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Due to the earthquake disaster in Türkiye on 06 February 2023,
the cover of the Journal is changed to black for this issue

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EDITORIAL

Dear Readers,

As we publish the second issue of our journal for the year 2023, we are deeply saddened by the recent massive earthquakes that struck Turkey and Syria on February 6, 2023, resulting in the loss of many lives. In this moment of grief, we have decided to dedicate the cover page of this issue of our journal to black as a symbol of mourning. Our thoughts and prayers are with those who have lost their lives and their loved ones, and we wish a speedy recovery to the wounded.

Despite this tragedy, we are presenting 13 Research Articles, 1 Short Communication, and 1 Case Report in this issue of our journal. Among the authors of these articles are our academic colleagues who unfortunately lost their lives in the earthquake disaster. The academic community, like many other sectors, has been deeply affected by this catastrophe, and we hope that the wounds caused by this disaster will heal soon. We are grateful for the support extended by both the affected countries and the global community during this challenging time. The recovery process will take time, but we are confident that with the support of all stakeholders, we will overcome this tragedy and emerge stronger. On behalf of the Editorial Board, we express our deepest gratitude to all the individuals and organizations who have extended support to the earthquake victims.

Finally, we extend our respects to all our readers, and we hope that such sufferings will never occur again.

Dr. Levent ALTINTAŞ
Editor in Chief
Ankara Üniversitesi Veteriner Fakültesi Dergisi

Determination of blood heavy metal concentrations and oxidant-antioxidant capacities in Angora cats at different age and gender

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ABSTRACT

This study was conducted to investigate heavy metal concentrations and oxidative status of plasma and erythrocytes in Angora cats at different ages and gender. Sixteen young (less than 1 year old) and 14 adult (1–6 years old) cats were also grouped according to gender as male (*n* = 17) and female (*n* = 13). The separated plasma samples from cat's blood were analyzed for selected heavy metals and total oxidant and antioxidant capacities (TOC and TAC) and calculated for oxidative stress index (OSI). The erythrocyte hemolysates were also evaluated for malondialdehyde (MDA), and super oxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Results of the study showed that most of the measured metals were not varied statistically according to age or gender. However, adult cats had significantly ($P<0.01$) higher Cu and lower Fe levels compared to young cats. Plasma levels of TOC, TAC and OSI, and erythrocyte MDA concentrations in young cats were significantly ($P<0.05$) higher than that of adults. While the SOD activity was decreased by the age, GPX activity was increased ($P<0.05$). However, the activity of CAT was changed by only gender, which was higher in males ($P<0.01$). In conclusion, metals, especially trace elements, are required for many kinds of physiological processes and the synthesis of antioxidant enzymes. Therefore, it can be suggested that the periodic measurement of metals and the addition of common antioxidant supplements to the diet of adult Angora cats will support weakening antioxidant mechanisms by age.

Introduction

The elements that had a density greater than 5 g/cm^3 water are called heavy metals. They have generally toxic effects even at low concentrations. While some of them are nonessential for the body such as arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb), some others are essential, such as aluminum (Al), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), selenium (Se), tin (Sn), and zinc (Zn). The latter is also defined as trace elements, which involve many kinds of metabolic events in humans and animals. However, both of them (essential and non-essential metals) may be toxic unless taken/given appropriate concentrations (20, 25).

Heavy metals are the most widespread chemical groups among the potential environmental contaminants because of an exponential increase in their usage in industry, agriculture, and technology. They are transported to the body through digestion, respiration and other routes as they are persistent in the environment for a long time, and threaten man and animals' health (20, 24). Pet animals may also be exposed to these chemicals continuously that resulting in bioaccumulation in many tissues in relation to the long lifespan of pets (30).

Moreover, canine and feline may have similar metabolic and clinical responses to toxic substances as humans (24). Exposure to high levels of heavy metals has toxic, teratogenic, and mutagenic effects and leads to the production of free radicals as a result of oxidative degradation of lipids (13, 20).

Although reactive oxygen species (ROS) are generated as a consequence of cellular metabolism in many physiological processes, the harmful effects of them are inhibited by endogenous antioxidant mechanisms, which are classified as nonenzymatic and enzymatic antioxidants (31). However, they can be potentially toxic and cause oxidative stress if the oxidant-antioxidant balance is disturbed. For instance, heavy metals may cause to the oxidative damage (13). Moreover, McMichael (23) reported an age-related increase in ROS formation in mitochondria during aging.

Angora cats that have gold/blue colored eyes and generally white hairs are one of the kind cat breeds lived in the Central Anatolia Region of Türkiye. Many studies have been conducted to get more information about them from all perspectives to save them as they are endangered (4, 14, 21, 26). In this study, it was aimed to investigate blood heavy metal concentrations and oxidant-antioxidant status in Angora cats, which drink tap water in Kırıkkale province at different age and gender.

Materials and Method

Animals: Thirty Angora cats were grouped according to age as young (less than 1 year old, n = 16) and adult (1–6 years old, n = 14) or gender as male (n = 17) and female (n = 13). The healthy and vaccinated cats housed in Kırıkkale University, Faculty of Veterinary Medicine at the same conditions were fed ad libitum with commercial dry cat food (Fit32, Royal Canin, France) and tap water during the investigation. All the procedures of this research were conducted with the approval of the Local Ethical Committee of Kırıkkale University (document number: 2019/110).

Sample collection and heavy metal analysis: Blood samples taken from *vena saphena medialis* into the heparinized test tubes were centrifuged at 1000 g for 10 min at 4 °C to obtain plasma and erythrocytes. Plasma samples were analyzed using inductively coupled plasma optical emission spectrometer (ICP-OES, Spectroblue, Germany) for Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sn, vanadium (V), Zn. Then the measured values were quantified by using calibration curves plotted from analytical standards (Merck, Germany) as previously described by Aluc and Ekici (3). The rest of the plasma and hemolysates prepared re-suspending of erythrocytes in an equal volume of phosphate-buffered solution (PBS, pH 7.4), were stored at −80° C for further analysis of the oxidant and antioxidants.

Evaluation of oxidative status of plasma: Plasma TOC levels of cats were measured via commercial Total Oxidant Status kits (Rel Assay Diagnostic, Türkiye) as previously described by Erel (16), and the results were expressed as µmol H_2O_2 equivalent/L. Plasma TAC level was also measured using commercial Total Antioxidant Status kits (Rel Assay Diagnostic, Türkiye) according to the novel automated method described by Erel (15), in which the results were expressed as mmol Trolox equivalent/L. The TOC/TAC ratio is expressed as oxidative stress index (OSI) (32). It was calculated according to the following formula: OSI (arbitrary unit, AU) = TOC (µmol H_2O_2 equivalent/L) / TAC (µmol Trolox equivalent/L).

Assessment of oxidative status of erythrocytes: The erythrocyte hemolysate was used to determine the level of cellular oxidant and antioxidants. The MDA concentrations, an indicator of lipid peroxidation, were measured as previously described by Buege and Aust (10). The SOD, CAT, and GPX activities of erythrocytes were analyzed with the same named commercial assay kits (Rel Assay Diagnostics, Türkiye) according to the manufacturer's instructions.

Data analysis: Descriptive analysis of all data was performed using SPSS 18.0 package program for Windows. After the distribution of normality of data checked by Shapiro-Wilk test, the effects of age and gender on the measured data were investigated using two-way ANOVA for statistical significance. The non-normally distributed data were analyzed using Mann–Whitney U test as a non-parametric test for young and adult, or male and female groups. $P<0.05$ was considered statistically significant.

Results

Plasma heavy metal accumulation in cats: Plasma heavy metal levels in cats at different ages and sex are shown in Table 1. Arsenic, Co, Cd, and Hg have not included in the table because they were under the detection limit of the used technique in all plasma samples. The limits of detection values of these metals were 1.60, 1.38, 0.79, and 1.60 ppb for As, Co, Cd, and Hg, respectively. It was observed that most of the measured elements were not varied statistically according to age or gender. However, the plasma levels of Cu in adults were significantly higher than the young cats ($P<0.01$). The concentrations of Fe in the plasma of young cats were higher compared to adults ($P<0.01$). Additionally, plasma Se levels in females ($P<0.01$), and V levels ($P<0.001$) in male Angora cats were significantly higher than those of the other gender, respectively.

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Table 1. Variations of plasma heavy metal concentrations according to age and gender in Angora cats.

Elements	Groups	n	Mean (mg/L)	SEM	Min.	Max.
Lead (Pb)	Young	16	0.206	0.028	0.149	0.616
	Adult	14	0.174	0.007	0.148	0.232
	Male	17	0.198	0.023	0.149	0.616
	Female	13	0.177	0.010	0.148	0.232
Aluminum (Al)	Young	16	3.380	0.159	2.159	4.578
	Adult	14	3.363	0.092	2.964	4.288
	Male	17	3.449	0.137	2.159	4.578
	Female	13	3.217	0.029	3.092	3.331
Chromium (Cr)	Young	16	0.316	0.049	0.217	0.977
	Adult	14	0.240	0.009	0.210	0.305
	Male	17	0.302	0.040	0.214	0.977
	Female	13	0.237	0.008	0.210	0.282
Copper (Cu)	Young	16	0.495	0.024	0.254	0.638
	Adult	14	0.687**	0.066	0.390	1.462
	Male	17	0.565	0.028	0.254	0.773
	Female	13	0.625	0.100	0.390	1.462
Iron (Fe)	Young	16	2.902**	0.354	1.517	6.804
	Adult	14	1.952	0.305	1.058	5.636
	Male	17	2.452	0.271	1.129	5.636
	Female	13	2.472	0.535	1.058	6.804
Manganese (Mn)	Young	16	0.035	0.004	0.022	0.086
	Adult	14	0.031	0.004	0.021	0.069
	Male	17	0.034	0.004	0.022	0.086
	Female	13	0.033	0.004	0.021	0.056
Nickel (Ni)	Young	16	0.129	0.019	0.092	0.409
	Adult	14	0.099	0.006	0.080	0.145
	Male	17	0.120	0.016	0.084	0.409
	Female	13	0.105	0.007	0.080	0.145
Selenium (Se)	Young	16	0.147	0.090	0.000	1.275
	Adult	14	0.277	0.188	0.000	1.979
	Male	17	0.000	0.000	0.000	0.000
	Female	13	0.622**	0.256	0.000	1.979
Tin (Sn)	Young	17	35.550	1.297	30.133	48.014
	Adult	13	34.425	0.945	31.306	43.232
	Male	16	35.304	1.152	30.133	48.014
	Female	14	34.467	0.859	31.306	38.138
Vanadium (V)	Young	17	0.009	0.002	0.000	0.022
	Adult	13	0.005	0.001	0.000	0.015
	Male	16	0.010***	0.002	0.000	0.022
	Female	14	0.001	0.001	0.000	0.004
Zinc (Zn)	Young	17	0.825	0.128	0.503	2.573
	Adult	13	0.651	0.023	0.546	0.868
	Male	16	0.777	0.104	0.503	2.573
	Female	14	0.676	0.031	0.558	0.868

The detection limits of the used technique for the metals were just like the following; Al: 0.47, As: 1.60, Cd: 0.79, Co: 1.38, Cr: 8.50, Cu: 0.09, Fe: 0.19, Hg: 1.60, Mn: 4.26, Ni: 0.29, Pb: 1.68, Se: 1.87, Sn: 0.13, V: 4.72, Zn: 0.31 ppb. The As, Cd, Co, and Hg were not included the table because they were under the limit of detection. SEM: Standard error mean, Asterisk represents the statistical significance, **: P<0.01, ***: P<0.001.

***Plasma oxidant and antioxidant status of cats*:** The TOC levels of plasma in young (P<0.001) and male cats (P<0.05) were significantly higher than that of the adult and female cats, respectively (Table 2). The plasma levels of TAC in young cats were significantly (P<0.001) higher than the adults. It was also higher in male cats but not statistically significant (P>0.05) according to females. The OSI of plasma in young cats was remarkably higher than that of adults (P<0.01). It was insignificantly different in males from females (P>0.05).

Erythrocyte oxidant and antioxidant status of cats: As shown in Table 3, the concentrations of MDA in erythrocytes of young cats were significantly higher than in adults (P<0.05). The increased level of MDA in male cats compared to females was not significant (P>0.05). While SOD activity of erythrocytes was higher in youngers than adults (P<0.001), GPX activity was higher in adults than in young cats (P<0.01). Additionally, CAT activity was higher in males than the female (P<0.001). Although GPX activity of female cats was higher than the male, it was not seen statistically significant because of the wide interval of the values.

Table 2. Plasma oxidative stress status of Angora cats according to age and gender.

	Groups	n	Mean	SEM	Min.	Max.
TOC (µmol/L)	Young	16	1.70***	0.13	0.89	2.60
	Adult	14	0.89	0.08	0.49	1.52
	Male	17	1.53*	0.15	0.74	2.60
	Female	13	1.05	0.11	0.49	1.71
TAC (mmol/L)	Young	16	1.61***	0.08	1.00	1.98
	Adult	14	1.30	0.10	0.86	1.84
	Male	17	1.53	0.07	1.00	1.95
	Female	13	1.38	0.13	0.86	1.98
OSI (arbitrary unite)	Young	16	0.11**	0.01	0.05	0.21
	Adult	14	0.07	0.00	0.05	0.10
	Male	17	0.11	0.01	0.05	0.21
	Female	13	0.08	0.00	0.05	0.10

SEM: Standard error mean, Asterisk represents the statistical significance, *: P<0.05, **: P<0.01, ***: P<0.001.

Table 3. Erythrocyte oxidative stress status of Angora cats according to age and gender.

	Groups	n	Mean	SEM	Min.	Max.
MDA (nmol/gHb)	Young	16	288.70*	45.43	114.02	489.34
	Adult	14	199.18	15.62	104.52	318.77
	Male	17	258.57	34.62	114.02	489.34
	Female	13	202.62	15.70	104.52	265.31
SOD (U/ gHb)	Young	16	1898.65***	43.51	1701.65	2064.36
	Adult	14	1639.72	36.18	1449.64	1911.93
	Male	17	1749.93	51.30	1449.64	2064.36
	Female	13	1720.72	50.23	1578.74	1911.93
CAT (U/ gHb)	Young	16	0.59	0.09	0.27	1.01
	Adult	14	0.53	0.08	0.13	0.89
	Male	17	0.68***	0.06	0.27	1.01
	Female	13	0.26	0.05	0.13	0.51
GPX (U/ gHb)	Young	16	3.02	0.45	0.63	5.13
	Adult	14	5.14**	0.37	2.85	7.76
	Male	17	4.01	0.43	0.63	7.76
	Female	13	5.00	0.58	2.85	6.61

SEM: Standard error mean, Asterisk represents the statistical significance, *: P<0.05, **: P<0.01, ***: P<0.001.

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Discussion and Conclusion

Angora cat is one of the special cat breeds grown and lived in Ankara and its surrounding provinces such as Kırıkkale and Çankırı of Türkiye. This study is conducted in Kırıkkale, which is a small industrial region containing arms factories, ammunition supply industry, and oil refineries. It is well known that industrial wastes contaminate environmental sources such as air, soil, and water with many kinds of pollutants including heavy metals. Kızılırmak, the longest river in Türkiye passes through Kırıkkale, and heavy metal contamination in the Kızılırmak river has been shown in several previous studies (7, 11). It has also been reported that some heavy metals were detected in soil, water and feedstuff samples collected from close to industrial areas in Kırıkkale (9). Therefore, in the present study, it was investigated plasma heavy metal concentrations and oxidant-antioxidant status of Angora cats, which drink tap water in Kırıkkale at different age and gender.

According to findings of this study, nonessential metals except Pb were not detected in plasma samples of cats. The levels of lead and most of the trace elements were varied, but not significant in cats' plasma at different age and gender as seen in Table 1. However, Cu concentration increased while Fe decreased by aging. Simsek et al. (27) has previously reported that serum Al, V, Mn, Ni, As and Sn levels were significantly higher in adult Angora goats that bred in Çankırı province compared to young goats while Cr, Fe, Co, Cu, Zn, Se, Cd, and Pb were similar levels in both. However, Esposito et al. (17) showed that Pb concentrations in the liver and kidney of stray cats in Naples, Italy were decreased by the age while Cd concentrations were increased. In another study, it was reported that while most of the metal levels accumulated in various organs and tissues were significantly higher in the adults than in the chicks, Ni and Pb in the brain, skeletal muscles from pectoral and femoral regions, liver, kidney and skin, and Cu in liver were seen lower concentrations in the adults compared to White Egret chicks (19). The only metal accumulated in the plasma by the age was Cu in our study. This situation was compatible with the findings of Doong et al. (12) who have reported that plasma Cu level in adult cats was higher than that of the kitten.

Heavy metal deposits can also differ according to gender. In this study, it was observed that plasma Se level in female cats were significantly higher than in males and, V levels in males were significantly higher than that of female cats. Altunok et al. (2) have shown that Ba, Al, Cu, Mn, and Sr were detected in high levels in female Van cats than in males, and none of the metals were differed statistically by the age as in Fe which was high level in young. Alternatively, Al-Kalidi et al. (1) demonstrated that the plasma Cu levels of female home cats was significantly higher than that of males while Fe levels did not differ according to gender. In accordance with our results, high Se levels in hair samples have been detected in female healthy cats compared to males (6). Altunok et al. (2) have also showed that serum Se level was higher in female Van cats compared to males, but was statistically insignificant. In another study, V level in the hair samples of male cats was higher compared to females (29), which is in agreement with our findings in terms of plasma V levels.

Exposure to inappropriate concentrations of heavy metals may have adverse effects even if they are required for several physiological processes (20). Although the detected concentrations of these metals in Angora cats are not high enough to cause acute or serious toxic effects, possible chronic toxicity cannot be ignored, since these chemicals can accumulate in the body over time. On the other hand, the measured levels of metal may be related to metal contamination of tap water, which can be affected by location. The study conducted by Behrooz and Poma (8) also supports this sight, in which different heavy metal levels were detected in plasma samples of wild cats taken from different regions.

Heavy metals may also lead to oxidative damages in the body (13). Although heavy metal levels did not appear high in young cats, the results of TOS, MDA and OSI show that the young cats have taken tap water had higher oxidative stress than their older ones in this study. It was seen that the TAC and SOD levels were also increasing against increased oxidative stress in young cats. A similar study conducted by Simsek et al. (26) showed that the concentration of MDA was higher in adult Angora goats compared to young goats, and SOD activity of goat erythrocytes was decreased by the age. Mitochondrial SOD plays a major role in defense mechanisms against oxidants. However, it is well known that the concentrations of mitochondrial antioxidants decrease by age (23). This situation may be the responsible lower level of SOD activity in adult cats.

Destructive effects of oxidative stress on biological macromolecules are inhibited by endogenous antioxidants such as GPX and CAT. Catalase is a heme protein located in peroxisomes and converts H_2O_2, generated in the cytosol or peroxisomes, to water and oxygen while GPX is protective against lipid peroxides (23). There are different reports of variations of enzymatic antioxidant activity according to age. For example, Tekeli et al. (31) have showed that serum MDA levels, and GSH and GPX activity of erythrocytes in Saanen goats were increased while the CAT activity was decreased by the age. In another study, it was found an age-related decrease in SOD activity while GPX increase in humans (22). Aydilek and Şimşek (5) have also reported increased GPX activity in mares and Gaál et al. (18) have revealed increased SOD

and GPX activity in dogs, while the latter researchers have showed decreased GPX and CAT activity in cows during aging as well. However, in the present study, adult cats had similar CAT and higher GPX activity compared to young cats in compliance with some previous studies. It is thought that oxidative stress during aging may increase the levels of GHS and enzymatic activities of GPX (28). Despite all the efforts, we have not found any study examining the heavy metal-oxidant and antioxidant relationship in cats or dogs depending on age or gender, and we think that our findings may be useful for future studies in this regard.

Finally, this study specified the age- and sex-related variations of some heavy metals and oxidant-antioxidant capacities of Angora cats given tap water and lived in Kırıkkale. It was revealed that toxic heavy metals such as As, Cd, Hg, and Co were not detected in plasma samples, and the levels of some others (Cu, Fe, Se, and V) changed according to age or gender. Additionally, young cats had higher levels of TOS, OSI, MDA, and SOD, whereas lower GPX activity compared to adults. Only CAT activity was differed by gender. In conclusion, metals, especially trace elements, are required for many kinds of physiological processes and the synthesis of antioxidant enzymes. Therefore, it can be suggested that the periodic measurement of metals and the addition of common antioxidant supplements to the diet of adult Angora cats will support weakening antioxidant mechanisms by the age.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

RK, AAY and HE conceived and planned the experiments. RK, YA and EK collected blood and plasma samples and carried out the experiments. YA and HE measured heavy matal concentratios of samples. RK and AAY analyzed the samples for oxidative stress parametes. RK, AAY and HE contributed to the interpretation of the results. RK and AAY took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Local Ethical Committee of Kırıkkale University with the document number of 2019/3-21.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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The assessment of effectiveness of a novel antidepressant, Agomelatine on anxiety and depression induced by fluoride intoxication by means of Open-Field and Hot-Plate tests in mouse model *(BalB-C)*

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ABSTRACT

It is well known that fluoride (F) poisoning causes anxiety and depression, and Agomelatine, an analogue of melatonin, has been reported to be effective on anxiety and depression. Therefore, the aim of this study is to investigate the short-term efficacy of Agomelatine application on anxiety and depression caused by F intoxication via Open-Field and Hot-Plate tests. Forty male *BalB-C* mice, aged 5-6 months, constituted the research material for this study. Subjects were randomly divided into 4 groups (Healthy-Control, Fluorosis-Control, 25 mg/kg Agomelatine, 50 mg/kg Agomelatine). Healthy-Control group (HC) received tap water, containing 0.3 ± 0.05 mgF/L. Fluorosis-Control group (F) received drinking water containing 40 mgF/L. Other two experimental groups (25 and 50) received drinking water containing 40 mgF/L and a single dose of Agomelatine (25 and 50 mg/kg respectively). The effect of Agomelatine on anxiety and depression induced by high dose F was evaluated using Open-Field and Hot-Plate tests compared to control groups. Fluorosis caused to decrease in Rearing, Grooming and Square numbers of Open-Field test and to increase Defecation counts ($P<0.05$). Agolematine applications enabled to normalize the Open-Field Test data. Similarly, according to the Hot-Plate findings, low reaction time caused by fluorosis increased in Agomelatine groups ($P<0.05$). According to those results, psychological improvement was observed in patients with fluorosis compared to the control group after Agomelatine applications. Consequently, according to Open-Field and Hot-Plate tests findings, it could be concluded that Agomelatine has a curative effect on anxiety and depression induced by F toxicity.

Introduction

It is well known that fluoride (F) is highly electronegative halogen (11, 19). Areas contaminated with F are reported to exist widely in both natural and industrial environments (7, 16, 31). Therefore, human and animal populations are under the risk of F toxicity and subsequent fluorosis. It is also well known that F poisoning damages metabolism, hormones (15, 23), hard (19-21) and soft tissues (10, 14) in animals and humans. Moreover, the brain and all other nervous system are also affected by F intoxication (11, 24, 28). In this case, it is an inevitable fact that brain functions and locomotor activities are also affected by F intoxication (7, 24, 30, 33, 37). Neurological and psychiatric disorders have also been reported in cases of F toxicity, such as; mental retardation, memory impairment, learning disruption, lethargy, memory, and concentration impairment, thinking difficulties etc. (7).

Agomelatine is a novel synthetic analogue of the melatonin hormone that has been used in the treatment of psychiatric disorders such as depression and anxiety (5, 29, 38). Its therapeutic effect is via its agonist effects on MT1 / MT2 receptors (1, 9, 26, 36) and antagonist effects on 5-hydroxytryptamine-2C (5-HT_{2C}) receptor (5, 6, 29).

It is reported that the Open-Field test is used as an indicator of the emotional state (depression, anxiety, etc.) in animal models with the conditions described above. The

Open-Field test is also used to investigate the effectiveness of antidepressant drugs on animal models (24, 28, 30, 33, 37). However, psychological problems such as depression and anxiety are known to reduce tolerance to distressing situations (2). The Hot-Plate test is used to evaluate the tolerance level to heat stress on animal models (3, 13, 22). In terms of its effect on 5-HT_{2C} receptors, Agomelatine is likely to have an effect on pain-heat tolerance (5, 6, 29, 36).

Depression and anxiety is an important problem in animals as well as humans and is often overlooked. The prevalence of fluoride toxicity with the increase of industrialization causes these two situations to interact with each other. There is rather limited information in the literature about Agomelatine, which has been recently used as a novel antidepressant in psychological state disorders after fluorine intoxication. This research will reveal the short-term effectiveness of Agomelatine in the treatment of these mental disorders. The results of this study will also guide further research examining the long-term use of Agomelatine in similar situations.

For the aforementioned reasons, the aim of this study was to investigate the short-term effects of Agomelatine on mice exposed to F intoxication and suffering from depression and anxiety diagnosed by Open-Field and Hot-Plate tests.

Materials and Methods

Experimental animals and design: This study was conducted in 40 male mice (*BalB-C*) 5-6 months old and weighing 25.0 ± 1.8 g. The subjects were divided into 4 equal groups randomly and kept under stable temperature (20 ± 0.5 C) and artificial lighting condition with tungsten lamp (12 hours dark and 12 hours light). Subject groups were designed as Healthy-Control group (Group HC), Fluorinated group (only exposed to 40 mgF/L, Group F), First experimental group received fluorinated drinking water and 25 mg/kg/bw Agomelatine (Group 25) and Second experimental group received fluorinated drinking water and 50 mg/kg/bw Agomelatine (Group 50). The amount of Agomelatine applied was determined as normal and maximum dose, considering the previous studies (1, 5, 6, 17, 26, 29, 38). Agomelatine were given the experimental groups in 0.3 ml 1% Hydroxyethyl-cellulose solution via intra peritoneal (1, 36). Intra peritoneal 0.3 ml 1% Hydroxyethyl-cellulose solution also applied the other control groups. Agomelatine application time accepted as first application start time for Open-Field and Hot-Plate tests (min. 0^{th}). All experimental groups except Group HC received drinking water containing 40 mgF/L for 3 mounts, control group received only tap water containing 0.3 ± 0.05 mgF/L. These drinking waters and commercial food were given *ad libitum* during experiment. Ingredients of Commercial food, purchased from Bayramoğlu Yem ve Un San. Tic. A.Ş. (ISO 9001:2000, ISO 22000:2005), were presented in Table 1.

Table 1. Nutritional content of commercial feed received by experimental animals, reported by commercial firm (Bayramoğlu A.Ş.).

Diet Composition	Amounts and Units	Diet Composition	Amounts and Units
Dry matter	88%	Phosphorus	0.75%
Crude protein	17%	NaCl	0.6%
Crude cellulose	12%	Vitamin A	5000 IU/kg
Crude ash	10%	Vitamin D_3	600 IU/kg
Acid insoluble ash	1%	Vitamin E	25 mg/kg
Calcium	1.5%	Metabolic energy	2600kcal/kg

Raw materials for this composition were barley, corn, corn chaff, corn gluten, wheat, rye chaff, cotton seed meal, sunflower meal, dicalcium phosphate, vitamins and minerals.

Timing design of Agomelatine application for Open-Field and Hot-Plate tests: It is reported that the plasma peak value of Agomelatine is between 45 and 90 minutes after a single dose administration and its half-life is approximately at 2 hours (1, 6, 17, 36, 38). Therefore, Open-Field test was performed at three times (0^{th}, 60^{th} and 90^{th} minutes) and "Hot-Plate" test was also carried out one-shot at 90^{th} minute to observe reactions of subjects after Agomelatine application. The Open-Field test does not hurt the subjects, but not Hot-Plate test. Therefore, three different effective time points for Open-Field test procedures following single dose of Agomeletine application were preferred. But, a single time point in the middle of the effective time interval was chosen for the Hot-Plate test application.

Open-Field Test and Procedures: The Open-Field test is used to assess behavioral changes such as anxiety and depression for animal models. It is also used to demonstrate the efficiency of drugs in such cases (24, 33, 37, 30). For Open-Field test application, a closed area of 80x80 cm with a transparent barrier was used, divided into 64 equal squares with permanent lines (18). The subjects were left in the middle of the test area and their behaviors were recorded with a video camera for 5 minutes. Each behavior (Rearing, Grooming, Crossed Squares and Defecation) was counted from this video.

Hot-Plate Test and Procedures: Hot-Plate test is an experimental method used to determine the pain threshold in an animal model (3, 13, 18, 22). For this purpose, a

heater plate adjusted to 50 ºC was used. The perimeter of the heating plate was surrounded by transparent material. "Hind Paw Licking" or "Jumping off" movements were identified as expected reaction. If any of these actions were observed, the test was terminated and the time recorded as "Reaction Time". In the event of no reaction, the test was scheduled to be terminated at 45 seconds and the Reaction Time considered as 45th second. The Hot-Plate test was performed in the 90th minute following the Agomelatine application.

Special Apparatus, chemicals and their preparations: Fluoridation of drinking water: Tap water already has included F in the level of 0.3 ± 0.05 mgF/L. The required reinforcement for 40 mgF/L level was provided by the addition of Sodium Fluoride (NaF, Merck 106449). The F content of the drinking water was confirmed by means of ion meter equipped with F ion selective electrode (Orion 4-Star portable ion meter and F ion-selective electrode - Orion 9609BNWP) (16, 19, 20, 21).

Agomelatine (N-[2-(7-Methoxy-1-naphthalenyl) ethyl]-acetamide, Sigma-Aldrich A1362) were used in 1% Hydroxyethyl-cellulose (Sigma-Aldrich 54290) intraperitoneally (IP) (1).

Statistical Analysis: Firstly, normality test (Kolmogorov-Smirnov) was performed for all test data (Hot-Plate and Open-Field) according to the groups.

One-Way ANOVA test was applied to check the significance of the difference between the Hot-Plate test data groups, which show normal distribution according to the groups (P>0.05). Homogeneity test result in One-Way ANOVA was insignificant (P>0.05). Tukey HSD was performed as Multiple Comparisons.

Normality test results for Open-Field test data were insignificant for Rearing and crossed Squares count data (P>0.05, Parametric data) and significant for Grooming and Defecation count data (P<0.05, Nonparametric data).

Repeat Measures (RM) ANOVA and Freadman tests were used to compare groups with each other over time (RM ANOVA for parametric, Friedman for nonparametric groups). Within RM ANOVA, Mauchly's test of Sphericity was significant (P<0.05) for Grooming and insignificant for crossed Squares groups. For this reason, Greenhouse-Geisser test was taken into consideration in the evaluation of Grooming data and Tamhane's T2 multiple comparisons test. But sphericity assumed for evaluation of crossed Squares data and Tukey HSD test for multiple comparisons was performed.

Grooming and Defecation data were illustrated in Figures 2 and 4 apparently; the course of the groups over time (HC, F, 25 and 50) was compared using the Friedman test and Wilcoxon test was used for multiple comparisons. The Kruskal-Wallis test was used to check for differences between the groups' data at the same time (0th, 60th and 90th minutes). Mann-Whitney U test was used for multiple comparisons of these time data. Bonferroni correction was performed manually for both Wilcoxon and Mann-Whitney U tests.

Results

All test data including Open-Field and Hot-Plate were analyzed for being normal distribution. Kolmogorov-Smirnov test results as the smallest values were as follows; for Open-Field test groups are as follows; Rearing; P>0.05, Grooming; P<0.05, Squares; P>0.05 and Defecation: P<0.05 and for the Hot-Plate data groups: P>0.05

The relationship among the Open-Field test parameters was examined with Spearman's Correlation test and the results are presented in Table 2.

Table 2. Spearman's Correlation test results (r) for Open-Field test parameters including Rearing, Grooming, Squars and Defecation.

	Grooming	Squars	Defecation
Rearing	0.322**	0.221*	-0.204*
Grooming		0.508**	-0.457**
Squars			-0.347**

*: P<0.05, **: P<0.01

For Rearing data, the RM ANOVA test was performed to reveal the relationship of groups with each other over time. Since the Sphericity value was found to be significant (P<0.01), the Greenhouse-Geisser test was taken into account for the interaction of time and groups (P<0.001). Tamhame's T2 Post-Hoc test was used for multiple comparisons of the groups. All of these test results are illustrated in Figure 1.

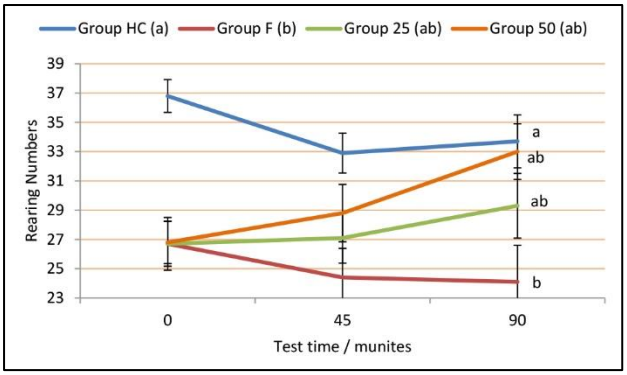

Figure 1. Course of Rearing groups over time and statistical comparisons with RM ANOVA test.
a,b: The difference between groups having different superscripts is statistically significant (P<0.01).

One-Way ANOVA test result of Rearing were as follows for minutes 0th; P<0.001 (Post Hoc; Group HC have statistical differences with all other groups) and minutes 90th; P<0.05 (Post Hoc; Group HC have no statistical differences with Groups 25 and 50, but not Group F).

Grooming and Defecation data, which are the two parameters of Open-Field test, did not show normal distribution (Kolmogrov Simirnov; P<0.05). Therefore, RM ANOVA, a parametric test, could not be used to analyze the relationship of the groups over time. Although the course of the groups can be seen clearly on the graphs, the situation should be clarified with statistical tests. For this purpose, nonparametric tests were used. Firstly, the course of each group (HC, F, 25 and 50) over time was evaluated separately with the Friedman test and results indicated with superscripts on the right side of the graphs (Figure 2 and 4). Then, Wilcoxon test was used to reveal Multiple Comparisons. Secondly, the differences of the groups (HC, F, 25 and 50) for each time point (0th, 45th and 90th) were demonstrated by the Kruskal-Wallis test and the results are shown in superscripts at the top of the graphs (Figure 2 and 4). Multiple Comparisons for these groups were performed by the Mann-Whitney U test (Table 3 and 4).

Wilcoxon test results (for Friedman test important groups) of Grooming data for both 25 and 50 mg Agomelatine groups are as follows: The difference between 0th -45th and 0th-90th minutes was found to be significant (P<0.01), but the difference between 45th- 90th minutes was not significant (P>0.05).

In case of Figure 2 and Table 3 are evaluated together, it was determined that the Grooming numbers were higher in the Healthy Control (HC) group than in the other Fluoride applied groups, and significant differences detected between them at the 0th minute (P<0.001). However, over time, a dose-dependent increase was observed in the treatment groups (Group 25 and 50). While the difference between the treatment groups and the F group gain a significancy in the 90th minute data (P<0.01), the differences between the treatment groups and the HC group decreased depending on the dose (P<0.05), and even the difference between the HC group and Group 50 was found to be statistically insignificant (P> 0.05).

For crossed Squares data, the RM ANOVA test was performed to reveal the relationship of groups with each other over time. Since the Sphericity value was found to be significant (P>0.05), "Sphericity assumed" option has been considered for the interaction of time and groups (P<0.001). Tukey HSD Post-Hoc test was employed for multiple comparisons of the groups. All test results are presented in Figure 3.

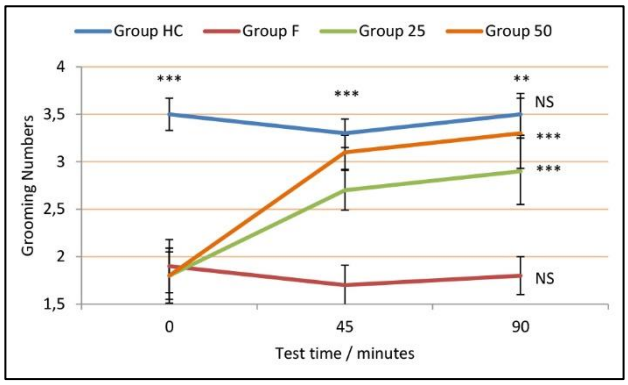

Figure 2. The course of the groups (HC, F, 25 and 50) within time (0th, 45 and 90th minutes) according to the number of Grooming data in the Open-Field test The asterisks at the top of the graph are used for statistical comparisons of independent variables (Kruskal-Wallis test) according to time points, while the asterisks on the right side of the graph are used to indicate the statistical differences of time dependent variables (Friedman test) of individual groups in their course over time.
NS: P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001.

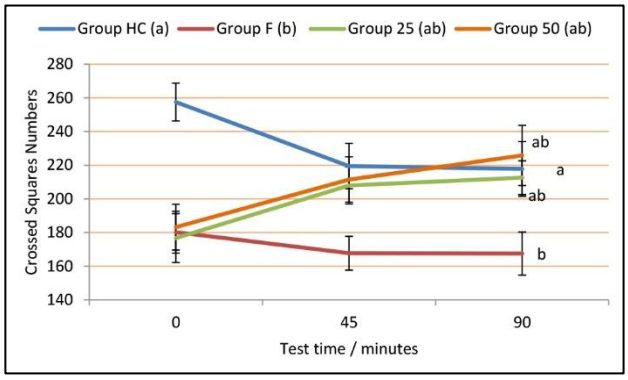

Figure 3. Course of Crossed Squares groups over time and statistical comparisons with RM ANOVA test.
a,b: The difference between groups having different superscripts is statistically significant (P<0.01).

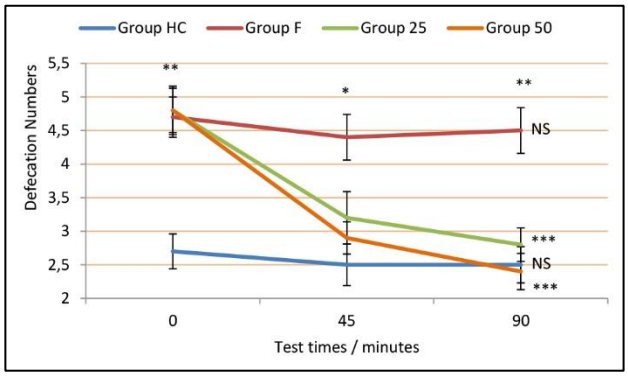

Figure 4. The course of the groups (HC, F, 25 and 50) within time (0th, 45 and 90th minutes) according to the number of Defecations data in the Open-Field test. The asterisks at the top of the graph are used for statistical comparisons of independent variables (Kruskal-Wallis test) according to time points, while the asterisks on the right side of the graph are used to indicate the statistical differences of time dependent variables (Friedman test) of individual groups in their course over time.
NS: P>0.05, *: P<0.05, **: P<0.01, ***: P<0.001.

Table 3. Mann-Withney U test results (for Kruskal-Wallis test important groups) for Grooming data.

	0th Minutes			45th Minutes			90th Minutes		
Groups	F	25	50	F	25	50	F	25	50
HC	***	***	***	***	*	NS	***	*	NS
F		NS	NS		**	***		**	**
25			NS			NS			NS

NS: Not Significant, *: P<0.05, **: P<0.01, ***: P<0.001.
Statistical comparisons were among groups HC, F, 25 and 50 within each time group (0th, 60th and 90th minutes).

Table 4. Mann-Withney U test results (for Kruskal-Wallis test important groups) for Defecation data.

	0th Minutes			45th Minutes			90th Minutes		
Groups	F	25	50	F	25	50	F	25	50
HC	***	***	***	**	*	NS	***	NS	NS
F		NS	NS		**	**		***	***
25			NS			NS			NS

NS: Not Significant, *: P<0.05, **: P<0.01, ***: P<0.001.
Statistical comparisons were among groups HC, F, 25 and 50 within each time group (0th, 60th and 90th minutes).

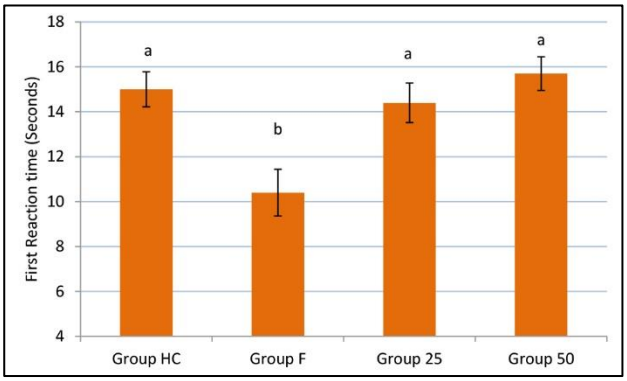

Figure 5. The first reaction time data of the groups in the hot-plate test and their statistical comparisons.
a,b: The difference between groups having different superscripts is statistically significant (P<0.01).

One-Way ANOVA test result of crossed Squares were as follows for minutes 0th; P<0.001 (Post Hoc; Group HC have statistical differences with all other groups) and minutes 90th; P<0.05 (Post Hoc; Group HC have no statistical differences with other groups).

Wilcoxon test results (for Friedman test important groups) of Defecation data for both 25 and 50 mg Agomelatine groups are as follows: The difference between 0th-45th and 0th-90th minutes was found to be significant (P<0.01), however, the difference between 45th- 90th minutes was significant with the P value of P<0.05 and P<0.01 for 25 mg/kg and 50 mg/kg Agomelatine groups respectively).

In the event of Figure 4 and Table 4 were consider together, it was observed that the defecation numbers of the Healthy Control (HC) group at the 0th minute were significantly lower than the other Fluoride applied groups (P<0.001). However, a dose-related decrease was observed in the treatment groups (Group 25 and 50) over time. While a significant difference was found between the treatment groups and the F group in the 90th minute data (P<0.001), it was observed that the difference between the treatment groups and the HC group became statistically insignificant depending on the dose (P>0.05).

Analysis of differences between the groups of Hot-Plate test data, which is parametric data, was performed employing the One-Way ANOVA test and Tukey HSD was performed as Post Hoc Multiple Comparisons. Analysis of Hot-Plate test results having homogeneous subsets is presented in Figure 5.

Discussion and Conclusion

First of all, in the research, it was determined that oral fluoride toxicity in mice caused some changes on the Open-Field and Hot-Plate tests findings. It is reported in the literature that similar findings are obtained in depression and anxiety states (3, 13, 22, 24, 28, 30, 33, 37). The second important outcome of this study is the determination that Agomelatine, a new antidepressant (8, 26, 38), brings the above-mentioned findings closer to normal levels in a short time depending on the dose.

Agomelatine (N-[2-(7-metoksinaftalen-1-yl)etil] acetamid) is a novel antidepressant agent that has been investigated for the treatment of depression and anxiety in recent years. It is reported that Agomelatine is a synthetic analogue of melatonin and has similar agonist effect on MT1 and MT2 receptors (8, 26, 38). It is also reported that Melatonin given exogenously in mental disorder work as an antidepressant and has a healing effect (9). At the same time, Agomelatine has an antagonist effect on 5-HT_{2C} receptors of serotonin, this effect strengthens its anti-depressant activity (5, 6, 29).

Open-Field test is one of the most used tests to determine the emotional state of the experimental animal before any procedure and the changes that may occur after the procedure (22, 12, 34). However, it is an animal model method used to assess loco-motor functions and hypo-locomotion has been reported in depressed and anxious (stressed) mice (18, 28, 33, 37). It has been reported that fluorosis affects the behavior of the subjects, thus changing the Open-Field test results (11, 28, 33).

In this context, there are some scientific studies on the Open-Field test results on different species of subjects who were exposed to toxic amounts of F intoxication with drinking water. In the shortest form, the results of some of these studies are as follows; The Open-Field test results of Oyagbemi et al. (30) showed an increase in motility with giving of 300 mgF/L in drinking water to Wistar rats. The test results of Lu et al. (25) showed a stability in motility with giving of 50 mgF/L in drinking water to mice. The results of Lopes et al. (24) were stable motility for 10-50 mgF/L in drinking water to mice. Mullenix et al. (28) reported a decrease in motility for Open-Field test results with given 75, 100, 125 mgF/L to Sprague-Dawley rat Weanlings. For the ICR mice given 100 mgF/L, Wang et al. (37) reported that a decrease in Open-Field Test results. As a motility data in Open-Field Test, Pereire et al. (33) reported a decrease for male rats given 100 mgF/L. Decreased data were reported by Kıvrak (18) for Open-Field test parameters including Rearing, Grooming and Crossed Square number except defecation count for the Swiss mice receiving 40 mgF/L. At the same time, Kıvrak stated that the number of defecation increased in mentioned study. As can be seen from the reported results, this situation is not clear, conflicting and causes confusion.

If one evaluates the effect of fluorosis alone on Open-Field test results in this study, for the first data (minute 0^{th}) of all parameters (Rearing Grooming, Square and Defecation), it will be clearly seen that there is a significant difference between the HC group and the F toxicity groups (F, 25 and 50) ($P<0.05$) (Figures 1-4). Open-Field test findings of this presented study were similar with some studies previously conducted by Mullenix et al., Wang et al. Pereira et al.ve Kıvrak (18, 28, 30, 33), nevertheless, contrasts with the findings reported by Oyagbemi et al., Lu et al. and Lopes et al. (24, 25, 30).

The different results in these studies above may be originated from different factors including the dose of F applied, the species and breed of subjects, environmental factors and personnel errors. However, the decline of some parameters (Rearing, Grooming and Crossed Square numbers) of this study presented may have been caused by developmental and neuro-developmental toxicity caused by F toxicity (7, 23, 28). On the other side, it has been reported that lethargy is an important symptom of neurological and psychiatric disorders (7, 18). Therefore, as a more logical reason, depression and anxiety induced by F intoxication may have also caused lethargy in subjects of the present study. As can be seen from the charts (Figure 1-4), it was determined that after the application of Agomelatine depending on the dose, the effects of F intoxication decreased and even situation reached in some parameters better than group of HC.

On the other hand, the defecation count findings of the Open-Field test of this study exhibited a different situation compared to other parameters (Figure 1-4). This parameter, unlike other parameters, showed an opposite course with bullish direction. Bowel movements are controlled by a complex mechanisms including sympathetic and parasympathetic nervous systems. Psychological conditions affect bowel movements through the sympathetic and parasympathetic nervous system. It has been reported that bowel movements increase with the increase of Vagal tone in cases of depression and anxiety (4, 32, 35). In this study presented similar to the above information, an increase in defecation was found in subjects exposed to F intoxication. In the advanced stages of the study, it was observed that Agomelatine applications neutralized the increase in defecation after F intoxication and returned it to the HC group data.

The relationship between Open-Field test parameters was examined using Spearsman's correlation test. Significant positive correlation was detected among the parameters Rearing Grooming and Square ($P<0.05$). However, there was significant negative correlation between the Defecation parameter and the others above ($P<0.05$) (Table 2). This correlation situation also confirms the opposite direction of motion mentioned above. The above-mentioned conditions of Open-Field test parameters of this presented study were also in line with the findings of previous studies (18, 28, 33).

A striking finding in the Open-Field test data of both control groups (Rearing, Grooming, Crossed Squares Number and Defecation) is that although a small decrease was observed from the data of minutes 0^{th} to 45^{th}, this decrease did not occur in the later time of the work (90^{th} minute) (Figures 1-4). Zador (39) uses the expression "*a long-lasting change in behavior that is the result of experience*" for the learning term in animal psychology. The reason for this initial fall in question may be because the subjects see an environment they do not know for the first time, and in later times they react more stable than they are used to the environment (12, 22, 27, 34). In fact, this first decline may have been in Agomelatine groups, but since the subjects were under the influence of the administered Agomelatine, these decreases may not have been noticed (Figures 1-4).

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On the other hand, the Hot-Plate test is a method that is generally used to investigate the effect of the analgesic agents on nociceptive system in animal models, based on the determination of the tolerance level against heat (3, 13, 22). However, there is very limited information about the use of the Hot-Plate test in psychiatric disorders such as depressive and anxious cases related to fluorosis. In the study presented, Hot-Plate test results also showed a significant difference according to reaction time between HC and other fluoridised groups ($P<0.01$). This also proves that in the case of depression and anxiety caused by F toxicity, mice have a lower tolerance to heat and react earlier.

Chenaf et al. (6) testified that Agomelatine administration reduces neuropathic pain in animal models through melatonergic, spinal 5-HT_{2C}, and Alpha-2 receptors. In this study also, in cases of depression and anxiety caused by fluorosis, it was observed that Hot-Plate test reaction time returned to normal in a short time depending on the dose after the administration of Agomelatine (Figure 5). These results reveal an interesting and novel effect of Agomeletine on the treatment of psychological disorders caused by F toxicity and prove applications neutralize the low tolerance to heat caused by fluorosis.

