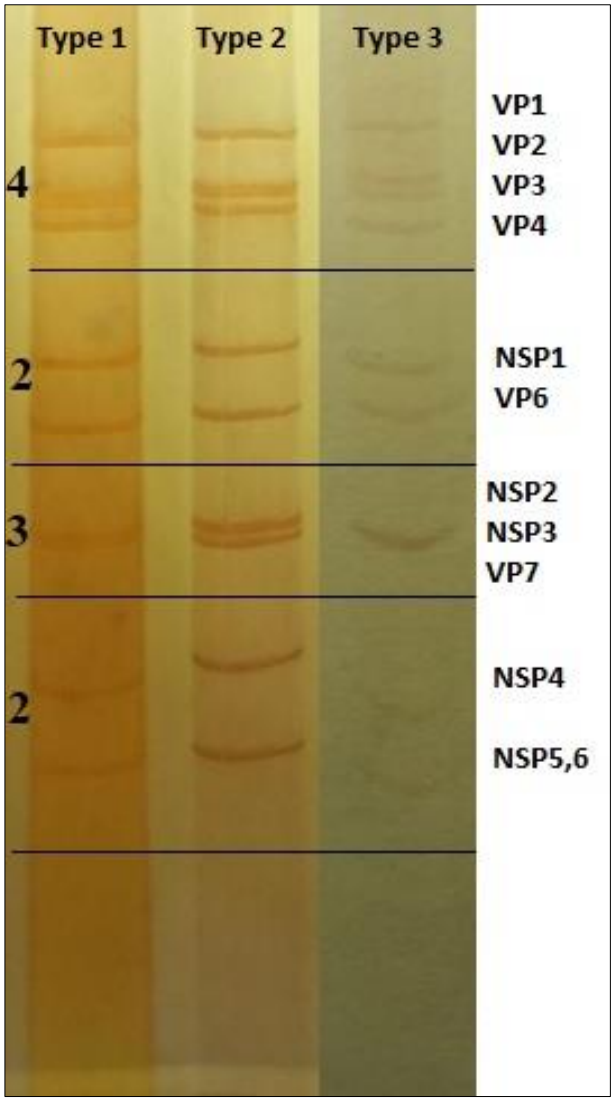

Figure 3: Comparison of three different electropherotypes. Type names given are located on top. Genome segment names are shown on the right. All types show 4/2/3/2 pattern indicating rotavirus group A.

DISCUSSION AND CONCLUSION

Bovine rotaviruses classified in group A are common agents in outbreaks of neonatal calf diarrhea and cross-species infections are reported from humans (da Costa Mendes et al 1993; Cabalar et al. 2001; Matthijnssens et al. 2009; Alkan et al. 2010; Martella et al. 2010; Chen et al. 2023; Carossino et al. 2024; Sadiq and Khan 2024). The most common genotype in Türkiye between 1997-2008 was reported as G6P[11] and G10P[11] (Alkan et al. 2010).

Only partial information is known at genomic level of how these combinations occur; this situation is the cause of the limited understanding of rotavirus infection between ruminants, humans, and other mammals (Karayel-Hacıoglu et al. 2022). Genetic reassortment seems to be the leading cause of interspecies transmission of rotaviruses, segment changes enable rotaviruses to infect different species. Transmission from bovine to other species transmission include rabbits and horses (Ghosh et al. 2013; Schoondermark-van et al. 2013).

In this study, rotaviruses were found to be an important causative agent of diarrhea in calves under six months of age in Erzurum (27/100 for positivity rate, 27%). It was found that 27% of animals with diarrhea symptoms belonged to Group A rotavirus. The rate of detection of rotavirus in 27% of diarrhea cases was higher than the 6.1% previously reported in Erzurum (Aydın and Timurkan 2018). This difference observed in our region suggests that there are ongoing problems with sanitation and lack of care for calf diarrhea in livestock holdings and may also be due to environmental or social factors. Additionally, the prevalence observed in this study was similar to previous studies conducted outside Erzurum in other regions of our country, where the prevalence reported in other studies ranged from 1.6% to 30%. Beside Erzurum, prevalence was reported as 1.6% (1/59, calves) in Van (Cabalar et al. 2001), 30% (9/30, calves) in Elazığ (Al and Balıkçı, 2012), and 18.75% (18/96, calves) in Konya-Afyon (Uyunmaz Saklı et al. 2019).

Reports from different parts of the world have shown electrophoretyping as a potential tool for studying the molecular epidemiology of rotavirus infections. Different electrophoretypes can be examined differently with two perspectives. Firstly we can determine and cluster the group (A, B, C etc) of the rotaviruses, which allows us evaluate the origin of the virus. Secondly, within the same group, there may be minor differences in patterns belonging to same group such as our study. This information can be considered as an indication of genetic changes including virulence, but the concept has not been studied extensively and regarded only preliminary (Özkul et al. 2002). In this context, electrophoretyping studies in humans are abundant. In one study (Ayolabi et al. 2013), 12 different electropherotypes (7 long E-types and 5 short E-types) were found and L5 was found to be dominant. Regardless of the country and region, the predominance of the long pattern over the short pattern is in accordance with the findings of other researchers and they considered this situation normal. The same study also reported that more electrophoretic patterns were observed in Nigeria than previously reported. In another study, in 1998, the African rotavirus study group reported 2 and 7 patterns from Jos and Zaria cities, respectively. However, 22 electrophoretic patterns were reported in Bangladesh and 7 patterns were reported in Kenya by the same study group (Ayub et al. 1993). Bukrinskaia et al. (1990) identified several mobility patterns of both long and short-migration types of rotavirus RNA during outbreaks in

children in Russia in the winter of 1988-1989. This reports the presence of antigenic variants of rotaviruses in an outbreak.

The mechanisms for generating wide genomic diversity among rotavirus strains (since they have segmented genomes) are mostly due to genetic reassortment. This genetic reassortment can occur either naturally or possibly due to suppression by the host immune system (Biryahwaho et al. 1987; Ward et al. 1988). This means that there are several strains circulating in the region in our study and this also emphasizes the need for regular rotavirus surveillance to detect these new/unusual and emerging strains that may have an impact on current vaccines. As important epidemiological data, it can be emphasized that the emergence of genomic variations and their carry-over to antigenic properties of circulating rotaviruses can be frequently encountered between herds at different intervals. In this study, 3 different rotavirus patterns were detected in the limited sample size. With more comprehensive studies, the relationship of these patterns with genotypes, the approach to the pathogenesis of the disease, and the similarity/difference with virus strains can be revealed.

PAGE allows distinguishing rotavirus groups without performing RT-PCR and sequencing. Group A rotaviruses are identified according to migration of the pattern the 11 segments of the genome as 4/2/3/2, while Group B has a migration pattern of 4/2/2/3, and Group C has 4/3/2/2 (WHO 2009; Ates and Yesilbag 2023). Even within the same group, like in our study all positive samples are group A, we can have different electropherotypes to interpret segment migration pattern changes to predict antigenic differences. A fast interpretation in this regard is advantageous when molecular diagnostic equipment/sequencing is not available. Three electropherotypes in our study are examined are mostly constant in segment patterns. Among positive samples type 1 was the dominant type and 22 of 27 positives were of this type. Whereas 4 of 27 were type 2 and only one sample was classified as type 3. The main difference is with type 3 is with segment 3 (VP3), it has migrated faster thus VP2 and VP3 is distinguishable. Type 2 has differed in segment 4 (VP4) has slower migration and segment 9 (VP7) is faster migration. Since VP7 is responsible for G genotype and VP4 is for P genotype is expected to have a different G type and P genotype than other strains if related genes were sequenced. Three main antigenic sites are defined by genes VP7, VP6 and VP4. Especially outer capsid proteins, VP7 and VP4, that independently generate serotype-specific neutralizing antibodies (Özkul et al. 2002; Ates and Yesilbag 2023). These segments can be examined with PAGE, although results can only be preliminary because there is not enough data which compare segment patterns/serological response.

There are two previous studies on rotaviruses that reported rotavirus presence detected with PAGE (Özkul et al. 2002; Ates and Yesilbag 2023). Özkul et al. (2002) determined 5 different electrophoretic types according to migration patterns in 83 stool samples in which rotavirus infection was detected. All of the rotaviruses examined in the study were found in group A and exhibited short migration patterns. They included 7 enterprises in their study and all of them showed a unique pattern, but in one enterprise, they detected rotaviruses with a pattern that changed over the years. They observed the greatest differences in electrophoretic migration in segments 2-4 and 6-9, but found that segments 1, 10 and 11 exhibited the most constant migration. Ates and Yesilbag (2023) worked with 20 stool samples and were able to isolate 2 strains in cell culture. These two isolates were examined with PAGE and researchers reported they belonged to rotavirus group A. Our study had similar results to these studies; all strains in this study also belonged to group A.

Bovine rotaviruses can impact herds by loss of calves when prevention measures are not taken carefully. The most important way of protection is vaccination, but since passive immunization is mostly acquired by colostrum vaccination of pregnant cows is the most effective form of prophylaxis (Alkan et al. 2010; Bulut et al. 2020).

In conclusion, the following data has been gathered in light of our results: Firstly, bovine rotavirus group A has been shown to still cause problems to breeders in the Erzurum region. Secondly when vaccine strains are taken into consideration current vaccine has been found suitable for field strains. Thirdly there is need for further research regarding antibody response for different electropherotypes since different types were found in our results.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGMENT

This project is supported by Ataturk University Scientific Research Projects (BAP) Coordination Office with number THD-2021-9090.

AUTHOR CONTRIBUTIONS

Idea / Concept: NC, MÖT
Supervision / Consultancy: MÖT
Data Collection and / or Processing: HA, MÖT, NC
Analysis and / or Interpretation: HA, MÖT, NC, VY
Writing the Article: NC, MÖT
Critical Review: NC, MÖT

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Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Sağun E, Ekici K, Sancak H, Yörük İH, Duyar HA (2024).** Quality Changes Occurring in Pearl Mullet (*Chalcalburnus tarichi*, Pallas 1811) Processed with Different Forms and Preserved at Cold (+4 °C) During the Preservation Period. *Van Vet J*, 35 (3), 173-183. DOI: https://doi.org/10.36483/vanvetj.1513376

ISSN: 2149-3359 **Araştırma Makalesi** e-ISSN: 2149-8644

Farklı Şekillerde İşlenerek Soğukta (+4 °C) Muhafaza Edilen İnci Kefalinde (*Chalcalburnus tarichi*, Pallas 1811) Muhafaza Süresince Meydana Gelen Kalite Değişiklikleri

Emrullah SAĞUN[1] Kamil EKİCİ[1] Hakan SANCAK[2,*] İbrahim Hakkı YÖRÜK[3] Hünkar Avni DUYAR[4]

[1] *Van Yüzüncü Yıl Üniversitesi, Veteriner Fakültesi, Besin Hijyeni ve Teknolojisi Anabilim Dalı, 65040, Van, Türkiye*
[2] *Bitlis Eren Üniversitesi, Organize Sanayi Bölgesi Meslek Yüksekokulu, Gıda İşleme Bölümü, 13200, Bitlis, Türkiye*
[3] *Van Yüzüncü Yıl Üniversitesi, Fen Fakültesi, Kimya Bölümü, 65040, Van, Türkiye*
[4] *Sinop Üniversitesi, Su Ürünleri Fakültesi, Su Ürünleri Avlama ve İşleme Teknolojisi Bölümü, 57000, Sinop, Türkiye*

Geliş Tarihi: 10.07.2024 Kabul Tarihi: 16.10.2024

ÖZ

Bu araştırmada; bütün halde ve temizlenerek vakumsuz/vakumlu ambalajlanıp 20 gün süreyle soğukta (+4 °C) muhafaza edilen inci kefalinde (*Chalcalburnus tarichi*, Pallas 1811) meydana gelen mikrobiyolojik, kimyasal ve duyusal değişiklikler incelenmiştir. Toplam aerob psikrofil mikroorganizmalar ve laktik asit bakterileri bütün gruplarda muhafaza süresince artarak 20. gün 7-8 log kob/g seviyelerine ulaşırken, *Pseudomonas* spp. sayıları ise sadece vakumsuz ambalajlanan örneklerde artmıştır (5-6 log kob/g). Örneklerin hiçbirinde fekal streptekoklara rastlanmamış, maya-küfler ve koliform grubu mikroorganizmalar muhafaza süresince düzensiz bir seyir izlemiştir. Toplam uçucu bazik azot (TVB-N) ve tiyobarbitürik asit (TBA) miktarları ile peroksit sayıları bütün gruplarda artarken, pH değerleri, vitamin (A, D_3) miktarları ve duyusal analiz puanları azalmıştır. Belirlenen TBA miktarlarının, TVB-N ve duyusal analiz bulguları ile uyum göstermediği görülmüştür. Bütün halde vakumlanarak ambalajlanan örneklerde, kan ve sindirim sisteminin içeriği hoşa gitmeyen bir görünüm ve kokuya neden olmuştur. Sonuç olarak; inci kefalinin soğukta bütün halde 7 gün, baş ve iç organları çıkarılarak ise 10 gün süreyle muhafaza edilebileceği belirlenmiştir. Duyusal özellikler ve raf ömrü üzerine balıkları temizleyerek vakumlu ambalajlamanın olumlu bir etkisinin bulunduğu ve bunun en uygun yöntem olabileceği değerlendirilmiştir. Ayrıca, az yağlı bir balık olan inci kefalinde TBA miktarı ve peroksit sayısının tazeliğin belirlenmesinde yararlanılabilecek güvenilir kriterler olmadığı kanaatine varılmıştır.

Anahtar Kelimeler: *Duyusal özellikler, inci kefali (Chalcalburnus tarichi), kimyasal kalite, mikrobiyolojik kalite, soğukta muhafaza, vakumlu ambalajlama.*

ABSTRACT

Quality Changes Occurring in Pearl Mullet (*Chalcalburnus tarichi*, Pallas 1811) Processed with Different Forms and Preserved at Cold (+4 °C) During the Preservation Period

In this study, microbiological, chemical, and sensory changes in whole and cleaned pearl mullet (*Chalcalburnus tarichi*, Pallas 1811) preserved in non-vacuumed/vacuumed packages and at cold (+4 °C) for 20 days were examined. While total aerobic psychrophil microorganisms and lactic acid bacteria increased in all groups during the preservation period and reached 7-8 log cfu/g levels on the 20th day, *Pseudomonas* spp. counts increased only in non-vacuumed package samples (5-6 log cfu/g). Fecal streptococci were not detected in any of the samples, and yeast-molds and coliforms followed an irregular course during the preservation period. While the total volatile basic nitrogen (TVB-N) and thiobarbituric acid (TBA) amounts and the peroxide counts increased in all groups, pH values, vitamin (A, D_3) amounts and sensory analysis scores decreased. It was observed that the determined TBA amounts were not compatible with TVB-N and sensory analysis findings. In samples vacuum-packaged as a whole, the contents of the blood and digestive system caused an unpleasant appearance and odor. As a result, it was determined that the pearl mullet can be preserved at cold for 7 days as a whole, and for 10 days with its head and internal organs removed. It has been evaluated that vacuum packaging of cleaned fish has a positive effect on sensory properties and shelf life and may be the most suitable method. In addition, it was convinced that the TBA amount and peroxide count in pearl mullet, which is a low-fat fish, are not reliable criteria that can be used to determine freshness.

Keywords: *Chemical quality, microbiological quality, pearl mullet (Chalcalburnus tarichi), preservation at cold, sensory properties, vacuum packaging.*

✉ *Corresponding author: hsancak@beu.edu.tr

GİRİŞ

İnsanların protein ihtiyacının karşılanmasında önemli bir gıda olan balık, kaliteli protein içeriğinin yanında yağ asitleri, vitaminler ve mineral maddeler yönünden de zengindir (Gregory 2017; Voyer 2022). Ancak rutubet miktarı yüksek olan bu gıda, diğer etlere göre daha çabuk bozulabilmekte ve önemli kalite kayıpları oluşabilmektedir (Velíšek ve ark. 2020). Balıkların gastro-intestinal sistemindeki enzimlerin neden olduğu otolitik bozulmalar ve balıkların kontaminasyonlara uğraması önemli kalite kayıplarına neden olmaktadır. Ayrıca balıkların depolandığı sıcaklık da, enzimatik ve mikrobiyal bozulmalarda rol oynayan önemli bir faktördür (Huss 1995; Forsythe 2020; Mishra 2022).

Balıkların kas dokusu normalde mikroorganizma içermezken; derisi, solungaçları ve sindirim sistemi birçok mikroorganizmanın ürediği kısımlardır (Huss 1995). Balıklar avlandıktan sonra da özellikle bu kısımlarda üreyen patojen mikroorganizmalar; çevresel şartlar, nakliye, işleme durumu ve muhafaza koşullarındaki olumsuzluklara bağlı olarak ilerleyen zamanlarda bütün vücuda yayılmakta ve bozulmada etkin rol oynamaktadır (Huss 1995; Forsythe 2020; Parthiban ve Felix 2022). Balıkların bozulmasının önlenebilmesi için, avlama yapıldıktan hemen sonra alınabilecek ilk önlem ürünün soğutulmasıdır (Connell 1995; Baygar ve Varlık 2004; Mishra 2022; Mueller 2023; Syanya ve ark. 2023). Ancak soğutmanın bozulmayı tamamen durdurmadığı, sadece belirli bir süre geciktirdiği ve kısa sürede tüketilen balıklar için tercih edilebilecek bir muhafaza şekli olduğu unutulmamalıdır.

İç su balıkçılığında üretimi sazandan sonra ikinci sırada yer alan inci kefalinin (*Chalcalburnus tarichi*, Pallas 1811) yaşadığı habitatın (Van Gölü) en yakın denizden yaklaşık 1000 km uzakta olması, bu balığın iç su balıkları içindeki önemini daha da artırmaktadır (Sarı 2001). Van Gölü çevresinde yaşayan halk tarafından sevilerek tüketilen, ucuz ve protein kalitesi yüksek olan inci kefali; uygun koşullarda muhafaza edilmemesi ve hijyenik şartlarda pazarlanmaması nedeniyle kısa sürede bozularak potansiyel bir halk sağlığı problemi oluşturmakta ve önemli ekonomik kayıplara neden olmaktadır (Sancak ve Sağun 2020). Yumurta bırakmak için Nisan/Temmuz ayları arasında akarsulara göç eden balıkların buralarda daha kolay avlanabilmesi, bol avlandığı için ucuz olması ve tüketicilerin yasak dönemde de balık tüketmek istemeleri avlanma yasağına uyulmamasına neden olan önemli faktörler olarak görülmektedir.

Yapılan bazı çalışmalarda (Demirci ve Orak 1999; Köse ve ark. 2000; Şengör ve ark. 2000; Kyrana ve Lougovois 2002; Taliadourou ve ark. 2003; Ekici ve Alisarli 2008; Çakmak ve Sancak 2023) farklı şekillerde muhafaza edilen balıklarda mikrobiyolojik ve kimyasal faktörlere bağlı olarak önemli kalite değişimlerinin belirlendiği, bazı çalışmalarda (Özogul ve ark. 2004; Stamatis ve Arkoudelos 2007; Vurat ve Kocatepe 2023) da vakumlu ambalajlamanın balıkların kimyasal ve duyusal özelliklerine olumlu etkiler yaptığı bildirilmiştir.

Bu araştırma, bütün ve temizlenmiş inci kefali örneklerinin vakumlu ve vakumsuz olarak ambalajlanarak soğukta muhafazası sırasında meydana gelen kalite değişimlerinin ve muhafaza süresinin bu değişimler üzerine etkisinin tespit edilmesi amacıyla yapılmıştır.

MATERYAL VE METOT

Bu araştırmada balıkçılar tarafından ticari satış amacıyla Van Gölü'nden avlanan inci kefali materyal olarak kullanılmış ve en kısa sürede soğutuculu araçlarla laboratuvara getirilen balıklar dört gruba ayrılmıştır. Bütün haldeki balıkların bir kısmı strafor tabaklarda üstleri streç film ile kaplanarak vakumsuz (A grubu) ve bir kısmı da polietilen torbalarda vakumlu (C grubu) olarak ambalajlanmıştır. Ayrıca baş ve iç organlarından ayırılan, mukoza ve kandan arındırılması için soğuk musluk suyu ile yıkanarak süzülen balıklar da bütün haldeki balıklar gibi vakumsuz (B grubu) ve vakumlu (D grubu) bir şekilde ambalajlanmıştır.

Farklı şekillerde ambalajlanan inci kefali örnekleri +4 °C'de muhafaza edilmiş ve muhafaza süresinin 0., 2., 4., 7., 10., 13., 16. ve 20. günlerinde mikrobiyolojik, kimyasal ve duyusal değişimlerin belirlenmesi amacıyla incelenmiştir. Her grupta bulunan ~10 adet balığın en az 5'inin dorsal kaslarından örnekler alınmış, tüm analizler üç tekerrürlü olmak üzere öncelikle mikrobiyolojik analizler ve sonra da diğer analizler gerçekleştirilmiştir.

Mikrobiyolojik Analizler

Aseptik koşullarda steril stomaher torbasına alınan 10 g örnek ve 90 ml tamponlanmış peptonlu su (Oxoid CM0509) stomaherde (IUL, 2373/400, İspanya) iki dakika süreyle homojenize edilerek 10^{-8}'e kadar desimal dilüsyonlar hazırlanmıştır. Mikrobiyolojik analizlerde kullanılan besiyerleri, uygulanan yöntemler ve inkübasyon koşulları Tablo 1'de sunulmuştur (Pichhardt 1998). İncelenen örneklerde fekal streptekoklar tespit edilmemiştir.

Tablo 1: Mikrobiyolojik analizlerde kullanılan besiyerleri ve inkübasyon koşulları.

Table 1: Used in microbiological analyses the media and incubation conditions.

Mikroorganizma	Besiyeri	Ekim yöntemi	İnkübasyon
TAPM	PCA (Oxoid, CM325)	Damla plak	7 °C (7-10 gün)
LAB	M17 Agar (Oxoid, CM785)	Dökme plak, çift kat	35 °C (48 h)
Maya-küf	PDA (Oxoid, CM139)	Damla plak	20-25 °C (5-7 gün)
Koliformlar	VRBA (Oxoid, CM107)	Dökme plak	37±1 °C (24-48 h)
Fekal streptokoklar	SBA (Oxoid, CM377)	Damla plak	37 °C (24 h)
***Pseudomonas* spp.**	PAB (Oxoid, CM559)	Damla plak	25 °C (48-72 h)

TAPM: Toplam aerob psikrofil mikroorganizmalar; **LAB:** Laktik asit bakterileri; **PCA**: Plate Count Agar; **PDA**: Potato Dextrose Agar; **VRBA**: Violet Red Bile Agar; **SBA:** Slanetz & Bartley Agar; **PAB:** Pseudomonas Agar Base (50 °C'ye kadar soğutulan sterilize besiyerine Pseudomonas Selective Supplement "Oxoid, SR103" ilave edilmiştir).

Kimyasal Analizler

Örneklerdeki toplam uçucu bazik azot (total volatile basic nitrogen, TVB-N) miktarı Antonacpoulos tarafından, peroksit sayısı ise Hadorn ve ark. tarafından modifiye edilen yönteme göre tespit edilmiştir. Tiyobarbitürik asit (thiobarbituric acid, TBA) miktarının belirlenebilmesi için; homojenize edilen 10 g örnek ve 50 ml distile su destilasyon balonuna alınmış, üzerine 47.5 ml distile su ve ortamın pH'sının 1.50'ye ayarlanabilmesi amacıyla da 4 N hidroklorik asitten 2.5 ml eklendikten sonra elde edilen karışım düzenekte destilasyona tabi tutulmuştur. Destilasyon sonucunda kapaklı tüplere alınan 5 ml destilat ve 5 ml TBA reaktifi homojenize edilerek su banyosunda (Nüve, ST 402, Belçika) 100 °C'nin altında 35 dakika bekletilmiş ve daha sonra bu karışımın 538 nm dalga boyunda köre karşı optik dansitesi belirlenmiştir. Belirlenen optik dansite 7.80 faktörü ile çarpılarak örneklerdeki TBA miktarı hesaplanmıştır (Varlık ve ark. 1993). Ayrıca örneklerdeki pH değerleri Honikel (2014)'e göre, vitamin A ve vitamin D_3 miktarları da Miller ve Yang (1985) tarafından bildirilen yüksek performanslı sıvı kromatografisi (High Performance Liquid Chromatography, HPLC) yöntemine göre belirlenmiştir.

Duyusal Analizler

Duyusal analizler Paulus ve ark. (1969) tarafından geliştirilen puanlama sistemine göre aynı kişilerden oluşan beş panelist tarafından gerçekleştirilmiştir. Örneklerin renk, koku, lezzet ve genel kabul edilebilirlik kriterleri hedonik skalaya göre (9.00-7.00 puan "çok iyi", 6.99-4.01 puan "iyi", 4.00 puan "tüketilebilir", <4.00 puan "bozulmuş") değerlendirilmiştir.

İstatistiksel Analiz

Çalışmanın örneklemini balıkçıların ticari amaçla Van Gölü'nden avladıkları inci kefali oluşturmuş ve balıklar temizleme/vakumlama durumlarına göre gruplara ayrılarak soğuk şartlarda (+4 °C) muhafaza edilmiştir. Mikrobiyolojik, kimyasal ve duyusal analizlerden elde edilen bulgular; bağımsız değişkenlerin ana etkilerini ve etkileşimlerini aynı anda test etme avantajı sunan faktöriyel deneme desenine göre SAS paket programında değerlendirilmiş (Stroup ve ark. 2018) ve muhafaza süresince balıkların bazı kalite özelliklerinin zamana göre değişimleri ile gruplar arasında oluşan farklılıklar ($p<0.05$) belirlenmiştir.

BULGULAR

İncelenen inci kefali örneklerinin mikrobiyolojik, kimyasal ve duyusal analiz bulguları ile bu bulguların grup ortalamaları ve zamana göre değişimleri Tablo 2-7'de gösterilmiştir.

Tablo 2: İnci kefali örneklerinin mikrobiyolojik analiz bulguları (log kob/g).

Table 2: Microbiological analysis findings of pearl mullet samples (log cfu/g).

	Zaman (gün)	GRUPLAR			
		A	B	C	D
TAPM	0	4.89±0.06 e	3.20±0.60 e	3.52±0.76 d	4.08±0.11 d
	2	5.38±0.20 Ade	4.27±0.21 Bd	5.42±0.11 Ac	4.59±0.25 Bd
	4	5.98±0.26 d	5.57±0.26 e	6.02±0.30 bc	5.83±0.15 c
	7	6.79±0.27 c	7.01±0.12 b	6.18±0.61 bc	6.72±0.15 abc
	10	7.62±0.21 b	7.82±0.09 ab	7.54±0.28 a	7.27±0.58 ab
	13	7.10±0.08 Bbc	7.47±0.04 Aab	6.68±0.01 Cabc	6.50±0.50 Cbc
	16	7.81±0.42 b	8.08±0.39 a	7.18±0.09 ab	7.45±0.41 a
	20	8.57±0.02 Aa	8.31±0.24 Aa	7.66±0.22 Ba	7.57±0.02 Ba
	Ortalama±sd	6.77±0.25 A	6.47±0.38 AB	6.27±0.28 B	6.25±0.27 B
LAB	0	4.22±0.44 Ae	2.60±0.60 Be	3.98±0.20 ABe	3.84±0.12 ABde
	2	4.43±0.32 Ae	4.22±0.04 Ad	4.07±0.03 ABe	3.37±0.23 Be
	4	5.02±0.08 Ad	4.44±0.03 Bd	4.49±0.06 Bd	4.31±0.23 Bd
	7	5.80±0.05 c	5.85±0.08 c	5.65±0.03 c	5.34±0.08 c
	10	6.49±0.15 b	6.70±0.06 b	6.36±0.17 b	6.34±0.16 b
	13	6.67±0.06 Ab	6.82±0.10 Ab	6.24±0.16 Bb	5.71±0.06 Cc
	16	6.23±0.12 Bbc	6.66±0.16 ABb	7.09±0.09 Aa	6.47±0.15 Bb
	20	8.02±0.03 Aa	7.67±0.11 ABa	7.41±0.21 BCa	7.02±0.21 Ca
	Ortalama±sd	5.93±0.25 A	5.82±0.33 A	5.73±0.26 A	5.36±0.26 B
Maya-küf	0	2.32±1.17 b	<2.30 d	2.30±1.15 b	<2.30
	2	4.36±0.22 ABa	3.79±0.28 Bab	4.48±0.15 Aa	<2.30 C
	4	5.20±0.10 Aa	4.70±0.26 ABa	4.28±0.28 Ba	2.30±0.00 C
	7	5.25±0.48 Aa	3.82±0.26 Bab	<2.30 Cc	<2.30 C
	10	4.56±0.86 Aa	3.40±0.10 Aab	<2.30 Bc	0.76±0.76 B
	13	4.06±0.18 Aab	4.12±0.21 Aab	2.30±0.00 Bb	0.76±0.76 C
	16	4.73±0.13 a	1.20±0.20 cd	2.20±1.24 b	1.32±1.32
	20	3.47±0.63 Aab	2.30±1.15 ABbc	<2.30 Bc	1.43±1.43 AB
	Ortalama±sd	4.24±0.25 A	2.91±0.36 B	1.94±0.40 C	0.82±0.28 D

Tablo 2 (devamı): İnci kefali örneklerinin mikrobiyolojik analiz bulguları (log kob/g).
Table 2 (continued): Microbiological analysis findings of pearl mullet samples (log cfu/g).

