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CONTENTS

Research Article

Case Report

Comparison of the fodder yield, nutritive value and cost of triticale and vetch mixtures under hydroponic condition

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ABSTRACT

This study aimed to determine the effects of different ratios of triticale and vetch seeds and the use of fertilizer on fodder yield, nutritive value, and the cost of grown fodder. The treatments included five ratios of triticale seeds to vetch seeds at 100:0, 90:10, 80:20, 70:30, and 0:100 (V0, V10, V20, V30, and V100, respectively) and a fertilizer treatment (with [+] or without [-] fertilizer). Considering yield performance, differences were found among treatments, with the highest yield (21.78 kg fresh weight m^{-2}) for V0+ treatment and the lowest (18.48 kg fresh weight m^{-2}) for V100- treatment. With increasing proportions of vetch seeds, ash, the content of crude protein, and acid detergent fiber increased linearly, whereas neutral detergent fiber and nitrogen-free extract decreased linearly. The content crude fiber was lowest (8.43%) for V0+ and highest (10.26%) for V100+ fodders, respectively. The highest neutral detergent fiber was observed for V0- fodder, while V100- fodder revealed the lowest neutral detergent fiber. Acid detergent fiber was lowest for V0+ fodder compared with all other treatments. The nitrogen-free extract content in V0+ fodder was gradually higher than that of other fodders. The fodder's energy value was significantly different among the treatments. The study results showed that the seed cost was the highest in the V0+ fodder to produce one kg fresh fodder. The higher expense for one kg CP and one unit of energy (MJ kg^{-1}) was obtained in V0- fodder as well. Taking those factors into account, the most profitable fodder seems to be vetch fodder grown individually or in combination with triticale (V30).

Introduction

The hydroponic system allows for the growth of plants without soil (31). It can be employed for green fodder production in a hygienic environment without the use of chemicals such as herbicides, insecticides, and fungicides. First discovered in 1860 in England and referred to as "Nutriculture," it emerged as an alternative to conventional agriculture. Agricultural and livestock businesses have been utilizing soilless agriculture equipment to maximize productivity (18, 33). A hydroponic production chamber for green fodder is a closed system where environmental parameters, including temperature, humidity, lighting values (1000 to 1500 microwatts cm^{-2}), pH, and electrical conductivity (EC) values of water, and the irrigation system, can be adjusted to desired values. These parameters are essential for faster and healthier plant growth (18). Hydroponic systems or incubation rooms do not require soil or nutrient mediums for green fodder growth. Optimal temperature, humidity, and light are necessary for improved germination, making green feed production much more manageable (22, 33). After germination, the roots interlock, forming a carpet-like structure, with stems reaching a height of 21-24 cm in 7-9 days, producing 7-9 times more fresh green fodder than the initial seed weight (31, 33). Soil-planted crops demand more time, tools, labor, and extensive field areas, and their growth can be affected by climate change. Utilizing hydroponic systems provides a simple solution for producing grass fodder in a few days to meet ruminant nutritional needs (31). Some researchers have reported

that the nutritional values of hydroponically grown green fodder are higher after 7 days of growth (2, 30). As fiber increases, energy levels and organic matter content decrease linearly after 7-8 days from planting (14, 15, 20). Throughout the sprouting process, the conversion of starch to sugar alters the nutritive value of fodder, resulting in an increase in ash and crude protein content, a decrease in dry matter content, and starch levels (29). Factors such as fertilization, temperature, seed quality, moisture, and seed density can influence the quality and yield of green fodder (31). It is worth noting that fungal and mold proliferation may occur due to the high water content of green fodder produced in hydroponic systems (31). On the other hand, there could be a challenge in using green fodder with low dry matter content for animal nutrition (17). While barley is commonly used in hydroponic systems (12-14, 21), studies on the nutritional values and plant heights of green fodders produced from other crops, such as sorghum, wheat (4), or oats (17), exist. However, few studies have focused on the comparison of green fodder performance of triticale and vetch seeding in a hydroponic system, and limited data is available on their nutritive value. Therefore, the aim of the study was to compare the fodder yield, nutritive value, and cost of triticale and vetch mixtures seeded in different ratios in a hydroponic system.

Materials and Methods

The experiment was conducted in a stainless steel hydroponic chamber at the Agriculture, Livestock, and Food Research and Application Center of Burdur (Southern Turkey, 30° 53' E, 36° 53' N and 950 m above sea level) Turkey in 2020. The intensive hydroponic system was constructed using a steel stand with dimensions of 2.80 m × 9 m × 7 m (H × L × W), equipped with seven shelves having a total capacity of 196 polyethylene trays (70 × 30 × 5 cm; 0.21 m^2). Hydroponic conditions were maintained with a temperature of 18-19 °C, relative humidity of 60%, a lighting time of 12 hours using yellow-colored lights, and irrigation every 2 hours for 90 seconds throughout the research period. The growing period lasted for 7 days after seed planting. The production chamber, trays, irrigation system, and necessary tools were sterilized with 10 % formaldehyde before planting. To mitigate the risk of mold formation, 50 ml of sodium hypochlorite (%20) was added to the irrigation water daily. Tap water was used for irrigation.

Triticale and vetch seeds were mixed at ratios of 100%:0%, 90%:10%, 80%:20%, 70%:30%, and 0%:100% (V0, V10, V20, V30, and V100, respectively). The experiment followed a completely randomized design with a 5 × 2 factorial treatment arrangement (five seed ratio treatments and two fertilizer treatments, with (+) and without (-)), with four replications. A total of 40 trays (five seed treatments x two fertilizer treatments x four replicates) were used in the hydroponic chamber. Liquid fertilizer NPK 8% N (1/8 Ammonium N, 1/8 Nitrate N, and 6/8 Urea N), 4% P_2O_5, and 3% K_2O (8:4:3) was applied at the rate of 10 ml into each tray per day, and each treatment received 350 ml of liquid fertilizer during the study. Seeds were pre-soaked in water separately for 24 hours to clean them from impurities, straw, etc., and to accelerate germination. In this study, seeds absorbed an average of 70% of their weight in water before planting, with an average of 1.7 kg of seed applied per tray (17). The dry seeding rate applied was approximately 1000 g per tray before soaking. The trays were placed on the shelves of the hydroponic chamber and allowed to grow for 7 days. At the end of the study, all trays were weighed to determine fresh weight (FW) and dry weight (DW) of fodder. The obtained dry fodder (kg) was divided by dry seed (kg) to determine the fodder produced (kg) per one kg dry seed (df/ds).

Feed samples were analyzed based on the AOAC (7) method for dry matter (DM, method 934.01), ash (method 942.05), ether extract (EE, method 920.39), and N (method 954.01) contents. ADF, NDF, and CF content were determined following the ANKOM (Ankom200 fiber analyzer, Ankom Technology Corp., Fairport, NY) methods. Non-fibrous carbohydrates (NFC =100 - (%NDF + %CP + %EE + %Ash) and nitrogen free extract (NFE= 100 - (%CF + %EE + %CP + %Ash) were calculated according to the standards of the National Research Council equation. The energy value of fodder was calculated according to Kirchgessner and Kellner (23) and MAFF (24). The unit production cost was calculated by using seeds and fertilizer expenses in the produced fodder under the same conditions, essentially dividing the seed plus fertilizer cost ($) by the produced fodder (kg ha^{-1}). $1 US Dollar is equal to 13.80 Turkish lira.

Statistical Analysis: A two-way ANOVA was applied using general linear model procedures of SPSS 22 (19) to compare fodder yield, nutritive value, and production cost of green fodder across treatments. Significant differences between means were calculated using the Tukey HSD test at α=0.05. The data were analyzed by independent-samples t-test to compare the nutritive value of both crop seed and their fodder.

Results

The effect of fertilizer and treatments on the FW (kg per tray) was significant ($P<0.001$). The highest FW (kg m^{-2}) was obtained by V0+ and V10+, while the lowest value was observed for V100- and V100+ ($P<0.001$). There was a significant treatment and fertilizer interaction for df/ds

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(P<0.05) and DM loss (P<0.01). The df/ds ratio ranged from 0.90 to 1.06 among all treatment groups. The dry fodder per unit dry seed (df/ds) was highest for V100+. The highest DM loss was observed in V20+, while the highest DM recovery was in V100+ (Table 1).

The nutritive values of green fodder are listed in Table 2. The content of crude protein (CP), ether extract (EE), and nitrogen-free extract (NFE) was significantly affected by seed ratio treatments, fertilizer addition, and their interaction (P<0.001). The fodders treated with

Table 1. Fodder yield from different ratios of triticale and vetch seeds with or without fertilizer grown in a hydroponic system.

Parameters[1]	F[2]	Treatments[3] (%)					SEM[4]	Impact[5]		
		V0	V10	V20	V30	V100		T	F	T×F
FW, kg/tray	-	4.52^{a}	4.48^{a}	4.46^{a}	4.50^{a}	3.89^{b}	0.02	***	***	ns
	+	4.57^{a}	4.53^{a}	4.51^{a}	4.54^{a}	3.95^{b}				
DW, kg/tray	-	0.85	0.90	0.88	0.91	0.87	0.03	ns	ns	ns
	+	0.94	0.89	0.84	0.89	0.99				
FW, kg m^{-2}	-	21.51^{a}	21.35^{a}	21.26^{a}	21.43^{a}	18.48^{b}	0.09	***	***	ns
	+	21.78^{a}	21.58^{a}	21.49^{a}	21.63^{a}	18.79^{b}				
DW, kg m^{-2}	-	4.00	4.30	4.18	4.35	4.19	0.15	ns	ns	ns
	+	4.49	4.24	4.01	4.24	4.71				
df/ds; kg/kg	-	0.91	0.97	0.94	0.98	0.94	0.04	ns	ns	*
	+	1.02	0.96	0.90	0.96	1.06				
DMloss, %	-	9.05-	2.75-	5.58-	1.97-	6.07-	2.20	ns	ns	**
	+	1.96+	4.12-	9.50-	4.28+	6.26+				

ns: not significant; *P<0.05; **P<0.01; ***P<0.001
[1]FW: Fresh weight, DW: Dry weight, df/ds: Dry fodder kg per dry seed kg, DMloss: Dry matter losses
[2]Fertilizer addition; 8 ml N (1 ml Ammonium N, 1 ml Nitrate N and 6 ml Urea N), 4 ml P_2O_5 and 3 ml K_2O each tray/per day
[3]Triticale and vetch was mixed at a ratio of 100:0 (V0), 90:10 (V10), 80:20 (V20), 70:30 (V30), and 0:100 (V100)
[4]SEM: Standard error mean
[5]T: Treatments, F: Fertilizer, T×F: The interaction between treatments × fertilizer

Table 2. Nutritive value of fodder from different ratios of triticale and vetch seed with or without fertilizer grown in a hydroponic system (DM basis, %).

Parameters[1]	F[2]	Treatments[3] (%)					SEM[4]	Impact[5]		
		V0	V10	V20	V30	V100		T	F	T×F
DM%	-	18.60	20.10	19.70	20.30	22.49	0.69	ns	***	*
	+	20.60	19.60	18.50	19.60	24.99				
Ash%	-	2.16^{c}	2.18^{c}	2.25^{bc}	2.40^{b}	3.00^{a}	0.05	***	ns	***
	+	1.98^{d}	2.19^{cd}	2.36^{bc}	2.52^{b}	3.15^{a}				
CP%	-	13.74^{c}	14.47^{bc}	14.49^{bc}	15.30^{b}	21.34^{a}	0.31	***	**	***
	+	11.94^{d}	14.29^{c}	16.20^{b}	17.03^{b}	22.86^{a}				
EE%	-	1.94^{ab}	1.86^{ab}	1.60^{b}	2.27^{a}	1.66^{b}	0.14	***	***	**
	+	2.51^{ab}	2.96^{a}	1.69^{c}	2.31^{abc}	1.84^{bc}				
CF%	-	8.97	9.04	9.38	9.12	8.99	0.39	ns	*	ns
	+	8.43	9.83	10.17	10.18	10.26				
ADF%	-	11.01^{b}	11.15^{b}	12.74^{ab}	13.56^{b}	14.16^{a}	0.53	***	ns	ns
	+	10.58	11.99	13.10	13.11	13.26				
NDF%	-	26.78^{a}	24.39^{ab}	25.91^{ab}	23.92^{b}	17.03^{c}	0.07	***	ns	ns
	+	24.68^{a}	25.00^{a}	24.21^{a}	22.96^{a}	18.65^{b}				
NFE%	-	73.12^{a}	72.51^{a}	72.33^{a}	70.91^{a}	63.48^{b}	0.68	***	*	**
	+	75.23^{a}	70.72^{b}	69.64^{b}	67.92^{b}	63.41^{c}				
NFC%	-	55.31	57.13	55.75	56.11	55.28	0.59	ns	ns	ns
	+	58.92	55.51	55.42	55.13	55.03				

ns: not significant; *P<0.05; **P<0.01; ***P<0.001
[1]DM: Dry matter, CP: Crude protein, EE: Ether extract, CF: Crude fiber, ADF: Acid detergent fiber, NDF: Neutral detergent fiber, NFE: Nitrogen free extract, NFC: Non-fiber carbohydrate
[2]Fertilizer
[3]Triticale and vetch was mixed at a ratio of 100:0 (V0), 90:10 (V10), 80:20 (V20), 70:30 (V30), and 0:100 (V100)
[4]SEM: Standard error mean
[5]T: Treatments, F: Fertilizer, T×F: The interaction between treatments × fertilizer

fertilizer had higher means of CP (16.46 vs. 15.86% DM) and means of EE (2.26 vs. 1.87% DM) but a lower concentration of means of NFE (69.37 vs. 70.44% DM) compared with fodders without fertilizer. A non-significant effect (P>0.05) of the treatment was observed on the DM, CF, and NFC. Lower fodder DM, on average, was observed in fodders without fertilizer than in fodders with fertilizer, but the differences were not significant (20.23 vs. 20.65; P=0.927). The highest CF content was found in V100+ fodder, and the lowest was in V0+ fodder. There was a significant increase in the ash content (P<0.001) with V100+ fodder having the highest ash content among treatments. Neither fertilizer addition nor the interaction of seed ratio and fertilizer affected ADF and NDF content (P>0.05). However, an effect of seed ratio on ADF (P<0.001) and NDF (P<0.001) contents was observed, with ADF increasing and NDF decreasing with a greater vetch seed ratio. The fodder energy content had a remarkable response, with the highest and lowest energy contents obtained from V0+ fodder and V100- fodder, respectively (Table 3).

Applied treatments and fertilizer addition showed a significant effect on the production cost of dry fodder (DF-c), fresh fodder (FF-c) without fertilizer, ranging from 0.249 to 0.431 and 0.056 to 0.080 $ for one kg fodder production fodder, respectively (Table 4). The production cost per kg of protein (CP-c) and per energy unit (ME-c) increased with the addition of fertilizer. When comparing the nutritive values of fodder and seed, the green fodder had higher CP and fiber value than its seeds, which had higher significantly NFE and NFC content (Table 5).

Table 3. Energy content of fodder from different ratios of triticale and vetch seed with or without fertilizer grown in a hydroponic system (DM basis, %).

Parameters[1]	F[2]	Treatments[3] (MJ/kg)					SEM[4]	Impact[5]		
		V0	V10	V20	V30	V100		T	F	T×F
ME_{CF}	-	12.51	12.50	12.45	12.33	12.29	0.06	ns	*	ns
	+	12.59	12.38	12.33	12.49	12.45				
ME_{ADF}	-	13.05^{a}	13.03^{a}	12.79^{ab}	12.67^{b}	12.58^{b}	0.09	***	ns	ns
	+	13.11	12.90	12.74	12.73	12.90				
ME_{CF+ADF}	-	12.87^{a}	12.84^{a}	12.50^{b}	12.27^{bc}	12.11^{c}	0.09	***	**	**
	+	12.91	12.73	12.50	12.50	12.77				
ME_{MAFF}	-	12.93^{a}	12.91^{a}	12.82^{a}	12.78^{a}	12.41^{b}	0.05	***	*	***
	+	13.15^{a}	13.03^{a}	12.72^{b}	12.92^{b}	12.71^{b}				

ns: not significant; *P<0.05; **P<0.01; ***P<0.001; ME_{CF}: calculated by using crude fiber ME_{CF+ADF}: Calculated by using crude fiber and acid detergent fiber; ME_{ADF}: Calculated by using acid detergent fiber: Calculated by Ministry of Agriculture, Forestry and Fisheries (ME_{MAFF}, MJ/kg DM = 0.12%CP + 0.31%EE + 0.05%CF + 0.14%NFE).
[1]ME: Metabolic energy, MJ kg^{-1}
[2]Fertilizer
[3]Triticale and vetch was mixed at a ratio of 100:0 (V0), 90:10 (V10), 80:20 (V20), 70:30 (V30), and 0:100 (V100)
[4]SEM: Standard error mean
[5]T: Treatments, F: Fertilizer, T×F: The interaction between treatments ×fertilizer

Table 4. Economic analyses of fodder production cost.