In the evaluation of both test results together, it was determined that F toxicity changed the results of Open-Field and Hot-Plate test, and Agomelatine applications neutralized the effects of F intoxication in the short term depending on the dose and even made them better than the healthy control group.

Agomelatine is reported to have acute and chronic treatment options (17). This presented study was conducted on the short-term effects of Agomelatine, namely its acute effects. Since psychiatric diseases such as depression are generally emotional diseases with a chronic course (7), experimental studies on the long-term use of Agomelatine are also needed.

As a result, it was observed that Agomelatine, a novel antidepressant, has a dose-dependent therapeutic effect on depression and anxiety caused by F intoxication in animal model (mouse). According to these results, it has been thought that new studies should be carried out in this direction that they may have positive effects on depression and anxiety caused by fluorosis in both humans and animals.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Animal Experiments Local Ethics Committee with the number KAÜ-HADYEK-014-047.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Effects of functional poly(ethylene terephthalate) nanofibers modified with sericin-capped silver nanoparticles on histopathological changes in parenchymal organs and oxidative stress in a rat burn wound model

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ABSTRACT

In this study, it was aimed to investigate the effect of a poly(ethylene terephthalate)-g-poly(hydroxyethyl methacrylate) (PET-g-HEMA) nanofiber wound dressing modified with sericin-coated silver nanoparticles (S-AgNPs) on internal organs, oxidative stress, and biochemical parameters. To establish a burn model, the backs of anesthetized rats were shaved and then third-degree burns were created with a round-bottomed stainless steel rod 2 cm in diameter kept in 100 °C water for 20 seconds. The wounds of the negative control group (G1) were covered with standard bandages; the wounds of the positive control group (G2) were covered with silvercel, used as burn wound material; and the wounds of the experimental group (G3) were covered with PET-based dressing material. Histopathological changes in organs (liver, kidneys, heart, pancreas, lungs), total oxidant status (TOS), total antioxidant status (TAS), nitric oxide (NO), and biochemical parameters (serum aspartate aminotransferase [AST], alanine aminotransferase [ALT], gamma glutamyl transpeptidase [GGT], creatine kinase, lactate dehydrogenase [LDH], total protein, albumin, globulin, urea) were examined. Compared with the G1 group, plasma AST, ALT, and GGT levels were found to be significantly decreased in G2 and G3 ($P<0.001$). Plasma TAS was found to be significantly increased in G2 and G3 compared to G1 ($P<0.05$). Compared to the G1 group, degenerative and necrotic changes in the liver, kidneys, and pancreas were found to be significantly reduced in G2 and G3 ($P<0.05$). In conclusion, this work demonstrates that the synthesized PET-based wound dressing material has the capacity to be used commercially.

Introduction

The skin is the largest organ of the body and has an important role in sensing the environment and maintaining body homeostasis and temperature control, as well as protecting against pathogens such as viruses, bacteria, toxins, and environmental contamination (42, 50). Wounds occur with the deterioration of the integrity of the skin as a result of deliberate or accidental causes or diseases (42). In general, wounds can develop due to many factors and conditions, including surgery, pressure, cuts, and diseases (diabetes or vascular) (33). Burn injuries are traumas whose time and place cannot be predicted, affecting many organs in addition to the skin (16, 25). They may be caused by friction, freezing, radiation, electrical currents (high voltage electrical), or chemicals. However, most burn injuries are caused by heat produced by extremely hot solid and/or liquid substances (25). Although it is difficult to classify burn wounds, they are generally divided into 4 groups according to the degree of damage to the layers of the skin (32). These are first-degree burns of the skin, second-degree superficial partial-thickness burns, third-degree full-thickness burns, and fourth-degree burns that affect deep tissues such as muscle or bone (25).

After an injury for any reason, the skin must immediately regain its integrity in order to continue its functions (13). The wound healing process occurs in three phases, which are interrelated and ordered consecutive steps. The first of these is inflammation. This phase is followed by the proliferative phase, and the final phase entails tissue maturation and remodeling (13, 17). After burn damage to the skin, which is the body's external barrier, the immune system deteriorates and susceptibility to infections increases, causing a delay in wound healing processes (47). In addition, after severe burns, free radicals (reactive oxygen species) are released, which exert local or systemic effects as a result of physiopathological responses. Thus, the release of increased levels of free radicals can cause immunosuppression, infection, sepsis, tissue damage, and multi-organ failure (29).

It is often difficult to choose the appropriate dressing after a burn wound; this decision depends on various factors such as the depth of the burn, the condition of the wound bed, the desired moisture retention and drainage of the wound, and the frequency of dressing changes (47). There are many specialty dressings available, some of which are designed for special occasions and for ease of use (22). Wound dressing products such as gauze, plasters, and bandages are used, but there are also modern dressing products. Among these modern products, there are many newly developed products such as bioactive dressings and medicated dressings (15). In recent years, wound dressing products have been created using nanotechnology (52). Silver compounds have been used in medical fields for centuries, but silver's importance as a suitable treatment option has recently increased in applications against infections in cases of burns, open wounds, and chronic ulcers (6, 41). However, it has recently been reported that silver compounds, which have serious cytotoxic activity against various host cells, may also delay the wound healing process (6, 27, 36). In addition to silver compounds such as silver sulfadiazine, widely used in burn treatment today, there are various newly developed compounds such as silver-coated nanoparticles (27, 36, 41). In general, silver has both beneficial properties and harmful cytotoxic effects that raise concern. It may pose a potential biohazard for health when used in various products. In addition, extensive production and application of silver will increase its release into aquatic environments such as rivers and lakes, thereby posing an environmental hazard (14). In our previous in vivo study, we obtained a functional and antimicrobial biomaterial surface to be used for wound dressing. Nanofiber membranes obtained from a poly(ethylene terephthalate)-g-poly(hydroxyethyl methacrylate) (PET-g-HEMA) copolymer with 55% HEMA grafting were coated with green synthesized sericin-coated silver nanoparticles (S-AgNPs) and the biomaterial was then successfully administered to experimental groups (19).

Subsequently, in the present study, S-AgNP-coated PET-g-HEMA nanofibers were used to cover burn wounds in a rat burn model. It was aimed to investigate the effects of the wound dressing material on internal organs and on oxidative stress and biochemical parameters.

Materials and Methods

Materials: In this study, to cover the burn wounds in the experimental group, PET-g-HEMA nanofibers coated with 10 mM S-AgNPs were used. The modified PET-g-HEMA nanofibers were synthesized and characterized as explained in our previous study (19). To cover the burn wounds in the positive control group, SILVERCEL® was used. Silvercel is a commercial alginate-based dressing material containing silver and it was obtained from Systagenix (UK). OctaCare® dressings (gauze) were used to cover the burn wounds of the negative control group.

Experimental Design and Rat Burn Model: Twenty-four male Sprague-Dawley rats (average weight: 275±25 g) were used. For sedation, xylazine (10 mg/kg, Xylazinbio 2%®, Bioveta, Czech Republic) and ketamine (90 mg/kg, Vetaketam®, Vetagro, Poland) were injected intraperitoneally just before the creation of burn wounds in the animals. Third-degree burn wounds were created in anesthetized rats using a round-bottomed stainless steel rod 2 cm in diameter, which was held in 100 °C water for 20 seconds (31). Animals were divided into 3 groups: a negative control group (G1; standard bandages), a positive control group (G2; silvercel, a commercial dressing material containing silver), and an experimental PET-based dressing material group (G3). The animals were randomly assigned to these groups after the burn wounds were created, with each group consisting of 8 rats. The period for the treatment of burn wounds in each group was 21 days and dressing materials were changed every 3 days. At the end of the study, blood was collected from anesthetized rats, and then the animals were immediately euthanized. Organ-tissue samples were taken from euthanized rats for histopathological examination.

Sample Collection and Analysis: The blood samples were centrifuged at 3000 rpm and 4 °C for 10 min to separate the plasma. The obtained plasma samples were stored in eppendorf tubes in a deep freezer at -80 °C until analysis. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), creatine kinase (CK), lactate dehydrogenase (LDH), total protein, albumin, total oxidant status (TOS), and total antioxidant status (TAS) in the plasma were determined with an autoanalyzer (Mindray BS400, China) using commercial test kits (Rel Assay Diagnostics, Türkiye). Nitric oxide (NO) levels were determined with a spectrometer (Shimadzu UV-1700, Japan) using a commercial test kit (Elabscience, USA).

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Histopathological Examination and Scoring of Lesions: In order to detect cellular damage and any histopathological changes, the collected tissues were fixed in a buffered neutral formalin solution (pH 7.2-7.4). After being trimmed and placed in disposable cassettes, a routine procedure was followed and tissues were embedded in paraffin wax. Sections of 5 µm in thickness were cut from each paraffin block. Routine hematoxylin and eosin (H&E) staining was applied for histopathological examination (34). The results were evaluated with a digital light microscope (Olympus BX51, Japan) in the bright field. Images at different magnifications were obtained from the required fields with an Olympus DP5 camera attachment. For degenerative-necrotic changes as well as hyperplastic changes, mean averages and standard errors were taken by counting 10 different fields at 400× magnification. After skin wounds were evaluated, the organs of interest were evaluated semi-quantitatively in terms of vascular and inflammatory changes as well as fibrosis and pigment accumulation. Average scores obtained from 10 field counts were categorized as 0%: negative, 10-30%: few changes, 30-45%: mild changes, 45-60%: mild to moderate changes, 60-75%: moderate changes, and 75-100%: strong changes.

Statistical Analysis: For statistical analysis of degeneration and hyperplasia, the differences between the groups (parametric distribution) were evaluated using GraphPad Prism 8.4.2 (GraphPad Software, USA; www.graphpad.com) and one-way analysis of variance (ANOVA) by comparing effects for each row (organ type) and column (name of group). Values of $P<0.05$ were considered statistically significant at 95% confidence intervals. Analysis of biochemical parameters was conducted with SPSS 18.0 (PASW Inc., USA). The normality of all data was assessed by the Shapiro-Wilk test. The levels of urea as a nonparametric variable were tested using the Kruskal-Wallis test to determine which of the three groups differed from the others, followed by the Mann-Whitney *U* test with Bonferroni adjustment ($P<0.001$). The other data (parametric distribution) were analyzed by one-way ANOVA testing. Duncan's multiple range test was conducted when *F* values were significant ($P<0.05$).

Results

Biochemical Parameters: The effects of S-AgNPs and functional PET-g-HEMA nanofibers on plasma enzyme profile and urea, total protein, and albumin levels are presented in Table 1. In comparison with the G1 group, plasma AST, ALT, and GGT activities were significantly decreased in G2 and G3 ($P<0.001$). Plasma urea levels were significantly decreased in G2 compared to G3 ($P<0.05$). Neither S-AgNPs nor PET-g-HEMA caused changes in plasma CK or LDH activities or total protein, albumin, or globulin levels ($P>0.05$).

Oxidative stress: The effects of S-AgNPs and PET-g-HEMA nanofibers on oxidative stress are presented in Table 2. Plasma TOS and NO levels were not statistically significant differences between control and experimental groups ($P>0.05$), while plasma TAS levels were found to be higher in G1 compared to G2 and G3 ($P\leq0.05$).

Table 1. Biochemical parameters of the groups (x ± Sx).

Parameters	Group-1	Group-2	Group-3	P
AST (U/L)	146.00 ±11.55^{a}	91.57±6.11^{b}	94.00±6.63^{b}	<0.001
ALT (U/L)	76.38±5.16^{a}	45.29±1.02^{b}	45.00±3.48^{b}	<0.001
GGT(U/L)	4.77±025^{a}	3.02±0.21^{b}	3.31±0.29^{b}	<0.001
CK (U/L)	586.63±53.55	425.14±33.77	474.86±65.37	>0.05
LDH (U/L)	1959.75±226.76	1633.71±170.32	1738.14±265.28	>0.05
Total protein (g/dl)	4.81±0.09	4.73±0.13	4.68±0.06	>0.05
Albumin (g/dl)	3.41±0.04	3.50±0.05	3.53±0.06	>0.05
Globulin (g/dl)	1.4±0.11	1.23±0.11	1.15±0.06	>0.05
Urea(g/dl)	54^{ab}	51^{b}	54^{a}	<0.05

The difference between the means with different letters in the same row is significant ($P<0.05$).

Table 2. TOS, TAS and NO levels of the groups (x ± Sx).

Parameters	Group-1	Group-2	Group-3	P
TOS (µmol/L)	12.55 ±1.14	11.87 ±1.40	10.79±1.21	>0.05
TAS (mmol Trolax Equiv/L)	1.19±0.03^{a}	1.13±0.01^{b}	1.13±0.01^{b}	<0.05
NO (µmol/L)	12.35±0.60	10.41±0.76	12.89±1.13	>0.05

The difference between the means with different letters in the same row is significant ($P<0.05$).

Histopathological Findings

Liver: In the G1 group, hepatic cords were dissociated and hepatocytes included different sizes of vacuoles in the cytoplasm as well as displaying karyopyknosis and karyolysis. In some areas, necrosis was also encountered in lobules. Kupffer cells were hyperplastic and active in many microscopic fields. Sinusoids and vessels were mildly hyperemic in some high-powered fields. In G2, these degenerative and necrotic changes were diminished within some foci in lobules. Hyperplastic Kupffer cells were increased; however, the increase in number was not as great as that in G1. Sinusoid and vessels were conspicuously hyperemic in many fields. In G3, a few degenerations were observed in lobules. Kupffer cells were neither hyperplastic nor active, and the numbers of cells were in the normal range in lobules. Hyperemic sinusoids and vessels were not dense as in the other groups (Figure 1, a-c).

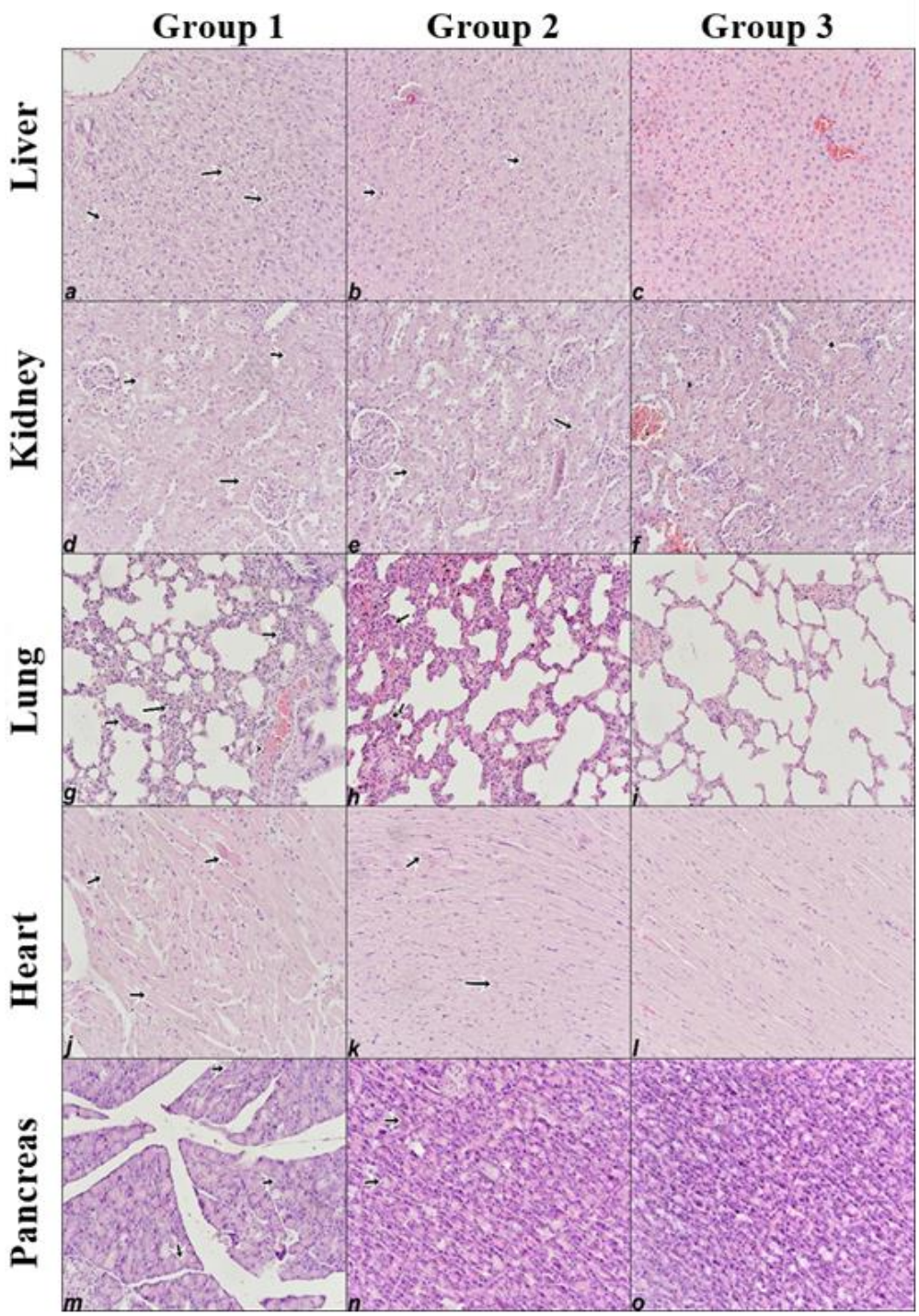

Figure 1. Acute cell swelling to severe hydropic degeneration (arrows) in hepatocytes (a-c), in cortical tubul epitheliums (d-f). Subacute inflammatory cell infiltration in interstitum (arrows) in lung (g-i), paranchymal degeneration and necrosis in cardiac muscles (arrows) in heart (j-l), cell swelling in pancreas (m-o), x100, Group I, II, and III. H&E staining.

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Kidney: In G1, cortical tubules were degenerative in general. The degenerative changes were due to acute cell swelling, as seen in many high-powered fields. Glomeruli were normal in appearance. There were no inflammatory or vascular reactions and protein droplets or deposition in either the cortical or medullar region. In G2, there were a few degenerations in the cortical tubule epithelium. In G3, acute cell swellings were decreased in a few fields. No other findings were seen (Figure 1, d-f).

Lung: In all groups, a few pneumocytes were degenerative per field. The capillaries and vessels were hyperemic. Edema was also observed in many fields. At the interstitium, neutrophils, lymphocytes, and monocytes infiltrated into the lumina of alveoli in many fields. In particular, inflammatory and vascular changes were dense in G1. However, in the other groups, there were relatively fewer vascular and edematous changes. Inflammatory cell infiltrations were common in many fields. Lymphocytes were predominantly observed compared to other cell types (Figure 1, g-i).

Heart: In all groups, parenchymal degeneration involving cytoplasmic shrinkage and karyopyknosis was seen in some areas in addition to mild vascular changes except in G2 (Figure 1, j-l).

Pancreas: In G1, acute cell swelling was observed in many high-powered fields. In G2, there were a few degenerations. In G3, there were no degenerative, vascular, or inflammatory changes. Mild hyperemic changes were only observed in G2 (Figure 1, m-o).

Degenerative and necrotic changes in each group are given in Table 3. Hyperplastic changes on the basis of experimental groups, as shown in Table 4. Semi-quantitative scores were also calculated according to vascular and humoral changes, inflammatory cells, fibrosis, and pigment accumulation for each group, as shown in Table 5. The 8 animals in each group constituted the sample size. The group effect was responsible for 31.25% of the variance and the contribution of group effect to the results was evaluated as F=88.32, DFn=2, DFd=105. The organ effect was responsible for 27.59% of total variance (F=38.99, DFn=4, DFd=105). Both effects may thus be considered as extremely significant for the results (P<0.0001).

Table 3. Degenerative and necrotic changes on the basis of experimental groups (Mean±Standard Error).

Organs	Group-1	Group-2	Group-3	P
Liver	18±1.86^{a}	5.5±0.34^{b}	1.8±0.44^{c}	<0.05
Kidney	10±1.73^{a}	2.4±1.89^{b}	0.6±0.33^{b}	<0.05
Lung	0.8±0.44	0.4±0.22	0.1±0.1	>0.05
Heart	0.5±0.22	0.7±0.21	0.4±0.22	>0.05
Pancreas	9.9±1.22^{a}	0.6±0.33^{b}	0.4±0.22^{b}	<0.05

The difference between the means with different letters in the same row is significant (P<0.05).

Table 4. Hyperplastic changes on the basis of experimental groups (Mean±Standard Error).

Organs	Group-1	Group-2	Group-3	P
Liver (Kupffer' cell)	30±0.52^{a}	28±1.13^{a}	11±0.21^{b}	<0.05
Lung (BALT)	1±0.12	1±0.19	1±0.12	>0.05

The difference between the means with different letters in the same row is significant (P<0.05).

Table 5. Semiquantitative score in vascular and humoral changes, inflammatory cells, fibrosis, pigment accumulation on the basis of experimental groups.

Findings	Vascular and humoral changes			Inflammatory changes				Fibrosis	Pigment accumulation
Groups	*Hyperemia*	*Haemorrhage*	*Edema*	*Neutrophil*	*Lymphocyte*	*Macrophage*	*Plasma cell*		
Group-1									
Liver	-/+	-	-	-/+	+	-/+	-	-	-
Kidney	-	-	-	-	-	-	-	-	+++
Lung	++	-	++	++	+++	++	-	-	-
Heart	-/+	-	-	-	-	-	-	-	-
Pancreas	-	-	-	-	-	-	-	-	-
Group-2									
Liver	+/++	-	-	-	-	-	-	-	-
Kidney	+	-	-	-	-	-	-	-	-
Lung	-	-	-	++	+++	++	-	-	++
Heart	-	-	-	-	-	-	-	-	-
Pancreas	-/+	-	-	-	-	-	-	-	-
Group-3									
Liver	-/+	-	-	-	-/+	-	-	-	-
Kidney	++	-	-	-	-	-	-	-	-
Lung	-	-	+	++	++	-	-	-	-
Heart	-/+	-	-	-	+	-	-	-	-
Pancreas	-	-	-	-	-	-	-	-	-

-: negative, -/+: a few positive, +: mild positive, +/++: mild to moderate positive ++: moderate positive, +++: strong positive.

Discussion and Conclusion

Severe burns can lead to multiple organ dysfunction and death as a result of the disruption of the body's homeostatic balance (7). Burn treatment is difficult, uncomfortable, and expensive for patients. Due to the antimicrobial effects of silver nanoparticles (AgNPs), they can prevent bacterial infection in wounds and accelerate wound healing (35). This study was carried out to determine the effects of burn treatment on changes in some biochemical parameters, oxidative stress, and histopathology in animals. The levels of these parameters are crucial in the physician's understanding of how the body is responding to the burn injury and the response to different possible treatments. The expected changes in the internal organs have been supported by the detection of liver and kidney function markers (28). Normally, aminotransferase enzymes are found in the liver and other tissues, possessing functions in energy metabolism, including transamination of aminocytes. However, in the event of cellular damage, AST and ALT leak into the circulation, and their activities in the blood increase (58). Khalil et al. (28) found that serum AST, ALT, and ALP activities increased while total protein and albumin levels decreased and bilirubin levels did not change in three burnt cattle. Anandani (5) found an increase in blood urea levels in people treated for burns. Jeschke et al. (24) stated that serum AST and ALT activities increased in rats with experimental burns. Şehirli et al. (54) stated that serum LDH activity increased in rats held in 90 °C water for 10 seconds and betaine administration decreased LDH activity. AgNPs are known to have many beneficial properties for wound management including antibacterial, anti-inflammatory, and healing properties (45). Adeyemi and Adewumi (2) investigated the biochemical effects of AgNPs in Wistar rats, administering AgNPs to the rats orally at doses of 100, 1000, and 5000 mg/kg for 7, 14, and 21 days. They observed that serum ALT activity had decreased at the 14th and 21st days with all three doses, as well as AST activity at the 7th day. Sulaiman et al. (53) showed that serum AST and ALP activities decreased in rats given 10, 50, 100 mg/kg AgNPs orally for 30 days, while ALT activity and total protein and urea levels decreased in rats given AgNPs at 50 and 100 mg/kg. It has been reported that oral administration of 30, 125, 300, and 700 mg/kg AgNPs to rats for 28 days did not affect serum ALP, ALT, or AST activities and total protein and albumin levels, and these enzymes remained in liver cells as under normal conditions (46). As a result of the antimicrobial efficacy of AgNPs, they can mitigate bacterial infection and accelerate the process of wound healing (35). In our study, the administration of AgNPs decreased the activities of AST and ALT, supporting the findings of the study conducted by Adeyemi and Adewumi (2). The decrease in AST activities caused by the nanoparticles was probably due to inactivation emerging from the affinity of AgNPs for thiol (eSH) groups, thereby causing changes in the functional state of proteins (3). Abbas et al. (1) reported that AgNPs inactivated amino transaminases by acting as inhibitors. The inactivation of enzymes or proteins may affect reactions in key metabolic processes with dire consequences for cellular integrity. Consistent with the findings of Pourhamzed et al. (46), no difference was observed in the present study between the groups in total protein or albumin levels. Similar to the findings of Sulaiman et al. (53), in this study, administration of AgNPs significantly decreased serum urea values.

Oxidative stress is the disruption of the balance between oxidants and antioxidants in favor of the oxidant system, which leads to cellular damage in the organism as a result of lipid peroxidation due to the release of free radicals/reactive oxygen products (9, 56). If the defense mechanisms of the organism (antioxidant mechanisms) are insufficient against those free radicals, oxidative damage develops in the cells and cell functions are significantly impaired. Antioxidants are known as natural or synthetic substances that can prevent or delay cell damage caused by oxidants (9).

Damage to the skin causes the formation of reactive oxygen products, reductions in various enzymatic and non-enzymatic free radical scavengers, and lipid peroxidation, affecting the healing process (18, 40). Reactive oxygen species impair the wound healing process due to their detrimental effects on cells and tissues (4). In various studies (7, 11, 30, 55, 57), topical applications of products with free radical scavenging properties were shown to be significantly effective in wound healing and protecting tissues from oxidative damage. Bahadır et al. (7) reported that minocycline treatment administered to animals burnt with exposure to a 90 °C water bath decreased TOS levels in comparison to the untreated burn group. Although the sham and burn groups did not differ in terms of TAC levels, minocycline treatment increased the TAC levels of the burnt animals in comparison to the sham group. Şener et al. (55) induced burn wounds by exposing the backs of rats to a 90 °C water bath for 10 seconds, revealing that while significant increases were observed in kidney tissue malondialdehyde (MDA) levels in both 6-hour and 24-hour burn groups, glutathione (GSH) levels decreased in both burn groups. However, following the administration of 2-mercaptoethane sulfonate, GSH levels significantly increased and MDA levels decreased. Hence, they concluded that 2-mercaptoethane sulfonate has protective effects against thermally induced oxidative kidney damage. Cell damage by free radical attack is typical of burn injuries. Burn injuries are known to trigger the formation and release of oxidative free radicals and pro-

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inflammatory mediators that mainly contribute to lipid peroxidation (44, 51).

In a previous study conducted with rats, scald burns were induced by pouring 90 °C water onto a patch of shaved dorsum 20 mm^2 in size. The rats were euthanized on the 21st day and levels of thiobarbituric acid reactive substances (TBARS) and GSH were measured in tissues and evaluated as markers of oxidative stress. A significant increase in TBARS and a decrease in GSH levels were found in the burn injury group. The application of an isoquercetin-based cream brought those increased levels closer to normal values again and tissue biochemical studies indicated a possible role of free radical scavenging of isoquercetin in wound healing. However, since burn injuries are considered acute dermatological problems representing multiple oxidative changes in a short period of time, the isoquercetin-based formulation was recommended for use in the treatment of burn injuries as early as possible to suppress free radical generation and oxidative stress (11). Bedlovičová et al. (9) reported that, in addition to the application of AgNPs in various fields, there have been many studies on the antioxidant properties of AgNPs in the last decade. For example, AgNPs are used in the treatment of Alzheimer's disease and cancer due to their antioxidant properties. The antioxidant properties of nanoparticles depend on the chemical composition of the chosen extracts. If the extracts are rich in phenolic compounds, the nanoparticles exhibit high levels of scavenging activity (9).

Kumandaş et al. (30) conducted a study to compare the effects of black seed oil and zinc-silver cream on wound healing by evaluating oxidative stress parameters in a rat wound model. They reported that the highest increase in plasma MDA levels was seen in the control group, while the lowest increase was in the silver-treated group. The results of that study showed that topical application of silver cream inhibits lipid peroxidation by increasing antioxidant activity. In the present study, the lowest plasma CAT level was observed in the zinc-silver group. This may have been due to anti-inflammatory effects. In our study, TAS values were higher in the G2 and G3 groups compared to G1 ($P<0.05$). The highest plasma TOS level was observed in the control group. Increases in plasma TAS levels may be a compensatory mechanism responding to the deteriorating oxidant-antioxidant balance. Kumandaş et al. (30) found that the application of zinc-silver cream to rats with dorsal wounds increased the animals' serum NO levels.

The production of NO is required for normal wound healing. While nitric oxide synthase inhibitors delay wound healing, the administration of NO accelerates wound healing (12, 59). Studies have shown that NO has a trophic effect on wound healing and plays an important role in collagen accumulation (10, 48).

Burn trauma may lead to multiple organ failure in addition to skin damage (38). Considering the microenvironment, increasing oxidative stress inevitably changes histological processes. A change in association with the histoarchitecture occurs in animals given thermal burns. After the burn trauma at skin level, cytokines and inflammatory cells, predominantly neutrophil leukocytes, disturb the microvascular dynamics at both the burn site and remote organs (20, 49). It is postulated that the increase in edema formation causes damage in the cells due to the release of several cellular enzymes. After longer exposure to thermal burns, a degenerative series can occur in the liver within the first week in proportion to the severity of the thermal injury. For instance, after the release of cellular enzymes, hepatocytes can move into necrosis and apoptosis within a short time because cellular homeostasis is disturbed more during degeneration processes (8, 24, 39). Likewise, based on pathogenetic knowledge, acute renal failure due to damage to tubular cells has been identified in the kidneys (21, 23). The cardiovascular system can also be affected by thermal injuries. As a result of decreased blood flow (i.e. oxygenation capacity), the tissue is not fed properly (37). An experimental study was performed regarding burn trauma modeling in rats and treatment with silver-containing wound dressings. Skin-protective silver particles were found mainly in the liver, kidneys, and spleen, as well as the brain, testes, lungs, heart, and muscle tissues (43). In another experimental study, the heart, liver, kidneys, spleen, lungs, and brain were evaluated histopathologically after 14 days in a rat burn model. Although the level of hepatotoxicity due to silver particles and Agicoat was higher, no pathological findings were identified in the brain, kidneys, spleen, heart, or lungs (26).

No findings in association with the spleen, lungs, and pancreas were noted after burn trauma in the present model. However, we obtained some notable histopathological results. We detected mainly vascular and humoral changes and inflammatory changes in all groups. However, the liver, heart, and lungs were more affected in the G1 group. Both vascular changes and edema, as well as inflammatory cell infiltrations were seen in those organs. In contrast, in the other groups, the distant organs were less affected by thermal injuries although liver injuries were seen. Severe hyperemia was also seen, although there were no inflammatory changes. The kidneys, heart, and pancreas were affected to a lesser extent. These findings all show us that the thermal injury group was more affected compared to the wound dressing group. Because of the imbalance in the microcirculation and deterioration in the cellular environment, degeneration and other alterations were seen more often

and that is why the liver, kidneys, heart, and lungs were more affected by such changes. In contrast, these organs were less affected in the other groups. Decreases in microvascular disturbances and inflammation confirmed that the nanoparticles were effective in treating side effects for organs. In particular, no inflammatory activity was seen in these groups. Only hyperemia and edema were observed in the lung tissues of both groups. Due to disturbances in the microcirculatory and microfluidic balance, cellular compositions were affected. Degeneration and necrosis occurred in all groups. However, lesions were seen to a lesser extent in G2 and G3, respectively. The most prominent differences were encountered in the liver and kidneys in terms of degeneration, while degeneration in the pancreas was also found to be more extensive in G1 compared to the other groups. Alterations in heart and lung tissues occurred to a lesser degree. Thus, we conclude that the liver and kidneys were the most affected organs in terms of vascularization and alterations, supporting the results of previously published studies. Accordingly, wound dressing materials containing s-AgNP-coated PET-g-HEMA nanofibers can be developed for commercial products such as SILVERCEL due to the occurrence of fewer lesions in organs, reduced enzymatic parameters such as blood ALT and AST, and high TAS values.

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Conflict of interest

The authors declared that there is no conflict of interest.

Author Contributions

YŞ, ZGG, and MEA conceived and planned the experiments. YŞ carried out the experiments. YŞ, ZGG, MEA and MÇ contributed to sample preparation. YŞ, ZGG, MEA and MÇ contributed to the interpretation of the results. YŞ, MEA and MÇ took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was carried out after the animal experiment was approved by Kırıkkale University Local Ethics Committee (Decision number: 2020-42).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Dose-dependent effects of simvastatin, atorvastatin and rosuvastatin on apoptosis and inflammation pathways on cancerous lung cells

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ABSTRACT

The aim of study was to investigate the anti-proliferative and inflammatory effects of atorvastatin, rosuvastatin, and simvastatin in lung cancer. The effects of statins were investigated in Vero, BEAS-2B, and A549 cell lines. In addition to expressions of *BAX, BCL-2, TNFα, IL-10, IL-6*, protein levels of TNFα, IL-10, IL-6 were determined. Cell viability and MDA were also measured. While the cell numbers in groups with low doses of statins were found to be approximately $1x10^6$/mL, proliferation was inhibited at higher rates containing high doses. Simvastatin, rosuvastatin, and high dose atorvastatin upregulated the *BAX*, while high dose of atorvastatin and both doses of rosuvastatin caused downregulation in *BCL-2*. All statin groups had higher MDA. Simvastatin and high dose rosuvastatin upregulated *TNFα*. While low dose simvastatin and atorvastatin and high dose atorvastatin and rosuvastatin upregulated *IL-10*, *IL-6* was upregulated with a low dose of rosuvastatin. TNFα was higher in simvastatin and rosuvastatin groups. IL-10 was highest in rosuvastatin groups. Atorvastatin groups had lower IL-6. Although cell numbers have been reduced by all statins, rosuvastatin is more effective on studied genes.

Introduction

Lung cancer is one of the most common malignant tumors and causes the death of thousands of people (9, 12). Although many new treatment methods are applied, there are still many people who lose their lives due to lung cancer, and its incidence and mortality have increased (18). For this reason, the possibilities of using more than one active substance in the treatment have been constantly investigated (4).

Statins, as HMG-CoA reductase inhibitors, are a group of drugs that decrease plasma cholesterol strongly (13). Statins lead to the production of isoprenoid, which has a vital function in the cell, and this situation affects cell development and differentiation with hypolipidemic effect. As a result of this pleiotropic effect, statins have an effect that prevents the growth of tumor cells (28).

Statins can act by increasing the sensitivity of tumor cells to traditional chemotherapy drugs. In this context, statins have been reported to have an anticancer impact on certain tumor cells (1, 6). Some studies report that statins may show different effects even in the same cell lines and do not cause cancer frequency (14). General knowledge is

that there is an important relationship between statins and cancer (2), but the molecular mechanisms by which this relationship can be controlled have not yet been fully elucidated. While the anticancer effects of statins are reported in the literature (6, 27), it is not known to what extent it affects the natural or synthetic statins on cell proliferation and inflammation in lung cancer.

In this study, the anti-proliferative and inflammatory effects of both synthetic (atorvastatin and rosuvastatin) and natural (simvastatin) statins in lung cancer cells were tested.

Materials and Methods

***Cell culture*:** Vero cell line (African Green Monkey Cells, ATCC CCL-81), human bronchial epithelial cell line (BEAS-2B, ATCC-CRL-9609) and human lung adenocarcinoma cell line (A549, ATCC CCL-185) were used. RPMI 1640 containing 10% fetal calf serum, 10 mM HEPES, 4mM glutamine, and 100 IU mL penicillin/ streptomycin was used as a cell culture medium. Incubation was carried out in an incubator at 37 °C, with 5% CO_2 and 95% air.

***Proliferation Assay*:** Primarily, non-toxic concentrations of statins in healthy cell lines (Vero and BEAS-2B cell lines) were determined. Then, activity studies on BEAS-2B and A549 cell lines were determined by MTT method as described in the literature (19). For this purpose, the effects of different concentrations of Simvastatin, Atorvastatin and Rosuvastatin on the cell proliferation of BEAS-2B and A549 cells were investigated by MTT cell proliferation method. After adding statins to the cells, they were incubated for 96 hours at 37°C in an incubator with 5% carbon dioxide. After the incubation, the culture medium was removed and 10 µl of MTT was added to each well and the plates were incubated under the same conditions for 4 hours to allow the formation of formazan crystals. The crystals formed were dissolved in DMSO added to each well and the optical density was measured spectrophotometrically at a wavelength of 570 nm. Proliferation was expressed as the ratio of cells in statin-treated wells to control cells. In addition, the following experiments were also performed to quantitatively evaluate the effects of statins on cell viability.

Cell culture studies were performed in 96-well, flat-bottomed sterile microplates. To investigate the activity of statins on BEAS-2B and A549, cell density was adjusted to $1x10^5$/ml cells. For cell adhesion, the plates were incubated for 6 hours under the same conditions as stated. Following the adhesion of the cells to the surface of the plate wells, different concentrations of statins were added to the culture medium. At the end of the 96 hours incubation, the cells in the culture vessel were collected in 0.25% trypsinization solution and transferred to tubes. Cells were centrifuged at +4 °C at 1250 rpm for 10 min. Cell number and viability were determined by hemocytometer.

Two different non-toxic concentrations (simvastatin: 40 and 80 µM, atorvastatin: 65 and 130 µM, rosuvastatin: 40 and 80 µM) of each statin were studied in the experiments. DMSO (Sigma, MI, USA) was chosen as the solvent in order to homogeneously dissolve the statins in the medium. The effects of DMSO on cell growth were evaluated daily with an inverted microscope, both morphologically and cell viability. In order to determine the non-toxic concentration of DMSO, different concentrations of DMSO (8, 4, 2, 1, 0.5%) were treated with cells for 96 hours. Cultures without DMSO (negative control) were left to incubation simultaneously as a control. Samples were collected and the number of viable cells was determined. The 1% concentration of DMSO was chosen as the solvent concentration, which did not show a significant difference in cell viability between the control and negative control groups. All experiments were performed in duplicate in 3 replicates.

***The groups were as follows*:** Control (Con), Atorvastatin Low (Ato-L, 65 µM), Atorvastatin High (Ato-H, 130 µM), Simvastatin Low (Sim-L, 40 µM), Simvastatin High (Sim-H, 80 µM), Rosuvastatin Low (Ros-L, 40 µM), Rosuvastatin High (Ros-H, 80 µM).

Cells were harvested after 48 hours. Half of the samples were homogenized with 1 mL TRIzol (Sigma-Aldrich, USA) and stored at –86 °C until RNA isolation. Other parts of samples were stored at –86 °C within PBS for ELISA.

***Total RNA isolation, cDNA synthesis, and qPCR application*:** After thawing samples at room temperature, total RNA isolation was performed (26). Following the chloroform-isopropyl alcohol and ethyl alcohol steps, the pellets were dried for about 10 min and dissolved with 30-100 µL nuclease free water (NFW). Concentrations, purities, and qualities were checked with a nucleic acid spectrophotometer (Merinton, SMA 1000) and gel electrophoresis (100 V and 30 min). After DNA digestion (DNase I, Thermo Scientific, USA), cDNA was synthesized (High-Capacity cDNA Reverse Transcription kit, Applied Biosystems, USA). Thermal cycler (BioRad T100, USA) protocol was as follows: Following the 10 min at 25 °C, samples were kept at 37 °C for 120 min. Then, the temperature was arranged the 85 °C for 5 min. After the reaction, samples were completed to 150 µL with NFW and stored at – 20 °C. SYBR Green Dye containing kit (Power SYBR® Green PCR Master, ThermoFisher Scientific, USA) was used to analyze the expressions of *TNFα*, *IL-10*, *IL-6*, *BAX*, and *BCL-2* (Table 1). Samples were studied as duplicated and *GAPDH* was the housekeeping gene (8). The protocol in qPCR (Bio-Rad CFX-96) was as follows: Following the 10 min at 95 °C, 95 °C for 10 sec, 60 °C for 60 sec, and 40 cycles.

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Table 1. Forward and reverse sequences of primers studied genes.

Gene	Primers	Product size (bp)	Reference
GAPDH	**F:** 5'-TGCACCACCAACTGCTTAGC-3' **R:** 5'-GGCATGGACTGTGGTCATGAG-3'	87	(7)
TNFα	**F:** 5'-AGAACTCACTGGGGCCTACA-3' **R:** 5'-GCTCCGTGTCTCAAGGAAGT-3'	177	*
IL-6	**F:** 5'-GGTACATCCTCGACGGCATCT-3' **R:** 5'-GTGCCTCTTTGCTGCTTTCAC-3'	81	(11)
IL-10	**F:** 5'-GGAGGTGATGCCCCAAGCTGA-3' **R:** 5'-AATCGATGACAGCGCCGTAGC-3'	111	(28)
BAX	**F:** 5'-TGGCAGCTGACATGTTTTCTGAC-3' **R:** 5'-TCACCCAACCACCCTGGTCTT-3'	195	(10)
BCL-2	**F:** 5'-CATGTGTGTGGAGAGCGTCAA-3' **R:** 5'-GCCGGTTCAGGTACTCAGTCA-3'	83	(20)

*: Designed by the current study.

***MDA analyzes and ELISA application*:** The levels of MDA were determined according to the Esterbauer ve Cheeseman method (5). TNFα, IL-10, IL-6 levels were determined with ELISA kits according to the manufacturer instructions (Bioassay Technology Laboratory, CHINA) via ELISA Reader (Thermo Multiskan GO) at 450 nm. Also, the total protein contents of samples were determined (16).

***Statistical analyses*:** Two-way analysis of variance was performed in order to identify the effects of cell line, and statin on dependent variables (protein levels of TNFα, IL-10, IL-6 and MDA, and cell counts):

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ij}$$

Where, Y_{ij}, dependent variable (TNFα, IL-10, IL-6, MDA or cell counts); μ, overall mean; α_i, effect of cell line (i = cancer and healthy); β_j, effect of statin (j = control, Ato-L, Ato-H, Sim-L, Sim-H, Ros-L, Ros-H); $(\alpha\beta)_{ij}$, two-way interaction term of cell line and statin; and e_{ij}, residual error. In case any interaction term was found statistically significant, simple effect analysis with Bonferroni correction was performed to find out the differences among the statin groups in each cell line and among the cell lines in each statin group. The normality of the data was checked using Shapiro Wilk Test in each level of the statin and cell line variables. In addition, the residuals of each model were controlled in terms of the normality assumption. Levene's Test of Equality of Error Variances was used to assess the assumption of homogeneity. For expression analysis $2^{-\Delta\Delta Ct}$ method was used (15). Groups which used different statins with different dose were compared with control in each cell line. All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 23.0. All descriptive statistics were given as mean ± SE and $P<0.05$ was considered as significant.

Results

The determination of DMSO concentrations that did not affect cell viability and growth were performed in three different cell lines. The 1% concentration of DMSO selected as a solvent was not toxic to cells in both the healthy cell lines (Vero and BEAS-2B cell lines) and the cancer cell line (A-549 cell line), (Figures 1-3).

The effects of statins on cell viability in Vero cells at different concentrations were given. At a concentration of 140 µM, atorvastatin was beginning to be toxic to cells. Therefore, a lower dose (130 µM) than 140 µM concentration was studied in the expression experiments. Concentrations of 100 µM of both Simvastatin and Rosuvastatin induced toxicity on cells. However, these two statins were not toxic at concentrations of 80 µM (Figure 4).

Concentrations of statins that did not affect cell viability were similar in BEAS cells as in Vero cells. Concentrations<140 µM for Atovastatin and 80 µM for Simvastatin and Rosuvastatin were non-toxic (Figure 5).

The cell numbers in statin groups were found to be lower than the control in BEAS-2B and A549 ($P<0.01$; $P<0.05$, respectively). Moreover, low-dose simvastatin and rosuvastatin had a similar effect on A549 as well as BEAS-2B. However, cell proliferation was inhibited at higher rates at most of the groups containing high doses of statins on A549 ($P<0.01$). In terms of MDA, the highest was Ato-H in BEAS-2B, while it was Sim-L in A549. While the high dose of rosuvastatin had similar MDA levels in both cell lines, it was determined that there were differences in other statin-treated groups ($P<0.001$) (Table 2).

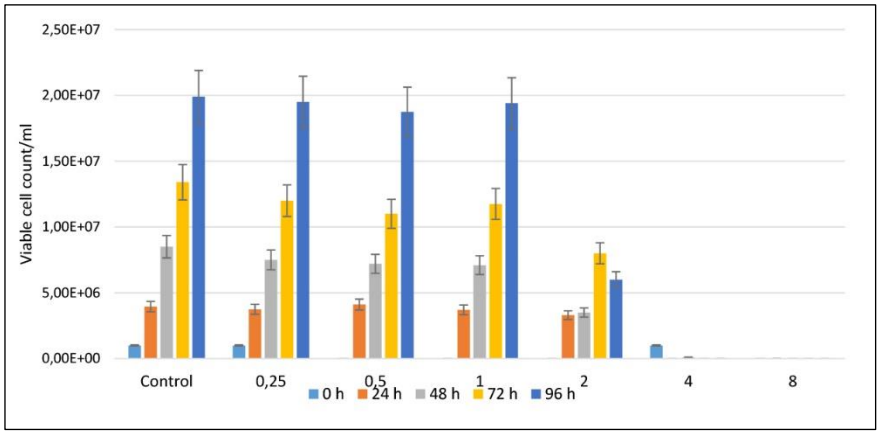

Figure 1. Determination of non-toxic concentrations of DMSO on Vero cells.

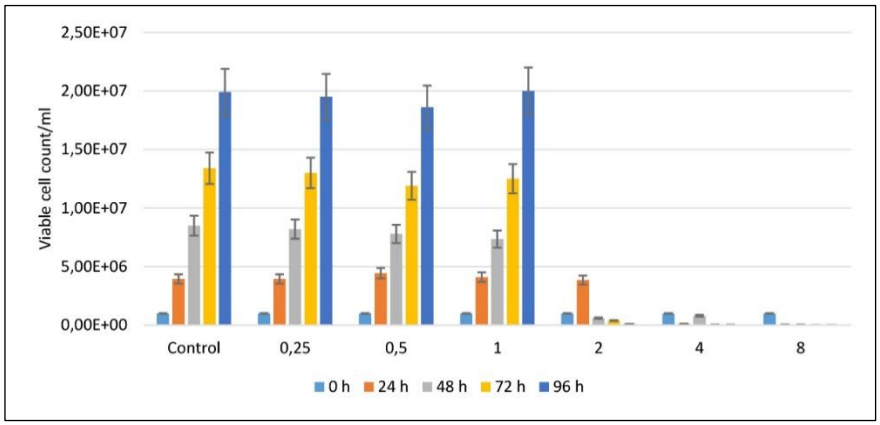

Figure 2. Determination of non-toxic concentrations of DMSO on BEAS-2B cells.

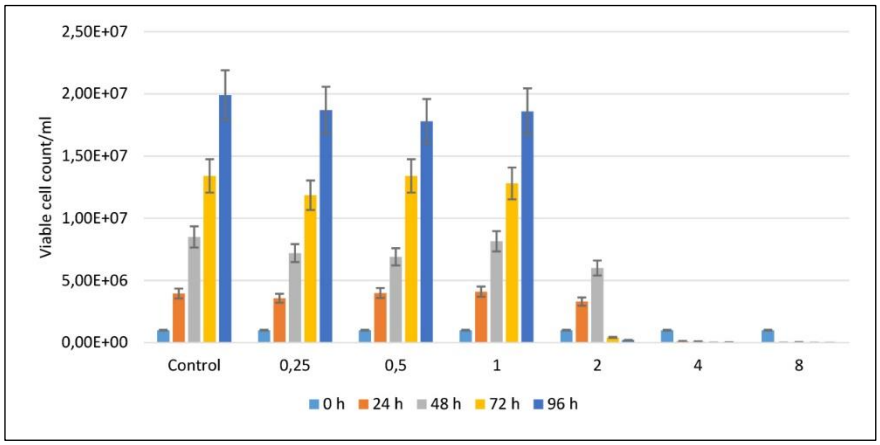

Figure 3. Determination of non-toxic concentrations of DMSO on A549 cells.