Koliformlar	0	<1.00 ^{c}	<1.00 ^{d}	<1.00 ^{c}	<1.00 ^{b}
	2	1.30±0.65 ^{Abc}	<1.00 ^{Bc}	<1.00 ^{Bc}	<1.00 ^{Bb}
	4	2.57±0.10 ^{Aab}	0.53±0.53 ^{BCcd}	1.77±0.88 ^{ABab}	<1.00 ^{Cb}
	7	3.29±0.21 ^{Aa}	0.56±0.56 ^{Ccd}	2.61±0.19 ^{ABa}	1.49±0.74 ^{BCab}
	10	2.33±1.16 ^{ab}	1.84±0.92 ^{abc}	0.76±0.76 ^{bc}	1.05±1.05 ^{ab}
	13	2.33±0.24 ^{ab}	0.90±0.90 ^{cd}	2.14±0.17 ^{ab}	1.37±0.73 ^{ab}
	16	2.08±0.03 ^{Bab}	2.67±0.09 ^{Aa}	2.50±0.17 ^{Aa}	2.67±0.10 ^{Aa}
	20	3.36±0.29 ^{Aa}	2.35±0.22 ^{Aab}	0.76±0.76 ^{Bbc}	2.50±0.28 ^{Aa}
	Ortalama±sd	2.15±0.25 ^{A}	1.11±0.25 ^{B}	1.32±0.25 ^{B}	1.14±0.26 ^{B}
***Pseudomonas* spp.**	0	3.08±0.18 ^{Abc}	2.35±0.05 ^{Bc}	2.50±0.10 ^{Bb}	2.40±0.10 ^{Bab}
	2	2.18±1.09 ^{c}	3.35±0.17 ^{abc}	2.21±1.10 ^{b}	1.00±1.00 ^{bc}
	4	4.21±0.26 ^{abc}	2.83±1.46 ^{bc}	4.56±0.04 ^{a}	2.02±1.02 ^{abc}
	7	5.05±0.35 ^{Aab}	5.22±0.34 ^{Aabc}	2.07±1.05 ^{Bb}	3.53±0.13 ^{ABa}
	10	4.39±0.31 ^{Aabc}	4.93±0.31 ^{Aabc}	<2.30 ^{Bc}	0.96±0.96 ^{Bbc}
	13	4.35±0.13 ^{Babc}	5.99±0.29 ^{Aa}	<2.30 ^{Cc}	<2.30 ^{Cc}
	16	3.10±1.81 ^{bc}	5.49±1.19 ^{ab}	3.12±0.08 ^{ab}	3.63±0.23 ^{a}
	20	6.73±0.67 ^{Aa}	5.65±1.67 ^{Aab}	<2.30 ^{Bc}	<2.30 ^{Bc}
	Ortalama±sd	4.13±0.36 ^{A}	4.47±0.38 ^{A}	1.80±0.36 ^{B}	1.69±0.33 ^{B}

A: Bütün/vakumsuz; **B:** Temizlenmiş/vakumsuz; **C:** Bütün/vakumlu; **D:** Temizlenmiş/vakumlu; **TAPM:** Toplam aerob psikrofil mikroorganizmalar; **LAB:** Laktik asit bakterileri; **sd:** Standard deviation (standart sapma); **^{ABC}:** Aynı satırdaki farklı harfler gruplar arasındaki farklılığı göstermektedir (p<0.05); **^{abcde}:** Aynı sütundaki farklı harfler zamanlar arasındaki farklılığı göstermektedir (p<0.05).

Tablo 3: İnci kefali örneklerindeki mikroorganizmaların grup ortalamaları ve zamana göre değişimleri (log kob/g).
Table 3: Group averages and their changes over time of microorganisms in pearl mullet samples (log cfu/g).

	n	**TAPM**	**LAB**	**Maya-küf**	**Koliformlar**	***Pseudomonas* spp.**
Muamele						
Bütün	48	6.52±0.19	5.83±0.18 ^{a}	3.09±0.28 ^{a}	1.74±0.19 ^{a}	2.97±0.30
Temiz	48	6.36±0.23	5.58±0.21 ^{b}	1.87±0.27 ^{b}	1.12±0.18 ^{b}	3.08±0.32
Ambalaj tipi						
Vakumsuz	48	6.62±0.22 ^{a}	5.88±0.20 ^{a}	3.58±0.24 ^{a}	1.63±0.19 ^{a}	4.30±0.26 ^{a}
Vakumlu	48	6.26±0.19 ^{b}	5.55±0.18 ^{b}	1.38±0.25 ^{b}	1.23±0.18 ^{b}	1.75±0.24 ^{b}
Zaman (gün)						
0	12	3.92±0.28 ^{e}	3.66±0.37 ^{f}	1.15±0.49 ^{d}	<1.00 ^{d}	2.58±0.10 ^{bc}
2	12	4.92±0.17 ^{d}	4.00±0.18 ^{e}	3.15±0.56 ^{b}	0.32±0.21 ^{d}	2.18±0.46 ^{c}
4	12	5.85±0.12 ^{c}	4.05±0.09 ^{d}	4.12±0.34 ^{a}	1.22±0.37 ^{c}	3.40±0.49 ^{ab}
7	12	6.68±0.17 ^{b}	5.66±0.06 ^{c}	2.26±0.71 ^{bc}	1.99±0.37 ^{abc}	3.96±0.45 ^{a}
10	12	7.56±0.15 ^{a}	6.47±0.07 ^{b}	2.18±0.61 ^{bc}	1.49±0.46 ^{bc}	2.57±0.68 ^{bc}
13	12	6.93±0.11 ^{b}	6.36±0.13 ^{b}	2.81±.045 ^{bc}	1.69±0.31 ^{abc}	2.58±0.80 ^{bc}
16	12	7.63±0.18 ^{a}	6.61±0.11 ^{b}	2.36±0.63 ^{bc}	2.48±0.08 ^{a}	3.83±0.55 ^{a}
20	12	8.03±0.14 ^{a}	7.53±0.13 ^{a}	1.80±0.56 ^{cd}	2.26±0.34 ^{ab}	3.09±1.01 ^{abc}

TAPM: Toplam aerob psikrofil mikroorganizmalar; **LAB:** Laktik asit bakterileri; **n:** Örnek sayısı; **^{abcdef}:** Aynı sütundaki farklı harfler balıklara yapılan temizleme/vakumlama işlemlerine göre oluşturulan gruplar ve zamanlar arasındaki farklılığı göstermektedir (p<0.05).

Tablo 4: İnci kefali örneklerinin kimyasal analiz bulguları ve pH değerleri.

Table 4: Chemical analysis findings and pH values of pearl mullet samples.

	Zaman (gün)	GRUPLAR			
		A	**B**	**C**	**D**
TVB-N (mg/100 g)	0	16.80±0.80 ^{Ad}	13.06±0.46 ^{Bd}	14.93±0.46 ^{Bc}	13.53±0.46 ^{Bf}
	2	20.06±2.59 ^{d}	14.46±0.46 ^{d}	16.80±1.40 ^{c}	17.26±1.68 ^{de}
	4	21.46±0.46 ^{Ad}	15.40±0.80 ^{Bd}	22.86±0.46 ^{Abc}	14.93±0.93 ^{Bef}
	7	27.53±1.68 ^{Ad}	17.26±1.23 ^{Bcd}	27.53±3.98 ^{Ab}	15.86±0.46 ^{Bef}
	10	44.33±1.68 ^{Bc}	23.80±1.61 ^{Cbc}	50.86±1.68 ^{Aa}	21.00±0.80 ^{Cc}
	13	65.80±5.65 ^{Ab}	29.40±1.40 ^{Bb}	57.86±1.23 ^{Aa}	19.60±0.00 ^{Cdc}
	16	59.73±4.45 ^{Ab}	28.46±3.36 ^{Bb}	57.40±4.91 ^{Aa}	27.53±0.46 ^{Ba}
	20	109.20±5.30 ^{Aa}	56.46±4.93 ^{Ba}	56.93±2.59 ^{Ba}	24.73±1.23 ^{Cb}
	Ortalama±sd	**45.61±6.26** ^{A}	**24.79±2.86** ^{C}	**38.15±3.84** ^{B}	**19.30±0.99** ^{D}
TBA (mg MA/kg)	0	0.66±0.33 ^{d}	0.59±0.01 ^{bc}	2.31±1.40 ^{bc}	0.64±0.06 ^{b}
	2	0.83±0.46 ^{ABd}	0.29±0.07 ^{Bc}	1.82±0.37 ^{Ac}	0.54±0.19 ^{Bb}
	4	1.65±0.29 ^{Bcd}	0.92±0.15 ^{Bbc}	3.90±0.61 ^{Ab}	0.62±0.10 ^{Bb}
	7	5.23±0.78 ^{Acd}	1.14±0.11 ^{Bb}	2.03±0.16 ^{Bc}	0.75±0.19 ^{Bb}
	10	2.83±0.60 ^{Ab}	0.48±0.12 ^{Bbc}	3.53±0.08 ^{Abc}	0.61±0.13 ^{Bb}
	13	3.39±0.43 ^{Ac}	0.70±0.04 ^{Bbc}	4.04±0.41 ^{Ab}	0.43±0.06 ^{Bb}
	16	3.25±0.35 ^{Acd}	1.16±0.20 ^{Cb}	2.52±0.15 ^{Bbc}	1.20±0.05 ^{Cb}
	20	6.75±0.41 ^{Aa}	4.93±0.48 ^{Ba}	7.53±0.40 ^{Aa}	4.19±0.60 ^{Ba}
	Ortalama±sd	**3.18±0.44** ^{A}	**1.30±0.31** ^{B}	**3.51±0.40** ^{A}	**1.14±0.26** ^{B}
Peroksit (mmol O_2/kg)	0	0.60±0.20 ^{d}	1.20±0.60 ^{b}	1.15±0.35 ^{c}	0.50±0.10 ^{c}
	2	0.55±0.22 ^{d}	0.82±0.09 ^{b}	1.95±0.73 ^{bc}	1.37±0.57 ^{bc}
	4	1.33±0.16 ^{Bcd}	1.33±0.16 ^{Bb}	2.16±0.16 ^{Abc}	1.50±0.28 ^{Bbc}
	7	1.66±0.44 ^{cd}	1.33±0.16 ^{b}	1.50±0.28 ^{c}	1.66±0.44 ^{bc}
	10	4.66±1.09 ^{Ab}	1.00±0.28 ^{Bb}	1.66±0.33 ^{Bc}	1.00±0.00 ^{Bc}
	13	2.35±0.19 ^{c}	2.44±0.29 ^{a}	3.20±0.23 ^{b}	2.65±0.59 ^{b}
	16	2.20±0.35 ^{cd}	1.54±0.08 ^{b}	2.18±0.22 ^{bc}	1.51±0.20 ^{bc}
	20	6.91±0.57 ^{Aa}	3.10±0.35 ^{Ca}	5.72±0.50 ^{ABa}	4.64±0.45 ^{BCa}
	Ortalama±sd	**2.62±0.46** ^{A}	**1.61±0.17** ^{B}	**2.49±0.31** ^{A}	**1.91±0.28** ^{B}
pH	0	6.77±0.02 ^{b}	6.77±0.01 ^{b}	6.74±0.03 ^{a}	6.78±0.02 ^{a}
	2	6.72±0.02 ^{b}	6.74±0.01 ^{bc}	6.69±0.02 ^{a}	6.74±0.02 ^{a}
	4	6.71±0.03 ^{Ab}	6.71±0.02 ^{Abcd}	6.66±0.02 ^{ABa}	6.61±0.02 ^{Abc}
	7	6.59±0.01 ^{Ac}	6.60±0.02 ^{Ae}	6.51±0.04 ^{Bbc}	6.50±0.01 ^{Bd}
	10	6.56±0.02 ^{Bc}	6.69±0.00 ^{Abcd}	6.48±0.01 ^{Cc}	6.50±0.01 ^{Cd}
	13	6.59±0.02 ^{Ac}	6.63±0.01 ^{Ade}	6.51±0.01 ^{Bbc}	6.50±0.00 ^{Bd}
	16	6.68±0.00 ^{Ab}	6.67±0.03 ^{Ade}	6.54±0.02 ^{Bbc}	6.56±0.02 ^{Bd}
	20	6.95±0.05 ^{Aa}	6.93±0.05 ^{Aa}	6.56±0.01 ^{Bb}	6.66±0.04 ^{Bb}
	Ortalama±sd	**6.70±0.02** ^{A}	**6.72±0.02** ^{A}	**6.58±0.02** ^{B}	**6.60±0.02** ^{B}

A: Bütün/vakumsuz; **B:** Temizlenmiş/vakumsuz; **C:** Bütün/vakumlu; **D:** Temizlenmiş/vakumlu; **TVB-N:** Total volatile basic nitrogen (Toplam uçucu bazik azot); **TBA:** Tiyobarbitürik asit; **sd:** Standard deviation (Standart sapma); ^{ABCD}**:** Aynı satırdaki farklı harfler gruplar arasındaki farklılığı göstermektedir ($p<0.05$); ^{abcde}**:** Aynı sütundaki farklı harfler zamanlar arasındaki farklılığı göstermektedir ($p<0.05$).

Tablo 5: İnci kefali örneklerindeki kimyasal analiz bulgularının ve pH değerlerinin grup ortalamaları ve zamana göre değişimleri.

Table 5: Group averages and their changes over time of chemical analysis findings and pH values in pearl mullet samples.

	n	TVB-N (mg/100 g)	TBA (mg MA/kg)	Peroksit (mmol O_2/kg)	pH
Muamele					
Bütün	48	41.88±3.67 ^{a}	3.34±0.29 ^{a}	2.55±0.27 ^{a}	6.64±0.01 ^{b}
Temiz	48	22.05±1.55 ^{b}	1.22±0.20 ^{b}	1.76±0.16 ^{b}	6.66±0.01 ^{a}
Ambalaj tipi					
Vakumsuz	48	35.20±3.73 ^{a}	2.24±0.30	2.11±0.25	6.71±0.01 ^{a}
Vakumlu	48	28.72±2.39 ^{b}	2.32±0.29	2.20±0.21	6.59±0.01 ^{b}

Tablo 5 (devamı): İnci kefali örneklerindeki kimyasal analiz bulgularının ve pH değerlerinin grup ortalamaları ve zamana göre değişimleri.

Table 5 (continued): Group averages and their changes over time of chemical analysis findings and pH values in pearl mullet samples.

Zaman (gün)					
0	12	14.58±0.50 ^{f}	1.05±0.38 ^{c}	0.86±0.18 ^{e}	6.76±0.01 ^{a}
2	12	17.15±0.94 ^{ef}	0.87±0.22 ^{c}	1.17±0.26 ^{de}	6.72±0.01 ^{b}
4	12	18.66±1.10 ^{de}	1.77±0.41 ^{b}	1.58±0.13 ^{cd}	6.67±0.01 ^{c}
7	12	22.05±1.91 ^{d}	2.29±0.56 ^{b}	1.54±0.15 ^{cd}	6.55±0.01 ^{e}
10	12	35.00±3.92 ^{c}	1.86±0.46 ^{b}	2.08±0.52 ^{bc}	6.56±0.02 ^{e}
13	12	43.16±5.92 ^{b}	2.14±0.49 ^{b}	2.66±0.18 ^{b}	6.55±0.01 ^{e}
16	12	43.28±4.88 ^{b}	2.03±0.28 ^{b}	1.86±0.14 ^{c}	6.61±0.02 ^{d}
20	12	61.83±9.28 ^{a}	5.85±0.45 ^{a}	5.09±0.46 ^{a}	6.77±0.05 ^{a}

TVB-N: Total volatile basic nitrogen (Toplam uçucu bazik azot); **TBA:** Tiyobarbitürik asit; **n:** Örnek sayısı; **^{abcdef}:** Aynı sütundaki farklı harfler balıklara yapılan temizleme/vakumlama işlemlerine göre oluşturulan gruplar ve zamanlar arasındaki farklılığı göstermektedir ($p<0.05$).

Tablo 6: İnci kefali örneklerinin vitamin A/D_3 miktarları (µg/100 g yaş doku) ve duyusal analiz puanları.

Table 6: Vitamin A/D_3 amounts (µg/100 g wet tissue) and sensory analysis scores of pearl mullet samples.

	Zaman (gün)	**GRUPLAR**			
		A	**B**	**C**	**D**
Vitamin A (µg/100 g)	0	42.43±1.45 ^{Ba}	61.06±2.66 ^{Aa}	57.10±2.38 ^{Aa}	54.63±5.22 ^{Aa}
	2	30.06±0.14 ^{b}	31.66±1.20 ^{b}	25.76±1.89 ^{c}	35.00±5.74 ^{b}
	4	27.03±3.24 ^{bc}	33.03±5.81 ^{b}	31.13±0.69 ^{b}	32.66±6.74 ^{b}
	7	21.30±4.02 ^{c}	19.33±2.30 ^{c}	15.90±0.75 ^{d}	16.45±3.55 ^{c}
	10	12.06±2.06 ^{d}	11.06±1.79 ^{cd}	14.33±0.24 ^{d}	13.40±1.81 ^{c}
	13	11.16±2.24 ^{d}	9.33±1.45 ^{d}	13.50±2.50 ^{d}	12.26±1.39 ^{c}
	16	8.70±1.68 ^{Ad}	7.50±0.86 ^{Bd}	13.00±2.00 ^{Ad}	9.83±1.48 ^{ABc}
	20	7.53±0.03 ^{d}	6.30±0.10 ^{d}	7.96±0.51 ^{e}	6.56±2.16 ^{c}
	Ortalama±sd	**20.03±2.51 ^{B}**	**23.11±3.85 ^{A}**	**22.72±3.24 ^{AB}**	**22.86±3.57 ^{AB}**
Vitamin D_3 (µg/100 g)	0	37.33±1.33 ^{b}	63.33±7.68 ^{ab}	50.33±18.20 ^{ab}	65.00±7.57 ^{a}
	2	30.00±4.16 ^{Bb}	28.66±4.09 ^{Bc}	32.00±4.04 ^{Bb}	53.00±8.02 ^{Aabc}
	4	57.00±10.78 ^{a}	62.33±11.66 ^{ab}	68.00±6.35 ^{a}	59.66±5.36 ^{ab}
	7	67.33±3.71 ^{a}	72.66±4.09 ^{a}	67.66±3.17 ^{a}	67.00±3.00 ^{a}
	10	40.67±4.05 ^{b}	32.67±16.42 ^{c}	46.67±2.40 ^{ab}	59.33±3.48 ^{ab}
	13	28.00±2.00 ^{Bb}	49.00±1.52 ^{Aabc}	29.00±1.00 ^{Bb}	44.00±3.05 ^{Abc}
	16	34.66±0.66 ^{Bb}	36.66±2.66 ^{Bbc}	49.33±5.69 ^{Aab}	41.33±1.33 ^{ABc}
	20	26.33±6.88 ^{b}	27.50±2.50 ^{c}	28.00±4.50 ^{b}	24.00±1.73 ^{d}
	Ortalama±sd	**40.16±3.24 ^{B}**	**47.43±4.27 ^{A}**	**47.13±3.92 ^{A}**	**51.00±3.21 ^{A}**
Duyusal analiz*	0	8.60±0.24 ^{a}	8.80±0.20 ^{a}	8.40±0.24 ^{a}	8.60±0.24 ^{a}
	2	7.80±0.37 ^{Aab}	8.20±0.20 ^{Aab}	7.00±0.00 ^{Bb}	8.00±0.31 ^{Aab}
	4	7.25±0.25 ^{Ab}	7.75±0.25 ^{Ab}	5.75±0.47 ^{Bc}	7.75±0.47 ^{Aab}
	7	4.80±0.20 ^{Bc}	7.40±0.50 ^{Ab}	4.60±0.40 ^{Bd}	7.40±0.40 ^{Ab}
	10	2.80±0.37 ^{Bd}	4.40±0.24 ^{Ac}	1.80±0.37 ^{Ce}	4.20±0.20 ^{Ac}
	13	2.00±0.31 ^{BCde}	2.80±0.20 ^{ABd}	1.20±0.20 ^{Ce}	3.20±0.37 ^{Ad}
	16	1.80±0.20 ^{Be}	3.00±0.31 ^{Ad}	1.40±0.24 ^{Be}	3.00±0.31 ^{Ad}
	20	Yapılmadı	Yapılmadı	Yapılmadı	Yapılmadı
	Ortalama±sd	**4.94±0.47 ^{B}**	**6.00±0.42 ^{A}**	**4.26±0.48 ^{C}**	**5.97±0.41 ^{A}**

A: Bütün/vakumsuz; **B:** Temizlenmiş/vakumsuz; **C:** Bütün/vakumlu; **D:** Temizlenmiş/vakumlu; ***:** 9.00-7.00 puan "çok iyi", 6.99-4.01 puan "iyi", 4.00 puan "tüketilebilir", <4.00 puan "bozulmuş"; **sd:** Standard deviation (Standart sapma); **^{ABC}:** Aynı satırdaki farklı harfler gruplar arasındaki farklılığı göstermektedir ($p<0.05$); **^{abcde}:** Aynı sütundaki farklı harfler zamanlar arasındaki farklılığı göstermektedir ($p<0.05$).

Tablo 7: İnci kefali örneklerindeki vitamin A/D_3 miktarlarının (µg/100 g yaş doku) ve duyusal analiz puanlarının grup ortalamaları ve zamana göre değişimleri.

Table 7: Group averages and their changes over time of vitamin A/D_3 amounts (µg/100 g wet tissue) and sensory analysis scores in pearl mullet samples.

	n	Vitamin A (µg/100 g)	Vitamin D_3 (µg/100 g)	Duyusal analiz*
Muamele				
Bütün	48	21.35±2.03	43.57±2.56 ^{b}	4.60±0.33 ^{b}
Temiz	48	22.99±2.59	49.21±2.65 ^{a}	5.98±0.29 ^{a}
Ambalaj tipi				
Vakumsuz	48	21.54±2.26	43.72±2.69 ^{b}	5.47±0.32 ^{a}
Vakumlu	48	22.79±2.39	49.06±2.52 ^{a}	5.11±0.33 ^{b}
Zaman (gün)				
0	12	53.80±2.51 ^{a}	54.00±5.63 ^{bc}	8.60±0.11 ^{a}
2	12	30.62±1.65 ^{b}	35.91±3.76 ^{de}	7.75±0.16 ^{b}
4	12	30.96±2.14 ^{b}	61.75±4.01 ^{ab}	7.12±0.27 ^{c}
7	12	18.40±1.38 ^{c}	68.81±1.71 ^{a}	6.05±0.35 ^{d}
10	12	12.71±0.79 ^{d}	44.83±4.73 ^{cd}	3.30±0.88 ^{e}
13	12	11.39±0.90 ^{d}	38.27±3.05 ^{d}	2.30±0.21 ^{f}
16	12	9.75±0.90 ^{de}	40.50±2.18 ^{d}	2.30±0.20 ^{f}
20	12	7.16±0.56 ^{e}	26.36±2.05 ^{e}	Yapılmadı

***:** 9.00-7.00 puan "çok iyi", 6.99-4.01 puan "iyi", 4.00 puan "tüketilebilir", <4.00 puan "bozulmuş"; **n:** Örnek sayısı; **^{abcdef}:** Aynı sütundaki farklı harfler balıklara yapılan temizleme/vakumlama işlemlerine göre oluşturulan gruplar ve zamanlar arasındaki farklılığı göstermektedir ($p<0.05$).

TARTIŞMA VE SONUÇ

Bu araştırma ile inci kefalinin bütün halde ve iç organları çıkarılıp vakumlu/vakumsuz olarak soğukta muhafaza edilme imkânları denenmiştir. Soğukta muhafaza edilen inci kefalindeki toplam aerob psikrofil mikroorganizma (TAPM) sayısı grupların hepsinde muhafaza süresince artmış, başlangıçta 3-4 log kob/g olan bu sayılar 7. gün bütün gruplarda 6-7 log kob/g seviyelerine ulaşmış ve 20. gün sırasıyla 8.57±0.02 log kob/g, 8.31±0.24 log kob/g, 7.66±0.22 log kob/g ve 7.57±0.02 log kob/g olarak belirlenmiştir (Tablo 2). Benzer şekilde farklı balıklarda yapılan bazı çalışmalarda da (Mol ve ark. 2007; Özpolat 2020; Çakmak ve Sancak 2023; Vurat ve Kocatepe 2023) psikrofil mikroorganizmaların arttığı bildirilmiştir. Bu araştırmada TAPM sayısı yönünden en fazla artış vakumsuz olarak ambalajlanan örneklerde (A, B) görülmüş ve bu mikroorganizma üzerine temizlemenin etkisi önemsiz bulunurken ambalaj tipi ile zamanın etkisi önemli ($p<0.05$) bulunmuştur (Tablo 3). Soccol ve ark. (2005) vakumsuz olarak ambalajlanan kontrol grubu tilapia (*Oreochromis niloticus*) filetolarındaki TAPM sayısını vakumlu olanlardan daha yüksek bulmakla birlikte iki grup arasında önemli bir fark olmadığını bildirmişlerdir. Balıkların avlanmasını takiben solungaç, deri ve sindirim kanalında bulunan psikrofil mikroorganizmalar hızla çoğalarak balığın tüm kaslarına yayılmaktadır. Nitekim balık etinin pH değerinin 6.60-6.70 arasında olması, bağ doku oranının az olması ve mukoza ile derinin yüksek oranda rutubet içermesi mikroorganizmaların üreyerek yayılmasını kolaylaştırmaktadır (Huss 1995; Forsythe 2020; Parthiban ve Felix 2022). Soccol ve ark. (2005) psikrofil mikroorganizmalarla ilgili bir sınır olmamakla birlikte fazla miktarda bulunan bu mikroorganizmaların ürünün raf ömrünün azalmasına neden olduğunu, Huss (1995) da psikrofil mikroorganizmaların 10^7-10^8 kob/g'a ulaştığında balığın bozulmuş olarak nitelendirilebileceğini belirtmişlerdir. İncelenen inci kefali örneklerindeki TAPM sayıları da muhafazanın 10. gününden itibaren yaklaşık olarak bu seviyelere ulaşmıştır (Tablo 2).

İnci kefali örneklerinde laktik asit bakterileri (LAB) muhafaza süresince bütün gruplarda artmış ($p<0.05$) ve en fazla artışın A grubunda (8.02±0.03 log kob/g) olduğu görülmüştür (Tablo 2). Ayrıca, LAB sayısı yönünden bütün ile temizlenmiş ve vakumsuz ile vakumlu örnekler arasındaki fark ve zamanın etkisi önemli ($p<0.05$) bulunmuştur (Tablo 3). Bu araştırmanın bulgularına benzer olarak farklı balıklarda yapılan bazı çalışmalarda da (İzgi ve Çiftçioğlu 1997; Stamatis ve Arkoudelos 2007; Çakmak ve Sancak 2023) LAB sayısının arttığı bildirilmiştir. Gram ve Huss (1996), balıkların bozulmasında laktik asit bakterilerinin etkili olduğunu bildirmişlerdir. Bu araştırmada da incelenen bütün gruplarda muhafazanın ilerleyen günlerinde LAB'nin yüksek miktarlarda belirlenmiş olması, bu mikroorganizmaların balıkların bozulmasında etkili olduğunu düşündürmektedir.

Maya-küf sayısı bütün gruplarda düzensiz bir seyir izlemiş, muhafaza süresinin ilerleyen günlerinde 5 log kob/g seviyesine kadar artan bu sayının daha sonraki günlerde azaldığı görülmüştür (Tablo 2). Maya-küf sayısı bakımından bütün ile temizlenmiş ve vakumsuz ile vakumlu örnekler arasında önemli farklılıklar ($p<0.05$) belirlenmiştir (Tablo 3). Örneklerde belirlenen maya-küfler, balıkların laboratuvara getirilmesi sırasındaki aşamalarda çevresel kaynaklardan bulaşmış olabilir. Nitekim soğukta muhafaza edilen balıklarda değişik miktarlarda maya-küf bulunabileceği ve balıklardaki küflerin bozulmalara neden olabileceği belirtilmektedir (Baygar ve Varlık 2004; Forsythe 2020). Bu araştırmada özellikle A grubunda yüksek miktarlarda belirlenen maya-küfler, bu gruptaki balıkların daha kısa sürede bozulmasında rol oynamış olabilir.

İncelenen örneklerin hiçbirinde fekal streptekoklara rastlanmamıştır. Koliform grubu mikroorganizmalar da

başlangıçta hiçbir grupta belirlenmemiş (<1.00 log kob/g), ancak muhafaza süresince bütün gruplarda düzensiz değişimler olduğu görülmüştür (Tablo 2). Bu durum kontaminasyon düzeyinin nispeten düşük olması ve mezofil olan bu mikroorganizmaların düşük muhafaza ısısında üreyememesinden kaynaklanmış olabilir. Koliform grubu mikroorganizmalar bakımından bütün ile temizlenmiş ve vakumsuz ile vakumlu örnekler arasındaki fark ve zamanın etkisi önemli ($p<0.05$) bulunmuştur (Tablo 3).