Parameters[1]	F[2]	Treatments[3] (%)					SEM[4]	Impact[5]		
		V0	V10	V20	V30	V100		T	F	T×F
DF-c	-	0.431^{a}	0.388^{ab}	0.380^{b}	0.351^{b}	0.249^{c}	0.07	**	*	ns
	+	0.537^{a}	0.555^{a}	0.565^{a}	0.518^{a}	0.362^{b}				
FF-c	-	0.080^{a}	0.077^{b}	0.074^{c}	0.071^{d}	0.056^{e}	0.01	**	**	ns
	+	0.110^{a}	0.102^{d}	0.105^{c}	0.108^{b}	0.091^{e}				
CP-c	-	0.066^{a}	0.056^{b}	0.055^{b}	0.048^{c}	0.025^{d}	0.02	*	*	**
	+	0.095^{a}	0.082^{b}	0.073^{bc}	0.064^{c}	0.033^{d}				
ME-c	-	0.033^{a}	0.031^{ab}	0.028^{b}	0.030^{b}	0.019^{c}	0.01	*	**	ns
	+	0.043^{a}	0.041^{a}	0.042^{a}	0.041^{a}	0.029^{b}				

ns: not significant; *P<0.05; **P<0.01; ***P<0.001
[1]DF-c: Production cost of dry fodder ($/kg ha^{-1}), FF-c: Production cost of fresh fodder ($/kg ha^{-1}), CP-c: Cost of producing one kg of protein ($/kg ha^{-1}), ME-c: Cost of producing one unit of energy ($/MJ ha^{-1}) by calculated using ADF content of fodder
[2]Fertilizer
[3]Triticale and vetch was mixed at a ratio of 100:0 (V0), 90:10 (V10), 80:20 (V20), 70:30 (V30), and 0:100 (V100)
[4]SEM: Standard error mean
[5]T: Treatments, F: Fertilizer, T×F: The interaction between treatments × fertilizer

Table 5. The comparison of the nutritive value of triticale and vetch seeds and their fodder (DM basis, g kg^{-1}).

Parameters[1]	Nutritive value[2]								
	DM	Ash	CP	EE	CF	ADF	NDF	NFE	NFC
TS	928.9	19.1	103.7	10.3	37.7	55.2	140.8	829.4	726.5
TF	186.0	22.5	137.4	19.4	89.7	110.1	267.8	730.1	553.8
P-value	***	ns	**	**	*	***	***	***	***
VS	931.3	29.1	197.1	15.2	50.6	103.0	159.6	707.1	599.0
VF	224.9	30.0	213.4	16.7	89.9	141.6	172.0	650.0	568.0
P-value	***	ns	**	*	***	***	***	**	***

ns: not significant; *P<0.05; **P<0.01; ***P<0.001

[1]TS: Triticale seeds, TF: Triticale fodder (V0-without fertilizers), VS: Vetch seeds, VF: Vetch fodder (V100-without fertilizers)

[2]DM: Dry matter, CP: Crude protein, EE: Ether extract, CF: Crude fiber, ADF: Acid detergent fiber, NDF: Neutral detergent fiber, NFE: Nitrogen free extract, NFC: Non-fiber carbohydrate.

Discussion and Conclusion

The inadequacy of good quality roughage in Türkiye has prompted the development of alternative methods. An alternative to conventional agriculture is the use of the hydroponic system, which allows the production of green fodder throughout the year irrespective of the climate changes (27). After 7 days of harvesting, the highest FW in V0+ fodder and DW in V100+ fodder were recorded at 4.57 and 0.99 kg per tray. The lowest FW and DW (kg m^{-2}) were obtained for V100- and V0- fodder, respectively. Higher DW was mainly due to the higher DM content in V100+ fodder. Özdemir and Temür (28) stated that a barley and vetch mix produced the lowest fresh yield but also produced the highest DM yield due to the mix having a higher DM concentration. The outcome of the present research agrees with those of Al-Karaki and Al-Momani (5), who reported that the FW of barley fodder ranged from 3.74 to 6.0 kg per kg of barley seeds. Gümüş and Bayır (17) showed that oat fodder had lower FW than barley-oat fodder. The DW of fodder ranged from 4.00 to 4.49 kg m^{-2}. Our study was comparable to those reported by Emam (12) and Assefa et al. (6), who obtained 7-8 kg of dry barley fodder and 4.58-6.63 kg of dry maize fodder per m^{-2}. Lower fodder FW, on average, was observed in fodders without fertilizer compared to those with fertilizer, but the differences were small (21.38 vs. 21.62 kg m^{-2}). The winter term generally lasts for 5 months in Türkiye, depending on topography (9). Assuming that quality fresh forage cannot be produced during winter, if fodder was produced in 7-day harvest cycles over 5 months using the current hydroponic chamber (41.16 m^2 of total trays), then about 17 tons of fresh triticale or triticale + vetch mixed fodder could be produced. A low level of DM in green fodder may limit its use for animal nutrition (3). However, it is worth pointing out that high-moisture forage such as pasture, silage, haylage, fodder, etc., have been consistently used in the ration as forage regardless of their DM content (17). The recommended (10, 32) optimal DM content of rations should be more than 45% and less than 60%. As higher DM content can reduce the ration intakes by dairy cattle (8). The present study showed that the fodder DM content was the highest (24.99%) for V100+ fodder and the lowest (18.5%) for V20+ fodder. Almost similar results were also found by researchers evaluating barley or oat fodder (12, 17). The reduction seen in DM content in V20 treatment along with an increase in DM loss is explained by the fact that the increment of germination of fodder results in a greater conversion of seeds and greater shoot height and root length. The results of our previous studies showed that the highest mean shoot height and root length value of fodder were recorded for V20 (3).

Regarding the nutritive value of fodder, the ash content increased for all treatments compared to the initial seeds. Similar results were obtained by Saidi and Omar (30) and Emam (12). The former stated that the ash content in the fodder was improved by using hydroponic production, while the latter stated that the ash content ranged from 2.27 to 3.43%. The reason for the increased ash content might be enhanced by root mineral uptake (26). Fazaeli et al. (13) suggested that the calcium, iron, and zinc content of fodder were significantly higher than that of original seeds, and it could be associated with higher ash content also due to the concentrating effect of the starch and sugars for growth, while the minerals are not lost. As expected, the highest CP content was found for V100+ (22.86%), probably due to its seeds having higher CP content than the other seeds and the addition of nitrogen increasing the protein content. The mean CP content in the fodder of V30+, V20+, V10+, and V0+ treatments were stated on level 17.03%, 16.2%, 14.29%, and 11.94% respectively. Our findings were in line with the results of Fazaeli et al. (14) who found that the CP content of barley fodder ranged from 13.7% to 14.5%. Contrary to the current study, Saidi and Omar (30) showed CP was 19.8% in barley fodder. It is worth stating that the findings were inconsistent possibly due to differences in research conditions, seed type, seed quality, addition of liquid fertilizer, and harvesting time. A study by Akbağ et al. (2) reported the effects of harvesting time on fodder CP,

with CP slightly higher at day 13 (17.6%) than at day 7 (17.1%), but lower than at day 10 (18.2%). One possible reason for higher protein content in the fodder might be the greater photosynthesis ability of young sprout and its higher DM losses (33). The results of El-Morsy et al. (11) indicated that an increased CP is associated with the positive effect of germination on enzyme activity, which leads to changes in the amino acid profile. Adding fertilizers, especially nitrate and ammonium, can improve the CP content of the fodder (16). In the current study, the fodders treated with fertilizer had a higher concentration of CP (16.46 vs. 15.86% DM) compared with fodders without fertilizer. The highest ether extract (EE) value was recorded in V10+ (2.96%) fodder, followed by V0+ (2.51%), V30+ (2.31%), V100+ (1.84%), and V20+ (1.69%) fodder in the current study. Gümüş and Bayır (17) found that the EE values of barley fodder and oat fodder were 3.22% and 5.23% of DM, respectively. However, ether extract of hydroponic fodder increases due to the increment of structural lipids and chlorophyll as the plant grows (16). Regarding the structural carbohydrates of fodder, it was expected that the CF, ADF, and NDF content of fodder would be higher compared to its seeds. The highest CF value was found as 10.26% of DM in V100+ fodder and the lowest was as 8.43% of DM in V0+ fodder. Emam (12) found that CF values of barley fodder ranged from 8.13 to 12.4% of DM which is similar to our results. These results are also in agreement with Fazaeli et al. (13), who reported that CF, ADF, and NDF content are increased, but NFC contents decrease in fodder compared to the seeds. For the fodder ADF, the highest value was found for V30- (13.56% of DM) and the lowest value was found as V0+ (10.58% of DM). Al-Karaki and Al-Monami (5) also obtained similar findings for barley fodder with the authors stating the fodder had better quality than alfalfa hay in terms of their use in animal nutrition. Fazaeli et al. (14) reported that structural carbohydrates increased due to late harvesting time. The mean value of NDF (14) with barley fodder ranged from 31.25% to 35.40% which is higher than the current study's range (17.03 - 26.78% of DM). Saidi and Omar (30) reported that the NDF content was 3.5 times higher in barley fodder compared to barley seeds which is greater than differences between fodder and seed in the current study. Also, fodder energy content was affected by seed ratios regardless of calculation methods with slightly lower energy contents as vetch seed share increased. Emam (12) stated that green fodder is highly digested by ruminant animals and has a greater protein and metabolic energy content. Afzalinia and Karimi (1) showed that the barley cultivar Behrokh had the highest energy productivity compared to the other cultivar due to its lower energy use during the growth process. As indicated by Girma and Gebremariam (16) during germination, the plant converts starch into sugars during respiration and therefore, a decreased energy content in fodder is expected as compared to grains.

Regarding the cost of production, V100- fodders had the lowest cost to produce one kg dry or fresh fodder among the groups. Assefa et al. (6) found that the cost of dry and fresh barley fodder ranged from $31.06 to $42.54 and $4.55 and $6.90 per 100 kg fodder, respectively. In the current study, the lowest ($0.025) and highest ($0.095) seed cost for the production of one kg CP of dry fodder was obtained for V100- and V0+ fodders. Compared to the present study, a lower cost per unit CP was found for pasture grass produced in a hydroponic chamber (6). The seed cost for energy production (ME/MJ) ranged from $0.019 and $0.043. Our results were in line with the results of Assefa et al. (6) who showed that energy pasture grass hay costs were $0.02 per MJ/ME.

In conclusion, the mixing of vetch seeds with triticale apparently changed the nutritive value of fodder and increased the DM, CP, CF, and ADF. The current results confirmed that the addition of vetch seeds can effectively decrease the FW of fodder. The highest DM loss was recorded in V20+ fodder with a value of 9.5%. The DM gain was observed in V0+ and V100+ fodder with fertilizer. The cost of dry fodder in the fertilized group was higher compared to the non-fertilized group. Further studies are required to investigate the use of hydroponic systems to produce green fodder from various seeds and mixture ratios in a hydroponic chamber taking into account economic efficiency.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Experimental design was created by MA and HG. Samples were collected by MA and HG led the manuscript writing. All authors have contributed to the revision of the manuscript.

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Evaluation of von Willebrand factor in dogs with parvoviral enteritis

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ABSTRACT

Canine parvoviral enteritis may lead to coagulopathy in various ways. In recent years, the importance of the von Willebrand factor has become the focus of more attention in infectious diseases. This study aimed to determine the significance of von Willebrand factor level and coagulation parameters in dogs with parvoviral enteritis. The experimental group of this study consisted of 20 dogs with parvoviral enteritis of different breeds aged 2-6 months, and the control group consisted of 10 healthy dogs aged 2-6 months. Blood samples were taken from the dogs included in the experimental group at the 0th hour, 24th hour and before discharge, and only at the 0th hour from the healthy puppies in the control group The 0th and 24th hour von Willebrand factor values of the trial were significantly higher than the control group. The 0th hour Prothrombin Time of the trial was significantly prolonged compared to the control group. The 0th hour Activated Partial Thromboplastin Time value of the trial was significantly prolonged compared to the before-discharge and control group. The before-discharge fibrinogen level was considerably lower than at 0th and 24th hours of the trial. In conclusion, von Willebrand factor concentrations, which increase significantly in dogs with parvoviral enteritis compared to healthy animals, can be evaluated as an acute phase protein. Prolongation of Prothrombin Time and Activated Partial Thromboplastin Time and no significant change in fibrinogen and D-dimer values demonstrated that dogs with parvoviral enteritis were in the hypercoagulation state without disseminated intravascular coagulation.

Introduction

Canine Parvovirus infection (CPV) is caused by two types of parvovirus; canine parvovirus type-1 (CPV-1) and canine parvovirus type-2 (CPV-2). CPV-1 or known as canine minute virus, are genetically and antigenically completely different from CPV-2. CPV-1 has low pathogenicity in dogs and can cause gastroenteritis, pneumonia or myocarditis, in puppies 1-3 weeks old. CPV-2 (or killer virus, infectious enteritis virus, intestinal flu virus or puppy heart virus) causes vomiting and bloody diarrhoea in puppies aged six weeks to 6 months. (1, 2, 7, 8, 15, 19, 26).

Clinical parvovirus infections have two clinical manifestations. These are the acute forms of hemorrhagic enteritis and myocarditis. Fetal contamination plays an important role in myocarditis form. Sudden death may occur in newborns infected with CPV shortly after birth as a result of interstitial myocarditis and congestive heart failure. Because the cardiac muscle cells of neonatal animals have high mitotic activity, and the virus directly infects the cardiac muscle cells (4, 5, 21).

The most characteristic clinical form of canine parvoviral enteritis is reported as hemorrhagic enteritis (8, 9, 11, 20, 22, 24). When Parvoviruses infect the germinal epithelium of intestinal crypts, they cause epithelial destruction and villous collapse. Bloody diarrhoea occurs in the small intestine as a result of impaired cell destruction leading to the characteristic pathological lesions that are shortened and atrophic. Clinical signs of the disease usually appear after an incubation period of 3-7 days. In cases with hemorrhagic enteritis, clinical signs are anorexia, depression, vomiting, mucoid or bloody diarrhoea, dehydration, and fever (1, 8, 9,20).

Von Willebrand factor (vWF) plays a crucial role in coagulation, platelet adhesion, and thrombus formation. vWF is a high molecular weight multimeric glycoprotein. This factor is a protein that provides the adhesion of platelets to the sub-endothelial tissue and thrombus formation. It also acts as a carrier for factor VIII. In addition, it has been found to increase in inflammation by acting as an acute phase protein in recent years. (6, 12).

One of its two most important roles in hemostasis is to help the adhesion and aggregation of platelets on the damaged vessel wall and the other is to transport factor VIII to the damaged area. vWF is required for platelet adhesion to the capillary vessel wall and subsequent aggregation. Therefore, in the VWF deficiency, bleeding occurs in the capillary-rich mucosal surface and skin (14, 17).

The present study was designed with the hypothesis that CPV may lead to changes in vWF concentrations in infected dogs. This study evaluated vWF factor, a disintegrin and metalloprotease with thrombospondin type motifs-13 (ADAMTS-13) and coagulation parameters in dogs with parvoviral enteritis.

Materials and Methods

Animal Material: This study consisted of 20 dogs with parvoviral enteritis (experimental group) and ten healthy dogs (control group) aged between 8-22 weeks admitted to the Department of Internal Medicine, Faculty of Veterinary Medicine, Selcuk University. Ethical approval (2017/011) was obtained from the Selcuk University Faculty of Veterinary Medicine Ethics Committee (SÜVFEK).

Clinical Examination: Anamnesis, physical and laboratory examination findings were recorded in dogs admitted to the animal hospital with complaints of vomiting, anorexia and bloody diarrhoea. The faecal rapid antigen test (Asan Easy Test PARVO, Asan Pharma, CO. LTD. Gyonggi-do Korea) was applied to the animals suspected of parvoviral infection. Dogs with positive test results were included in the study. During the routine clinical examinations of the dogs with parvoviral enteritis; body temperature, heart and respiratory rates, mucous membrane colour, peripheral pulse quality, hydration and mental status were recorded. Routine clinical and laboratory (hemogram, blood gases and serum biochemistry) examinations and faeces test were applied to dogs. Dogs with normal clinical, laboratory findings and negative faeces parvovirus antigen test were considered healthy and were included in the control group.

Collection, storage and analysis of blood samples: Blood samples were taken from the anterior leg vena cephalica antebrachii, with anticoagulant (K3-EDTA), sodium-citrate and heparin) and without anticoagulant, before treatment (0^{th} hour), 24^{th} hours after treatment and before discharge. Hemogram and blood gas analyses were immediately performed. Melet Schloesing MS4-e Hematology Analyzer (France) was used for hemogram analysis. ABL 90 flx Radiometer (radiometer medical ApS Aakandevej 21, DK-2700 Bronshoji, Denmark) was used for blood gas analysis.

Measurement of vWF and coagulation profile: Blood samples were centrifuged at 5000 rpm for 5 minutes. Plasma samples were stored at -20°C freezer until the measurement for vWF, activated partial thromboplastin time (APTT), prothrombin time (PT), D-Dimer and fibrinogen. Intra-assay (within-assay) and inter-assay (inter-assay) coefficient of variation (CV) reported for von Willebrand factor <8% and <12%, respectively, detection range 0.15-70 ng Intra-assay and inter-assay coefficient of variation reported for /mL (vWF, (Catalog # MBS286695, Lot # L06RVW25, My BioSource, USA). Canine specific vWF was measured in ELISA devices with commercial test kits. Coagulation profiles were PT (catalogue no: OUHP-49, Siemens Innovin Germany), APTT (catalogue no: OQGS-29 Actin Germany), D-dimer (catalogue no: OPBP-03, Innovance) d-dimer Germany) and fibrinogen (catalogue no: OWZG-15, Multifibrin U Germany) levels were also measured in a Sysmex CA 150 device (Siemens) in a private laboratory.