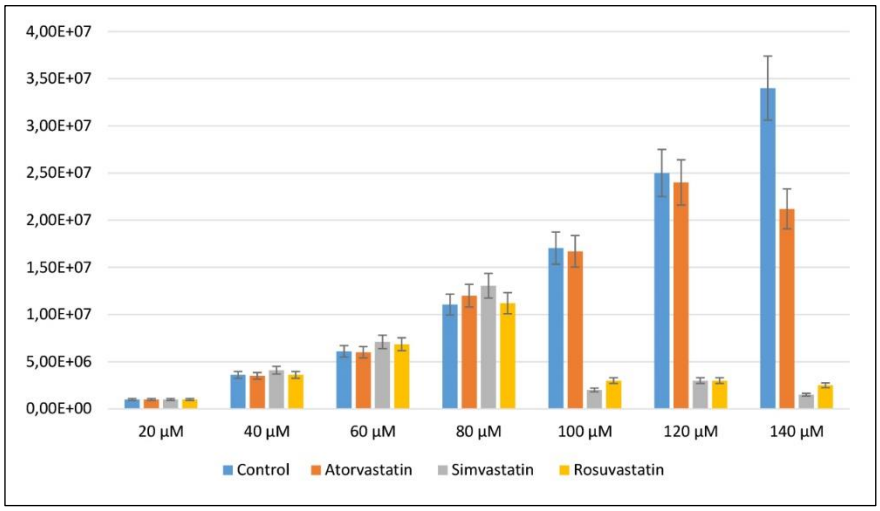

Figure 4. Effects of the three Statins on cell viability in Vero cells compared to the control group.

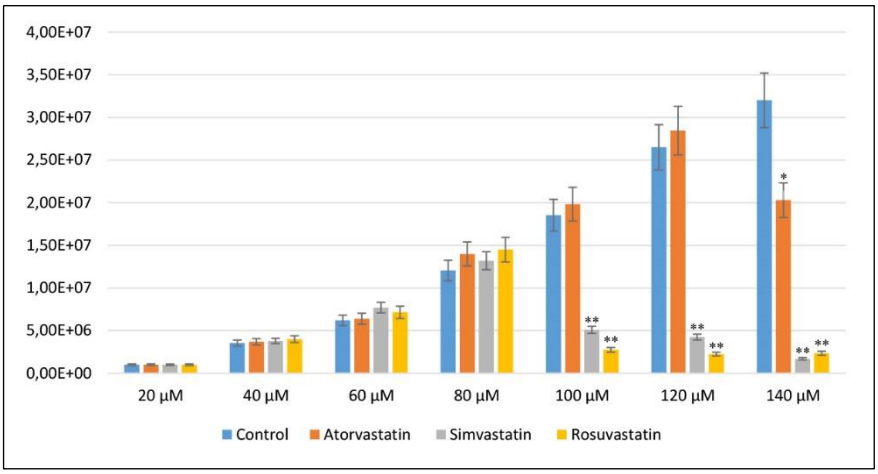

Figure 5. Effects of the three Statins on cell viability in BEAS-2B cells compared to the control group.
*: P<0.05, **: P<0.01.

Table 2. Cell counts and MDA levels in BEAS-2B and A549 cell lines.

Cell Line	Group	Cell Counts (x10^3/mL)	MDA (nmol/mg protein)
BEAS-2B	**Con**	1394.00±93.56^{a}	80.08±0.74^{e,B}
	Sim-L	476.67±40.55^{bc}	277.90±3.67^{b,B}
	Sim-H	318.33±22.42^{c}	272.60±3.16^{b,A}
	Ato-L	639.33±34.80^{b}	232.48±1.14^{c,A}
	Ato-H	500.00±34.64^{bc}	463.40±1.99^{a,B}
	Ros-L	743.33±53.64^{b}	114.88±0.22^{d,B}
	Ros-H	410.00±35.12^{bc}	219.63±2.67^{c}
A549	**Con**	1316.33±139.56^{a}	96.13±1.43^{e,A}
	Sim-L	870.00±64.29^{b}	289.56±5.02^{a,A}
	Sim-H	336.67±63.60^{c}	92.68±4.47^{e,B}
	Ato-L	510.00±94.52^{c}	150.14±0.75^{d,B}
	Ato-H	400.00±80.83^{c}	162.29±6.59^{d,B}
	Ros-L	943.33±74.46^{b}	248.73±7.24^{b,A}
	Ros-H	380.00±87.18^{c}	216.11±2.19^{c}
P	**Cell Line**	0.423	<0.001
	Statin	<0.001	<0.001
	Cell Line*Statin	0.007	<0.001

Con: Control; **Sim-L**: Low dose of simvastatin; **Sim-H**: High dose of simvastatin; **Ato-L**: Low dose of atorvastatin: **Ato-H**: High dose of atorvastatin; **Ros-L**: Low dose of rosuvastatin; **Ros-H**: High dose of rosuvastatin.
a,b: Different lower-case superscript letters indicate significant difference among Statin groups. A,B: Different upper-case superscript letters indicate significant difference among Cell line groups.

High doses of simvastatin and atorvastatin caused upregulation of the *BAX*, while high dose of rosuvastatin caused downregulation in BEAS-2B (P<0.05). *BAX* upregulated in all statin groups in A549. *BCL-2* was downregulated in BEAS-2B with low doses of all statins. However, *BCL-2* downregulated in Ato-H, Ros-L, and Ros-H groups in A549 (P<0.05). In addition, *TNFα* upregulated in all groups except for Ato-L and Ros-H in BEAS-2B (P < 0.05). While this gene was downregulated in Ato-L, it was upregulated in most of the groups in A549. *IL-10* was similar in all groups with control except for Ato-H in BEAS-2B. In A549, it was upregulated in Ato-L, Ato-H, Sim-L, Ros-H (P<0.05). In BEAS-2B, IL-6 was only upregulated in Sim-L (P<0.001). However, it was lowest in Ros-L (P<0.05). But, *IL-6* in Ros-L was upregulated almost 2 folds in A549 (P<0.05) (Figure 6).

TNFα protein levels increased approximately twice as much at both doses of simvastatin while it decreased in Ato-H in BEAS-2B (P<0.05). Also, TNFα increased in Ato-L and Ros-L. In A549, all groups treated with simvastatin and rosuvastatin had higher TNFα protein levels. However, Ato-L had lower TNFα levels (P<0.01). IL-10 levels were increased in the groups using statins except for Ato-H in BEAS-2B (P<0.05). However, it had the highest levels in Ros-L and Ros-H in A549 (P<0.001). IL-6 was found the highest in Sim-L in BEAS-2B (P<0.05).

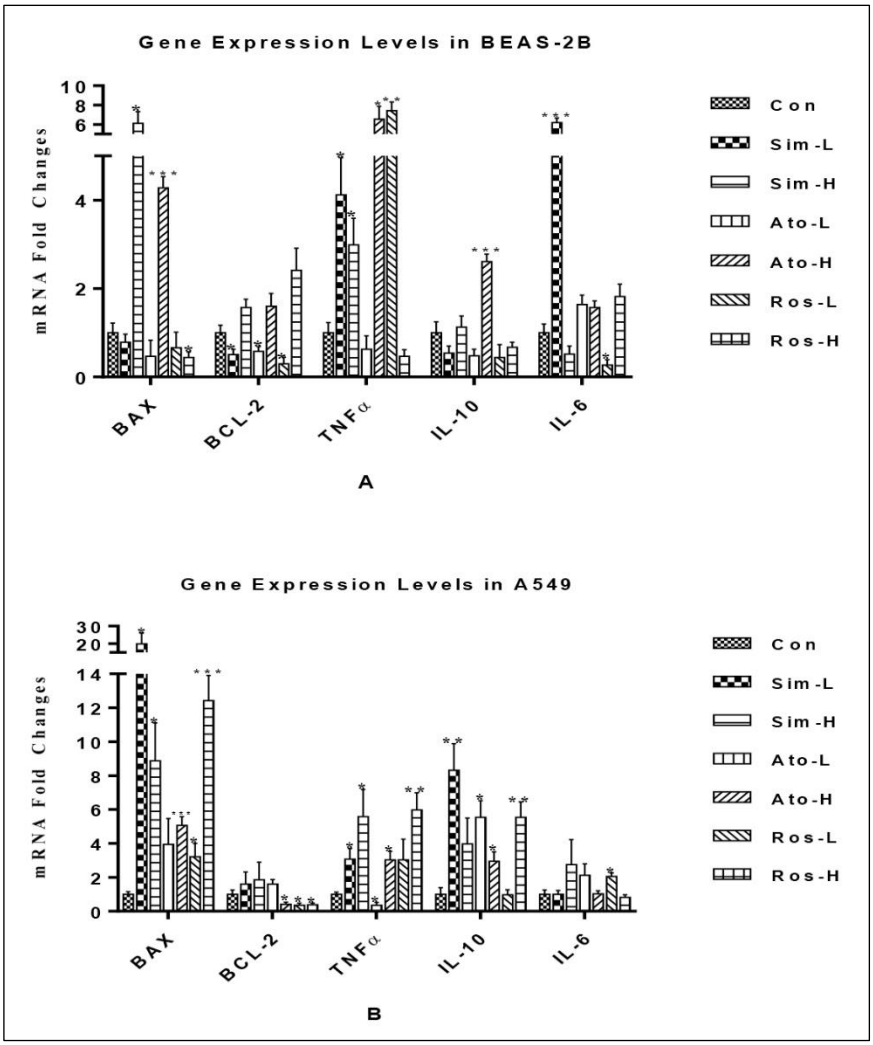

Figure 6. Gene expression levels in BEAS-2B and A549 cell lines.
A: Gene expression levels in BEAS-2B cell line; **B:** Gene expression levels in A-549cell line; *: P<0.05, **: P<0.01, ***: P<0.001; **Con**: Control; **Sim-L**: Low dose of simvastatin; **Sim-H**: High dose of simvastatin; **Ato-L**: Low dose of atorvastatin: **Ato-H**: High dose of atorvastatin; **Ros-L**: Low dose of rosuvastatin; **Ros-H**: High dose of rosuvastatin.

Table 3. Protein levels (ng/mg protein) in BEAS-2B and A549 cell lines.

Cell Line	Group	TNFα	IL-10	IL-6
BEAS-2B	Con	15.34±0,51^{c,A}	8.99±0.35^{d,A}	7.40±0.04^{de,A}
	Sim-L	27.40±1,02^{a,A}	31.25±0.47^{a,A}	16.70±0.25^{a,A}
	Sim-H	26.59±0,26^{a,A}	23.11±0.60^{b,A}	13.05±0.25^{b,A}
	Ato-L	21.44±0,90^{b,A}	23.25±0.37^{b,A}	7.04±0.20^{e,A}
	Ato-H	11.63±0,48^{d,B}	8.17±0.54^{d}	4.32±0.15^{f,A}
	Ros-L	20.54±0,59^{b,B}	13.71±0.50^{c,B}	9.39±0.16^{c}
	Ros-H	16.36±0,59^{c,B}	11.97±0.10^{c,B}	8.41±0.10^{cd,A}
A549	Con	12.39±0,75^{c,B}	5.36±0.30^{d,B}	5.74±0.33^{c,B}
	Sim-L	22.05±0,59^{b,B}	12.23±0.52^{b,B}	5.89±0.09^{c,B}
	Sim-H	20.22±1,25^{b,B}	16.83±1.03^{a,B}	7.66±0.47^{b,B}
	Ato-L	6.21±0,15^{d,B}	5.31±0.21^{d,B}	1.68±0.14^{e,B}
	Ato-H	14.81±0,77^{c,A}	8.39±0.67^{c}	2.76±0.21^{d,B}
	Ros-L	25.62±0,36^{a,A}	18.12±0.39^{a,A}	9.84±0.13^{a}
	Ros-H	27.41±0,43^{a,A}	18.73±0.44^{a,A}	6.75±0.24b^{c,B}
P	Cell Line	<0.001	<0.001	<0.001
	Statin	<0.001	<0.001	<0.001
	Cell Line*Statin	<0.001	<0.001	<0.001

Con: Control; **Sim-L**: Low dose of simvastatin; **Sim-H**: High dose of simvastatin; **Ato-L**: Low dose of atorvastatin: **Ato-H**: High dose of atorvastatin; **Ros-L**: Low dose of rosuvastatin; **Ros-H**: High dose of rosuvastatin.
a,b: Different lower-case superscript letters indicate significant difference among Statin groups. A,B: Different upper-case superscript letters indicate significant difference among Cell line groups.

However, it was significantly lower in Ato-H. In A549, both doses of atorvastatin had lower IL-6 ($P<0.05$). On the other hand, both doses of simvastatin and a high dose of rosuvastatin had the same effect on IL-6 in A549. The highest IL-6 was in Ros-L ($P<0.001$) (Table 3).

Discussion and Conclusion

Recent studies have focused the effectiveness of statins in the treatment of diseases develop due to inflammation and cancer (24). Studies on the effects of statins on the organism are mostly conducted on cell lines as well as on human and experimental animals. There are some studies controversial results on the effects of statins (4, 7, 30, 31). However, in cell line studies, mostly healthy cell lines are tested (31). However, it has been reported in a study that statins do not affect the growth of normal human embryonic stem cells, but inhibit the growth of cancer cells (6). In another study, the statin family of drugs have been reported as triggers of tumor-specific apoptosis (30). This suggests that effects of statins on cells proliferative activity change depending on cell type and distinctive conditions such as cancer (30, 31).

Oxidative damage is mostly determined by the detection of MDA level and is considered a significant marker of inflammation in the cell (29). Atorvastatin and simvastatin were stated to increase MDA levels in tissues in mice and rats (20, 22). In this study, it was determined that both doses of rosuvastatin had a similar effect on MDA, which is indicator of oxidative stress and inflammation in cell, in BEAS-2B and A549, however, the effects of simvastatin and atorvastatin were varied in different cell lines (20).

The apoptotic activities of statins were reported to be dose and type dependent (31). In a study in which 5 µM of simvastatin was applied to breast cancer cells, it was reported that *BCL-2* decreased, while *BAX* was unchanged, although apoptosis was induced (10, 21). This suggests that apoptosis might be regulated independently of the activity *BAX* with statin treatment. It was reported that simvastatin administration caused inconsistent activation of *BCL-2* and *BAX* (7). It was stated that apoptosis and *BAX* increased in MCF7 cells, while *BCL-2* decreased with 20 µM simvastatin treatment. On the other hand, no effect as stated above was observed in healthy cells (27). It was reported in a study that low and high doses of atorvastatin and simvastatin in ViBo cells showed similar effects on cell proliferation, but high doses in CaSki cell line showed anti-proliferative effect (2). It was understood that the effects of statins on cell proliferation and apoptosis are dose-dependent as well as tissue and cancer type.

It was reported a positive relationship between TNFα levels and the rate of apoptosis (3). In the related study, TNFα increased apoptosis by suppressing cell proliferation with its activities at both mRNA and protein levels. In our study, the suppression in cell numbers might have been caused by TNFα-induced apoptosis as reported (3). Metastatic melanoma cells were reported to be sensitive in apoptosis induced by simvastatin, and IL-6 was reported to act as a growth inhibitor in the early melanoma stage (17). In this study, it was determined that simvastatin increased IL-6 gene and protein expression in healthy cell lines, while high dose simvastatin increased IL-6 protein levels in both cell lines. However, IL-6 in the cancer cell line was found significantly higher in Sim-H, Ros-L, and Ros-H. Both doses of rosuvastatin were more effective than other statins in terms of IL-6 activity. Rosuvastatin groups were the highest groups in terms of TNFα, IL-10, and IL-6 levels in the cancer cell line. Possible reasons for the difference between protein and gene expression levels might be due to some post-transcriptional factors such as miRNAs (25). The activity of cytokines changes in macrophages and monocytes with statin activity and the cholesterol biosynthesis pathway regulates the IL-10 activity (23). Statins regulate IL-10 as well as TNFα and IL-6 in cancer cell lines. In a study, it has been reported that IL-10 levels increased with higher dose of atorvastatin (23).

In conclusion, although all statins have been shown to reduce cell numbers in cancerous cell lines, it may be said both doses of rosuvastatin are more effective on genes in both apoptosis and inflammation pathways. The findings of this study give important insights about drugs and target receptors to be used with and without statins in cancer treatment. More studies are needed regarding the dose-dependent activity of rosuvastatin.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

NDi and AY planned the experiments and took the lead in writing the manuscript. HÖ and BÇ practiced the gene expression analyses. NDu, EA and FÇA carried out cell culture steps. PA made statistic analyses. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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Pathological and molecular findings of visceral gout caused by Israel variant 2 (IS/1494/06) genotype of infectious bronchitis virus in chickens

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ABSTRACT

The aim of this study was to investigate pathological lesions and the presence of of Israel variant 2 (IS /1494/06) genotype of infectious bronchitis virus (IBV) in chickens with visceral gout. Sudden deaths were observed in broiler breeders and layer hens belonging to two different flocks located in eastern Türkiye. Broiler chickens were previously vaccinated with a vaccine containing strains of IBV Massachusetts serotype, but no information was available about the vaccination history of laying hens. At necropsy, there was accumulation of white chalk-like material on the serosal surface of the heart, liver, spleen and air sacs. The kidneys were unilaterally or bilaterally enlarged and pale in color and, ureters were also enlarged. Pooled organ samples from diseased chickens and kidneys alone were examined by virus isolation, real-time reverse transcription polymerase chain reaction (rRT-PCR), nucleotide sequencing analysis and histological methods. Israel variant 2 genotype of IBV was detected in the samples of both flocks as a result of virus isolation, rRT-PCR and DNA sequencing analysis. Histological examination revealed multifocal and randomly distributed crystal deposits in the renal tubulus and adjacent interstitium. Mild to moderate crystalline deposits surrounded by heterophils and macrophages were detected in the serosal layers of the heart, spleen, liver, and air sacs. The findings of this study indicated that IBV should be taken into consideration in visceral gout cases of chickens, and detection of IBV genotypes in the field will enable us to use vaccines compatible with these genotypes in order to control the disease more efficiently.

Introduction

Gout is a disorder characterized by increased uric acid in the blood and the accumulation of urates in organs due to impaired renal function (15). The disease is not seen in mammals except humans and anthropoid apes, because uric acid, the end product of purine metabolism, is converted to allantoin by the uricase enzyme. Owing to the absence of uricase enzyme in poultry, conversion of uric acid to allantoin, which is water soluble, does not occur which makes these animals prone to gout (39). In healthy poultry, uric acid binds with a specific protein in the proximal tubules of the kidney that prevents it from crystallizing in the kidneys, but disruption of kidney functions may lead to the occurrence of gout in birds (9, 28, 39). There are two forms of gout known as visceral gout and articular gout. Articular gout is rare in birds and has little economical significance (15). On the other hand, visceral gout is a multifactorial disease and many factors that cause kidney damage and impaired kidney functions may play role in the etiology of visceral gut. In visceral gout, urates accumulate on the serous surface of visceral organs such as the kidneys, heart, liver, spleen,

mesenterium, air sacs and peritoneum. Atrophy in one or both kidneys and dilatation in ureters with diffuse urate deposits may also be detected. Eventually, sudden and gradual deaths are seen in affected poultry (6, 7, 15).

The visceral gout has been linked with nutritional and toxic reasons as well as infections (9, 19). Among infectious agents, infectious bronchitis virus (IBV), avian nephritis virus (ANV) and chicken astrovirus (CAstV) have been showed to cause visceral gout (9, 10). IBV, the etiological agent of infectious bronchitis (IB), is an enveloped, single-stranded, positive sense RNA virus and takes place under Gammacoronavirus genus, subfamily of Coronavirinae and Coronaviridae family (20). IB is a highly contagious disease that occurs worldwide and causes huge economic losses in the poultry industry (17). The spike (S) protein of the virus has two subunits called as S1 and S2, the former provides the binding of the virus to the receptor while the latter performs the fusion between the virus and the cell membrane. Serotype and genotype classification is made depending on the changes in the S1 protein which shows the highest variability among the structural proteins (16). The S1 protein gene has multivariable regions responsible for the production of neutralizing and serotype specific antibodies (13, 27). The IBV has a large number of serotypes and new variants emerge due to frequent point mutation and recombination events in the genome of the virus. Although serotyping of IBV isolates is carried out by haemagglutination inhibition and virus neutralization tests (2), molecular methods have found widespread use in the diagnosis of the disease in recent years. Genetic identification of IBV is performed by replicating the S1 protein gene region by reverse transcription polymerase chain reaction (RT-PCR) and DNA sequence analysis (24).

In spite of the fact that the most effective method in controlling the disease is vaccination, the lack or insufficiency of cross protection against infections caused by genotypes different from the vaccine strains used in the field makes the control of IB difficult (36). Therefore, determining the genotypes of field isolates is very important in terms of monitoring new variants and evaluating vaccination programs. In Türkiye, there are a limited number of studies on frequency of IBV genotypes, in particular Israel Variant 2 (IS-var 2) which is nephropathogenic. Also, the role of nephropathogenic IBV in the etiology of visceral gout cases is overlooked. This study was therefore carried out to investigate pathological lesions and the presence of IS-var 2 genotype of IBV in broiler and laying hens with visceral gout.

Materials and Methods

Samples: Three dead and two live chickens from a broiler flock in addition to two dead chickens from a layer hen flock (totally five deaths and two live chickens) were submitted to the Department of Pathology at Fırat University located in Elazığ province. There were a total number of 850 chickens of Isa-Tinted breed at the average age of 18 weeks and the mortality rate was approximately 7% in the laying hen flock. No information was available on whether the animals had previously been vaccinated with IBV vaccine. In the broiler flock, there were a total number of 35.000 chickens of Ross 308 breed at the average age of 8 weeks and the mortality rate was approximately 6%. Animals in this flock were vaccinated with a vaccine containing strains of IBV Massachusetts serotypes. Sudden deaths were reported in both flocks. Pooled organ samples (kidney, heart, liver, spleen, sinus, air sac, larynx, trachea, lungs and genital organs) in addition to solely kidney samples collected from necropsied chickens were examined for the presence of IBV genotypes at the diagnostic laboratories of Bornova Veterinary Control Institute, İzmir.

Necropsy and histopathological examination: Systemic necropsy was performed in submitted dead and live chickens euthazised by servical dislocation under ether anesthesia, and tissue samples of kidneys, heart, liver, spleen, sinus, larynx, air sacs, lungs, trachea, cecal toncils and genital tract organs were collected, then fixed in 10 % neutral formalin solution. After processing routine procedures, prepared paraffin blocks were cut into 5 µm thick, stained with hematoxylin and eosin (H&E) and evaluated by light microscopy.

Virus isolation: Each chicken was assessed separately for virus isolation. Pooled organs and kidney samples were mixed with Phosphate Buffer Saline (PBS) (Sigma-Aldrich) containing penicillin (2000 units/ml), streptomycin (2 mg/ml) and gentamicin (50 µg/ml) antibiotics and, Mycostatin (1000 units/ml). The organs were homogenized using a MagNA Lyser (Roche) according to the manufacturer's instructions, followed by centrifugation at 3000 rpm for 10 min. The supernatants passed through a 0.45µm filter membrane were used for virus isolation and screened by real time reverse transcription polymerase chain reaction (rRT-PCR). 0.2 ml of the supernatants were inoculated onto the chorioallantoic cavity of ten 9-11 day-old specific pathogen free (SPF) eggs and were incubated at 37°C. Inoculated eggs were checked twice daily and those that died within 24h after inoculation were removed. Deaths between 2 and 7 days post inoculation (PI) were accepted as virus specific. The chorioallantoic fluid was picked up aseptically from embryos died between 48 and 72h PI, providing that the fluid did not demonstrate Hemagglutination (HA) activity. Dead embryos were investigated for the occurrence of embryo stunting, curling, urate in the mesonephros, or focal necrosis in the

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liver. Also, five live embryos taken from the incubator on day 3 PI were kept at 4°C for 24h and, the chorioallantoic fluids belonging to the embryos were used for the next passage (2, 33).

RNA extraction and cDNA synthesis: The chorioallantoic fluids collected on day 4 PI were serially diluted and used in Reverse Transcription-Polymerase Chain Reaction (RT-PCR). High Pure Viral Nucleic Acid Kit (Roche) was employed to extract total RNA from 200 µL of the chorioallantoic fluids according to the manufacturer's instruction. The extracted RNA was stored at -40 °C until PCR was performed. Viral RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) and the obtained cDNAs were stored at -20°C until use.

Real-time reverse transription polymerase chain reaction (rRT-PCR): The rRT-PCR was performed on LightCycler480 (Roche, Mannheim, Germany) by using the Kylt IB-aCo Kit for the detection of Avian Coronaviruses, and the Kylt IBV-Variant O2 Kit for the detection of IBV Middle-East GI-23 lineage (Var2-like). The rRT PCR tests were conducted according to the manufacturer's instruction (AniCon Labor, Hoeltinghausen, Germany).

Partial sequencing of S1 gene: The cDNA samples detected as positive for IBV by rRT-PCR were subjected to partial sequencing of S1 gene by using two pairs of primers SX1: CACCTAGAGGTTTGYTWGCATG and SX2: TCCACCTCTATAAACACCYTTAC; SX3: TAATACTGGYAATTTTTCAGATGG and SX4: AATACAGATTGCTTACAACCACC (4). The first pair of primers (SX1 and SX2) were selected for use in the initial PCR and the other pair (SX3 and SX4) for nested PCR. First round amplification was performed in a final volume of 20 µL (2 µL D.W, 13 µL Norgenbiotek 2X PCR master mix (Canada), 2 µL of SX1 and SX2 primers and 3 µL of cDNA) with a thermal profile of one step denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 15 sec, 58 °C for 30 sec, 72 °C for 30 sec, and a final step of synthesis at 72 °C for 10 min. Amplifications were performed in an Eppendorf master cycler gradient thermocycler (Eppendorf, Hamburg, Germany). Nested-PCR reactions (total volume: 20 uL) were performed using 1 µL of the first PCR product. The reaction mixture was the same as the abovementioned PCR with the addition of nested primers (SX3 and SX4). The amplification products were analyzed by electrophoresis in 1.5% agarose gels in Tris-Acetate-EDTA (TAE) buffer, stained with GelRed™ (Biotium, USA) and visualized under UV light (33).

Nucleotide sequencing, alignment analysis and phylogenetic tree: The rRT-PCR products were purified and sequenced by ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, USA) in a forward direction using primer SX3 and in a reverse direction using primer SX4. The sequences obtained (345 bp) were compared with the IBV sequences in GenBank database and similarities were analyzed by BLAST. Multiple sequence alignments were carried out with Clustal W and phylogenetic tree was constructed with MEGA software (version 7; Biodesign Institute, Tempe, USA) using the neighbour-joining method and Tamura-Nei model with 1000 bootstrap (40).

Results

Gross findings: There was a diffuse accumulation of interspersed, white chalk like material on the serosal surface of the heart, liver, spleen and air sacs (Figure 1A). The kidneys were often markedly enlarged unilaterally or bilaterally with some atrophied lobes, pale in color, and the ureters were enlarged containing a white semi-fluid content that gave a cord-like appearance to ureters (Figure 1B).

Histopathological findings: There were similar histopathological findings in all cases. In kidneys, multifocally and randomly distributed radiating crystalline accumulations were seen in renal tubulus and adjacent intersititium, surounded by mild heterophils and macrophages (Figure 1C). In some areas, tubules exhibited acutely necrotic with degenerated or intact heterophils, while other tubules had eosinophilic protenous casts in the lumen. Renal intersitium contained prominent hemorrhage and congestion in addition to multifocal, moderate inflammatory infiltration including lymphocytes and macrophages (Figure 1D). Severe tubular dilation with flattened epithelium were observed. There were multiple fibrosis and mononuclear cell infiltratins in the wall and surrounded the ureters. Serosal layers of the heart, spleen, liver and air sacs showed crystalline depositoins surrounded by mild to moderate heterophils and macrophages. Myocardium adjacent epicardium also included mononuclear cell infiltrations and oedema, congestion, and myocardial degeneration/ necrosis. Trachea exhibited mild tracheitis characterised with mild mononuclear cell infiltrations in the propria mucosa, and the epithelium was intact (Figure 1E). In lungs, there was multifocal, mild mononuclear cell infiltrations localised in perivascular areas, and pleura was thickened due to moderate lymphocyte infiltrations (Figure 1F). There were no significant histopathological findings in the other organs.

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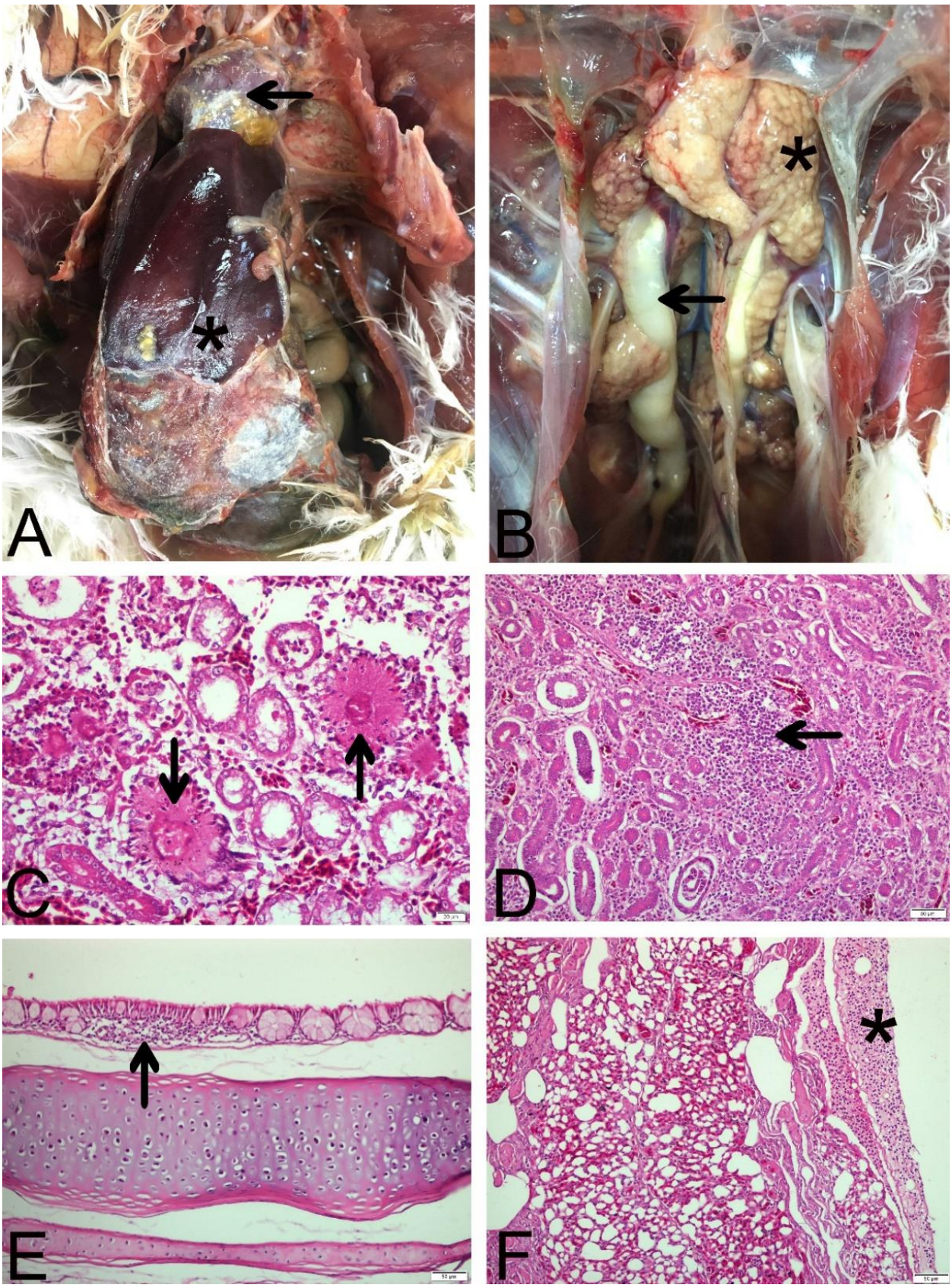

Figure 1. A. White chalk like material accumulations on the serosal surface of the liver (asterix) and heart (arrow). B. Enlargement and paleness in kidneys (asterix), enlarged ureters with a cord-like appearance (arrow). C. Radiating crystalline accumulations in renal tubulus (arrows), H&E staining, Scale bar = 20 µm. D. Intersititiel nephritis characterized with moderate inflammatory infiltration including lymphocytes and macrophages (arrow), H&E staining, Scale bar = 50 µm. E. Tracheitis characterised with mild mononuclear cell infiltrations (arrow) in the propria mucosa, H&E staining, Scale bar = 50 µm. F. Thickened pleura due to moderate lymphocyte infiltrations in lung (asterix), H&E staining, Scale bar = 50 µm.

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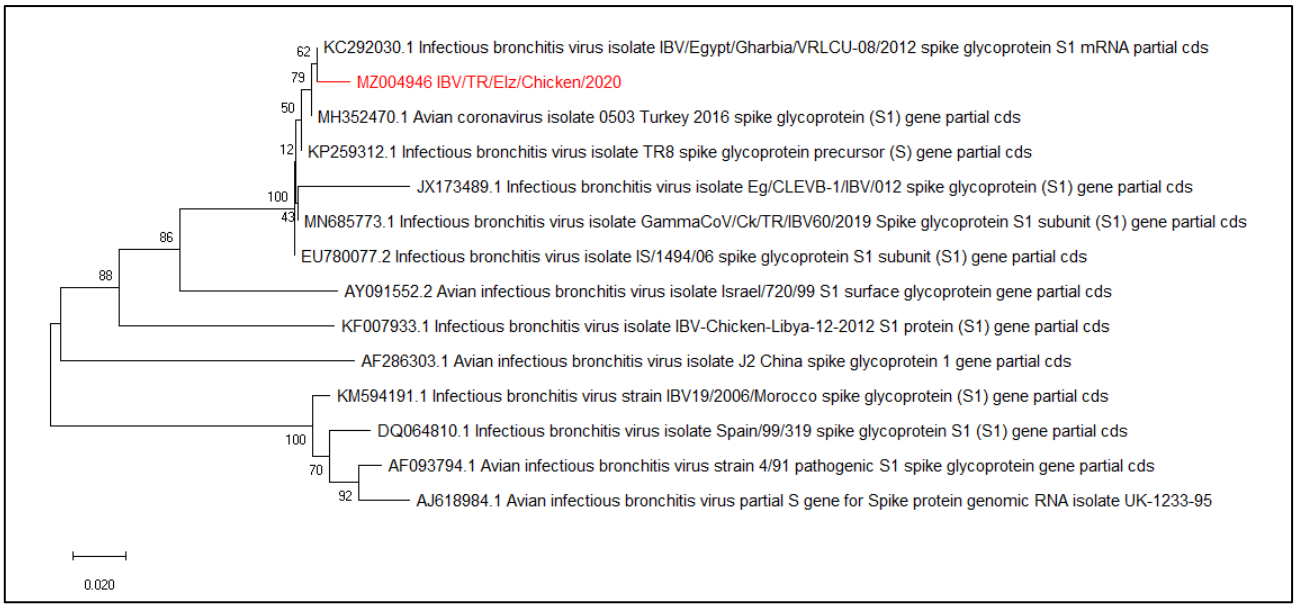

Figure 2. The phylogenetic tree of the sample determined as IBV/TR/Elz/Chicken/2020 (Accession number: MZ004946) constructed by neighbor-joining method.

Egg passage: After the seventh passage in SPF embryonated eggs, no specific lesions for IBV such as stunting, curling and uric acid deposition in the kidneys and ureter were observed. The chorioallantoic fluid of inoculated eggs were found to be negative for Newcastle disease virus and Avian Influenza virus by Hemagglutination (HA) Test.

rRT-PCR: The rRT-PCR analysis of homogenized organ samples showed positive results, with a threshold cycle value of 29.97 for Avian Coronaviruses, 32.70 for IBV Middle-East GI-23 lineage (Var 02). But, analysis of the infected chorioallantoic fluid samples could not yield any positive results.

Nucleotide sequencing, alignment analysis and phylogenetic tree: The results of S1 gene sequencing belonging to two isolates showed great similarity (approximately 98-99 %) with IBV IS-var2 (Accession No: MT270490.1). The phylogenetic tree of the sample determined as IS-Var2 (IBV/TR/Elz/Chicken/2020-GenBank Accession Number: MZ004946) was constructed by using the neighbour-joining method and Tamura-Nei model. Our isolates were detected to belong to genotype I lineage 23 (GI-23) based on the phylogenetic analysis (Figure 2).

Discussion and Conclusion

This study was carried out to describe patohological and virological findings of gout cases detected in chicken flocks. Gout can develop due to non-infectious and infectious causes. Non-infectious factors such as high calcium and protein diets, excessive salt, sodium bicarbonate intoxication, administration of high doses of gentamicin, vitamin A and D deficiency, imbalance between Ca-P levels, dehydration, mycotoxins and some administrative stress factors (9, 15, 38) can cause gout in poultry. In the present study, commercial ration and automatic watering regime were applied to the chickens in both flocks. When the feed composition was examined, it was observed that the protein, Vitamins A and D, calcium and phosphorus ratios in the ration were within normal levels. Mycotoxin analysis was not performed in feeds, but pathological examination revealed no findings specific for mycotoxicosis such as enlargement and paleness of liver, fatty degeneration, vacuolar degeneration, bile duct hyperplasia, fibrosis and enlargement of the bile duct (1). In addition, nephrotoxic antibiotics such as gentamicin were not administered to the animals in these poultry flocks. All these data suggested that non-infectious causes did not play a role in the occurrence of gout in chickens sampled in the present study.

IBV and astroviruses are considered among the infectious causes of gout (10). In this study, the samples were not examined in terms of astroviruses which were reported to cause disease mainly in turkeys (44). However, some astroviruses have been linked with enteric and kidney diseases in chickens (37). A few days/weeks old chickens have been reported to be more susceptible to astrovirus infection, and the disease was usually observed in animals up to 4 weeks of age (11, 23). Also, it has been reported that astrovirus can be found in healthy chickens (11). The average age of the chickens sampled here was 18 weeks for laying hens and 8 weeks for broilers. Also, due to the reports of previous studies conducted in Türkiye

that IBV infections were very common in chickens of all ages (25, 33, 43), gout cases in two flocks were examined for the presence of IBV infection only, in the current study.

IBV can cause lesions in the respiratory, urinary, genital and digestive systems in general (16, 17). Recently, nephropathogenic IBV has been detected in birds with visceral gout (19, 24, 30). In infections caused by nephropathogenic IBV, respiratory system is initially affected followed by severe kidney infection (36). In the present study, kidney enlargement, ureter dilatation, and uric acid crystals in the kidney, liver, heart, spleen and air sac were detected at necropsy. Microscopic examination revealed mild mononuclear cell infiltrates in the trachea and lungs, as well as multifocal and randomly distributed crystal accumulations in the renal tubulus and adjacent interstitium. Mild to moderate heterophiles and crystalline deposits surrounded by macrophages were observed in the serosal layers of the heart, spleen, liver, and air sacs. These findings suggested that some IBV strains involved in visceral gout had a strong affinity (tropism) to the kidney. Kidney damage caused by hyperuricemia can cause urates to accumulate on the surface of different visceral organs (39). According to the findings of this study, it is plausible to suggest that some IBV strains can disrupt kidney function and cause visceral gout by substantially proliferating in renal epithelial cells. In an experimental study investigating the reason for kidney affinity of IBV strains, nephropathogenic (B1648) and respiratory system pathogenic (Massachusetts-M41) IBV strains were compared and unlike the M41 strain, B1648 strain was found to grow better in peripheral blood monocytes cells and spread from the blood to internal organs (36). Nonstructural proteins such as Nsp1 and Nsp11, which are encoded by the ORF 1a gene in the genome of the nephropathogenic IBV, have been put forward to be responsible for nephropathogenicity (35). It has been determined that key genes in kidney tissue were associated with nephropathogenic IBV infection (42). However, additional studies are required to fully understand the kidney affinity (tropism) mechanism of some IBV strains.

Virus isolation requires several passages in embryonated eggs until stunting, curling or other signs are detected in the embryos (45). In the present study, the homogenized organ samples were positive by rRT-PCR for IBV. However, the virus could not be isolated from the samples and specific lesions for IBV were not detected in the embryos despite several passages. The failure of isolation might be due to the virus being inactive during the storage or transport of samples to the laboratory (45). In the present study, IS-var 2 genotype of IBV was detected in the examination of both pooled and kidney samples collected in two flocks with gout cases. The sequencing of hyper variable region (HVR) of the S1 gene (2) revealed that our isolates had similarity at about 98-99% with IS- var2-like isolates (IS/1494/06). The isolates were detected to belong to GI-23 lineage based on the phylogenetic analysis. Recently, GI-23 lineage has been reported to be widespread in many countries including Türkiye (22, 25, 33, 43). The first IS-var 2 IBV causing respiratory and nephropathogenic lesions was detected in Israel in 2004 (31). Later, this genotype has been reported in Lebanon, Libya, Jordan, Egypt and other countries in the Middle East (3, 5, 25, 32, 34). Although Mass41, 4/91, D274, Italy 02 and QX IBV are considered as the most common serotypes in Europe (12, 14), IS-var 2 genotype has been detected in recent studies conducted in European countries (18, 29, 41). The presence of this variant was reported in laying and broiler flocks in Türkiye (25, 33, 43). The determination of IS-var 2 genotype of Middle East origin in Türkiye and many other countries suggested that this genotype has spread to different continents through various sources. Uncontrolled human movements, animal tradeship and wild birds might be responsible for the spread of the disease between countries and continents (25). The fact that wild birds can carry Gammacoronaviruses asymptomatically may result in the emergence of new pathogenic IBV strains in poultry (26). Despite this, the use of live vaccines against a previously undetected strain in a region is not recommended, because possible genetic diversity of the virus can cause emergence of new strains due to recombination between field and vaccine strains (24). In Türkiye, it is known that Massachusetts (Ma5, M41, H120) and 793/B (4/91) serotypes vaccines are commonly used in chickens (43). In the present study, there was no information about vaccination history of the laying hen flock. On ther other hand, animals in the broiler flock were vaccinated with vaccines containing strains of IBV Massachusetts serotype, but no data were available on the protective antibody levels following vaccination. The finding of this study that IS-var 2 was detected in broiler chickens vaccinated against infectious bronchitis was similar to the results of previous studies conducted in different countries (5, 18, 32, 33). It is therefore suggested that the IBV vaccines widely applied in Türkiye do not provide cross protection against IS-var2 genotype. Significant difference between nucleotide sequences of S1 gene encoding immunogenic antigen of IS-var2 IBV detected in the current study and those of IBV strains within the vaccines widely used in Türkiye might explain inefficiency of the vaccines against infections caused by this genotype. However, it has been reported that live vaccines containing H120, D274 and QX IBV genotypes provided 50-70% protection against IS-Var2 IBV infection (8). Also in an experimental study, cross protection against IS-var2 (IS/1494/06) genotype was provided in chickens vaccinated with the combination of

H120-H120 and H-120-1/96 (793/B like) strains, though a complete protection was not noted (21).

It was concluded that nephropathogenic IBV should be considered in visceral gout cases of poultry. The fact that new variant strains may emerge depending on the changes in the genetic structure of the virus urges conducting large scaled epidemiological studies toward investigating variant strains circulating in both domesticated and wild birds. This will enable more effective and up to date vaccines against IS-var 2 IBV genotype to be included in poultry vaccination programs in Türkiye.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

NT, HO and HK conceived and planned the experiments. HO, HK, BK and FC carried out the experiments. NT, HO and HK planned and carried out the simulations. NT, AC and BK contributed to sample preparation. NT, HO, HK, HE and BC contributed to the interpretation of the results. NT, HK and BC took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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A comparative study on egg cholesterol contents and eggshell protoporphyrin and biliverdin pigments of different poultry species

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ABSTRACT

This study compared the cholesterol levels and shell pigments (protoporphyrin and biliverdin) of chicken (conventional and organic), quail, pheasant, and goose eggs. The material for the study was chicken (organic system - Lohmann Brown and conventional system - HyLine Brown) eggs, quail (*Coturnix coturnix japonica*), goose (local), and pheasant (*Phasianus colchicus*) eggs homogeneously selected with a subjective scoring. High-performance liquid chromatography with photodiode array detection was used to analyze the samples (HPLC-PDA). There were no significant differences in the yolk cholesterol content of eggs between species. Based on mg/g of yolk, different poultry species had comparable amounts of cholesterol. Quail eggshells contained significantly more protoporphyrin (81.92 M/g) than chicken (conventional-organic) and pheasant eggshells (P<0.01), but conventional chicken eggshells contained less protoporphyrin (10.73 M/g) than other species (P<0.01). Biliverdin was found only in the eggshells of quail (2.83 M/g) and pheasant (1.02 M/g) (P<0.01). It was observed that white shelled goose eggs had no detectable pigment. Research is required to elucidate the role of diet, age, stressor, strain, and housing systems on protoporphyrin and biliverdin pigment concentrations and cholesterol in table eggs and breeder eggs production.

Introduction

Besides supplying entire nutrients for normal embryo development, egg offers a great source of nutrients in the daily diets of humans (2). Undoubtedly, a chicken egg is the most widely consumed type of egg (37). It is preferred mainly because of its high nutritional value, better digestibility, low cost and easy accessibility (4). Despite insufficient official data, it was thought that eggs of various other poultry species were served to markets for table consumptions. Besides small-size eggs, like quail eggs, large-size eggs, like goose and duck eggs, are mostly consumed as gourmand materials. Just because of seasonal production, consumption of goose and pheasant eggs is quite limited (37). There has been an increasing interest in quail eggs (22). The chemical composition of eggs of different poultry species is similar to each other, but rational distributions are different (9). Among the egg constituents, cholesterol is a highly significant biological molecule playing a precursor role in cell membrane structure and synthesis of gender and adrenal hormones, bile acids and vitamin D (11). Although egg is related to cardiovascular diseases over high cholesterol levels, *in vivo* and *in vitro* studies revealed a weak correlation between egg cholesterol levels and cardiovascular disease risk (28,31).

Cholesterol intake through the diets is at minimum levels in laying hens. The liver and ovary are the primary organs for cholesterol biosynthesis. Although most yolk cholesterol is synthesized in the liver, transported through the blood in the form of lipoprotein and accumulated in follicles, plasma cholesterol level was not found to be related to egg yolk cholesterol concentration (11). In

poultry species, the egg is the primary means of cholesterol removal. Fecal neutral and acidic sterols represent a secondary means of cholesterol removal (11). Zemkova et al. (42) reported whole egg and egg yolk cholesterol levels respectively as 228.3 mg and 13.3 mg. Kasapidou et al. (18) reported lower yolk cholesterol levels (12.5 g/g) in enriched cases than in litter bed systems (14.1 mg/g). Although it was reported that yolk cholesterol levels were influenced by species, race, age, growing systems and diets (2), it was indicated that cholesterol levels were resistant against the changes in these factors (11).

Eggshell pigmentation was reported as efficient selection-genetic progress in some poultry species (35) and it was also reported that hatching performance was significantly influenced by shell pigmentation (10). Additionally, eggshell pigmentation was also used as an assessment criterion for stress and disease conditions resulting in lighter shell colors in commercial laying hens (29). Although there is a slight or no correlation between brown egg color and nutrient composition (33), brown eggshell color is considered a quality indicator by consumers in several countries (29). The primary shell pigments, protoporphyrin and biliverdin, are the products of heme catabolism; therefore, these molecules could directly be derived from red blood cells, or they can be de nova synthesized in the uterus (34). While protoporphyrin pigment forms red and brown colors on eggshell, green and blue-green eggshell colors are formed by biliverdin (17). Protoporphyrin acts as a prooxidant (16) and increases the breaking resistance of eggshell (5). Biliverdin acts as an essential cytoprotectant and it is a metabolically-produced antioxidant pigment (44). However, the number of studies about the concentration of both pigments in eggshells of chicken and the other poultry species is quite limited.

There is a substantial scientific literature on egg quality, biological value, eggshell pigment synthesis, and deposition (7), but there have been few comparative studies on the egg cholesterol content and eggshell protoporphyrin and biliverdin pigments of chicken, goose, quail, and pheasant. Chicken and quail eggs are primarily used for table egg and breeder, whereas goose and pheasant eggs are occasionally used as table egg, and their eggs vary in color. Therefore, we aim to determine the pigments (biliverdin and protoporphyrin) in chicken, goose, quail, and pheasant. We also measured the egg cholesterol concentration to elucidate the cholesterol level in these species.