Vakumsuz muhafaza edilen inci kefali örneklerinde (A, B) muhafazanın sonunda *Pseudomonas* spp. sayılarında artış görülürken, vakumlu örneklerde (C, D) ise bu sayıların azaldığı gözlenmiştir (Tablo 2). Bu mikroorganizma yönünden bütün ile temizlenmiş örnekler arasında önemli bir fark görülmezken, vakumsuz ile vakumlu örnekler arasındaki fark önemli ($p<0.05$) bulunmuştur (Tablo 3). Bu araştırmanın bulgularına benzer olarak, Ekici ve Alisarli (2008) tarafından yapılan bir çalışmada da inci kefalinde aerob şartlarda muhafaza süresince *Pseudomonas* spp.'nin arttığı bildirilmiştir. Stamatis ve Arkoudelos (2007) sardunya filetolarında *Pseudomonas* spp.'nin muhafaza süresince vakumlu ve vakumsuz örneklerde de arttığını bildirmişler, ancak bu araştırmada sadece vakumsuz örneklerde artış olmuştur. Sardunyada *Pseudomonas* spp. belirlenmediğini bildiren Erkan ve ark. (2006)'nın bulguları ise bu araştırmanın bulgularından farklıdır. Depolama süresine bağlı olarak balığın mikroflora kompozisyonu değişmekte, aerob şartlarda depolanan balıklarda *Pseudomonas* spp. 1-2 hafta sonra artmakta ve bu sayı vakumlu muhafaza edilen balıklarda aerob şartlarda depolanan balıklardan daha düşük olmaktadır (Huss 1995). Bu araştırmada da vakumlu örneklerdeki *Pseudomonas* spp. sayısı, Huss (1995)'un ifadelerine paralel olarak daha düşük belirlenmiştir. Ayrıca balıkların bozulmasında, *Pseudomonas* spp.'nin etkili olduğu belirtilmektedir (Huss 1995; Gram ve Huss 1996). Bu araştırmada *Pseudomonas* spp.'nin A ve B gruplarında muhafaza süresince artış göstermiş olması, bu mikroorganizmanın vakumsuz olarak ambalajlanan balıkların daha kısa sürede bozulmasında etkili olduğunu düşündürmektedir.

Su ürünlerinin tazeliğinin belirlenmesinde önemli olan TVB-N miktarı bozulmaya paralel olarak artış göstermektedir (Lang 1983). Bu araştırmada TVB-N miktarları muhafaza süresince bütün gruplarda artmış (Tablo 4), gruplar arasındaki fark ve zamanın etkisi de önemli ($p<0.05$) bulunmuştur (Tablo 5). Benzer şekilde bazı çalışmalarda da (Şengör ve ark. 2000; Mol ve ark. 2007; Alice ve ark. 2020; Das ve ark. 2021; Çakmak ve Sancak 2023; Vurat ve Kocatepe 2023) incelenen balıklarda muhafaza süresince TVB-N miktarlarının arttığı belirtilmiştir. Connell (1995) ve Huss (1995) yeni avlanmış balıklardaki TVB-N miktarının 5-20 mg/100 g arasında olabileceğini belirtmişler ve bu araştırmada da başlangıçta belirlenen TVB-N miktarları (13.06-16.80 mg/100 g) bu sınırlar içerisinde kalmakla birlikte, Sarı ve ark. (2004)'nın taze inci kefalinde bildirdikleri ortalama değerden (10.24 mg/100 g) yüksek bulunmuştur. Ayrıca bu araştırmada belirlenen TVB-N miktarları, muhafaza süresince tilapia filetolarında artış olmadığını ve soğukta muhafaza edilen vakumlu/vakumsuz örnekler arasında fark olmadığını belirten Soccol ve ark. (2005)'nın bulgularından farklıdır. Bu farklılıklar incelenen su ürünlerinin türü, beslenme durumları, avlanma mevsimleri, bölgeleri ve derinlikleri ile muhafaza sürelerinden kaynaklanmış olabilir.

Kietzmann ve ark. (1969) TVB-N miktarına göre balık ve su ürünlerinin kalite yönünden "çok iyi" (25 mg/100 g), "iyi" (30 mg/100 g), "pazarlanabilir" (35 mg/100 g) ve "bozulmuş" (>35 mg/100 g) olarak sınıflandırılabileceğini ifade etmişlerdir. Bu değerlendirmeye göre temizlenerek vakumlanmış örnekler (D) muhafaza süresinin sonuna kadar "çok iyi" sınıfına girmekle birlikte, bütün halde vakumsuz olarak muhafaza edilen örneklerin (A) 10. gün, bütün halde vakumlanarak muhafaza edilen örneklerin (C) 13. gün ve iç organları çıkarılıp temizlenerek vakumsuz bir şekilde muhafaza edilen örneklerin (B) ise 20. gün "bozulmuş" sınıfına girdiği görülmüştür. Tatlı su balıklarında TVB-N miktarları yönünden tüketilebilirlik sınır değerinin 32-36 mg/100 g olduğu ve bunun balık türlerine göre farklılık gösterebileceği belirtilmektedir (Lang 1983). Bunlarla birlikte köpek balığı ve vatoz gibi üre yönünden zengin balıklar için ise TVB-N miktarları yönünden tüketilebilirlik sınır değeri 50 mg/100 g olarak bildirilmiştir (Ludorff ve Meyer 1973). İnci kefali de alkali göl suyunda yaşayabilmek ve iyon dengesini koruyabilmek için vücudunda üre biriktirmektedir (Sarı ve ark. 2004). Bu durumlar dikkate alınarak TVB-N için Kietzman ve ark. (1969)'nın bildirdikleri tüketilebilir sınır değerleri yerine, diğer araştırmacılar tarafından belirtilen değerlerin alınması ve duyusal test puanlarıyla da desteklenerek bu değerlerin bir miktar üzerine çıkılması daha uygun olabilir. Ayrıca bölge halkının beslenmesi ve ekonomik kalkınmasına katkı sağlayan inci kefali için uygulanabilecek TVB-N sınır değerlerinin belirlenebilmesi açısından daha kapsamlı çalışmaların yapılması da faydalı olacaktır.

TBA miktarları tüm gruplarda düzensiz bir seyir izleyerek, muhafaza süresinin sonunda artış göstermiştir. Bu araştırmada belirlenen TBA miktarları yönünden bütün olarak ambalajlanan gruplardaki (A, C) artış, iç organları çıkarılarak ambalajlanan gruplardakinden (B, D) daha fazla olmuştur (Tablo 4). Ayrıca, TBA bakımından bütün ile temizlenmiş örnekler arasındaki fark ile örneklerin muhafaza edildiği sürelerin etkisi önemli ($p<0.05$) bulunmuştur (Tablo 5). Bu araştırmanın bulgularına benzer şekilde bazı araştırmacılar (Demirci ve Orak 1999; Köse ve ark. 2000; Erkan ve ark. 2006) inceledikleri örneklerde TBA miktarlarının düzensiz bir seyir izlediğini ve muhafaza süresince arttığını bildirmişlerdir. Balıktaki fazla yağ ve protein miktarının balık etindeki lipit peroksidasyon hassasiyetini artırabileceği belirtilmektedir (Kyrana ve Lougovois 2002). Aubourg (1993) da malonaldehitin balık vücudundaki farklı komponentlerle (proteinler, aminler, amino asitler, nükleotidler, nükleik asitler, fosfolipitler, diğer aldehitler) interaksiyona girebildiği ve bu interaksiyonların farklı balıklara göre büyük değişiklikler gösterebildiği için sadece TBA miktarı değerlendirilerek lipit oksidasyonunun gerçek oranının açıklanamayabileceğini ifade etmiştir.

Peroksit sayısının lipit oksidasyonunun erken aşamalarını gösterdiği ve oluşan lipit oksidasyonunun yağlı balıkların raf ömrünü sınırlandırdığı bildirilmektedir (Pacheco-Aguilar ve ark. 2000). Bu araştırmada dört grupta da düzensiz bir seyir izleyen peroksit sayıları muhafaza süresinin sonunda artmış, en yüksek değer (6.91±0.57 mmol O_2/kg) A grubunda 20. günde belirlenmiş (Tablo 4) ve bütün ile temizlenmiş örnekler arasındaki fark önemli ($p<0.05$) bulunmuştur (Tablo 5). Bu araştırmanın bulgularına benzer şekilde, yapılan farklı çalışmalarda (Pacheco-Aguilar ve ark. 2000; Erkan ve ark. 2006) balıklarda peroksit sayısının düzensiz bir seyir izleyerek muhafazanın sonunda yükseldiği bildirilmiştir. İnci kefaline göre biraz daha yağlı bir balık olan istavrit üzerine yapılan bir çalışmada da (Demirci ve Orak 1999), farklı soğutma ortamlarında 18 gün süreyle muhafaza edilen

örneklerde başlangıçta tespit edilemeyen peroksit sayısının muhafaza süresinin sonunda arttığı belirtilmiştir.

Örneklerde belirlenen pH değerleri (6.48-6.95) tüm gruplarda muhafazanın sonuna doğru düşme eğilimi göstermiş (Tablo 4), benzer bulgular Stamatis ve Arkoudelos (2007) tarafından da bildirilmiştir. pH değerlerinin balıklara göre değişebildiği ve balık eti için tüketilebilirlik değerlerinin 6.80-7.00 arasında olabileceği belirtilmektedir (Ludorff ve Meyer 1973; Connell 1995). Balık etinde bulunan ve tamponlama etkisi olan çözünebilir proteinler, peptidler ve trimetilamin pH değişimini maskeleyebilmektedir (Soccol ve ark. 2005). pH değerini etkileyen birçok faktör olduğundan, Köse ve ark. (2000)'nın da ifade ettikleri gibi bu değer tek başına kesin bir kriter olarak değerlendirilmemeli ve diğer kalite kontrol parametrelerini destekleyici olarak kullanılmalıdır. Nitekim bu araştırmada da belirlenen pH değerleri incelenen örneklerin tüketilebilirlik sınırları içerisinde kaldığına işaret etse de, TVB-N miktarları ve duyusal analiz puanları balıkların bozulduğunu göstermektedir. İzgi ve Çiftçioğlu da (1997) inceledikleri ürünlerde benzer bir duruma rastladıklarını belirtmişlerdir. Bu durumlar katabolik olaylar sırasında nötrleşmelerin olabileceğini düşündürmektedir.

Gıdalardaki vitamin A'nın yıkımlanması genellikle doymamış yağ asitlerinin oksidatif parçalanmasına paralel olarak veya serbest radikallerin dolaylı etkisiyle oluşmaktadır. Vitamin D_3 de oksidatif bozulmaya hassas olmakla birlikte gıdalardaki kaybı çok fazla olmamaktadır (Gregory 2017). Bu araştırmada da farklı şekillerde soğukta muhafaza edilen inci kefalinde muhafaza süresince vitamin A miktarları azalırken vitamin D_3 miktarları düzensiz bir seyir izlemiş, ayrıca vitamin D_3 kaybı vitamin A'ya göre daha az olmuştur (Tablo 6). Bununla birlikte vitamin A miktarları yönünden bütün ile temizlenmiş ve vakumsuz ile vakumlu örnekler arasında istatistiksel olarak bir fark görülmezken zamanın etkisi önemli ($p<0.05$) bulunmuş, vitamin D_3 miktarları yönünden ise hem gruplar arasındaki farkın hem de zamanın etkisinin önemli ($p<0.05$) olduğu görülmüştür (Tablo 7).

Panelistler tarafından inci kefali örneklerine verilen duyusal analiz puanları icelendiğinde (Tablo 6), tüm gruplarda başlangıçta 8.40-8.80 arasında olan puanların muhafaza süresince düştüğü ve bütün halde ambalajlanan gruplardaki düşüşün daha fazla olduğu (1.40±0.24, 1.80±0.20) görülmektedir. Duyusal analiz puanları bakımından bütün ile temizlenmiş ve vakumsuz ile vakumlu örnekler arasındaki fark ve muhafaza süresinin etkisi önemli ($p<0.05$) bulunmuştur (Tablo 7). Yapılan bazı çalışmalarda (İzgi ve Çiftçioğlu 1997; Erkan ve ark. 2006; Stamatis ve Arkoudelos 2007), vakumlu olarak veya modifiye atmosferde ambalajlanan örneklerin duyusal analiz puanlarının vakumsuz olarak ambalajlananlardan daha iyi olduğu belirtilmiştir. Özogul ve ark. (2004) inceledikleri vakumlu ambalajlanan sardunyalardaki raf ömrünün (12 gün) vakumsuz ambalajlananlardan (3 gün) daha fazla olduğunu, Soccol ve ark. (2005) ise vakumsuz ve vakumlu ambalajlanan tilapia filetoları arasında önemli bir fark olmadığını bildirmişlerdir. Bu araştırmada duyusal analiz puanlarına göre; A grubundaki örnekler 4. güne kadar "çok iyi", 7. güne kadar "iyi" ve 10. gün "bozulmuş"; B ve D grubundaki örnekler 7. güne kadar "çok iyi", 10. güne kadar "iyi" ve 13. gün "bozulmuş"; C grubundaki örnekler ise 2. güne kadar "çok iyi", 7. güne kadar "iyi" ve 10. gün "bozulmuş" olarak değerlendirilmiştir (Tablo 6). Bütün halde vakumlu olarak ambalajlanan grupta (C) vakumlamanın etkisiyle balıkların solungaçlarından çıkan kanlı sıvı ve sindirim sisteminden çıkan içerik, daha muhafazanın ilk günlerinden itibaren iyi karşılanmayan bir görünüm ve arzu edilmeyen bir kokunun şekillenmesine neden olmuştur.

Gıdaların kalite kontrolünde, duyusal analizler önemli bir parametre olarak kabul edilmektedir. Kietzmann ve ark. (1969), depolanan ürünlerin kalitesini belirleyen en önemli kriterin duyusal analiz sonuçları olduğunu, kimyasal veya mikrobiyolojik kalite parametreleri yönünden kabul edilebilir nitelikte olan bir ürünün duyusal özellikler açısından kabul edilemez bir nitelik taşıyorsa o ürünün tüketilemeyeceğini bildirmişlerdir. Ayrıca Taliadourou ve ark. (2003) mikrobiyolojik, kimyasal ve duyusal analizler arasında daima iyi bir korelasyon olmadığını, Tejada ve Huidobra (2002) da TVB-N miktarının balıklarda geç arttığını belirtmişlerdir. Bu araştırmada belirlenen TVB-N miktarları duyusal analiz bulgularını desteklemekle birlikte, bulgular arasında tam bir uyum görülmemektedir. Nitekim D grubunda duyusal analiz puanlarına göre 13. gün "bozulmuş" olarak değerlendirilen balıkların aynı günkü TVB-N miktarlarına göre "iyi" sınıfa girmesi, diğer araştırmacıların görüşleri ile paralellik göstermektedir.

TBA miktarının çok iyi bir materyalde "3'den az olması", iyi bir materyalde "5'den fazla olmaması" ve tüketilebilir sınır değerlerinin ise "7-8 mg MA/kg" olması (Schormüller ve Heimann 1969); peroksit sayısının da çok iyi bir materyalde "2'den az olması", iyi bir materyalde "5'den fazla olmaması" ve tüketilebilir sınır değerlerinin ise "8-10 mmol O_2/kg" olması (Ludorff ve Meyer 1973) gerektiği bildirilmiştir. Bu araştırmada TBA miktarları ve peroksit sayıları düzensiz bir seyir izlemiş, muhafaza süresince belli bir artış olmuşsa da balıklar kokuştuğu halde bu bulgular tüketilebilirlik sınırlarının çok altında kalmıştır (Tablo 4). Belirlenen TBA miktarları duyusal analiz bulgularını desteklemediği gibi, TVB-N miktarları ile de uyum göstermemiştir. Benzer şekilde Sarı ve ark. (2004) yeni bir yöntemle tuzlanan inci kefali örneklerinde TBA miktarını tüketilebilirlik sınırları içerisinde (3.56-3.69 mg MA/kg), TVB-N miktarını ise bu sınırın üzerinde (42.15-45.37 mg/100 g) tespit edildiğini bildirmişlerdir. Köse ve ark. (2000) da buzdolabında muhafaza edilen mezgit, tirsi ve hamsilerde TBA miktarlarının düzensiz bir seyir izlediğini ve TBA miktarlarının sadece hamsi örneklerinde bozulmayı desteklediğini bildirmişler, ayrıca pH ve TBA parametrelerinin balık türleri ile diğer koşullara göre değişiklik gösterebileceğini ve bu parametrelerin güvenilir olmadığını belirtmişlerdir. Pacheco-Aguilar ve ark. (2000) yaptıkları bir çalışmada TBA miktarı ve peroksit sayısının lipit oksidasyonunun bir indikatörü olarak kabul edildiğini belirtmişlerdir. Şengör ve ark. (2000) ise az yağlı balıklarda yağ oksidasyonundan ziyade, mikroorganizma faaliyeti ve enzimlerin etkisiyle oluşan kimyasal değişimlerin incelenmesini tavsiye etmişlerdir. İnci kefalindeki yağ miktarı da %3.34-%3.81 arasında değiştiğinden (Sarı ve ark. 2004), az yağlı bir balık olan inci kefalinde TBA miktarı ve peroksit sayısının tazeliğin belirlenmesinde kullanılabilecek güvenilir kriterler olmadığı düşünülmektedir.

Sonuç olarak; yapılan tüm analizlerden elde edilen bulgular göz önüne alındığında soğukta muhafaza edilen inci kefalinin bütün halde 7 gün, baş ve iç organları çıkarıldığında ise 10 gün süreyle kalite kaybına uğramadan muhafaza edilebileceği değerlendirilmiştir. Ayrıca inci kefalinin temizlendikten sonra vakumla ambalajlanması, kabul edilebilir duyusal özellikler ve raf ömrü bakımından daha olumlu etkiler göstermiştir. Bunlarla birlikte, bütün haldeki inci kefalinin vakumlanarak ambalajlanmasının

soğukta muhafaza için uygun bir yöntem olmadığı kanaatine varılmıştır. Nitekim vakumlamanın etkisiyle bütün haldeki balıkların solungaçlarından çıkan kanlı sıvı ve sindirim sisteminden çıkan içerik ambalajın içinde birikmiş ve muhtemelen bu ortamda üreyen mikroorganizmalar arzu edilmeyen bir görünüm ile kötü bir koku oluşturmuştur. Buna bağlı olarak da muhafazanın ilerleyen günlerinde ambalajlarda vakum yapılmamış gibi gevşemeler oluştuğu görülmüştür. Baş ve iç organları çıkarılarak temizlenmiş balıkların piyasada bulunmasının, temizleme zahmetini ortadan kaldıracağı için tüketiciler açısından balık tüketimini teşvik edebileceği düşünülmektedir. İşlenmiş ürünler daha az yer kaplayacağından taşıma/pazarlama işlemleri kolaylaşabilir ve ambalajlanmış ürünler de dış etkilerden korunduğu için balıklar en az kalite kaybı ile tüketicilere sunulabilir. Böylece bölgede hijyenden uzak bir şekilde sürdürülen pazarlama şekilleri terk edilerek balıkların ambalajlı bir şekilde ve hijyenik şartlarda uygun teknolojik alt yapıya sahip satış yerlerinde pazarlanmasına katkıda bulunulabilir.

ÇIKAR ÇATIŞMASI

Yazarlar bu çalışma için herhangi bir çıkar çatışması olmadığını beyan ederler.

TEŞEKKÜR VE BİLGİLENDİRME

Bu araştırma TÜBİTAK Çevre, Atmosfer, Yer ve Deniz Bilimleri Araştırma Grubu tarafından "102Y094" nolu proje olarak desteklenmiştir.

Bu araştırmanın gerçekleşmesinde önemli katkıları bulunan Prof. Dr. Candan VARLIK, Prof. Dr. Suphi DENİZ, Prof. Dr. Özkan ÖZDEN, Prof. Dr. Taçnur BAYGAR'a ve istatistiksel analizlerin yapılmasında yardımcı olan Prof. Dr. Ecevit EYDURAN'a teşekkür ederiz.

YAZAR KATKILARI

Fikir/Kavram: ES
Denetleme/Danışmanlık: ES, KE, HS, İHY, HAD
Veri Toplama ve/veya İşleme: ES, KE, HS
Analiz ve/veya Yorum: ES, KE, HS, İHY, HAD
Makalenin Yazımı: ES, HS
Eleştirel İnceleme: ES, KE, HS, İHY, HAD

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Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Okur S, Yanmaz LE, Orhun ÖT et al. (2024).** The Agreement of Intraocular Pressure Measurement in Healthy Merinos Sheep Using Rebound Tonometer (Tonovet®) and Applanation Tonometer (Tono-Pen Vet™). *Van Vet J*, 35 (3), 184-188. DOI: https://doi.org/10.36483/vanvetj.1513839

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

The Agreement of Intraocular Pressure Measurement in Healthy Merinos Sheep Using Rebound Tonometer (Tonovet®) and Applanation Tonometer (Tono-Pen Vet™)

Sıtkıcan OKUR[1,*] Latif Emrah YANMAZ[2] Ömer Tarık ORHUN[1] Yakup KOCAMAN[1] Ayşe GÖLGELİ BEDİR[1] Ferda TURGUT[1] Mumin Gökhan ŞENOCAK[1] Esra MODOĞLU[1] Uğur ERSÖZ[1]

[1] *Atatürk University, Faculty of Veterinary Medicine, Department of Surgery, 25240, Erzurum, Türkiye*
[2] *Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Surgery, 15030, Burdur, Türkiye*

Received: 10.07.2024 Accepted: 07.11.2024

ABSTRACT The assessment of intraocular pressure (IOP) holds significant importance in ophthalmology as a crucial diagnostic tool for various ocular disorders. This study aimed to evaluate the agreement between rebound (TonoVet®, TV) and applanation (Tono-Pen Vet™, TPV) tonometers in measuring IOP in healthy Merino sheep. 155 healthy Merinos (80 males, 75 females) with a mean weight of 54.4±8.7 kg, aged 24±6 months, were included in the study. IOP was measured between 9:00 and 11:00 am using both the rebound and applanation tonometers. The rebound tonometer was used first, followed by the applanation tonometer. A total of 620 readings (310 readings for each tonometer) were obtained from the two devices. No statistical differences were noted between the mean IOP measurements of the right and left eyes for both tonometers ($p>0.05$). However, there was a significant difference in the mean IOP measurements between the TV (11.8±2.3 mmHg) and the TPV (13.9±2.9 mmHg) tonometers ($p<0.001$). The concordance correlation coefficient indicated weak agreement strength ($\rho c=0.319$, CI 95% = -0.169 to 0.455) between the TV and TPV. The mean difference in bias and the 95% limits of agreement for the differences between TV and TPV were -2.1 mmHg (-9.0 to 3.5 mmHg). The regression equation derived from a Bland-Altman plot, describing the relationship between the two tonometers, was Y = 1.43 - 0.33X (Y = TV and X = TPV). In conclusion, the TPV measured higher IOP values compared to the TV, and due to the significant bias and limits of agreement, the two tonometers should not be used interchangeably for IOP measurement in Merino sheep.

Keywords: *Agreement, Intraocular pressure, Ophthalmology, Sheep, Tonometer.*

ÖZ

Sağlıklı Merinos Koyunlarında Rebound Tonometre (Tonovet®) ve Applanasyon Tonometresi (Tono-Pen Vet™) Kullanılarak Yapılan Göz İçi Basıncı Ölçümlerinin Uyumu

Göz içi basıncının (GİB) değerlendirilmesi, çeşitli oküler hastalıklar için önemli bir teşhis aracı olarak oftalmolojide büyük önem taşımaktadır. Bu çalışmanın amacı, sağlıklı Merinos koyunlarında GİB ölçümünde rebound (TonoVet®, TV) ve aplanasyon (Tono-Pen Vet™, TPV) tonometreleri arasındaki uyumu değerlendirmektir. Ortalama 54.4±8.7 kg ağırlığında, 24±6 aylık, 155 sağlıklı Merinos (80 erkek, 75 dişi) koyunu çalışmaya dahil edilmiştir. GİB sabah 9:00 ile 11:00 arasında hem rebound hem de aplanasyon tonometreleri kullanılarak ölçülmüştür. IOP ölçümleri için önce rebound, ardından aplanasyon tonometresi kullanıldı. İki cihazdan toplam 620 okuma (göz başına 310 okuma) elde edilmiştir. Her iki tonometre için sağ ve sol gözlerin ortalama GİB ölçümleri arasında istatistiksel bir fark kaydedilmemiştir ($p>0.05$). Ancak, TV (11.8±2.3 mmHg) ve TPV (13.9±2.9 mmHg) tonometreleri arasında ortalama GİB ölçümleri açısından anlamlı bir fark belirlendi ($p<0.001$). Çalışma verileri, korelasyon katsayısının TV ve TPV arasında zayıf uyum gücü ($\rho c=0.319$, CI %95 =-0.169 ila 0.455) olduğunu göstermiştir. TV ve TPV arasındaki farklar için ortalama yanlılık farkı ve %95 uyum sınırları -2.1 mmHg (-9.0 ila 3.5 mmHg) olarak belirlendi. İki tonometre arasındaki ilişkiyi tanımlayan Bland-Altman grafiğinden elde edilen regresyon denklemi Y = 1.43 – 0.33X (Y = TV ve X = TPV) olarak tanımlandı. Sonuç olarak, TPV, TV'ye kıyasla daha yüksek GİB değerleri ölçmüştür ve önemli sapma ve uyum sınırları nedeniyle, iki tonometre Merinos koyunlarında GİB ölçümü için birbirinin yerine kullanılmamalıdır.

Anahtar Kelimeler: *İntraoküler basınç, Koyun, Oftalmoloji, Tonometre, Uyum.*

INTRODUCTION

Ciliary activity is responsible for the production of aqueous humor, which occurs through active secretion, as

✉ *Corresponding author: sitkican.okur@atauni.edu.tr

well as passive processes, such as plasma diffusion and ultrafiltration. The balance between the production and outflow of aqueous humor is a determinant of intraocular pressure (IOP). Measurement of IOP is an essential component of ophthalmic examinations, serving as an important indicator of ocular diseases (Gum et al. 2007; Broadwater et al. 2008). The study of ocular function in sheep has considerable relevance within comparative ophthalmology research. Sheep serve as a suitable model for investigating the mechanisms underlying glaucoma, specifically corticosteroid-induced glaucoma (Gerometta et al. 2009; Gerometta et al. 2010). Additionally, the size and volume of the sheep's eye are comparable to that of humans, for instance, with an anterior-posterior axis of approximately 27 mm and an equatorial axis of approximately 30 mm (Gerometta et al. 2010). Although manometry is the only method to achieve correct IOP measurements, it is invasive and unsuitable for clinical examination. For this reason, tonometry, which is an indirect non-invasive measuring method, is commonly used as a device for measuring IOP obtained using indentation, applanation, or rebound methods (Bertens et al. 2021). The most widely used commercial tonometers in veterinary ophthalmology are the TonoVet® (rebound, TV) and Tono-Pen Vet™ (applanation, TPV) tonometer models (Lewin and Miller 2017). The TV tonometer included three different modes as follows; 'h' for horses, 'd' for dogs and cats, and 'p' for other species (Pereira et al. 2011). The TPV tonometer measures the force necessary to flatten a constant area of the central corneal surface, which indirectly measures IOP (Ahn et al. 2012), while the TV tonometer measures IOP depending on the deceleration of a moving magnetized probe as it comes into contact with the cornea. The magnet action in the probe induces a voltage in the solenoid; simultaneously, the moving parameters of the object are monitored as a digital signal (Chihara 2008). Previous studies compared the IOP measurement of TV and TPV in cats (von Spiessen et al. 2015), dogs (Kulualp et al. 2018), farm animals (Peche and Eule 2018), rabbits (Gloe et al. 2019), and porcine (Lewin and Miller 2017). However, to the author's knowledge, there have been no reported studies comparing the IOP measurement obtained by TV and TPV in Merinos sheep. Although spontaneous glaucoma is uncommon in sheep, they were used as a model of glaucoma (Gerometta et al. 2009). Therefore, it is important to assess the agreement of the tonometers for IOP measurement in Merinos sheep and to develop strategies for reducing potential biases in clinical and research assessments. The aim of the current study was to use the Bland–Altman limits of agreement technique to assess and compare the IOP measurement TV with TPV in healthy Merinos sheep.

MATERIAL AND METHODS

The protocol (2022/15) was approved by Atatürk University Local Board of Ethics Committee for Animal Experiments. The experimental study was carried out under the guidelines of the Association for Research in Vision and Ophthalmology Statement for the use of animal research in ophthalmic and vision research.