Treatment Protocol: Dogs with parvoviral enteritis were taken to the infection or intensive care unit according to the severity of the disease and hospitalised for 5-8 days. Treatment was initiated following the detection of parvovirus in the test results.. For those with low blood pH, 0.9% NaCl (Poliflex 0.9% isotonic sodium chloride, Polifarma) 20 mL/kg intravenously, Ringer's lactate (Poliflex lactated Ringer's, Polifarma) 65 mL/kg/ and 5% dextrose (Poliflex 5% dextrose, Polifarma) 5mL/kg intravenously was applied as a standard. The patients were given b complex and amino acid solutions (Duphalyte, Zoetis) and vitamin C (Vita C Vetoquinol) as supportive treatment. In addition, Metoclopramide (Metpamid, Recordanti) at a dose of 0.03 mg/kg intravenously every 8 hours or as a continuous infusion at a dose of 0.1-0.2 mg/kg/day intravenously and Ranitidine (Ranixel, Menta Pharma) 1-5 mg/kg intravenously given every 8 hours were given to patients. Cefazolin (Iespor®, İbrahim Ethem, Ulagay or Equizolin®, Tüm-Ekip İlaç AŞ) 22 mg/kg intravenously, TID and Enrofloxacin (Baytril® Bayer) 5 mg/kg intravenously, SID, combination was administered to patients with fluid therapy. The recovered dogs were discharged from the hospital.

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Statistical Analysis: SPSS 25 (IBM Corp®, 2017, Armonk, NY, USA) statistical program was used to evaluate the data. The Kolmogorov–Smirnov test determined the variables' normality and the variances' homogeneity. Since the variables do not have a normal distribution, the study data are presented as median (min/max) and were evaluated using the Friedman test. Statistical significance was considered as $P<0.05$.

Results

Clinical Examination Findings: At the first clinical examination, all patients had complaints of anorexia and stagnation. During the first clinical examination of the patients, vomiting in 15 cases and hemorrhagic diarrhoea in 8 cases were detected. However, hemorrhagic diarrhoea and vomiting were observed in all patients after 1-2 days. Fever (>39.2°C) was detected in only 9 cases during the initial examination. At the initial examination, the degree of dehydration was >6% in 15 of the 20 dogs. Twenty four hours after first examination, the number of cases of hemorrhagic diarrhea increased to 17, the number of cases with vomiting increased to 19, and the number of cases with high body temperature increased to 10. While the body temperature of only one patient was low during the first examination, this number increased to 3 after 24 hours. Despite treatment, one patient died within the first 24 hours. Forty eight hours after examination, all cases had hemorrhagic diarrhoea and vomiting. During this time, a dog died. Vomiting disappeared in all 18 surviving dogs before discharge, and body temperature, appetite and stool returned to normal.

Hemogram and Blood Gases Findings: Although granulocyte values were significantly lower in the experimental group than in the control group at the 0^{th} and 24^{th} hours, they increased until discharge and were insignificantly lower than the control group. The erythrocyte values of the experimental group at the 0^{th} and 24^{th} hours were insignificant compared to the control group. However, the 0^{th} hour erythrocyte values of the experimental group were significantly higher than the before discharge values. The hematocrit values in the experimental group at the 0^{th} hour were significantly higher than those in the control group and before discharge. Before discharge hematocrit values were significantly lower than the 0^{th} and 24^{th} hours of the experimental group. Hemoglobin values of the experimental group at the 0^{th} and 24^{th} hours were significantly higher than those in the before discharge and control groups (Table 1).

Table 1. Hemogram values in dogs with parvoviral enteritis and healthy.

Parameter	Control Group	Experimental Group		
		0^{th} hour	**24^{th} hour**	**Before Discharge**
WBC ($x10^3$ /mm^3)	12.88 (6.93/21.75)	10.13 (2.28/9.04)	8.72 (1.12/22.30)	13.15 (4.16/38.54)
Lym ($x10^3$ /mm^3)	4.19 (2.50/8.80)	4.13 (1.27/15.65)	3.20 (0.97/12.98)	4.94 (0.87/22.5)
Mon ($x10^3$ /mm^3)	0.58 (0.25/1.48)	0.41 (0.04/4.35)	0.42 (0.03/5.44)	1.64 (0.15/7.39)
Gran ($x10^3$ /mm^3) ($P<0.02$)	**8.15 (2.19/12.08)^a**	**3.24 (0.21/18.77)^b**	**3.33 (0.12/9.05)^b**	**4.49 (1.87/11.18)^{ab}**
MCV (fl)	59.96 (53.3/65.4)	61.81 (55.9/68.2)	61.09 (53.6/67.8)	58.47 (52.8/64.9)
RBC ($x10^6$ /mm^3) ($P<0.03$)	**6.58 (5.61/8.22)^{ab}**	**7.32 (5.43/10.05)^a**	**6.77 (4.7/9.2)^{ab}**	**5.81 (3.85/7.6)^b**
HCT (%) ($P<0.01$)	**38.23 (33.1/46.1)^{bc}**	**45.03 (3.5/58.4)^a**	**41.18 (27.2/52.7)^{ab}**	**33.78 (23.9/43.3)^c**
MCHC(g/dl)	30.20 (28.7/31.8)	29.85 (28.10/58.90)	30.50 (27.9/49)	32.20 (21.10/37.80)
RDW	11.46 (9.6/13.8)	12.67 (9.4/23.6)	12.68 (9.4/23.6)	13.58 (9.7/24.9)
Hb (g/dl) ($P<0.05$)	**11.62 (9.5/13.9)^{ab}**	**13.42 (9.1/22.7)^a**	**13.50 (8.3/25.2)^a**	**10.82 (7.9/13.4)^b**
THR ($x10^3$ /mm^3)	313.30 (136/514)	425.10 (103/898)	348.21 (40/880)	281.94 (47/598)

The blood pH value at the 0th hour was found to be significantly lower than before discharge. Before discharge, the potassium (K^+) value was significantly lower than the control group. The sodium (Na^+) value at the 0th hour was found to be statistically significantly lower than before discharge. The before discharge calcium (Ca^{++}) concentration was also significantly lower than the 0th and 24th hours and the control group. Blood glucose concentration at the 0th hour was statistically significantly higher than the before discharge values (Table 2).

vWF and Coagulation profile Findings: The experimental group's 0th and 24th hour vWF values were significantly higher than the control group. While experimental group's 0th hour PT and APTT values were significantly higher than the control group, the APTT value at the 24th hour of the experiment remained significantly higher than the control group. However, the PT before discharge value showed insignificant changes compared to the control group (Table 3.4). The fibrinogen concentrations before discharge were significantly lower than in the 0th and 24th hour, and the control group (Table 3).

Table 2. Blood gas values in dogs with parvoviral enteritis and healthy.

Parameter	Control Group	Experimental Group		
		0th hour	**24th hour**	**Before Discharge**
Ph (P<0.04)	**7.38 (7.34/7.41)^{ab}**	**7.34 (7.12/7.46)^{b}**	**7.38 (7.28/7.44)^{ab}**	**7.43 (7.38/7.48)^{a}**
K (mmol/L) (P<0.04)	**3.83 (2.8/4.5)^{a}**	**3.52 (2.6/4)^{ab}**	**3.57 (2.7/4.4)^{ab}**	**3.22 (2.2/4.3)^{b}**
Na (mmol/L) (P<0.03)	**149.80 (145/167)^{ab}**	**145.33 (139/157)^{b}**	**148.17 (139/165)^{ab}**	**153.92 (145/172)^{a}**
Ca (mmol/L) (P<0.04)	**1.19 (0.63/1.45)^{a}**	**1.02 (0.46/1.26)^{a}**	**1.00 (0.65/1.35)^{a}**	**0.77 (0.54/1.1)^{b}**
Cl (mmol/L)	110.80 (107/117)	106.44 (94/115)	107.52 (99/115)	111.14 (102/122)
Laktat (mmol/L)	1.38 (0.7/2)	1.83 (0.5/4.4)	1.68 (0.7/2.7)	1.30 (0.4/3)
BE (mmol/L)	-2.02 (-5.1/2.3)	-2.96 (-13/9.7)	-0.54 (-8/6.6)	-0.42 (-6.4/5.1)
Hco3 (mmol/L)	22.24 (19.6/25.5)	21.37 (13.8/31.6)	23.27 (18.2/29.2)	23.62 (19.1/27.7)

Table 3. Von Willebrand and coagulation parameters values in dogs with parvoviral enteritis and health.

Parameter	Control Group	Experimental Group		
		0th hour	**24th hour**	**Before Discharge**
vWF (ng/ml) (P<0.01)	**2.30 (0.1/5.04)^{b}**	**6.19 (0.1/13.96)^{a}**	**5.29 (0.1/16.23)^{a}**	**4.91 (2.12/9.05)^{ab}**
PT (sn) (P<0.01)	**9.43 (8/12.3)^{b}**	**13.68 (9.2/21.4)^{a}**	**12.62 (8/24)^{ab}**	**10.44 (8/12.60)^{ab}**
APTT (sn) (P<0.02)	**17.15 (14.90/37.10)^{a}**	**32 (22.50/40.30)^{b}**	**28.70 (15.80/105.40)^{bc}**	**17 (14.90/31.20)^{ac}**
D-DİMER (mg/dl)	1.20 (0.51/2.46)	1.18 (0.19/2.31)	0.92 (0.13/1.83)	1.38 (0.51/2.13)
FIBRINOJEN (mg/dl) (P<0.05)	**430.29 (120/684)^{ab}**	**518.14 (334/684)^{b}**	**492.08 (312/684)^{b}**	**276.43 (179/359)^{a}**

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Discussion and Conclusion

In canine parvoviral infections, leukopenia due to neutropenia (granulocytopenia) and lymphopenia is a prominent haematological abnormality. Some researchers (9, 23, 27) reported that 85% of dogs with parvoviral enteritis developed leukopenia due to granulocytopenia (neutropenia) within 72th hours. The granulocytopenia in our study was consistent with their findings (3, 9, 24).

In the presented study, erythrocyte (RBC) and haemoglobin (Hb) concentrations at the 0th and 24th hours were higher than the control group, although not statistically significant. Whereas a significant hyperviscosity is observed due to impaired perfusion as a result of polycythemia in hemorrhagic gastroenteritis (HGE) syndromes, severe anaemia and polycythemia are rarely observed in hemorrhagic diarrhoea due to parvoviral enteritis (30) in various studies on parvoviral enteritis (1, 3, 8, 13). RBC and Hb concentrations were in average reference values, although they were found to be higher than the control group. In the presented study, high RBC and Hb concentrations were consistent with the above mentioned investigators and may be related to splenic contraction and dehydration.

In dogs with parvoviral enteritis, changes in blood gases and serum biochemistry are usually nonspecific (8, 20). In the present study, the blood pH at the 0th hour in dogs with parvoviral enteritis was lower than in the control group, although not statistically significant. However, the pH value at the 0th hour was found to be statistically significantly lower than the values before discharge. Blood K^+, Na^+ and ionised Ca^{++} concentrations were also lower than the control group at 0th hours, although not statistically significant. However, Na^+ and Ca^{++} values at the 0th hour were statistically significantly higher and lower than before discharge. In other words, the animals were evaluated to have metabolic acidosis at admission. The fact that metabolic acidosis was observed in patients at the 0th hour is due to H^+ retention and HCO_3 loss due to diarrhoea. The significant decrease in the K^+ value in dogs with parvoviral enteritis compared to control group is due to the loss of potassium due to vomiting. The decline in Ca++ concentration before discharge may be related transforming free calcium into protein-bound calcium by binding due to increased blood pH (7.43). Our study's nonspecific blood gas results are also consistent with the results of other researchers (1, 8, 13, 20).

Hypercoagulopathy without dissemined intravascular coagulopathy (DIC) has been demonstrated in dogs with canine parvoviral enteritis. Increased vWF levels associated with inflammation and endothelial damage are observed in glomerulonephritis, infectious diseases, arteritis, and sepsis. VWF levels are increased during an acute response of the endothelium. VWF is acute phase reactant indicating endothelial dysfunction and inflammation (28). This suggests that vWF acts like an acute phase protein (6, 10, 18). Acute phase proteins are a non-specific tissue reaction secreted as the body's acute response to inflammation (25). Many researchers (3, 16, 17) reported that acute phase proteins are increased in dogs with parvoviral enteritis and may be a prognostic factor evaluating clinical improvement.. Previous studies (8) found prolongation in PT and APTT and a significant increase in fibrinogen and D-dimer levels in dogs with parvoviral enteritis. They reported that this may indicate of DIC in dogs with parvoviral enteritis. Likewise, another study (13) determined prolongation of PT and APTT, a decrease in fibrinogen level and increase in D-dimer levels in parvoviral enteritis and sepsis. In the present study, vWF levels at the 0th and 24th hours showed a significant increase in dogs with parvoviral enteritis compared to the control group. At the same time, their before discharge values decreased and did not show a significant change compared to the control group. Likewise, our study observed a significant prolongation in PT and APTT at the 0th hour. On the other hand, the D-dimer level did not change statistically. This may be related to the development of hypercoagulopathy without the occurrence of DIC (9, 24). In dogs with parvoviral infection, high concentrations of vWF at the 0th hour and 24th hours act as an acute phase protein, as shown by other authors (6, 10, 18) and it can be considered as the body's acute response to inflammation (25).

In conclusion, vWF concentrations, which increase significantly in dogs with parvoviral enteritis compared to healthy animals, can be evaluated as an acute phase protein. Prolongation of PT and APTT and no significant change in fibrinogen and D-dimer values demonstrated that dogs with parvoviral enteritis were in the hypercoagulation state without DIC.

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Ethical Statement

This study was carried out after the animal experiment was approved by Selcuk University Faculty of Veterinary Medicine Ethics Committee (SÜVFEK) (Decision number: 2017/011).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

E.K., and M.S. contributed to conception and design of the study. E.K., M.S. performed the experiments. M.S. and E.K. organized the database. M.S., E.K. performed the statistical analysis. M.S. and E.K. wrote the first draft of the manuscript. E.K. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

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

Animal Welfare

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

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Characterization of the complete mitogenomic and phylogenetic of the *Columba livia* breed Şebap pigeon

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ABSTRACT

The initial phase of the studies focused on the conservation of gene resources involves the phenotypic and genetic characterization of existing breeds. By elucidating the differences at the mitochondrial DNA (mtDNA) level, a particularly suitable source for genetic analyses, it becomes feasible to calculate genetic distances between local breeds, identify potential domestication centers through evolutionary analyses, and construct phylogeny trees depicting kinship relationships between among breeds. Şanlıurfa holds significance in pigeon breeding due to its prolonged history of traditional pigeon breeding, establishing itself as one of the pigeon domestication centers. This study aims to genetically characterize Şebap pigeon breed which is specific to Şanlıurfa province in Türkiye. The mitogenome of the Şebap pigeon was analyzed by next generation sequencing and determined to be 17,232 bp in size, comprising 13 Protein-Coding Genes (PCGs), two Ribosomal RNAs (rRNAs), 22 Transfer RNAs (tRNAs). The overall mitogenome composition includes 29.6% for A, 24.5% for T, 31.2% for C, and 14.7% for G, with a notable A–T richness of 54.1%. Notably, ND6 is encoded on the L strand while the others are encoded on the H strand. Our study has provided a comprehensive examination of whole mitochondrial DNA. Using this information, we have constructed a phylogenetic tree, locating the Şebap pigeon within the rock pigeon lineage.

Introduction

Prior to the development of contemporary molecular genetic methodologies for detecting variants at DNA-level, breed differentiation was dependent exclusively on phenotypic traits; nowadays, DNA-level variances serve to enhance phenotypic distinctions. Analyzing genetic diversity enables the calculation of genetic distances between local breeds, which sheds light on vicariance biogeography (6), identifies potential domestication centers through evolutionary analysis, and constructs phylogenetic trees illustrating relationships among breeds (20). Mitochondrial DNA (mtDNA) is stands out as a valuable genetic resource in research, particularly in phylogenetic investigations, owing to its non-recombining and uniparental inheritance characteristics, which enable the elucidation of evolutionary relationships across animal species and identification of domestication origins (7, 20).

Although the mitochondrial genome size in pigeons varies across breeds, it generally consists of 2 ribosomal RNA (rRNA) genes, 13 protein-coding genes (PCG), 22 transfer RNA (tRNA) genes and a non-coding control region (D-loop) (8, 14, 17, 21). While comprehensive whole-genome mtDNA analyses remain notably absent for pigeon breeds in Türkiye, various studies conducted in different countries have shed light on the mtDNA characteristics of pigeon breeds (20**)**. Notably, the

mitochondrial genome size varies among pigeon breeds across the globe. For instance, in Egyptian rock pigeons (specifically, the *Columba livia* breed Egyptian swift), the mtDNA size is reported as 17,239 base pairs (bp) (15). Similarly, in king pigeon breed (*Columba livia* breed king) which is selectively developed for meat production over several years, the mtDNA size is documented as 17,221 bp (23). Additionally, Chinese pigeons, as studied by Kan et al. (10), exhibit a mtDNA size of 17,229 bp. These findings underscore the need for a comprehensive exploration of the mtDNA landscape in Turkish pigeons to contribute to the broader understanding of global pigeon genetics.