Materials and Methods

The material for the study was chicken (organic system - Lohmann Brown and conventional system - HyLine Brown) eggs, quail (*Coturnix coturnix japonica*), goose (local), and pheasant (*Phasianus colchicus*) eggs homogeneously selected with a subjective scoring. Apparent colors of eggshells under daylight: Lohmann Brown and HyLine Brown: brown color; quail: dark brownish white-spotted; goose: white color, and pheasant: dark brown color. Based on the previous result (3) and using α=0.05 and power=90, the projected sample size was approximately 76 for egg yolk cholesterol in total. Thus, we estimated that a sample size of 100 eggs (20 eggs for each poultry species) would be more than adequate to investigate our primary objectives in this study. The eggs used in the experiments were daily eggs and they were not cold-stored. Egg weights were measured with a precise balance (±0.001 g). Chicken (conventional – organic), quail and goose eggs were supplied from a commercial poultry facility in Samsun province and pheasant eggs were supplied from the Pheasant Production Station of the Ministry of Agriculture and Forestry. Homogeneous color and weights were taken into consideration in the selection of the eggs.

***Care and feeding conditions of the facilities from where the eggs were supplied*:** Conventional chickens and quails were housed in cages, pheasants were housed in ground pens (4 m × 5 m) and geese were housed in free-spaces with natural plant cover. Five chickens/m^2 indoor space was provided for organic chickens, and one chicken/4 m^2 outdoor space without plant cover was provided. Conventional chickens were supplied with a ration containing 17% CP and 2800 kcal ME/kg; quails with a ration containing 20% CP and 2900 kcal ME/kg; pheasants with a ration containing 15% CP and 2660 kcal ME/kg. Organic chickens were supplied with an organic poultry feed containing 16% CP and 2627 kcal ME/kg energy and organically grown ground alfalfa hay. Feed and water were supplied ad libitum.

Preparation and extraction of egg yolk samples for cholesterol analysis: Fresh eggs were individually broken and the yolk component was carefully separated. The yolk was washed with distilled water and then rolled on a filter paper to remove adhering white component. The whole yolk was then transferred to a test tube and the yolk membrane was punctured and vortex-mixed for the weighing process. About 2 g yolk sample was weighed into a 50 mL test tube and diluted with 20 mL of distilled water. After vortex-mixing vigorously, 1 mL of diluted sample was transferred into a 15 mL test tube and 1 mL of 95% ethanol was added and the mixture was vortex-mixed vigorously. Then 2 mL of diethyl ether was added into the tube and vortex-mixed vigorously. The former step was repeated with 2 mL of petroleum ether. After holding on for 30 min at ambient temperature, 0.5 ml of the organic phase was pipetted into a new tube and evaporated to

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dryness under a nitrogen stream at 45 °C. The residue was dissolved with 0.5 mL of ethanol, vortex-mixed vigorously and transferred into a 1.5 mL amber vial for HPLC analysis (43).

***HPLC-PDA analysis of egg yolk cholesterol*:** Cholesterol content of the yolk samples was measured by an HPLC system (Prominence LC-20A, Shimadzu, Kyoto, Japan) equipped with a PDA detector (SPD M20A, Shimadzu, Kyoto, Japan) fixed at 208 nm wavelength. A reversed-phase C18 column (Inertsil ODS-3V, 4.6 x 250 mm, 5 µm, GL Science, Tokyo, Japan) was used for separation at an oven temperature of 30 °C. The mobile phase consisted of acetonitrile and 2-propanol (4:1, v/v) isocratically. The flow rate was 0.6 mL/min and the injection volume was 10 µl. The run time was 35 min. A stock solution (1 mg/mL) of cholesterol standard was prepared in ethanol and then eight calibration solutions were adjusted by diluting the stock solutions between the ranges of 0.05-0.40 mg/mL (43).

***Preparation and extraction of eggshell samples for protoporphyrin and biliverdin analysis*:** The eggshell of each egg was broken into small pieces in a mortar and then 100 mg of sample was weighed into a 1.5mL centrifuge tube. An aliquot of 0.5 mL of disodium EDTA solution (100 mg/mL, pH 7.2 using NaOH) was added into the tube, vortex-mixed for 1 min, and the cap was carefully loosened. After reducing effervescence, the tube was centrifuged for 2 min at 15,000 rpm and the supernatant was discarded. These procedures were repeated three times, each allowing the eggshell fragments and EDTA solution to have a contact time of 5 min. Following this step, 1 mL of acetonitrile-acetic acid (4:1, v/v) mixture was added to the tubes and vortex-mixed vigorously for 2 min in 30 s intervals, uncapping the tubes to flow out of CO_2. The supernatant was transferred to a clean tube after centrifugation for 4 min at 15,000 rpm. Finally, the supernatant was filtered through a 0.45 µm PTFE disc filter into a 1.5 mL amber vial, making it ready for HPLC analysis (13).

***HPLC-PDA analysis of eggshell protoporphyrin and biliverdin*:** Protoporphyrin and biliverdin amounts of the eggshell samples were measured by an HPLC system (Prominence LC-20A, Shimadzu, Kyoto, Japan) equipped with a PDA detector (SPD M20A, Shimadzu, Kyoto, Japan) set at 400 and 376 nm wavelengths for protoporphyrin and biliverdin analysis, respectively. Analyses were separated on a Lichrosorb RP-8 column (4 x 250 mm, 5 µm, Merck, Darmstadt, Germany) at oven temperature of 25 °C. The mobile phase A was 100 mM ammonium acetate (pH 5.5 with ortho-phosphoric acid), 2-methoxy ethanol and methanol (45:5:50, v/v) and the mobile phase B was 2-methoxy ethanol and methanol (5:95, v/v). Gradient elution was applied as 100% mobile phase A to 100% mobile phase B over 11 min at a flow rate of 1.4 mL/min and the injection volume was 20 µl. The run time was 15 min. Stock solutions (500 µM) of protoporphyrin and biliverdin standards were prepared and then six calibration solutions (mixed) were adjusted by diluting the stock solutions between the ranges of 0.3-10 µM (19).

Statistical analyses: All data were subjected to analysis of variance (one-way ANOVA) by employing the general linear model procedure of SPSS 21.0 (16). The group means, which are given in Table 1 as mean ± standard deviation, were considered significantly different at the level of $P<0.05$.

Results

Mean values for egg weight, yolk weight and cholesterol levels of chicken (conventional system – organic system), quail, goose and pheasant eggs are provided in Table 1. Naturally, goose eggs had the greatest egg weight and quail eggs had the lowest egg weight. There were no significant differences in egg weights of conventional and organic chickens. The average egg weight was 53.28 g for conventional chicken eggs, 51.04 g for organic chicken eggs, 10.10 g for quail eggs, 128.28 g for goose eggs and 32.72 g for pheasant eggs. There were significant differences in egg yolk weights and total cholesterol levels of investigated species ($P<0.01$) (Table 1, Figure 1). The greatest total cholesterol content was obtained from goose egg (709.45 mg/egg) followed by the conventional chicken egg (218.38 mg/egg), organic chicken egg (200.50 mg/egg), pheasant egg (170.78 mg/egg) and quail egg (55.16 mg/egg). Although total cholesterol level was lower in an organic chicken egg (200.50 mg/egg) than in a conventional chicken egg (218.38 mg/egg), such a difference was not significant. Significant differences were not observed in cholesterol level of 1 g of egg yolk in investigated species.

As can be inferred from Table 1 and Figure 2 and 3, biliverdin and protoporphyrin were not encountered in goose eggshell. The greatest eggshell biliverdin (2.83 µM/g eggshell) and protoporphyrin (81.92 µM/g eggshell) contents were observed in quail eggs ($P<0.01$). As compared to organic chicken eggshells, conventional chicken eggshells had lower ($P<0.01$) protoporphyrin levels (10.73 µM/g eggshell). Pheasant eggshell biliverdin level (1.02 µM/g eggshell) was significantly lower than quail eggshells and pheasant eggshell protoporphyrin level (23.32 µM/g eggshell) was similar with the protoporphyrin level organic chicken eggshell (23.23 µM/g eggshell). It was found that white shelled goose eggs contained no detectable pigment.

Table 1. Egg weight, yolk weight and egg yolk cholesterol, eggshell protoporphyrin and biliverdin pigments values in different poultry species.

Genotype	Egg weight (g)	Yolk weight (g)	Cholesterol (mg/egg)	Cholesterol (mg/g yolk)	Protoporphyrin (µM/g eggshell)	Biliverdin (µM/g eggshell)
C. Chicken	$53.28^{b} \pm 7.71$	$14.66^{b} \pm 2.29$	$218.38^{b} \pm 45.08$	14.96 ± 2.36	$10.73^{c} \pm 10.52$	ND
O. Chicken	$51.04^{b} \pm 2.84$	$12.28^{c} \pm 1.43$	$200.50^{bc} \pm 30.57$	16.96 ± 1.68	$23.23^{b} \pm 14.54$	ND
Quail	$10.10^{d} \pm 1.01$	$3.33^{e} \pm 0.50$	$55.16^{d} \pm 12.79$	16.43 ± 2.06	$81.92^{a} \pm 39.08$	$2.83^{a} \pm 0.98$
Goose	$128.28^{a} \pm 8.38$	$43.10^{a} \pm 6.00$	$709.45^{a} \pm 103.83$	16.61 ± 2.56	ND	ND
Pheasant	$32.72^{c} \pm 2.11$	$10.30^{d} \pm 0.78$	$170.78^{c} \pm 25.53$	16.54 ± 1.96	$23.32^{b} \pm 11.29$	$1.02^{b} \pm 0.60$
P	**	**	**	NS	**	**

C: Conventional; O: Organic; Data are expressed as mean ± standard deviation; $^{a\text{-}e}$: Different superscript letters in a column indicate significant difference; **: P<0.01; ND: not determined; NS: not-significant

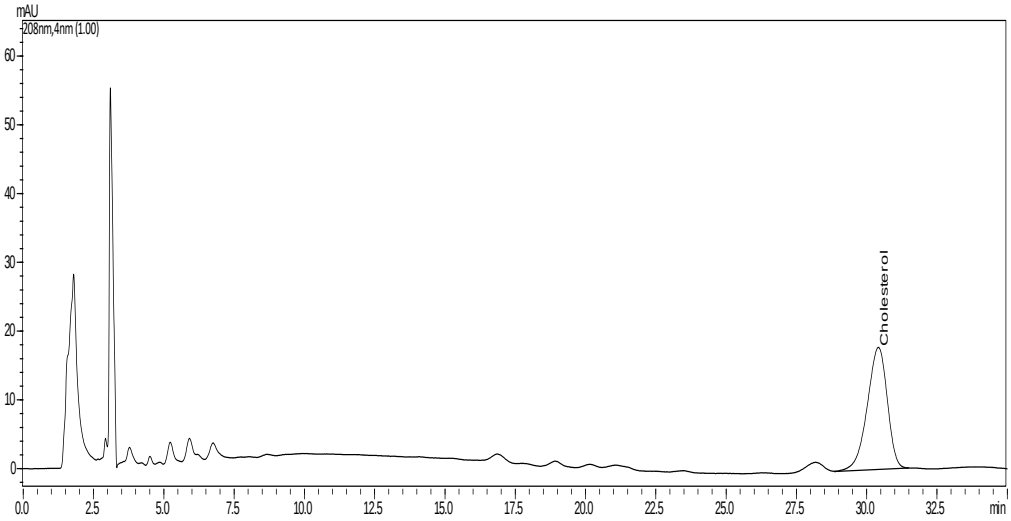

Figure 1. A sample HPLC-PDA chromatogram corresponding to the analysis of egg yolk cholesterol at 208 nm.

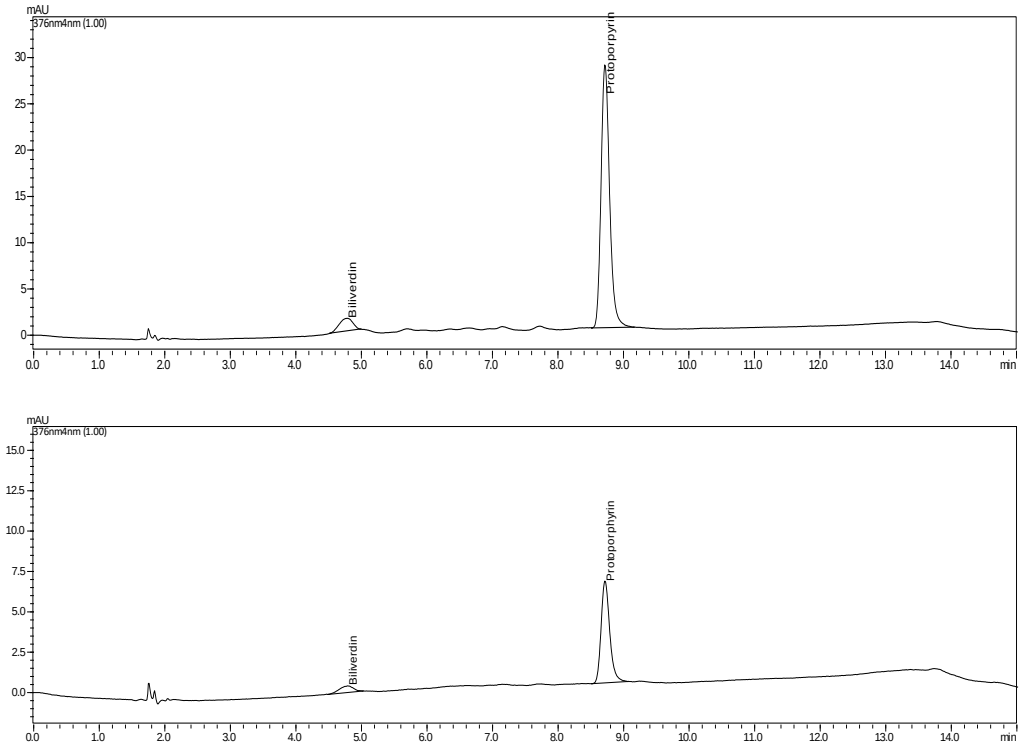

Figure 2. A sample HPLC-PDA chromatogram is corresponding to the analysis of biliverdin in quail eggshell (a) and pheasant eggshell (b) at 376 nm.

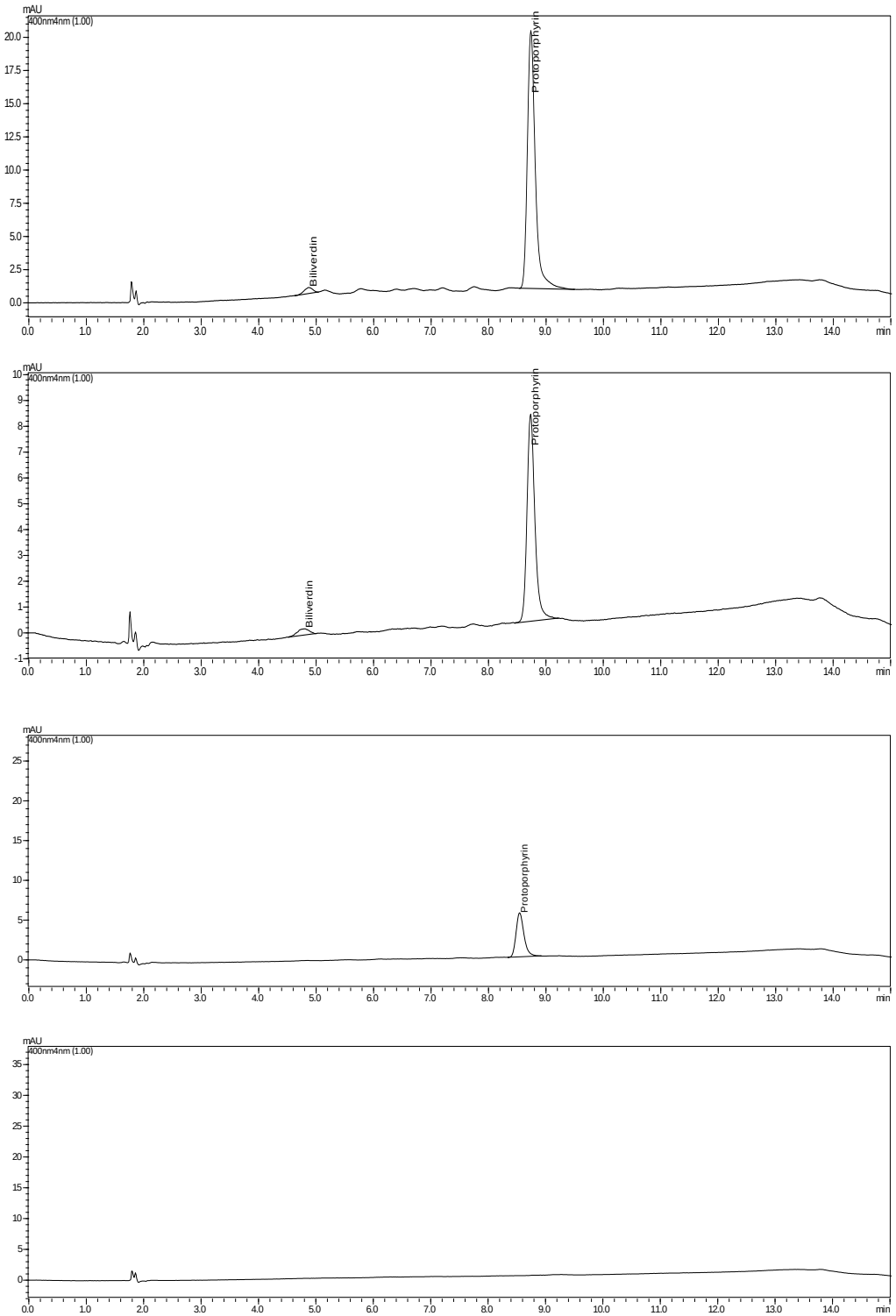

Figure 3. A sample HPLC-PDA chromatogram is corresponding to the analysis of protoporphyrin in quail eggshell (a), pheasant eggshell (b), chicken (organic) eggshell (c) and goose eggshell (d) at 400 nm.

Discussion and Conclusion

As can be inferred from Table 2, there were significant differences in whole egg and egg yolk weights. There is a positive correlation between live weight and egg size of poultry species (25). In previous studies, chicken egg weights were reported as between 60.05 - 67.41 g (25, 36), quail egg weights as between 10.40 - 13.19 g (3, 24), peasant egg weights as between 31.89 - 32.53 g (21, 38) and goose egg weights as between 120 - 195 g (22). In the present study, as compared to conventional chicken eggs, organic chicken eggs had lower egg and egg yolk weights. However, the differences in egg weights were not found to be significant.

In this experiment, the cholesterol content of eggs varies greatly between species when expressed as mg/egg. On an egg basis, it is expected that there will be a difference in cholesterol results. It was found that the cholesterol content of eggs increased with egg weight. These findings are consistent to those of Faitarone et al. (12), who claimed that egg cholesterol levels are positively related to poultry genetics and age, egg weight and yolk weight, and negatively related to lay percentage and dietary protein levels. On the other hand, the cholesterol content of organic chicken eggs (200 mg/g egg) was numerically lower than that of conventional chicken eggs (218.38 mg/g egg). This is thought to be due to the layer diet, which includes herbs from the organic system (23).

The statistical analyses in the current study revealed no significant differences in the yolk cholesterol levels of eggs (mg/g yolk) produced by chickens, quail, goose, and phasant. Adeniyi et al. (1) found a higher average yolk cholesterol content in chicken and quail eggs (33.67 and 46.83 mg/g, respectively) than the current study. Kazmierska et al. (20) observed that chicken, quail, and pheasant eggs had lower yolk cholesterol content (13.91, 7.78, and 6.82 mg/g, respectively) than the current findings. Ukachukwu et al. (39) showed that the overall value for egg yolk cholesterol in quail eggs was 6.79 mg/g, while it was 4.03 mg/g in chicken eggs. Antova et al. (2) and Yalçn et al. (41) reported lower mean values of egg yolk cholesterol (9.95 and 12.52 mg/g, respectively) in white Leghorn and Hyline Brown chickens than the current study. Aziz et al. (4) reported mean values for egg yolk cholesterol in chicken and quail eggs of 7.65 and 16.05 mg/g, respectively. The existing literature mentions various levels of cholesterol in pheasant eggs. Choi et al. (6) reported much higher values (approximately 20 mg/g), while Gugala et al. (6) reported significantly lower (6.8 mg/g of yolk) (15). The difference in egg yolk cholesterol content between the current study and the previous studies could be due to a variety of factors, including laying hen age and diet, production systems, or assay methods (15). Due to limited availability of comparable studies on goose yolk cholesterol, comparing the current study's findings to those in the literature is difficult. According to a previous study (22), the yolk cholesterol content of goose eggs (13.94 mg/g yolk) was lower than that of chicken, quail, and pheasant eggs.

It is of particular importance with respect to sexual signaling and the physiological and mechanical properties of shell pigment (27). The eggshell pigments are influenced by age and genetics (40), and the housing system and nutrition have only a minor impact (8). However, there is much less data on shell pigment concentration. The primary pigment responsible for brown egg coloration is protoporphyrin. Brown eggshells contain traces of biliverdin, which can affect egg color (26). Polin (30) observed that the eggshell glands of brown egg-laying hens have a greater capacity to convert -aminolevulinic acid to porphyrin than other tissues. Protoporphyrin was found to be present in chicken eggs with brown shells in our study. In our study, it was observed that chicken eggs with brown shells were characterized by protoporphyrin. Eggs from organically raised chickens, on the other hand, contained a higher concentration of protoporphyrin than eggs from conventionally raised chickens. In this regard, chickens in the organic system may have produced more protoporphyrin pigments because they were raised in a better environment with appropriate temperature and humidity, as well as under less physiological stress. As a result, if hens are to be raised in a cage system, the conditions required to resolve the problem of poor eggshell color obtained in these systems should be considered. The analysis of eggshell pigment concentration revealed that quail and pheasant eggshells are pigmented with protoporphyrin and, to a lesser extent, biliverdin. Uğurlu et al. (38) found higher biliverdin levels in dark brown, light brown, and green shell colors of pheasant eggs (5.24, 3.72, and 4.72 M/g, respectively) and lower protoporphyrin levels (14.87, 9.44, and 8.68 M/g, respectively) than the current values. Gorchein et al. (14) found protoporphyrin and biliverdin levels in quail eggshells to be 1.66 - 2.17 and 0.25 - 0.40 nmol/mg, respectively. Samiullah and Roberts (32) measured protoporphyrin concentrations in brown eggshells at 33, 50, and 67 weeks as 1.304 10-8, 1.898 10-8, and 1.806 10-8 mM/g, respectively. Changes in eggshell pigment concentrations may reflect physiological conditions such as egg-laying or nesting, but they may also be caused by exogenous (environmental) factors.

In conclusion, non-significant differences in egg yolk cholesterol content were found between species. Quail eggshells contained much higher levels of protoporphyrin than chicken (conventional-organic) and pheasant eggshells, but conventional chicken eggshells contained less protoporphyrin than other species. Only quail and pheasant eggshells contained biliverdin. Research is required to elucidate the role of diet, age, stressor, strain, and housing systems on protoporphyrin and biliverdin pigment concentrations and cholesterol in table eggs and breeder eggs production.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

HM, EA, and AA conceived and planned the experiments. HM, EA, and AA carried out the experiments. HM, EA, and AA planned and carried out the simulations. HM, EA, and AA contributed to sample preparation. HM, EA, and AA contributed to the interpretation of the results. HM took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Factors affecting the choice of marketing channel by beekeepers in Türkiye

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ABSTRACT

This study aims to analyze the factors affecting the marketing channel choices of beekeepers in the sale of strained honey. The primary data was collected with questionnaires conducted with 162 bee breeders in Türkiye. When beekeeper characteristics by marketing channel selections were compared, it was determined that breeder's education status, income other than beekeeping, the status of getting support, payment method, satisfaction with the marketing channel, way of determining the price, the source of information, and credit usage status were the variables influential in choosing a marketing channel. As a result of comparing the group selling strained honey through the direct channel and the group selling it through the indirect channel, significant differences were found between the groups in terms of beekeeper's age, the share of beekeeping in annual income, the number of hives, the share of strained honey in beekeeping income, and the selling price of honey. Providing training for beekeepers, ensuring their access to market information, improving infrastructure conditions, and encouraging the production of bee products and cooperative membership will increase their income.

Introduction

Beekeeping activities contribute to the economic and social development of countries by providing self-employment and additional income opportunities. Beekeeping is brought to the fore by some significant features: it is not dependent on soil, can be accomplished with a small amount of capital, and requires less labor than other agricultural branches (30). Beekeeping is mainly performed as small scale family businesses and contributes to rural development. Türkiye possesses an important potential with its rich flora, suitable ecological conditions, and existing colonies. Beekeeping has become a sector that has made significant progress in recent years in Türkiye as well as all over the world (29). As of 2019, there are 8 128 360 beehives belonging to 80 675 beekeeping enterprises in Türkiye, and 109 330 tons of honey is produced (35).

There are many studies on beekeeping based on original data in the world and Türkiye, both technically and economically (2, 6, 10, 11, 17). As a result of the studies carried out in various regions of Türkiye, the problems faced by the producers were revealed. Beekeeping enterprises in Türkiye encounter important technical, economic and marketing-related problems. The primary marketing-related problems of beekeeping enterprises are that the products cannot be sold at the desired time and for the desired price, the quality-price relationship cannot be established for honey, and consumers' level of knowledge about quality honey is low. In a study (13), it was observed that 83.9% of beekeepers experienced problems in marketing honey. These were reported as low honey prices (80.6%), unfair competition (38.7%), fluctuations in market prices (27.4%), and inability to access information about the market (21%).

Agricultural marketing plays an important role in reducing poverty sustainably and ensuring food security, especially in developing countries (16). Honey marketing in Türkiye has a traditional structure, and various marketing channels can be found in its marketing. These marketing channels are usually in the way that producer-

consumer and producer-wholesaler-retailer-consumer, producer-exporter. In Türkiye, the traditional marketing structure reduces the efficiency of marketing other beekeeping products, especially honey, causes a high price difference between producer price and consumer price, and does not satisfy the producer in terms of their income. Marketing channel selection is one of the critical components of the successful marketing of products. Marketing costs incurred by different marketing channels and revenues from different marketing channels differ. Marketing channels used for selling the products have an impact on breeders' incomes (38). Therefore, studies carried out about the decisions regarding marketing channel selection are highly important, especially when there are many alternative market channels.

In the studies conducted, the factors affecting the decisions of the beekeepers to choose a marketing channel in different agricultural enterprises in rural areas were discussed (8, 9, 12, 18, 27). In a study conducted in beekeeping enterprises related to the subject (22), it was reported that beekeeper's average monthly income, previous agreement with buyers, and market knowledge factors affected the choice of local collector channel; age, beekeeping experience, distance to the nearest market, and market information variables affected the choice of retailer channel.

In Tarekegn's (34) study, it was found that the majority of beekeepers sold their honey and bee products to cooperatives. Through the econometric model developed, it was revealed that the amount of honey sold, extension activities, beekeeping experience, distance to the nearest market, access to market information, cooperative membership, and trust in buyers determined the marketing channel choices of the honey producers in the study area.

In another study that analyzed the factors affecting honey marketing channel choices of small-scale beekeepers in Ethiopia's Tigray Region (36), it was reported that the inadequacy of credit access and the distance from the market increase the probability of selling to the local market and merchants in comparison with industrial processors, while the size of the enterprise and the number of beehives reduce the possibility of using the local market in comparison with the industrial processors.

The number of studies examining the factors affecting the marketing channel choices of beekeeping enterprises in Türkiye is insufficient. A great majority of the studies on beekeeping are aimed at determining the economic structures of the enterprises. However, studies on the marketing of honey and bee products are limited. This study was aimed to reveal the marketing channels used by beekeepers in the marketing of strained honey and the factors affecting their channel selection.

Materials and Methods

The research data were obtained using a questionnaire structured between September 2019 and February 2020. The studies of Maspaitella et al. (21), Nyaupane and Gillespie (25), Tarekegn et al. (33), Thamthanakoon (37), Tsourgiannis et al. (38) were utilized to prepare the questionnaire.

Sampling: The research material consisted of the data related to the socio-economic characteristics and production and marketing activities of the breeders affiliated with a total of 162 beekeeping enterprises in Türkiye. In order to determine the enterprises to be included in the scope of the research, the total number of beekeeping businesses in Türkiye (37 329 units) and the regions and provinces where the enterprises were concentrated were determined utilizing the records of the Turkish Association of Beekeepers (TAB). According to the TAB records, these regions, which had 69.59% of all the beekeeping enterprises in Türkiye, were the Aegean (Cities of İzmir, Muğla, Afyon, Denizli, Manisa), Black Sea (Zonguldak, Kastamonu, Samsun, Ordu, Düzce), Central Anatolia (Ankara, Konya, Aksaray, Kayseri, Sivas), Eastern Anatolia (Kars, Ardahan, Erzurum, Tunceli, Bingöl), and Southeastern Anatolia (Şanlıurfa, Mardin, Diyarbakır, Gaziantep, Adıyaman) regions.

For collecting the data, two-stage purposive and convenience sampling techniques were employed (21). The regions where beekeeping activities and honey production were intensely carried out were determined through the purposive sampling method. In cooperation with the Bee Farmers Unions in the provinces of these regions, the data were collected from beekeepers who came to visit the union and agreed to participate in the survey through the convenience sampling technique. Businesses with incomplete data were removed from consideration, and the data of 162 enterprises in total were included in the analysis.

Statistical analysis: The marketing channels used by the bee breeders were divided into two groups as direct (direct consumer marketing channel) and indirect channels (wholesaler, broker, contractor company, association, cooperative). The explanatory variables affecting the marketing channel selection discussed in this study were examined in three parts. These are the socio-economic characteristics of the breeders, the general characteristics of the business, and the features of its marketing and operation. Socioeconomic characteristics include gender, education, income other than beekeeping, and monthly income; enterprise characteristics involve whether products other than strained honey were produced or not, key production issues, government support, and the level of contentment from beekeeping activities; marketing

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features cover the payment method, satisfaction with the channel used, the way of price determination, the source of learning the market information, the status of using credit, the status of receiving training, the statuses of cooperative membership and association membership.

SPSS 25 statistical software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) was employed to assess the data. The variables were expressed using mean, ± standard deviation, percentage, and frequency values. The variables were evaluated after checking the prerequisites of normality and homogeneity of variances (the Shapiro-Wilk Test and Levene's Test). When analyzing the data, the Independent 2-group t-test (Student's t-test) was used to compare two groups; if the prerequisites were not met, the Mann-Whitney U test was applied. Categorical data were analyzed using Fisher's Exact Test and the Chi-Square Test. In cases where the expected frequencies were less than 20%, the "Monte Carlo Simulation Method" was used for the evaluation to include these frequencies in the analysis. For the significance level of the tests, $P<0.05$ and $P<0.01$ values were accepted.

Results

Mean values and standard deviations of some characteristics of beekeepers are given in Table 1.

In this study, it was determined that beekeepers were on average 49 years old and had 19 years of beekeeping experience, the share of beekeeping in their annual income was 57%, the average number of hives was 167, they earned 70% of their total beekeeping income from the sale of strained honey, and the average sale price of honey was 36 TL/kg (Table 1).

In this study, it was found that 63% of the bee breeders sold their products through direct channels and 37% through indirect channels.

The explanatory variables in the study were analyzed and compared in terms of the two existing marketing channels. The findings obtained from the comparison of the two marketing channels in terms of socio-economic characteristics are given in Table 2. It was determined that beekeepers were generally male and predominantly primary school graduates (49.1%). When the findings regarding the breeders' earnings were examined, it was determined that 71% of them also generated income from

Table 1. Mean values and standard deviations of some characteristics of beekeepers.

Characteristics	n	Min.	Max.	Mean	S. D.
Age (years)	155.00	21.00	74.00	49.39	11.08
Experience (years)	161.00	1.00	50.00	19.19	11.62
Share of beekeeping in annual income (%)	157.00	1.00	100.00	57.01	32.38
Number of hives (units)	161.00	12.00	920.00	167.35	135.42
The share of the income of strained honey in the income of beekeeping (%)	143.00	0.00	100.00	70.27	24.73
Price (TL)	162.00	10.00	100.00	36.23	23.74

Table 2. Relationships between some selected socio-economic characteristics and the marketing channels.

Characteristics	Direct Channel		Indirect Channel		Total		P value
	n	%	n	%	n	%	
Gender							
Male	97	62.60	58	37.40	155	100.00	0.635
Female	5	71.40	2	28.60	7	100.00	
Educational							
Literate	1	33.30	2	66.70	3	100.00	
Primary School	29	50.00	29	50.00	58	100.00	
Middle School	14	66.70	7	33.30	21	100.00	0.008**
High School	21	60.00	14	40.00	35	100.00	
University	24	85.70	4	14.30	28	100.00	
Income other than beekeeping							
Yes	79	68.70	36	31.30	115	100.00	0.021*
No	23	48.90	24	51.10	47	100.00	
Monthly income (TL)							
≤ 2,000	20	66.70	10	33.30	30	100.00	
2,001-3,000	32	61.50	20	38.50	52	100.00	0.9
3,001-4,000	27	65.90	14	34.10	41	100.00	
4,001-5,000	9	52.90	8	47.10	17	100.00	
≥ 5,001	13	61.90	8	38.10	21	100.00	

* P<0.05 ** P<0.01.

activities other than beekeeping and hence beekeeping was a side business, and 29%, on the other hand, generated income only from bee breeding. Within the income level brackets, it was observed that the breeders with an income between 2,001-3,000 TL were more than the others (32%). It was determined that the differences between the distributions of "educational status" (P<0.01) and "income other than beekeeping" (P<0.05) according to the marketing channels were statistically significant (Table 2).

Relationships between some selected enterprise characteristics and honey marketing channels were analyzed; the results obtained are given in Table 3.

It was identified that 80% of the breeders participating in the survey obtained bee products other than strained honey, and the breeders regarded diseases and breeding (41%) and marketing (26%) as the two most important problems. It was determined that 86% of the bee breeders benefited from state support, and the majority of the bee breeders were content with their activities. The differences between the distributions of the breeders' statuses of receiving state support (P<0.05) according to the marketing channels were found to be statistically significant (Table 3).

The statistical relationships between the marketing channels and the organizational status of the enterprises, financial and training statuses were analyzed; the results are presented in Table 4.

It was determined that the beekeeping enterprises examined were predominantly members of the Beekeepers Association, however, the rate of cooperative membership was very low (14.3%); breeders preferred the advance payment channel for marketing their products (71%); in product sales, the prices were mostly set by the buyer and the seller together (43%); 38% of the breeders obtained their market knowledge from other producers; the majority of the breeders (81%) received training on beekeeping and were satisfied with the marketing channel they used (71%); the credit utilization rate was 49% (Table 4).

It was identified that the differences between the distributions of "the mode of the payment made to breeders at the sale" (P<0.01), "the form of price determination" (P<0.01), "satisfaction with the marketing channel" (P<0.05), "the source of knowledge" (P<0.01), and "credit usage status" (P<0.01) according to direct or indirect honey marketing channels were significant at different levels (P<0.05; P<0.01). On the other hand, in terms of other characteristics, no statistical difference was determined between the groups according to the marketing channels (P<0.05) (Table 4).

Statistical properties between the marketing channels and some variables are given in Table 5.

As a result of comparing the group selling strained honey through the direct channel and the group selling it through the indirect channel, significant differences were found between the groups in terms of the variables beekeeper's age (P<0.01), the share of beekeeping in annual income (P<0.01), the number of hives (P<0.01), the share of strained honey in beekeeping income (P<0.05), and the selling price of honey (P<0.01) (Table 5).

Table 3. Relationships between some selected enterprise characteristics and honey marketing channels.

Characteristics	Direct Channel		Indirect Channel		Total		P value
	n	%	n	%	n	%	
Product other than strained honey							
Yes	87	66.40	44	33.60	131	100.00	0.067
No	15	48.40	16	51.60	31	100.00	
The most important problem							
Marketing	28	66.70	14	33.30	42	100.00	
Fake Honey	16	61.50	10	38.50	26	100.00	
Honey Prices	9	52.90	8	47.10	17	100.00	0.844
Apiary Location	8	72.70	3	27.30	11	100.00	
Diseases and Breeding	41	62.10	25	37.90	66	100.00	
Government support							
Yes	77	57.90	56	42.10	133	100.00	0.033*
No	18	81.80	4	18.20	22	100.00	
Contentment from beekeeping activity							
Yes	87	64.40	48	35.60	135	100.00	0.358
No	6	50.00	6	50.00	12	100.00	

* P<0.05 ** P<0.01.

Table 4. Relationships of the marketing channels with the organizational status of enterprises and some economic variables.

Characteristics	Direct Channel		Indirect Channel		Total		P value
	n	%	n	%	n	%	
Cooperative membership							
Yes	14	60.90	9	39.10	23	100.00	0.842
No	87	63.00	51	37.00	138	100.00	
Association membership							
Yes	95	62.10	58	37.90	153	100.00	0.462
No	6	75.00	2	25.00	8	100.00	
Payment method							
Advance	85	73.90	30	26.10	115	100.00	0.001**
Deferred	17	36.20	30	63.80	47	100.00	
Satisfaction with the channel used							
Yes	76	69.70	33	30.30	109	100.00	0.028*
No	23	51.10	22	48.90	45	100.00	
Determination of price by							
Buyer	20	42.60	27	57.40	47	100.00	0.001**
Seller	26	92.90	2	7.10	28	100.00	
Both	42	64.60	23	35.40	65	100.00	
Other	6	60.00	4	40.00	10	100.00	
The source of information							
Market vizit	8	42.10	11	57.90	19	100.00	0.001**
Other producer	36	67.90	17	32.10	53	100.00	
Friends	11	37.90	18	62.10	29	100.00	
Association	16	88.90	2	11.10	18	100.00	
Government officials	1	100.00	0	0.00	1	100.00	
Internet	1	100.00	0	0.00	1	100.00	
Other	12	80.00	3	20.00	15	100.00	
Credit usage status							
Yes	37	50.00	37	50.00	74	100.00	0.001**
No	58	76.30	18	23.70	76	100.00	
Training status							
Yes	81	65.90	42	34.10	123	100.00	0.156
No	15	51.70	14	48.30	29	100.00	

* P<0.05 ** P<0.01.

Table 5. Comparison of beekeeper characteristics according to different channels.

Characteristics	Direct Channel n= 98	Indirect Channel n= 57	P
Age	51.31±11.63	46.11±9.25	0.001**
Experience	20.4±13.09	17.17±8.33	0.060
Share of beekeeping in annual income	48.12±31.83	71.78±27.76	0.001**
Number of hives	130.9±122.33	228.72±135.13	0.001**
Share of the product in beekeeping income	66.39±26.06	76.3±21.36	0.010*
Price	46.01±22.95	19.62±13.77	0.001**

* P<0.05 ** P<0.01.

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Discussion and Conclusion

In this research, the marketing channels used by beekeepers were separably examined into two groups. These are the direct channel where products are sold directly to consumers and the indirect channel through which intermediaries enter between consumers and breeders. The research showed that the breeders preferred the direct channel with a higher rate (63%). Saner et al. (28) reported in their study that the enterprises were producing strained honey sold honey in the market in retail at a rate of 48.28% and through indirect channels at a rate of 51.72%.

In the present study, when beekeeper characteristics by marketing channel selections were compared, it was determined that breeder's education status, income other than beekeeping, the status of getting support, payment method, satisfaction with the channel, way of determining the price, the source of information, and credit usage status were the variables influential in choosing a marketing channel. As a result of comparing the group selling strained honey through the direct channel and the group selling it through the indirect channel, significant differences were found between the groups in terms of beekeeper's age, the share of beekeeping in annual income, the number of hives, the share of strained honey in beekeeping income, and the selling price of honey.

Previous studies reported that the ages of breeders had a significant effect on their choices of marketing channels (1, 22, 38). In our study, it was determined that older bee breeders preferred the direct channel more. In Mehari's (22) study, it was determined that the older the beekeepers, the higher the buyers' perception that bee breeders would produce better quality honey, and the establishment of good relationships increased the selection of the retail channel. In Adu's (1) study, it was reported that as breeders got older, they tended to avoid risk, so they would be less likely to cover the costs associated with participating in the direct marketing channel. This situation could also be interpreted as older and experienced producers, who had been operating in the sector for a long time, could create a certain customer potential.

The education level of a beekeeper is one of the factors affecting the choice of marketing channel (7,15,18). Mutura et al. (24) reported in their study that farmers' education levels affected the interpretation of market knowledge and thus their level of market participation; as the education level of farmers increased, they were more likely to spend less time on marketing activities; therefore, they preferred to sell through cooperatives rather than intermediaries. In another study (18), it was shown that the increased education level of the farmers increased the possibility of access to wholesale and supermarket outlets. Similarly, in our study, the effect of education was found to be significant. When evaluated in terms of all breeders, it was determined that, in terms of education, those with a higher education level than a primary school preferred the direct consumer channel at a higher rate.

If the breeders generate income from activities other than beekeeping, it enables them to decide without feeling under pressure when choosing channels. It was reported in Muthini's (23) study that farmers who had a source of income outside of the beekeping enterprise were less likely to sell to brokers because they had no cash restrictions and could delay sales in order to find a good market. Similarly, it can be inferred that the breeders who receive state support do not have to prefer the indirect channel either since they create an additional income opportunity for themselves and they can use direct channels more. In the present study, it was determined that the variable of income other than beekeeping was effective in the marketing channel preference. Those with other sources of income; beneficiaries of state support preferred the direct consumer channel at a higher rate.

Girma and Abebaw (15) reported in their study that credit usage positively and significantly affected the selection of consumers and other farmer markets. In our study, it was determined that the rate of preferring the direct consumer channel was higher among those who do not use bank loans.

In our study, it was determined that the beekeepers with a higher share of beekeeping in their annual income are more likely to choose the indirect channel to meet their cash needs in a short time due to their limited additional income other than beekeeping. This result is also similar to the findings of Nyaupane et al. (26).

In the present study, it was determined that payment methods had a significant effect on the choice of marketing channel. When evaluated in terms of all breeders, it was determined that,in terms of payment method, those selling the strained honey with cash payment preferred the direct consumer channel at a higher rate. The relationship between payment method and marketing channel selection shows similarity with the study results of researchers like Tsourgiannis et al. (38), Siddique (32), and Adu (1). It was stated in Siddique's (32) study that breeders tended to prefer a channel making advance payment as they found it safe. In another research (1), it was reported that rice producers were more likely to sell to buyers who paid for the product immediately, independent of the price offered.

In the studies conducted, bargaining power was determined as another factor influential in marketing channel selection (1, 4, 38). Breeders' possession of additional sources of income (3) or large-scale enterprises (39) are factors increasing their bargaining power. In our study, it was identified that the beekeepers determining the

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price of honey by the consensus of buyer and seller were more likely to prefer the direct consumer channel.

Bee breeders' access to information about market prices, time of sale, and place of sale have a directing effect on channel selection (15, 23). In their research, Kuma et al. (19) and Mehari (22) stated that lack of market information or difficulties in accessing high-priced markets forced small-scale farmers to use marketing channels offering low prices. The source from which market information is obtained is also important (23). In Muthini's (23) study, it was expressed that receiving market knowledge from the buyers coming to the farm gates did not provide any benefits to the breeders. It was determined in our study that breeders who obtained the market knowledge from other producers preferred the direct consumer channel at a higher rate.

In this study, it was identified that the increase in the number of beehives increased the breeders' likelihood of choosing the indirect channel. The relationship between enterprise size and channel selection was also shown in the studies of researchers such as Adu (1), Bardhan et al., (5), Benedek (7), Mehari (22) and Tesfamariam et al. (36).

In Adu's (1) study, it was stated that the scale size of an enterprise tended to increase farmers' likelihood of selling their rice to processors. Martey et al. (20), explained that farmers making more production preferred to sell in nearby markets to prevent the loss, especially if the product was not durable. Shilpi and Umali-Deininger (31) stated that especially breeders with poor physical market infrastructure had to cover very high transaction costs to sell to the market rather than to the merchants, and this situation made it difficult for the grower to sell large quantities of products directly on the market. However, if the breeders are members of a marketing group or a cooperative, they can sell large quantities of their products to these groups at the gate of the enterprise (direct channel) (5, 23, 33).

Beekeepers who have a lower rate of strained honey in their beekeeping income obtain more bee products (pollen, royal jelly, propolis). Breeders can produce various products to provide additional income and avoid market risks. Nyaupane and Gillespie (25) revealed in their study that diversification affected the choice of marketing channel. In addition, breeders who had too many bee products were also likely to prefer the direct marketing channel where they could sell bee products at higher prices. In our study, the breeders who had a lower share of strained honey in their beekeeping income preferred the direct canal to a greater extent.

The difference between the channels was found significant in terms of the price of strained honey. Breeders tend to choose the marketing channel that offers the highest price (14, 20, 21, 23, 38). The present study revealed that a better price was obtained in the direct marketing channel than in the indirect marketing channel, and producers could partially regulate the price advantage in their favor in direct marketing. Siddique et al. (32) argued that price was not the only determinant of farmers' participation in a marketing channel, but also factors other than price equally and significantly affected marketing decisions.

In the present study, it was determined that marketing channel satisfaction had a significant effect on the choice of marketing channel. When evaluated in terms of all breeders, it was determined that, in terms of marketing channel satisfaction, those who were satisfied with the marketing channel they used preferred the direct consumer channel at a higher rate. The breeders' high levels of satisfaction with the marketing channels they use can be explained in association with the fact that the breeders who use the direct marketing channel sell their products at a higher price. In Thamthanakoon's study (37), it was indicated that the satisfaction of the breeders from the marketing channel, depends on the reliability of the channel as well as cash payment, easy accessibility and contract flexibility.

As a result of this study, the factors affecting the selection of marketing channels for strained honey were revealed by evaluating the survey data of 162 beekeepers in different regions of Türkiye. In our research, it was found that 63% of the breeders sold their products through direct marketing channels and 37% through indirect marketing channels. The bee breeders preferred to sell their products directly to the consumer and in cash in order to get a better price for their products. In these circumstances, direct consumer access opportunities should be enhanced for bee breeders to increase their incomes. Breeders must possess the economic power to afford their marketing expenses and have the products of appropriate quantity and quality to compete in the market so that they can sell their products for cash price. In this context, it is important to ensure beekeepers' access to training opportunities and market information essential for increasing the production amount and product quality, to improve infrastructure conditions for their easy access to markets, and to encourage cooperative membership to enhance their competitiveness. Furthermore, supporting the production of bee products other than strained honey by state institutions in terms of education and financing is another factor that will contribute to breeders' earnings.

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Conflict of Interest

The author declare that they have no conflict of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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Retrospective investigation of Newcastle disease reported in Türkiye between 2017-2019

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ABSTRACT

This study was aimed to understand the spatial and seasonal epidemiology of Newcastle disease (ND) in Türkiye using the outbreak data between 2017-2019 and also to calculate the case-fatality rates of this disease. It was also aimed to produce the maps by using Geographical Information Systems (GIS). Data were obtained from the World Animal Health Information System (WAHIS) database of the World Organization for Animal Health (OIE). Total number of 220 outbreaks of ND were registered in 47 provinces of Türkiye between this years. Accordingly, 88,372 poultry birds transmitted the disease. The highest number of ND outbreaks, cases and deaths was reported in The Black Sea Region. According to the regions there was a statistically significant difference in the number of outbreaks ($P<0.05$), but there was no statistically significant difference in terms of cases and deaths ($P>0.05$). On the other hand the highest number of ND outbreaks, cases and deaths was reported in the spring season. As a result of the comparisons according to the seasons, there was a statistically significant difference in the number of deaths ($P<0.05$), but there was no statistically significant difference in terms of outbreaks and cases ($P>0.05$). The spatial and seasonal distributions identified in this study should be taken into account while attempting to control the disease. Also, it is thought that the creation of spatial maps based on ND outbreaks that are common in Türkiye will contribute to the determination of the areas where precautions should be taken against the disease.

Introduction

Newcastle disease (ND) is caused by strains of Avian Paramyxovirus (APMV-1) in the family Paramyxoviridae, a subfamily of the order Mononegavirales (5, 15). Transmission of the disease occurs through direct contact of infected birds with each other, alimentary route, and inhalation of infected particles (27). The incubation period varies between 2-15 days (average 5-6); some species may be over 20 days (21). In the rapid spread of the disease, factors such as legal or illegal movement of infected birds, migrating wild birds, contaminated litter, manure and water are effective. The importance of these factors varies according to the situation (10, 20).

The clinical signs of the disease vary depending on the virulence of the virus, tissue affinity, route of infection, poultry species, age and immune status (4, 12).

ND is a highly contagious viral disease that can be seen in nearly all domestic and wild bird species, affecting more than 250 bird species worldwide (2). It causes serious economic losses and epidemiological threats in the poultry industry with its high morbidity and mortality rate (1, 8). The creation of active surveillance systems, rationalizing fast, effective, and reliable prevention strategies in disease control, is necessitated by the disease's high death rate (6, 13).

It has been reported that the first outbreaks of ND were seen on the Indonesian island of Java in 1926, followed by the British town of Newcastle upon Tyne, where it was first described (3). Except for Oceania countries, ND is widespread in most of the countries worldwide (11).