Animals

This study enrolled one hundred fifty-five (80 males and 75 females) healthy Merinos sheep, which were randomly assigned from the Food and Livestock Application and Research Center of Atatürk University. All of sheep in the study were investigated concerning non-pregnancy and were housed in the same pen under the same environmental, nutritional, and management conditions. Sheep were considered healthy based on physical examinations, complete blood counts, and ophthalmic examinations, including direct and indirect ophthalmoscopy (Aesculap AC635 C, Braun, Tuttlingen, Germany), rebound tonometry (Tonovet, Icare, Vantaa, Finland), Schirmer's tear test, and fluorescein test.

Study Design

The right and left eyes selected for the current study were randomly determined by a randomizer (Excel, Microsoft cooperation, Redmond, WA, USA) and the same eye measured first in all subsequent reading with both tonometers. The IOP measurement of each eye in sheep was first used with the rebound tonometer (TonoVet® [TV], using the "d" setting), which was programmed to achieve six successive rebounds and demonstrate the mean value of IOP. The TV tonometer was retained in a vertical position and the distance between the tip of probe and the central cornea was kept close to 4-8 mm as possible for each sheep. After the TV measurements, two drops of the local anesthetic agent 0.5% proparacaine hydrochloride (Alcaine®; Alcon-Couvreur, Puurs, Belgium) were administered to each eye. The applanation tonometer (Topo-Pen Vet™ [TPV], Reichert Technologies, Depew, New York, USA) was used to measure IOP in each eye following two min local anesthetic agent instilled. The probe tip of TPV was touched gently with the central cornea to obtain IOP measurement. All measurements IOP were recorded between 09:00 and 11:00 a.m. to minimize diurnal variations. Both tonometers were performed by the same operator to minimize individual variations. During the study, all measurements were employed with the animals in a sternal position and their heads and necks were gently restrained upright ahead to avoid potential incorrect readings in IOP measurement. The IOP measurements in both tonometers were repeated an error sign was encountered due to excessive deviation. If a third attempt to measure the animals' IOP on either tonometer were unsuccessful, then the animal was excluded from the study. Only readings with a maximum 5% deviation were collected with both tonometers. Three sequential readings (The average IOP measurement obtained after six rebounds for TV and the IOP measurement obtained by corneal touching for TVP were counted as one reading) were recorded in each eye using both tonometers. Tonometers were calibrated as instructed in the manufacturer recommendation and a new probe for TV and an ocu-film tip cover for TPV were replaced prior to use of each animal.

Statistical Analyses

A power calculation (PS-Power and Sample Size Calculation, Version 3.1.2, Vanderbilt University, TN, USA), conducted based on information reported by previous study (Peche and Eule, 2018), determined that a total of 310 readings for each tonometer (Type I error [α] of 5%, Type II [Power, β] of 95%) to detect a 20% difference (Standard deviation of ± 3 mmHg) in the IOP between tonometers.

All data were analyzed using the Medcalc version 20.015 (Medcalc Software, Ostend, Belgium). The normality distribution of the data was evaluated by a Kolmogorov–Smirnov test. A paired Student's t-test was performed to detect differences between the IOP measurement of the right and left eyes and the mean difference of IOP measurement in TV and TPV. The result of the data was presented as mean ± standard deviation. The concordance correlation coefficient was tested for the IOP measurement

of the tonometers, to evaluate relationship power. Agreement between TV and TPV was assessed using the Bland-Altman plot, in which the differences between tonometers were plotted against their mean IOP measurements and the limits of agreement (mean ± 1.96 × SD) (Bland and Altman 1986). The level of statistical significance was set at $p < 0.05$.

RESULTS

The mean weight of the sheep was 54.4±8.7 kg and they were aged 24±6 months. All the measurements of IOP with both tonometers were completed successfully. No signs of ocular discomfort or pain were noted throughout the study. A total of 310 readings for each tonometer were recorded. All data were stated in units based on mmHg. There were no statistical differences between the IOP measurements of right and left eyes in both tonometers ($p>0.05$, Table 1). Hence, the IOP measurements were recorded bilaterally on each sheep and their average measurement were used.

Table 1: The mean intraocular pressure (IOP, mmHg) measurements in the right and left eyes of sheep were measured using the rebound tonometer, TonoVet® (TV), and the applanation tonometer, Topo-Pen Vet™ (TPV).

Device	Eye	n	Mean± Standard Deviation (IOP, mmHg)	95% Cl for the mean	p
TV	Right	155	11.1±2.45	10.7 to 11.5	0.460
	Left	155	11.2±2.86	10.8 to 11.7	
TPV	Right	155	13.9±3.51	13.4 to 14.5	0.742
	Left	155	13.8±3.31	13.3 to 14.4	

Data were expressed as mean ± standard deviation. Cl; confidence interval

A significant difference was observed between TV (11.8±2.3 mmHg) and TPV (13.9±2.9 mmHg) tonometers in the mean IOP measurements ($p<0.001$, Fig. 1). The lowest (7 mmHg) IOP was measured by TV, while the highest (24 mmHg) was measured by TPV in sheep. The concordance correlation coefficient determined that poor strength of agreement was observed between tonometers. ($\rho c=0.319$, Cl 95%= -0.169 to 0.455, Fig. 2).

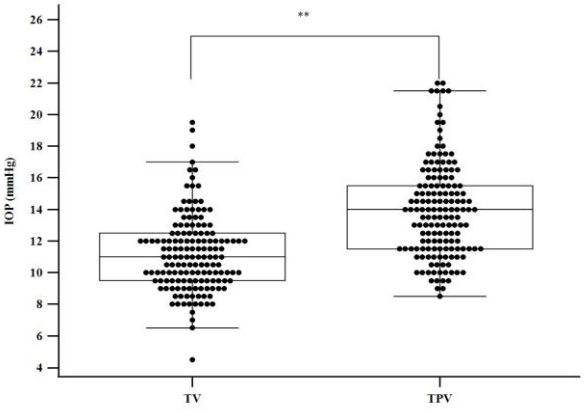

Figure 1: The minimum, mean, and maximum IOP measurements were ascertained differences between the rebound tonometer, TonoVet® (TV), and the applanation tonometer, Topo-Pen Vet™ (TPV). ** indicated significantly different between tonometer ($p<0.001$).

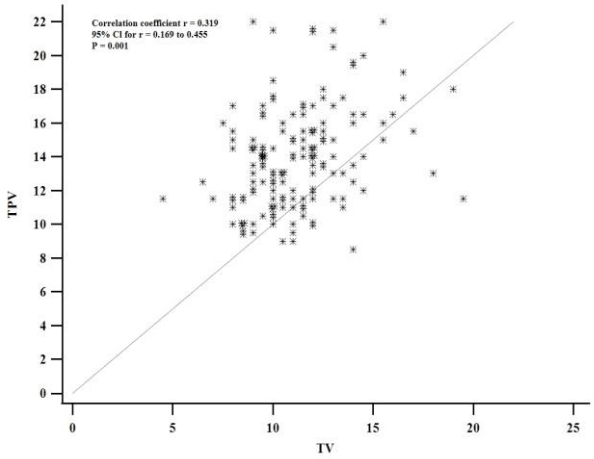

Figure 2: Correlation analysis of the rebound tonometer, TonoVet® (TV), and the applanation tonometer, Topo-Pen Vet™ (TPV). Cl; confidence interval.

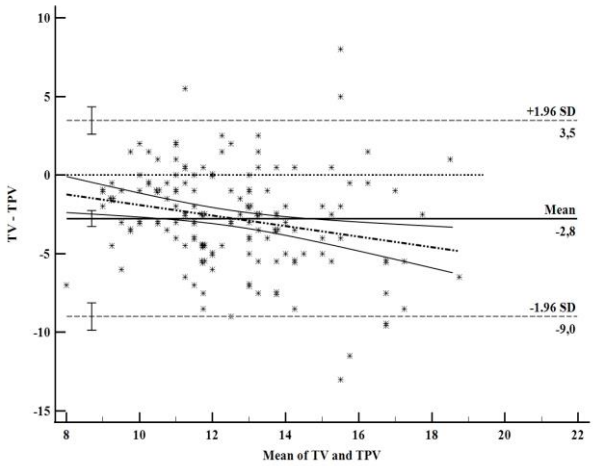

Figure 3: Bland-Altman limits of agreement plots comparing the rebound tonometer, TonoVet® (TV), and the applanation tonometer, Topo-Pen Vet™ (TPV). The solid black line is the mean difference in intraocular pressure (IOP, mmHg) with its 95% confidence interval (Cl) marked by the dotted black lines. The dotted horizontal line account for zero difference. The dashed-dot sloping black line depict the slope of the regression line for the linear relationship between IOP difference and mean of IOP with its 95% Cl.

Bland-Altman analysis for IOP measurement in the TV and TPV was depicted in Fig. 3. We found a significant difference in bias between the TV and TPV tonometers ($p<0.001$). Over the range of IOP measurement reported in the current study, the model expected that the mean IOP measure in the TPV was higher TV with the mean bias and 95% Cl for tonometer difference demonstrated in Fig. 3. According to the Bland-Altman plots, the differences in IOP (TV-TPV) are not near to zero, indicating that the studied methods do lack agreement. The mean difference and 95% limits of agreement for the differences between TV and TPV were -2.8 mmHg (-9.0 – 3.5 mmHg). The analysis of the regression relationship between the IOP difference and the mean IOP measurement, suggested that as the mean IOP increased, hence the IOP in the TPV increased relative to TV (Table 2). The models estimate that the IOP measured in TPV was mostly higher than TV, but this difference decreased as IOP increased.

Table 2: Bland-Altman analysis comparing intraocular pressure (IOP, mmHg) measured using the rebound tonometer, TonoVet® (TV), and the applanation tonometer, Topo-Pen Vet™ (TPV) and results of regressing the IOP difference TV (*Y*) and TPA (*X*) to quantify changes in bias.

Parameter estimate	Difference TPV (*X*) compared to TV (*Y*) TV - TPV
Mean Difference (95% Cl)	-2.76 (-3.26 to -2.25)
Limit of agreement	-9.0 to 3.5
$SE_{y.x}$	0.114
Lower / Upper Limit (95% Cl)	-8.9 (-9.8 to -8.1) / 3.5 (2.6 to 4.3)
Regression equation	*Y*=1.43-0.33*X*
Slope coefficient (95% Cl)	-0.33 (-0.56 to -0.10)
p*	<0.001

*Significance test for difference in slop from zero. Cl; confidence interval. Sy.x; standard error of estimate.

DISCUSSION AND CONCLUSION

The main focus of this study was to compare TV with TPV tonometer in healthy Merinos sheep. Although the result of the currents study indicated that both tonometers provided consistent results of IOP measurement individually, our findings suggested that both tonometers cannot be used interchangeably for IOP measurement in Merinos sheep due to the high bias and limit of agreement, and the concordance correlation coefficient indicated that the TV and TPV showed week strength of agreement. As previous studies have reported that the position of the head and body has a substantial effect on the IOP (Broadwater et al. 2008; Ghaffari and Gherekhloo 2018), all readings of the IOP were performed in a sternal position with the head and neck gently restrained in a normal and upright position for each sheep. In a previous study carried out sheep reported that TV provided more valuable readings on IOP with calibrated 'd' setting (Peche and Eule 2018). Thus, all measurement IOP in TV was used 'd' setting in the current study due to lack of specific setting for sheep. In addition, no topical anesthetic drug was used for the IOP measurement of TV without discomfort or corneal pain, in agreement with previous studies on other species (Leiva et al. 2006; Yanmaz et al. 2016; Lewin and Miller 2017; Bertens et al. 2021). The IOP measurement of TV was obtained prior to TPV, as a previous study indicated that rebound tonometry might be altered by topical anesthesia (Baudouin and Gastaud 1994). For both TV and TPV tonometers, the first measurement attempt failed because of blinking or error signs in five readings and a second attempt was performed to obtain readings. However, a repeated measurement may cause underestimation of IOP (Morris et al. 2006), and the first measurement attempt was noted to avoid or reduce this false reading of IOP. A previous study reported that differences in distance between the probe tip of TV and the cornea surface could be affected the IOP reading (Rodrigues et al 2021). For this reason, during the IOP measurement of TV was maintained the same distance between the probe tip and cornea surface by the same operator. In this study, the IOP measurement of TPV tended to be higher than TV (mean difference [95% Cl] = 2.8 mmHg). A similar result was previously reported in rabbits (Pereira et al. 2011), and dogs (Leiva et al. 2006) comparing rebound and applanation tonometer. This may be due to the instillation of topical anesthesia for IOP measurement of TPV. Although the effect of topical anesthetic on IOP is unknown in sheep, a previous study demonstrated that proparacaine hydrochloride caused a slight increase in IOP immediately after administration of the drug in dogs (Sarchahi and Eskandari 2019). Our results were contradictory with the research of Peche and Eule (2018), who found that the IOP measurement of applanation tonometer (Tono-Pen AVIA®) tended to be lower than TV. Although the TV measurements in both studies were consistent with each other (in our study; 11.8 ± 2.3 mmHg, Peche and Eule (2018); 12.2±3.1 mmHg), the applanation tonometers in both studies were inconsistent with each other (in our study; 13.9±2.9 mmHg, Peche and Eule (2018); 10.1±2.5 mmHg). This inconsistency could be due to the type of applanation tonometer that previously reported significant variability between IOP measured using different tonometers (Guresh et al. 2021). Additionally, Peche and Eule (2018) administered a different topical anesthetic drug (oxybuprocaine hydrochloride) compared with our study (proparacaine hydrochloride) prior to the measured IOP of the applanation tonometer. A previous study has reported that the different topical anesthetics have an effect on IOP measurement (Sarchahi and Eskandari 2019). Moreover, a study carried out in humans reported that oxybuprocaine significant decreases IOP (Almubrad and Ogbuehi 2007). Although spontaneous glaucoma is uncommon in sheep, experimental animal researches are still an essential part of developing new treatment procedures in glaucoma studies. Many different tonometers are presently utilized in animal experiments, the accuracy of which has previously been established in many species (Bertens et al. 2021; Lewin and Miller 2017; Kulualp et al. 2018). In this study, both tonometers were easily used for IOP measurement and well-tolerated in sheep. The TV tonometer was more rapid reading IOP compared with the TPV tonometer due to did not require administration of the topical anesthetic prior to IOP measurement. The disadvantage of the TV tonometer required to be held in a vertical position to the cornea with the tip of the probe parallel to the cornea surface, while the TPV can be utilized regardless of device position. In the present study, the Bland-Altman bias plot demonstrated that the level of agreement between IOP measurements using two tonometers was high in sheep. Additionally, we found that the correlation coefficient between TV and TPV was poor agreement strength. This incompatibility between the two tonometers can be explained in the same order of tonometers (first TV, second TPV) for IOP measurements in each sheep. During the study, the first IOP measurement was performed with TV by handling the animal which might have been caused by elevated mental stress levels in the sheep. Therefore, IOP measurements performed secondly to TPV may tend to be higher than TV in sheep. In addition, physical stress due to immobilization increased IOP, as reported in a study conducted with rabbits (Miyazaki et al. 2000). Another possible explanation is that both tonometers have different working mechanisms (rebound vs applanation). The main limitation of this study was not evaluating manometry, which is the most accurate method of IOP measurement, due to instrumental limitations. The IOP measurement could be influenced by central corneal thickness (CCT), as reported in previous studies (Yanmaz et al. 2016; Martin-suarez et al. 2014). Thus, the lack of CCT measurement can be considered as a limitation. A further limitation is that the repeatability and inter-operator variability were not evaluated in both tonometers. Another limitation of the study is that topical

anesthetic were used during TPV measurements, while no topical anesthetic was used during TV measurements. This may be a factor affecting the agreement between each other. In conclusion, given the data obtained in the current study, it is suggested by the authors that although both tonometers were easy to employ and consistent results individually, they cannot be used interchangeably for IOP measurement in healthy sheep due to the high bias and poor agreement strength according to the correlation coefficient.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: SO, LEY, AG
Supervision / Consultancy: SO, MGS, ÖTO, YK, FT
Data collection: SO, AG, UE, EM
Writing the Article: SO, AG
Critical Review: LEY, MGS, AG, FT

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Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Köse Sİ, Gönenci R (2024).** Evaluation of Cardiologic Alterations in Radiographs of Dogs with Bronchopneumonia Before and After Treatment. *Van Vet J*, 35 (3), 189-195. DOI: https://doi.org/10.36483/vanvetj.1538872

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

Evaluation of Cardiologic Alterations in Radiographs of Dogs with Bronchopneumonia Before and After Treatment

Serkan İrfan KÖSE[1,*] Ramazan GÖNENCİ[2]

[1] *Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Internal Medicine, 31060, Hatay, Türkiye*
[2] *Necmettin Erbakan University, Faculty of Veterinary Medicine, Department of Surgery, 42310, Konya, Türkiye*

Received: 26.08.2024 Accepted: 19.10.2024

ABSTRACT Pneumonia, a common respiratory disease in small animals, causes significant hypoxemia in dogs due to reduced lung capacity and cardiac output. In addition, pneumonia may lead to cardiac complications. This study aimed to evaluate the alterations in cardiac silhouette and main vessel views in dogs that underwent bronchopneumonia treatment, and the alterations were evaluated in radiographs of the dogs with bronchopneumonia before and after treatment. For this purpose, radiographic data from 26 dogs diagnosed with and treated for bronchopneumonia were analyzed. The measurements included left atrial size, long axis, short axis, the fourth thoracic vertebra length, aorta width, caudal vena cava width, and thoracic width. Radiographs obtained before and after treatment indicated no changes in the long and short axes of the vertebral hearth score, the fourth thoracic vertebrae length, aorta and caudal vena cava width, and thoracic width. However, in pre-treatment radiographs, the left atrial size was found to be larger compared to post-treatment radiographs. A strong positive correlation was observed between measurements of LAs and LAs/TV4 in both pre- and post-treatment radiographs, as well as in the measurement differences between pre- and post-treatment radiographs. As a consequence, the radiographs employed to monitor bronchopneumonia treatment may serve as an initial indicator of potential cardiac complications in affected dogs.

Keywords: *Dog, bronchopneumonia, radiograph, cardiac alterations, left atrial size.*

ÖZ

Bronkopnömonili Köpeklerin Tedavi Öncesi ve Sonrası Radyografilerinde Kardiyolojik Değişikliklerin Değerlendirilmesi

Küçük hayvanlarda yaygın bir solunum yolu hastalığı olan pnömoni, akciğer kapasitesinin ve kalp debisinin azalması nedeniyle köpeklerde önemli hipoksemiye neden olur. Ayrıca, pnömoni kardiyak komplikasyonlara da yol açabilir. Bu çalışmada bronkopnömoni tedavisi uygulanan köpeklerde kardiyak siluet ve ana damar görünümlerindeki değişikliklerin değerlendirilmesi amaçlanmış ve bronkopnömonili köpeklerin tedavi öncesi ve sonrası radyografilerindeki değişiklikler değerlendirilmiştir. Bu amaçla, bronkopnömoni teşhisi konulan ve tedavi edilen 26 köpeğe ait radyografik veriler analiz edilmiştir. Ölçümler sol atriyal boyut, uzun eksen, kısa eksen, 4. torasik vertebra uzunluğu, aort genişliği, kaudal vena kava genişliği ve torasik genişliği içeriyordu. Tedavi öncesi ve sonrasında çekilen radyografilerde vertebral kalp skorunun uzun ve kısa eksenlerinde, dördüncü torasik vertebra uzunluğunda, aort ve kaudal vena kava genişliğinde ve torasik genişlikte herhangi bir değişiklik saptanmamıştır. Ancak, tedavi öncesi radyografilerde sol atriyal boyut tedavi sonrası radyografilere kıyasla daha büyük bulunmuştur. Hem tedavi öncesi hem de tedavi sonrası radyografilerde LAs ve LAs/TV4 ölçümleri arasında ve tedavi öncesi ile sonrası radyografiler arasındaki ölçüm farklılıklarında güçlü bir pozitif korelasyon gözlenmiştir. Sonuç olarak, bronkopnömoni tedavisini izlemek için kullanılan radyografiler, etkilenen köpeklerde potansiyel kardiyak komplikasyonların ilk göstergesi olarak hizmet edebilir.

Anahtar Kelimeler: *Köpek, bronkopnömoni, radyografi, kardiyak değişiklikler, sol atriyal boyut.*

INTRODUCTION

In small animals, pneumonia is defined as inflammation of the lower airways and is one of the most commonly diagnosed respiratory diseases (Brady 2004; Ayodhya et al. 2013; Köse et al. 2021). Pneumonia causes variable degrees of ventilation/perfusion mismatch, potentially

✉ *Corresponding author: serkanirfankose@mku.edu.tr

leading to hypoxemia (Brady 2004). Significant hypoxemia in dogs with pneumonia has been reported in the literature (Wingfield et al. 1997; Brady 2004; Kogan et al. 2008). It is stated that in dogs with pneumonia, total lung capacity and functional residual capacity are severely reduced, and there is also an increase in cardiac output and a decrease in mean systemic arterial pressure and vascular resistance due to hypoxia (Hanly and Light 1987). Respiratory diseases and/or hypoxia (RD/H) are reported as the most common causes of pulmonary hypertension (PH) in dogs (Jaffey et al. 2019). A presumptive diagnosis of RD/H in dogs with PH is typically made entirely on the existence of respiratory symptoms and the exclusion of left-sided heart disease in undiagnosed dogs to determine the underlying cause (Jaffey et al. 2019). The systemic vasculature responds to hypoxia with vasodilation to more effectively perfuse hypoxic tissue, but the pulmonary vasculature responds with pulmonary artery vasoconstriction. Thus, the pulmonary arteries contract in order to take blood away from the diseased lungs while maintaining arterial oxygen concentration (Kellihan and Stepien 2010).

Thoracic radiography is a valuable technique for evaluating both thoracic and systemic illness (Rudorf et al. 2008). So, the pleural space, the mediastinum, the trachea and carina, the bronchi, the cardiac silhouette, the aorta, caudal vena cava, and pulmonary vasculature, and the lung can be easily evaluated thanks to thoracic radiographs (Rudorf et al. 2008; Bradley 2013). Although echocardiography is the gold standard for measuring heart dimensions and internal structures, thoracic radiographs remain the primary clinical diagnostic tool for dogs with suspected cardiac diseases and alterations (Lamb and Boswood 2002; Mostafa and Berry 2017; Mostafa et al. 2020; Tangpakornsak et al. 2023). The most prevalent abnormalities investigated by radiography for the heart are cardiomegaly, pericardial effusion, and chamber enlargements, especially left atrial enlargement (Mostafa et al. 2020).

A vertebral heart score (VHS) is a radiographic procedure to determine the cardiac size by using some measurements and calculations on the cardiac silhouette in the thoracic radiography (Buchanan and Bücheler 1995; Mostafa et al. 2020). However, in the detection of individual cardiac chamber enlargements, this measurement technique is reported as relatively poor (Buchanan and Bücheler 1995; Jojima et al. 2019). So, some researchers have modified the VHS technique to evaluate individual left atrial enlargement and added some new measurements (Sanchez et al. 2013; Jojima et al. 2019). Left atrium size can be measured on lateral radiographs by using a new line drawn at 45 degrees from the intersection of the long axis and short axis of the VHS (Sanchez et al. 2013; Jojima et al. 2019).

Although the heart and lungs are considered individual organs, they are strongly related as a part of the cardiorespiratory system. When abnormalities occur in these organs, they directly affect each other (Corrales-Medina et al. 2013; Restrepo and Reyes 2018). With this insight, in this study, it was aimed to evaluate the alterations in cardiac silhouette and main vessel views in dogs that underwent treatment for bronchopneumonia with the hypothesis that some radiographic alterations in cardiac silhouette and main vessel views (including the aorta and caudal vena cava) might occur in dogs with bronchopneumonia.

MATERIAL AND METHODS

The study was approved by the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (Approval number, 2015/10-10; Approval date, 30 Dec 2015).

A total of 116 dogs with respiratory complaints (cough, respiratory distress, nasal discharge, etc.) were evaluated at Hatay Mustafa Kemal University Veterinary Health, Practice, and Research Center Department of Internal Medicine between January 2017 and December 2018. A total of 41 dogs of different breeds, ages, and sexes with signs of lower respiratory tract disease (purulent nasal discharge, intermittent cough, wet rales, crackles, and wheezes on auscultation) were selected within these admissions. These animals underwent radiographic examination with a presumptive diagnosis of bronchopneumonia and were diagnosed and started on their treatment for bronchopneumonia based on their clinical presentations and radiographs. Of these 41 dogs that began treatment for bronchopneumonia, pre- and post-treatment radiographs of 26 dogs were selected according to the inclusion and exclusion criteria and constituted the material for this study.

Inclusion criteria were 1) radiographs in the left lateral and ventrodorsal position to evaluate the diagnosis and treatment outcome in radiographs, and 2) radiographic images of the same treated dog both before and after treatment.

Exclusion criteria for the study were 1) the dog's treatment was not completed for any reason (such as death, inability to reach the owner, or not brought in by the owner), 2) one of the lateral and/or ventrodorsal radiographs is missing, 3) the presence of additional radiographic findings such as cardiac-noncardiac pulmonary edema, pleural effusion, or pericardial effusion that may affect the cardiac silhouette and main vessel appearance on radiography, and 4) that the quality of the radiographic images was poor to measure the heart silhouette, main vessel views, and thoracic width.

Radiographic Evaluations

Thoracic radiographs in DICOM format were obtained from the patient database. The left lateral (LL) and ventrodorsal (VD) radiographs (60-80 kV and 5-20 mAs, Regius ∑II, Konica Minolta, Tokyo, Japan) were used to evaluate the cardiological views. The same expert veterinary surgeon evaluated all radiographs by using PDI DICOM Viewer V2.20, Konica Minolta, Inc. The cardiological views in radiographs of dogs with bronchopneumonia were evaluated before and after treatment. Left Atrial Size (LAs), Long Axis (LA), Short Axis (SA), 4th Thoracic Vertebra Length (TV4), Aorta Width (Aw), Caudal Vena Cava Width (CVCw), Thoracic Width (Tw), and the ratios of Left Atrial Size to Aorta Width (LAs/Aw), Left Atrial Size to Caudal Vena Cava Width (LAs/CVCw), and Left Atrial Size to 4th Thoracic Vertebra Length (LAs/TV4) were measured and calculated in the radiographs as the cardiological views. Left Atrial Size, LA, SA, 4TV, Aw, and CVCw were measured in the LL radiographs (Figure 1), and Tw was measured in the ventrodorsal radiographs (Buchanan and Bücheler 1995; Estrada and Fox-Alvarez 2016) (Figure 2). All measurements were performed as described by Buchanan and Bücheler (1995), Hansson et al. (2005), and Jojima et al. (2019).

Statistical Analysis

For categorical variables, descriptive statistics were presented as frequencies and percentages, and for continuous variables, as arithmetic means and standard errors. The normality assumption was checked with the Shapiro-Wilk Test. Comparison of the data of radiographical views in pre- and post-treatment radiographs was performed with a paired t-test if the data held the normality assumption or a Wilcoxon rank test if the data did not hold the normality assumption. The relationships between data of radiographical views were specified with Spearman's rank correlation coefficient due to the normality assumption not holding. The association was classified as weak, moderate, strong, or perfect when the correlation coefficient value was .1-.3, .4-.6, .7-.9, or 1, respectively (Bagardi et al. 2021). A p value of <0.05 was considered statistically significant. All statistical analyses were performed using SPSS Statistics for Windows, Version 26.0.

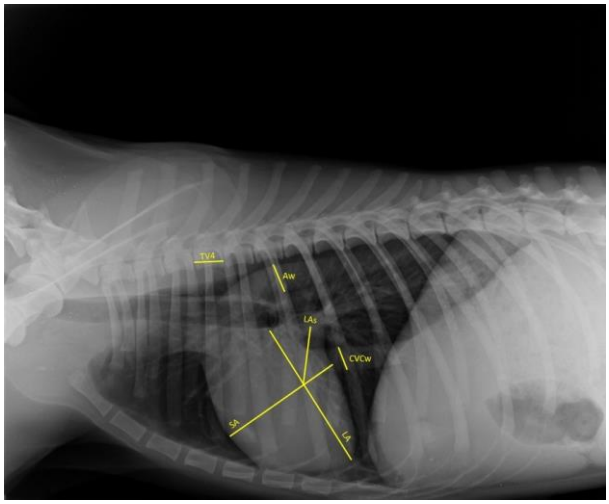

Figure 1: Measured cardiac views in L/L radiograph.