Pigeon breeding in Şanlıurfa province which resides in south-eastern part of Türkiye, holds a distinctive position, deeply rooted in its longstanding tradition and recognized role as a significant pigeon domestication center. Tracing back to the pigeons of Şanlıurfa the city's heritage intertwines with the genetic lineage of numerous pigeon breeds across Türkiye. Boasting a vibrant community of pigeon breeders, the city also hosts dedicated venues for pigeon shows and trading activities, establishing itself not only as a hub for enthusiasts but also as a thriving commercial venture in the region (12, 16). While Şanlıurfa's pivotal role in pigeon breeding and trade is undeniable, it's notable that the city currently lacks formal registration for its unique pigeon breeds. This gap is particularly intriguing given the historical and geographical significance of Anatolia, renowned for its rich genetic resources, including the pigeons, traditionally bred in these lands for centuries (5, 17). Türkiye, with its plethora of indigenous pigeon breeds, stands as a testament to the nation's diverse avian heritage (1). Çelik (4) reported the morphological characteristics of the Şebap pigeon, which are typically used to classify pigeon breeds based on various traits globally; however, Türkiye's classification system prioritizes flight display traits, underscoring the cultural and practical importance of pigeons within Turkish tradition and society. This study aims to elucidate the genetic composition of the Şebap pigeon, a distinct breed specific to the Şanlıurfa province, thereby contributing to the enhancement of our knowledge regarding Türkiye's avian genetic diversity.

Materials and Methods

The animal material of the study consisted of 30 pigeons from Şanlıurfa (37° 9′ 30″ N; 38° 47′ 30″ E), sourced from the Şebap Pigeon Association and local breeders within the Şanlıurfa province. For each pigeon the roots of 5 feathers were collected, cut into 0.5-1 cm lengths, and transferred to a 1.5 ml centrifuge tube. The DNA isolation process utilized the phenol-chloroform extraction procedure as reported by Sambrook (19). In the tube 360 µl Lysis solution (10 mM TRIS-HCl, 1 mM EDTA, %0.1 Sodium Dodecyl Sulphate, pH:8.0), 50 µl DTT, 50 µl Proteinase K (10 mg/ml) were added followed by an overnight incubation in a thermo-shaker TS-100C (Kisker biotech, Germany) device at 56 °C. Subsequently, 400 µl of phenol and 400 µl chloroform was introduced to the lysate followed by gentle shaking and a 30-minute incubation at room temperature. After centrifugation at 10,000 rpm for 4 minutes at 4 °C the upper phase containing DNA was carefully transferred to a new tube. A second step involved the addition of 800 µl chloroform to remove the DNA from phenol residues. After vigorous shaking for 5 seconds, the mixture was left at room temperature for 10 minutes followed by centrifugation at 10,000 rpm for 4 minutes at 4 °C. The upper phase was transferred to a new tube and an equal volume of isopropanol was added and centrifuged at 14,500 rpm for 30 minutes for precipitation of the DNA. The DNA pellet was washed with %70 ethanol by centrifuging for 5 minutes at 14,500 rpm. After drying the DNA pellet, it was diluted in 50 µl ddH_2O (19). Concentrations of the DNA samples were set to 100 ng/µl.

Oligonucleotide design involved the creation of primers for polymerase chain reaction (PCR) utilizing the reference Pigeon mtDNA NC_013978.1 from National Center for Biotechnology Information (NCBI) database as a template. The design and selection of candidate primers were accomplished with Primer3Plus (22). Subsequently, the chosen candidate primers were evaluated for their capacity to create secondary structures using IDT OligoAnalyzer (10). The specificity of the primers was tested by "Primer-BLAST" tool within the relevant databases. Two sets of oligonucleotides were used for each sample (Table 1).

Table 1. Oligonucleotide sequences and amplicon lengths.

Name	Sequence	Amplicon lengths
RD_mtDNA_F1	ATAACCTCCCCGACGCATTC	10505bp
RD_mtDNA_R1	CAGTAACACCGGAAGCGAGT	
RD_mtDNA_F2	TCCTACTCGCCCTTCCATCA	9661bp
RD_mtDNA_R2	GTTTTGGACAATTGATGAGTGAAAA	

Oligonucleotide PCR optimization was conducted utilizing the Master Cycler epgradient (Eppendorf, Germany). The amplification of long DNA segments within mtDNA was achieved with the Phire II enzyme (Thermo, Germany). The $MgCl_2$ concentration in the buffer solutions provided with enzyme for long-region amplification was maintained as standard 2.5mM. Annealing at a variable temperature ranging from 58 to 60°C for a duration of 3 minutes were tried for optimization. The PCR amplification protocol began with denaturation at 98°C for 60 seconds, followed by a second denaturation step at the same temperature but for a shorter duration of 30 seconds. Subsequently, annealing temperature of 60°C for 30 seconds followed by the elongation phase took place at 72°C for 3 minutes, and a final elongation step is carried out at the same temperature but extended to 5 minutes. The PCR components included using Buffer containing $MgCl_2$ 5X at a 1X concentration, 200nM for dNTP, 1.25 units of Phire II, 50ng/µl for DNA, and 0.5µM for both oligonucleotides. The reaction mixture is completed with ddH_2O to 25µl. Adhering to these specified conditions ensures the successful execution of the PCR process, leading to the desired replication of the target DNA sequence.

Bioinformatics analyses were carried out on mtDNA data obtained through next-generation sequencing in the “fastq” file format. The Geneious Prime Bioinformatics Software v2023.2.1 package program (https://www.geneious.com) was utilized for various analysis. Trimming and quality filtering operations, including the removal of adapters, low-quality bases (quality score less than 30) at the ends, and short reads (less than 50 bp) from the raw reads, were performed with the BBDuk trimming tool within the same program. Duplicated readings were removed using the "Remove Duplicate Reads" tool and erroneous readings were addressed with the "Error Correct & Normalize Reads" tool.

The same process was independently applied to each sample and consensus results were obtained for 20 clean samples. These consensus results were then aligned in Geneious Prime using the MAFFT v.7 (11) procedure based on the multiple alignment algorithm found in “Allign/Assemble”. The clean and high-quality reads aligned to the latest pigeon mitochondrial genome (NC_013978.1), were obtained using the "Map to Reference" algorithm in "Align/Assemble" with parameters set to "Sensitivity: Highest sensitivity/Medium, Fine Tuning: Iterate up to 25 times." This process enabled the acquisition of the entire mtDNA genome sequence of Şebap pigeons, followed by gene annotation.

We used 19 reference nucleotide sequences to construct the phylogenetic tree. *Didunculus strigirostris* was used as the outgroup. The neighbor-joining tree (18) was constructed with the Tamura-Nei Model using the "Tree" algorithm in the Geneious Prime® v2023.2.1 package program.

Results

In this study, we present the first-ever sequence of the mitochondrial genome of the Şebap pigeon (*Columba livia* breed Şebap). The complete mitochondrial genome spans 17,232 bp and comprises 37 genes encoding 13 proteins, 2 rRNAs, 22 tRNAs, and a control region. The mitogenome has an overall composition of 29.6% A, 24.5% T, 31.2% C, and 14.7% G, with a significant A–T richness at 54.1%.

The protein-coding genes (PCG) are distributed across both strands, with ND6 encoded on the light (L) strand and the remaining protein-coding genes on the heavy (H) strand. Of the 13 protein-coding genes, ten genes initiate with the common start codon ATG, while COX1 uses GTG, ND5 employs GGG, and ND3 utilizes AGT as initiation codons (Table 2). The lengths of the 12s rRNA and 16s rRNA genes were determined as 973 base pairs (bp) and 1,586 bp, respectively. Besides these genes are positioned between the tRNAPhe and tRNALeu genes and separated by the tRNAVal gene.

The control region (D-Loop) of the Şebap pigeon’s mtDNA spans 1,662 bp long and is located between the tRNAPhe and the tRNAGlu genes. L-stranded genes included ND6 gene and eight tRNAs (tRNA-Ala, tRNA-Cys, tRNA-Glu, tRNA Asn, tRNA-Gln, tRNA-Pro, tRNA-Ser and tRNA-Tyr) while all other mitochondrial genes are H-stranded (heavy in the helix) (Figure 1 and Table 3).

In the mitochondrial genome of Şebap pigeons, a total of twenty-two tRNA genes were identified. These tRNA genes are distributed across the genome, exhibiting sizes that vary from 65 nucleotides for tRNAHis to 74 nucleotides for tRNALeu (L2) (Table 3, Figure 2 and 3). Notably, tRNAPro, tRNAArg, and tRNAGly, undetected by tRNAscan-SE, were successfully identified through comparative analysis with their respective counterparts. Importantly, all tRNA gene sequences demonstrated the potential to form characteristic cloverleaf secondary structures, as illustrated in Figure 4. The DHU and TΨC arms of these tRNA structures exhibit variability, ranging from two to five nucleotide pairs. The neighbor-joining tree the Şebap pigeon located within the *Columba livia* branch as expected (Figure 5).

Table 2. Annotation of the complete mitochondrial genome of Şebap pigeon.

	Position			Nucleotide composition %				
Gene	**Start**	**End**	**Size**	**A**	**C**	**G**	**T**	**Strand**
tRNA-Phe	1	69	69	29.0	34.8	23.2	13.0	H
12S-rRNA	70	1042	973	31.2	27.5	20.1	21.1	H
tRNA-Val	1043	1114	72	31.9	27.8	19.4	20.8	H
16S-rRNA	1115	2700	1586	33.8	25.4	18.8	22.0	H
tRNA-Leu	2701	2774	74	24.3	25.7	24.3	25.7	H
ND1 CDS	2786	3751	966	26.0	34.2	12.9	26.9	H
tRNA-Ile	3769	3839	71	32.4	22.5	19.7	25.4	H
tRNA-GIn	3845	3915	71	38.0	22.5	9.9	29.6	L
tRNA-Met	3915	3983	69	29.0	30.4	18.8	21.7	H
ND2 CDS	3984	5023	1040	32.0	34.0	10.0	23.9	H
tRNA-Trp	5024	5094	71	35.2	25.4	16.9	22.5	H
tRNA-Ala	5096	5164	69	31.9	26.1	15.9	26.1	L
tRNA-Asn	5167	5239	73	30.1	31.5	17.8	20.5	L
tRNA-Cys	5242	5308	67	31.3	28.4	16.4	23.9	L
tRNA-Tyr	5308	5379	72	36.1	26.4	15.3	22.2	L
C0X1 CDS	5381	6931	1551	26.2	32.0	16.1	25.7	H
tRNA-Ser	6923	6996	74	29.7	28.4	17.6	24.3	L
tRNA-Asp	6999	7067	69	34.8	21.7	15.9	27.5	H
C0X2 CDS	7070	7753	684	30.4	30.8	14.6	24.1	H
tRNA-Lys	7555	7825	71	29.6	23.9	21.1	25.4	H
ATP8 CDS	7827	7994	168	32.1	38.7	5.4	23.8	H
ATP6 CDS	7985	8668	684	28.4	35.1	10.1	26.5	H
COX3 CDS	8668	9451	784	26.7	33.0	15.3	25.0	H
tRNA-GIy	9452	9520	69	27.5	23.2	14.5	34.8	H
ND3 CDS	9521	9871	350	23.1	30.0	14.9	32.0	H
tRNA-Arg	9873	9941	69	26.1	26.1	17.4	30.4	H
ND4LCDS	9943	10239	297	25.6	33.3	14.5	26.6	H
ND4CDS	10233	11609	1377	29.7	33.0	13.1	24.1	H
tRNA-His	11610	11678	69	31.9	23.2	15.9	29.0	H
tRNA-Ser	11679	11744	66	30.3	27.3	21.2	21.2	H
tRNA-Leu	11744	11815	72	34.7	18.1	18.1	29.2	H
ND5CDS	11816	13628	1813	29.7	32.8	13.6	23.9	H
CYTB CDS	13640	14784	1145	25.9	34.4	14.8	24.8	H
tRNA-Thr	14785	14853	69	34.8	21.7	17.4	26.1	H
tRNA-Pro	14860	14929	70	25.7	22.9	22.9	28.6	L
ND6CDS	14975	15496	522	38.7	35.8	12.5	13.0	L
tRNA-Glu	15500	15570	71	21.1	32.4	22.5	23.9	L
Control region	15571	17232	1662	30.6	28.6	13.3	27.5	H

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Table 3. Codon number and relative synonymous codon usage (RSCU) in Şebap pigeon mitochondrial PCGs.

Codon	AA	Count	RSCU	Codon	AA	Count	RSCU	Codon	AA	Count	RSCU
GCA	A	64	1.08	AGC	S	65	1.09	CTA	L	182	1.96
GCC	A	111	1.87	AGT	S	24	0.40	CTC	L	145	1.56
GCG	A	12	0.20	TCA	S	87	1.45	CTG	L	51	0.55
GCT	A	50	0.84	TCC	S	108	1.81	CTT	L	88	0.95
TGC	C	34	1.33	TCG	S	15	0.25	TTA	L	62	0.67
TGT	C	17	0.67	TCT	S	60	1.00	TTG	L	30	0.32
GAC	D	46	1.31	ACA	T	114	1.47	ATA	M	85	1.88
GAT	D	24	0.69	ACC	T	110	1.41	ATG	M	50	1.10
GAA	E	71	1.51	ACG	T	5	0.06	GTG	M	1	0.02
GAG	E	23	0.49	ACT	T	82	1.05	AAC	N	98	1.26
TTC	F	133	1.41	GTA	V	42	1.13	AAT	N	57	0.74
TTT	F	56	0.59	GTC	V	47	1.26	CCA	P	113	1.42
GGA	G	52	1.19	GTG	V	25	0.67	CCC	P	92	1.16
GGC	G	56	1.28	GTT	V	35	0.94	CCG	P	12	0.15
GGG	G	39	0.89	TGA	W	81	1.69	CCT	P	101	1.27
GGT	G	28	0.64	TGG	W	15	0.31	CAA	Q	81	1.38
CAC	H	88	1.11	TAC	Y	75	1.36	CAG	Q	36	0.62
CAT	H	70	0.89	TAT	Y	35	0.64	CGA	R	29	1.38
ATC	I	151	1.32	AGA	*	24	1.05	CGC	R	30	1.43
ATT	I	78	0.68	AGG	*	24	1.05	CGG	R	12	0.57
AAA	K	85	1.63	TAA	*	25	1.10	CGT	R	13	0.62
AAG	K	19	0.37	TAG	*	18	0.79				

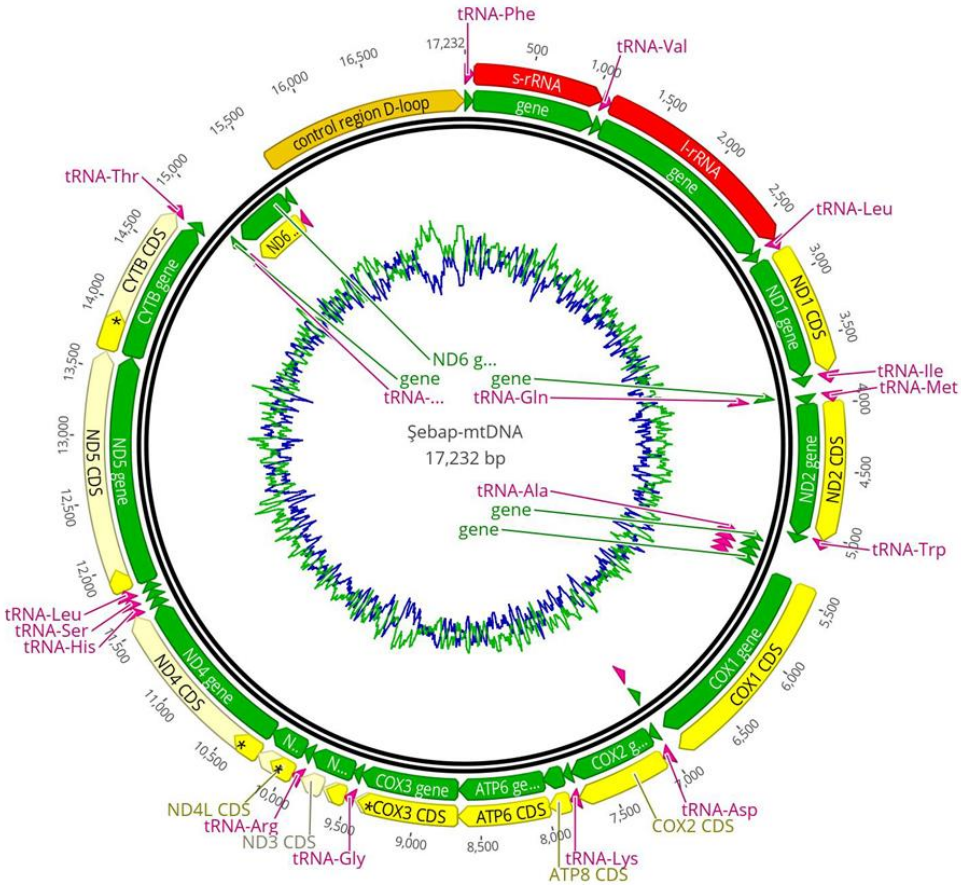

Figure 1. Gene map of the mitochondrial genome of Şebap pigeons (Columba livia breed Şebap).