Fighting epidemics like Newcastle disease is an important task in terms of ensuring food security and nutrition, for strengthening national economies. Combating outbreaks is one of the most basic research areas of epidemiology (26). Although the history of

epidemiology is very old, recently geographic information systems (GIS) has become an innovative and important component of many researches in the field of epidemiology. The widespread use of GIS in epidemiology has also led to an increase in spatial epidemiology research (17).

With the development of GIS, the importance of spatial analysis studies has increased. Prevention measures can be adopted by identifying unusually high-risk areas with disease mapping. Furthermore, creating a reliable disease risk map allows for better resource consideration and risk assessment (18).

On visualizing the data instead of tables and graphs using thematic maps with GIS the decision-making units easily determine the regions that need to be taken precautions. In this sense, GIS remains the most useful application in basic disease mapping (23, 25).

ND is one of the most important poultry diseases in the world. The large number of birds affected by the disease has a serious economic impact on the poultry industry. The aim of this study is to determine spatial and seasonal the distribution of ND in Türkiye by conducting a registry-based study, to guide the eradication following development of control programs against the disease. In addition, it is to produce maps by using Geographic Information Systems (GIS) and to guide in determining the areas that need to be taken precautions.

Materials and Methods

The material for the study consisted of ND outbreak data between 2017-2019, publicly published in the World Animal Health Information System (WAHIS) database and Provincial level shapefile (.shp extension) data.

Statistical analysis: The Kolmogorov–Smirnov and Shapiro–Wilk tests for normality of data was done and the Levene test for homogeneity of variances to determine whether to use parametric or non-parametric statistical tests before performing the statistical analysis. As the parametric test assumptions are violated, the Kruskal Wallis test was utilized to test the difference between groups. For the significant differences, multiple comparison tests were utilized as a post hoc approach. A probability value of less than 0.05 was considered significant, unless otherwise noted. SPSS 14.01 (License No: 9869264) was used for statistical analysis. Distribution of ND, according to geographical regions, seasons and years were evaluated.

Geographical analysis: Thematic maps were needed to determine ND sensative regions in Türkiye. In this respect, a database based on Geographic Information Systems (GIS) was created and firstly, the area to be studied was determined. As the study area, Türkiye which is located in the northern hemisphere, between 36-42° north latitude and 26-45° east longitude, was targeted. Provincial level shapefile (.shp extension) data were used to be used in GIS software for spatial analysis and mapping of outputs. The shapefile format is a digital vector storage format for storing geometric location and associated attribute information. In order for the shapefile format to be displayed in CBS software, shp, .shx and .dbf file formats must be in the same folder. Here we used, the GIS program QGIS™ 3.6 was used to visualize the spatial data of the ND outbreaks.

Results

ND recorded a total of 220 (100%) outbreaks, 81 (36.82%) in 2017, 99 (45.00%) in 2018, and 40 (18.18%) in 2019. The highest number of outbreaks were seen in 2018, and the lowest was in 2019. When the number of cases in ND outbreaks was evaluated, a total of 88372 cases were identified, as 15823 (17.91%) in 2017, 66015 (74.70%) in 2018, and 6534 (7.39%) in 2019. When the number of deaths was examined, a total of 75436 deaths were identified, 9519 (12.62%) in 2017, 60298 (79.93) in 2018, and 5619 (7.45%) in 2019. As a result of the comparisons according to the years, there was no statistically significant difference ($P>0.05$) in terms of the number of outbreaks, cases and deaths (Table 1).

Between 2017 and 2019, the first outbreak was reported in Samsun and Ordu provinces and subsequently, the infection was spread to 47 provinces of Türkiye, probably as a result of movements of infected poultry from the infected farms. While the highest number of outbreaks were reported in Samsun in total, no outbreaks were reported in 34 provinces (Figure 1).

When the outbreak data were evaluated according to geographical regions, the highest number of outbreaks, cases and deaths were determined in the Black Sea region. The least number of ND outbreaks was reported in The Southeastern Anatolia and The Marmara Region on these dates. As a result of the comparisons according to the regions, there was a statistically significant difference ($P<0.05$) in the number of outbreaks, but there was no statistically significant difference ($P>0.05$) in terms of cases and deaths (Table 1). On the other hand the outbreak data were evaluated according to seasons, the highest number of outbreaks, cases and deaths were determined in spring season. The least number of ND outbreaks was reported in autumn season. As a result of the comparisons according to the seasons, there was a statistically significant difference ($P<0.05$) in the number of deaths, but there was no statistically significant difference ($P>0.05$) in terms of outbreaks and cases (Table 1). The peak month of Newcastle disease was in May, the lowest month was October (Figure 2).

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Table 1. Newcastle outbreaks, cases, deaths poultry by years, geographical regions and seasons.

	Outbreaks			Cases			Deaths		
	n	**%**	**Med (Min - Max)**	**n**	**%**	**Med (Min - Max)**	**n**	**%**	**Med (Min - Max)**
Year									
2017	81	36.82	1(1-4)	15823	17.91	125(8-2200)	9519	12.62	70(3-1400)
2018	99	45	1(1-6)	66015	74.7	100(2-51122)	60298	79.93	85(2-48035)
2019	40	18.18	1(1-2)	6534	7.39	80(6-849)	5619	7.45	68(4-766)
P		0.164			0.427			0.788	
Region									
Mediterranean	24	10.91	1(1-2) [ab]	3158	3.57	100(8-543)	2107	2.79	60(3-520)
Eastern	22	10	1(1-3) [ab]	3317	3.75	123(2-744)	2750	3.65	80(2-714)
Aegean	21	9.55	1(1-2) [b]	4221	4.78	120(8-1369)	2983	3.95	59(8-840)
Southeastern	2	0.91	1(1-1) [b]	760	0.86	380(320-440)	558	0.74	279(168-390)
Central Anatolia	38	17.27	1(1-2) [b]	10684	12.09	114.5(4-2200)	7609	10.09	82.5(4-1400)
Black Sea	111	50.45	1(1-6) [a]	65501	74.12	97(4-51122)	58857	78.02	74.5(3-48035)
Marmara	2	0.91	1(1-1) [b]	731	0.83	365.5(20-711)	572	0.76	286(18-554)
P		**0.01**			0.683			0.721	
Season									
Spring	89	40.45	1(1-4)	66709	75.49	88(2-51122)	58345	77.34	45(2-48035) [b]
Winter	63	28.64	1(1-6)	11986	13.56	131.5(5-1820)	9442	12.52	100(3-1390) [a]
Autumn	30	13.64	1(1-3)	4495	5.09	120(16-744)	3539	4.69	79(11-714) [ab]
Summer	38	17.27	1(1-6)	5182	5.86	103(4-861)	4110	5.45	90(4-825) [ab]
P		0.669			0.199			**0.025**	

[a,b] Values within a column with different superscripts differ significantly at P<0.05.

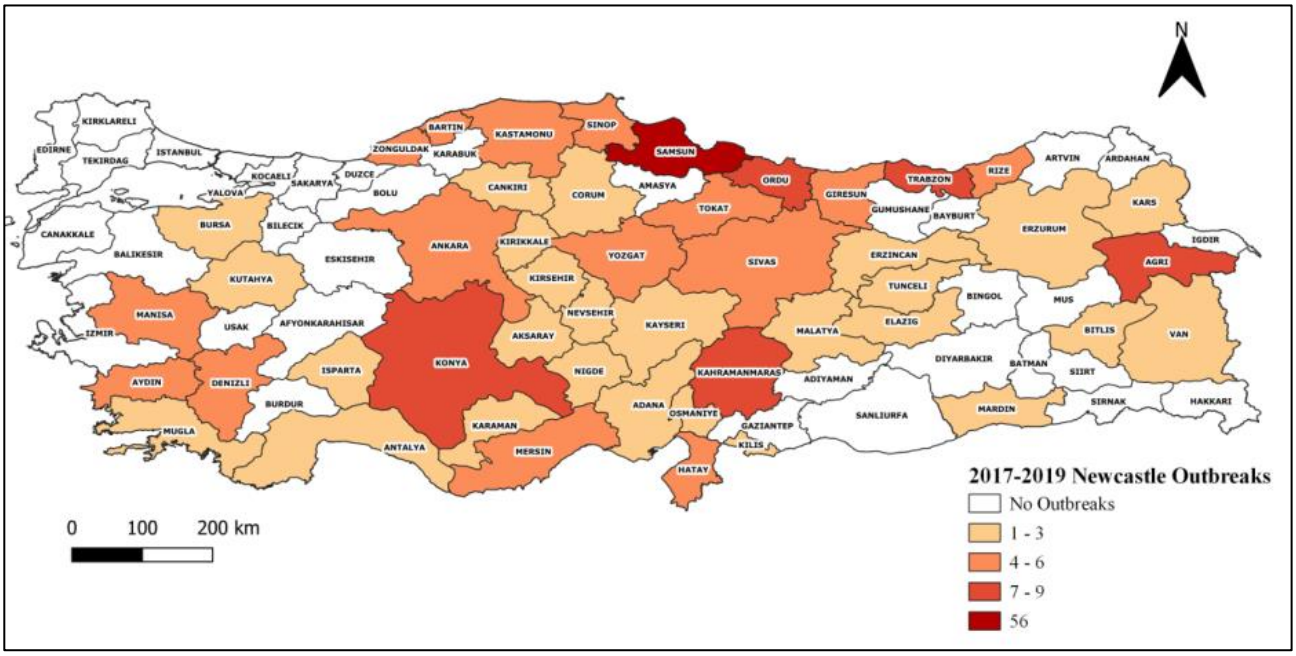

Figure 1. Map produced for Türkiye according to Newcastle outbreaks between 2017-2019.

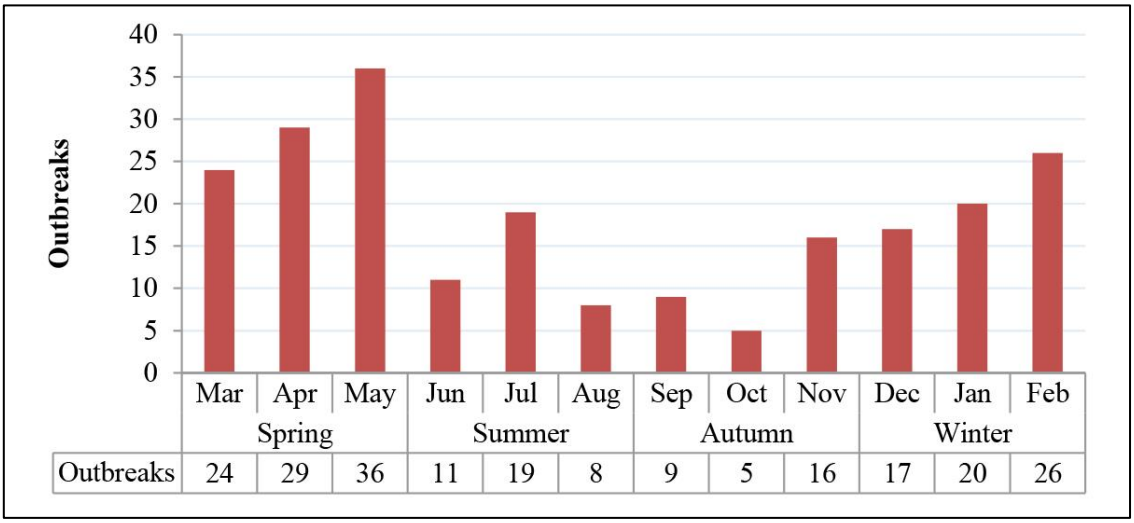

Figure 2. Newcastle outbreaks by seasonal in Türkiye between 2017-2019.

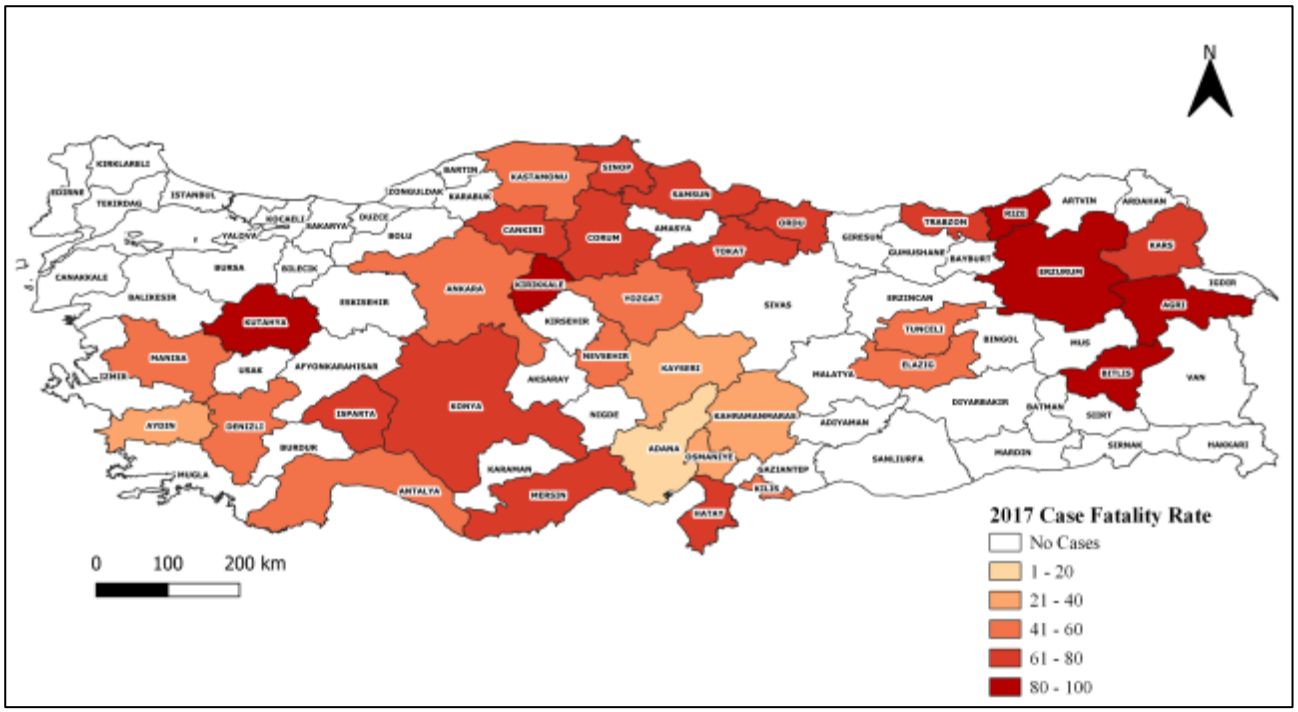

Figure 3. Map produced for Türkiye according to Newcastle case fatality rates 2017.

When the number of cases between 2017-2019 was evaluated, it was seen that the highest number of cases was in Samsun in 2017, Bartın in 2018 and Yozgat in 2019. According to the case and death numbers reported in Türkiye between these dates, the case-fatality rates are calculated. It is determined that the highest case-fatality rate is in the province of Bitlis and Rize in 2017 (100%), in the province of Çankırı, Erzurum and Hatay in 2018 (100%), in the province of Trabzon in 2019 (100%), the number of cases in these provinces is not very high. When we evaluate the Newcastle cases and deaths, cases were seen in 33 provinces in 2017 and no cases were observed in 48 provinces. While cases were seen in all six geographical regions of Türkiye, no cases were reported in the Marmara region. It was determined that the case-fatality rate was very high in six provinces where the outbreak was seen (Figure 3). In 2018, cases were seen in 34 provinces and no cases were seen in 47 provinces. The outbreak was seen in all regions of Türkiye. Case-fatality rates were calculated to be high in many provinces (Figure 4). In 2019, cases were seen in 24 provinces and no cases were seen in 57 provinces. While the cases decreased considerably in 2019, case-fatality rates remained high (Figure 5).

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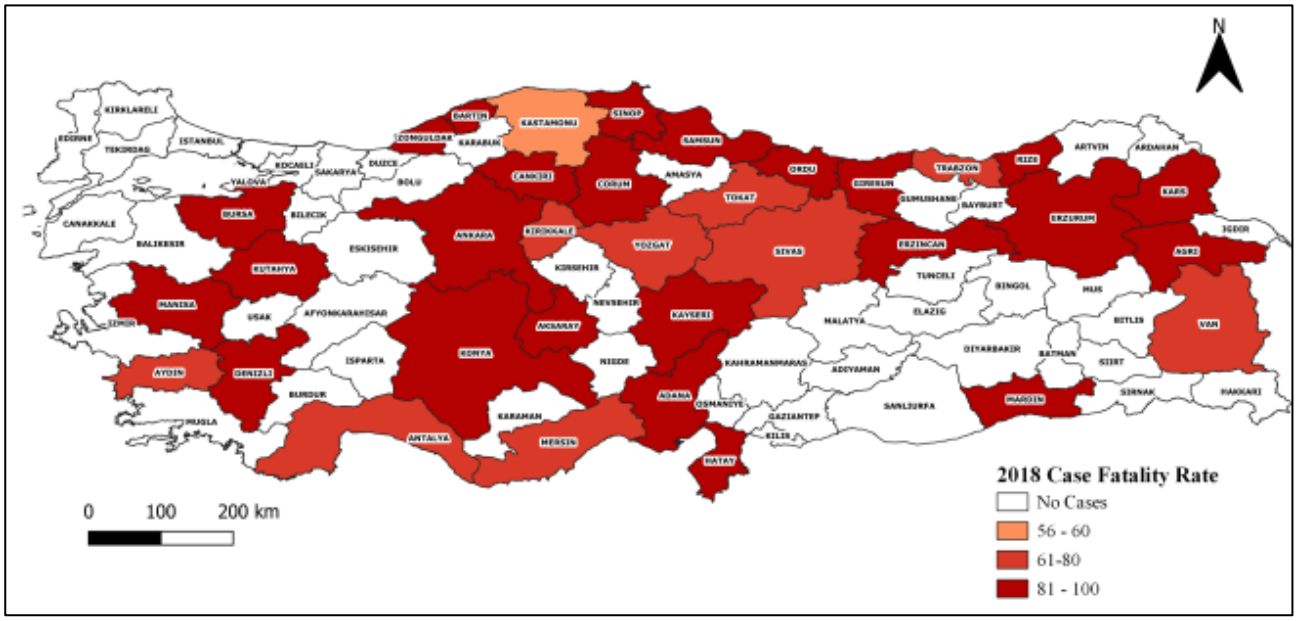

Figure 4. Map produced for Türkiye according to Newcastle case fatality rates 2018.

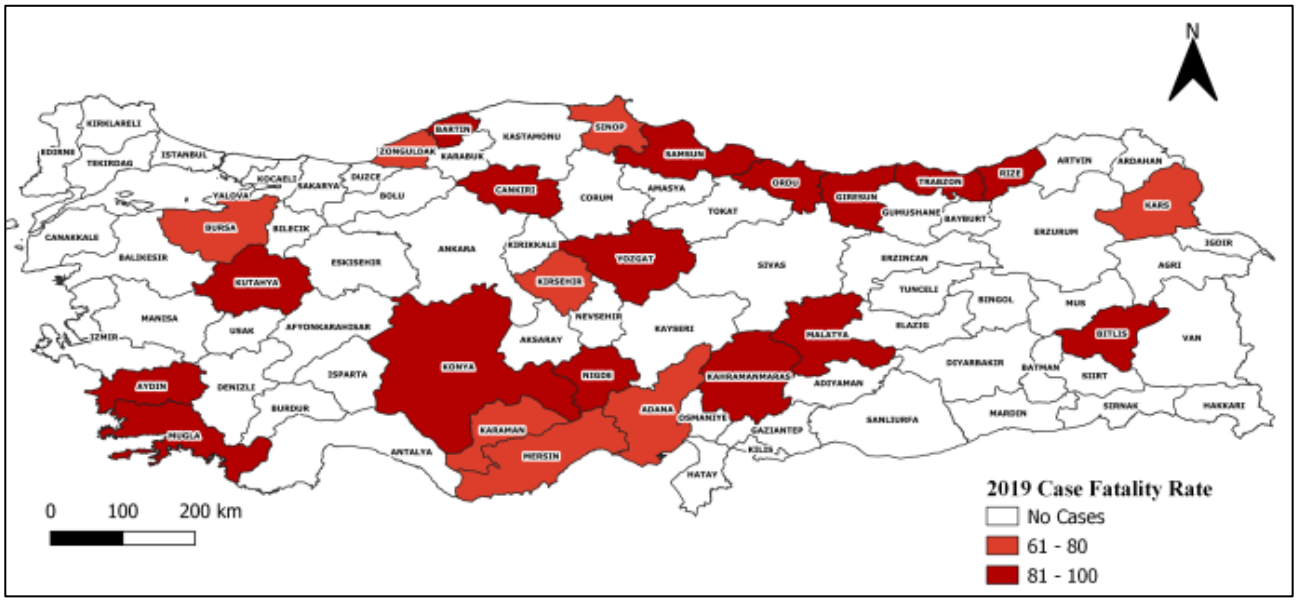

Figure 5. Map produced for Türkiye according to Newcastle case fatality rates 2019.

Discussion and Conclusion

Evaluation of the spatial and seasonal distribution of Newcastle disease is critical for continued surveillance of the disease, as it is located on the main transit route for migratory birds in Türkiye, Europe and Asia, and the simultaneous different seasonal characteristics. In this study, ND was evaluated according to spatial and seasonal.

When the disease is examined spatially, 36 outbreaks were seen in 8 provinces in 2017, 59 outbreaks in 11 provinces in 2018 and 16 outbreaks in 8 provinces in 2019 in the Black Sea region. When the Black Sea region was evaluated in detail, it was determined that the area where the outbreaks were intense was the province of Samsun. Similarly, in the study conducted in previous years on ND status in Türkiye, it was reported that the disease is common in Samsun province, which is located in the Black Sea Region (16). As a result, it has been determined that the Black Sea Region is the region with the highest number of outbreaks for all years. It is thought that giving priority to this area with the measures to be taken will contribute to the eradication of the disease.

When the number of deaths was evaluated, it was determined that the area that caused the most deaths in 2017 and 2018 was in the provinces of Samsun and Bartın in the Black Sea Region, but the area that caused the most

deaths in 2019 was in Yozgat, which is in the Central Anatolia region. When the number of provinces affected by the outbreak in Türkiye was examined, it was determined that there were 33 provinces in 2017, 34 provinces in 2018 and 24 provinces in 2019. In a study conducted in previous years, it was reported that the number of provinces affected by the outbreak was 48 in 2013 and 20 in 2014 (16). In this context, it cannot be said that the number of affected provinces has decreased consistently over the years and that active surveillance systems have yielded results.

Also in this study ND was evaluated seasonally. It has been reported that ND outbreaks occur throughout the year in Türkiye and the area causing the most deaths in all seasons was the Black Sea region. Similarly, ND is said to occur throughout the year in the rural poultry populations in most countries. However, it has been reported by many authors that it is important in the seasonal incidence and severity of the disease (8). A study in Thailand reported that ND cases occur throughout the year, but the incidence peaks at the end of the season between February and April (22). Similarly, at the end of April, the highest number of outbreaks was observed in May (36, %16.36) Türkiye.

Another study reported ND outbreaks in Mauritania throughout the year, particularly during the warm season starting in March (9). Similarly, ND outbreaks were reported to be high in our country, especially in March (24, 10.91%), April (29, 13.18%), and May (36%, 16.36%) with the onset of hot seasons.

In different studies, it has been reported that ND outbreaks are more common in winter (7) and hot and dry season (September-November) and hot humid season (January-March) (24). A review concluded that ND outbreaks are often associated with seasonal change, particularly at the onset of the rainy season, with cold and hot weather (19). In Türkiye, the most outbreaks were seen in the spring (89, %40.45) and the least in the autumn (30, %13.63). When the literature is reviewed, it can be said that the ND outbreaks is not associated with a specific season, but rather with climatic stress periods.

With this research, the geographical distribution of the disease was examined, it was determined that the epidemics were intense in the Black Sea region and the least number of epidemics was observed in the Marmara region and Southeastern Anatolia. In addition, with the maps created using geographic information systems, the areas where the disease is seen are shown on the map. It has been tried to show that besides standard methodological approaches, spatial mapping can be used to gather information about the places where the disease occurs and can be useful in identifying priority areas where precautions should be taken. As a result, the importance of a systematic approach has emerged before, during and after the eradication program. It has also been concluded that the improvement of prevention and control strategies for ND in endemic countries is necessary (14).

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

TB collected and organized ND outbreak data, drafted the manuscript and wrote the article. ISG participated in the design of the study, performed the statistical analysis, and has given final approval for the version to be published.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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Meat yield and chemical composition of freshwater crab (*Potamon persicum* Pretzmann, 1962)

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ABSTRACT

In this study, morphometric measurement, meat yield, moisture, pH, protein, fat, fatty acids and ash content were determined in a total of 102 (15 female and 87 male) freshwater crab (*Potamon persicum* Pretzmann,1962) caught from Aşağı and Yukarı Çay of Pertek, Tunceli. Meat yield in male and in female crabs were found to be as 12.75±0.38% and 10.93±0.32%, respectively. It has been observed that moisture and protein amounts were higher in female crabs than in male crabs. The amounts of fat were 0.96±0.31% in male crabs and 0.97±0.35% in female crabs. The amount of ash was 2.68±0.04% in male crabs and 2.66±0.03% in female crabs. It was determined that the content of monounsaturated fatty acids (female:male 33.56%:37.44%) in female and male crabs were higher than polyunsaturated (female:male 24.19%:21.62%) and saturated fatty acid (female:male 28.11%:32.85%) content. The highest fatty acid was found to be as omega-9, in terms of omega-3 (male crabs 8.54%, female crabs 14.85%), omega-6 (male crabs 10.04%, female crabs 5.46%) and omega-9 fatty acids (male crabs 23.65%, female crabs 19.14%) in freshwater crab (*Potamon persicum* Pretzmann, 1962) meat.

Introduction

Aquatic creatures are one of the primary sources of animal protein. Aquatic foods, such as fish and shellfish, are deemed remarkable as they contain nine essential amino acids and have sufficient levels of Omega-3 and polyunsaturated fatty acids. In terms of such foods, crabs are counted as one of the cheapest shellfish products (26). It is also considered a healthy diet due to its high-quality protein and low-fat content (24).

Marine species of crabs are usually consumed in many countries (China, France, Indonesia, Japan, Philippines, Spain, and Thailand). In the world, 22 crab species are used directly as food and feed additives (16). In addition, crabs bear medical and pharmacological significance in the production of chitin and chitosan.

While crabs can be fished in their natural habitat, crab cultivation is also widespread in various countries (Japan, Poland, Australia, Norway) (18). Various studies demonstrated that crab meat contains high protein, carbohydrate, and fiber, as well as low fat, and is a rich source of sodium, potassium, magnesium, calcium, and phosphorus (22, 24). Türkiye's inland waters host 12 crab species belonging to the *genus Potamon*. According to the morphology of the freshwater crabs gonopods living in Türkiye, those living in the Black Sea, Marmara and Aegean Regions are included in *Potamon ibericum tauricum* (Czerniavsky, 1884) subspecies, those living in the Mediterranean and South East Anatolia Regions are included in *Potamon potamios* (Oliver, 1804) subspecies, and those living in Lake Amik and its associated waters is included in the subspecies of *Potamon potamios setiger* (Bott, 1970) (17). The research subject, *Potamon persicum* Pretzmann, 1962, it is from the family of *Potamidae* and is commonly seen in Sivas, Kayseri,

Malatya, Elazığ, Tunceli, Diyarbakir, Hakkari, Siirt, Van and Erzurum (17).

Since the review of the relevant literature revealed the shortage of studies on *P. persicum*, a freshwater crab, the present study aimed to research some properties and nutritional value of the mentioned crab and contribute to its consumption rates. For this purpose, the study investigated protein, moisture, fat, and ash contents, fatty acid composition, and pH of *P. persicum* fished in Aşağı and Yukarı Çay zone of Pertek district in Tunceli city, as well as its meat yield.

Materials and Methods

Materials: The research subject crabs (15 females, 87 males), were purchased from the fishers in the region (coordinates of the region: 38°59'20.7"N 39°18'22.7"E) and immediately brought to the laboratory in August-October 2011. After separating according to sex, the crabs were weighed, and their morphometric measurements (carapace width (CW), carapace length (CL), pincer length, and pincer width) were taken with a caliper. Then, crab meat was extracted, and the meat yield and the chemical composition of meat (humidity, pH, protein, fat, fatty acids, and ash content) were determined.

Sample preparation: The crabs separated based on sex were given numbers, packed in polyethylene bags, and subjected to heat treatment for 5-6 min. in 95-100 ^{o}C water.

Proximate composition analysis: The meat was picked from the body, pincers, and legs of the crabs and weighed, and the results were indicated as %. Meat yield was determined according to the following formula.

Meat yield % = Meat weight (g) / Total weight (g) x 100

The moisture content of the samples was determined by TS 1743 ISO 1442 (33), pH-values were identified with pH meter (Metler Toledo, FE 20); and protein amounts were measured with the LECO FP 528 automatic nitrogen analyzer by the AOAC 955.04-1998 method (2). While TS 1744 method was used for the determination of fat in the samples (34), ash content was determined using TS 1746 ISO 936/2001 method (35).

Fatty Acid Analysis: Fatty acids were determined by the International Olive Council COI/T.20/Doc.no.28/2010 (19). For this purpose, a Flame Ionization Detector (FID) and Clarus 500 (Perkin Elmer, USA) gas chromatography device with autosampler containing DB-23 (50% -Cyanopropyl)-methylpolysiloxane (60 m x 0.25 mm x 0.25 µm) GC column were used. The sample was thoroughly mixed and homogenized, and approximately 60 mg of test sample was weighed into the test tube on a precision balance. 10 ml of n-heptane was added to the test tube and then 0.5 ml of methanolic KOH solution was added and the cap of the tube was closed. After shaking vigorously for 30 seconds and standing for one hour, the upper clear portion was removed. This part was put into 2 ml vials, made ready for injection and injected into the device. Mix standard was also injected into the device and the peaks were read. The content of methyl esters in the sample is expressed as a percent by mass, relative to the ratio of the area of the corresponding peak to the sum of all peak areas. The temperatures of the injector and FID detector were set to 220 °C and 280 °C, respectively. The furnace temperature was set to 200 °C, starting at 100 °C for the first 5 minutes, then by increasing the temperature by 5 °C per minute until 180 °C and by 2 °C per minute until 200 °C. 1µL was extracted from the samples and injection was carried out at a split ratio of 1:25 (19).

Statistical analysis: All analyzes (carapace length, carapace width, live weight, pincer length, pincer width, meat yield, moisture content, pH-value, protein ratio, total fat and crude ash) in the study were carried out in triplicate. Statistical analysis of data was carried out applying the basic statistic tests using MiniTab 19. The t-test (independent t test) was used to compare male and female crab meats. The results are presented as mean ± standard deviation.

Results

Meat yield in male and in female crabs were found to be as 12.75 ±0.38% g, 10.93 ±0.32% g respectively (Table 1). The amounts of fat were 0.96±0.31% in male crabs and 0.97±0.35% in female crabs (Table 1). The ash contents were found to be as 2.68±0.04% and 2.66±0.03% in male and female crabs, respectively (Table 1). While male crabs have higher meat yield values than females, the situation is the opposite in terms of moisture content of the meat. Statistical difference was observed for both ($P<0.05$). Differences in other parameters were found to be insignificant. It was determined that the content of monounsaturated fatty acids in female and male crabs (33.56%:37.44%) were higher than the content of polyunsaturated (24.19%:21.62%) and saturated fatty acid (28.11%: 32.85%) content (Table 2). The highest fatty acid was found to be as omega-9, in terms of omega-3 (8.54%, 14.85%), omega-6 (10.04%, 5.46%) and omega-9 fatty acids (23.65%, 19.14%) in male and female freshwater crab meat (*Potamon persicum* Pretzmann, 1962), respectively (Table 2). Chromatograms of fatty acids of male and female crab meat are given in Figure 1-2, respectively.

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Table 1. The mean morphometric values and chemical composition of crab meat (n: 102).

	Male	Female
Carapace length (cm)	3.65±0.73	3.67±0.60
Carapace width (cm)	4.68±1.62	4.70±0.90
Average live weight (g)	54.78±20.12	53.80±12.95
Pincer length (cm)	5.50±1.90	5.05±1.05
Pincer width (cm)	1.03±0.20	1.05±0.15
Meat yield (%)	12.75±0.38[a]	10.93±0.32[b]
Amount of moisture (%)	80.23±2.26[b]	81.22±1.12[a]
pH value	8.16±0.12	8.21±0.03
Protein ratio (%)	12.99±0.20	13.26±0.08
Total fat (%)	0.96±0.31	0.97±0.35
Crude ashes (%)	2.68±0.04	2.66±0.03

a,b: Indicates statistically significant difference between the groups (P<0.05).
The differences in results obtain from female and male crabs (t- test).

Table 2. The composition of fatty acids determined in crab meat.

Number	Composition of Fatty Acids	Fatty Acids of Male Crabs (%)	Fatty Acids of Female Crabs (%)
	Saturated Fatty Acids (ΣSFAs)	32.85	28.11
1	Lauric acid (C12:0)	1.18	0.66
2	Tridecanoic Acid (C13:0)	0.02	0.06
3	Myristic acid (C14:0)	2.30	1.73
4	Cis-10 Pentadecanoic acid (C15:0)	0.39	0.30
5	Palmitic acid (C16:0)	15.82	11.91
6	Heptadecanoic acid (Margaric) (C17:0)	0.37	0.36
7	Stearic acid (C18:0)	4.16	2.62
8	Arachidic acid (eicosanoic) (C20:0)	2.73	5.46
9	Behenic acid (C22:0)	0.12	0.11
10	Lignoceric acid (C24:0)	5.76	4.90
	Unsaturated Fatty Acids	59.06	57.75
	Monounsaturated Fatty Acids (ΣMUFAs)	37.44	33.56
11	Myristoleic acid (C14:1)	0.45	0.40
12	Pentadecanoic acid (C15:1)	0.68	2.29
13	Palmitoleic acid (C16:1)	10.51	9.30
14	Heptadecenoic acid (Margoleic acid) (C17:1)	0.95	0.90
15	Oleic acid (C18:1 n9)	23.65	19.14
16	Eicosenoic acid (Gadeloic) (C20:1)	0.80	1.08
17	Erucic acid (C22:1 n9)	0.40	0.45
	Polyunsaturated Fatty Acids (ΣPUFAs)	21.62	24.19
18	Linoleic acid (C18:2 n6)	10.04	5.46
19	Linolenic acid (18:3 n3)	8.54	14.85
20	11C,14C Eicosadienoic acid (C20:2)	0.57	0.78
21	8C,11C,14C Eicosatrienoic acid (C20:3 n6)	0.06	0.07
22	11C,14C,17C Eicosatrienoic acid (C20:3 6)	0.00	0.10
23	Arachidonic acid (C20:4 n6)	2.41	2.93
	Unidentified	8.09	14.14
	ratio of saturated fatty acids / ratio of unsaturated fatty acids	0.56	0.49
	Σω6	12.51	8.56
	Σω3	8.54	14.85
	ω3/ω6	0.68	1.73
	ω6/ω3	1.46	0.58

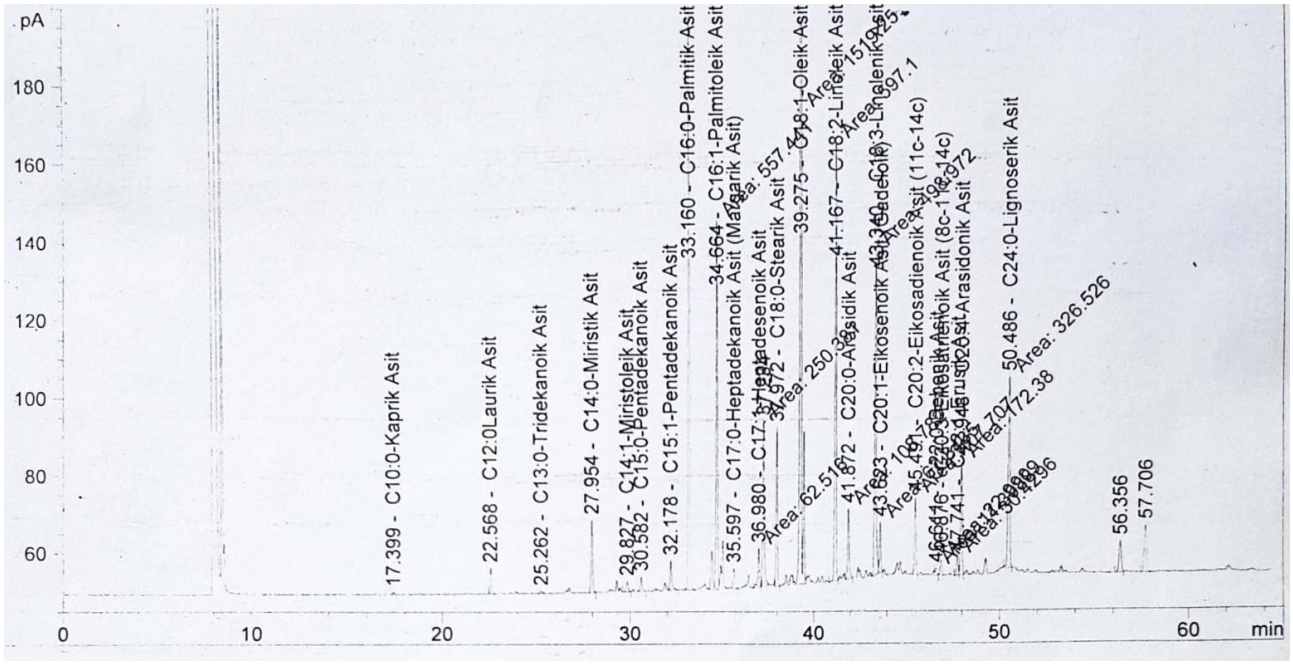

Figure 1. Fatty acid chromatogram of male crab meat.

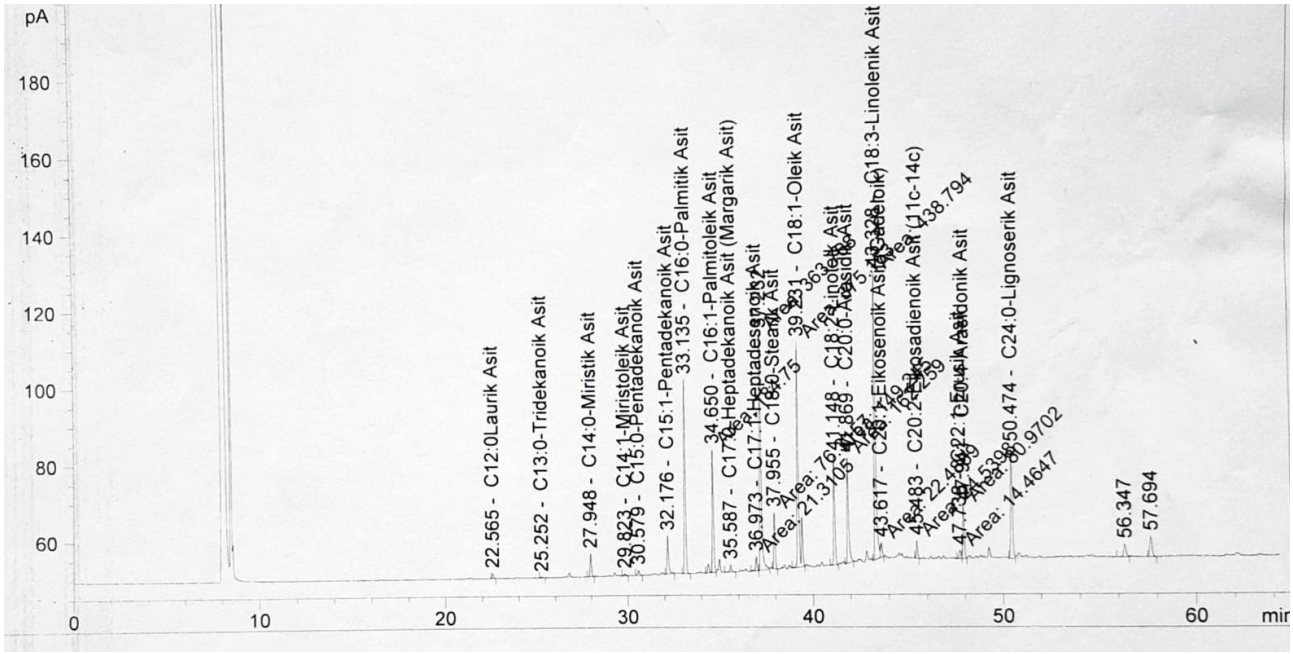

Figure 2. Fatty acid chromatogram of female crab meat.

Discussion and Conclusion

Since the relevant literature lacks studies on freshwater crab *P. persicum*, the acquired data were discussed considering the research on other crab species. In their study, Gökoğlu and Yerlikaya (15) measured the mean carapace width and length of *C. sapidus* individuals as 9.62 cm and 4.85 cm, and of *P. pelagicus* individuals as 13.25 cm and 6.15 cm, respectively. Therefore, it is thought that the variability with the results in our study is likely to be due to species differences. Türeli et al. (37) determined the total meat yield as 28.23% for female blue crabs and 41.99% for male blue crabs. The meat yield varies by species, sex, age, breeding season, feeding, and stomach content when fished (13). Eggs constitute 30-40% of a female crab's body weight at the time of spawning, which may reduce the meat yield. The low meat yield in the crabs investigated in the study can be attributed to their being in the spawning season (12). In a study by Ünlüsayın (38), meat proportions of *P. potamios* and *Ocypode cursor L.* were found to be 12.61±4.60% and

6.51±1.03%, respectively. Besides, Sachindra et al. (31) found the meat yield of the large sea crab *Charybdis cruciata* to be 29.7%.

In our study, the mean moisture content of crabs was found to be 81.22±1.12% for females and 80.23±2.26% for males (Table 1). Bilgin (6) found the highest moisture content of *Potamon potamios* (Olivier, 1804) in spring with a value of 81.03 ± 0.160%. In his study investigating how muscle tissue/water level would be affected by seasons, Ayas (3) recorded the lowest moisture content in spring and the highest value in autumn and uttered an inverse ratio between protein/lipid level and water. Ünlüsayın (38) determined the moisture content of *P. potamios* as 74.20% for females and 77.64% for males. Naczk et al. (28) also found 79.10%-82.30% moisture content in the European green crabs, and such values are relatively close to our findings. Gökoğlu (14) delivered an increase in the water amount in lean fish due to the depletion of nutrients and energy reserves during spawning. Hence, it was deemed quite normal to measure high moisture content in the study crabs since they were in the spawning period and about to change their shells in autumn when they were fished.

The mean pH-values of the study crabs was determined as 8.21±0.03 for females and 8.16±0.12 for males (Table 1). Dima et al. (9), determined the pH of crab (*Ovalipes trimaculatus*) pincer meat to be 7.3. On the other hand, Degnan et al. (8) the pH value in blue crab meat found as 8.1 (*Callinectes sapidus*). Ultimately, it is considered that such pH differences may be caused by the feeding patterns, habitats, and physiologies of the animals.

In our study, the protein amounts were found higher in females than males, and the mean values were 13.26 ± 0.08% and 12.99 ± 0.20%, respectively (Table 1). In their study on blue crabs fished in Iskenderun Bay, Türeli et al. (37) determined the mean protein amounts of breast and pincer meat as 15.51% and 16.81% for males, while they were 16.67% and 14.26% for females. However, such values are higher than our findings. Similarly, Ayas and Özoğul (3) and Kuley et al. (21), measured the mean protein amounts for female crabs as 22.45% (breast meat), 26.51% (pincer meat) and for male crabs as 21.40% (breast meat) and 30.31% (pincer meat), respectively. These values are also higher than the findings we obtained from *P. Persicum*. The variability in the findings is thought to be due to the species differences and the body parts analyzed in the studies.

Musaiger and Al-Rumaidh (27) determined the mean protein values (*P. pelagicus*) as 19.80% for females and 19.80% for males in raw meat. In Atlantic blue crabs (*Callinectes sapidus* Rathbun, 1896), Ağbaş (1) found the highest crude protein value in male pincer meat with 16.10% and the lowest in female breast meat with 12%. Skonberg and Perkins (32) found the mean protein value of green crabs to be 17.1%. Cherif et al. (7) found the protein value in pincer meat of *Carcinus mediterraneus* between 17.80-18.20%, while Gökoğlu and Yerlikaya (14) determined the mean protein values of *C. sapidus* and *P. pelagicus* as 15.00% and 21.54%, respectively. Moreover, the crude protein values of the crabs determined in the study of Moronkola et al. (25) are higher (19.2-28.3 g/100g) than those determined in this research.

Low protein levels in this study might have stemmed from many factors such as sex, season, species, size, differences in sexual maturation, fishing area, feeding characteristics, and carapace change time (3, 26). Crabs change carapace once a year thanks to the growth. They hold water in their muscles before the change, which increases the water ratio in muscles, leading to a reduction in the protein ratio (5). As a matter of fact, in his study, where the effects of sex and season on crab meat were investigated, Ayas (3) found the highest protein value in spring and the lowest value in autumn and associated the result with carapace change.

Pati et al. (30) found the protein content as 30-59%, the fat content as 7-11%, the ash content as 38-39% (dry weight), and moisture content as 71-79% in their study, in which they examined the effects of spawning period and season on female crab meat. When compared with the values in the study of Pati et al. (30), except for moisture content, the results of our study were determined to be higher depending on the season and spawning period.

Ayas and Özoğul (5) determined the fat amounts as 0.96% for female blue crabs and 1.11% for male blue crabs, and such findings are relatively close to the results obtained in our study. Ayas (3) found the lipid level of crabs higher in winter than in other seasons. He attributed this to the reproductive and spawning periods lasting in spring, summer and autumn and also argued that carapace change in autumn might influence the higher levels of lipids in crabs. In Atlantic blue crabs, Ağbaş (1) determined the highest fat content with 2.97% in male breast meat and the lowest with 1.01% in male pincer meat. Türeli et al. (36) stated that the fat rates were 1.16% for male blue crabs and 2.26% for female blue crabs, while they were 1.45% for male sand crabs and 1.16% for female sand crabs. In addition, Kuley et al. (21) determined the fat proportions as 1.62% for female crabs and 1.64% for male crabs. In contrast to our study, Ünlüsayın (38) reported higher fat proportions in crabs fished from Lake Eğirdir as 4.63% for males and 2.66% for females. These differences are thought to be due to many factors such as species, size, sex, feeding, habitat, spawning period, carapace change, and season (4).

On the other hand, the mean ash amounts were found to be 2.66 ± 0.03% for females and 2.68 ± 0.04% for males. In Atlantic blue crabs, Ağbaş (1) determined the highest ash content with 2.37% in female pincer meat and

the lowest with 1.79% in male pincer meat. These values are slightly lower than our findings. In blue crabs caught in Iskenderun Bay in winter, Türeli et al. (37) determined ash level as 3.28% in female breast meat. Ünlüsayın (38) determined ash amounts as 1.95% for females and 2.67% for males, while Kuley et al. (18) determined it to be lower than our findings as 1.16% for females and 1.10% for males.

Despite high numbers of crabs in seas and inland waters, crab meat consumption is not common due to cuisine traditions and lack of information. As a result of the relevant examinations, it was concluded that the species could be a good dietary food item, especially because it contains high-quality protein, has a balanced fatty acid profile, is a sufficient source of minerals, and contains low fat. Therefore, it is thought that increasing crab meat consumption at the national and international level will be of great importance.

It is well-known that essential fatty acids are involved in maintaining certain physiological functions in the human body, providing energy, and helping maintain body temperature (11). Nevertheless, since such fatty acids cannot be synthesized within the body, they must be taken ready-made with food. Our research revealed that crab meat might be necessary for a balanced diet since crab meat contains essential fatty acids.

Our study found that the amounts of monounsaturated fatty acids (37.44%) were higher than those of saturated fatty acids (32.85%) and polyunsaturated fatty acids (21.62%) in the crabs (Table 2). Palmitic acid (C16:0) had the highest ratio as saturated fatty acid in both female and male crabs we examined. As evident in Table 2, it was determined that male crabs contained more saturated fatty acids than females. In terms of monounsaturated fatty acids, oleic acid (omega-9) was found to have the highest amount. In contrast, linolenic acid (omega-3) and linoleic acid (omega-6) were the highest amounts in terms of polyunsaturated fatty acids. While the amounts of monounsaturated fatty acids were higher in male crabs, the amounts of polyunsaturated fatty acids were higher in female crabs (Table 2). Moruf and Lawal-Are (26) analyzed the fatty acid profiles of *Callinectes amnicola* and *Portunus validus* crabs, and, similar to our results, palmitic and oleic acids were the highest amounts as saturated fatty acid and monounsaturated fatty acid, respectively. Reporting that palmitic acid was the highest-amount saturated fatty acid in *Carcinus maneus* species, Naczk et al. (28) had obtained similar results with our research. Cherif et al. (7) revealed that palmitic and stearic acids, oleic acid and arachidonic acid had the highest-amount fats in *Carcinus mediterraneus* species. It is considered that the resulting differences may arise from the species difference. In their study with blue crabs and swimming crabs fished in Mersin Bay, Özoğul et al. (29) reported lower amounts of saturated fatty acids and monounsaturated fatty acids than our study but revealed higher polyunsaturated fatty acids than ours. Nevertheless, similar to our study, Keivandokht et al. (20) found higher amounts of palmitic acid from saturated fatty acids; oleic acid from monounsaturated fatty acids; and alpha-linoleic acid from polyunsaturated fatty acids.