LAs: Left Atrial Size, LA: Long Axis, SA: Short Axis, TV4: 4th Thoracic Vertebra, Aw: Aorta Width, CVCw: Caudal Vena Cava Width

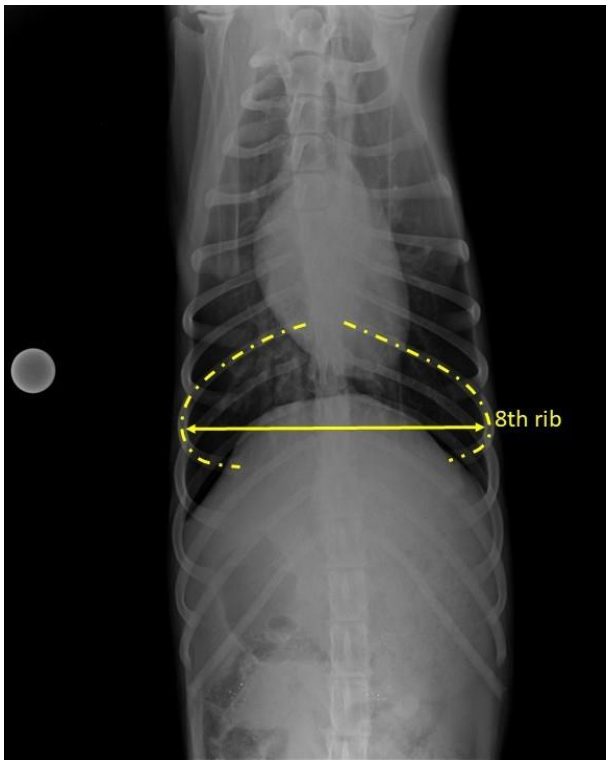

Figure 2: Measuring of thoracic width in V/D radiograph.

RESULTS

The radiographic data from 26 dogs that underwent bronchopneumonia treatment were retrospectively analyzed. No difference between pre-treatment and post-treatment radiographs was detected in the long and short axes of VHS, 4th Thoracic Vertebra, the aorta and caudal vena cava width, and thoracic width (Table 1). On the other hand, the left atrial size was observed as approximately nine percent larger in pre-treatment radiographs than in post-treatment radiographs (Table 1). Pre-treatment radiographs showed higher ratios of left atrium size to aorta width, caudal vena cava width, and the fourth thoracic vertebra than post-treatment ones (Table 1). Correlations between measured parameters both in pre-treatment and post-treatment radiographs were given in Tables 2 and 3. The difference in measurement of left atrial size between pre- and post-treatment radiographs had a strong positive correlation with the ratio of fourth vertebrae and aortic width, but a moderate positive correlation with the ratio of caudal vena cava width (Table 4).

DISCUSSION AND CONCLUSION

As explained above, pneumonia may lead to cardiovascular complications by various pathways. In this study, some changes were observed in pre- and post-treatment radiographs from the dogs with bronchopneumonia, suggesting that pneumonia may cause cardiac changes, especially left atrial size. In this study, changes in the silhouette of the heart and the appearance of the main blood vessels were assessed in the radiographs of dogs treated for bronchopneumonia before and after treatment. The left atrial size was found to be larger in the pre-treatment radiographs than in the post-treatment radiographs. A strong positive correlation was identified between the measurement differences performed in the pre- and post-treatment radiographs for LAs and LAs/Aw, as well as LAs/TV4.

Whereas the heart and lungs are separate organs, they are inextricably linked as part of the cardiorespiratory system (Corrales-Medina et al. 2013; Restrepo and Reyes 2018). So, pneumonia and cardiovascular diseases are reported as causes of morbidity and mortality in veterinary medicine as well as in human medicine (Ilten et al. 2003; Kogan et al. 2008; Corrales-Medina et al. 2013; Jaffey et al. 2019; Restrepo and Reyes 2018). The occurrence of functional alterations in the cardiovascular system in the process of respiratory diseases, especially pneumonia, is supported by various studies that have been done with both humans and animals (Hanly and Light 1987; Ilten et al. 2003; Jaffey et al. 2019). These alterations are explained by the effects of pneumonia-related myocardial microorganism invasion, hypoxemia-related myocardial ischemia or infarction, deterioration in the hemostatic functions of the vascular endothelium, and renal dysfunction (Corrales-Medina et al. 2013).

Many respiratory disorders in dogs, such as pneumonia, infiltrative pulmonary disease, and tracheobronchial disease, have been linked to pulmonary hypertension (PHT) (Campbell 2007; Morita et al. 2018). PHT is a multifactorial disorder characterized by increased pulmonary vascular resistance due to vasoconstriction and vascular remodeling, triggered by hypoxemia, and the release of endogenous vasoactive mediators and mitogens from diseased pulmonary cells, activated platelets, and inflammatory cells (Voelkel and Tuder 1995; Campbell

2007). In dogs with severe respiratory disease, compensatory polycythemia develops in response to hypoxemia, increasing red blood cell concentration. This causes an exponential increase in blood viscosity, resulting in increased pulmonary vascular resistance and exacerbation of PHT (Campbell 2007). On the other hand, it is obvious that an increase in proteolysis, formation of thrombus, and generation of reactive oxygen species, all of which may raise the risk of significant cardiac events, occur in pneumonia in both animals and humans (Stotts et al. 2023).

The vertebral heart scale system (or vertebral heart size) is designed to objectively evaluate the size of the heart both in dogs of various breeds and thoracic conformations and in cats (Estrada and Fox-Alvarez 2016) by measuring and calculating the cardiac appearance in thoracic radiography (Buchanan and Bücheler 1995; Mostafa et al. 2020). On a lateral radiograph, the line drawn from the carina to the most ventral aspect of the heart is the long axis. The line drawn perpendicular to the long axis at the widest point of the heart and extending to the craniocaudal limits is the short axis (Figure 1) (Buchanan and Bücheler 1995; Hansson et al. 2005; Estrada and Fox-Alvarez 2016; Jojima et al. 2019). In addition to research showing that there is no change in VHS measurements in the lateral radiographic position (Buchanan and Bücheler 1995), researches have shown that the measurements are greater (approximately 0.3 vertebrae) in the right lateral radiographic position (Greco et al 2008; Jojima et al. 2019). The published range for a normal VHS on a lateral radiograph is 9.2 to 10.9 (Buchanan and Bücheler 1995; Gülanber et al 2005; Estrada and Fox-Alvarez 2016). In a study, VHS in dogs without cardiac disease is reported as 10.5±0.7 on the right lateral radiographs (Mostafa et al. 2020). Birks et al. (2017) report that the left lateral VHS was detected as 10.1 vertebrae. In the presented study, similar to previous studies (Buchanan and Bücheler 1995; Gülanber et al. 2005; Estrada and Fox-Alvarez 2016; Birks et al. 2019; Mostafa et al. 2020), pre- and post-treatment VHS on radiographs were detected as 10.22±0.13 and 10.32±0.12 vertebrae, respectively. Therefore, there was no statistical difference (p=0.191) between the pre- and post-treatment radiographs for VHS. The fact that there was no difference in the vertebral heart scale in the pre- and post-treatment radiographs confirms that there was no difference in the vertebral heart scale parameters: long axis (p=0.959), short axis (p=0.07), and length of the fourth thoracic vertebra (p=0.574) (Table 1).

Table 1: Comparison of pre-treatment and post-treatment X-Ray measurements and ratio calculations of cardiac views (mean±SEM).

	LAs	LA	SA	TV4	Aw	CVCw	Tw	LAs/Aw	LAs /CVCw	LAs/TV4
PreT	35.877 ±0.913	114.904 ±2.428	96.438 ±1.844	20.719 ±0.380	19.358 ±0.543	17.935 ±0.524	165.281 ±3.816	1.876 ±0.055	2.025 ±0.057	1.738 ±0.043
PostT	32.800 ±0.860	114.973 ±2.180	98.423 ±1.953	20.715 ±0.380	19.831 ±0.653	18.204 ±0.573	167.558 ±3.814	1.685 ±0.055	1.829 ±0.054	1.592 ±0.043
P value	***0.003***[1]	0.959	0.070	0.574	0.236	0.488	0.195	***0.002***[1]	***0.003***[1]	***0.005***[2]

[1]Paired t-test; [2]Wilcoxon test

PreT: Pre-treatment, PostT: Post-treatment, LAs: Left Atrial Size, LA: Long Axis, SA: Short Axis, TV4: 4th Thoracic Vertebra, Aw: Aorta Width, CVCw: Caudal Vena Cava Width, Tw: Thoracic Width

Table 2: Correlations in Pre-treatment X-Ray measurements and ratio calculations.

Parameters	LAs	LA	SA	TV4	Aw	CVCw	Tw	LAs/Aw	LAs /CVCw	LAs/TV4
LAs	1	**.576****	**.636****	**.429***	**.409***	**.529****	.057	**.482***	.373	**.730****
LA		1	**.622****	**.811****	**.726****	**.537****	.298	-.187	-.033	.000
SA			1	**.529****	**.693****	**.465***	.126	-.087	.131	.282
TV4				1	**.793****	**.578****	**.611****	-.373	-.212	-.302
Aw					1	**.714****	**.488***	**-.589****	-.360	-.170
CVCw						1	.356	-.213	**-.584****	.109
Tw							1	**-.414***	-.354	**-.405***
LAs/Aw								1	**.664****	**.795****
LAs /CVCw									1	**.568****
LAs/TV4										1

**Correlation is significant at the 0.01 level.

*Correlation is significant at the 0.05 level.

LAs: Left Atrial Size, LA: Long Axis, SA: Short Axis, TV4: 4th Thoracic Vertebra, Aw: Aorta Width, CVCw: Caudal Vena Cava Width, Tw: Thoracic Width

Table 3: Correlations in Post-treatment X-Ray measurements and ratio calculations.

Parameters	LAs	LA	SA	TV4	Aw	CVCw	Tw	LAs/Aw	LAs /CVCw	LAs/TV4
LAs	1	**.468***	**.654****	.279	**.472***	**.520****	-.105	.303	.292	**.757****
LA		1	**.757****	**.746****	**.704****	**.544****	**.492***	-.350	-.165	-.067
SA			1	**.677****	**.800****	**.647****	.266	-.322	-.130	.160
TV4				1	**.666****	**.439***	**.654****	**-.457***	-.160	**-.413***
Aw					1	**.780****	.324	**-.673****	**-.433***	-.013
CVCw						1	.137	-.357	**-.647****	.196
Tw							1	**-.418***	-.151	**-.529****
LAs/Aw								1	**.646****	**.616****
LAs /CVCw									1	.386
LAs/TV4										1

**Correlation is significant at the 0.01 level.

*Correlation is significant at the 0.05 level.

LAs: Left Atrial Size, LA: Long Axis, SA: Short Axis, TV4: 4th Thoracic Vertebra, Aw: Aorta Width, CVCw: Caudal Vena Cava Width, Tw: Thoracic Width

Table 4: Correlations in difference of Pre-treatment and Post-treatment X-Ray measurements and ratio calculations.

Parameters	LAs	LA	SA	TV4	Aw	CVCw	Tw	LAs/Aw	LAs /CVCw	LAs/TV4
LAs	1	.113	.260	-.224	.156	.311	-.017	**.725****	**.624****	**.996****
LA		1	-.172	-.002	.113	-.044	-.057	.015	.107	.128
SA			1	.000	.145	.165	.347	.107	.166	.272
TV4				1	-.025	-.056	.114	-.178	-.141	-.237
Aw					1	**.486***	-.003	**-.553****	-.256	.149
CVCw						1	.094	-.049	**-.525****	.302
Tw							1	.025	-.007	-.007
LAs/Aw								1	**.683****	**.735****
LAs /CVCw									1	**.637****
LAs/TV4										1

**Correlation is significant at the 0.01 level.

*Correlation is significant at the 0.05 level.

LAs: Left Atrial Size, LA: Long Axis, SA: Short Axis, TV4: 4th Thoracic Vertebra, Aw: Aorta Width, CVCw: Caudal Vena Cava Width, Tw: Thoracic Width

The absence of a difference in these parameters may be attributed to the utilization of left-sided radiographs. Furthermore, in consideration of the p-value approaching the significance level for the short axis of VHS, the small sample size may also be responsible for the absence of a difference in VHS.

In addition to mechanical function, the left atrium performs endocrine and regulatory roles (Boudoulas et al. 2014). Cardiopulmonary baroreceptors situated in the atria, pulmonary veins, and systemic vena cava help to regulate cardiac output by controlling venous return, pulmonary arterial and venous pressure, and pulmonary capillary blood flow (Aviado and Guevera Aviado 2001; Boudoulas et al. 2014). The aging process, neurohumoral stimulation, and chronic atrial stretch engage several signaling pathways, resulting in histological alterations in the atria (Boudoulas et al. 2014). Atrial remodeling refers to histologic changes such as myocyte hypertrophy, fibroblast proliferation, and complex alterations of the extracellular matrix, which may affect LA function (Nattel et al. 2008; Boudoulas et al. 2014). As a consequence of atrial remodeling, LA dilation, LA hypocontractility, and atrial fibrillation are likely to occur (Boudoulas et al. 2014). In dogs, some studies evaluate the vertebral left atrial size in radiographs to determine reference intervals for the evaluation of atrial enlargement (Malcolm et al. 2018; Sánchez Salguero et al. 2018; Vezzosi et al. 2020). In healthy dogs, Bagardi et al. (2022) report that the radiographic left atrial dimension on left lateral radiographs was determined to be 1.37±0.20 vertebrae, as well as 1.20±0.34 vertebrae on the right side. On the other hand, while it ranges between 2.1 and 2.8 vertebrae, the cut-off value has been reported to be ≥2.3 vertebrae for dogs with left atrial enlargement (Malcolm et al. 2018; Vezzosi et al. 2020). In this study, there was no difference between pre- and post-treatment cardiac and vascular

views in radiographs, except for the left atrial size (Table 1). Even though the left atrium size in the pre-treatment radiograph seems larger (Z: -2.831, p=0.005) than in the post-treatment radiograph, the left atrium size in both radiographs (pre-treatment=1.74±0.04 vertebrae; post-treatment=1.59±0.04 vertebrae) (Table 1) remained below the reference value (Malcolm et al. 2018; Vezzosi et al. 2020). Therefore, explaining this condition as a compensatory response of the heart to PHT triggered by hypoxemia that is likely to develop as a result of bronchopneumonia may be a more accurate approach than atrial enlargement.

In veterinary medicine, the most often used technique for assessing left atrial size is the left atrial-to-aortic root ratio, which gives a body weight-independent measurement of left atrial size (Wesselowski et al. 2014). In the recent studies (Sanchez et al. 2013; Mostafa and Berry 2017; Mostafa et al. 2020; Tangpakornsak et al. 2023), the fourth thoracic vertebra length is used for the evaluation of cardiac alterations in radiographs, especially for left atrial enlargement. With this approach, in this study, the left atrial size measurement was ratioed to aorta width, caudal vena cava width, and fourth thoracic vertebra length, which were obtained on the thoracic radiograph. The difference was detected for LAs/Aw, LAs/CVCw, and LAs/TV4 ratios between pre- and post-treatment radiographs. All ratios were observed to be higher in the pre-treatment radiographs than the post-treatment radiographs (Table 1). Authors have reported that the left atrial size in the left lateral radiographs was weakly correlated (r=0.60) with the left atrium to aorta ratio detected by using echocardiographic measurement (Jojima et al. 2019). In a similar methodology to that employed by Jojima et al. (2019), Bagardi et al. (2021) report a moderate correlation (r=0.591) between the left atrial dimension as observed in right lateral radiographs and the left atrium to aorta ratio. In this study, contrary to Jojima et al. (2019) and Bagardi et al. (2021), a strong positive correlation (r=0.725) was identified between the LAs and the LAs/Aw ratio in measurement differences between pre- and post-treatment radiographs (Table 4). The dissimilarity in findings may be attributable to a discrepancy in methodology, whereby Jojima et al. (2019) and Bagardi et al. (2021) employed a comparison of radiographic measurements (LAs) with echocardiographic measurements (LA/Ao), in contrast to the approach taken in our study.

A strong positive correlation was detected between LAs and LAs/TV4 in pre- and post-treatment measurements (Tables 2 and 3), as well as for measurement differences between pre- and post-treatment radiographs (Table 4). The strong correlation between the differences in LAs and LAs/TV4 ratio measurements in pre-treatment and post-treatment radiographs (Table 4) may be related to the changes that are likely to occur in the aforementioned left atrium (Aviado and Guevera Aviado 2001; Boudoulas et al. 2014) rather than the length of the 4th thoracic vertebra, which showed almost no difference in both measurements (Table 1). In fact, the difference in the size of the left atrium pre- and post-treatment radiographs may also support this idea (Table 1). Therefore, the 4th thoracic vertebra length ratio may be usable to assess changes in the left atrial silhouette.

The main limitation of this study designed as a retrospective study may be the lack of echo or Doppler ultrasonographic examination that would allow comparison of changes in cardiac and vascular silhouettes in radiographs. Another limitation of this study may be the lack of etiology, symptom duration and blood gas data.

Finally, in dogs with bronchopneumonia, it is thought that the radiographs used to monitor the treatment process may provide an early warning of dogs with cardiac complications. In addition, it is also thought that further comprehensive studies consisting of echo and Doppler ultrasound examinations, right lateral thoracic radiographs, and laboratory analyses (blood gases analyses, canine troponin I, NT-proBNP etc.) should be performed for the evidence of internal and functional cardiac alterations in dogs with pneumonia.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGMENT

We would like to thank all MSc students for their assistance in clinical processes.

The radiographic data, which were obtained from the dogs previously used in the project supported by Hatay Mustafa Kemal University Scientific Research Projects Coordination Unit with grant number 15244, were used in this study.

AUTHOR CONTRIBUTIONS

Idea / Concept: SİK
Supervision / Consultancy: SİK, RG
Data Collection and / or Processing: SİK
Analysis and / or Interpretation: SİK, RG
Writing the Article: SİK, RG
Critical Review: RG

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Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Aktaş MS, Eren E, Aydın Ö et al. (2024).** Research on hematology, inflammatory and antimicrobial peptide levels according to clinical scoring in calves with Bovine Respiratory Disease (BRD). *Van Vet J*, 35 (3), 196-201.
DOI: https://doi.org/10.36483/vanvetj.1543947

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

Research on Hematology, Inflammatory and Antimicrobial Peptide Levels according to Clinical Scoring in Calves with Bovine Respiratory Disease (BRD)

Mustafa Sinan AKTAŞ[1] Emre EREN[1,*] Ömer AYDIN[1] Kerim Emre YANAR[1] Sefa KÜÇÜKLER[2] Muhammed Sertaç EROĞLU[1] Şükrü DEĞİRMENÇAY[1] Murat İLGÜN[3]

[1] *Atatürk University, Faculty of Veterinary Medicine, Department of Internal Medicine, 25010, Erzurum, Türkiye*
[2] *Atatürk University, Faculty of Veterinary Medicine, Department of Biochemistry, 25010, Erzurum, Türkiye*
[3] *DVM, Veterinarian, Pethome Veterinary Clinic, 34290, İstanbul, Türkiye*

Received: 05.09.2024 Accepted: 04.10.2024

ABSTRACT Bovine respiratory disease (BRD) is a significant and costly disease in cattle, characterized by various infections with distinct causes and clinical signs. This study focuses on investigating some hematological and inflammatory parameters, as well as cathelicidin antimicrobial peptide (CAMP) parameters, in calves with BRD. Forty-two calves were allocated to three groups based on clinical scoring: Group I (n=10, healthy, score 0), Group II (n=16, BRD, score 3), and Group III (n=16, BRD, score 4). Some hematological (WBC, NEU, LYM, NLR, and PLT), inflammatory (IL-1β, TNF-α, NF-κB, and IL-10), and CAMP parameters were evaluated in all groups. Group III had significantly higher WBC, NEU, and NLR concentrations than Group I, while concentrations of PLT in Group II were higher than Group I ($p<0.05$). NF-κB, TNF-α, and CAMP levels were enhanced in Group III compared to Group I, and CAMP levels were higher in Group II than in Group I $p<0.05$). Strong positive correlations were found between NEU and WBC and NLR. Weak positive correlations existed between WBC, NEU, IL-10, and CAMP, as well as between LYM and IL-1β and NF-κB and CAMP. In conclusion, the most severe inflammation was observed in Group III, aligning with clinical scoring in BRD-affected calves. CAMP was identified as a reliable marker for inflammation assessment. Additionally, NLR, being low-cost and easily measurable, showed promise as an inflammation indicator.

Keywords: *Anti-inflammatory, Bovine Respiratory Disease, Calf, Cathelicidin antimicrobial peptide, Neutrophil-Lymphocyte Ratio, Pro-inflammatory.*

ÖZ

Sığır Solunum Yolu Hastalıklı (BRD) Buzağılarda Klinik Skorlamaya Göre Hematoloji, İnflamatuar ve Antimikrobiyal Peptid Düzeylerinin Araştırılması

Sığır solunum yolu hastalığı (BRD), sığırlarda farklı nedenleri ve klinik belirtileri olan çeşitli enfeksiyonlarla karakterize önemli ve maliyetli bir hastalıktır. Bu çalışma, BRD'li buzağılarda bazı hematolojik ve inflamatuvar parametrelerin yanısıra katelisidin antimikrobiyal peptid (CAMP) parametrelerinin araştırılmasına odaklanmıştır. 42 buzağı klinik skorlamaya gore üç gruba ayrılmıştır: Grup I (n=10, sağlıklı, skor 0), Grup II (n=16, BRD, skor 3) ve Grup III (n=16, BRD, skor 4). Bazı hematolojik (WBC, NEU, LYM, NLR ve PLT), inflamatuvar (IL-1β, TNF-α, NF-κBve IL-10) ve CAMP parametreleri değerlendirilmiştir. Grup III'te WBC, NEU ve NLR konsantrasyonları Grup I'e göre istatistiksel olarak yüksekken, Grup II'de PLT konsantrasyonları Grup I'e göre istatistiksel olarak daha yüksekti ($p<0.05$). NF-κB, TNF-α ve CAMP düzeyleri Grup III'te Grup I'e kıyasla artarken, CAMP düzeyleri Grup II'de Grup I'e kıyasla daha yüksekti ($p<0.05$). NEU ile WBC ve NLR arasında güçlü pozitif korelasyonlar bulundu. WBC, NEU, IL-10 ve CAMP arasında ve ayrıca LYM ile IL-1β ve NF-κB ile CAMP arasında zayıf pozitif korelasyonlar mevcuttu. Sonuç olarak, BRD'den etkilenen buzağılarda klinik skorlama ile uyumlu olarak en şiddetli inflamasyon Grup III'te gözlenmiştir. CAMP, inflamasyon değerlendirmesi için güvenilir bir belirteç olarak tanımlanmıştır. Ayrıca, düşük maliyetli ve kolay ölçülebilir NLR, bir yangı göstergesi olarak umut vaat etmektedir.

Anahtar Kelimeler: *Anti-inflamatuar, Sığır Solunum Hastalığı, Buzağı, Cathelicidin antimikrobiyal peptid, Nötrofil-Lenfosit Oranı, Pro-inflamatuar.*

✉ *Corresponding author: emreeren@atauni.edu.tr

INTRODUCTION

Respiratory tract infections in livestock farming cause serious economic losses such as mortality, loss of yield, and growth retardation. Bovine respiratory disease (BRD) of calves is defined as a multi-etiological disease condition in which many bacterial and viral agents play a role (Edwards 2010). The etiology of this complex is predominantly caused by bacterial and viral agents (Panciera and Confer 2010). During the initial stages of the disease, rapid and shallow breathing (hyperventilation) is evident. Dyspnea occurs when the disease progresses and most of the lung tissue loses its function. Another important finding is cough. Nasal and ocular discharges, fever, anorexia, depression, and increased respiratory rate may accompany these symptoms. As the disease worsens, dyspnea, severe lacrimation, and closed nostrils are observed. Hoarse sounds are heard on lung auscultation. The animal shows the posture of extending its neck forward and moving its elbows away from its body (Guterbock 2014). The clinical severity of the disease can also be scored by using the above-mentioned clinical signs (Perino and Apley 1998).

The neutrophil-lymphocyte ratio (NLR) is a cost-effective and easily accessible metric that provides information on both the cellular immunological and systemic inflammatory response. It is calculated by dividing the number of neutrophils (NEU) by the number of lymphocytes (LYM). NLR represents the equilibrium between NEU, the inherent immune response, and LYM, the acquired immune response, during inflammatory and diseased conditions. It stands as a crucial parameter for identifying sepsis, inflammation, and infections. NLR monitoring has been shown to be beneficial, especially in severe pneumonia cases (Buonacera et al. 2022).

Cytokines are small proteins that provide intercellular communication. These proteins contain anti-inflammatory cytokines, as well as pro-inflammatory cytokines. Proinflammatory cytokines are mostly produced by activated macrophages. While these increase in case of inflammatory reaction, anti-inflammatory cytokines provide an immunoregulatory effect regulating pro-inflammatory cytokines (Winter et al. 2007). NF-κB plays a pivotal function in tissue inflammation and the activation of the immune system. NF-κB selectively attaches to the exclusive DNA-binding sites of genes associated with pro-inflammatory cytokines, including TNF-α and IL-1β (Gloire et al. 2006). The activation of NF-κB is stimulated by various factors, potentially including pro-inflammatory cytokines like TNF-α and IL-1β. TNF-α is the foremost cytokine in bacterial sepsis or endotoxemia (Batra et al. 2011). Because of the increased TNF-α level in bacterial sepsis in previous animal models (Hesse et al. 1988), it was thought that there may be a relationship between the severity of the disease and TNF-α level in calves with BRD. IL-1β plays a crucial function in stimulating the immune system during inflammation associated with fever infectious diseases (Zhang and An 2007). IL-10 is a cytokine with anti-inflammatory properties and important functions in the immune response against pathogenic factors, providing homeostasis and preventing damage to the host (Iyer and Cheng 2012). Cathelicidins are small cationic peptides synthesized by innate immune cells and epithelial cells, and they serve crucial functions in defending the airway's epithelial surfaces. Cathelicidins can prevent infection directly by stimulating the recruitment of immune cells in the respiratory tract. They exhibit extensive antimicrobial activity against bacteria, viruses, and parasites (Tecle et al. 2010).

Identifying the causative agent of respiratory tract infections in calves and routinely diagnosing the disease remain formidable challenges (de Carvalho et al. 2016). The objective of this research was to examine the levels of pro-inflammatory and anti-inflammatory cytokines, antimicrobial peptide concentrations, and NLR, along with clinical scoring, as a supplementary diagnostic method for evaluating the extent of inflammation in BRD-affected calves.

MATERIAL AND METHODS

The experimental study was carried out with the authorization of the Animal Experiments Local Ethics Committee of Atatürk University on January 26, 2023, under the reference number 2023/23.

Animals

The animal material consists of calves aged between 30 and 45 days of different breeds and genders.

Experimental Groups

To create the calf groups included in the study, the "Severity Score Criteria For Undifferentiated Respiratory Disease" protocol determined by Perino and Apley (1998) was used (Table 1).

The study consisted of 3 groups:

Group I (n=10): This is the healthy control group. Calves with a clinical score of 0 were included.

Group II (n=16): Calves with a clinical score of 3 were included in this group.

Group III (n=16): Calves with a clinical score of 4 were included in this group.

Table 1: Severity score criteria for undifferentiated respiratory disease.

Score	Clinical Signs
0	Normal, no signs of disease
1	Noticeable depression, signs of weakness are usually not apparent
2	Marked depression, moderate signs of weakness may be apparent but without significantly altered gait
3	Severe depression accompanied by signs of weakness such as altered gait or lowered head
4	Moribund, unable to rise

Clinical Examination

The calves' respiratory and heart rate, and rectal temperature were assessed, additionally clinical outcomes were documented using the scoring parameters listed in Table 1.

Blood Sample Collection

Blood samples were obtained from *v. jugularis externa* of all the calves involved in the study and collected in tubes with EDTA(EDTA K3, Pty Ltd., Adelaide, SA, Australia) as well as serum (Vacutainer, Becton Dickinson Co. USA) tubes. A series of blood samples were obtained in serum tubes, left to coagulate for 30 minutes at ambient temperature, subjected to centrifugal force at 3000

revolutions per minute for 10 minutes, and then stored at a temperature of -80 °C until analysis.

Hematological Analysis

White blood cell (WBC), lymphocyte (LYM), neutrophil (NEU), NLR (NEU/LYM ratio) and platelet (PLT) parameters in blood samples collected in EDTA tubes were analysed using a haemogram device (Abacus Junior Vet 5, Diatron, Hungary).

Biochemical Analysis

Serum concentrations of NF-κB[a], TNF-α[b], IL-1β[c], IL-10[d], and Cathelicidin[e] levels were determined by using bovine-specific ELISA kits (BT-LAB Bioassay Technology Laboratory) according to manufacturer's recommen dations ([a]Cat. No. E0314Bo, [b]Cat. No. E0019Bo, [c]Cat. No. E0197Bo, [d]Cat. No. E0252Bo, and [e]Cat. No. E0400Bo).

Statistical Analysis

Analysis of the study data was conducted using SPSS software (version 25.0, IBM Software, Inc. in Chicago, United States). Prior to analyzing the data of the study, the Shapiro-Wilk test was used to ascertain the normal distribution of the data. An analysis of variance (ANOVA) was conducted to identify the differences in the parameters among the groups. Subsequently, the *post hoc* Tukey test was used. A Pearson correlation analysis was conducted to determine the correlation among the values in the provided data. A significance level of 0.05 was used to determine statistical significance, and the results were reported as the mean ± standard deviation (SD).

RESULTS

Clinical Findings

Respiratory rate, heart rate and rectal temperature of all calves are shown in Table 2. Respiratory rate and rectal temperature were higher in group II and group III than in group I ($p<0.001$).

Table 2: Clinical findings of the groups.