Genes encoded in heavy or light strands are shown outside and inside the circular gene map, respectively. Twenty-two tRNA genes are indicated along with their amino acid codes. This graph was obtained using the Geneious Prime® v2023.2.1 package program.

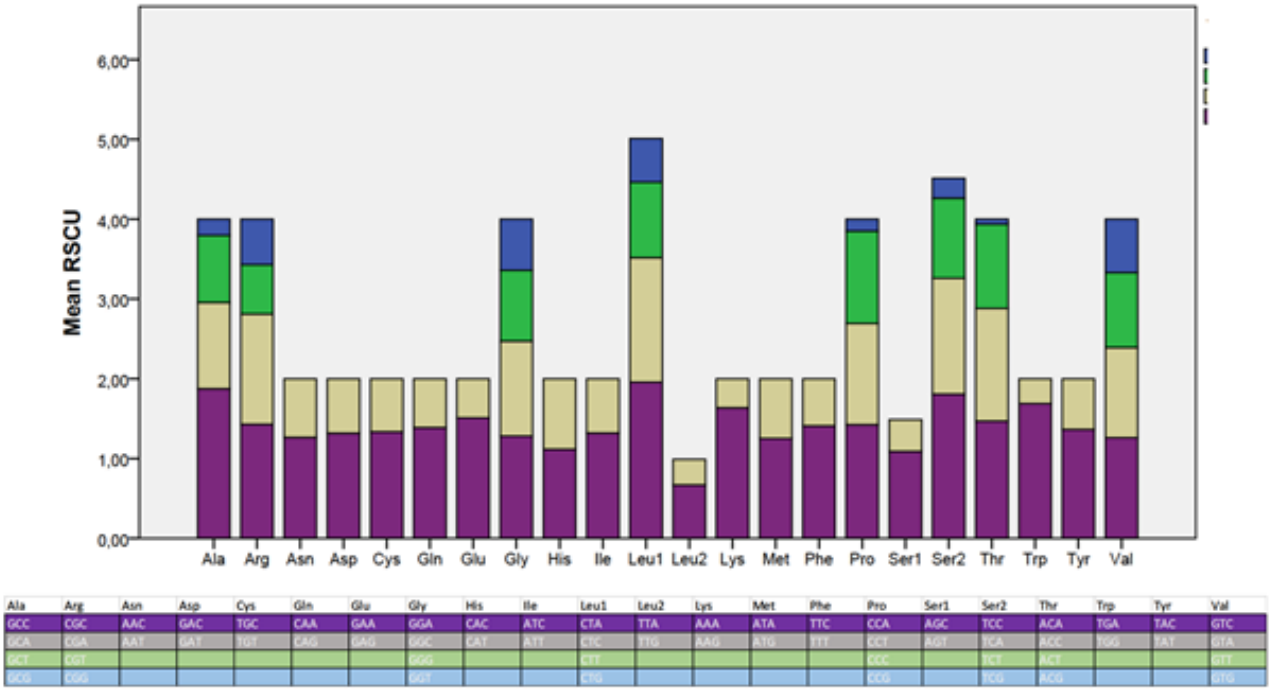

Figure 2. Codon distribution in the Şebap pigeon mitogenome.

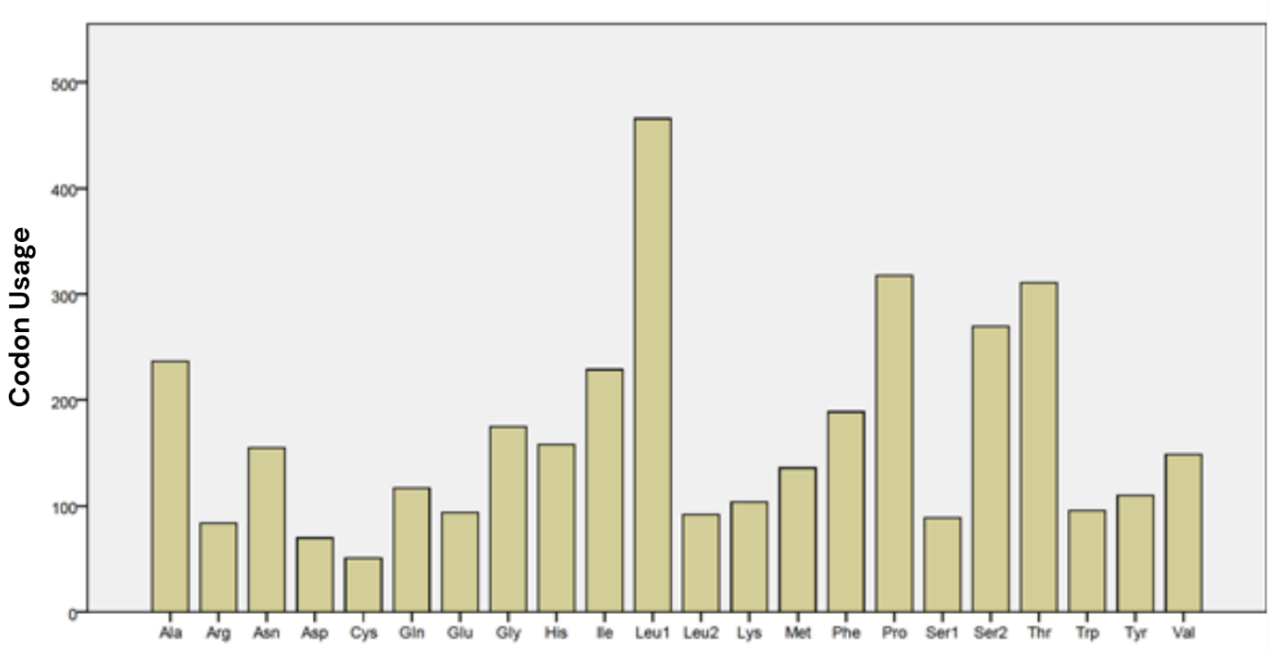

Figure 3. The relative synonymous codon usage (RSCU) in the Şebap mitogenome.

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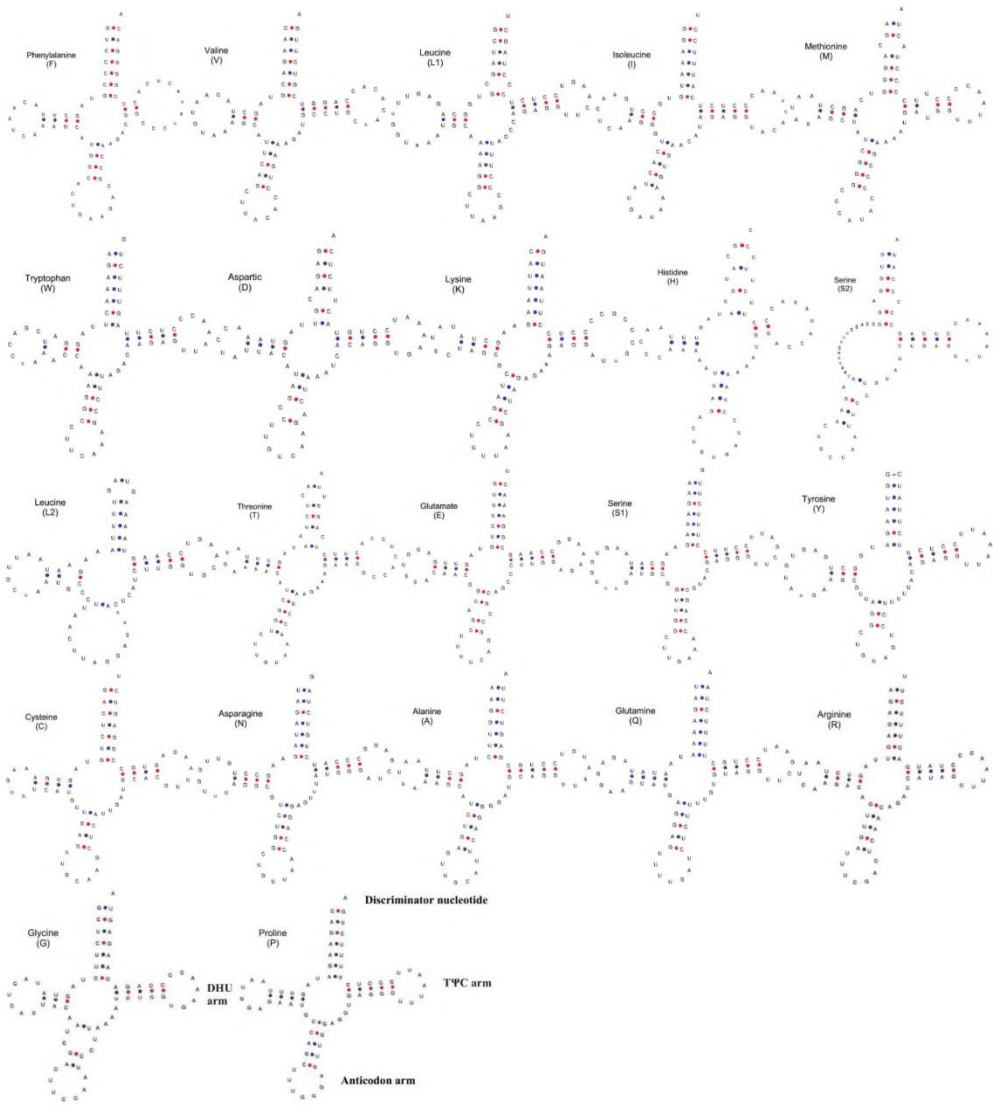

Figure 4. These are the secondary structures of the 22 tRNAs in the *Columba livia* breed Şebap mitochondrial genome.

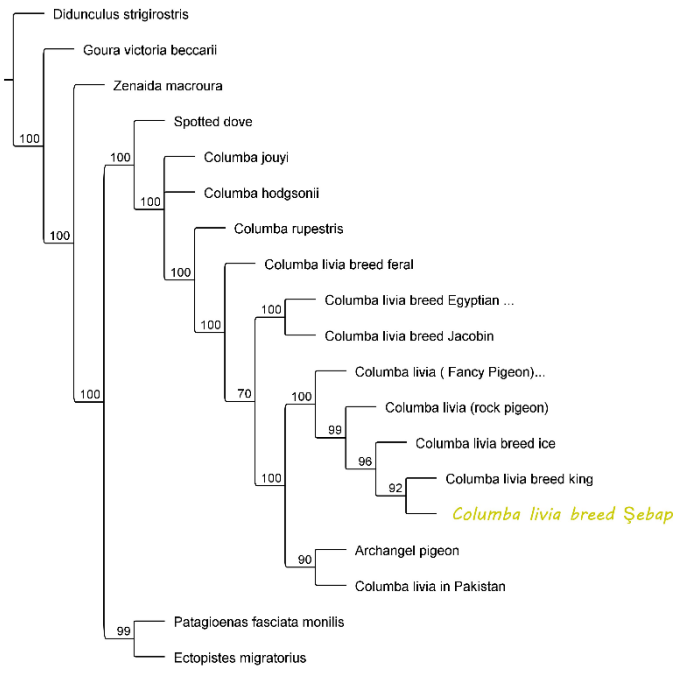

Figure 5. Nucleotide-based phylogenetic tree of 19 species and breeds belonging to the Columbidae family, with the bird *Didunculus strigirostris* as the outg.

Discussion and Conclusion

The Şebap pigeon, a domesticated breed of the rock pigeon (*Columba livia*), represents a lineage within the Columbidae bird family, encompassing domestic pigeon, feral pigeon, and wild rock pigeon. This species has undergone several millennia of domestication, resulting in the emergence of diverse domestic breeds across various geographical locations (20, 24). Domestic pigeon descendants of wild rock pigeons have successfully adapted to urban habitats. This comprehensive analysis provides valuable insights into mitogenome diversity and features genes within the mitochondrial genome of Şebap pigeons.

The Protein coding genes (PCG) of the pigeon consist encoding *Cytochrome C Oxidases* (COXI, COXII and COXIII), two ATPases (ATP6 and ATP8), seven NADH dehydrogenases (ND1-6 and ND4L), and Cytochrome *b*. In the study of Kan et. al (6) on the rock pigeon (*Columba livia*), they found that the total length of 13 protein-coding genes in *C. livia* mtDNA was 11,388 bp, corresponding to 66.1% of the entire mitochondrial genome. Kan et al. (10) observed that the length of 13 protein-coding genes found in other bird species ranged from 11,379 bp for *Rhynochetos jubatus* to 11,412 bp for *Branta canadensis*. Additionally, the length of the 13 protein-coding genes found in *C. livia* mtDNA is similar to most other bird species. The longest protein-coding gene of *C. livia* mtDNA was determined to be the NAD5 gene (1,815 bp), and the shortest was ATP8 (168 bp). In a similar study on *Columba hodgsonii*, Liu et al. (16) determined that the total length of 13 protein-coding genes in mtDNA was 11,385 bp, corresponding to 65.1% of the entire mitochondrial genome. In our study the total length of 13 protein-coding genes on the mitochondrial genome of Şebap pigeons was 11,411 bp and corresponded to 66.2% of the entire mitochondrial genome. The general features, including mitochondrial genome organization and gene regulation pattern, align with those observed in other bird species (7, 8, 10, 15, 16) (Figure 1).

Biray et al. (3) and Khan et. al (13) who focused on *COI* and D-loop regions in *Columba livia* mtDNA emphasized the challenges establishing the phylogeny of domestic pigeon lineages using mtDNA. Our study, leveraging whole-genome next generation sequencing, provides a more comprehensive analysis of all mitochondrial genes revealing the Şebap pigeon's domesticated status within the rock pigeon lineage.

Balog et al. (2) studied the partial sequencing of COI region of mtDNA in 7 Hungarian and 35 international pigeon breeds and detected no significant difference between Hungarian and foreign breeds.

In conclusion, our study not only contributes to the understanding of the mitochondrial genome of the Şebap pigeon but also highlights the importance of comprehensive genomic analyses for a more accurate portrayal of genetic relationships within and between pigeon breeds. It is significant as it reveals the entire mitogenomic sequence of the Şebap pigeon breed of *C. livia* for the first time. The circular mitogenome, spanning 17,232 bp, exhibits the canonical configuration of 37 genes, including 13 protein-coding genes (PCGs), two rRNAs, and 22 tRNAs—features typical of Columbidae species. The nucleotide composition of the mitogenome of Şebap pigeon showed a distribution of 29.6% for A, 24.5% for T, 31.2% for C, and 14.7% for G, emphasizing a pronounced A–T richness at 54.1%. Notably, the encoding of ND6 on the L strand, in contrast to the H strand encoding of other genes, adds a layer of complexity to the genomic landscape. This comprehensive characterization significantly enhances our understanding of the mitochondrial dynamics in *C. livia* breed Şebap, laying a robust foundation for future investigations into its genetic and evolutionary intricacies.

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Ethical Statement

This research was approved by the Harran University Animal Experiments Local Ethics Committee (Approval no: 2021.005.04).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

RÇ, NB, and ŞG conceived the presented idea. FB developed the theoretical framework and conducted the computations. NB and RÇ designed the study. RÇ and AY were involved in data collection. FK and MYA contributed to the laboratory experiments. NB performed the data analysis. AY, FB, and NB contributed to the interpretation of the results. All authors discussed the findings and contributed to the preparation of the final manuscript.

Data Availability Statement

The datasets analyzed during the current study are available from the corresponding author on a reasonable request.

Animal Welfare

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

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Antimicrobial and antibiofilm activities of *Capparis ovata* Desf. methanolic extracts

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ABSTRACT

This study aimed to investigate the antibacterial, antifungal, and antibiofilm activities of methanol extract of *Capparis ovata* against a range of foodborn pathogenic bacteria as well as *Candida tropicalis*. The polyphenolic content of the extract was determined using high pressure liquid chromatography. Liquid microdilution, and microplate methods were used for the evaluation of antimicrobial and antibiofilm activities, respectively. The Minimum Inhibitory Concentration (MIC) values for *C. ovata* extract were determined as follow: 8 mg/mL against *Bacillus cereus*, 16 mg/mL against *Enterococcus faecalis,* and *C. tropicalis*, 32 mg/mL against *Staphylococcus aureus* strains, *S. epidermidis*, *Escherichia coli* 43895, *Listeria monocytogenes* strains, *L. innocua*, and 64 mg/mL against *E. coli* 35150 and *Salmonella* Typhimurium. The Minimum Bactericidal Concentration (MBC) values were 16 mg/mL (*B. cereus*), 64 mg/mL (*S. aureus* strains (except 13552), *E. faecalis*, *C. tropicalis*). For *E. coli* (43895 and 35150), *L. monocytogenes* strains, *L. innocua*, *S.* Typhimurium, *S. aureus* NCTC 13552, *S. epidermidis*, the MBC value was 128 mg/mL. The inhibition of biofilm formation by *C. ovata* extract was found at the following concentrations (in mg/mL): 8 for *S. aureus* strains (except 13552), *S. epidermidis*, *B. cereus*, and *C. tropicalis*, 16 for *L. innocua* and *E. faecalis*, 32 for *E. coli* 43895, *L. monocytogenes* strains, and *S. aureus* NCTC 13552, and 64 for *E. coli* 35150 and *S.* Typhimurium. Overall, these results indicate that *C. ovata* extract possesses potent antimicrobial and antibiofilm activities on the tested pathogenic microorganisms. Due to these properties, *C. ovata* extract can be considered as a promising plant-based antimicrobial agent for potential application in the food industry.