Dvoretsky et al. (10), determined the amount of monounsatured, saturated and polyunsatured faty acids as 17.2%, 27.6% and 55.2% in Barents Sea red king crab meat (*Paralithodes camtschaticus*), respectively. The amount of monounsaturated faty acids and saturated faty acids found in Barents Sea red king crab meat by Dvoretsky et al. (10), is lower compared to our findings, while the amount of polyunsatured faty acids is higher. The amount of palmitic acid (C:16:0) detected in red king crab meat (16.2%) by Dvoretsky et al. (10), is close to the value we determined in male crab meat (15.82%). Lian et al. (23), determined the polyunsatured, monounsatured and saturated fatty acids in cooked leg meat of red king crab (*Paralithodes camtschaticus*) as 50.9%, 27.0% and 22.1%, respectively. The researchers determined the amount of palmitic acid (C16:0) as 15.4% and the amount of stearic acid (C18:0) as 4.1%. These findings are close to the values found in male crab meat in the current study (15.82% and 4.16%, respectively).

In our study, essential fatty acids were determined as palmitic, oleic, palmitoleic, linoleic, and linolenic acids. Compared with the other studies, different results in our study can be related to many factors such as species, environmental factors, feeding, and spawning period. Ghazali et al. (13), stated in their study that the fatty acid profile was near related to feeding and ovarian maturity phases. Besides, it was found out that there were no statistically significant differences between freshwater female and male crabs by the chemical composition of their meat ($P>0.05$). However, sex-based differences were suggested in terms of the fatty acid profile.

Overall, despite high numbers of crabs in seas and inland waters, crab meat consumption is not typical due to cuisine traditions and lack of information. It was concluded that crab meat could be a food item with high nutritional quality because this species contains high-quality protein, has a balanced fatty acid profile, is a sufficient source of minerals, and contains low fat. At the same time, benefiting from crabs abundant in the seas and inland waters will both contribute to the country's economy and create new employment opportunities with the establishment of crab meat processing factories. Finally, it is thought that the findings obtained in this study will contribute to the relevant literature on the morphological and chemical composition of *P. persicum*.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

AA, SK, HY and PK conceived and planned the experiments. SK, AA and HY carried out the experiments. SK, AA, HY and PK planned and carried out the simulations. SK, AA, HY and PK contributed to sample preparation. AA, HY, SK and PK contributed to the interpretation of the results. SK took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study does not present any ethical concerns.

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A field study on the profile of veterinary students in Türkiye: example of Ankara University Faculty of Veterinary Medicine

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ABSTRACT

This research was carried out to determine the general profile of the students enrolled in Turkish and English programs of Ankara University, Faculty of Veterinary Medicine (AUFVM). Determination of the demographic characteristics, socioeconomic statuses, pre-university education levels, foreign language levels of students are aimed. Additionally, reasons for choosing the veterinary profession, career expectations, views on post-graduate education, leisure time preferences, and participation in sportive and artistic activities are evaluated. A survey consisting of questions prepared for the purpose of the study was conducted with 545 students studying in the 1st, 2nd, 3rd, 4th, and 5th grades enrolled in Ankara University Faculty of Veterinary Medicine, 2019-2020 Spring Semester of Turkish and English programs. After the data analysis, it has been determined that the students are generally of urban origin and come from families with low income of the Central Anatolian Region. Accordingly, more than half of the students chose the veterinary faculty program as their first choice. They are satisfied with being a veterinary faculty student and a candidate for the profession. In addition, it has been highlighted that more than half of the students want to continue their post-graduate education, "sometimes" have the chance to participate in sportive and cultural activities, and have a low rate of reading books. As a result, it is thought that presenting a general overview of the student profile will be beneficial for both university and faculty administration and academicians in order to provide the opportunity to know the students better.

Introduction

The profile is the whole extent of distinguishing features for a person or an object, an attitude or tendency (36), and means examining the individual or object by considering all internal and external factors (25). Profile research describes the current situation of the target audience in terms of various variables (10, 39). Profile researches about individuals in all fields of education provide essential data about cultural contexts, socio-demographic factors and individual characteristics, etc. (10). Studies to define the student profile include the determination and statistical expression of the common characteristics of individuals in different fields of education that everyone can observe (28, 39). Studies conducted to determine student profiles are very essential in specifying the socio-economic roots of students, their views on academic and social life in the university/faculty, and their future expectations (30). It has been determined that these studies have played an essential role in increasing the quality of education by strengthening the communication between the student-lecturers, and university administration, as they also serve as a feedback tool by ensuring better recognition of the student population (30, 41). It is very essential to know the profiles of the students in making decisions that are directive and open to improvement (6). Educational institutions, which are in constant development, frequently collect student data and make forward-looking plans and programs. Many organizations are working for this purpose in developed countries. For example, *Observatoire Nationale de la Vie Etudiante* (OVE) in France, *Deutsches Studentenwerk* (DSW) in Germany, and *Fondazione Della Residentia Universitaria Italiana* (RUI) in Italy are organizations working for this objective (8, 28). There are student statistics available in

Türkiye since 2016 published by the *Council of Higher Education* (YÖK) (42). However, these statistics cover only higher education input indicators. They do not provide detailed information about student profiles. After a literature review, it was seen that there are many studies about student profiles both in Türkiye and abroad (6, 21, 25). Some studies are for all departments or several faculties of the universities, and some are for all classes of a faculty or only one class (2, 10, 17). Although profile studies have been conducted in different fields and scopes, they serve the same purpose. In Türkiye, students are placed in veterinary medicine programs via the *Transition to Higher Education Examination* (YKS) conducted by *Student Selection and Placement Center* (ÖSYM). Students who choose the faculty of veterinary medicine programs come from various social-cultural and economic strata. A limited number of profile studies are conducted in Türkiye for veterinarians and veterinary candidates (21, 26, 27, 35). This study aimed was to determine the profiles, demographics, family structures, socio-economic levels, reasons for choosing the veterinary profession, pre-university education statutes, post-graduation career plans, satisfaction with the veterinary profession, and attitudes about participation in sportive and artistic activities of students of Ankara University, Faculty of Veterinary Medicine (AUFVM). The study aims to contribute to the archives of Ankara University Faculty of Veterinary Medicine and the veterinary profession in general.

Materials and Methods

***Study Design*:** AUFVM provides education in two separate programs as Turkish and English. The research universe consists of all students enrolled in the AUFVM, 2019-2020 Spring Semester, Turkish and English programs for 1st, 2nd, 3rd, 4th and 5th grades. The sample size of the study was determined by the random sampling method. No sample selection was made in the study, and all students who agreed to participate in the study were included in the sample group. A web-based survey implementation that is proven effective over traditional survey methods was used in data collection (12, 24, 44). Some of the questions in the studies of Küçükaslan and İlhami (21) regarding veterinary medicine were used while preparing the data collection tool. The questionnaire form prepared using the "Google Forms" application was delivered to volunteers by sharing the link. A total of 575 students were surveyed. The questionnaire form used in the study consists of three parts. The first part includes 23 questions about age, gender, family structure of parents, education and professional status of parents, the total income of the family, number of siblings, employment statuses, etc. In the second part, 11 questions about reasons for choosing university, post-graduation goals, professional satisfaction levels, etc. were included. The last part consisted of six questions about students' socio-cultural and sportive characteristics etc.

Statistical analysis: The research is a survey (descriptive survey) model. The survey model is based on reflecting the current situation as it is. Descriptive statistics related to the obtained data were calculated and shown using frequency (n) and percentage (%) slices. In the statistical evaluation of the relationship between categorical variables, Pearson Chi-square and Fisher-Freeman-Holton analyzes were used. $P<0.05$ criterion was used in all statistical evaluations. SPSS 21 package program was used for statistical analysis.

Results

Findings, including the demographic characteristics of the students of all classes of the 2019-2020 academic year in AUFVM are shown in Table 1. Data about the settlement where most of the pre-higher education life passed with the participants' families are given in Table 2. A significant difference was found between the programs in terms of the place of residence in the family. It has been determined that this difference arising from the people living in the village (8.6%) favours of the Turkish program.

Findings of the education levels and professions of the parents of the students are given in Table 3. It is seen from the findings that the education levels of the fathers are significantly higher than that of the mothers. Findings related to the socio-economic statutes of the students are given in Table 4. Students' high school information and language levels are given in Table 5. The rate of English speaking has been determined higher in favour of students enrolled in the English program. The answers regarding the veterinary faculty program preferences and how students have preliminary information about the veterinary profession are given in Table 6 and 7, respectively. Findings regarding whether the students would like to choose the veterinary medicine program again are shown in Table 8, and findings regarding the reason for preferring the veterinary medicine program are shown in Table 9.

The findings of the students' interests and enthusiasm for the veterinary profession are given in Table 10, and findings of which field they want to work in after graduation are given in Table 11. Findings, including the students' views on postgraduate education are given in Table 12. According to Table 12, a statistically significant difference was found between the programs. The findings of the students regarding socio-cultural activities and leisure time preferences in Table 13, and the findings of the preferences of reading professional and non-professional books are given in Table 14.

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Table 1. Findings of the personal information of participants.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
Gender	Male	223 (42.6)	23 (45.1)	246 (42.8)	0.212	0.899
	Female	300 (57.3)	28 (54.9)	328 (57.0)		
	Other	1 (0.2)	0 (0.0)	1 (0.2)		
Grade	1	120 (22.9)	16 (31.4)	136 (23.7)	8.439	0.077
	2	93 (17.7)	12 (23.5)	105 (18.3)		
	3	78 (14.9)	11 (21.6)	89 (15.5)		
	4	165 (31.5)	9 (17.6)	174 (30.3)		
	5	68 (13.0)	3 (5.9)	71 (12.3)		
Place where you live with your family	City center	341 (65.3)[a]	35 (68.6)[a]	376 (65.6)	6.006	0.047*
	District**	136 (26.1)[a]	16 (31.4)[a]	152 (26.5)		
	Village	45 (8.6)[a]	0 (0.0)[b]	45 (7.9)		

*indicates statistically significant difference between groups (P<0.05), [a,b]: Different subscript letters indicate statistically significant difference between Turkish and English programs at the 0.05 level.
**non-centrals.

Table 2. Findings of the region where most of the pre-higher education life was spent with the families.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
Region where you live with your family	Mediterranean	73 (13.9)	5 (9.8)	79 (13.6)	8.926	0.207
	Eastern Anatolia	21 (4.0)	0 (0.0)	21 (3.7)		
	Aegean	89 (17.0)	9 (17.6)	98 (17.0)		
	Southeastern Anatolia	14 (2.7)	0 (0.0)	14 (2.4)		
	Central Anatolia	219 (41.8)	24 (47.1)	243 (42.3)		
	Black Sea	62 (11.8)	4 (7.8)	66 (11.5)		
	Marmara	38 (7.3)	9 (17.6)	47 (8.2)		
	Abroad	8 (1.5)	0 (0.0)	8 (1.4)		

Table 3. Findings related to the education levels and professions of the parents.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
Education status of your mother	Illiterate	20 (3.8)	0 (0.0)	20 (3.5)	7.343	0.244
	Primary school	133 (25.4)	8 (15.7)	141 (24.5)		
	Secondary school	46 (8.8)	3 (5.9)	49 (8.5)		
	High school and equivalent	140 (26.7)	15 (29.4)	155 (27.0)		
	University	159 (30.3)	21 (41.2)	180 (31.3)		
	Master's degree	22 (4.2)	3 (5.9)	25 (4.3)		
	Ph.D.	4 (0.8)	1 (2.0)	5 (0.9)		
Education status of your father	Illiterate	1 (0.2)	0 (0.0)	1 (0.2)	11.358	0.070
	Primary school	79 (15.1)	3 (5.9)	82 (14.3)		
	Secondary school	59 (11.3)	3 (5.9)	62 (10.8)		
	High school and equivalent	122 (23.3)	11 (21.6)	133 (23.1)		
	University	222 (42.4)	27 (52.9)	249 (43.3)		
	Master's degree	31 (5.9)	3 (5.9)	34 (5.9)		
	Ph.D.	10 (1.9)	4 (7.8)	14 (2.4)		

Table 4. Findings regarding the socio-economic statuses of participants.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
House of your family	Owned	407 (77.7)	41 (80.4)	448 (77.9)	0.200	0.655
	Rent	117 (22.3)	10 (19.6)	127 (22.1)		
Number of siblings	1	259 (49.4)	29 (56.9)	288 (50.1)	3.807	0.577
	2	117 (22.3)	10 (19.6)	127 (22.1)		
	3	47 (9.0)	6 (11.8)	53 (9.2)		
	4	20 (3.8)	1 (2.0)	21 (3.7)		
	5 and above	23 (4.4)	0 (0.0)	23 (4.0)		
	None	58 (11.1)	5 (9.8)	63 (11.0)		
Who works in the family	Mother	36 (6.9)	7 (13.7)	43 (7.5)	7.327	0.120
	Father	229 (43.7)	19 (37.3)	248 (43.1)		
	Both	169 (32.3)	20 (39.2)	189 (32.9)		
	None	67 (12.8)	2 (3.9)	69 (12.0)		
	Siblings	23 (4.4)	3 (5.9)	26 (4.5)		
Family's total monthly income (TL)	Less than 2500	86 (16.5)	2 (3.9)	88 (15.4)	8.302	0.140
	2501-3500	81 (15.5)	5 (9.8)	86 (15.0)		
	3501-4500	62 11.9)	8 (15.7)	70 (12.2)		
	4501-5500	77 (14.8)	10 (19.6)	87 (15.2)		
	5501-6500	76 (14.6)	8 (15.7)	84 (14.7)		
	6501 and above	140 (26.8)	18 (35.3)	158 (27.6)		
Your monthly income (TL)	Less than 550	194 (37.4)	17 (33.3)	211 (37.0)	0.970	0.914
	551-1000	192 (37.0)	18 (35.3)	210 (36.8)		
	1001-1500	79 (15.2)	10 (19.6)	89 (15.6)		
	1501-2000	24 (4.6)	3 (5.9)	27 (4.7)		
	2001 and above	30 (5.8)	3 (5.9)	33 (5.8)		
Place you live during your education period (In student life)	Homestay	161 (30.7)	22 (43.1)	183 (31.8)	4.470	0.603
	Relatives	8 (1.5)	1 (2.0)	9 (1.6)		
	Government dorm	114 (21.8)	10 (19.6)	124 (21.6)		
	Rent	152 (29.0)	12 (23.5)	164 (28.5)		
	Private dormitory	75 (14.3)	5 (9.8)	80 (13.9)		
	Hotel/hostel	1 (0.2)	0 (0.0)	1 (0.2)		
	Other	13 (2.5)	1 (2.0)	14 (2.4)		
Do you have an insured or uninsured job to have additional income?	Yes	97 (18.5)	9 (17.6)	106 (18.4)	0.023	0.879
	No	427 (81.5)	42 (82.4)	469 (81.6)		
Do you get a scholarship?	Yes	145 (27.8)	13 (25.5)	158 (27.6)	0.122	0.727
	No	377(72.2)	38 (74.5)	415 (72.4)		

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Table 5. Students' high school information and language levels.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
Type of high school you graduated from	Science High School	74 (14.1)	10 (19.6)	84 (14.6)	7.766	0.514
	Anatolian High School	294 (56.1)	25 (49.0)	319 (55.5)		
	Social Sciences High School	2 (0.4)	0 (0.0)	2 (0.3)		
	Veterinary Health Vocational High School	1 (0.2)	0 (0.0)	1 (0.2)		
	Anatolian Imam Hatip High School	8 (1.5)	0 (0.0)	8 (1.4)		
	Vocational and Technical Anatolian High School	4 (0.8)	0 (0.0)	4 (0.7)		
	Private Science High School	14 (2.7)	4 (7.8)	18 (3.1)		
	Private Basic High School	55 (10.5)	6 (11.8)	61 (10.6)		
	Private High School Teaching in a Foreign Language/Private Anatolian High School	13 (2.5)	2 (3.9)	15 (2.6)		
	Other	59 (11.3)	4 (7.8)	63 (11.0)		
Level of foreign language	I don't know	15^{a} (2.9)	0^{a} (0.0)	15 (2.6)	69.133	0.001*
	Very little	160^{a} (30.5)	1^{b} (2.0)	161 (28.0)		
	At a level to sustain daily conversations	289^{a} (55.2)	19^{b} (37.)	308 (53.6)		
	Very good	60^{a} (11.5)	31^{b} (60.8)	91 (15.8)		

* indicates statistically significant difference between groups (P<0.05), ^{a,b}: Different subscript letters indicate statistically significant difference between Turkish and English programs at the 0.05 level.

Table 6. Findings regarding the veterinary faculty program preferences of the participants.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
How many times did you take the entrance exam to be placed in the veterinary faculty program?	First	347^{a} (66.3)	45^{b} (90.0)	392 (68.4)	12.471	0.005*
	Second	159^{a} (30.4)	5^{b} (10.0)	164 (28.6)		
	Third	15^{a} (2.9)	0^{a} (0.0)	15 (2.6)		
	Fourth and above	2^{a} (0.4)	0^{a} (0.0)	2 (0.3)		
What is the rank of your preference of veterinary faculty program	1-5	415 (79.3)	37 (74.0)	452 (78.9)	4.348	0.361
	6-10	49 (9.4)	5 (10.0)	54 (9.4)		
	11-15	30 (5.7)	2 (4.0)	32 (5.6)		
	16-20	6 (1.1)	2 (4.0)	8 (1.4)		
	21 and above	23 (4.4)	4 (8.0)	27 (4.7)		

* indicates statistically significant difference between groups (P<0.05), ^{a,b}: Different subscript letters indicate statistically significant difference between Turkish and English programs at the 0.05 level.

Table 7. Findings on how the participants have prior knowledge of the veterinary profession.

		Program of Veterinary Faculty			Chi Square	P
		Turkish n (%)	English n (%)	Total n (%)		
Did you have enough prior knowledge when choosing the veterinary profession?	Yes	304 (58.0)	28 (54.9)	332 (57.7)	2.193	0.334
	No	155 (29.6)	13 (25.5)	168 (29.2)		
	Indecisive	65 (12.4)	10 (19.6)	75 (13.0)		
If your answer to the previous question is yes, how did you get enough prior knowledge when choosing the veterinary profession?	Family	30 (9.9)	5 (17.9)	35 (10.5)	3.619	0.460
	Teachers	13 (4.3)	1 (3.6)	14 (4.2)		
	Entourage	95 (31.3)	6 (21.4)	101 (30.4)		
	Social media	75 (24.7)	5 (17.9)	80 (24.1)		
	Other	91 (29.9)	11 (39.3)	102 (30.7)		

Table 8. Findings regarding whether the participants would like to re-prefer the veterinary medicine program.

		Program of Veterinary Faculty			Chi Square	P
		Turkish n (%)	English n (%)	Total n (%)		
Would you consider changing the program by taking the university entrance exam again?	Yes	51 (9.7)	5 (9.8)	56 (9.7)	0163	0.922
	No	372 (71.0)	35 (68.6)	407 (70.8)		
	Indecisive	101 (19.3)	11 (21.6)	112 (19.5)		

Table 9. Findings on the reason for the participants to choose the veterinary medicine program.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
Why did you choose the veterinary faculty program? (Please tick the only option that is a priority for you)	My love for animals	133 (25.4)	19 (37.3)	152 (26.4)	9.760	0.378
	Job opportunities	85 (16.2)	4 (7.8)	89 (15.5)		
	Being a profession suitable for my abilities	107 (20.4)	12 (23.5)	119 (20.7)		
	Being a profession that offers good economic conditions	50 (9.5)	4 (7.8)	54 (9.4)		
	At the request of my family	9 (1.7)	2 (3.9)	11 (1.9)		
	Having a family (mother, father, relative, etc.) profession	5 (1.0)	1 (2.0)	6 (1.0)		
	The influence of the environment - friend, teacher etc.	25 (4.8)	1 (2.0)	26 (4.5)		
	It is a profession that provides social dignity	3 (0.6)	0 (0.0)	3 (0.5)		
	I didn't have a better choice	73 (13.9)	4 (7.8)	77 (13.4)		
	Wrong preference order	4 (0.8)	0 (0.0)	4 (0.7)		
	Other	30 (5.7)	4 (7.8)	34 (5.9)		

Table 10. Findings regarding the interest and enthusiasm of the participants for the veterinary profession.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
What is your level of satisfaction with being a veterinary faculty student and candidate of profession? (Please tick the only option that is a priority for you)	I'm getting more and more interested and excited	264 (50.4)	22 (43.1)	286 (49.7)	3.628	0.429
	Nothing changed	94 (17.9)	12 (23.5)	106 (18.4)		
	It has decreased since I started faculty	128 (24.4)	16 (31.4)	144 (25.0)		
	I am not sure about continuing the program	18 (3.4)	0 (0.0)	18 (3.1)		
	Other	20 (3.8)	1 (2.0)	21 (3.7)		

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Table 11. Findings regarding in which field the participants would like to work after graduation.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
What field do you want to work in after graduation?	I want to be an academician	85[a] (16.2)	8[a] (15.7)	93 (16.2)	21.634	0.006*
	I want to be a clinician	155[a] (29.6)	7[b] (13.7)	162 (28.2)		
	I want to work at Ministry of Agriculture and Forestry	50[a] (9.5)	1[a] (2.0)	51 (8.9)		
	I want to work in food industry	22[a] (4.2)	2[a] (3.9)	24 (4.2)		
	I want to work in pharmaceutical industry	12[a] (2.3)	0[a] (0.0)	12 (2.1)		
	I will consider the opportunities to work abroad	74[a] (14.1)	15[b] (29.4)	89 (15.5)		
	I will not work as a veterinarian	8[a] (1.5)	0[a] (0.0)	8 (1.4)		
	I haven't decided yet	89[a] (17.0)	16[b] (31.4)	105 (18.3)		
	Other	29[a] (5.5)	2[a] (3.9)	31 (5.4)		

*indicates statistically significant difference between groups (P<0.05), [a,b]: Different subscript letters indicate statistically significant difference between Turkish and English programs at the 0.05 level.

Table 12. Findings regarding the students' views on postgraduate education.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish n (%)	English n (%)	Total n (%)		
Do you want to have postgraduate education (Master's/Ph.D.)?	Yes	322[a] (61.5)	29[a] (56.9)	351 (61.0)	7.768	0.021*
	No	74[a] (14.2)	2[b] (3.9)	76 (13.2)		
	Indecisive	128[a] (24.4)	20[b] (39.2)	148 (25.7)		

*indicates statistically significant difference between groups (P<0.05), [a,b]: Different subscript letters indicate statistically significant difference between Turkish and English programs at the 0.05 level.

Table 13. Findings regarding students' socio-cultural activities and leisure time preferences.

		Program of Veterinary Faculty			Chi-Square	P
		Turkish	English	Total		
		n (%)	n (%)	n (%)		
How often do you participate in socio-cultural activities	Never	36 (6.9)	2 (3.9)	38 (6.6)	1.487	0.685
	Rarely	188 (35.9)	19 (37.3)	207 (36.1)		
	Sometimes	240 (45.9)	22 (43.1)	262 (45.6)		
	Very	59 (11.3)	8 (15.7)	67 (11.7)		
What type of cultural events you attend the most?	Concert	93[a] (18.1)	10[a] (20.0)	103 (18.3)	10.471	0.333*
	Cinema	204[a] (39.8)	22[a] (44.0)	226 (40.1)		
	Theater	82[a] (16.0)	4[a] (8.0)	86 (15.3)		
	Exhibition	5[a] (1.0)	3[b] (6.0)	8 (1.4)		
	Other	129[a] (25.1)	11[a] (22.0)	140 (24.9)		
Please tick the most appropriate option regarding your participation in sports activities.	I don't do any sports	93 (17.8)	6 (11.8)	99 (17.2)	6.036	0.110
	I only watch sports	49 (9.4)	3 (5.9)	52 (9.1)		
	I do sports whenever I have the opportunity	302 (57.7)	28 (54.9)	330 (57.5)		
	I do sports regularly	79 (15.1)	14 (27.5)	93 (16.2)		
Please tick the most appropriate option for your leisure time preferences.	Reading books	57 (10.9)	7 (13.7)	64 (11.1)	4.933	0.424
	Listening to music	48 (9.2)	4 (7.8)	52 (9.1)		
	Spending time with my friends	227 (43.4)	22 (43.1)	249 (43.4)		
	Going to the mall	6 (1.1)	1 (2.0)	7 (1.2)		
	I don't have free time due to the intensity of the courses	141 (27.0)	9 (17.6)	150 (26.1)		
	Other	44 (8.4)	8 (15.7)	52 (9.1)		

* indicates statistically significant difference between groups (P<0.05), [a,b]: Different subscript letters indicate statistically significant difference between Turkish and English programs at the 0.05 level.

Table 14. Findings about the preferences of reading professional and non-professional books.

		Program of Veterinary Faculty				
		Turkish n (%)	English n (%)	Total n (%)	Chi-Square	P
How many professional books have you read in the last year? (except lecture notes)	None	166 (31.7)	13 (25.5)	179 (31.2)	3.126	0.373
	1-3	256 (48.9)	28 (54.9)	284 (49.5)		
	4-5	55 (10.5)	3 (5.9)	58 (10.1)		
	6 and above	46 (8.8)	7 (13.7)	53 (9.2)		
How many non-professional books have you read in the last year?	None	43 (8.2)	5 (9.8)	48 (8.4)	4.499	0.212
	1-3	128 (24.5)	18 (35.3)	146 (25.5)		
	4-5	99 (19.0)	5 (9.8)	104 (18.2)		
	6 and above	252 (48.3)	23 (45.1)	275 (48.0)		

Discussion and Conclusion

Looking at the gender distribution of students studying at AUFVM, it was found that the rate of females in both Turkish and English programs is higher than males (Table 1). It is thought that the number of female students being higher shows that the old perception of the veterinary profession being male-dominated is broken in Türkiye, relatedly in Europe, and the United States of America (19). In Canada and the United States, women make up about 80% of the student population today, and the momentum that started in the 1950s reached a high level in the 1970s and has continued to increase until this time (18, 29). As an example of Türkiye, AUFVM's findings support the idea that veterinary medicine is no longer a male-dominated profession. When the findings were examined in terms of where the students lived with their families, it was found that the highest rate (42.3%) lived in the Central Anatolia Region (Table 2). As Ankara is close to the Aegean (17.0%), Mediterranean (13.6%), and Black Sea regions (11.5%) due to its geographical location, it is determined that the students mostly come to the veterinary faculty from these regions. After a literature search on the factors affecting the university selection of students, it was found that many criteria affect university preferences, and students mostly prefer universities that were close to where their families live. Relevant literature is found to be supportive of our study's findings (7, 11, 14, 32). When the educational status of the parents was examined, it was found that mothers (31.3%) and fathers (43.3%) were university graduates in high ratios (Table 3). The ratio of university graduates rose from 5.5% to 13.9 % since 2008 according to the TSI (*Turkish Statistical Institute*) National Education Statistics (37). This result was consistent with the findings that the parents of AUFVM students are university graduates (Table 3). The findings on mother's literacy levels being lower than father's is found to be consistent with the general literacy levels of Türkiye. In general, literacy levels are lower among women, in rural areas, and in the eastern Türkiye (3, 13). Besides, all the variables have much more effects on women than men (3, 13). Examining Table 4, which includes information on the socio-economic status of the students, it was found that 50.1% of the students were single siblings. In Türkiye, the average number of children for a family is 2.6. Families who have higher education levels prefer a smaller number of children, with an average of 1.2 in university graduate families (9). The family structures of AUFVM students are found to be compatible with Turkish families in general. The highest rate to the question of who is working in the family was 43.1%, with the father's answer. This is consistent with the tradition in Türkiye that fathers mostly are responsible for providing the living expenses, and sustenance of the family is associated with men (22). When Table 4, which shows the monthly income levels of the families was analyzed, it was determined that 15.4% of the families were below 2500 TL and 27.6% of them were over 6500 TL. According to the results of the TÜRK-İŞ Research (*Confederation of Turkish Trade Unions*) for May 2020, the poverty line for a family of four is 7942.17 TL, and the hunger limit is 2438.24 TL (38). It has been concluded that families of all students of AUFVM are at the poverty line and 30.4% are at the hunger limit. These results are compatible with the literature (9, 30) and Küçükaslan and Bulut's (14) study on socio-demographic levels of university students. The data that 37.0% of the students have less than 550 TL monthly income, 81.6% do not work and only 27.6% have scholarship mean that students do not have sufficient financial resources. Although their monthly income was quite low in Türkiye's conditions, 72.4 % of the students answering negatively to the scholarship question shows that university students in Türkiye have a limited number of scholarship opportunities (9). The finding that 55.5% of the students are graduates of the Anatolian High Schools (Table 5) is found to be compatible with the common presence of these schools in general (35% of all high schools in Türkiye, according to the Ministry of National Education 2018 and 2020 university preference guides)

(22, 23). When Table 5, the results of the students' knowledge of English were examined, it was found that more than half of the students enrolled in the English program have very good levels of English. Thus, it is concluded that they made the right choices and can understand the courses without being limited by the language barrier. Students enrolled in the Turkish program can be suggested to improve their English levels. The finding that more than half of the students (68.4%) of the veterinary faculty have entered the faculty at the first try and their order of preference was 1-5 for 78.9% of the students (Table 6) shows that they were aware and informed of their preferences and the profession. The finding that 57.7 % of the students had prior knowledge and 30.4% received the preliminary information from their entourage shows a conscious choice (Table 7).

Considering the participants' opinions on whether they would like to change their program (Table 8), it is seen that most of the students (70 %) are satisfied with being AUFVM students. Looking at why they preferred the veterinary profession, they stated that their love for animals (26.4%) was decisive, and it was a profession suitable for their abilities (20.7%) (Table 9). These results show that students made conscious choices that will make them happy throughout their professional life. The fact that students prioritise their abilities (20.7%) instead of job opportunities (9.4%) leads us to think that they consider the financial gains as secondary. Career choice is significant in terms of directing the future of individuals. This appears to be a positive attitude in professional life, as prioritizing financial concerns in career choices may overshadow the feeling of job satisfaction (4). The individual will be successful, productive, and happy in the field chosen as a profession in line with their talents, interests, and desires. For this reason, when choosing a profession, one should pay attention to the compatibility between his/her characteristics and the qualifications of the profession (31, 40). Results that 13.4% of the students did not have a better chance, and 0.7% of them preferred the faculty due to the wrong choice indicate that there may be unwanted outcomes and a decrease in job satisfaction in the long term if the program selection is not made carefully (Table 9). The fact that students answered "I am getting more and more interested and excited" (49.7 %) to the question "what is your satisfaction level with being a veterinary faculty student and a candidate of the profession?" highlights that they are not disappointed with their choices and have a positive impression for the faculty (Table 10). The results that 28.2% of the students want to work in clinical veterinary medicine (Table 11) leads us to think that they want to practice their profession freely in their workplaces. This finding seems to be compatible with the research of Küçükaslan and Bulut (21). More than half of the students want to have postgraduate education (Table 12) leading us to think that students give importance to post graduation education. Continuous increase in the ratio of postgraduate students, in general, seems to be coherent with these findings (16). Utilization of leisure time has been defined as participation in certain free time activities (33). Examples of these active and organized activities include sports, cultural activities, and hobbies (20, 34). Answers that AUFVM students "sometimes" attend to socio-cultural activities, as going to see a movie being the most, (40.1%) (Table 13) found to be consistent with other university students' leisure time activities (1, 5).

In Türkiye, many university students spend their free time reading newspapers, books, magazines, going to the cinema and theatre, watching TV and sports events, wandering, or chatting with friends. Studies have shown that university students who engage in educational, cultural, or artistic activities are in the minority (1, 15, 34, 43). Considering how veterinary students spend their free time (Table 13), it has been determined that the most preferred activity is "spending time with friends" of % 43.4 of all students. These answers were found to be compatible with literature findings. Considering that 26.1% of the students responded as not having much free time leads us to think that they cannot find free time due to the intensity of their curriculum. It is striking that students read one to three professional and six or above non-professional books in a year (Table 14). This situation shows that we are a society that does not value reading books enough. This has also reflected in our universities. Although students understand the importance of reading books, they admit that they have not read enough books. Findings are consistent with the paper of Arslan et al. (2).

Consequently, a field study was conducted to determine the general profile of AUFVM students. In line with this purpose, it was tried to determine the demographics of the students studying at the AUVFM, their socio-economic statuses, general information about their pre-university education life and their families, reasons for choosing the veterinary profession, career goals, socio-cultural activities, leisure time activities, etc. It is found that the socio-economic and cultural statuses of the students of AUFVM represent Türkiye's average. Additionally, it is concluded that students of AUFVM are mostly placed to their first preference of university, and are content with being veterinary faculty students.

It can be concluded that this research, which is conducted to provide student profiles, will contribute to both the administrators of the institutions and the academic staff in terms of having the opportunity to know their students.

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Conflict of Interest

The author declared that there is no conflict of interest.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

Official permissions were obtained from the AUVFM Dean's Office and Ankara University Ethics Committee (Approval number: 2020-10-146) for the questionnaire to be applied to the students.

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Investigations on the incidence of deafness in Van cats and its distribution by eye color

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ABSTRACT

This study aims to demonstrate the incidence of deafness in Van cats and its distribution by eye color. A total of 300 Van cats aged between 2 months and 8 years were classified into three separate groups (equal in number) subjected to hearing tests using Clinical ABR (Auditory Brain Response) device. In this study, the incidence of deafness in Van cats was found to be 14.33%. Moreover, it was determined that deafness was not related to sex and hair length. Van cats with spots on their heads did not have deafness. The hearing thresholds showed that most Van cats had a very good level of hearing (0-10 dB nHL). The incidence of unilateral deafness was much lower in Van cats than it was in other white cat breeds. By means of this study, the breeding of the cats found to be deaf according to the results of ABR test will be prevented, and in this way we believe that the incidence of deafness in Van cats will decrease in future. This study is the first deafness study conducted in Van cats and it is worth to present as the findings of the study will shed light on future studies.

Introduction

Cats are good models for deafness studies because they can hear low frequency sounds like humans, and making it possible to evaluate the incidence of deafness in them (19). In cats deafness is often caused by a genetic disorder. It has long been known that congenital deafness is common in white cats with blue eyes (24). Owing to genetic factors Van, Ankara and other white cat breeds show the presence. Deafness which has been determined to be associated with the White (W) gene that is responsible for producing white fur in these cats (25).

Two dominant genes, W and White spotting (S), are responsible for the white color. The W gene, which is found in many cat species, is an autosomal dominant gene and not found to be associated with albinism. Cats carrying the W gene are completely white and some have spots on their heads that disappear with age. Furthermore, the prevalence of deafness is high in white cat populations carrying the W gene (19, 22). The W gene is pleiotropic and white coats, blue iris and deafness, all three effect of the W gene are reported to be associated with the absence or abnormality of melanocytes (19). Additionally, hereditary sensorineural deafness is associated with the W pigment gene (6). Cats may have blue eyes even though they do not have the W gene. This is caused by the Siamese (c^s) pigment gene and it is reported that this gene does not lead to deafness (23). Another reason for white fur in cats is the S gene called Piebald. According to reports, this gene is also not associated with deafness (6).

Certain melanocyte populations in the inner ear are essential for hearing, and absence of functional melanocytes will lead to deafness and changes in the pigment. Melanocytes are produced from the neural crest and located in the cochlear region called the stria vascularis which is rich in blood vessels. In the absence of melanocytes in the inner ear, the stria vascularis typically does not undergo development and acquire functionality. Thus, cochlear hair cells as well as auditory neurons degenerate, resulting in congenital deafness and changes in the pigment of the iris and the fur (16).

The best objective evaluation of deafness can be performed using the Auditory Brainstem Response (ABR). ABR is evoked by sound, is a non-invasive and records the electrical activity that occurs in the auditory pathways in response to click-like sound impulses via electrodes placed on the scalp (4, 14, 22). It is an accurate and reproducible method for assessing deafness in dogs and cats. It provides valuable information on treatment protocol and prognosis and distinguishes conductive and sensorineural deafness (3). Since ABR is affected by movement, the tests are usually performed during sleep, under sedation or general anesthesia (10). To record ABR, a "click" sound is sent to the ear, consequently, 4-7 characteristic waves are formed in the brain in response to the stimulus. Each of these waveforms, expressed in Roman numerals, is related to a specific structure or region within the auditory canal (27). For diagnostic purposes, wave crests I, III and V are evaluated and it has been reported that the wave V, which is the largest peak in individuals with normal hearing, is the most emphasized component in clinical practice (8, 9).

The Van cat is one of the most important cat species in the world. However recently, it has been decreasing in number and therefore, is under protection. The Van cat, highly valuable to Türkiye and the province of Van, is known for its different eye colors, friendliness, white and silky fur and interest in water (7, 11). All scientific studies conducted on Van cats would contribute toward a better understanding of this breed, reveal its difference from other cat breeds, determine its reference values, and lead to a better understanding of its value in Türkiye and in the world. Although many studies on deafness have been conducted for white cats in Türkiye and in the world, the absence of scientific studies on the incidence of deafness in Van cats and its relationship with eye color, gender, hair length and spots on the head has encouraged us to perform this study.

Therefore, the purpose of this study is to demonstrate the incidence of deafness in Van cats with one blue and one amber eye, two blue eyes, and two amber eyes, and the relationship of deafness with eye color, sex, hair length and spots on the head.

Materials and Methods

This study included 300 male and female Van cats [cats with two blue eyes (n = 100), two amber eyes (n = 100) and one blue eye and one amber eye (n = 100)] of 2 months-8 years in age having no health problems as per screening test results and located at the Van Yüzüncü Yıl University Van Cat Research and Application Center within the borders of the Van province. A Clinical ABR device (*Otometrics ICS Chartr Ep 200*) was used to determine the hearing status of the cats. It is reported that the ear canal of cats and dogs do not open immediately after birth but open 3–4 weeks later, therefore, tests performed at earlier ages are not reliable (22).

Cats to be tested were kept under fasting conditions for 12 h. The cats determined to be that healthy based on general examination result were anesthetized using atropine (0.06–0.1 ml/4 kg), xylazine (0.1 ml/kg) and ketamine (0.1 mg/kg). Electrodes and headphones were prepared while the cats were under anesthesia. For disinfection, the electrodes were first disinfected with povidone-iodine solution, then zefiran was used and the electrodes were dried with sterile gauze. Care was taken so that the electric and headphone cables did not touch each other. The description of the cat to be tested was entered on the ABR device. Once the cats were anesthesized, they were placed on their chest, and electrodes were subcutaneously placed in the area surrounding the right and left mastoid bones of the cats, and on the front and base of the neck (Figure 1). Disposable insert headphones suitable for cat ears were used for performing the hearing test. Although the ABR device had the capacity to mask ambient sounds, an environment that was quiet and free of external stimuli was created for the test.

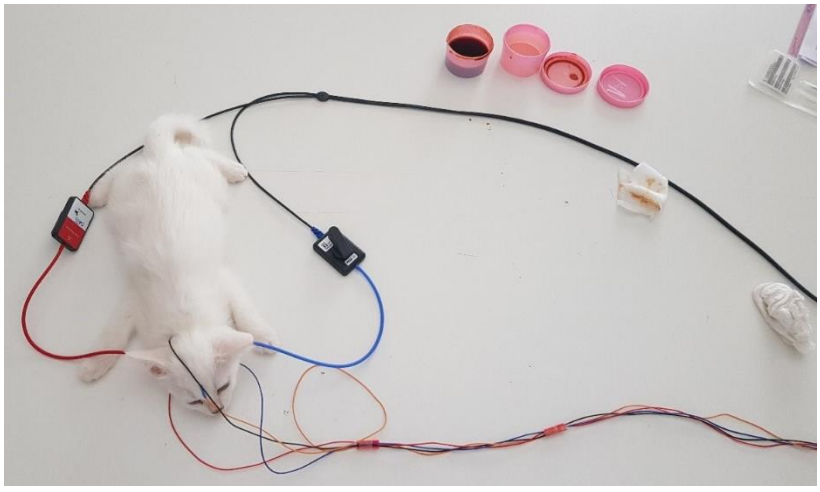

Figure 1. Electrode and headphone placement in a Van cat undergoing ABR testing.

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First, 1000 click stimuli with an intensity of 50 dB nHL were sent to the ear of the cat under test. If the wave V was detected at this sound intensity, the sound intensity was decreased, and a click stimulus with an intensity of 30 dB nHL was sent. Similarly, if the wave V was detected at this sound intensity, the sound intensity was decreased to 10 dB nHL this time. If the wave V was detected once again, the last click was sent with a 0 dB nHL intensity, and the graphics were recorded. If there was no wave V at an intensity of 50 dB nHL, a click stimulus with an intensity of 70 dB nHL was sent. If there was no wave V, the sound intensity was increased to 90 dB nHL, and the click stimulus was sent again (Figure 2, 3, 4, and 5). Thus, the hearing threshold of the cat was determined (Table 1).

Table 1. Classification based on the audiological findings of Van cats.

Hearing loss range	Degree of hearing loss
≤30 dB nHL	Normal hearing
50–70 dB nHL	Moderate hearing
70–90 dB nHL	Poor hearing
>90 dB	No hearing

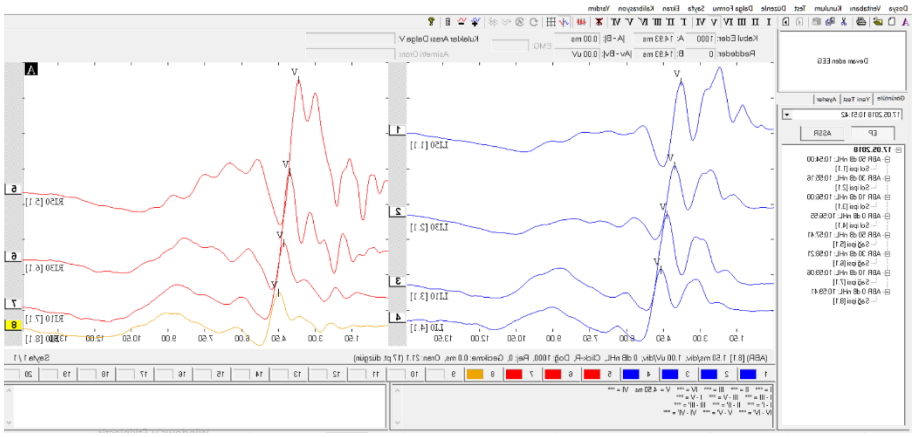

Figure 2. ABR performed for the right and left ears of a Van cat with two blue eyes and unimpaired hearing function (0, 10, 30, and 50 dB nHL).

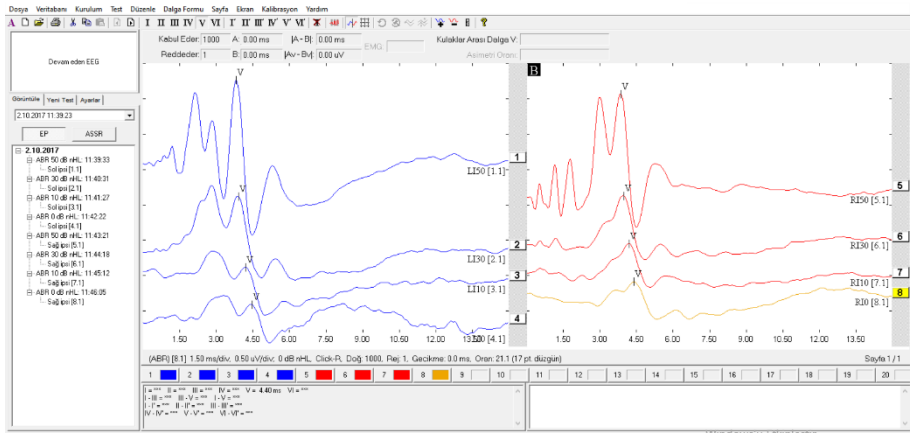

Figure 3. ABR performed for the right and left ears of a Van cat with two amber eyes and unimpaired hearing function (0, 10, 30, and 50 dB nHL).

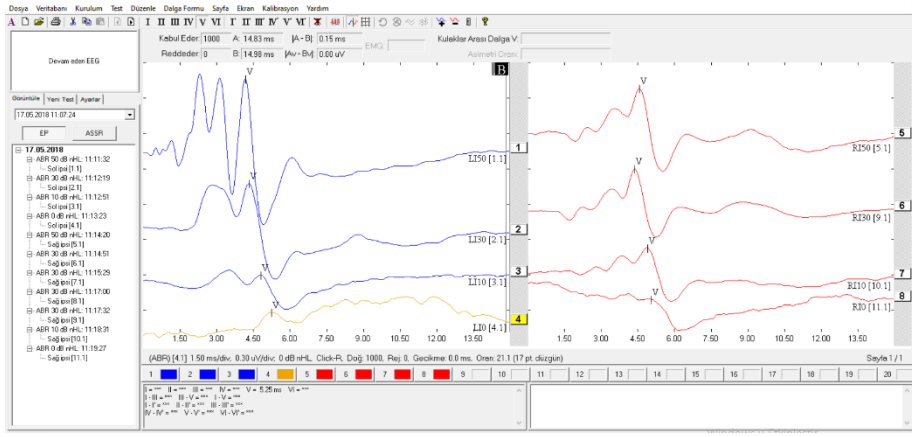

Figure 4. ABR performed for the right and left ears of a Van cat with one amber and one blue eye and unimpaired hearing function (0, 10, 30, and 50 dB nHL).

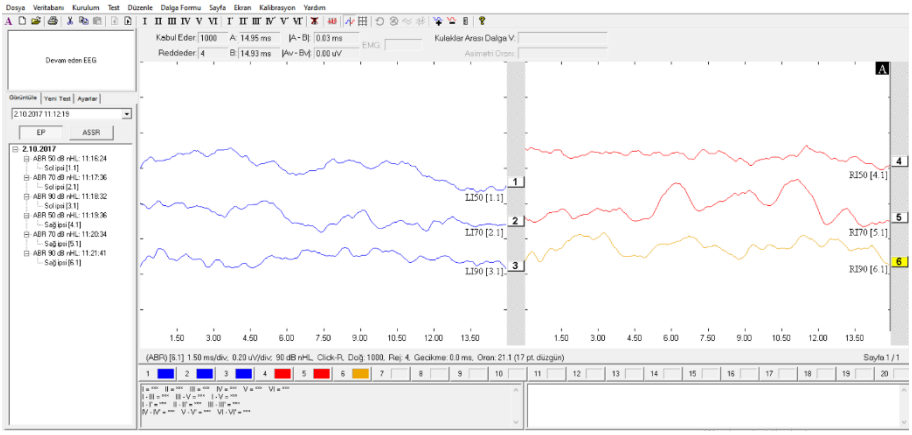

Figure 5. ABR performed for the right and left ears of a deaf Van cat with one amber and one blue eye (50, 70, and 90 dB nHL).

Statistical analysis: Descriptive variables were expressed as mean, and standard deviation, continuous as minimum and maximum values and categorical variables as number and percentage. One-way analysis of variance was used for comparing the groups in terms of continuous variables. Pearson correlation coefficients were calculated to determine the relationship between continuous variables. Chi-square test and multiple correspondence analysis were used to determine the relationship between the groups and categorical variables. Additionally, logistic regression analysis was performed to determine the variables that may have an effect on deafness. Furthermore a statistical significance level 5% was considered, and the SPSS (ver: 21) statistics package software was used for calculations.

Results

No health problems were reported in the routine clinical examination result of the animals included in the study. Body temperature and respiratory and pulse rates were within the normal reference ranges reported for cats.

Considering the relationship of hearing function with age, weight, body temperature, pulse and respiration, it was shown that the relationship of these parameters with deafness was statistically insignificant (Table 2).