Clinical Findings	Group	Mean ± SD	p - Values
Respiration Rate (min)	Group I*	35.1±3.25[a]	< 0.001
	Group II**	55.2±5.01[b]	
	Group III***	57.4±4.73[b]	
Heart Rate (min)	Group I*	97.2±3.97	> 0.05
	Group II**	99.1±10.7	
	Group III***	97.9±8.65	
Rectal Temperature (°C)	Group I*	38.6±0.35[a]	< 0.001
	Group II**	39.6±0.29[b]	
	Group III***	39.7±0.25[b]	

* score 0 (Normal, no signs of disease), **score 3 (Severe depression accompanied by signs of weakness such as altered gait or lowered head), ***score 4 (Moribund, unable to rise).

Haematological Findings

Haematological results for animals are summarized in Table 3 in all groups. Group III showed higher WBC, LYM, NEU and NLR levels than group I, while group II had higher PLT levels ($p<0.05$).

Table 3: Hematological findings of the groups.

Parameters /Groups	Group	Mean ± SD	p - Values
WBC (10^{-3}/µl)	Group I*	11.26±3.01[a]	< 0.01
	Group II**	13.76±6.94[ab]	
	Group III***	20.23±8.65[b]	
LYM (10^{-3}/µl)	Group I*	6.19±2.07	> 0.05
	Group II**	6.08±1.58	
	Group III***	7.01±4.29	
NEU (10^{-3}/µl)	Group I*	4.77±2.32[a]	< 0.01
	Group II**	7.2±5.7[ab]	
	Group III***	12.82±8.03[b]	
NLR	Group I*	0.84±0.55[a]	< 0.01
	Group II**	1.17±0.81[a]	
	Group III***	2.10±1.01[b]	
PLT (10^{-3}/µl)	Group I*	272±75[a]	< 0.01
	Group II**	608±297[b]	
	Group III***	449±205[ab]	

* score 0 (Normal, no signs of disease), **score 3 (Severe depression accompanied by signs of weakness such as altered gait or lowered head), ***score 4 (Moribund, unable to rise). WBC: Leukocyte, LYM: Lymphocyte, NEU: Neutrophil, NLR: Neutrophil-lymphocyte ratio, PLT: Platelet. Data are presented as the mean ± SD, SD: standard deviation. [a, b]: The means shown in different lowercase letters between the groups (on the line) are statistically significant ($p<0.05$).

Biochemical Findings

The calf samples from all the groups are summarised in Table 4, which shows that the levels of NF-kB, TNF-α and cathelicidin were higher in the III group than in the I group. In addition, the levels of cathelicidin were higher in group II compared to group I ($p<0.05$). Our correlation results, represented in Table 5, indicate a strong positive correlation between NEU and both WBC ($r=0.924$, $p<0.05$) and NLR ($r=0.872$, $p<0.05$), and a strong positive correlation between WBC and NLR ($r=0.706$, $p<0.05$). Moderate positive associations were found between WBC and LYM and between TNF-α, NLR and NF-kB. Additionally, weak positive correlations were discovered between WBC and IL-10 and CAMP and between NEU and IL-10 and CAMP. Lastly, weak positive correlations were observed between LYM and IL-B, as well as between NF-κB and CAMP.

Table 4: Biochemical findings of the groups.

Parameters/Groups	Group	Mean ± SD	p - Values
NF-kB (ng/mL)	Group I*	1.26±0.40[a]	< 0.01
	Group II**	1.59±0.33[ab]	
	Group III***	1.83±0.33[b]	
TNF-α (ng/L)	Group I*	120±38.9[a]	< 0.05
	Group II**	142±31.91[ab]	
	Group III***	156±28.09[b]	
IL-1β (ng/L)	Group I*	6.01±1.52	> 0.05
	Group II**	6.17±1.62	
	Group III***	6.97±2.76	
IL-10 (ng/L)	Group I*	135±16.49	> 0.05
	Group II**	145±27.8	
	Group III***	155±28.39	
CAMP (nmol/mL)	Group I*	16.41±9.64[a]	< 0.01
	Group II**	23.86±5.35[b]	
	Group III***	25.28±4.62[b]	

* score 0 (Normal, no signs of disease), **score 3 (Severe depression accompanied by signs of weakness such as altered gait or lowered head), ***score 4 (Moribund, unable to rise). NF-kB = Nuclear Factor Kappa B, TNF-a = Tumor Necrosis Factor Alpha, IL-1B = Interleukin-1 Beta, IL-10 = Interleukin-10, CAMP: Cathelicidin Antimicrobial Peptide. Data are presented as the mean ± SD, SD: standard deviation. Different letters in the same line are statistically significant ($p<0.05$).

Table 5: Correlation of parameters between groups (Pearson Correlation).

Parameters	WBC	NEU	LYM	NLR	PLT	NF-κB	TNF-α	IL-1β	IL-10	CAMP
WBC	1.000	0.924**	0.437**	0.706*	0.088	0.175	0.247	0.305	0.352*	0.361*
NEU		1.000	0.064	0.872**	0.090	0.059	0.291	0.207	0.375*	0.344*
LYM			1.000	-0.209	-0.006	0.302	-0.048	0.346*	0.071	0.116
NLR				1.000	0.069	0.008	0.412*	0.189	0.286	0.280
PLT					1.000	-0.007	-0.031	0.028	-0.018	0.142
NF-κB						1.000	0.417**	0.192	0.094	0.368*
TNF-α							1.000	0.214	0.153	0.250
IL-1β								1.000	-0.049	0.203
IL-10									1.000	0.149
CAMP										1.000

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

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

DISCUSSION AND CONCLUSION

Bovine respiratory disease (BRD) is a significant ailment that leads to substantial mortality rates among beef and dairy calves globally. Although there are many factors that cause BRD, this disease is caused by the interaction of bacteria and viruses (Panciera and Confer 2010). This study purposed to assess pro-inflammatory and anti-inflammatory cytokines, antimicrobial peptide concentrations and NLR according to clinical scoring as an auxiliary diagnostic tool in determining the severity of inflammation in BRD in calves.

Symptoms such as high fever and tachypnea can be seen in animals with BRD. The formed fever causes the calf to tachypnea. At the same time, the cause of tachypnea is explained by the increased respiratory rate due to the non-functionality of a large part of the infected lung tissue (Guterbock 2014). In the present study, in line with the

above information, it was found that both respiratory rates and rectal temperatures of calves in group I were lower than in the other groups. This could be due to a fever caused by an infection, as well as a major portion of the lung tissue becoming dysfunctional.

It was reported that the leukocyte counts of the groups with BRD were higher than the control group in calves (Howard et al. 1976). Martin and Lumsden (1987) reported that a table of leukocytosis was formed in their study in calves with BRD and this was probably due to acute respiratory tract inflammation due to infection. In this study, both WBC and NEU counts were higher in infected calves than in healthy calves. Compared to group I, this increase was found to be significant in group III ($p<0.01$). The possible cause of the high leukocyte elevation is thought to be neutrophilic leukocytosis formed due to acute inflammation. At the same time, the very strong positive correlation between WBC and NEU supports this.

According to one study, NEUs in cattle can boost the production of pro-inflammatory cytokines such as TNF- α and IL-1β (Sohn et al. 2007). According to research comparing M. bovis, which causes respiratory disease, and NEUs, the count of NEUs increased. This is explained by the fact that M. bovis stimulates TNF-α production by promoting NEU apoptosis (Howard et al. 1976). In this study, we found that concentrations of NEUs and TNF-α were higher in calves of groups II and III in proportion to I. This aligns with previous research. This can be explained by pathogen agents enhancing TNF-α production by stimulating NEU death, as previously indicated.

Recent research has highlighted the significance of NLR as a marker for identifying inflammation and predicting the course of the disease (Zahorec 2021). It has been reported that NLR levels are increased in goats with pneumonia (Jarikre et al. 2016). In this study, group II and III had greater NLR levels than group I. group III had a statistically significant rise compared to group I. Consistent with the aforementioned reports, it was discovered that the NLR level increased as BRD severity increased, in this study. This could be attributed to an increase in WBC, NEU and TNF-α. In addition, the high positive association between NLR level and WBC and NEU as well as the moderate positive correlation between NLR level and TNF-α provide support for this assumption. Primary thrombocytosis is a condition caused by adrenaline-induced splenic contraction. Seconder (reactive) thrombocytosis is triggered by the release of cytokines in the body and is seen in conditions such as stress and inflammation (Jones and Allison 2007). In the study, according to clinical scoring, PLT levels in group II and III animals were shown to be greater than in group I.

Pro-inflammatory cytokines have a significant impact on initiating the activation of acute-phase proteins (APPs) throughout the body. Each of these cytokine groups induces a different APP (Baumann and Gauldie 1994). Therefore, the study focused on different pro-inflammatory cytokines, such as NF-κB, TNF-α, and IL-1β. NF-κB facilitates the activation of pro-inflammatory cytokines like IL-1β and TNF-α, and anti-inflammatory cytokines such as IL-10, by binding to the nucleic regions that are crucial for cytokine initiation. Pro-inflammatory cytokines also cause NEU and PLT adhesion and binding to endothelial cells (Celik et al. 2013). Studies conducted on humans have reported that the level of NF-κB increases in case of pneumonia (Wahl et al. 2003; Devaney et al. 2013). In this study, NF-κB levels in the calves of group II and III were greater than those in group I. In fact, this elevation was shown to be statistically significant in group III compared to group I. It is thought that the possible reason for this elevation is macrophage activation in infected lymphoid cells, causing an increase in both NF-κB and TNF-α production. The moderate positive correlation between NF-κB and TNF-α supports this hypothesis.

TNF-α and IL-1β play a crucial role in the activation of the acute phase response. Under conditions of inflammation, the pro-inflammatory cytokines TNF-α and IL-1β, which are secreted by macrophages and monocytes, act as triggers for the production of anti-inflammatory proteins (APPs) by liver cells (Baumann and Gauldie 1994). A study by Zhang et al. (2019) reported the detection of pro-inflammatory cytokines, including TNF-α and IL-1β, being released by inflammatory cells in the alveoli during bacterial lung infections. An explanation for this rise was provided by the fact that pathogenic agents stimulated the activation of monocytes and macrophages, leading to an increase in TNF-α synthesis (Thacker 2006). Corroborating other studies, the calves in groups II and III had much higher levels of TNF-a compared to the calves in group I. The observed increase was determined to be statistically significant in group III as compared to group I. With increasing illness severity, the numerical values of IL-1β were seen to be greater in groups II and III compared to group I, but this difference was not statistically significant. The aforementioned outcome is believed to be caused by the stimulation of monocytes and macrophages by infectious pathogens in pneumonias. Furthermore, when the intensity of the infection becomes more severe, the levels of pro-inflammatory cytokines likewise rise. This notion is supported by the substantial positive connection between TNF-α and NLR.

IL-10 helps regulate pro-inflammatory cytokines and is produced by immune system cells to control tissue damage (Zhang and An 2007). However, with increasing clinical severity, IL-10 levels were higher for groups II and III. One potential explanation for this increase is believed to be the increased production of IL-10 in infected cells, which inhibits macrophages and T cells, as reported by Brown et al. (2008). This is supported by the fact that both WBC and NEU concentrations of calves in groups II and III were statistically higher than those in group I. The positive correlation between IL-10 and WBC and NEU in this study supports this hypothesis.

Cathelicidins are related components of inherited immunity that are responsible for activating cells responsible for inflammation, such as macrophages, NEUs, and B lymphocytes (Tecle et al. 2010). There are very few studies evaluating CAMP in calves with BRD. CAMP levels were reported to be higher in the patient group than in the healthy group in a study of calves with BRD (Koçer 2022). In an in vitro study, it has been documented that CAMP possesses antiviral activity against respiratory syncytial virus. Additionally, in both in vivo studies conducted in mice and humans, CAMP was found to serve a protective role (Leite et al. 2002). Tomasinsig et al. (2010) reported that cathelicidins trigger the release of TNF-α in bovine mammary cells. In this study, in parallel with the above studies, TNF-α levels were found to be higher in infected calves compared to healthy calves. This increase was found to be significant in group III compared to I. Lippolis and Reinhardt (2005) reported that cathelicidins are of NEU origin and that bovine NEUs produce cathelicidin. In our study, both NEU and WBC were positively correlated with CAMP, and in group II and III, NEU and WBC concentration of calves were increased compared with

group I. This elevation was found to be statistically significant in group III. Moreover, CAMP concentration of group II and III were also enhanced compared with group I. The observed rise is believed to be caused by the loss of epithelial cells and infection in the respiratory tract of ill calves, increased protection against respiratory infections, increased expression of Toll-like receptors 3, 7, and 9, and activation of immune systems (Martin and Lumsden 1987).

As a result, elevated WBC, NEU, NLR, NF-κB, TNF-α, and CAMP levels were found in group III, which consisted of animals with the highest severity score according to clinical scoring in BRD calves. NLR is seen as a potential marker since it is a low-cost, easily quantifiable, and repeatable assay for detecting/assessing inflammation.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: MSA
Supervision / Consultancy: MSA
Data Collection and / or Processing: MSE, Mİ
Analysis and / or Interpretation: ÖA, SK
Writing the Article: EE, KEY
Critical Review: ŞD

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Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Yüksek V, Görmez G (2024).** Evaluation of the Chemotherapeutic Potential of Medicinal Plant Mespilus germanica Fruit Extract: Cell Death Pathways and DNA Damage Mechanism. *Van Vet J*, 35 (3), 202-207. DOI: https://doi.org/10.36483/vanvetj.1556349

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

Evaluation of the Chemotherapeutic Potential of Medicinal Plant *Mespilus germanica* Fruit Extract: Cell Death Pathways and DNA Damage Mechanism

Veysel YÜKSEK[1, *] Gül GÖRMEZ[2]

[1] *Van Yuzuncu Yil University, Özalp Vocational High School, Department of Medical Laboratory, 65080, Van, Türkiye*
[2] *Van Yuzuncu Yil University, Faculty of Health Sciences, Department of Nutrition and Dietetics, 65080, Van, Türkiye*

Received: 26.09.2024 Accepted: 20.11.2024

ABSTRACT Plant extracts are a mixture of natural complex compounds containing various biological activities, including anticancer properties. The fact that they have fewer side effects than synthetic drugs has made plant extracts an important strategy in cancer treatment The purpose of this study was to explore the chemotherapeutic potential of Mespilus germanica (medlar) fruit extract. The compound content of the extract was determined by HPLC. The proliferative concentration (PRO) and the concentration inhibiting the proliferation of half of the cells (IC50) were determined by the MTT viability test. PRO and IC50 concentrations were treated to A549 lung cancer cells for 48 hours. The study groups were determined as 3 groups: control, PRO, and IC50. Total mRNA was obtained from the cells by using the Trizol Reagent-chloroform method. cDNA synthesis was performed from total mRNA. mRNA gene expression levels of programmed cell death markers were detected by RT-qPCR. For all group studies, $p<0.05$ was considered statistically significant. It was detected in the extract content analysis, chlorogenic acid, ellagic acid, quercetin, and gallic acid polyphenolic compounds. As a result of MTT, IC50 was detected at 540 µg/ml, and PRO was 100 µg/ml. It was revealed that IC50 concentration significantly increased ($p<0.05$) the expression of *ATG5* (autophagic) and *RIPK1* (necrotic) genes. In addition, it was observed that the expression of proteases C*aspase-8, BAX, Apaf-1, Caspase-9, Caspase-3, and Caspase-7*, as well as genes associated with genotoxic damage, *PARP-1* and *P53*, increased significantly ($p<0.05$). As a result, it was determined that *Mespilus germanica* triggered the programmed cell death pathways in the A549 cancer cell line. It was concluded that adequate consumption of *Mespilus germanica* fruit can reduce or inhibit cancer cell proliferation. An experimental administration with an in vivo phase should be administered to reveal these results definitively.

Keywords: *Cancer, Cell death pathway, DNA damage, Mespilus germanica.*

ÖZ

Tıbbi Bitki *Mespilus Germanica* Meyve Ekstraktının Kemoterapötik Potansiyelinin Değerlendirilmesi: Hücre Ölüm Yolakları ve DNA Hasar Mekanizması

Bitki ekstarktları antikanser dahil olmak üzere çeşitli biyolojik aktiviteler içeren doğal kompleks bileşiklerin bir karışımıdır. Sentetik ilaçlara göre daha az yan etkiye sahip olmaları, bitki ekstraktlarını kanser tedavisinde önemli bir strateji haline getirmiştir. Bu çalışmanın amacı, *Mespilus germanica* (Muşmula) meyve ekstraktın kemoterapötik potansiyelinin araştırılmasıdır. Elde edilen ekstraktın bileşik içeriği HPLC ile tespit edildi. MTT canlılık testi yoluyla proliferatif konsantrasyon (PRO) ve hücrelerin yarısının çoğalmasını inhibe eden konsantrasyon (IC50) belirlendi. PRO ve IC50 konsantrasyonlar 48 saat boyunca A549 akciğer kanser hücresine uygulandı. Çalışma grupları kontrol, PRO ve IC50 olmak üzere 3 grup olarak belirlendi. Elde edilen hücrelerden Trizol reagent-kloroform yöntemi kullanılarak toplam mRNA elde edildi. Toplam mRNA'dan cDNA sentezi yapıldı. Programlanmış hücre ölümü belirteçlerinin mRNA gen ekspresyon seviyeleri RT-qPCR ile tespit edildi. Tüm grup çalışmaları için $p<0.05$ istatistiksel olarak anlamlı kabul edildi. Ekstrakt içeriği analizinde klorojenik asit, elajik asit, kuersetin ve gallik asit polifenolik birleşikler tespit edildi. MTT sonucu IC50 540 µg/ml ve PRO 100 µg/ml olarak tespit edildi. IC50 konsantrasyonunun, *ATG5* (otofajik) ve *RIPK1* (nekrotik) genlerin ekspresyonunu önemli ölçüde arttırdığı ($p<0.05$) ortaya konuldu. Ayrıca *Kaspaz-8, BAX, Apaf-1, Kaspaz-9, Kaspaz-3* ve *Kaspaz-7* proteazlarının yanı sıra, genotoksik hasarla ilişkili genler olan *PARP-1* ve *P53*'ün ekspresyonunun da önemli ölçüde arttığı gözlendi ($p<0.05$). Sonuç olarak *Mespilus germenica* A549 kanser hücrelerinin ortadan kaldırılması için istenilen programlanmış hücre ölüm yollaklarını tetiklediği tespit edildi. *Mespilus germenica* meyvesinin yeterli oranda tüketilmesinin kanser hücre çoğalmasını azaltabileceğini veya tamamen ortadan kaldırabileceği kanaatine varıldı. Elde edilen bu sonuçların kesin olarak ortaya konması için *in vivo* fazlı deneysel bir uygulamanın yapılması gerekeceği düşünülmektedir.

Anahtar Kelimeler: *DNA hasarı, Hücre ölüm yolağı, Kanser, Mespilus Germanica.*

✉ *Corresponding author: veyselyuksek@yyu.edu.tr

INTRODUCTION

The side effects of therapeutic plants are less than those of artificial drugs. The synergistic effects of therapeutic plant ingredients are becoming increasingly important in the treatment of diseases. Therefore, the therapeutic effects of herbs are necessary to reveal (Dar et al. 2017). The chemotherapeutic effects of plants containing high amounts of phenolic compounds have been intensively investigated in recent years. At this point, studies conducted with plant extracts have yielded promising results. Intensive research is being carried out on this subject in various countries worldwide (Oruganti and Meriga 2021; Ng et al. 2022). Metabolites such as alkaloids, tannins, flavonoids, and phenolics synthesized in plants are beneficial therapeutically (Rawat et al. 2018; Ginwala et al. 2019; Top et al. 2019). *Mespilus germanica* is a plant belonging to the family *Rosaceae* (Shulaev et al. 2008), mainly growing on rocks. The edible fruits of the *Rosacea* family are rich in compounds including potent antioxidant activities such as L-ascorbic acid, phenolics, flavonoids, and other phytochemicals beneficial for health (Rop et al. 2011). *Mespilus germanica* is popularly consumed as a fruit and used to treat enteritis (Glew et al. 2003), constipation, diuretics, and kidney and bladder stones (Baytop 1999). It has been reported in previous studies that Mespilus germanica has abundant mineral and trace elements (Ayaz et al. 2002; Glew et al. 2003), its fruits are rich in organic acids, amino acids and tannins (Rop et al. 2011) and it has high polyphenol content (Ercisli et al. 2008). This study was carried out to evaluate the anticancer effect of the extract obtained from the fruit of *Mespilus germanica*, which is popularly consumed in Türkiye and also used in the treatment of various diseases *in vitro*. Despite the disease-curing properties and rich content of Mespilus germanica, studies on its effects on cancer cell lines remain limited.

MATERIAL AND METHODS

Materials

The proposed research project does not need Animal Research Ethics Committee Approval (Date: 29/08/2024 Decision number: 2024/08-12). This study was performed using the A549 (ATCC® CCL-185™) lung cancer cell line. Fruits of *Mespilus germanica* were collected from Van province, Türkiye, during the young fruiting period in July. *Mespilus germanica* trees were grown without insecticides or herbicides for two fruiting seasons. Lyophilized extracts obtained from the fruits of *Mespilus germanica* were extracted with solvents of different polarities and applied to cancer cell lines.

Preparation of Plant Extract

Mespilus germanica fruits were treated with liquid nitrogen and lyophilized at -51 °C and 50 millitorr pressure for 96 hours. Sequentially lyophilized hydrophilic fractions were prepared according to a modified version of the methods (Dai and Mumper 2010; Dalar et al. 2013). The mixture was kept in a sonicator at +40 °C for half an hour, then at +4 °C for 24 h. Then it was centrifuged (Hitachi-High speed refrigerated centrifuge-CR22N) at 15.320 x *g* (10.000 rpm) for 30 min at 4 °C. The supernatant obtained was removed from the solvent by the evaporator and lyophilized. Acidified ethanol was added to the remaining pellet and centrifuged. The ethanol extract obtained was kept in a lyophilizer at -51 °C and 50 millitorr pressure for three days and then stored at -20 °C until analysis. Lyophilized extracts were filtered through a 0.45 µm filter and made ready for analysis.

HPLC Conditions

A quantity of 1000 µg/mL methanol was added to *Mespilus germanica* extract for HPLC analysis. Fruit extracts were made with slight modifications in compliance with earlier investigations (Dalar et al. 2012). Phenolic compounds were detected by using HPLC system that was equipped with a C18 column (5 µm particle size, 150 mm L x 4.6 mm I.D, Kromasil, Nouryon SE–445 80 Bohus, Sweden) and a Thermo Scientific Finnigan Surveyor diode array detector (system controler SCL-10A, LC-10ADVP pump, DGU-12A degasser, CTO-1-ADVP column oven). 1.25 ml/min was the flow rate. The HPLC analyses were conducted using an injection volume of 20 µL and an oven temperature of 40 °C. 28% (v/v) of methanol, 2% (v/v) of acetic acid, and 70% (v/v) of purified water made up the mobile phase. The elution profile of the HPLC was isocratic. The gradient employed to elude the sample was 1 ml/min for 1–12 min, 1.25 ml/min for 12–15 min, and 1.5 ml/min for 15–40 min afterward. Before injection, all standards were dissolved in HPLC-quality methanol. They employed concentration ranges of 1.0–40.0 ng/µl. By contrasting the retention periods of extracts with those of pure standards, phenolic components in the extracts were identified. The percentage of each compound from the phenolic compounds was used to clarify the results.

Cell Culture

Frozen cells war thawed rapidly (< 1 minute) in a 37 °C water bath. The cell series were grown in their specific media at 37 ^{o}C, 5% CO_2, and 95% humidity. The cell was purchased commercially. For MTT, 10^5 cells per well were seeded in plates (96 wells). 10^6 were seeded per flask (25 cm^2) for the study groups. The cells were allowed to adhere to the surface for 24 hours. At the end of 24 hours, plant extracts were treated to the cells according to the MTT result and the concentrations in the study groups.

Preparation of Plant Extract Solutions Treated

Since the extraction was prepared in a solvent, the stock solution was dissolved in DMSO. Final concentrations (100, 200, 400, 600, 800, and 1000 µg/ml) were prepared by dilution with the medium. In this dilution, the DMSO ratio was ready to have a nontoxic (≤0.005) effect (Sangweni et al. 2021).

Cytotoxicity (MTT Cell Viability) Test

In cell culture studies, IC50 (half maximal inhibitory concentration), i.e., the cytotoxic concentration that inhibits the proliferation of 50% of the cells, is used to determine the active substance or extract with the best potential. Among all drug candidates with this potency, the active substance with the lowest IC50 value is preferred. In this study, extracts prepared at different concentrations (0, 100, 200, 400, 400, 600, 800, and 1000 µg/ml) were treated to cancer cells for 48 hours, and their cytotoxic effects were determined. MTT viability test was performed at the end of the administration. The best proliferative concentration of the plant extract with the lowest IC50 value was also determined. The determined IC50 and proliferative concentrations were treated to the cell lines for 48 hours. The study was carried out in 3 groups in cell line: control, IC50, and PRO.

RNA Extraction and cDNA Synthesis

At the end of the administration periods, the cells were removed from the flask surface by using trypsinization. The cells were collected in 15 ml sterile falcon tubes. The collected cells were centrifuged at 1500 rpm for 5 minutes at 4 °C to remove the top medium, washed with sterile PBS, and centrifuged again to remove the PBS.

Total mRNA was obtained from the obtained cells by using the Trizol Reagent-chloroform method (Chomczynski and Mackey 1995). The amount and purity of the obtained mRNAs were measured in the spectrometer (BioDrop µLITE, England). Total mRNA 260/280 ratio was determined as 2 and above. No traces of DNA were found. Therefore, DNase I enzyme application could not be performed. cDNA synthesis was performed from total mRNA using a High-Capacity cDNA Reverse Transcription isolation kit (Applied Biosystems™ Cat: 4368814, Lithuania).

Real Time-qPCR Analysis

The mRNA transcription levels of the target genes, for which the primer sequences are provided in Table 1, were assessed by utilizing the complementary DNAs (cDNAs) acquired. The optimal conditions for primer design for each gene were found. The provided information on the reaction conditions for RT-qPCR may be found in Table 2. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted using the ROTOR-GENE Q instrument manufactured by Qiagen in Germany. To ascertain the patterns of gene expression, the transcription levels of several key genes involved in autophagic and necrotic death pathways (*ATG5, ATG3*, and *RIPK1*), as well as genes belonging to the caspase enzyme systems implicated in the apoptotic pathway (*BCL-2, BAX*, *Apaf1, Caspase-8, Caspase-9, Caspase-3, and Caspase*-7), and the *TP53* gene, a marker for DNA damage, were assessed. Actin Beta (ACTB) was used as a control gene in expression analysis. SYBR Green master mix (ENZO Life Science cat: ENZ-NUC104–0200) was used for amplification detection in the study. The primer list of target genes is given in Table 1. Primers were purchased commercially. Each sample was repeated in 3 independent replicates, with one cycle threshold (Ct) determined at the beginning of the logarithmic phase of the amplifications. Evaluation of target gene products was performed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2011). Differences between groups were evaluated according to the comparison of the increase-decrease fold changes in the expression of the control gene.

Statistical Analysis

SPSS version 22.0 package program was used for all statistical analyses. Since all our data showed normal distribution, One-way ANOVA and post-hoc Tukey test were used for descriptive statistics between groups. All parameters are presented as mean ± standard deviation. For all group studies, $p<0.05$ was considered statistically significant.

RESULTS

Ellagic acid and quercetin were detected at 254 nm, while gallic and chlorogenic acid were detected at 280 nm (Figure 1). Ellagic acid, chlorogenic acid, quercetin and gallic acid were determined in the extract. (Table 3).

The effect of the extracts obtained from the fruits of the *Mespilus germanica* plant collected during the seasonal period with solvents of different polarities on the A549 cell line was investigated. The results are given in Figure 2. The graph of cell viability values corresponding to each concentration treated to the cells was generated in CurveExpert (Professional 2.7.3). It was determined that IC50 was 540 µg/ml and proliferative concentration 100 µg/ml. These concentrations were treated to the cell line for 48 hours.

Figure 3 shows the effect of extract administration to A549 lines at both IC50 and proliferative concentrations on significant genes in *ATG3, ATG5* (autophagic), and *RIPK1* (necrotic) pathways compared to the control. Up and down-regulation of gene expression was done according to the control gene. It was determined that the *ATG3* gene was up-regulated 2 fold in the PRO group, and down-regulated 0,6 fold in the IC50 group. ATG5 gene was up-regulated 19 times in the IC50 group. It was determined that the *RIPK1* gene expression increased by 10 fold in the IC50 group. Differences were considered significant when $p<0.05$ compared to the control and PRO groups, there was an increase ($p<0.05$) in *ATG5* and *RIPK1* in the IC50-treated group.