Introduction

The treatment of infections that threaten public health has become quite complex due to the increasing number of bacteria developing resistance to antibiotics (55). Recently, antibiotic resistance has become a serious global health problem. The growing prevalence of infection caused by resistant microorganims impacts human health by increasing mortality rates and hospitalizations, leading to higher economic costs (32). Yeasts can also colonize the human body and cause serious health problems ranging from simple infections to life-threatening conditions. The pathogenicity of *Candida* species is closely linked to their ability to form biofilms and adhere to host cells (55). Today, the increased use of antifungal drugs has also led to the emergence of drug-resistant strains (34).

Microbial biofilms, particularly those formed by pathogenic bacteria (31, 41) consist of microorganisms embedded in an extracellular matrix composed of polysaccharides, proteins, nucleic acids and lipids. These biofilms adhere to both living and non-living surfaces, serving to protect the bacteria from environmental threats (16, 32, 55). It's well known that biofilms play a crucial role in the pathogenesis of various infectious diseases, posing significant challenges in treatment and severely limiting therapeutic. Biofilms are considered as a key

virulence factor, causing persistent, chronic and recurrent infections (32), and they also represent a major source of bacterial contamination in the food industry (17, 25). Of particular concern is the prevention and elimination of biofilms formaed by *S. aureus* strain on surfaces (28), as these are highly resistant to antibiotics and can evade host immune response by preventing phagocytosis (54). The most common mechanism of resistance within microbial biofilms is the inability of antimicrobial agents to penetrate the biofilm barrier and reach the embedded microorganims. In biofilm formation, resistance genes are more easily transferred from one microorganism to another (55).

Antibiotics used in the treatment of biofilm-related infections are not able to fully eliminate the biofilm, but they can reduce its presence. To effectively remove the biofilm layer, higher concentrations of chlorine-based antimicrobials are needed. For many reasons, "biofilm inhibition" is considered the main drug-targeted approach for treating a wide range of infections (32). Recently, the overuse of synthetic antimicrobials to prevent bacterial infections has posed serious threat to public health by contributing to the rise of drug resistance. In addition, various synthetic food additives commonly used in the food industry to preserve foods and extend their shelf life cause public health concerns. For this reason, there is a growing recommendation to use natural products as alternatives to conventional synthetic antimicrobials. Although new antibacterial and antifungal drugs can be developed, various new therapeutic agents specifically targeting biofilm formations, as these continue to cause significant health problems (55).

It is well established that microbial pathogens can develop resistance to many commonly used drugs. Along with such multidrug resistant pathogens, it can lead to treatment failures, increasing mortality rates, and higher healthcare costs. In addition, the problem of synthetic antimicrobials leaving residues in food is another problem. The problem of resistance and residue encourages researchers to develop natural antimicrobial products. In recent years, interest in the natural products of plants has been increasing. This situation forces researchers to develop drugs derived from natural origin. In recent years, there has been a growing interest in the plant-based natural products (30).

Capparis species, belonging to the *Capparis* genus within the *Capparaceae* family, are among the important perennial shrubs. There are approximately 39 genera and 650 species within the *Capparis* genus worldwide (22). These plants grow naturally in temperate (tropical/subtropical) regions across all continents, especially in Mediterranean countries (8, 34). The caper, widely used in Mediterranean cuisine (42), is generally consumed as pickle due to its economic and nutritional benefits (34).

Different parts of the caper have been used for nutritional, cosmetic and medicinal purposes since ancient times. *C. spinosa*, *C. ovata* and *C. decidua* are among the most extensively studied species for their therapeutic and nutritional properties (22). *Capparis* species are used in traditional medicine to treat various illnesses, including rheumatic, stomach problems, headaches and toothaches, gout, joint inflammation, and anemia (33, 34). Additionnaly, *Capparis* species are commonly used as diuretics, appetite stimulant, vitamin C supplement in scurvy (6, 19), analgesic, and wound healer (5). Experimental studies conducted on various parts of caper species have demonstrated a wide range of biological and pharmacological properties, such as antitumoral, antioxidant, anti-inflammatory, antidiabetic, antihyperglycemic, antiatherosclerotic, antidepressant, antiallergic, antihyperlipidemic, antihypertensive, antifungal, antimicrobial, antiviral, and antihepatotoxic effects. These species are also used to enhance liver functions, treat stroke and hemophilia. It has been reported that they have a protective effect in preventing epileptic attacks and are effective in the treatment of skin and hair diseases (2, 5, 12, 20, 22, 33, 34, 40, 45).

In fact, several phytochemical studies have shown that many species from *Capparis* genus are rich in a wide range of nutritional and/or bioactive compounds. These include vitamins, lipids (such as fatty acids), terpenes, terpenoids, alkaloids (such as spermidine alkaloid), sugars, glucosinolates (such as glucocapparin), isothiocyanate glucosides, glycosides, phytosterols, and various beneficial compounds (saponins, tannins and sulfurs). They contain also some phenolic compounds with antioxidant properties (such as flavonoids, polyphenols and phenolic acids), such as tocopherols and carotenoids (such as lutein, β-carotene) (1, 3, 15, 21, 22, 29, 30, 34, 46, 49, 50, 52), many volatile compounds and essential oils (13). Capers are particularly rich in flavonoids such as kaempferol, quercetin, (13) and rutin (Vitamin P) (22). The most common flavonoids found in *Capparis* species are rutin (46) and quercetin (7). Studies have also revealed that capers are a rich source of many useful chemical compounds, such as campesterol, catechin, luteolin, coumarin, resveratrol, stigmasterol, caffeic acid, chlorogenic acid, ferulic acid, shergic acid, vanillic acid, aliphatic, and triterpenic alcohols (27, 47, 48, 49, 51, 52, 53). Moreover, the presence of glucocleomine distinguishes *Capparis* species from other fruits and vegetables (36). Toxicity studies have shown that the ethanolic extract of *Capparis sepiaria* leaves is not toxic at 300-5000 mg/kg in the experimental model (38). *Capparis spinosa* seed oils have been determined to have low toxicity and do not show signs of acute toxicity. Oral

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and dermal exposures have been determined to have mean lethal doses of 7000 mg/kg and 3000 mg/kg, respectively (37). The results of these study indicate that this plant may have potential use in medical applications. Although there are studies investigating the polyphenolic content analysis and antimicrobial activities of various species of Capari, studies investigating the content analysis and antimicrobial activities of *Cappari ovata* are very limited. In this study, the *in vitro* antimicrobial and antibiofilm-forming activity of the methanol extract of *C. ovata* were investigated against some foodborn pathogenic microorganisms.

Materials and Methods

***Plant Material and Extraction*:** *Capparis ovata* (Marmara University, Faculty of Arts and Sciences, Herbarium no: 5091) was obtained from a local plant market (Aşçı Murat Kapari Ar-Ge Gıda Ürt. Mrk.) in Burdur Province of Türkiye in June-2023. *C. ovata* methanol extract was prepared according to the method described by Özkan *et al*. (35). According to this method, the plant samples were collected and cleaned. The aerial of the plant were dried in the shade. The dried samples were pulverized using a grinder (Arzum, Türkiye). 20 g of *C. ovata* powder was placed in a Soxhlet appratus (Caliskan Cam Teknik) with cartridge, and extracted with 500 mL of methanol at 50 °C for 3 hours. The obtained extracts were filtered using Whatman no: 4 filter paper, and the solvent in the extract was evaporated at 40 °C using a rotary evaporator (IKA RV 10, Germany). The lyophilization was carried by using a freeze dryer (Labconco, Model: Freezone 6 plus). The obtained crude extract was then stored at -20 °C until use.

***Polyphenolic Compound Analysis*:** Polyphenolic compound analysis of the extract was performed using Shimadzu Prominence Brand HPLC system (Japan) equipped with LC20 AT pump, CTO-10ASVp column oven, DAD detector (SPD-M20A), and a Zorbax C18 column (250 × 4.6 mm, 5 µm). The mobile phase consisted of 3% formic acid (A) and methanol solvents (B), and the results were evaluated using the LC solution software. The content of 11 phenolic compounds, including gallic acid, vanillic acid, caffeic acid, epicatechin, p-coumaric acid, ferulic acid, rutin, ellagic acid, naringin, cinnamic acid, and quercetin were evaluated. For the analysis, 0.2 g of *C. ovata* extract was dissolved in 1 mL of mobile phase, filtred through a 0.45 µm membrane, and then injected into the HPLC system (9).

***Antimicrobial and Antibiofilm Activities of the Extract*:** The antimicrobial activity was evaluated using the disc diffusion and microdilution methods, whereas the antibiofilm-forming activity was assessed using the microplate method (43).

***Test Microorganisms*:** Standard microorganisms were obtained from Burdur Mehmet Akif Ersoy University, Department of Food Hygiene and Technology. Various Gram-positive bacteria including *Staphylococcus aureus* ATCC 25923, *S. aureus* (MRSA) ATCC 43300, *S. aureus* (Enterotoxin E) FRI 918, *S. aureus* NCTC 13552, *S. epidermidis* ATCC 12228, *Bacillus cereus* NRRL-B-3711, *Enterococcus faecalis* ATCC 29212, *Listeria innocua* ATCC 33090, *L. monocytogenes* RSKK 02028, and *L. monocytogenes* RSKK 472 have been used. The Gram negative bacteria used were *Escherichia coli* O157:H7 ATCC 35150, *E. coli* O157:H7 ATCC 43895, and *Salmonella* Typhimurium ATCC 14028. Additionally, the yeast fungus; *Candida tropicalis* ATCC 13803 has been included.

***Minimum Inhibitory Concentration (MIC)*:** 200 µL of Mueller-Hinton II Broth containing *C. ovata* extracts at various concentrations (2.00, 4.00, 8.00, 16.00, 32.00, 64.00, 128.00 and 256.00 mg/mL) was placed into each well of a 96-well plate. Subsequently, 20 µL of a microorganism suspension was added to each wells. Wells containing only Mueller-Hinton II Broth were used as controls. The plates were then incubated for 24 hours at 37 °C. After incubation, the absorbance was measured at 600 nm using a spectrophotometer (Varioskan Lux, Thermo, Finland). The experiment was performed in triplicate.

***In Vitro Disc Diffusion Test*:** Each bacterial strain was cultured on Tryptic Soy Agar (TSA) (Merck) for 18 hours at 37 °C. The turbidity of the bacterial cultures was then adjusted to 0.5 McFarland standard using a densitometer (Biosan, Latvia) in 0.9% NaCl. 100 µL of the adjusted bacterial suspension was spread uniformly onto the surface of Mueller-Hinton Agar petri dishes using a sterile spreader. 10 µL of *C. ovata* extracts prepared at concentrations of 25 mg/mL and 50 mg/mL were adsorbed onto 6 mm sterile discs (Whatman™ 2017–006). The discs containing the test substances were placed on the inoculated agar plates, which were then incubated at 37 °C for 48 hours. After incubation, the diameters of the inhibition zones (mean ± SD) were measured in milimeter (mm) (43).

***Minimum Bactericidal Concentration (MBC)*:** After incubation, 10 µL of the suspension from each well was inoculated onto fresh TSA plates. The TSA plates were then incubated at 37 °C for 24 h. The MBC was identified as the lowest concentration of *C. ovata* extract at which no bacterial growth was observed (43).

***In Vitro Antibiofilm Activity*:** For the biofilm assay, pathogenic microorganisms were cultured in TSA at 37 °C for 16 h. The cultured bacteria were collected using a sterile swab and adjusted to 0.5 McFarland standard using a densitometer (Alla, France) in 10 mL of 0.9% NaCI. Then, 20 µL of this bacterial suspension was added to 200 µL Mueller Hinton Broth containing different doses of *C. ovata* extracts, and the plates were incubated at 37 °C for 24 h. Following incubation, the microplate was emptied and washed 3 times with 200 µL phosphate-buffered saline (PBS) to remove non-adherent bacteria. Adherent bacteria were fixed by adding 200 µL of methanol for 15 min. After discarding the methanol the plates were dried at 55 °C for 1 h. Bacteria adhered to the wells were stained with 200 µL of crystal violet for 5 min. Then, the stained microplates were washed with tap water to remove excess stain and dried again. The retained dye was solubilized 200 µL of 33% acetic acid and the absorbance was measured at 590 nm (45).

Results

Phenolic compounds of the extract and wavelengths, retention times, and LOD values for the analytes were given Table 1. Standard and sample chromatograms are shown in Figures 1 and 2, respectively. The analysis showed that *C. ovata* metanolic extract was pedominated by caffeic acid (15220.493 µg/g), rutin (8431.761 µg/g), ellagic acid (7487.660 µg/g), epicatechin (5710.737 µg/g), naringin (1835.020 µg/g).

The *in vitro* antimicrobial activity of *C. ovata* methanol extract against the tested microorganisms was determined using disc diffusion and microdilution methods, along with its *in vitro* antibiofilm effects (43). The MIC values for the tested bacterial strains and the yeast strain (*C. tropicalis* ATCC 13803) are presented in Figure 3. MIC, MBC and antibiofilm activity are summarized in Table 2.

Table 1. Concentration of major phenolic compounds in the extract, and LOD, wavelength and retention time values for analysis.

Compounds	Concentration (µg/g)	LOD (ppm)	Wavelength (nm)	Retention time (min)
Gallic acid	91.130	0.01	280	6.8
Vanillic acid	485.940	0.11	320	19.2
Caffeic acid	15220.493	0.01	280	22.7
Epicatechin	5710.737	0.43	260	21.3
p-Coumaric acid	72.964	0.01	320	26.1
Ferulic acid	189.094	0.01	320	30.1
Rutin	8431.761	0.57	360	45.6
Ellagic acid	7487.660	0.57	240	47.7
Naringin	1835.020	0.40	280	49.7
Cinnamic acid	910.190	0.01	280	71.1
Quercetin	366.202	0.57	360	70.4

Note: This table focuses on major phenolic compounds, showcasing their concentrations, limits of detection (LOD), wavelength values, and retention times for a comprehensive overview. Standard and sample chromatograms are illustrated in Figures 1 and 2, respectively.

Table 2. Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), and antibiofilm values of *C. ovata* methanol extract.

	Concentrations (mg/mL)		
	MIC	MBC	Antibiofilm
***B. cereus* NRRL-B-3711**	8	16	8
***S. aureus* ATCC 25923**	32	64	8
***S. aureus* (MRSA) ATCC 43300**	32	64	8
***S. aureus* (enterotoksin E) FRI 918**	32	64	8
***E. faecalis* ATCC 29212**	16	64	16
***C. tropicalis* ATCC 13803**	16	64	8
***E. coli* O157:H7 ATCC 43895**	32	128	32
***E. coli* O157:H7 ATCC 35150**	64	128	64
***L. monocytogenes* RSKK 02028**	32	128	32
***L. monocytogenes* RSKK 472**	32	128	32
***L. innocua* ATCC 33090**	32	128	16
***S.* Typhimurium ATCC 14028**	64	128	64
***S. aureus* NCTC 13552**	32	128	32
***S. epidermidis* ATCC 12228**	32	128	8

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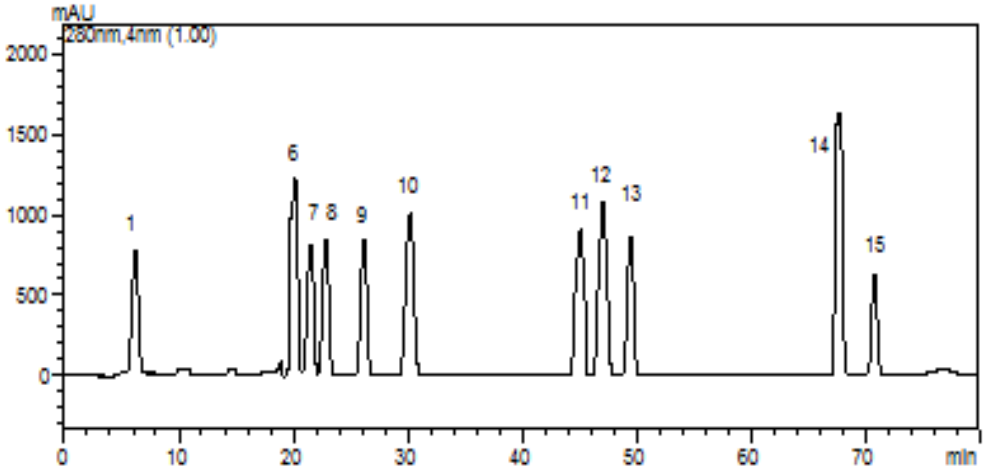

Figure 1. Standard Chromatogram.
Gallic acid (1), Vanillic acid (6), Caffeic acid (8), Epicatechin (7), p-Coumaric acid (9), Ferulic acid (10), Rutin (11), Ellagic acid (12), Naringin (13), Cinnamic acid (14), and Quercetin (15).