The hearing thresholds of the cats subjected to the ABR test were found to be different. Of the cats included (n = 300), 62% (n = 186) reponded to the 0–10-dB nHL sound stimuli sent in the right ear, 18% (n = 54) reponded to the 30–50-dB nHL range, and 5.35% (n = 17) reponded to the 70–90-dB nHL range. Furthermore, 14.33% (n = 43) cats did not show wave V responses for sounds > 90 dB nHL. For the left ear, 63% (n= 190) cats showed wave V response for sounds between 0 and 10 dB nHL, 14.33% (n= 43) for sounds between 30 and 50 dB nHL, 8% (n= 24) for sounds between 70 and 90 dB nHL, and 14.33% (n= 43) did not show wave V response for sounds > 90 dB nHL (Table 3). With respect to hearing thresholds, there was no remarkable difference between the right and left ears, and the majority of Van cats had very good hearing.

On evaluating the hearing thresholds of the cats (n = 300), it was determined that 17.66% of the cats could hear even at 0 dB nHL. In terms of eye color, of the cats who showed response at 0 dB sound 10 were with two blue eyes, 23 with two amber eyes, and 20 with one blue and one amber eye. Based on this, it was found that the group of cats with two amber eyes and that of cats with one blue and one amber eye had higher numbers of cats with very good hearing compared to the group of cats with two blue eyes.

The rate of deafness in male cats was 53% and that in females was 47%. This showed that male cats had a slightly higher rate of deafness compared to females; however, the difference was not statistically significant (P>0.10) (Table 2 and 4).

In the three study groups that each consisted of 100 female and/or male Van cats, the rate of deafness in Van cats with two blue eyes was 25%, with two amber eyes was 6% and with one blue and one amber eye was 14%. The overall deafness rate was 14.33%. The highest rate of deafness was in those with two blue eyes, and the lowest rate of deafness was in those with two amber eyes. The relationship between hearing status and eye color, was found to have a high level of statistical significance (P<0.001) (Table 2 and 5).

On evaluating the cats in terms of hair length, 17 out of 119 long-haired cats and 26 out of 181 short-haired cats were found to be deaf. When the relationship between short-haired and long-haired cats, was evaluated, it was found to be statistically insignificant (P>0.10) (Table 2 and 6).

Among the three groups (n = 300), unilateral or bilateral deafness was not present in a total of 30 (10%) cats with black spots on their heads. The relationship between the presence of spots and hearing status was statistically significant (P<0.01) (Table 2 and 7).

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Table 2. Logistic regression.

Variables in the Equation							
		B	S.E.	Wald	df	Sig.	Exp (B)
Step 0	Constant	-1.788	0.165	117.748	1	0.000	0.167

Model Summary			
Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	220.575[a]	0.083	0.148

a. Estimation terminated at iteration number 20 because maximum iterations has been reached. Final solution cannot be found.
Method = Enter

Variables in the Equation							
		B	St. Hata	Wald	df	P	OR
	Sex (1)	-0.012	0.360	0.001	1	0.972	0.988
	Age month	0.007	0.015	0.217	1	0.642	1.007
	Weight	0.000	0.000	0.171	1	0.679	1.000
	Eye color			9.389	2	0.009	
	Eye color (1)	0.969	0.448	4.679	1	0.031	2.636
	Eye color (2)	-0.608	0.567	1.149	1	0.284	0.544
	Hair (1)	-0.150	0.364	0.170	1	0.680	0.861
	Body temperature	0.438	0.296	2.189	1	0.139	1.550
	Pulse	0.003	0.016	0.033	1	0.856	1.003
	Respiration	-0.017	0.022	0.582	1	0.446	0.983
	Spot (1)	-19.197	7177.001	0.000	1	0.998	0.000
Step 1[a]	Constant	-18.412	11.637	2.503	1	0.114	0.000

Table 3. The hearing threshold of Van cats in study groups.

Hearing threshold (dB nHL)	Hearing classification	Right ear	Left ear	Mean	%
0–10	Very good hearing	186	190	188	62.66
30–50	Good hearing	54	43	49	16.33
70–90	Poor hearing	17	24	21	7
>90	Deaf	43	43	43	14.33

Table 4. Statistical results according to crosstabs: hearing * sex.

			Sex		Total
			Female	Male	
Hearing	Normal	Count	127	130	257
		% within hearing	49.4%	50.6%	100%
		% within sex	86.4%	85%	85.7%
		% of Total	42.3%	43.3%	85.7%
	Deaf	Count	20	23	43
		% within hearing	46.5%	53.5%	100%
		% within sex	13.6%	15%	14.3%
		% of Total	6.7%	7.7%	14.3%
Total		Count	147	153	300
		% within hearing	49%	51%	100%
		% within sex	100%	100%	100%
		% of Total	49%	51%	100%

Ki-kare=0.124
P=0.724

Table 5. Statistical results according to crosstabs: hearing * eye color.

			Eye color			Total
			Blue eyes	Amber eyes	Blue-amber eyes	
Hearing	Normal	Count	75	94	88	257
		% within hearing	29.2%	36.6%	34.2%	100%
		% within eye color	75%	94%	88%	85.7%
		% of Total	25%	31.3%	29.3%	85.7%
	Deaf	Count	25	6	12	43
		% within hearing	58.1%	14%	27.9%	100%
		% within eye color	25%	6%	12%	14.3%
		% of Total	8.3%	2%	4%	14.3%
Total		Count	100	100	100	300
		% within hearing	33.3%	33.3%	33.3%	100%
		% within eye color	100%	100%	100%	100%
		% of Total	33.3%	33.3%	33.3%	100%

Ki-kare=15.365
P=0.000

Table 6. Statistical results according to crosstabs: hearing * hair.

			Hair		Total
			Long	Short	
Hearing	Normal	Count	102	155	257
		% within hearing	39.7%	60.3%	100%
		% within hair	85.7%	85.6%	85.7%
		% of Total	34%	51.7%	85.7%
	Deaf	Count	17	26	43
		% within hearing	39.5%	60.5%	100%
		% within hair	14.3%	14.4%	14.3%
		% of Total	5.7%	8.7%	14.3%
Total		Count	119	181	300
		% within hearing	39.7%	60.3%	100%
		% within hair	100%	100%	100%
		% of Total	39.7%	60.3%	100%

Ki-kare=0.000
P=0.985

Table 7. Statistical results according to crosstabs: hearing * spot.

			Spot		Total
			Spotted	Unspotted	
Hearing	Normal	Count	30	227	257
		% within hearing	11.7%	88.3%	100%
		% within spot	100%	84.1%	85.7%
		% of Total	10%	75.7%	85.7%
	Deaf	Count	0	43	43
		% within hearing	0%	100%	100%
		% within spot	0%	15.9%	14.3%
		% of Total	0%	14.3%	14.3%
Total		Count	30	270	300
		% within hearing	10%	90%	100%
		% within spot	100%	100%	100%
		% of Total	10%	90%	100%

Ki-kare=5.577
P=0.018

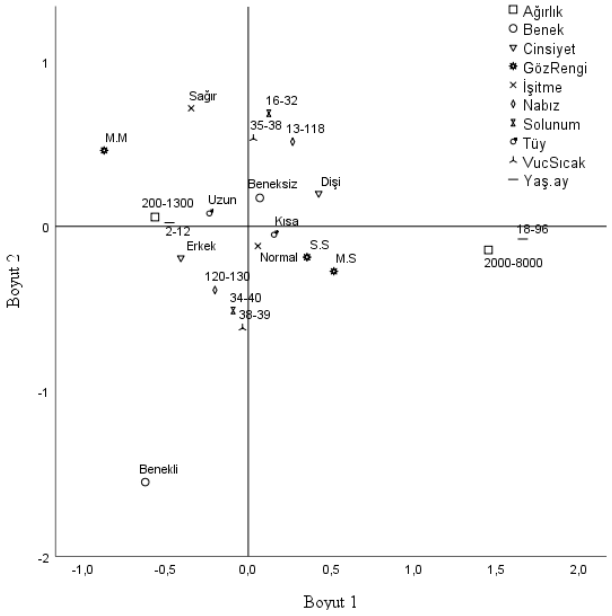

Figure 6. Multiple correspondence analysis.

Multiple correspondence: Based on the multiple correspondence analysis, there was a positive correlation between the rate of deafness and presence of long hair in cats with two blue eyes. The rate of deafness was low in cats with two amber eyes and those with one blue and one amber eye, and male cats were more prone to deafness than female cats, Furthermore, deafness was not observed in cats with spots on their head (Fig. 6).

Discussion and Conclusion

Hearing, which is a physiological process, is one of the most effective means of communication among living things. Hearing loss occurs as a result of the impairment of this process due to pathological reasons and can range from total loss of hearing to a slightest decrease in hearing ability (12, 26).

Deafness in white cats is mostly the result of a genetic disorder (24). It is reported that Ankara, Van, and other white cat breeds are deaf due to genetic factors, and that this condition is associated with the White (W) gene which produces white fur in cats (19, 22). To evaluate the incidence of deafness in the Van cat breed, which is among the pure white cat breeds, the cats must be evaluated first in terms of genetic and other characteristics (1). Although Van cats are shown to be among the white cat breeds in which deafness is common, there is no scientific data regarding the characteristics and incidence of deafness in Van cats. Therefore, the purpose of this study was to demonstrate the incidence of deafness in Van cats and its distribution by eye color using scientific data.

When performing the ABR test, it is important to sedate or anesthetize the animal for ensuring test (10). In one study, intramuscular ketamine HCl and intramuscular acepromazine were administered for sedating the cats to be subjected to the ABR test (17). In a thesis for a study on Angora cats, it was reported that the cats were anesthesized using atropine, xylazine and ketamine for the ABR test (25). In another study, it was reported that mice were anesthetized using intraperitoneal ketamine and xylazine injection before performing the ABR test to determine hearing status (14). However, although it was reported that some clinicians preferred sedation while performing the ABR test, some studies reported that sedation or anesthesia was not required (19). In our study, it was determined that when the anesthesized cats blinked or the cats tails were lifted even slightly, it resulted in the deterioration of the ABR waves. Therefore, it was suggested that the animal to be tested should be under sedation or anesthesia in order to obtain smooth ABR waves and evaluate the results properly, and even though the ABR device has the capacity to mask ambient sounds, the test will yield better result when it is performed in a quiet environment.

Audiometers used in audiological diagnosis are now calibrated according to ISO-1969 standards, taking into account the hearing level (HL). In the calibration of audiometers, instead of the previously; used dB sound pressure level (SPL) the dB HL, which is the lowest sound intensity perceived by the human ear at different frequencies, has been accepted, resulting in the introduction of the concept of audiometric zero (20). The most important component emphasized while evaluating the clinical applications of ABR is the wave V (8). This wave forms the highest peak in an individual with normal hearing, and delay or absence of wave formation may indicate neurological or cochlear defects (2, 9). Considering these data, the dB HL unit was used in the evaluations in our study, and based on the audiological results, cats responding with the wave V at sound levels < 30 dB nHL and between 50-70 dB nHL, and between 70-90 dB nHL had good, moderate and poor hearing respectively. Cats with no response to the click stimulus at sound levels > 90 dB were classified as deaf. In our study, of the total 300 cats included, 188 (62.66%) undergoing the ABR test had very good hearing in the 0–10 dB nHL range, 49 (16.33%) had good hearing in the 30–50 dB nHL range, 21 (7%) had poor hearing in the 70–90 dB nHL range, and 43 (14.33%) were deaf and had no wave V response above 90 dB nHL. This study determined the hearing thresholds of Van cats. And showed that there was no remarkable difference between the right and left ears in terms of hearing thresholds, and the majority of Van cats had very good hearing.

Deafness can be unilateral or bilateral (26). In three simultaneous studies conducted in 1984, the rate of deafness was analyzed in a total of 256 crossbred cat breeds, and it was found that 12.1% of these cats had

unilateral deafness, and 37.9% had bilateral deafness. In a thesis for a study on Angora cats, the ABR test was performed in nine cats, of which, seven had unilateral deafness. It was reported that there was no bilateral deafness, and the prevalence of unilateral deafness was 77.7% in Angora cats (25). In another study, the prevalence of deafness was found to be 20.2% based on the results of the ABR test performed in 84 white cats; 9.5% of these cats had unilateral deafness, and 10.7% of them had bilateral deafness. The combined prevalence of unilateral or bilateral deafness in cats was reported to be 44.4%. This rate was 20% in cats with different eye colors and 18.9% in others (6, 24). Furthermore, evidence that the ear on the side of the blue eye is more affected in unilaterally and bilaterally deaf animals has been reported (18). In our study, it was determined that there were 43 cat with bilateral (14.33%, n = 300) deafness and 1 ear of 2 cats among the study groups showed a wave V response to a sound at 0 dB nHL and the other ear at 90 dB nHL. Additionally, the unilateral deafness rate in Van cats was found to be much lower compared to that in other white breed cats.

Based on the ABR test results of a study conducted with white cats, the prevalence of deafness was reported to be 20.2%. This study revealed that deafness was not associated with gender (6, 24). Based on the statistical evaluations conducted in our study, it was revealed that the relationship between gender and deafness was not significant (P>0.10). However, based on the multiple correspondence analysis, it was determined that male Van cats may be more prone to deafness. These results are consistent with the literature (Figure 6).

The most common locus that produces long hair in mammals is the *Long (L)* locus, which is controlled by *fibroblast growth factor-5 (FGF-5)* and the most important factor for cat hair length (15). It was suggested that the rates of deafness and having blue eyes were higher in long-haired cats compared to short-haired cats; however no related evidence was reported (22). In our study, it was revealed that the relationship between deafness and hair length was statistically insignificant (P>0.10). On performing multiple correspondence analysis, it was determined that long-haired blue-eyed Van cats may be more prone to deafness (Figure 6), and the data obtained are consistent with the literature.

Deafness can occur congenitally or later after birth (22, 26). Congenital deafness is more common in blue-eyed white cats. Charles Darwin stated in a book that he wrote in 1868 that blue-eyed cats were mostly deaf. He reported that the eyes of these cats were blue due to the lack of melanocytes, and the rate of deafness in blue-eyed cats was approximately 80% (19, 22). Deafness was analyzed in a total of 256 crossbred white cat breeds in three simultaneous studies conducted in 1984; two of these studies examined the effect of blue eye on deafness. It was reported the rate of deafness in cats with two blue eyes was 64.9%–85%, and it was 39.1%–40% in those with one blue eye and 16.7%–22% in those without blue eyes (21). Another study investigated whether there was a gene responsible for deafness in blue eye and reported that the rate of deafness in the experimental colony was 67%. Bivariate analysis results showed that polygenic effects could also have played an important role in addition to the effect of the main gene (22). Based on the results of the ABR test performed in 84 white cats, the rate of deafness in blue-eyed cats was found to be higher than that in others (6, 24). In a thesis study conducted on Angora cats, the ABR test was performed in a total of 9 cats; of which 5 two blue eyes and 2 different eye colors, and it was reported that unilateral deafness was not correlated with eye color and gender (25). It is widely accepted that there is a relationship between deafness and animal pigmentation (5). A study tested hearing function in dog breeds at risk of pigment-related congenital sensorineural deafness and found that deafness in English Setter and English Cocker Spaniel dogs was associated with blue eye, and deafness was more common in white Bull Terriers than in colored Bull Terriers (22). It was also shown that the risk of being prone to congenital sensorineural deafness was higher in blue-eyed Dalmatian dogs compared to brown-eyed dogs (13). In a study conducted on 1031 Dalmatian dogs, a positive relationship was found between deafness and having one or two blue eyes or not having pigments in the tapetum lucidum. This relationship was also demonstrated in other dog breeds and white cats (22). There was a positive correlation between deafness in the offspring of those with unilateral or bilateral deafness in one or both parents. Another study showed that there was a positive correlation between blue eye color and deafness, and that blue-eyed cats were more prone to deafness than amber-eyed cats those with different eye colors (6, 24). In our three study groups (n_{total} = 300), the rate of deafness in Van cats with two blue eyes was 25%, and it was 6% in those with two amber eyes and 14% in those with one blue and one amber eye. The mean deafness rate for all the three groups was 14.33%. Our study found that 43 out of 300 cats were deaf. Based on these data, the highest rate of deafness was found in Van cats with two blue eyes and the lowest rate of deafness was in those with two amber eyes. Furthermore, the relationship between eye color and deafness was determined to be highly significant (P<0.001). These data support the view in the literature that deafness is more common in cats with two blue eyes and less common in cats with two amber eyes and those with different eye colors in both the eyes (21). It was demonstrated that the incidence of deafness in Van cats was lower compared that in other pure white cat breeds of the same category.

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White cats are among the animal species in which congenital deafness is common. It has been found that this is associated with the White (W) gene which is responsible for producing white fur in cats (19, 22). Cats carrying the W gene are not always completely white, and some may have colored spots on their heads that disappear with age. It is reported that if a white kitten has even a small amount of discoloration (a speck or spot), this significantly reduces the likelihood of deafness in the cat. Having spots is an indication that the cat has more functional melanocytes (albeit few) than non-spotted cats. It has been reported in the literature that deafness is less common in animals with spots (19, 22). In our study on Van cats, unilateral or bilateral deafness was not found in 30 (10%) cats with black spots on the head in all three groups. This result is consistent with the literature.

If the genes responsible for deafness are not known and marker tests are not feasible, it is very important to use ABR records to reduce genetic hearing disorders, prevent deaf cats from breeding and develop selection plans. It is reported that breeding cats with hereditary deafness will result in the continuation of defects in the gene pool of the breed, and that the rate of deaf litter will be higher than that of normal litter. Thus, even if only one parent is deaf, the vast majority of the litter will be born deaf and have white fur (25). Therefore, this study is important as it aims to detect deaf cats to help prevent the transmission of deafness to future generations by discontinuing the breeding of deaf animals.

As a result of this study, we believe the breeding of cats evaluated to be deaf on the basis of the ABR test results and located at the Van Cat Research and Application Center; in the province of Van and its surroundings can be prevented. Furthermore, we believe that in future, the incidence of deafness in Van cats will decrease and the deafness rate determined in this study will decrease even more via the discontinuation of the breeding of deaf cats. Our study is very important since, it is the first study on deafness in Van cats and shows the rate of deafness in them.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

FAÇ and AK conceived and planned the experiments. FAÇ carried out the experiments. FAÇ and AK contributed to the interpretation of the results. FAÇ took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee (dated 02.05.2019 and numbered 2019/04).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Thoracoscopic partial pericardiectomy for the treatment of pericardial effusion in dogs

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ABSTRACT

Thoracoscopy is a minimally invasive imaging method used in the imaging of the thoracic cavity. In comparison with thoracotomy, thoracoscopy provides better visualization of even the smallest lesions localized in the thoracic cavity. With the use of thoracoscopy, the operation stress in the patient and tissue trauma are reduced, and operating time is shortened. The aim of this study was to evaluate 20 dogs with severe pericardial effusion, which manifested as severe circulation disorder and respiratory distress. The patients determined with pericardial effusion as a result of radiographic and echocardiographic examinations were applied with partial pericardiectomy to achieve permanent health and it was decided to apply this with the minimally invasive thoracoscopic method. The paraxiphoid-transdiaphragmatic approach was applied to the first ten patients and the intercostal approach to the latter. Applicability of thoracoscopic partial pericardiectomy, advantages compared to open surgery, differences between two approach techniques, disadvantages, complications, and success rates were evaluated. It was concluded that this procedure was a successful procedure for dogs and the transdiaphragmatic approach was more useful. In conclusion, thoracoscopic partial pericardiectomy was determined to be easy to apply and more advantageous than open thoracotomy operations.

Introduction

Pericardial effusion (PE), which is defined as abnormal fluid accumulation within the pericardial sac (7, 11), is a well-described clinical condition in dogs. The increase in pericardial fluid levels leads to elevate intrapericardial pressure, and when this pressure increases to a level greater than or equal to right ventricular pressure, cardiac tamponade occurs. Right-sided congestive heart failure and reduced cardiac tamponade are consistent with clinical signs. Studies shows that exercise intolerance (70%), muffled heart sound (60%) and ascites (50%) are common clinical findings (16, 23). Pericardiocentesis is the most effective and fastest way to resolve clinical symptoms and is often performed as an emergency procedure. Pericardial fluid obtained by pericardiocentesis may also be helpful for diagnosis (9, 13).

Pericardial effusion is idiopathic or caused by a neoplastic process (mesothelioma), the most common acquired pericardial disease in dogs (15, 16). In the majority of affected cases, pericardiocentesis is initially indicated for fast patient stabilization (11). Pericardiectomy is indicated in recurrent PE of idiopathic or neoplastic origin to facilitate drainage into the pleural space. In this way, it reduces intrapericardial pressure and cardiac compression (13, 25). Prolonged survival is possible ensuing pericardiectomy.

Traditionally, pericardiectomy is performed with open thoracic surgery, either by median sternotomy or via an intercostal approach. This kind of procedure is often limited to more specific applications and may be rejected by owners due to the associated costs and invasive methods (1, 2).

Thoracic access to the thoracic cavity is used in lateral intercostal, transdiaphragmatic or paraxiphoid, and cranial or thoracic inlet entrance areas. The advantages and disadvantages of the first two entry zones compared to each other are completely related to the experience of the surgeon. Thoracic inlet is a technique taken from the human surgical technique and has a limited use for access to the cranial mediastinum (14).

Thoracoscopic partial pericardiectomy has the additional advantage of offering better visualization than conventional open thoracotomy by improving illumination and magnification to previously unapproachable areas (27). It is also associated with less postoperative pain and lower morbidity. Previous studies have shown thoracoscopic partial pericardiectomy in dogs has less pain and the recovery time of patients is much shorter (18, 26). Thoracoscopic surgery is associated with several advantages compared with open thoracotomy, including reduced postoperative pain, faster return to function, and reduced wound complications (24). It can be used in many surgical procedures such as lobectomy, ductus thoracicus ligation, and removal of pulmonary masses (3, 5, 18, 19, 22, 24).

Thoracoscopic partial pericardiectomy has the added benefit of offering better visualization than traditional open thoracotomy by improving illumination and magnification of areas inaccessible with other methods. It is also associated with less postoperative pain and lower morbidity (2, 8, 26). The aim of this study was to determine the advantages, complications, and success rates of thoracoscopic partial pericardiectomy applied to dogs.

Materials and Method

The study sample was formed of 20 dogs of different ages, sex, and breeds, determined with pericardial effusion on radiographic and echocardiographic examinations. In the clinical examination of the dogs, respiratory distress, anorexia, and cough were determined. Preoperatively, serum biochemistry and full blood analyses were performed together with clotting profile, urine analysis, pericardial effusion fluid sample cytology, and thorax and abdomen radiography, ultrasonography, and echocardiography evaluations.

Radiography was performed in latero-lateral and ventro-dorsal planes to dogs and vertebral heart scale (VHS) evaluations were made. Patients with high VHS scores were applied with echocardiographic examinations and left and right parasternal long axis images were taken. An anechoic space between pericardia and epicardium was accepted as proof of pericardial effusion. Patients were intubated, connected to a mechanical ventilator, and laid in dorsal recumbence. Radiographs were taken using an digital radiography device (Dynamic X-Ray, Türkiye), with 1000 milliamps equivalent to 600 mA HF frequency, and 150 kV power. Patients observed with cardiomegaly underwent echocardiographic examination. An evaluation was made of pericardial effusion from the left and right window, the cause, and heart diseases.

Echocardiographic examinations were made with 2.5, 5, 7 MHz multifrequency probes in color Doppler machine (ESAOTE AU5, Italy).

A endoscopic camera (Kalz Storz Telecam SL II, Germany), endoscopic light source (Kalz Storz Xenon Nova 175, Germany), aspiration machine (Kalz Storz Vetpump 2, Germany), Hopkins II, 0^0, rigid, 30 mm length 5 mm diameter telescope (Kalz Storz 62046 AA, Germany), were used to access the abdominal cavity for the telescope and the thoracoscopic surgical equipment used were a 6 mm diameter, 10.5 cm long Ternamian Endotip 3 spiral trocar (Kalz Storz 60160 MTR, Germany), thoracoscopic scissors (Kalz Storz 34310 MA, Germany), grasper forceps (Kalz Storz 33310 ME, Germany) and a suction catheter (Kalz Storz 26173 BN, Germany).

All animals received pre-emptive analgesia with morphine HCl (0.12 mg/kg), and general anesthesia was provided with propofol (4 mg/kg, IV) and isoflurane (2-4%, using cuffed endotracheal tubes), setting the ventilator to “pressure-controlled ventilation” mode with an inspiratory pressure value of 10 cm H_2O, with respiratory rates of 16 breaths/minute. PEEP (positive end-expiratory pressure) mode was activated and was set to 2 cm H_2O for recruitment maneuver and to prevent atelectasis. All the dogs recovered from anesthesia in the intensive care unit, received fluids, analgesics, oxygen and were monitored for hypothermia, pain and dyspnea. Postoperatively advanced ampicillin (30 mg/kg IV TID), Tramadol (4 mg/kg SC QID) and Dipyron (25 mg/kg SC TID) were administered. Analgesics were administered to all the dogs at 6 and 12 hours after surgery.

The thoracoscopic partial pericardiectomy procedure was applied with the paraxiphoid-trandiaphragmatic approach to the first ten patients and with the intercostal approach to the remaining ten (10). The first trocar was positioned immediately below the xiphoidal cartilage, directed to the thorax and when the thoracic cavity was reached, the trocar valve was opened and pneumothorax was created. The telescope was advanced to the thoracic cavity from this port. The first operative toll was introduced from the left 6^{th} or 7^{th} intercostal space and the second operative toll was introduced from the left 9^{th} or 10^{th} intercostal space under telescope guidance.

The pericardium was grasped with grasping forceps. In some cases, pericardium was too tight to grasp because of effusion, so in these cases some of the fluid was drained with pericardiocentesis. The samples obtained were sent

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to the pathology laboratory for cytological evaluation. A 1 cm diameter incision was made in the pericardia with thoracoscopic scissors. The fluid discharged to the pleural cavity was drained with a suction catheter. A piece approximately 4x4 cm in size was taken from the pericardium. The telescope was then inserted from the intercostal ports and the space between the pericardium and epicardium was examined for any signs of neoplasia. After completion of the procedure, all the equipment was removed, a thoracic drain was placed in the 9th or 10th intercostal space and thoracic incision sites were closed with simple interrupted sutures using nonabsorbable polypropylene (prolene) 2/0 monofilament suture material. The thoracic drain was connected with a 3-way stopcock. Free air in the thoracic cavity was evacuated with a 3-way stopcock and negative pressure was achieved. All the dogs received a single dose of meloxicam (0,2 mg/kg, Meloxicam, Bavet, Türkiye) subcutaneously immediately after the procedure.

All dogs were examined at 3, 6, 12 and 24 months after the operation. Radiographic and echocardiographic examinations were repeated in patients on these dates. If the patient could not attend the examination, the owner was contacted by telephone.

Results

The evaluation was made of 20 dogs, comprising 12 females and 8 males with a mean age of 7 years (range, 2-12 years). The dog breeds were West Highland white terrier (WHWT) (*n*= 4), German Shepherd (*n*= 3), Golden Retriever (*n*= 2), Kangal Shepherd (*n*= 1), Miniature Pinscher (*n*= 1), Pekingese (*n*= 1), Maltese Terrier (*n*= 1), Boxer (*n*= 1) and street mixed breed (SMB) (*n*= 6).

It was learned in the anamnesis that 5 patients with cough and respiratory stress were diagnosed with asthma in 4 (nos. 2, 6, 11, 17) and heart failure in 1 (no. 7), and medical treatment was performed. One case (no. 15) had received treatment for 3 weeks for a diagnosis of lymphoma. Except for one case (no. 15) with a low platelet count, no abnormalities were determined in the preoperative blood analysis. In the same patient, growth of the intra-abdominal lymph nodes and increased spleen dimensions were noticed on ultrasonography.

On the echocardiography examination, severe pericardial effusion was determined in 12 dogs and cardiac tamponade in 5 (Figure 1). In case number 19 a neoplasia suspected lesion was seen in the right atrium (Figure 2), and in case number 7, dilated cardiomyopathy (DCM) was seen together with pericardial effusion.

The transdiaphragmatic approach was used in ten randomly selected cases and the intercostal approach was used in the other ten cases (Figure 3). In one patient, the close proximity of the lungs to the placement of the camera and thoracoscopic instruments providing the

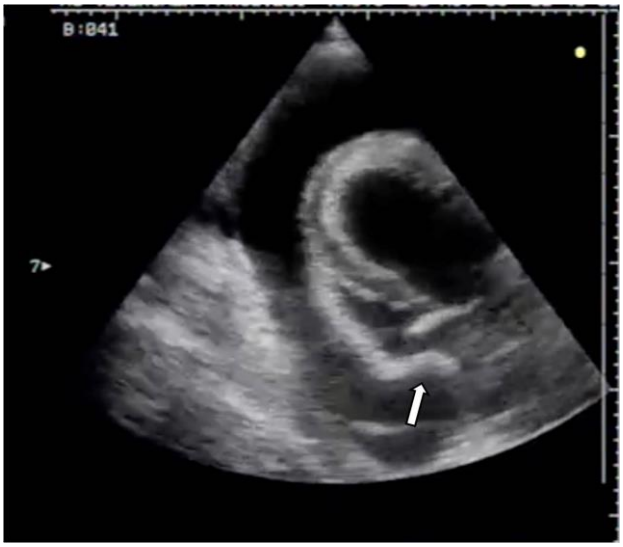

Figure 1. Severe pericardial effusion and cardiac tamponade image (arrow) in ultrasonographic examination of case number 14.

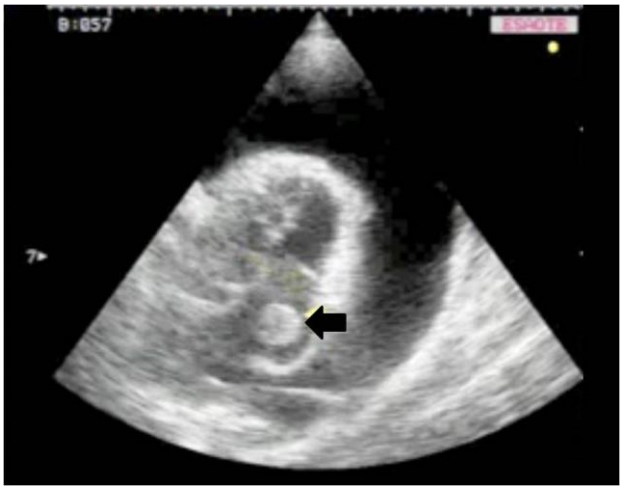

Figure 2. Left window, long axis four chamber view, myocardial neoplasia in the right atrium (arrow), case 13.

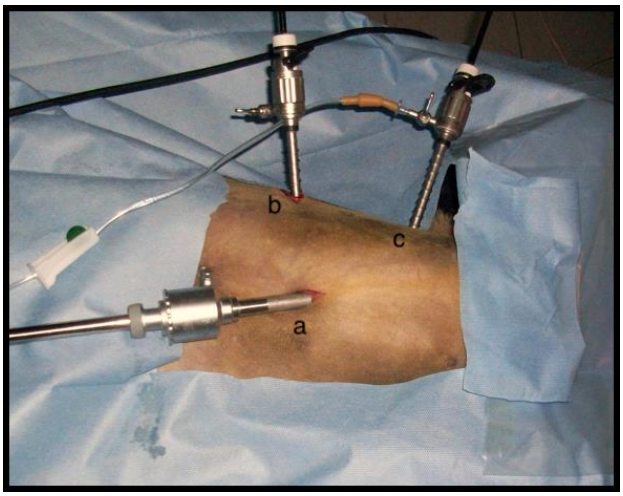

Figure 3. The position of trocar units for thoracoscopic partial pericardiectomy operation of case number 11. Paraxiphoid port used for telescope entrance (a). Other ports in left 6th (c) and 10th intercostal space (b) used for thoracoscopic equipment entry.

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visualization angle necessary for the operation prevented comfortable work. Therefore, the intercostal approach was converted to the paraxiphoid trans-diaphragmatic approach because of poor visualization of the working area. Inflammatory pericardial thickening was seen in 5 of 20 cases. In three cases thickening in the parietal pleura was also seen (Figure 4). In case number 3, a mass originating from the myocardium was seen and the pathology report was of rhabdomyosarcoma (Figure 5). In case number 15, thoracic lymph nodes were enlarged, and in case number 16, an abscess approximately 3 cm in diameter was seen in the left cranial lung lobe. Patients with confirmed negative pressure were hospitalized and monitored for one day then discharged from the hospital. In one case (number 9) hypothermia and dyspnea were observed and the patient was kept in an oxygen tent for 48 hours. After the dog recovered, it was discharged from the hospital. In case no. 5, pulmonary laceration occurred through surgeon error, so partial pulmonary lobectomy and partial pericardiectomy were applied. The case was not withdrawn from the study so that the complication could be discussed. The operating time was recorded as mean 32 mins for the paraxiphoid-transdiaphragmatic approach and mean 48 mins for the intercostal approach.

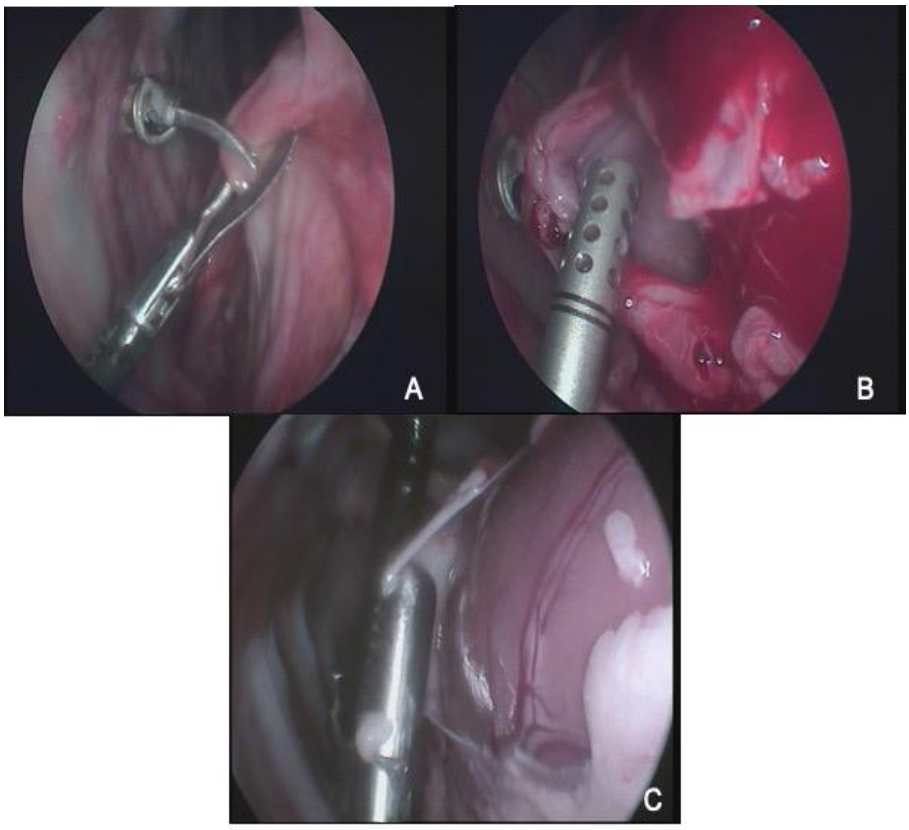

Figure 4. A and B = Severe thickening in parietal pleura and pericardium (case 2 and 13). C=A normal pericardium (case 10).

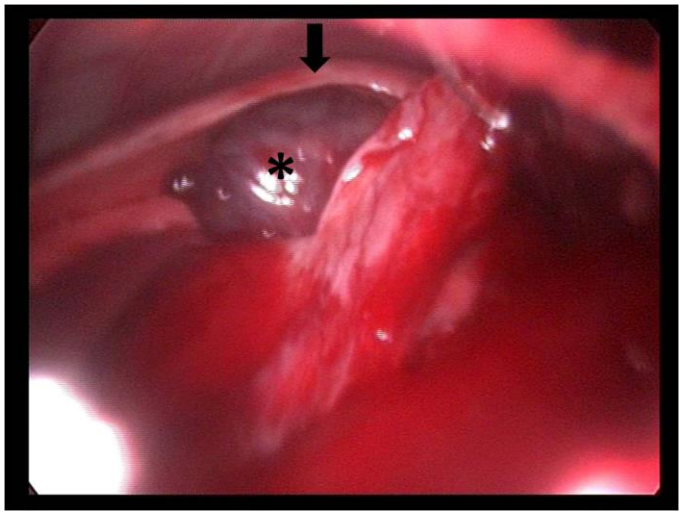

Figure 5. Case 3, Rhabdomyosarcoma. Pericardium (arrow), tumor (*).

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Table 1. Echocardiography results, thoracoscopic approach methods, findings and outcomes of cases.

Case	Breed	Age	Echocardiography results	Thoracoscopic approach	Thoracoscopic findings	Outcome
1	Golden Retriever	4	Severe pericardial effusion, cardiac output	PTD	Thoracic cavity, pleura and pericardium were normal	No complications
2	Maltese terrier	2	Pericardial and pleural effusion	PTD	Pleural and pericardial thickening	No complications were seen
3	Pekingese	8	Severe pericardial effusion, cardiac output pleural effusion	I	Mass formation	No complications were seen. The patient died due to pulmonary metastases at 4 months postoperatively
4	Boxer	5	Pericardial and pleural effusion	I	Pleural and pericardial thickening	No complications were seen
5	Miniature Pinscher	5	Pericardial effusion	I	Thoracic cavity, pleura and pericardium were normal	Pulmonary laceration occurred during procedure, then converted to open surgery
6	SMB	7	Pericardial effusion	First I, later PTD	Thoracic cavity, pleura and pericardium were normal	No complications were seen
7	WHWT	6	Pericardial effusion, pleural effusion, Dilate cardiomyopathy	PTD	Thoracic cavity, pleura and pericardium were normal	No complications were seen. The patient died due to kidney failure at 8 months postoperatively.
8	SMB	9	Pericardial effusion	PTD	Thoracic cavity, pleura and pericardium were normal	No complications were seen.
9	Golden Retriever	12	Pericardial effusion	I	Pericardial thickening	Respiratory stress developed after surgery, respiration stabilized after six hours
10	SMB	2	Pericardial effusion	I	Thoracic cavity, pleura and pericardium were normal	No complications were seen.
11	SMB	3	Severe pericardial effusion, cardiac output	PTD	Thoracic cavity, pleura and pericardium were normal	No complications. The patient died when struck by a car after 1 year
12	German Shepherd	4	Pericardial and pleural effusion	I	Thoracic cavity, pleura and pericardium were normal	No complications.
13	Kangal Shepherd	7	Pericardial and pleural effusion, mass-like lesion in left ventriculi	PTD	Pleura and Pericardial thickening	No complications.
14	German Shepherd	10	Severe pericardial effusion, cardiac output	PTD	Thoracic cavity, pleura and pericardium were normal	No complications. Hypothermia and hypoxia but stabilized after 4 hours
15	WHWT	9	Pericardial and pleural effusion	I	Thoracic lymph nodes were enlarged	No complications.
16	SMB	11	Pericardial and pleural effusion	I	Abscess in cranial lung lobe	No complications.
17	WHWT	12	Pericardial effusion	I	Thoracic cavity, pleura and pericardium were normal	No complications.
18	German Shepherd	9	Pericardial effusion	PDT	Thoracic cavity, pleura and pericardium were normal	No complications.
19	SMB	8	Severe pericardial effusion, cardiac output	PDT	Pericardial thickening	No complications.
20	WHWT	6	Pericardial effusion	PDT	Thoracic cavity, pleura and pericardium were normal	Pleural effusion recurrence at 8 months postoperatively and the patient died

I: Intercostal approach.
PDT: Trans- diaphragmatic approach.
SMB: Street mix breed.
West Highland white terrier: WHWT.

In four cases, the dogs came from another city and follow-up was made with telephone calls to the owners and the well-being of the dogs was confirmed. One year after the operation, one dog (case number 11) died when struck by a car, and one dog (case number 7) died from kidney failure. Case number 20 was brought to the clinic after eight months with severe respiratory distress. Ultrasonographic examination showed a reoccurrence of pericardial effusion and the dog died after that day.

The only complications seen were lung laceration in case no. 5, postoperative hypothermia and hypoxia in case no.9, and a recurrence of pericardial effusion because of closure of the opened pericardial window in case no. 20.

Discussion and Conclusion

The common goal of all treatment methods in pericardial effusion is to resolve it, determine its etiology, and avoid recurrences, all with the least possible morbidity and mortality. There are three fundamental surgical possibilities: complete pericardiectomy, subxiphoid pericardiotomy and pericardial window (thoracotomic or thoracoscopic approach). The application times of the techniques are generally close to each other and the thoracotomy takes a little longer. The possibility of thoroughly exploring the pericardial cavity and pleural space and on the possibility of obtaining sufficient study material is effective in choosing a technique (8, 12). A thoracoscopic partial pericardiectomy is a very effective operation for the elimination of the clinical symptoms of pericardial effusion (14). Thoracoscopic partial pericardiectomy has a better diagnostic capacity in these aspects than the other techniques, as it enables to explore the pleural cavity thoroughly and visualize and obtain samples of suspicious lesions and associated effusions (17).

Jacksons et al. (14) reported that opening a 4-5 cm diameter pericardial window is sufficient to eliminate clinical symptoms in thoracic partial pericardiectomy. It has been reported that a more widely formed pericardial window cup could cause clotting from this hole (10). In this study, a 3x3 cm pericardial window was opened in a Miniature Pinscher. Due to the small size of the Miniature Pinscher, a small window size was preferred to prevent herniation of the heart. In the other 19 dogs, a pericardial window 4 x 4cm was opened and recurrence was only seen in one dog, which was attributed to adhesions having closed the opened window. However, there were no histopathological findings to support this theory.

Recent studies have claimed subphrenic pericardiectomy facilitates thinning of the heart surface and prolongs life compared to partial pericardiectomy (6, 7). In another study, classic open thoracotomies (intercostal, median sternotomy) and thoracoscopic pericardiectomy methods (subtotal (subphrenic) partial) were compared in respect of pain, survival time, length of stay in the hospital, and wound management. Thoracoscopic subphrenic pericardiectomy has proven to be the most advantageous method (20). In the current study, the partial pericardiectomy method was preferred due to a lack of experience of the with subphrenic method.

Walsh et al. (26), reported that thoracoscopic partial pericardiectomy has several advantages compared with open thoracotomy including less pain and shorter recovery time. In the current study, all except one dog were discharged from the hospital after 24 hours and no pain or respiratory distress was seen. Some cases (nos. 8, 15, 16) had a VATS time of 117.5 minutes. A study by Burton (5) reported open surgery time as 109 minutes. In this study, the mean VATS time was 40 minutes, which was much shorter than open surgery times stated in other studies. This period could be shortened with practice.

It has been reported that scar complications at the thoracoscope entry site, phrenic nerve damage, lung lacerations and intraoperative bleeding can be seen as complications of thoracoscopy (14). Case et al. (7) reported that fatal hypotension occurred in 2 of 36 dogs. In this current study, lung laceration occurred during thoracic entry in only one case, a Miniature Pinscher dog (case 5), and that case was converted to open surgery. It was concluded that in small breed dogs, pneumothorax must be ensured before starting thoracoscopic surgery.

To understand whether or not thoracic valves are in the pleural cavity, the valve must be removed. The pulmonary automatic ventilation device should be inflated as little as possible.

A high recurrence rate has been reported in the pericardiocentesis procedure alone. Surgical pericardial windowing offer the best long-term results minimizing pericardial effusion recurrence (14, 21). Of the 20 dogs included in this study, no recurrence occurred in 19 (96%) throughout one year. Case number 20 had recurrent pericardial effusion at 8 months after the procedure and the patient died that day. In one case (case number 9) hypotension and respiratory distress occurred but did not become fatal. In one study, a dog with similar symptoms was euthanized because it did not improve with medical treatment (2).

Brisson et al. (4), reported that in a patient with mesothelioma, metastases occurred in the thoracic entry site. in the current study, (case number 3), pericardial effusion had developed because of rhabdomyosarcoma but no metastasis or recurrence was seen. However, in pericardial effusions of neoplastic origin, metastases can be expected because effusion opens and drains from the thoracic cavity. Modified pericardiectomy procedures should be developed to minimize the risk of metastasis in these kinds of patients. In this case pulmonary metastatic areas were seen on the postoperative 3rd month

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radiography and the patient died at 4 months postoperatively due to severe respiratory problems. Hartmann et al. (11) reported that a cardiac mass was not observed on ultrasonography but the mass was subsequently observed on thoracoscopic examination. In the current study, a rhabdomyosarcoma mass was not seen on ultrasonography but was detected later on thoracoscopic examination. Both of these studies demonstrate thoracoscopy is a superior method for the visualization of mass lesions causing pericardial effusion.

In the current study, the tumoral formation (rhabdomyosarcoma) in one case that could not be seen on echocardiography was able to be visualized during the partial pericardiectomy. This can be considered fortunate because the tumor localization could be seen from the site where the pericardial window was opened. As stated by Carvajal et al (6), this proved that if the partial pericardiectomy method is to be applied to a patient, a detailed evaluation of the pericardial/epicardial surface with pericardioscopy is necessary.

Dogs with presumptive idiopathic pericardial effusion treated with a subtotal pericardiectomy via thoracotomy have been shown to have longer median survival times (MST) compared with dogs treated with a thoracoscopic pericardial window. The discrepancy in long-term outcomes between surgical techniques has been suggested to be the result of non-durable decompression of the pericardial space for dogs with chronic effusion, and the inaccuracy of the initial diagnosis, possibly because of the limited visualization of gross pericardial/epicardial pathology provided by the method of choice (6).

For dogs with presumptive idiopathic pericardial effusion applied with the pericardial window technique, McCarthy (20) and Walsh et al. (26) performed pericardiectomy with the patients in lateral recumbence. In the current study, dorsal recumbence was preferred as it increased the field of vision, and facilitated pericardial incision and dissection because the lung stayed in the dorsal field because of gravity.

In patients where the para-xiphoid trans-diaphragmatic approach was used, the heart was seen in angulation and the desired area could be easily reached by advancing the thoracoscopic tools from the left and right intercostal segments. Visualization of the thoracic cavity and the use of thoracoscopic instruments in the intercostal approach in small breed patients may be difficult. The authors believe that depending on the experience of the surgeon, the paraxiphoid-transdiaphragmatic approach is more useful in small animals. Balsa (3) suggested that pericardiocentesis should be performed in small dogs with severe pericardial effusion to provide a comfortable field of vision before thoracoscopy, and the same result was reached in this study.

From our experience, the paraxiphoid trans-diaphragmatic technique provides a more comfortable visual angle and the movements of the thoracoscopic manual instruments can be followed more easily from this visualization. In the intercostal approach, the visual window is narrow, but manual instrument-camera coordination can be made more easily. With further studies, the operation time may be shortened. From the experience of this study, it was concluded that the ease of working in both techniques was completely operator-dependent.

In conclusion, this study showed that thoracoscopic partial pericardiectomy for the treatment of pericardial effusion is very successful in dogs. Because thoracoscopic partial pericardiectomy is a minimally invasive procedure, it causes less pain and has a shorter recovery time. This could also be the first step for thoracoscopic subtotal pericardiectomy, which has better results than this method, both for the patient and surgeon.

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Conflict of Interests

The authors have no conflict of interests to declare.

Author Contributions

YŞ, AB and BBÖ operated and evaluated the patients with the technique mentioned in the article. OOŞ determined the anesthesia protocol of the application and managed the anesthesia and pain relief aspect of his patients. AEH took part in the diagnosis and surgical referral of pericardial effusion. YŞ and İB did the image recording of the patients, the drafting of the publication and the English translation of the article. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was conducted with the approval of the Animal Experiments and Local Ethics Committee of Ankara University, decision numbered 2008-31-143.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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The Continuum of Eustachian Tube Obstruction in Cats: A temporal bone study

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ABSTRACT

The Eustachian tube is a canal from the tympanic cavity to the nasopharynx that is responsible for the aeration, drainage, and pressure equalization of the middle ear. Partial or complete blockage of the tube can trigger severe ear disease. We evaluated pathologic changes from Eustachian tube obstruction (ETO) in 15 temporal bones from cats with bilateral ETO from the temporal bone collection at the University of Minnesota Medical School Otopathology Laboratory. The samples were evaluated for histopathological changes to investigate the continuum of the disease at intervals of 2 days, 1-week, 2-weeks and 4-weeks. Temporal bones were sectioned in the horizontal plane and every 10th section was stained with hematoxylin and eosin. One section from each ear was stained with periodic acid-Schiff and Alcian blue. Sections were studied under light microscopy. The results revealed moderate hyperplasia equally developed throughout the epithelial layer surrounding the middle ear and neutrophil-rich inflammatory cell infiltration. As the duration of obstruction prolonged to the 4th week, compositional change of the middle ear effusion from serous to mucoid that was accompanied with granulation tissue formation was observed. In conclusion, the severity of the findings related to ETO are directly proportional to the duration of the disease. Therefore, patients presenting with long-lasting complaints of ear diseases should be examined for dysfunction or blockage of the Eustachian tube.