A549 lung cancer cell lines were treated with *Mespilus germanica* extract for 48 hours. At the end of the treatment, the effect on the mRNA expression of apoptotic genes is given in Figure 4. Up and down regulation of gene expression was done according to the control gene. It was determined that *BAX* and *Apaf-1* were approximately up-regulated 8-fold compared to the control group in the IC50 group. It was detected that the *BAX/BCL-2* ratio increased 4 fold in the PRO group and 5-fold in the IC50 group. *Caspase-8, Caspase-9*, *Caspase-3*, and *Caspase-7* were also respectively determined to be up-regulated 7-fold, 4-fold, 9-fold and 7-fold. The mRNA levels of apoptotic genes *BAX, Caspase-9, Caspase-8, Apaf-1, Caspase-7* and *Caspase-3* significantly increased compared to the control group ($p<0.05$). It was determined that no change occurred in the level of *BCL-2*, which is antiapoptotic.

The effect of *Mespilus germanica* fruit extract applied to A549 lung cancer cell lines for 48 hours on the mRNA expression of DNA repair genes is given in Figure 5. *PARP-1* gene expression was down-regulated 0.6-fold in the PRO group and up-regulated 7-fold in the IC50 group. It was determined that *P53* was down-regulated 0.7-fold in the PRO group and up-regulated 8 fold in the IC50 group. The mRNA levels of *PARP1* and *P53* genes significantly increased ($p<0.05$) compared to the control group in IC50 group.

Table 1: Primer sequence of the target genes.

The name of the gene	Primer sequence	
	F. 5'-3'	**R: 5'-3'**
Actin Beta (ACTB)	ACTCTTCCAGCCTTCCTTC	ATCTCCTTCTGCATCCTGTC
ATG3	GAGATCACCTAGTCCACCAC	GCTTCCGTTATTCCTGTAATACC
ATG5	GAGACAAGAAGACATTAGTGAG	GATATTCCATGAGTTTCCGA
RIPK1	TGAGCTTCCGCTAGACA	CTGAAGCTCAACGCCCA
Caspase-8	GATGTTATTCCAGAGACTCCAG	GGTAGGTAATCAGCAAATCCA
Caspase-3	ATGGAAGCGAATCAATGGAC	AAACATCACGCATCAATTCC

Table 1 (continued): Primer sequence of the target genes.

The name of the gene	Primer sequence	
	F. 5'-3'	**R: 5'-3'**
BCL2	GTGGTGGAGGAACTCTTCAG	GTTCCACAAAGGCATCCCAG
Caspase-9	GGCTCTTCCTTTGTTCATCTCC	TCACCAAATCCTCCAGAACCA
BAX	AGCAAACTGGTGCTCAAGGC	CCACAAAGATGGTCACTGTC
Caspase-7	GGCTTGTATTGAAGAGCAGGG	CTGATCTTGTATCGAGGATTAGCA
Apaf-1	CCCTTTGTGTCCAGTAGTGGG	CTCTGTCTCGCCACATACCC
PARP-1	CACCAAAAAGGAGGTGGAAA	CAACTCCTGAAGGCTCTTGG
P53	CCCAGGTCCAGATGAAGCTC	CATGTAGTTGTAGTGGATGGTGGT

Table 2: Real-Time qPCR reaction conditions.

Reaction content	For one sample	Reaction cycle
Buffer (2X)	10 µl	95 °C 2' denaturation
Primers	Forward : 0.5 µl Reverse : 0.5µl	95°C 5'' *58 °C -60 °C } 40 cycle
dH_2O	8.4 µl	
cDNA	0.6 µl	
Total	20 µl	* The binding temperature varied according to the primers. Melting Curve Ramp: 50-99 (1 degree increment) 90 °C 5 seconds

Table 3: Retention time and concentrations of phenolics detected in *Mespilus germanica* fruit extract.

Phenolics	**Retention time(min)**	**Concentration (mg/ml) mean ± SD**
Chlorogenic acid	5.064	3.85±0.089
Ellagic acid	29.999	4.68±0.1701
Quercetin	47.00	3.68±0.096
Gallic acid	3.204	1.75±0.125

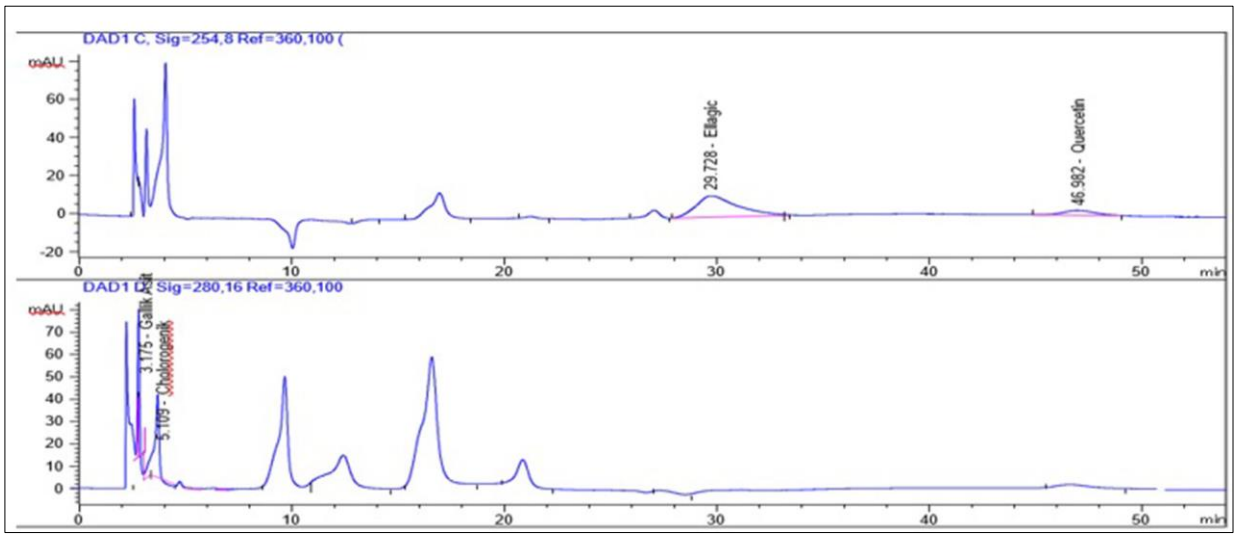

Figure 1: HPLC chromatogram of phenolic acid standards.

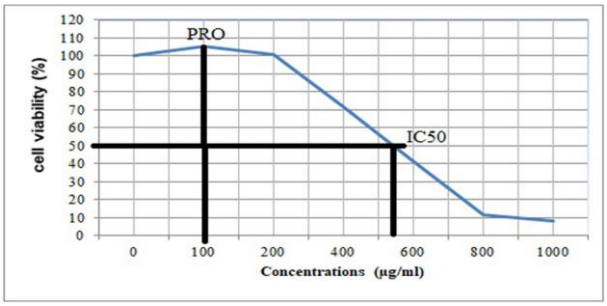

Figure 2: Graph of MTT viability test showing the effect of the extract treated at different concentrations on A549 cells.

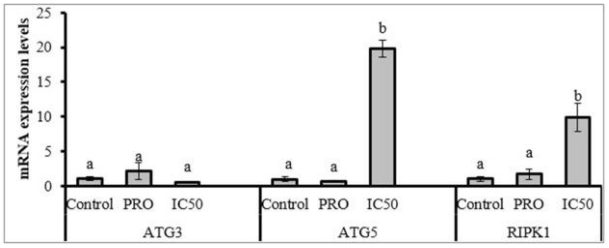

Figure 3: The mRNA transcript levels of *ATG3*, *ATG5* and *RIPK1* in the A549 cell line at the 48th hour**:** Represent the relative mRNA expression levels of *ATG3*, *ATG5* and *RIPK1* in the A549 cell line at the 48th hour. All data were expressed as mean±SD. Different letters (a–b) on the columns show a statistical difference ($p<0.05$).

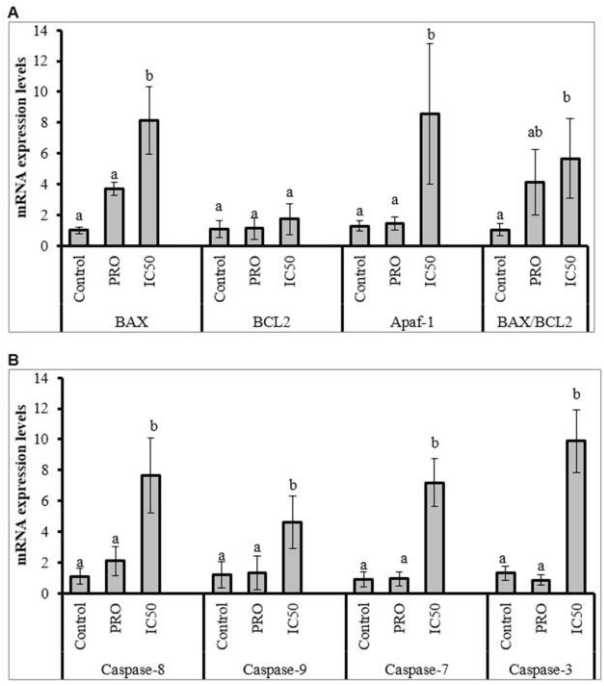

Figure 4: (A–B) The mRNA transcript levels of *BAX, BCL2, Apaf-1, Caspase-8, Caspase-9, Caspase-7* and *Caspase-3*, and *BAX/BCL-2* ration in the A549 cell line at the 48th hour: (A) Represent the relative mRNA expression levels of *BAX, BCL-2* and *Apaf-1*, and *BAX/BCL-2* ration in the A549 cell line at the 48th hour. (B) Represent the relative mRNA expression levels *Caspase-8, Caspase-9, Caspase-7* and *Caspase-3* in the A549 cell line at the 48th hour. All data were expressed as mean±SD. Different letters (a–b) on the columns show a statistical difference ($p<0.05$).

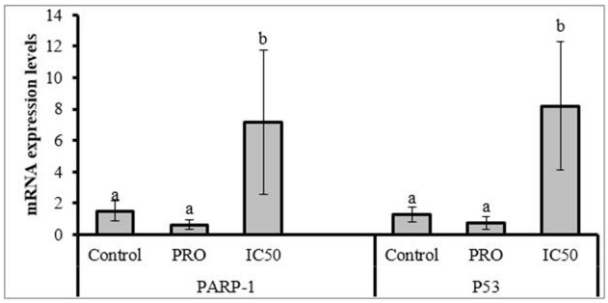

Figure 5: The mRNA transcript levels of *PARP-1* and *P53* in the A549 cell line at the 48th hour: Represent the relative mRNA expression levels of *PARP-1* and *P53* in the A549 cell line at the 48th hour. All data were expressed as mean±SD. Different letters (a–b) on the columns show a statistical difference ($p<0.05$).

DISCUSSION AND CONCLUSION

Chlorogenic acid is a dietary phenolic acid compound synthesized by various plant species. Current reports have revealed that chlorogenic acid shows anticancer effects by inhibiting the cell cycle and initiating apoptosis (Hayakawa et al. 2022). In another study, chlorogenic acid inhibited the proliferation of the A549 cell line (Wang et al. 2020; Gupta et al. 2022). Ellagic acid is a polyphenol compound found naturally in various fruits and vegetables. This compound has been found to inhibit the growth of tumourigenic structures by reducing cell growth and inducing apoptosis (Duan et al. 2020). Another reported study stated that Ellagic acid induced apoptosis in A549 cells by inhibiting the PI3K/Akt signalling pathway (Liu et al. 2018). The anticancer potential of quercetin has been documented in numerous *in vivo and in vitro* studies involving various animal models and cell lines (Almatroodi et al. 2021). Quercetin was found to dose-dependently inhibit cell viability and induce mitochondria-dependent apoptosis in both A549 and H1299 cells. The mRNA levels of *LC3-II*, *beclin 1, Atg5, Atg7,* and *Atg12* were upregulated by quercetin treatment (Guo et al. 2021). In this study, the content analysis of the *Mespilus germanica* fruit extract we obtained was determined by the HPLC method. According to HPLC standard comparison, phenolic compounds such as chlorogenic acid, ellagic acid quercetin, and gallic acid were detected (Table 3). As a result of the application of this extract to the cells, it was determined that cell proliferation was stopped according to the MTT result, which is a cell viability test. It has been determined that the extract obtained from *Mespilus germanica* fruit is rich in molecules that reduce the proliferation of cancer cells and stop or inhibit the proliferation of cells. Therefore, these compounds prevent or inhibit the proliferation of cancer cells. The given information above is consistent with studies by Gupta et al. (2022), Almatroodi et al. (2021) and Wang et al. (2020). In the Sadeghinejad et al. (2022) study, different *Mespilus germanica* fruits were identified as suitable natural sources containing important antioxidants and phenolic compounds. Yunusa and Ozturk (2024) conducted a study on *Mespilus germanica* leaves and fruit, showing that this plant extract has a significant cytotoxic effect on cancer cell lines and may have an anti-cancer effect. In this study, it was determined that *Mespilus germanica* fruit contains important phenolic components. Our results were found to be compatible with the research conducted by Sadeghinejad et al. (2022). In this study, *Mespilus germanica* fruit extract caused significant cytotoxicity in the A549 cell line. The results are compatible with other studies (Yunusa and Ozturk 2024). Cytotoxic concentrations of *Mespilus germanica* extract obtained by MTT were treated to cells to determine the expression levels of genes that are markers of programmed cell death pathways and DNA damage. It was revealed that *ATG5*, an autophagic marker, reached a very high expression level. However, an increase in *RIPK1* mRNA level, a necrotic marker, was also found. The rise in *ATG5* level, in particular, leads the cells to prefer autophagy, a resting phase, to save themselves. Afterwards, it was revealed that this situation did not improve. The increase in the *BAX/BCL-2* ratio in the direction of *BAX* in the results of this study further supports the activation of caspase mechanisms involved in the programmed cell death pathway. The second process is initiated by loss of membrane integrity and mitochondrial depolarisation, regulated by members of the Bcl-2 protein family, which triggers the release of cytochrome c into the cytosol, activating caspase-3 as an effector. *Poly (ADP-ribose) polymerase-1 (PARP-1)* is another molecule involved in many critical biological processes, including apoptosis, cell proliferation control, replication, and DNA damage repair. *PARP-1* targets caspase protease activity and is associated with apoptosis (Calaf et al. 2018). Excessive DNA damage causes massive poly (ADP-ribosyl) action by *PARP-1*, which can activate death programs (Hong et al. 2013). According to the RT-qPCR results obtained in this study, it was determined that there was a significant increase in the expression of *PARP-1* and *P53* genes along with increased *BAX, Caspase-8, 9, 7*, and *3* and decreased *BCL-2*. The rise in *PARP-1* and *P53* mRNA expression levels parallels the effector and lethal proteases involved in apoptotic pathways. The literature review found no study on apoptotic, autophagic, necrotic, and

DNA repair mechanisms related to the *Mespilus germanica* plant or fruit. These increases were found to be consistent with the literature. Drugs used in cancer treatment are obtained by various chemical means. The medicines obtained in this way cannot completely stop the proliferation of cancerous cells and cause side effects on healthy cells. Studies that can be an alternative to treatment processes and eliminate or at least reduce these side effects should be continued rapidly. The most important active substances used in this alternative treatment are polyphenolic compounds and derivatives in other living species that can be compatible with human nature. In this study, the first of our research initiated for this purpose, we found that the fruit of *Mespillus germenica*, which we used in this study, has a rich polyphenolic content. As a result of the application of the extract to the A549 lung cancer cell line under in vitro conditions, it was determined that the cells died. Drugs used in cancer treatment target apoptosis, a programmed death pathway. This study determined that *Mespillus germanica* fruit can trigger both cytoplasmic and mitochondrial pathways of apoptosis. MTT viability test and mRNA expression results support each other at this point.

As a result, it can be concluded that adequate consumption of *mespillus germenica* fruit may be beneficial in preventing or treating cancer development. It plans to experiment with an in vivo phase to demonstrate these results definitively.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: VY, GG
Supervision / Consultancy: VY
Data Collection and / or Processing: VY, GG
Analysis and / or Interpretation: VY, GG
Writing the Article: VY
Critical Review: VY

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Van Vet J, 2024, 35 (3) 208-212

Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Gülaydın Ö, Yeşilyurt M, Gülaydın A (2024).** Determination of Antimicrobial Susceptibility and Some Virulence Genes of *Staphylococococcus* spp. Strains Isolated from Keratoconjunctivitis Cases in Sheep and Goats. *Van Vet J*, 35 (3), 208-212. DOI: https://doi.org/10.36483/vanvetj.1558036

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

Determination of Antimicrobial Susceptibility and Some Virulence Genes of *Staphylococococcus* spp. Strains Isolated from Keratoconjunctivitis Cases in Sheep and Goats

Özgül GÜLAYDIN[1,*] Muazzez YEŞİLYURT[1] Ali GÜLAYDIN[2]

[1] *Siirt University, Faculty of Veterinary Medicine, Department of Microbiology, 56100, Siirt, Türkiye*
[2] *Siirt University, Faculty of Veterinary Medicine, Department of Surgery, 56100, Siirt, Türkiye*

Received: 29.09.2024 Accepted: 20.10.2024

ABSTRACT This study aimed to isolate *Staphylococcus* spp. from sheep and goats clinically diagnosed with keratoconjunctivitis, and to determine their antimicrobial susceptibility. Phenotypic and genotypic characterisation of biofilm forming ability of the isolates obtained was carried out. *Staphylococcus* spp. strains isolated from 288 ocular swab samples were identified by MALDI-TOF MS. Antimicrobial susceptibilities of the isolates were determined by the using disk diffusion method. The Congo-red agar method was used to determine the ability to form biofilms. The presence of genes associated to formation of biofilm and toxin synthesis was investigated by PCR. While *Staphylococcus aureus* was identified in 2 of the 35 strains identified in the research, the remaining isolates were found to be coagulase-negative *Staphylococcus* spp. The most frequently isolated coagulase-negative strain was identified to be *Staphylococcus equorum*. The strains were susceptible to enrofloxacin, gentamicin, and tobramycin. While 68.57% of the isolates phenotypically formed biofilms, the *AtlE* gene had a high positivity rate. Furthermore, the presence of genes responsible for toxin synthesis was not identified in the strains analysed. Based on the findings of the study, it was determined that *Staphylococcus* spp. isolates should be considered for small ruminant keratoconjunctivitis cases. It was concluded that antimicrobial agents such as enrofloxacin, gentamicin, and tobramycin would achieve success in the treatment of the disease caused by the causative agents.

Keywords: *Biofilm, Goat, Keratoconjunctivitis, Sheep, Staphylococcus* spp.

ÖZ

Koyun ve Keçilerde Keratokonjunktivitis Olgularından İzole Edilen *Staphylococococcus* Spp. Suşlarının Antimikrobiyal Duyarlılığının ve Bazı Virülens Genlerinin Belirlenmesi

Bu çalışmada koyun ve keçilerde klinik olarak tespit edilen keratokonjunktivitis olgularından *Staphylococcus* spp. izolasyonu ve antimikrobiyal duyarlılığının belirlenmesi amaçlandı. Elde edilen izolatlarda biyofilm oluşturma yeteneğinin fenotipik ve genotipik karakterizasyonu gerçekleştirildi. Koyun ve keçilerden alınan 288 adet göz svabı örneğinden izole edilen *Staphylococcus* spp. suşları MALDI-TOF MS yöntemiyle identifiye edildi. İzolatların antimikrobiyal duyarlılıkları disk difüzyon testi ile belirlendi. Biyofilm oluşturma yeteneğinin belirlenmesinde Kongo-red agar yöntemi kullanıldı. Biyofilm oluşumundan ve toksin sentezinden sorumlu gen varlığı ise PCR ile araştırıldı. Araştırmada izole edilen 35 suşun 2'si *Staphylococcus aureus* olarak identifiye edilirken, geri kalan izolatların koagulaz negatif *Staphylococcus* spp. olduğu belirlendi. Koagulaz negatif türler arasında çoğunlukla izole edilen türün *Staphylococcus equorum* olduğu tespit edildi. Suşlar, enrofloksasin, gentamisin ve tobramisine duyarlı bulundu. İzolatların %68.57'sinin fenotipik olarak biyofilm oluşturduğu belirlenirken, *AtlE* gen pozitiflik oranının da yüksek olduğu görüldü. Ancak, çalışmada biyofilm oluşturma yeteneği ile antimikrobiyal direnç profili arasında herhangi bir ilişki saptanmadı. Bununla birlikte incelenen suşlarda toksin sentezinden sorumlu gen varlığı da tespit edilmedi. Çalışmadan elde edilen bulgular doğrultusunda küçük ruminant keratokonjunktivitis olgularında *Staphylococcus* spp. izolatlarının göz önünde bulundurulması gerektiği belirlendi. Etkenlerin neden olduğu enfeksiyonların tedavisinde enrofloksasin, gentamisin ve tobramisin gibi antimikrobiyal maddelerin başarı sağlayacağı kanısına varıldı.

Anahtar Kelimeler: *Biofilm, Keçi, Keratokonjuktivitis, Koyun, Staphylooccus* spp.

✉ *Corresponding author: ozgul.gulaydin@siirt.edu.tr

INTRODUCTION

Infectious keratoconjunctivitis (IKC), which negatively affects animal well-being in small ruminants, is a contagious disease characterised by clinical findings such as redness on conjunctiva, purulent or serous discharge, and blepharospasm. The disease not only causes a drop in yield in sheep and goats but also leads to serious conditions such as pregnancy toxaemia as a result of lack of feed conversion (Işik et al. 2018). It has been reported that bacterial agents such as *Moraxella ovis*, *Chlamydia* spp., *Mycoplasma* spp. *Staphylococcus aureus* and *Listeria monocytogenes* are frequently isolated and identified from disease cases (Fernandez-Aguilar et al. 2017). Since *Staphylococcus* spp. isolates are opportunistic pathogens and are available in the natural flora of mucous membranes, they are considered to be less important than other bacterial agents in determining the a etiology of keratoconjunctivitis cases diagnosed in small ruminants. However, the strains that develop resistance to topical and/or systematic antibiotics used especially in the treatment of keratoconjunctivitis cases negatively affect the prognosis of the disease and animal well-being (Udegbunam et al. 2014). *Staphylococcus* spp. strains have various virulence factors that allow them to evade the host immune system and play a role in the occurrence of infection. These virulence factors are synthesised in bacteria under the control of some enzymes (Karahan et al. 2009). The formation of biofilm in staphylococcal isolates is among the important virulence factors. The mechanism of biofilm formation plays a role in the development of antimicrobial resistance of the agents against both antibiotics and phagocytosis mediated by macrophages (Watkins and Unnikrishnan 2020; Gurler et al. 2022). The biofilm formation in *Staphylococcus* spp. isolates is induced by the icaABCD operon (Arciola et al. 2015). However, it has been known that various adhesin proteins (AtlE, aap) are involved in biofilm formation in isolates. These proteins mediate the accumulation and adhesion stages of biofilm (Soumya et al. 2017). It has been reported that panton-valentine leukocidin and exfoliative toxin synthesised by the agent play a role, especially in soft tissue infections, and these toxins are induced by *luk-PV*, as well as *eta*, *etb* and *etd* genes, respectively (Boyle-Vavra and Daum 2007). In numerous studies, different bacterial agents were isolated and identified from clinically diagnosed conjunctivitis cases in sheep and goats, and different treatment procedures were applied (Angelos and Rowe 2014; Biswas and Saifuddin 2017; Athira et al. 2018). Previous studies showed that antimicrobial agents such as chloramphenicol, tetracycline, enrofloxacin, ceftiofur, gentamicin, and tobramycin could be used topically and/or systemically in small ruminant conjunctivitis cases (Angelos and Rowe 2014; Jesse et al. 2017; Athira et al. 2018). This study was aimed to determine the antimicrobial susceptibility of *Staphylococcus* spp., which are frequently isolated from conjunctivitis cases in sheep and goats to antimicrobial agents frequently used in the treatment of conjunctivitis cases with current data. Furthermore, it is also aimed to investigate the virulence associated genes that play a role in the occurring of the disease.

MATERIAL AND METHODS

This study was approved by the Local Ethics Committee for Animal Experiments at Siirt University with decision number 2024/04/22 on 30/04/2024.

Ocular swab samples collected from 188 Hamdani sheep and 100 hair goats clinically diagnosed with keratoconjunctivitis between 2019 and 2023 were used. The ages of the sheep and goats were found to range from 1 to 3 years. 134 (72.27%) of the sheep were ewe and 54 (28.72%) were rams, while 71 (71.00%) of the goats were does and 29 (29.00%) were billy.

Collection of Swab Samples

The swabs were collected from the affected eye and/or eyes of animals diagnosed with keratoconjunctivitis based on clinical examination by following the rules of asepsis and antisepsis. The lower eyelid was gently turned upside down, and a cotton-tipped swab was rubbed on the conjunctiva, and samples were collected. If both eyes were found to be infected in the same animal, the same swab was used for sampling from both eyes.

Isolation and Identification of *Staphylococcus* spp.

The swabs were inoculated on Columbia blood agar (Oxoid, CM 03331, UK) and Mannitol Salt Agar (Oxoid, CM85, UK) media containing 5-7% defibrinated sheep blood. The inoculated media were allowed to incubate at 37 °C in an aerobic environment for 24-48 hours. Pure cultures were obtained from the formed colonies. The obtained isolates were analysed according to Gram staining, microscopic morphology, catalase reaction, ability to grow on mannitol salt agar medium, and tube coagulase test results (Quinn et al. 2011).

The isolates were identified by using Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) Microorganism Identification device. Ethanol formic acid extraction method was used to obtain the protein profiles. The identification process was completed by comparing the spectra obtained with the flex control software (Biotyper 3.0; Microflex LT; Bruker Daltonics GmbH, Bremen, Germany) with the Maldi Biotyper Real-Time Classification (RTC) software (version.9) (Uysal et al. 2019).

Determination of Antimicrobial Susceptibility

Antimicrobial susceptibility of the isolates was determined by the disk diffusion method using chloramphenicol (30 µg), tetracycline (30 µg), enrofloxacin (5 µg), ceftiofur (30 µg), gentamicin (10 µg), and tobramycin (10 µg) disks. The criteria reported in CLSI (2018) were taken into consideration in the performance and analysis of the test. The isolates were categorized as susceptible (S), intermediate (I), or resistant (R).

Determination of the Biofilm-Forming Ability by Congo-Red agar Method

Congo-Red Agar method was used to determine the ability of biofilm-forming in the strains. To prepare Congo red agar, 0.8 g of Congo red and 36 g of sucrose were added to 1 liter of brain heart infusion agar medium (Merck 1.10493, Germany). The prepared media was inoculated and then, incubated at 37 °C for 24 hours. After that, the plates were kept at room temperature for 48 hours. Isolates forming black colour were considered as biofilm-forming, and strains forming burgundy or red colour were considered as non-biofilm-forming strains (Gurler et al. 2022).

Identification of Genes Responsible for Biofilm and Toxin Synthesis

The genes responsible for toxin synthesis and biofilm formation in the isolates were determined by PCR using specific primers. Genomic DNA used in the PCR method was extracted by the boiling method. PCR mixture was

Table 1: Primer sequences for investigating the presence of genes associated with toxin synthesis and biofilm formation in isolates by PCR.

Gene	Oligonucleotide (5'-3')	Amplicon size (bp)	Denaturation - annealing - extension (35 cycles)	References
Biofilm and adhesion factors to hydrophobic surfaces				
AtlE	F: CAACTGCTCAACCGAGAACA R: TTTGTAGATGTTGTGCCCCA	682	94°C / 1 min. - 51°C / 1 min. - 72°C / 1 min.	Frebourg et al., 2000
icaAB	F: TTATCAATGCCGCAGTTGTC R: GTTTAACGCGAGTGCGCTAT	546	94°C / 1 min. - 51°C / 1 min. - 72°C / 1 min.	Frebourg et al., 2000
aap	F: ATACAACTGGTGCAGATGGTTG R: GTAGCCGTCCAAGTTTTACCAG	399	94°C / 1 min. - 54°C / 1 min. - 72°C / 1 min.	Vandecasteele et al., 2003
icaA	F: CCTAACTAACGAAAGGTAG R: AAGATATAGCGATAAGTGC	1315	94°C / 1 min. - 51°C / 1 min. - 72°C / 1 min.	Vandecasteele et al., 2003
icaD	F: AAACGTAAGAGAGGTGG R: GGCAATATGATCAAGATAC	381	94°C / 1 min. - 50°C / 1 min. - 72°C / 1 min.	Vandecasteele et al., 2003
Toxin genes				
Exfoliative toxin				
eta	F: CTATTTACTGTAGGAGCTAG R: ATTTATTTGATGCTCTCTAT	741	94°C / 1 min. - 45°C / 1 min. - 72°C / 1 min.	Yamaguchi et al., 2002
etb	F: ATACACACATTACGGATAAT R: CAAAGTGTCCAAAAGTAT	629	94°C / 1 min. - 45°C / 1 min. - 72°C / 1 min.	Yamaguchi et al., 2002
etd	F: AACTATCATGTATCAAGG R: CAGAATTTCCCGACTCAG	376	94°C / 1 min. - 45°C / 1 min. - 72°C / 1 min.	Yamaguchi et al., 2002
Panton-Valentine leukocidin toxin				
luk-PV	F: ATCATTAGGTAAAATGTCTGGACATGATCCA R: GCATCAASTGTATTGGATAGCAAAAGC	433	94°C / 1 min. - 57°C / 1 min. - 72°C / 1 min.	Lina et al., 1999

prepared using a commercial mastermix (2X PCR Mastermix, ABT®, Ankara, Turkey). The mix was consisted of 5 µl of genomic DNA, 1.5 µl of each of primers (10 µM) and 12.5 µl of mastermix. The total volume of PCR mix was completed up to 25 µl with PCR water. Table 1 shown primer sequences and PCR process. DNA-free PCR water was used as a negative control in the assay. The amplicons were electrophoresed at 80 V for 1.5 h in agarose gel. Amplicons were compared with DNA markers and analysed in the gel imaging device (Gen-Box ImagER, Ankara, Türkiye).