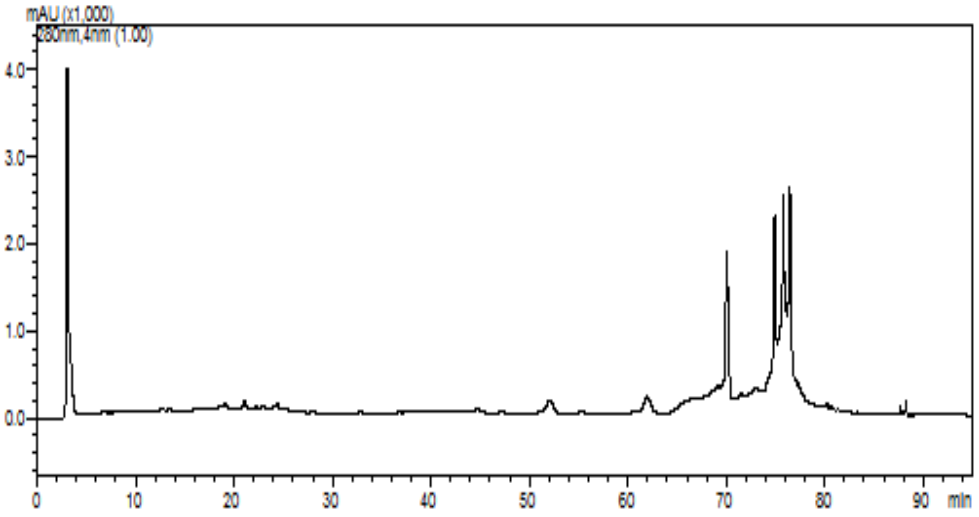

Figure 2. Sample Chromatogram of *C. ovata* extract.

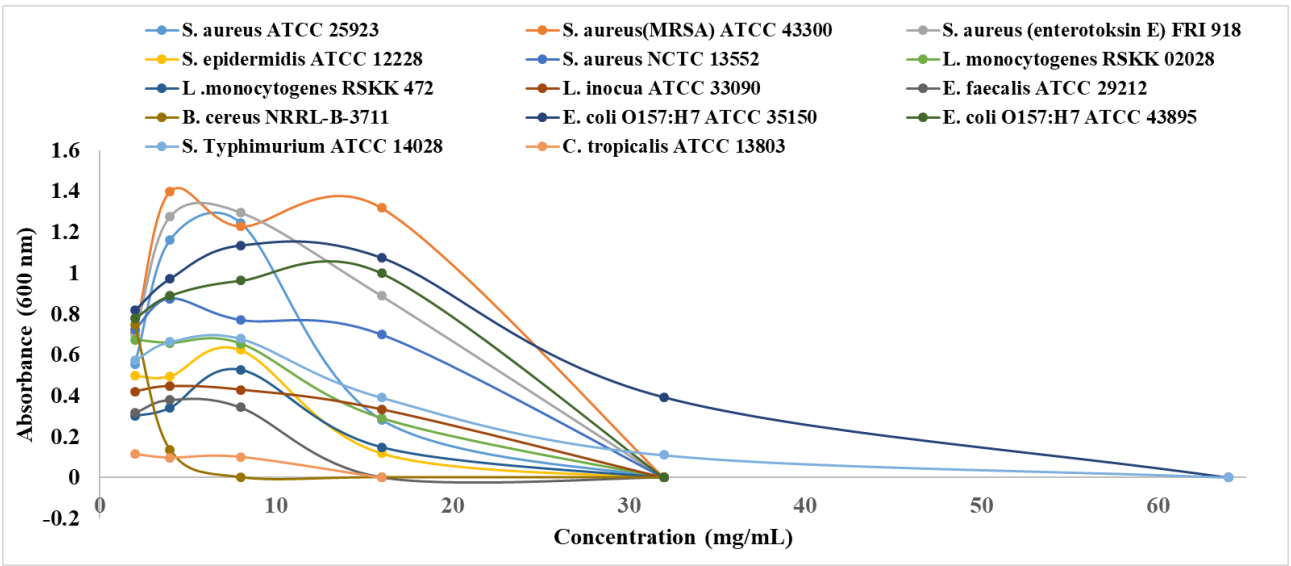

Figure 3. MIC values of all tested bacteri and *C. tropicalis*.

Table 3. The mean ± SD diameter of inhibition zones (mm) of the extract of *C. ovata* against the bacteria.

Bacteria	Concentration of *C. ovata* extract	
	25 mg/mL	50 mg/mL
S. aureus ATCC 25923	6.6 ± 0.14	7.15 ± 0.07
B. cereus NRRL-B-3711	7.8 ± 0.14	12.65 ± 0.21
C. tropicalis ATCC 13803	-	7.45 ± 0.21

The MIC values for *C. ovata* extract were determined as follows: 8 mg/mL for *B. cereus* NRRL-B-3711, 16 mg/mL for *E. faecalis* ATCC 29212, and *C. tropicalis* ATCC 13803, 32 mg/mL for *S. aureus* ATCC 25923, *S. aureus* (MRSA) ATCC 43300, *S. aureus* (enterotoxin E) FRI 918, *E. coli* O157:H7 ATCC 43895, *L. monocytogenes* RSKK (02028, and 472), and *L. innocua* ATCC 33090, *S. aureus* NCTC 13552, *S. epidermidis* ATCC 12228, and 64 mg/mL for *E. coli* O157:H7 ATCC 35150, and *S.* Typhimurium ATCC 14028 strains.

The MBC values were found to be 16 mg/mL for *B. cereus* NRRL-B-3711, 64 mg/mL for *S. aureus* ATCC 25923, *S. aureus* (MRSA) ATCC 43300, *S. aureus* (Enterotoxin E) FRI 918, *E. faecalis* ATCC 29212, and *C. tropicalis* ATCC 13803, 128 mg/mL for *E. coli* O157:H7 ATCC 43895, *E. coli* O157:H7 ATCC 35150, *L. monocytogenes* RSKK (02028, and 472), *L. innocua* ATCC 33090, *S.* Typhimurium ATCC 14028, *S. aureus* NCTC 13552, and *S. epidermidis* ATCC 12228 strains.

The methanol extract of *C. ovata* inhibited biofilm formation effectively**.** At a concentration of 8 mg/mL, it prevented biofilms in *S. aureus* ATCC 25923, *S. aureus* (MRSA) ATCC 43300, *S. aureus* (enterotoxin E) FRI 918, *B. cereus* NRRL-B-3711, *C. tropicalis* ATCC 13803, and *S. epidermidis* ATCC 12228. At 16 mg/mL, it was effective against *L. innocua* ATCC 33090 and *E. faecalis* ATCC 29212. A higher concentration of 32 mg/mL was needed for *S. aureus* NCTC 13552, *E. coli* O157 ATCC 43895, and both strains of *L. monocytogenes* RSKK 02028 and RSKK 472. The highest concentration tested, 64 mg/mL, was required to inhibit biofilm formation in *E. coli* O157 ATCC 35150 and *S.* Typhimurium ATCC 14028. According to our findings, biofilm formation persisted in other tested bacteria under our experimental conditions. The extract was most effective against *B. cereus* NRRL-B-3711, *S. aureus* ATCC 25923, *S. aureus* (MRSA) ATCC 43300, *S. aureus* (enterotoxin E) FRI 918, *C. tropicalis* ATCC 13803, and *S. epidermidis* ATCC 12228 strains at the lowest concentration of 8 mg/mL. However, it exhibited the least inhibitory effect on *E. coli* O157 ATCC 35150 and *S.* Typhimurium ATCC 14028 at the highest concentration of 64 mg/mL.

The disc diffusion mean values for *C. ovata* methanol extract were determined as follows: *S. aureus* ATCC 25923 for 25 mg/mL; 6.6 ± 0.14 mm, for 50 mg/mL; 7.15 ± 0.07 mm, *B. cereus* NRRL-B-3711 for 25 mg/mL; 7.8 ± 0.14 mm, for 50 mg/mL; 12.65 ± 0.21 mm, *C. tropicalis* ATCC 13803 only for 50 mg/mL; 7.45 ± 0.21 mm (Table 3).

Discussion and Conclusion

In the current study, the methanol extract of *C. ovata* was assessed for its antimicrobial and antibiofilm activities against a panel of foodborne pathogenic microorganisms. The results showed the metanolic extract of *C. ovata* demonstrated effective activities against some pathogenic bacteria, as well as against the yeast fungus *C. tropicalis.* As with various parts and extracts of the genus *Capparis* (22, 39, 49), the species *C. ovata* species is recognized for its antibacterial effects (10) and contain phenolic acids with documented antibacterial properties (39, 49). It has been suggested that the antimicrobial and antifungal potential of this medicinal plants may be due to phenolic compounds, quaternary ammonium and glycosinolates in the plant (22). Additionnaly, terpenes, flavonoids, tannins, alkaloids and reducing sugars have been identified as having antimicrobial activities and could contribute to their antibacterial effects (30). It has been reported that capers exhibit antimicrobial and disinfectant effects due to their long-chain alkyl groups (26). However, it is important to consider various factors that affect the chemical composition and biological activities of these plants (30). The leaves and buds of the Caper plant are exceptionally rich in phenolic compounds (26). In the current study, high amounts of caffeic acid, rutin, ellagic acid, epicatechin, and naringin were found in the extract of *C. ovata*. Studies indicate that caffeic acid, rutin, ellagic acid, and naringin have high antimicrobial activity (11, 18, 23, 44). The antimicrobial activity in the current study can be attributed to these high amounts of compounds in the extract.

In an *in vitro* study conducted by Muhaidat *et al.* (30), the antibacterial activity of *C. ovata* extract and essential oils extracted using various organic solvents (butanol, aqueous methanol, hexane) and methods was evaluated. The study found that the highest antibacterial activity among all tested fractions was against the Gram-positive *Streptococcus faecalis* (ATCC 29212). Conversely, the lowest activity was observed against the Gram-negative *S.* Typhimurium (ATCC 13311) when

using the petroleum ether fraction. In the current study, the highest antibacterial activity was found against *B. cereus* NRRL-B-3711 with MIC value of 8 mg/mL. In contrast, the lowest activity was noticed against *E. coli* O157:H7 ATCC 35150 and *S.* Typhimurium ATCC 14028 with MIC value of 64 mg/mL. The MIC values from the current study were higher than results of aquous methanol extraction of *C. ovata* by Muhaidat *et al.* (30). The region where the plant was collected and the extraction method may have changed the content of the extract and thus caused differences in antibacterial activity.

Studies have demostrated that various *Capparis* species possess antifungal activity against various fungal pathogens such as *Valsa vali* and *Rhizoctonia solani* (4, 24, 34). The methanolic extract of *C. ovata* was found to exhibit strong antifungal activity against *C. albicans* (ATCC 90028), *Candida parapsilosis* (ATCC22019), *Candida krusei* (ATCC 6258), *Malassezia pachydermatis* yeast strains and filamentous fungal strains *Microsporum canis*, *Microsporum gypseum*, *Microsporum nanum* and *Trichophyton mentagrophytes* (MIC values: 100-200 mg/mL) (34). In the current study, methanolic extract of *C. ovata* showed antifungal activity against *C. tropicalis* ATCC 13803 (Table 1). In our present research, the *C. ovata* extract effectively inhibited biofilm formation in all bacterial strains and the *C. tropicalis* ATCC 13803 strain tested, as detailed in Table 1. This study confirmed the antifungal activity of the methanolic extract of *C. ovate* against Candida species.

To date, there are no studies specifically addressing the antibiofilm activity of the *C. ovata* species. Reports on other *Capparis* species demonstrating such activity are also scarce. Notably, El-Subeyhi *et al.* (14) discovered that potassium oxide nanoparticles (K2O NPs), employed as reducing and capping agents in *C. spinosa* flower extract, exhibited significant antibiofilm activity. In the current study, *C. ovari* methanolic extracts exhibited anti-biofilm acitivities against all tested bacteria.

In conclusion, this study demonstrate that the methanolic extract of *C. ovata* holds potential as a natural antimicrobial agent for therapeutic formulations aimed at controlling the tested microorganisms and treating associated diseases. The bacteria investigated in this study are predominantly associated with food-derived products, suggesting that *C. ovata* could serve as a plant-based alternative to chemical preservatives for food preservation and safety. Furthermore, it may offer a viable option for biocontrol strategies and maintaining biofilm-free systems. However, additional studies are necessary to further confirm the role of this plant in herbal medicine and to explore its potential for discovering new natural bioactive compounds.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

MB, AS, HY and AK conceived and planned the experiments. MB, AK and AS contributed to extraction. MB and AK conducted the extract content analyses. HY and AS conducted antimicrobial activity studies. MB made the first drift of the manuscript. MB, AS, HY and AK contributed to the interpretation of the results. 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.

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Confirmation of epidural anesthesia with bupivakain in cats by infrared thermographic imaging and SEP

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ABSTRACT

In this study, the usability of electromyography and infrared thermography was tested to confirm the success of epidural anesthesia. The cats were randomly divided into 2 groups as experimental and control groups. The cats were intubated and placed under inhalation anesthesia using an anesthesia device. SEP (Somatosensory Evoked Potentials) recordings were obtained at the L7-S1, L6-L7, L6-L5, and L5-L4 intervals before epidural injection. Before epidural injection, infrared thermographic images and rectal temperatures were taken from a distance of 50 cm, where the areas where surface temperatures were to be measured were clearly visible. Following asepsis and antisepsis, 0.5% bupivacaine in the experimental group and saline in the control group were injected into the lumbosacral region. Infrared thermograms and rectal temperatures were obtained from each cat seven times (0.min, 5.min, 10.min, 15.min, 20.min, 25.min and 30.min) for 30 min at 5 min intervals after injection. After the last infrared thermogram was recorded, post-epidural SEP was recorded. Rectal temperature values decreased gradually in all cats throughout the anesthesia period, and no difference was observed between the groups. A decrease in potential duration and an increase in latency values were recorded in 10 cats administered epidural 0.5% bupivacaine compared with 10 cats administered epidural saline. Although there were only statistically significant values, the amplitude values were not kinetically significant. No clinically or statistically significant difference was observed in the infrared thermograms obtained before and 5 min after epidural injection in both groups.

Introduction

It is a common technique used to perform surgical procedures in veterinary medicine since the 1950s in North America and Europe (15, 27). In the late 1980s, with the recognition of the analgesic effects of opioids on the spinal cord, the use of epidural analgesia became an important re-emerging tool in intra- and postoperative epidural techniques to provide analgesia and anesthesia in veterinary medicine (27).

The efficacy of epidural anesthesia in cats was first confirmed experimentally in 1969 (9). Epidural anesthesia has been used successfully in cats for a variety of purposes (27). Epidural injections are most easily and safely performed in the lumbosacral (L7-sacrum [L-S]) intervertebral space in small animals because the spinal cord and dura end cranially in this region. Although it is optional to perform the technique under general anesthesia in the dog, general anesthesia is considered indicated in the cat (15). Considered by some to be the gold standard for preemptive analgesia, epidural anesthesia reduces central sensitization, intraoperative inhalant and opioid requirements, and stress responses during surgery (21, 25). The most commonly used agents were bupivacaine and lidocaine (15).

Infrared thermography has been used in many veterinary medicine studies, such as for inflammation detection and herd inspection, and its use is expanding. (5, 13, 20, 29). Infrared thermographic changes after epidural

bupivacaine injection have been investigated in various animals and humans (4, 5, 10, 12, 16, 17, 28). However, the use of thermography to confirm the efficacy of epidural administration has not been widely studied in veterinary medicine (5).

Electrodiagnostic methods have been used in the field of neurology for approximately 70 years, and have been used in veterinary neurology since the 1960s and are now developing as a sub-branch. In the field of veterinary neurology, it was first isolated from a dog in 1966 (6). Somatosensory evoked potentials (SEP) have been routinely used for many years to evaluate the somatosensory pathway and thus complete the diagnostic process when history, neurological examination, and imaging are not entirely conclusive (11, 19, 22). The effects of epidural bupivacaine administration on SEP have been investigated in humans (18).

This study aimed to evaluate the somatosensoryevoked potential and segmental warming of cats epidurally anesthetized with bupivacaine using infrared thermography and to confirmepidural anesthesia because failure of the epidural technique was estimated to be as high as 9% in cats (27). Techniques that demonstrate the accuracy of epidural injection may be useful.

Materials and Methods

The study subjects were 20 2-5 years old and 3-4 kg mongrel cats, which were brought to Burdur Mehmet Akif Ersoy University of Veterinary Medicine Animal Hospital for castration, were in class I or II according to ASA classification, and no hind limb or spine fracture or cardiovascular disease was detected in the clinical examination. Before the operation, all patients were premedicated with intravenous diazepam (Diazem, Deva Holding A.Ş., Türkiye) at a dose of 0.5 mg/kg by opening the vascular access with an appropriately sized intravenous catheter. After premedication, anesthesia was induced with intravenous propofol (Propofol® 1% Fresenius, Fresenius Kabi) at a dose of 4 mg/kg. Following induction, the patient was intubated with an endotracheal tube (Bıçakçılar A.Ş., Istanbul) of the appropriate size, and the patient was connected to the anesthesia device (Dräger, Primus, Lübeck, Germany). Until the end of the operation, sevoflurane (Sevorane®, Abbott Laboratuvarları İthalat İhracat Tic. Ltd. Şti. Istanbul, Türkiye) was used to maintain anesthesia with mechanical ventilation. Also, the oxygen concentration was maintained at 100%.