Introduction

The Eustachian tube connects the tympanic cavity to the nasopharynx and functions in the ventilation, drainage, and pressure equalization of the middle ear (14, 20). Failure of the Eustachian tube to perform these functions defines Eustachian tube dysfunction. Since the functions of the Eustachian tube are quite complex and multifactorial, the etiology of Eustachian tube dysfunction is not fully understood (14, 20).

Previous studies (1, 6, 14), have identified a link between viral upper respiratory infections leading to Eustachian tube dysfunction, accompanied by an increased nasal inflammatory response. In later studies, allergen induced dysfunction of the Eustachian tube presenting with inflammation and edema of the middle ear epithelium has been described (1, 9, 10, 21). In addition to allergen stimuli, benign or malignant neoplastic formations originating from the middle ear and eustachian tube can also cause complete or partial obstruction of the Eustachian tube leading to nasopharyngeal signs and otitis (5, 8, 11). In Eustachian tube obstruction (ETO) or blockage, negative pressure increases and triggers the transition of plasma from mucosal vessels to the middle ear cavity, and undrained accumulated fluid results in serous otitis media (2, 4, 14). In this study, we describe the histopathologic changes in the middle and inner ears of cats from 2 days to 4 weeks after ETO.

Materials and Methods

Our temporal bone samples consisted of 15 cats with bilateral ETO from the animal temporal bone collection at the Otopathology Laboratory, University of Minnesota. The study was performed and the samples were added to the collection in the 1970s and at that time the temporal bone study was approved by the institutional review board.

In order to histopathologically identify the changes caused by ETO in the middle ear, 15 healthy cats were purchased from and housed individually by Research Animal Resources (RAR). Animals were given food and water ad libitum. All cats underwent detailed general examinations, including otoscopy, and cats that were determined to be healthy weighting 2 to 5.5 kg. They were pre-anesthetized with atropine (0.04 mg/kg) and continued with ketamine (10 mg/kg) and acepromazine (0.1 mg/kg). Before the procedure butorphanol (0.5 mg/kg) was injected subcutaneously for analgesia and oxytetracycline ophthalmic ointment was used for each eye. A midline incision was made in the soft palates to expose the orifices of the Eustachian tubes. Multiple pieces of silastic sponge were pushed into the Eustachian tubes bilaterally and the soft palates sutured with chromic catgut suture. Analgesic and antibiotic treatment (Penicilin G BID, 25000 U/kg) was continued for 3 days after surgery. The cats' vitals and pain conditions were monitored every day. Pain management was established due to physiological and behavioral evaluation by trying to keep them content and quiet, comfortable when resting and interested about environmental surroundings. The cats were fed with soft food to be able to minimalize the pain on the surgery site.

To be able to follow the continuum of the histological changes, the animals were divided into 4 groups and euthanized at 2 days (n=3), and 1 (n=4), 2 (n=4), and 4 (n=4) weeks after obstruction. The temporal bones were removed and fixed in 10% formalin, dehydrated in a graded series of ethanol, decalcified with trichloroacetic acid, and embedded in celloidin. They were serially sectioned in the horizontal plane at a thickness of 20 µm. Every 10th section was stained with hematoxylin and eosin. Celloidin was removed from an additional section from each ear and stained with periodic acid-Schiff and Alcian blue as described previously (17). Sections were studied under light microscopy.

The mid modiolar section was used to measurethe hickness of the middle ear mucosa at the inferior, anterior, and posterior wall using a digital camera and image analysis software (SPOT Advanced; SPOT Imaging Solutions, Sterling Heights, MI, USA). Three measurments were taken at each location and their values averaged.

Results

Two days after obstruction, there was very slight widening of the subepithelial layer of the middle ear. No other abnormalities of the middle ear structures were observed.

One week after obstruction, there was an increase in the thickness of the subepithelial layer compared to the 2-day obstructed group. Hyperplasia was equally developed throughout the epithelial layer surrounding the middle ear. Serous effusion filled the majority of the middle ear cleft. There was mild neutrophil-rich inflammatory cell infiltration mainly in the area around the round and oval window membranes.

At 2 weeks after obstruction, intense thickening, edema, hyper vascularization, and hyper dilatation of the vessels of the subepithelial layer of the middle ear were observed. Serous effusion filled the majority of the tympanic cavity while effusion with higher viscosity was observed around the Eustachian tube. There was neutrophil-rich inflammatory cell infiltration in other locations of the middle ear cavity. Additionally, neutrophil granulocyte and mononuclear cell infiltration were seen in the subepithelial space. The epithelium of the round window membrane that faces the tympanic cavity was covered with inflammatory cells. Goblet cells stained with Periodic acid Shiff and Alcian blue appeared magenta in color demonstrating the presence of neutral mucins.

After 4 weeks of obstruction, there was a transition of the middle ear effusion from serous to mucoid, with increased infiltration of plasma cells and other mononuclear cells. There was severe hyperplasia of the epithelial layer and hypervascularization of the subepithelial layer (Figure 1). Goblet cells that were predominantly in the mesotympanum and Eustachian tube stained dark blue to purple with periodic acid Shiff and Alcian blue indicating the presence of both neutral and acidic mucins (Figure 2). Some goblet cells had expelled their mucous contents and contained empty vacuoles. Mucoid effusion was mainly around the opening of the Eustachian tube in the middle ear cavity. Extensive granulation tissue was observed in the middle ear cavity (Figure 3). Although the small number of animals in each group did not permit statistical analysis of mucosal thickness, it did show a trend to increase overtime (Figure 4).

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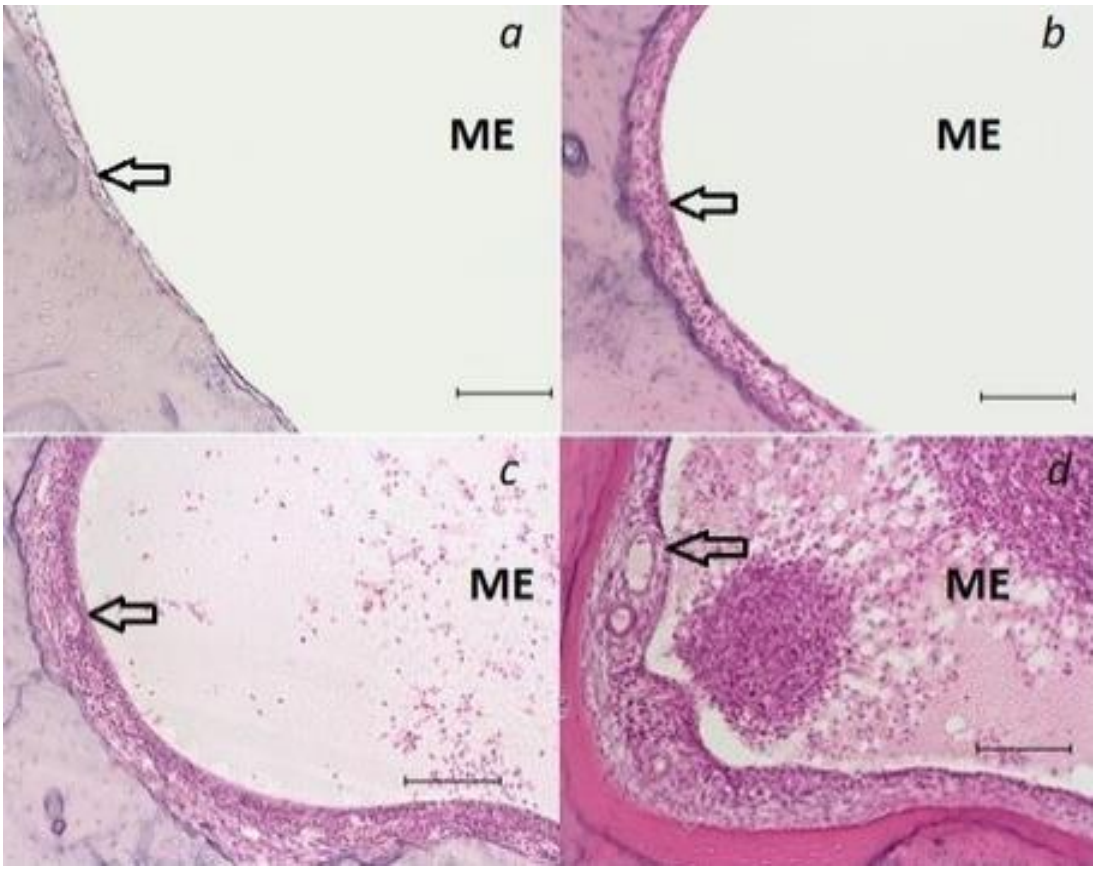

Figure 1. There is slight widening of the sub-epithelial layer of the middle ear after 2 days after Eustachian tube obstruction (a), that increases in weeks 1 (b), 2 (c), and 4 (d) Stained with haematoxylin and eosin. (ME=middle ear, hollow arrow: sub-epithelial layer of the middle ear). *Scale bar: 100 µm*.

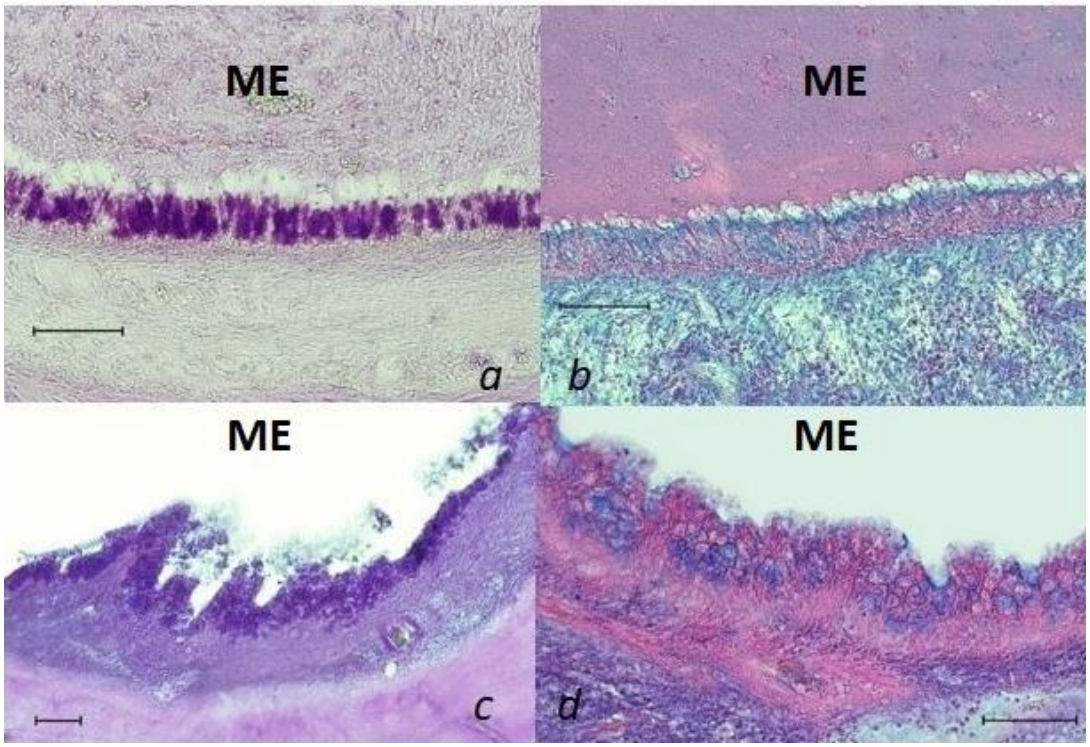

Figure 2. a & b: After two weeks of obstruction goblet cells appear magenta in color indicating the presence of neutral mucins. There is increased activity of goblet cells. c & d: four weeks after obstruction periodic acid Shiff and Alcian blue staining that are dark blue to purple in color demonstrating continued goblet cells activity with a greater content of acidic mucins (ME= middle ear). *Scale bar: 100 µm.*

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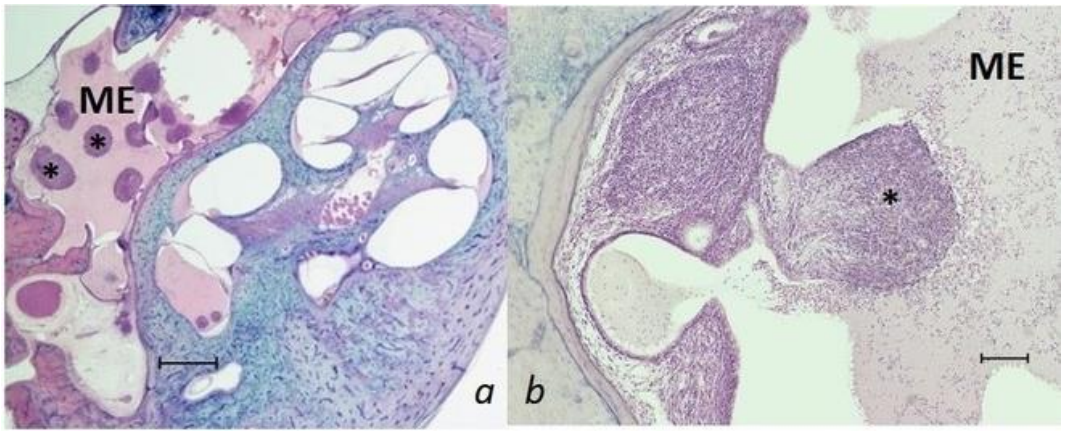

Figure 3. a: Middle ear effusion and granulation tissue formation in the middle ear and round window niche occulted at 4 weeks post Eustachian tube obstruction (ME = middle ear, star = granulation tissue). *Scale bar: 1.0 mm.* b: Granulation tissue formation in the middle ear cavity 4 weeks after obstruction (ME = middle ear, star = granulation tissue). *Scale bar: 100 µm.*

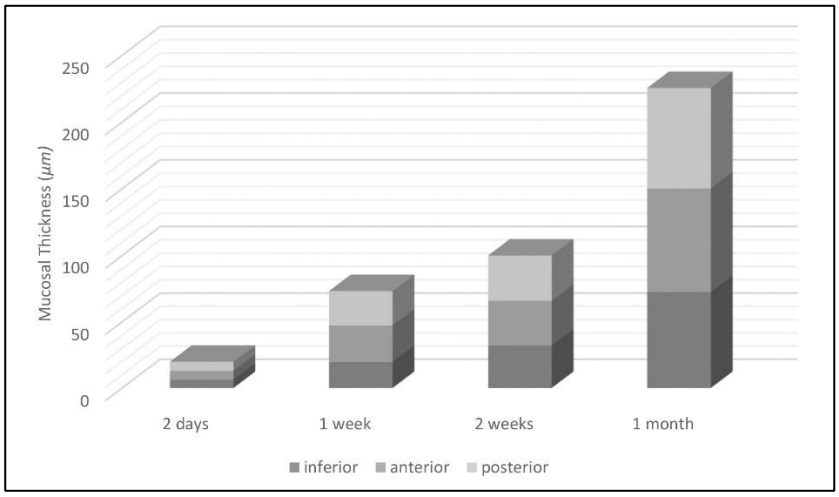

Figure 4. Graph showing the increase in middle ear mucosal thickness in the inferior, anterior and posterior wall of the middle ear in the study groups.

Discussion and Conclusion

Nasopharyngeal masses, cleft palate, neurologic and pulmonary diseases, and nasal and ocular discharge have been suggested to trigger Eustachian tube obstruction and serous otitis media (3, 7, 14, 19). In our study, we found that, Eustachian tube obstruction causes serous otitis media as early as 1 week post obstruction. We have also observed widening of the subepithelial layer in all temporal bone samples. Due to the small number of samples, it was not possible to do statistical analysis in this study. However, we have observed that the mucosa thickened with increasing periods of obstruction in the early periods from edema and at later periods from inflammatory infiltration. We have also revealed the hyperactivation of goblet cells, accumulation of exudate, capillary dilatation, and granulation tissue formation at later time intervals of obstruction. These findings are consistent with previous studies and associated with the inflammatory process (12, 13).

Previous studies have shown that the number and activity of goblet cells can increase in a very short time in association with the inflammatory or immune response (12, 16). In our study, we observed a dramatic increase in the activation of goblet cells in the middle ear mucosa in the first and second weeks. In the fourth week when the effusion transformed from sero-mucoid to mucoid, goblet cells were still active. Our histochemical evaluation using Alcian blue and periodic acid-Shiff staining showed neutral mucins at 2 weeks that by 4 weeks also contained acid mucins. It may be that the increase in acid mucins and viscosity are related.

We observed granulation tissue formation as early as 4 weeks after obstruction. Granulation tissue formation is often present in chronic otitis media (18). The phenomenon of granulation tissue formation was summarized in three steps: Rupture in the surface epithelium and accompanying cellular infiltration from the lamina propria to the middle ear cavity, expulsion through the ruptured region of the epithelial cell surface of the separated lamina propria and finally the beginning of re-epithelialization (18). Negative ear pressure causes a more severe decrease in sound transmission than positive

pressure and leads to conductive hearing loss. When effusion and granulation tissue formation coexist in the middle ear space, there is a reduction in the compliance of the tympanic membrane and a further reduction in sound transmission (15).

Dysfunction of the Eustachian tube should be considered as a disease with many complications. In this study, epithelial hyperplasia and the composition of middle ear effusion are directly proportional to the duration of Eustachian tube obstruction and untreated cases may develop chronic ear infections with granulation tissue formation. Considering the major impact of Eustachian tube dysfunction on otitis media and related surgeries, the early symptoms should be carefully addressed and followed-up.

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The authors have no financial relationships to disclose.

Conflict of Interest

The authors have no financial relationships, or conflicts of interest to disclose.

Author Contributions

NKY, PAS, MMP and SC conceived and planned the study, NKY, PAS, MMP carried out the experiments and collected the data, NKY, PAS, İGS performed the data analysis, NKY, PAS and SC took the lead in writing the paper. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the institutional review board of the University of Minnesota (No:#00003249).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Using Mealworms (Arthropoda: Tenebrionidae) to Prepare Rat Skeleton

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ABSTRACT

In anatomy, various methods such as boiling, maceration, and dissection are used to prepare the skeleton. However, while the existing methods are used in the skeleton preparation stage of small animals, the integrity of the skeleton is impaired, and there are difficulties in the reassembly of the bones. For this reason, it is planned to create small animal skeletons without loss and damage by using mealworms. In the study, 1100 mealworms (Arthropoda: Dermestidae) with a total weight of approximately 110 grams and three rat carcasses with a weight of approximately 177 grams were used. To observe the carcass cleaning performance of the mealworms, the initial weight and final weight of the carcass were measured with a precision scale. It was observed that on days of 3rd-5th, the meat on the rib was eaten and the ribs appeared. The vertebral column became more evident in the 6th-8th days. In the 6th-9th days, the ribs were completely cleared, and the extremity bones were visible. The skull and extremities were evident in all their details between the 9th-12th days. At the end of the 15th day, it was determined that mealworms had completely exposed the rat skeleton. As a result, in this study, it was observed that approximately 1100 mealworms consumed 159 g of meat in 15 days, resulting in a lossless and undamaged rat skeleton. In addition, the advantages and disadvantages of creating small animal skeleton using mealworms were determined in this study.

Boiling, maceration, burying and dissection methods are frequently used in the preparation of skeletal material in anatomy (9). The boiling method requires large equipment and a special area. During this process, which takes a lot of time, a bad odor is emitted into the environment. It also needs to be constantly checked by the employee (3, 23). In the maceration method using additional chemicals, it is mentioned that the bones are removed from the soft tissue in a short time and the bones obtained are quite clean. However, it should not be forgotten that there may be a risk of damage to the bones by chemicals during this method (1, 17). Again, in this method, manual removal of the tissues adhered to the bone is often considered a waste of time. In addition, another disadvantage of the maceration method is the high cost of chemicals and the bad odor produced by bacteria that reproduce in the water or chemical solution (22). Creating a skeleton with another method that is the embedding method takes time. In this method, while the preservation of bone integrity is an advantage, there is also the possibility of damaging the bones of some carnivorous animals (20). The biggest disadvantage of the dissection method, in which a skeleton can be formed in a short time with minimum equipment, is that the tools are used to damage the worker and the bone (15, 23).

Since these methods mentioned above usually require intensive work and equipment, some insect species such as *Dermestes maculatus larvae* are used in forensic entomology, zoology, anthropology and museums for cleaning bones for skeleton construction (14, 18). While the use of these insects saves time and work, it does not cause any damage to bone morphology. The most important issue for the establishment of the *Dermestes maculatus* (Arthropoda: Dermestidae) colony and the continuity of the colony is the creation of suitable environmental conditions (11, 15, 18). In addition, other processes are needed to purify the obtained bones from insects (16).

Apart from all these methods, in 1950, mealworms were tried in species such as marmoset, monkey, wildcat,

and raccoon to obtain skull bone, and it was shown that mealworms (Arthropoda: Tenebrionidae) cleaned the head and exposed the bone (2). After that, no study was found on this subject. Mealworms are easy to obtain, maintain and reproduce (8, 21). The life cycle of mealworms consists of four stages: egg, larva, pupa, and adult, and the whole process takes place in the same ecosystem (5). This study was targeted to create skeletons in small animals using mealworms and to reveal the advantages and disadvantages of this method compared to other classical methods. In addition, we aimed to determine the meat consumption amount and duration of these arthropods and contribute them to the literature.

Approximately 1100 mealworms (Arthropoda: Tenebrionidae) weighing 110 grams were commercially purchased for 8 dollars. In the study, performed in the Department of Pathology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, three rats used in the control group were used.

Commercially purchased mealworms (Arthropoda: Tenebrionidae) larvae were stored in a plastic container at room temperature. 500 g crumbled bran was placed on the bottom of the plastic container as a substrate. Photographs were taken every two days until the carcass was cleaned and turned into a skeleton. In order to determine the meat consumption rate and amount of mealworms larvae, the initial weight and final weight of the carcass were measured with a precision scale. The carcass was wrapped in a damp cloth to prevent the muscle tissue from drying out.

Small pieces of meat were given for 7 days to increase the adaptation of mealworms, which are known to eat vegetables as food. Afterward, rat carcasses, whose skin and organs were removed, were used. In addition, during this process, the container containing the carcass was examined every day, and the mealworms larvae that became pupae and adults were removed from the colony and kept in a separate container. Large pieces of vegetables (potatoes, cucumbers) were kept in the container to meet the water needs of the colony and to balance the ambient humidity.

The mean weight of three rat carcasses whose skin and internal organs were removed, before being fed to the mealworms was 177±12.24 g. It was observed that the worms started to feed on the first day when rat carcasses were given to the adapted mealworms (Fig. 1). It was observed that the meat was eaten on the ribs and the ribs appeared on average 3^{rd}-5^{th} days for all carcass. In 6^{th}-8^{th} days vertebral column became more prominent. By 6^{th} -9^{th} days the ribs were completely cleared and the extremity bones were visible. Between days the 9^{th} and 12^{th}, the cranium and extremities were evident in all their details. At the end of the 15^{th} day, it was determined that the mealworms revealed the rat skeleton as a whole without any damage to the bone structure. The final weight at this stage was 18.3 ± 2.08 g on average. It was observed that the consumption rate slowed in the ligaments and the skin parts of the tail, where mealworms quickly consumed the muscle and adipose tissue of the carcass. The consumption rate was decreasing due to drying on the carcass over time. During this process, there was no offensive odor. Mealworms in the colony were not prone to escape and were easy to care for. It was observed that mealworms, which started their life cycle with eggs, continued as larvae, pupae and adults (Fig. 2). It was determined that while the larva and adult form in the life cycle ate meat, the pupa form was in minimum motion and did not consume meat. It was remarkable that as the number of pupae in the colony increased, the meat consumption rate decreased, and mealworms from different life cycles had to be present in the colony to obtain a constant consumption rate. In this way, it was observed that the colony consumption rate could be kept constant without adding new mealworms.

Various methods have been used to prepare skeletons from past to present (1, 18, 20, 23). It has been reported that these methods have disadvantages such as time, cost, labor, bad odor, bone damage, bone loss, and deterioration of skeletal integrity (1, 6, 12, 20, 23). In this study, a complete skeleton was obtained in rats without bone loss by using mealworms (7). The literature states that apart from the methods mentioned above, insects such as *Dermestes* spp. are also used to prepare skeletons (7, 10, 13). It has been reported that the skeleton was obtained without causing any damage to the bone with the use of these species (18). However, the disadvantage of this method is the difficulty of obtaining *Dermestes* spp. and maintaining the colony (4, 19). In the study, it was revealed that mealworms, which are readily available, can be easily obtained skeletons from small animals without requiring a particular area and special care.

Obtained bone using mealworms (Arthropoda: Tenebrionidae), but they did not mention the advantages or disadvantages of this method in their studies (2). Again in this study, data about the total time, colony living conditions, number of mealworms in the colony, and consumption rate were not included. For the first time in our study, the approximate number of mealworms to be used to form a rat skeleton with mealworms was determined as 1100 pieces, and the meat consumption rate of these worms was determined as 159 gr.

In conclusion, with this study, the advantages and disadvantages of using mealworms in the creation of small animal skeletons such as rats were determined.

While this method has the advantages of being low-cost, easy to obtain and care for mealworms, not causing any damage to the bone, being an odorless method, and requiring minimum labor, the disadvantage of the method meat consumption rate decreased due to an increase in the number of pupae in the colony. With the data we obtained from this study, we believe that the mealworms used in the preparation of small animal skeletons can also be used in cleaning the bones of large animals.

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Figure 1. Stages of formation of rat skeleton using mealworm.
A: Rat carcass, skin, and internal organs removed and were given to mealworms; B: The view of the carcass on the 3rd-5th days; C: The view of the carcass on the 6th-8th days; D: The view of the carcass on the 8th-9th days; E: The view of the carcass on the 9th-12th days; F: Emergence of rat skeleton at the end of day 15th.

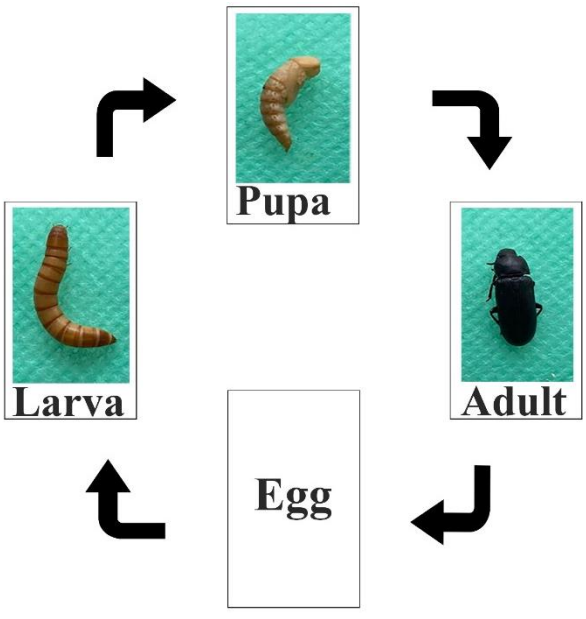

Figure 2. Mealworms life cycle.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

SSS, SK, BO, and MK conceived and planned this study. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

Since no live animals were used in the study, there is no need for an ethics committee.

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First case of chronic systemic spironucleosis in Freshwater Angelfish (*Pterophyllum scalare* Schultze, 1823) in Türkiye

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ABSTRACT

This study aimed to identify the cause of sustained mortality in freshwater angelfish (*Pterophyllum scalare* Schultze, 1823) kept at an ornamental fish production facility in Türkiye. Parasitological, bacteriological and histopathological examination were performed on moribund hybrid angelfish individuals. The moribund fish had haemorrhaged eyes, darkened skin, scale loss, ascites and false faeces. A pale liver, splenomegaly and a thinning of the intestinal wall were observed internally. The parasitological examination revealed flagellated protozoan endoparasite *Spironucleus* sp. in the intestine. A number of histopathological changes were observed including lipid degeneration in the liver, hemosiderin deposits as well as granulomas in the spleen, a large number of mast cells in the lamina propria of the intestine and enteritis. Numerous *Spironucleus* parasites were seen in the intestinal wall. According to physiological and biochemical tests, the bacterial isolates obtained from the visceral organs of some fish were identified as *Citrobacter freundii*. After oral metronidazole treatment, with a dose of 50 mg/kg fish daily for 5 days, a decrease in fish mortality and resumed feeding were noted. A chronic spironucleosis, which systemically affects fish by penetrating the intestinal mucosa, was identified as the cause of this sustained mortality in freshwater angelfish.

The total global value of ornamental fish trade is 15 billion US$ and deals with more than two billion live ornamental fish mostly from freshwater (21). Ornamental fish farming, which started out as a private enterprise in Türkiye, has now become a major industry. Freshwater angelfish (*Pterophyllum scalare* Schultze, 1823), originally imported from Amazon River of Brazil and Peru, Colombia, French Guiana and eastern Ecuador, are the most important commercial cichlid species (7, 16). Freshwater angelfish adults can reach 15 cm in length and females can lay up to 1000 eggs. Individuals are considered monogamous because they tend to mate with a single partner (29). These fish require good water quality, so care must be taken to avoid large fluctuations in conditions and partial water changes must be made regularly. The majority of reported disease outbreaks are either caused by systemic iridovirus (22) or protozoan parasite *Spironucleus* infections (14).

Diplomonad flagellates of the genus *Spironucleus* have been reported to cause serious systemic infections in both aquaculture and wild fish since the first systemic spironucleosis outbreak was reported in Norwegian Atlantic salmon (*Salmo salar*) in 1989 (13, 30). *Spironucleus salmonicida* (basonym *S. barkhanus*) has been identified in grayling (*Thymallus thymallus*), brown trout (*Salmo trutta*), Arctic char (*Salvelinus alpinus*) and *Oncorhynchus namaykush* as the causative agent of spironucleosis (8, 9, 25). *Spironucleus salmonis* was reported in brook trout (*Salvelinus fontinalis*) (18), Chinook salmon (*O. tshawytscha*) (11), rainbow trout (*O. mykiss*) (1, 2). *Spironucleus torosus* was reported in Atlantic cod (*Gadus morhus*), haddock (*Melanogrammus aeglefinus*) (18), burbot (*Lota lota*) (24). *Spironucleus elegans* has been detected in *Titurus alpestris* and freshwater angelfish (12). *Spironucleus vortens* was found in freshwater angelfish (15), ide or orfe (*Leuciscus idus*)

(26) and discus (*Symphysodon discus*) (17). They can cause cellular damage in the intestinal tract of fishes with severe infections (13, 15, 17).

In Türkiye, *Capillaria* sp. (28), hexamitiasis (10) and edwardsiellosis (27) were previously reported in angelfish. The purpose of the present study was to identify the cause of sustained mortality in freshwater angelfish kept at an ornamental fish production facility in Türkiye.

Eight moribund individuals of each hybrid type (silver, zebra, koi, and black lace angelfish) were examined. Individuals that were immobile at the bottom of the aquarium and showing clinical signs were collected and necropsy was performed. The behavior of the moribund fish was monitored and the anamnesis of the epizootic was obtained from the fish farm executives. The body cavity, all internal organs, the gills, the eyes, the skin, and the fins were examined for parasites. Some visceral organs such as kidney, spleen, liver were placed on a slide with a drop of water to make a tissue squash. Along with these, the intestine and bile contents were examined under light microscope (14). Tissue samples were fixed in 10% buffered formaldehyde for histopathological processing and then tissue samples were dehydrated in ethanol series, cleared in trichloromethane and embedded in paraffin. Finally, the tissue sections were stained with haematoxylin-eosin (5). Photomicrographs were taken using a microscope with an imaging system. Swabs taken from internal organs were streaked onto Tryptic Soy Agar (TSA) and incubated at 22°C for 48h. After incubation, the bacterial isolates were examined using standard laboratory protocols and biochemical test were performed. The isolates were also evaluated for antimicrobial susceptibility using the Kirby-Bauer disc diffusion method and analyzed according to recommendations of the Clinical and Laboratory Standards Institute (3).

The moribund fish stopped feeding and had hemorrhaged eyes, darkened skin, scale loss (Figure 1a, b, c, d), ascites and false faeces. A pale liver, splenomegaly and thinning of the intestinal wall were observed internally (Figure 1 c, d). Highly motile flagellated protozoan endoparasites of the genus *Spironucleus* were found in intestines (Figure 2) and bile contents. These findings are very similar to previous reports of the intestinal form of this disease. Here in contrast, *Spironucleus* sp. was also found in the bile content. A yellowish mucoid fluid was also observed in the intestines of black lace angelfish hybrids in this study.

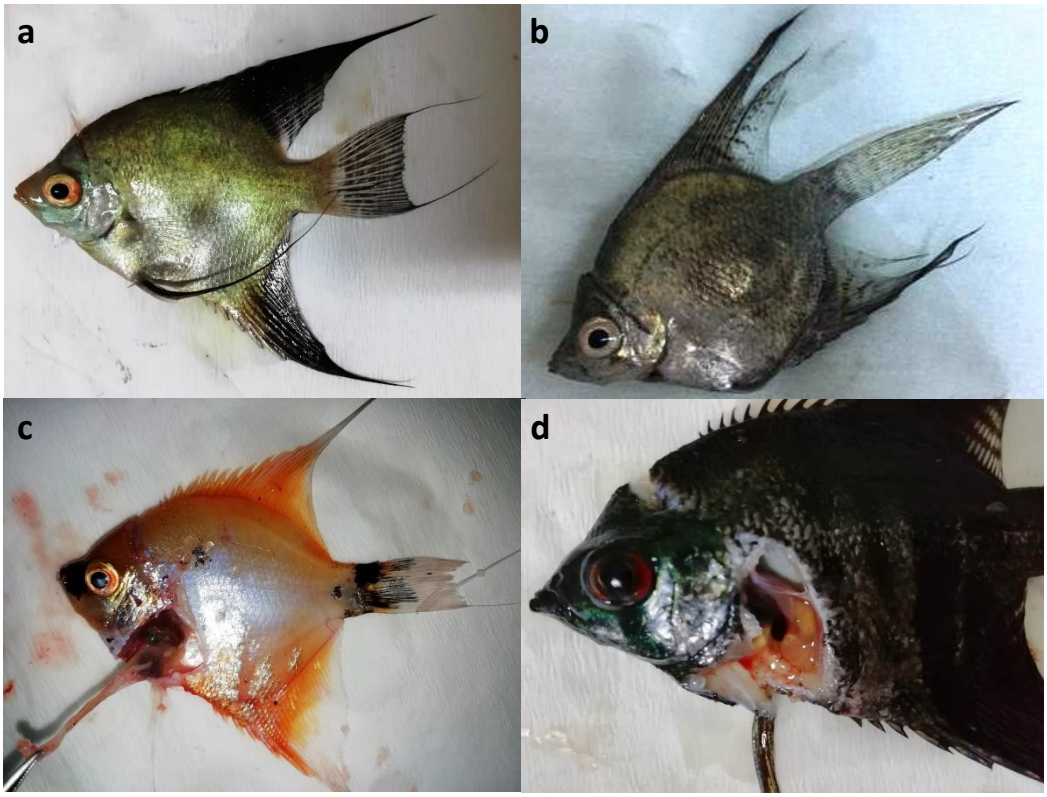

Figure 1. Moribund freshwater angelfish hybrids.
(a) Zebra angelfish display hemorrhaged eyes and scale lose
(b) Silver angelfish had dark skin pigmentations, scale lose
(c) Koi angelfish examination revealed hemorrhaged eyes, scale lose and an internally pale liver
(d) Black lace angelfish observations included splenomegaly, thinning of the intestinal wall and mucoid yellowish fluid in the intestine.

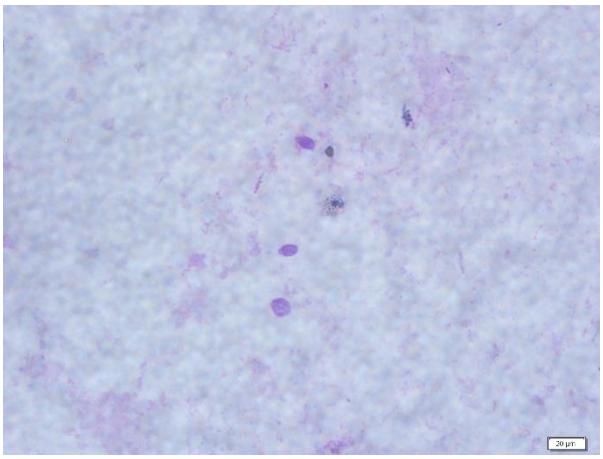

Figure 2. The flagellate protozoan *Spironucleus* sp. stained with Giemsa in intestine contents.

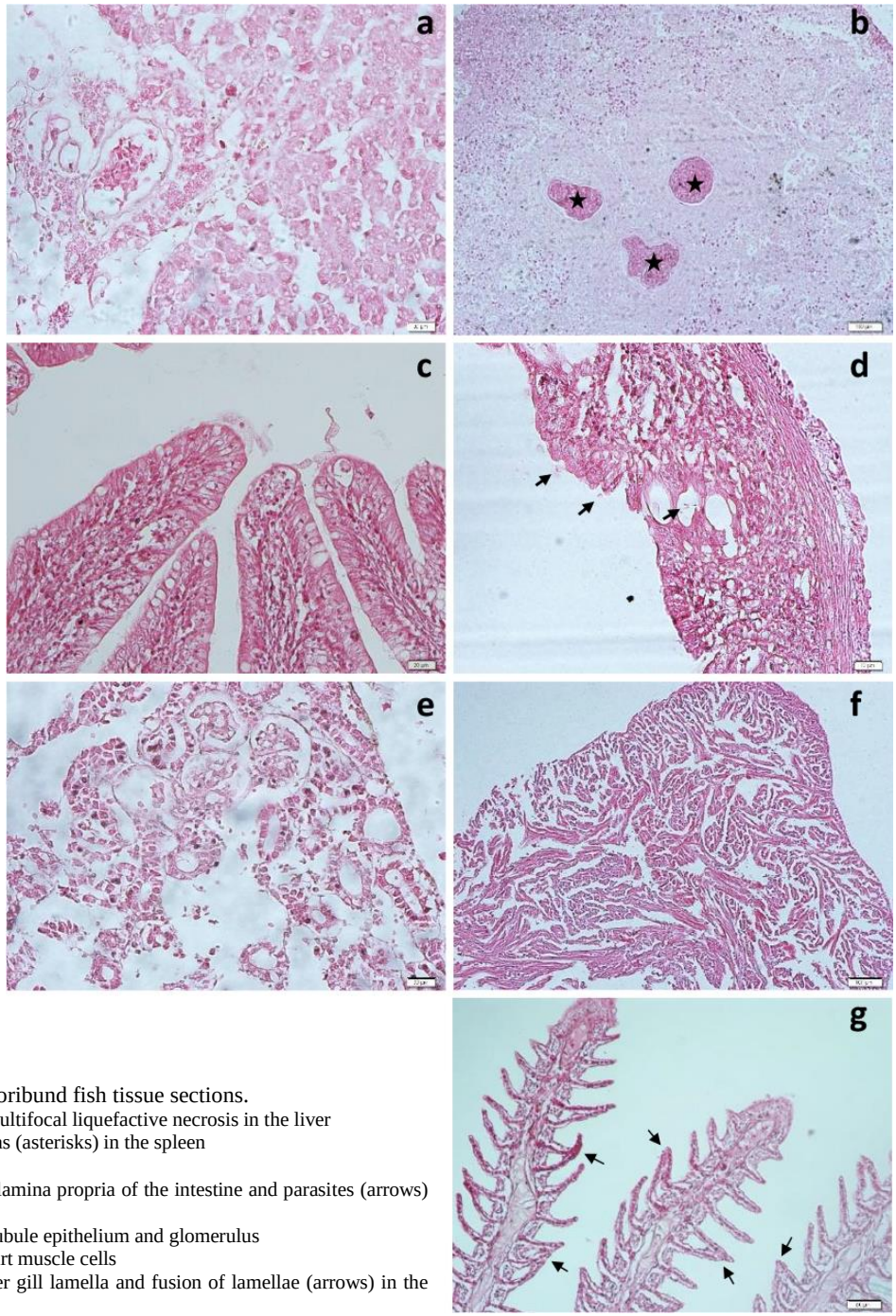

Figure 3. A photomicrograph of moribund fish tissue sections.
(a) lipid degeneration hepatocyte and multifocal liquefactive necrosis in the liver
(b) hemosiderin deposits and granulomas (asterisks) in the spleen
(c) enteritis
(d) a large number of mast cells in the lamina propria of the intestine and parasites (arrows) attached on basement membrane
(e) liquefactive necrosis in the kidney tubule epithelium and glomerulus
(f) necrosis and haemorrhage in the heart muscle cells
(g) distal tip hyperplasia in the seconder gill lamella and fusion of lamellae (arrows) in the gills.

Other observations included lipid degeneration and multifocal liquefactive necrosis in the liver (Figure 3a), hemosiderin deposits and granulomas in the spleen (Figure 3 b) and a large number of mast cells in the lamina propria of the intestine and enteritis (Figure 3c, d). These findings were similar in all examined hybrids. Numerous *Spironucleus* parasites were seen in the intestinal wall. Although it was similar to the intestinal form of the disease, a systemic infection was evident in this study. Liquefactive necrosis in the kidney tubule epithelium and glomerulus (Figure 3e), necrosis and hemorrhage in the heart muscle cells (Figure 3f), distal tip hyperplasia and fusion of lamellae in the gills (Figure 3g) were also detected. Because granuloma formation in the spleen and other findings in the internal organs resembles the systemic form of the disease, a chronic systemic spironucleosis was diagnosed in this study.

In addition to loss of appetite and excessive nervousness (1, 2), enteritis and cytoplasmic blebbing in the gastrointestinal epithelium have been reported as clinical signs of this parasitic disease (31). It has been reported in many studies that disease in farmed salmonids correlates with parasite density as well as environmental factors and nutritional deficiency. Symptomatically spironucleosis has been divided into two main variants; an intestinal and a systemic form. The intestinal form of this disease is benign. Lethargia, exophthalmos, ascites, and false faeces were typical both in salmonids (1, 2) and ornamental fish. Although the symptoms are not specific, the parasite has been found in large numbers in the intestine (1, 2). Intestinal spironucleosis is often associated with locomotive disorder and increased mortality. In the systemic form of spironucleosis, on the other hand, lesions in the head region have been reported and these necrotic lesions with many parasites are located symmetrically along the lateral line (30). In cichlids such as discus and angelfish, it is reported that these parasite-filled necrotic lesions in the head area are combined to form larger lesions and cause a yellow mucoid discharge. This parasite has been reported in many internal organs such as the heart, the liver and the kidney but not in the intestine in the systemic form of the disease. Atlantic salmon infected with *S. salmonicida* reveal the typical histopathology of the systemic form of this disease. These findings include severe epicarditis, large caseonecrotic areas with the formation of a granulomatous response in the kidney, liver and spleen (8, 25).

Spironucleosis causes heavy economic losses, especially in cichlids. *S. elegans* (11) and *S. vortens* (25) have so far been identified as the causative agent of spironucleosis in the posterior intestine of angelfish. Although host specificity and causing granulomatous lesions in the current study point to some particular species within the genus, it is impossible to determine *Spironucleus* species by light microscopy. It is therefore deemed necessary to use scanning electron microscopy [SEM] or transmission electron microscopy [TEM] for accurate identification of the species (8, 18, 19, 24). In this study, the parasites causing disease could only be identified to genus level.

Spironucleosis is treated with chemicals such as dimetridazole, metronidazole, pyrimethamine, albendazole, fenbendazole, mebendazole and magnesium sulfate (23). Besides that treatment with medicinal aromatic plant extracts, including tetterwort (*Chelidonium majus*), purple coneflower (*Echinacea purpurea*), garlic (*Allium sativum*), chestnut (*Aesculus hippocastanum*), horseradish (*Armoracia rusticana*), *Bryophyllum pinnatum* (*Kalanchoe pinnata*), oregano (*Origanum vulgare*), tansy (*Tanacetum vulgare*), thyme (*Thymus vulgaris*), yarrow (*Achillea millefolium*) (20) and wormwood (*Artemisia campestris*) (4), have also yielded successful results. Diler et al. (4) reported good results when ethanol extract of the wormwood plant (*Artemisia campestris)* was applied *in vivo* (21 days) to rainbow trout juveniles in the treatment of *S. salmonis*. In another study conducted in Türkiye, Balta and Balta (1) reported that albendazole and metronidazole were the most effective *in vivo* treatments against *S. salmonis* in the rainbow trout juveniles. Metronidazole is the active compound of nitroimidazole used in the treatment of infections caused by anaerobic bacteria and protozoa, thus are prohibited from use in food-producing animals in member states in accordance with the European Union Commission Regulation No 37/2010. Since angelfish is not used for human consumption, it was recommended metronidazole treatment to the ornamental fish company officials. After oral metronidazole treatment at a dose of 50 mg/kg fish daily for 5 days, a decrease in fish mortality and resumed feeding were noted and further losses ended following parasite treatment.

Cream colored bacterial colonies were observed on TSA plates after 48h incubation at 20°C. Isolates were identified as Gram negative, motile, catalase positive, cytochrome oxidase negative, fermentative and resistant to O/129. Biochemical tests results are shown (Table 1). According to their morphological and biochemical characteristics, all isolated bacteria were identified as *Citrobacter freundii*. Likewise, in two other studies (6, 28), only the ability to degrade urea was found variable between bacterial isolates. Furthermore, we determined that all strains were erythromycin, oxytetracycline, sulfamethoxazole/trimethoprim and ampicillin resistant but sensitive to ciprofloxacin, enrofloxacin and intermediate sensitive to florphenicol (Table 2). These three antibiotics can thus be used in disease control. However, additional antibiotic treatment was not required because losses ended shortly after the treatment.

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Table 1. Morphological and phenotypical characteristics of *Citrobacter freundii*.

Morphology	B	Arginine Dihydrolase	+
Motility	+	Lysine Decarboxylase	-
Gram Staining	-	Ornithine Decarboxylase	-
Catalase	+	Degradation of Urea	V
Cytochrome Oxidase	-	ONPG	+
O/129 Resistance (150ig)	R	Esculin	-
O/F	F	Nitrate Reduction	+
Indole	-	Citrate	+
Voges Proskauer Reaction	-	Hemolysis Blood Agar	+
Methyl Red	+	TSI	+
Acid Production of			
Glucose	+	Trehalose	+
Cellobiose	+	Saccharose	+
Mannose	+	Fructose	+
Arabinose	+	Rhamnose	+
Growth on			
1,5% NaCl	+	40ºC	+

B: bacilli
+: positive reaction
-: negative reaction
F: fermentative
R: resistant
V: variable

Table 2. Antibiotic susceptibility of *C. freundii* according to disk diffusion test.

Antibiotic	**Test result**
Florphenicol	I
Oxytetracycline	R
Enrofloxacin	S
Ciprofloxacin	S
Ampicillin	R
Sulfamethoxazole/ trimethoprim	R
Erythromycin	R

I: intermediate
R: resistant
S: sensitive

In short, spironucleosis, which systemically affects fish by penetrating the intestinal mucosa, was identified as the cause of sustained mortality in freshwater angelfish. *Citrobacter freundii* was likely a secondary opportunistic pathogen associated with this case of chronic spironucleosis.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

REY and ET examined the samples and performed the lab work. REY, ET, and ST interpreted and reviewed the results. All authors contributed equally to the writing of the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Istanbul University Animal Experiments Local Ethics Committee (2016).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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Instruction to Authors

1. The Journal of the Faculty of Veterinary Medicine, Ankara University is a peer-reviewed general veterinary medical journal being published 4 times a year and its abbreviation is "Ankara Univ Vet Fak Derg".
2. The language of the journal is English.
3. Original research articles, reviews, case reports and short communications on all aspects of veterinary science, which had not been previously published elsewhere in whole or in part except abstract not exceeding 250 words, are published in the journal. Review articles are only be submitted by invitation.
4. Manuscripts (including footnotes, references, figure legends, and tables) should be prepared with the following attributes: 12-point Times New Roman, double-space typed, 3-cm ample margins, sequential line numbering, and A4 page size. Page numbers should also be written on the top-middle of each page except first page. Manuscripts including figures and tables should not be exceeding 30 pages for original research articles, 30 pages for review articles, 15 pages for case reports and short communications.
5. The manuscripts have to be submitted online from this web page: "vetjournal.ankara.edu.tr". Once a manuscript has been submitted electronically via online system, the order of authorship (including adding or removing authors) cannot be changed.
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   Title should be short and clear, and be written with small letters. Explanation/s regarding the study should be indicated as footnotes.
   Author/s should be indicated as first and last name. Last name/s should be written with capital letters.
   Abstract should be written as a single paragraph not exceeding 250 words.
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