RESULTS

Staphylococcus spp. was isolated in 35 (12.15%) swab samples. *S. arlettoe* was identified in 1 (2.85%) of the isolates, *S. cohnii* was identified in 1 (2.85%) isolate, *S. lentus* was identified in 1 (2.85%) isolate, *S. sciuri* was identified in 1 (2.85%) isolate, *S. aureus* was identified in 2 (5.71%) isolates, *S. haemolyticus* was identified in 2 (5.71%) isolates, *S. simulans* was identified in 2 (5.71%) isolates, *S. xylosus* was identified in 2 (5.71%) isolates, *S. chromogenes* was identified in 4 (11.42%) isolates, *S. vitulinus* was identified in 7 (20.00%) isolates, and *S. equorum* was identified in 12 (34.28%) isolates. All *Staphylococcus* spp. isolates were susceptible to enrofloxacin, gentamicin, and tobramycin. While 2.85% of the isolates were resistant to tetracycline and ceftiofur, 11.42% and 2.85% of the isolates were found to be moderately susceptible to tetracycline and chloramphenicol, respectively. Moreover, 24 (68.57%) of *Staphylococcus* spp. isolates phenotypically formed biofilm. The *aap* gene was identified in 6 (25.0%) of the strains that were able to form biofilms, *AtlE* gene in 9 (37.5%), and *icaA* gene in 2 (8.33%). The *icaD* and *icaAB* genes responsible for biofilm formation were not identified in the isolates. One of each of the strains resistant to tetracycline and ceftiofur formed biofilm. Table 2 shows the distribution of the presence of genes responsible for biofilm formation in strains found to phenotypically form biofilm. No *eta*, *etb*, *etd*, or *luk-PV* genes responsible for toxin production were identified in the strains analysed in the study.

Table 2: Distribution of the gene profile associated with biofilm formation in strains determined to form biofilms phenotypically (n=24).

Gene profile	Number of the isolates	%
aap+AtlE+icaA+icaB+icaAB	11	45.83
aap	4	16.66
aap+AtlE	2	8.33
AtlE	5*	20.83
AtlE+icaA	2**	8.33

*: 1 isolate resistant to ceftiofur; **: 1 isolate resistant to tetracycline

DISCUSSION AND CONCLUSION

Staphylococci are agents that are available in the natural flora of mucous membranes; therefore, *Mycoplasma* spp., *Moraxella* spp., and *Chlamydia* spp. strains are considered to play a role in the aetiology of keratoconjunctivitis cases in sheep and goats (Fernandez-Aguilar et al. 2017; Gulaydin et al. 2024). However, the transfer of antibiotic resistance developed, especially in staphylococcal strains, to other bacterial agents in the flora jeopardises both animal and human health and brings challenges in the treatment of the disease (Udegbunam et al. 2014). Therefore, it is critical to continuously monitor antimicrobial resistance in bacteria using up-to-date data. Various studies have analysed the presence of *Staphylococcus* spp. isolates in ocular swab samples collected from small ruminants. The study conducted by Hammadi (2015) in Iraq reported that they isolated *Staphylococcus* spp. in 30.5% of 200 ocular swab samples collected from sheep. In another study conducted in the

same country, *S. aureus* was isolated in 6.8% of ocular swab samples collected from goats and *S. epidermidis* and *S. saprophyticus* were isolated in 4.5%. On the other hand, it was reported that the isolation rates of the agents were 25.9%, 11.1%, and 3.7%, respectively, in samples collected from sheep (Rhaymah et al. 2013). A study conducted in Nigeria reported that *S. aureus* was identified in 49.2% of ocular swab samples collected from ruminants (Udegbunam et al. 2014). In a study conducted in the United States of America, *Staphylococcus* spp. strains were identified in most of the samples collected from goats (Meekins et al. 2017). The related studies have revealed that the isolation rate of *Staphylococcus* spp. varies in ocular swab samples collected from small ruminants. In this study, *Staphylococcus* spp. was isolated in 12.15% of the samples collected from clinically diagnosed keratoconjunctivitis cases in small ruminants raised in the Siirt region, and most of the isolates were identified as coagulase-negative species. Although the isolation rate was generally similar to other studies, it was lower than the data obtained by Udegbunam et al. (2014) and Meekins et al. (2017). Regional differences, breed, age, and disease conditions of the animals examined in the study, the sampling process, and the applied laboratory test methods were considered to cause differences in the isolation rates achieved in the studies. It has been reported that the use of antimicrobial agents such as oxytetracycline, chloramphenicol, ceftiofur, and gatifloxacin as eye drops locally or systemically in the treatment of keratoconjunctivitis cases in small ruminants yielded successful outcomes in the treatment of the disease (Biswas and Saifuddin 2017; Athira et al. 2018). Although chloramphenicol resistance in *Staphylococcus* spp. strains isolated from ocular swab samples of sheep and goat were reported to be low by Hammadi (2015) and Rhayman et al. (2013), Udegbunam et al. (2014) reported that 71% of the isolates were resistant to chloramphenicol. On the other hand, no resistance to chloramphenicol was found in the isolates obtained in this study. Likewise, the study revealed that all of the isolates were susceptible to gentamicin. However, this result was different from the data reported by Hammadi (2015) and Rhayman et al. (2013). While only one of the *Staphylococcus* spp. isolates isolated from the ocular swab samples was resistant to tetracycline, this result is compatible with the data obtained by Rhayman et al. (2013). On the other hand, another study reported that the rate of resistance to tetracycline group antimicrobial agents was quite high in *Staphylococcus* spp. isolates (Udegbunam et al. 2014). It was thought that the differences in antibiotic use habits in livestock breeding in different geographical regions may have caused differences between the results obtained in the studies. It was also concluded that the nomadic breeding in the Siirt region posed a problem with access to antibiotics, and consequently, antibiotic resistance rates in bacterial agents may have been limited. The biofilm-forming ability has been one of the important virulence factors in *Staphylococcus* spp. isolates. The biofilm formation has been known to play a role in the evasion of the agent from the host defence system and in the development of resistance to antimicrobials (Andrade et al. 2021). The biofilm-forming ability in staphylococcal strains isolated from sheep and goats has been mostly investigated in isolates obtained from milk samples. No studies have been found to investigate the biofilm formation and the presence of genes related to biofilm formation in *Staphylococcus* spp. isolates obtained from ocular samples. In their study, Andrade et al. (2021) reported that 75% of 137 *Staphylococcus* spp. isolates formed biofilm. They reported that the *icaA* gene was identified in 15.90% of 44 isolates and the *icaD* gene was identified in 43.18% (Andrade et al. 2021). Lira et al. (2016) determined that 28% (n=17) of the strains isolated from milk samples formed biofilm, while 82% of these isolates tested positive for the *icaD* gene. Lianou et al. (2023) showed that the majority (71.8%) of *Staphylococcus* spp. strains that they isolated from tank milk in sheep and goat herds were able to form biofilm. The present study revealed biofilm formation in 68.57% of the isolates obtained, as in other studies. Unlike the strains isolated from milk samples, the *icaD* gene was not identified in the isolates that this study examined. However, biofilm formation was not significantly correlated with antibiotic resistance in this study. This result was compatible with the data obtained by Lianou et al. (2021). Conclusively, this study revealed that it is necessary to consider especially coagulase-negative *Staphylococcus* spp. isolates in the cases of keratoconjunctivitis leading to well-being problems in small ruminants. It was demonstrated that antimicrobial agents such as enrofloxacin, gentamicin, and tobramycin can be used for the effective treatment of cases induced by the causative agents. While biofilm formation was found in most of the isolates, it was observed that this was ineffective in the formation of an antimicrobial resistance profile. It was considered that the gathered data would contribute to the studies on the subject.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

ACKNOWLEDGMENT

The abstract of this study was presented as a poster at the 18th Veterinary Surgery Congress.

AUTHOR CONTRIBUTIONS

Idea / Concept: ÖG
Supervision / Consultancy: ÖG
Data Collection and / or Processing: AG
Analysis and / or Interpretation: MY
Writing the Article: ÖG
Critical Review: ÖG

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Van Veterinary Journal

https://dergipark.org.tr/tr/pub/vanvetj

Cite this article as: **Keleş ÖF, Bati B (2024).** Histopathological Evaluation of the Anti-Obesity Effects of the Plant Kenger (*Gundelia tournefortii* L.) in an Experimental Model of Obesity Induced in Rats. *Van Vet J*, 35 (3), 213-217. DOI: https://doi.org/10.36483/vanvetj.1558632

ISSN: 2149-3359 **Original Article** e-ISSN: 2149-8644

Histopathological Evaluation of the Anti-Obesity Effects of the Plant Kenger (*Gundelia tournefortii* L.) in an Experimental Model of Obesity Induced in Rats

Ömer Faruk KELEŞ[1,*] Bedia BATİ[2]

[1]*Van Yuzuncu Yil University, Faculty of Veterinary Medicine, Department of Pathology, 65040, Van, Türkiye*
[2]*Van Yuzuncu Yil University, Faculty of Education, Department of Mathematics and Science Education, 65040, Van, Türkiye*

Received: 01.10.2024 Accepted: 15.11.2024

ABSTRACT In this study, the anti-obesity effects of *Gundelia tournefortii* extract were histopathologically investigated in experimental obesity induced by a high-calorie diet in rats. For this purpose, Wistar-Albino male rats were divided into four groups, each consisting of 10 rats: Control (C), High-Calorie Diet (HC), High-Calorie Diet + *Gundelia tournefortii* 200 mg/kg (HCG1), and High-Calorie Diet + *Gundelia tournefortii* 400 mg/kg (HCG2). The study was conducted over a period of three months. Histopathological analyses of liver tissue samples revealed that the HC group exhibited fatty degeneration, with coagulation necrosis observed in hepatocytes. In the HCG1 group, the liver showed macro-microvesicular fat vacuoles in hepatocytes of the pericentral regions, although this accumulation was significantly milder compared to the HC group. Conversely, the HCG2 group displayed a histological appearance close to that of the control group, with only rare microvesicular fat vacuoles in hepatocytes. As a result, it is evaluated that the *Gundelia tournefortii* extract given with a high-calorie diet in rats has a hepatoprotective effect.

Keywords: *Gundelia tournefortii, Histopathology, Obesity.*

ÖZ

Deneysel Obezite Modeli Oluşturulan Ratlarda Kenger (*Gundelia tournefortii* L.) Bitkisinin Antiobezite Etkisinin Histopatolojik Olarak Değerlendirilmesi

Bu çalışmada, yüksek kalorili diyet ile deneysel obezite oluşturulan ratlarda, kenger (*Gundelia tournefortii*) bitki ektresinin antiobezite etkisinin histopatolojik olarak araştırılması amaçlanmıştır. Bu amaçla Wistar-Albino ırkı erkek ratlar, her grupta 10 rat olacak şekilde toplam 4 gruba Kontrol (K), Yüksek Kalorili Diyet (YK), Yüksek Kalorili Diyet + *Gundelia tournefortii* 200mg/kg (YKG1), Yüksek Kalorili Diyet + *Gundelia tournefortii* 400 mg/kg (YKG2) ayrıldı ve çalışma 3 ay süre ile yürütüldü. Elde edilen sonuçlara göre, karaciğerlerden alınan doku örneklerinden yapılan histopatolojik analizler sonucunda; YK grubunda yağlanma ile hepatositlerin bazısında ise koagulasyon nekrozu izlenmiştir. Ayrıca YKG1 grubundaki ratların karaciğerlerinde YK grubunda olduğu gibi lopçukların periasiner bölgelerindeki hepatositlerde makro-mikroveziküler yağ vakuollerinin bulunduğu, ancak bu birikimlerin YK grubuna göre çok daha hafif olduğu gözlenmiştir. Diğer yandan, YKG2 grubunda histolojik görünümün kontrol grubuna yakın olmakla birlikte hepatositlerde çok seyrek olarak mikroveziküler yağ vakuollerinin bulunduğu tespit edilmiştir. Sonuç olarak; ratlarda yüksek kalorili diyet ile birlikte verilen kenger bitki ekstresinin, hepatoprotektif etkisinin olduğu değerlendirilmektedir.

Anahtar Kelimeler: *Gundelia tournefortii, Histopatoloji, Obesite.*

INTRODUCTION

Obesity emerges as a complex disease that threatens both developed and developing countries, affecting all age groups with its social and psychological aspects (WHO 2017). The primary factors leading to obesity are unbalanced nutrition and a lack of physical activity. Moreover, it has been recognized that genetic, biochemical, physiological, psychological, neurological, environmental, and socio-cultural factors also impact the development of obesity (Chakrabarti 2009; Wright and Aronne 2012).

In addition to medical, behavioral, and physical treatments, various interventions such as dietary measures, surgical procedures, acupuncture, and hypnosis are available for obesity management (Yanovski 2011; Apovian et al. 2015; Bautista et al. 2019). With the growing emphasis on ethnopharmacology, the selection of plant species for testing the safety, efficacy, and quality of pharmacological effects against obesity has become an essential scientific tool in both in vivo and in vitro studies (de Freitas and de Almeida 2017). Epidemiological and

✉ *Corresponding author: ofkeles@yyu.edu.tr

clinical studies have shown that consuming bioactive components, mainlythose rich in secondary metabolites, provides therapeutic properties against obesity-related problems (Mir et al. 2019). It has been suggested that diets rich in active components can be used as an alternative dietary option to suppress oxidative stress and inflammation in the adipose tissues of obese individuals (Mir et al. 2019). Additionally, plant phenols and polyphenols play a role in preventing various pathological conditions due to their antioxidant properties (Losso et al. 2007; Haghi 2011).

The plant *Gundelia tournefortii*, used in this study, has been traditionally favored in medicine. Belonging to the Asteraceae family, it primarily grows in the temperate regions of Asia, including Turkey, Iran, Egypt, Jordan, Cyprus, and Turkmenistan (Çoruh et al. 2007; Matthäus and Özcan 2011). It is particularly rich in phenolic content, including derivatives of caffeoylquinic acid, quercetin gallic acid, and other components responsible for the plant's biological activity (Hajizadeh-Sharafabad et al. 2016; Konak et al. 2017). Additionally, the stalks of this plant are thought to have hepatoprotective properties (Konak et al. 2017). The liver, a significant metabolic organ, plays a crucial role in regulating homeostasis through carbohydrate, protein, and fat metabolism, bile acid production, and drug detoxification (Sapmaz et al. 2015; Koike 2018; Matz-Soja 2019). Therefore, this study aims to histopathologically investigate the effects of *Gundelia tournefortii* (Kenger) plant extract on the development of obesity in rats fed a high-calorie diet.

MATERIAL AND METHODS

The study was conducted with the approval of the Van Yuzuncu Yil University Laboratory Animal Ethics Committee (Date: 31.01.2023, Decision No: 2023/03-15).

Preparation of Plant Material

The plant material used in the study, *Gundelia tournefortii* L., was collected around the province of Van in May. The lyophilized aqueous extract of the plant was prepared according to a modified version of the method by Dalar and Konczak (2013). After the collected plant sample was divided into pieces, 100 grams were weighed and blended with 500 ml of distilled water. The obtained extract was homogenized at +4 °C for 2 hours using a shaker. After homogenization, the mixture was centrifuged for 20 minutes at 10,000 rpm, and the supernatants were separated from the solvent using an evaporator at +37 °C. The concentrated extract was frozen in distilled water, and then lyophilized at -51 °C and 50 millitorr pressure for one week. The obtained lyophilized aqueous fraction was stored at -20 °C until the analysis procedures began.

Experimental Animals

The live subjects used in our research, male Wistar albino rats, were obtained from the Van Yuzuncu Yil University Laboratory Animal Unit. Throughout the experiment, the rats were housed at a temperature of 25±1 °C with a 12-hour light/12-hour dark cycle and were fed ad libitum.

Preparation of Experimental Groups

The rats used in the study were divided into four equal groups. The groups were as follows:

1. Control (C) group: Rats were fed standard rat chow and tap water for three months.

2. High-Calorie Diet (HC) group: Rats were fed a high-calorie rat chow and tap water for three months.

3. High-Calorie Diet + *Gundelia tournefortii* 200 mg/kg (HCG1) group: In addition to the high-calorie rat chow, rats were administered *Gundelia tournefortii* plant extract (200 mg/kg) orally via gavage for the last four weeks.

4. High-Calorie Diet + *Gundelia tournefortii* 400 mg/kg (HCG2) group: In addition to the high-calorie rat chow, rats were administered *Gundelia tournefortii* plant extract (400 mg/kg) orally via gavage for the last four weeks.

All feeds used in the study were obtained from Research Diet. The dosages of the plant extract used in the study (200 and 400 mg/kg) were determined according to the acute toxicity test guidelines of OECD 425 (Organization for Economic Cooperation and Development) (OECD 2008).

Histopathological Examination

At the end of the study, the rats were sacrificed under anesthesia. Liver tissue samples from the rats were fixed for 72 hours in a 10% buffered formaldehyde solution. The tissue samples were then routinely processed and embedded in paraffin blocks. Sections of 4 µm thickness were cut from these blocks using a microtome (Leica RM 2135) and stained using Hematoxylin-Eosin (H.E.) and Oil Red O fat staining techniques. The sections were examined under a light microscope (Nikon80i-DS-RI2).

RESULTS

In the control group, microscopic examination of the rats' livers showed a normal histological appearance. The structure of hepatocytes and portal areas appeared normal, with hepatocytes forming regular remark cords around the central vein, and the sinusoids between these cords were of standardwidth (Fig 1A).

In the (HC) group, a microscopic examination of the rats' livers revealed the presence of macro or microvesicular vacuoles of various sizes with sharp boundaries in the cytoplasm of hepatocytes (Fig 1B-C). Additionally, staining with the special Oil Red O fat stain confirmed these vacuoles contained orange-colored fat deposits (Fig 1D). These morphological changes were localized in the periacinar regions of the lobules. Particularly, coagulation necrosis was observed in many of these hepatocytes. The necrotic hepatocytes typically had darkly stained, pyknotic, and flattened nuclei. Other common microscopic findings included congestion in the central veins and sinusoid dilation. Inflammatory reactions consisting of focal mononuclear cell infiltrations were observed in some portal areas.

In the (HCG1) group, sharp-bordered macro or micro vesicular fat vacuoles were found in hepatocytes of the periacinar regions of lobules (Fig 1E), similar to the HC group. However, these morphological changes were significantly milder compared to the HC group. Moreover, the necrotic changes and congestion in the central veins observed in the HC group were not present in this group. In the (HCG2) group, the histological appearance of all rats' livers was close to that of the control group. However, micro vesicular fat vacuoles were rarely observed in hepatocytes, but neither the necrotic changes in hepatocytes nor congestion in the central veins were detected (Fig 1F).

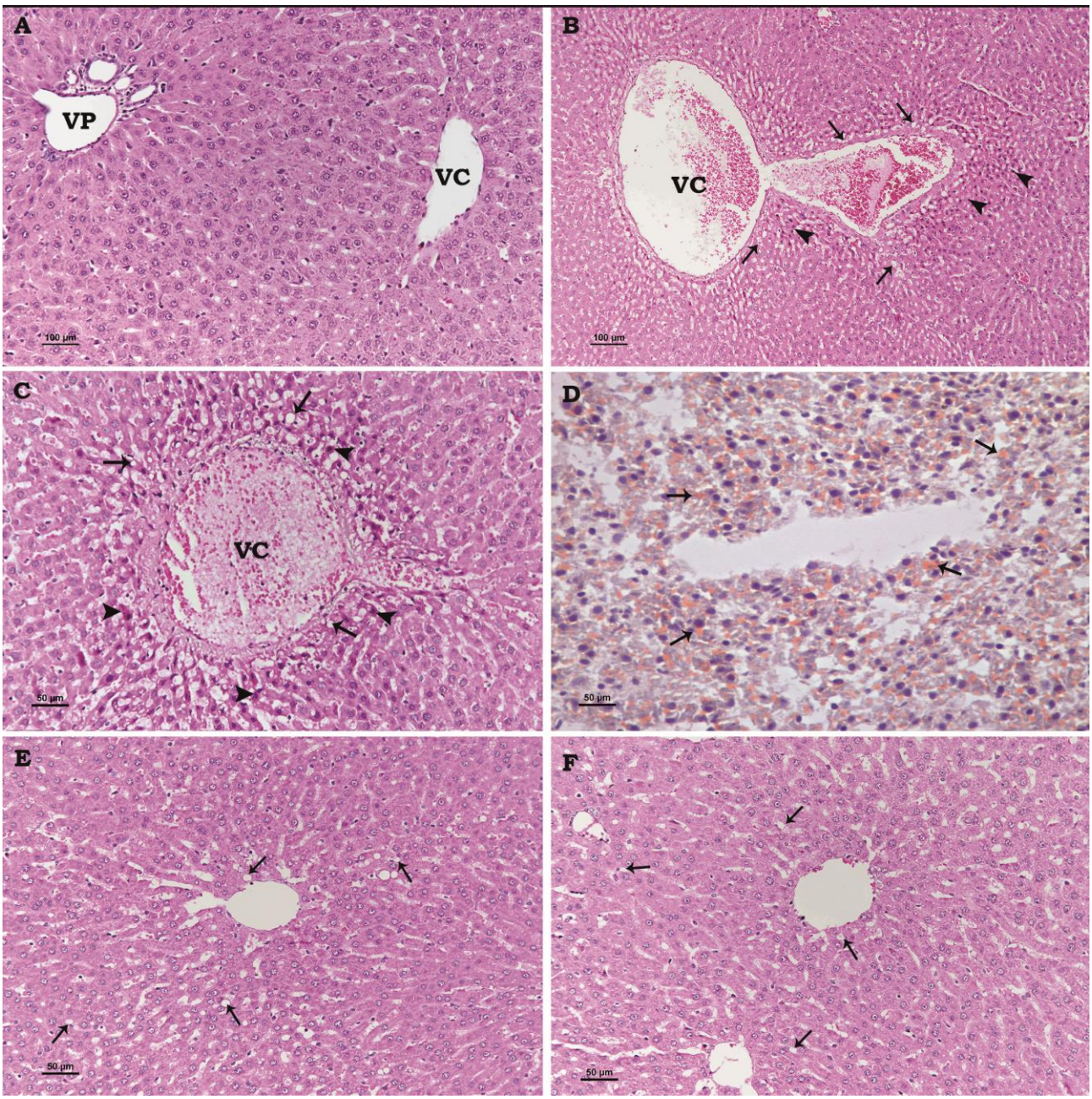

Figure 1: Histopathological images for all groups.

A) C group: The normal microscopic appearance of the liver is observed, with central veins (VC) and portal vein (VP). H.E. Bar; 100 µm. B) HC group: Congestion in central veins (VC) and vacuolar degeneration in centrilobular hepatocytes (arrows) along with coagulation necrosis (arrowheads) are observed in the liver. H.E. Bar; 100 µm. C) HC group: Centrilobular hepatocytes show sharply defined vacuoles of varying sizes (arrows) and coagulation necrosis in hepatocytes (arrowheads). H.E. Bar; 50 µm. D) HC group: Hepatocytes exhibit orange-colored lipid deposits of varying sizes (arrows) in the liver. Oil Red O. Bar; 50 µm. E) HCG1 group: Centrilobular hepatocytes display sharply defined vacuoles (arrows). H.E. Bar; 50 µm. F) HCG2 group: Centrilobular hepatocytes show sharply defined vacuoles (arrows). H.E. Bar; 50 µm.

DISCUSSION AND CONCLUSION

Obesity is recognized globally as a significant public health issue, rapidly increasing in prevalence. It is a major risk factor for the development of many chronic diseases such as heart disease, diabetes, hypertension, certain types of cancer, and musculoskeletal disorders (WHO 2020). According to WHO data, since 1975, the global obesity rate has tripled. In 2016, 39% of adults worldwide aged 18 and over were classified as overweight, and 13% were classified as obese (WHO 2016). Obesity is a multifactorial condition arising from the interaction of genetic, environmental, behavioral, and psychosocial factors. An imbalance between energy intake and expenditure is one of the fundamental causes of obesity. The prevalence of high-calorie, processed foods and a decrease in physical activity contributes to excess energy, leading to fat accumulation (Swinburn et al. 2011). Obesity is associated with a range of serious health problems. Cardiovascular diseases, type 2 diabetes, hypertension, and certain cancer types are strongly linked to obesity. For instance, obesity can lead to insulin resistance, resulting in diabetes (Zimmet et al. 2021). Additionally, obesity increases the risk of hypertension and atherosclerosis, leading to serious complications such as heart attacks and strokes (Lavie et al. 2009). Obesity is also related to musculoskeletal issues like osteoarthritis and psychological disorders such as sleep apnea (Haslam and James 2005).

Gundelia tournefortii is rich in various bioactive components such as phenolic compounds, flavonoids, and essential oils. The high antioxidant capacity of phenolic compounds is known to reduce cellular oxidative stress

(Öztürk and Özçelik 2011). Reducing oxidative stress significantly alleviates insulin resistance, inflammation, and cardiovascular complications associated with obesity (Furukawa et al. 2004).

Gundelia tournefortii also stands out for its anti-inflammatory properties. Obesity is characterized by low-level chronic inflammation, which plays a critical role in the development of insulin resistance, type 2 diabetes, and cardiovascular diseases (Gregor and Hotamisligil 2011). The anti-inflammatory effects of *Gundelia tournefortii* may help prevent such metabolic complications.

Considering the results obtained, significant fat degeneration and coagulation necrosis around the central veins, and congestion in the central veins were observed in the HC group. In contrast, only the HCG1 treatment group showed partially evident fat degeneration around the central veins, but neither necrosis nor congestion was observed. In the other treatment group, hepatocellular degeneration was rare and did not concentrate in any particular area, staying at minimal levels, with no signs of necrosis or congestion observed. It is thought that this hepatoprotective effect may be due to the antioxidant effects of the plants and their beneficial effects on the circulatory system. The likely reason for fat degeneration occurring particularly around the central veins in the liver of the HC group is due to hepatocytes in these areas being most susceptible to hypoxia, and a high-calorie diet possibly exacerbating circulation issues more significantly around the central veins. The reduction in lipolytic activity of hepatocytes due to hypoxia is also considered. Indeed, the congestion in the central veins, which could be interpreted as a circulatory disturbance, was observed only in the HC group, supporting this interpretation.

Similar to our findings, a study by Keleş (2019) on rats fed a high-fat diet also showed sharp-bordered fat vacuoles of various sizes in the centrilobular hepatocytes. Additionally, while coagulation necrosis was observed in some of these hepatocytes, it was prevented in the group fed with Silymarin along with a high-fat diet (Obesity + Silymarin), despite the high-fat diet. In another study, Işık (2019) observed nearly similar morphological changes in the livers of rats on a high-fat diet, noting sharp-bordered fat vacuoles in particularly the centrilobular hepatocytes. However, despite a high-fat diet, *Nigella sativa* fed to one group prevented liver steatosis and degenerative necrotic changes. Furthermore, our study found that the *Gundelia tournefortii* plant extract had a positive effect on the liver tissue of rats fed a high-calorie diet, and these results paralleled the histopathological findings of previous studies (Ejaz et al. 2009; Uyar and Esim 2018). From these studies, Ejaz et al. (2009) showed that curcumin administration and a fatty diet reduced hepatic steatosis in mice. Another study indicated that Mate tea, given in a high-fat diet, could prevent liver damage (Uyar and Esim 2018). Additionally, many studies on plants have reported that foods rich in phytochemicals have hepatoprotective effects against various toxic agents (Bati et al. 2015; Turan and Çelik 2016; Yaman et al. 2016). Polat (2019) noted in their study that treatment with Shepherd's purse in rats with ethanol-induced toxicity reduced some histopathological findings in the liver and improved some liver serum enzyme activities. Moreover, due to their various biological effects, plant-derived compounds have attracted significant attention for their anti-obesity, anti-cancer, and anti-diabetic properties (Engin et al. 2018; Sharifi-Rad et al. 2018; Salehi et al. 2019; Islam et al. 2020). In our study, the experimental groups given the plant extract showed more favorable results in terms of fat degeneration observed in hepatocyte tissues compared to the HC group. Therefore, it was determined that the administration of the plant extract reduced liver steatosis, and these results support the literature. In conclusion, it has been determined that the hepatoprotective effect of the *Gundelia tournefortii* L. plant extract in rats formed with a high-calorie diet may be due to the antioxidant properties of its bioactive constituents.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: ÖFK, BB
Supervision / Consultancy: ÖFK, BB
Data Collection and / or Processing: ÖFK, BB
Analysis and / or Interpretation: ÖFK, BB
Writing the Article: ÖFK, BB
Critical Review: ÖFK, BB

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