A 22G spinal needle (MediSpine, Olgun Medikal İNŞ ve TİC. LTD.ŞTİ) was used for the epidural catheterization. For epidural anesthesia, 0.5% bupivacaine (Marcaine 0.5%, Zentiva Sağlık Ürünleri Sanayi ve Ticaret A.ş., Türkiye) was administered at a dose of 1 mg/kg.

With an electromyography (EMG) device (Synergy CareFusion 5-channel EMG-EP®), SEP parameters were evaluated and recorded with Teflon-coated stainless-steel needles in the S1-L7, L7-L6, L6-L5, and L5-L4 intervals before epidural anaesthesia and at 30 minutes after epidural injection.

Immediately before epidural anesthesia, an infrared thermographic image was taken with an infrared thermal camera (FLIR® C5, USA) using a tripod at a right angle at a distance of 50 cm from the operating table and rectal temperature was recorded (Figure 1). Subsequently, infrared thermographic images and rectal temperature measurements were obtained seven times (0. minute, 5. minute, 10. minute, 15. minute, 20. min, 25. min, and 30. min) at 5-minute intervals after epidural anesthesia. The thermal images were evaluated using the Flir Ignite application and superficial temperature values of seven different regions (Figure 2., Figure 3.) in each image was recorded.

Figure 1. Taking thermographic images.

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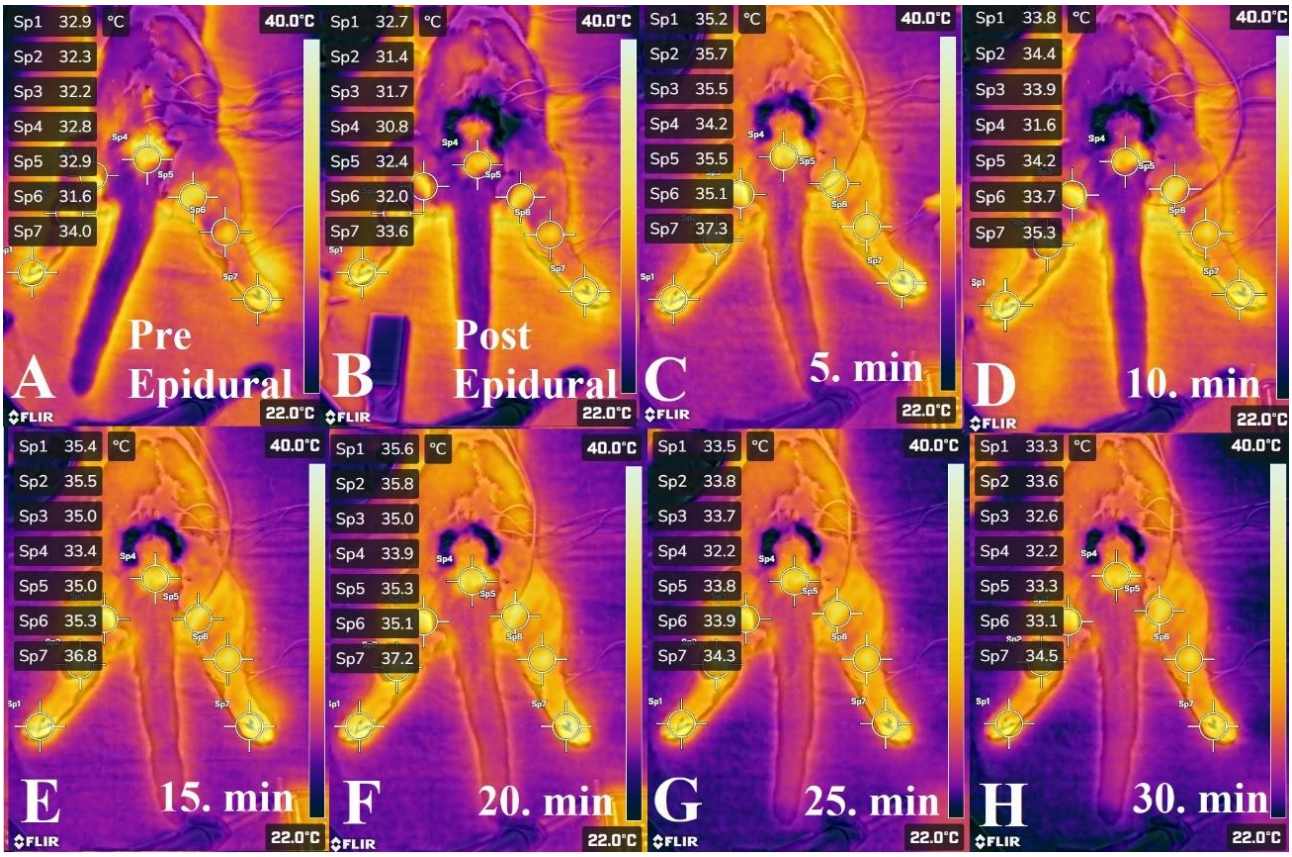

Figure 2. Infrared thermographic images of experimental group, 2-year-old female cat with epidural injection of bupivacaine.
(A) Thermography taken before epidural injection. (B, C, D, E, F, G, H) Thermographic images taken 5 minutes after epidural injection, respectively.

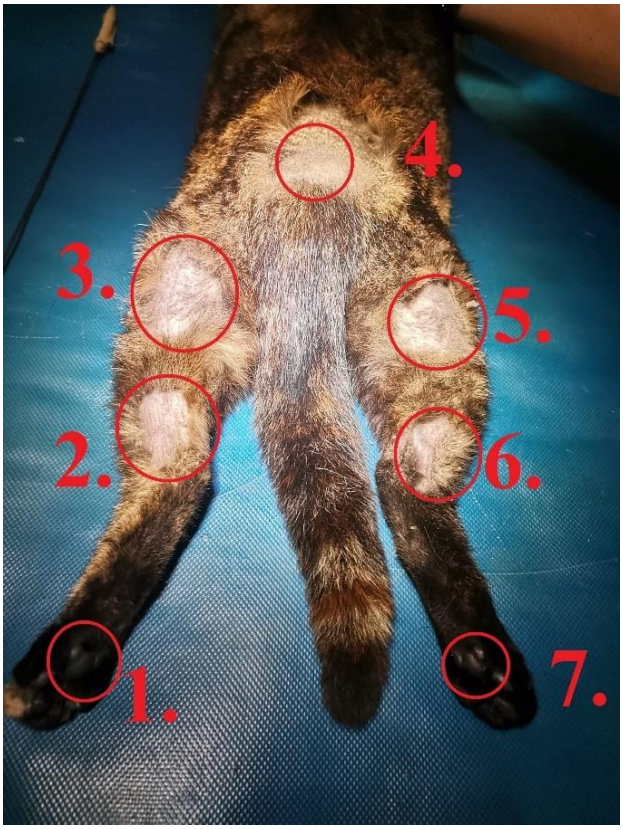

Figure 3. Regions to measure temperature in Infrared Thermographic images.

In all cases, after the radiopaque material was injected into the epidural catheter, radiography of the region in the laterolateral position was performed to confirm whether the needle was in the right place. In the experimental group, 0.5% bupivacaine (Marcaine 0.5%, Zentiva Sağlık Ürünleri Sanayi ve Ticaret A. Ş., Türkiye) at a dose of 1.0 mg/kg was used to induce epidural anesthesia. The total amount of fluid administered into the epidural space was limited to 0.4 ml/kg. If the drug dose did not meet the total amount of fluid administered, 0.9% isotonic NaCl (Polifarma İlaç San. ve Tic. A.Ş., Tekirdağ, Türkiye) was added, not exceeding 1 ml in total. After confirming that the spinal needle was in the epidural space, the control group received 1 mL 0.9% isotonic NaCl.

Statistical Analysis: The Shapiro-Wilk test was used to test whether the variables fit a normal distribution. Variables conforming to normal distribution are given as mean±standard deviation, and an independent sample t-test was used for comparisons between two independent groups. Variables that did not fit the normal distribution were presented as median (minimum-maximum) values, and the Mann-Whitney U test was used for comparisons between two independent groups. Statistical analyses were performed using the IBM SPSS Statistics 22.0 program. The significance level was set at α=0.05.

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Results

This study tested the usefulness of EMG and infrared thermography in confirming the success of epidural anesthesia. Rectal temperature values decreased gradually during the anesthesia period in all cats, and no difference was observed between the groups. Epidural administration of 0.5% bupivacaine decreased the potential duration and increased the latency values in 10 cats compared to 10 cats receiving epidural saline, but the amplitude values were not clinically and statistically significant. No clinically or statistically significant difference in temperature change was observed in the infrared thermograms taken beforeand 30 min after epidural injection in either group.

The groups within the scope of the study showed statistically significant differences in terms of the amount of change in pre-test, post-test amplitudes S1-L7, L7-L6, and L5-L4 ($P<0.05$). The decreases in amplitude S1-L7, amplitude L7-L6, and amplitude L5-L4 values of the cats in the experimental group were higher than those in the control group. The change in pre-test, post-test amplitude L6-L5 values did not show a statistically significant difference between the groups ($P>0.05$) (Table 1).

When we consider the amplitude values, we see that the L6-L7 and L4-L5 potentials recorded in the experimental group decreased, as expected, at the 30th minute of epidural bupivacaine administration. However, a decrease in the 3rd and 1st potentials. The potentials recorded in the control group showed a slight increase 30 min after saline injection. (Figure 4.)

Table 1. Examination of pre-test - post-test amplitude change amounts of the cats included in the study.

	Group	N	Avarage.±SS*/Median(Min-Maks)**	t*/Z**	P
Amplitud S1-L7 yd*	Test	10	-0.23±0.24	-2.784	0.012*
	Control	10	0.15±0.36		
Amplitud L7-L6 yd**	Test	10	-0.24(-0.33-0.12)	-2.57	0.010*
	Control	10	0.05(-0.19-0.50)		
Amplitud L6-L5 yd**	Test	10	-0.13(-0.61-0.47)	-1.470	0.141
	Control	10	0.01(-0.09-0.17)		
Amplitud L5-L4 yd*	Test	10	-0.23±0.15	-3.292	0.004*
	Control	10	0.07±0.25		

*P<0.05, yd: Percentage Change, *Independent Sample t-Test, **Mann Whitney U Test*

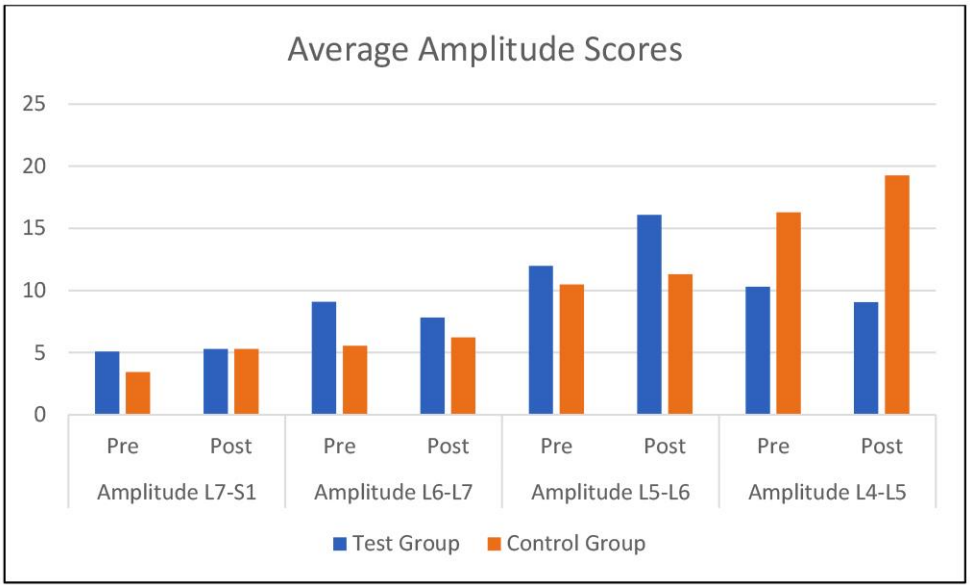

Figure 4. The bar graph shows average amplitude scores for both pre and post epidural injection SEP measurement.

The groups within the scope of the study did not show statistically significant differences in terms of the amount of change in pre-test,post-test latency S1-L7, L7-L6, L6-L5, and L5-L4. (P>0,05) (Table 2).

In the experimental group, the potential latencies measured at each interval after the epidural bupivacaine injection increased, as expected. However, the control group did not show such a significant increase, as observed in the experimental group (Figure 5). When changes in the mean potential duration were examined, a decrease was observed in the measurements obtained from the experimental group. In contrast, an increase was recorded in the control group measurements, except for the second interval (Figure 5). However, the groups within the scope of the research did not show statistically significant differences in terms of the pre-test-post-test duration S1-L7, duration L7-L6, duration L6-L5, and duration L5-L4 change amounts (P>0,05) (Table 3).

Table 2. Examination of the pre-test, post-test latency change amounts of the cats included in the study.

	Group	N	Average.±SS*/Median(Min-Maks)**	t*/Z**	P
Latans S1-L7 yd**	Test	10	0.13(-0.20-0.30)	-0.267	0.796
	Control	10	0.04(0-0.31)		
Latans L7-L6 yd**	Test	10	0.09(-0.20-0.14)	-1.328	0.218
	Control	10	0(-0.06-0.10)		
Latans L6-L5 yd**	Test	10	0.08(-0.19-0.33)	-2.061	0.052
	Control	10	0(-0,11-0)		
Latans L5-L4 yd*	Test	10	0.04±0.13	-0.558	0.584
	Control	10	0.07±0.12		

*P<0.05, yd: Percentage Change, *Independent Sample t-Test, **Mann Whitney U Test*

Table 3. Examination of the pre-test - post-test duration change amounts of the cats included in the study.

	Group	N	Average.±SS*/Median(Min-Maks)**	t*/Z**	P
Duration S1-L7 yd**	Test	10	-0.002(-0.26-0.33)	-0.982	0.356
	Control	10	0.03(-0.11-1.19)		
Duration L7-L6 yd**	Test	10	-0.08(-1.2-0.52)	-1.063	0.315
	Control	10	0(-4.77-1.4)		
Duration L6-L5 yd**	Test	10	-0.07(-0.21-0.25)	-1.798	0.079
	Control	10	-0.001(-0.7-0.05)		
Duration L5-L4 yd*	Test	10	-0.09±0.32	-0.753	0.462
	Control	10	-0.009±0.14		

*P<0.05, yd: Percentage Change, *Independent Sample t-Test, **Mann Whitney U Test*

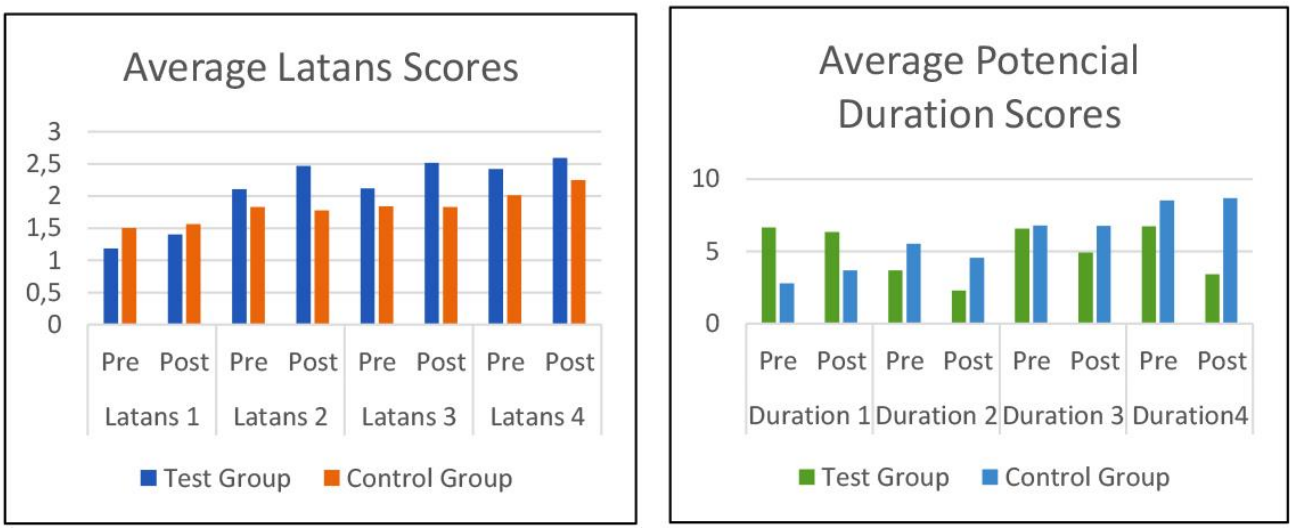

Figure 5. Column graph showing the comparison of latency (Left) and potencial duration (Right) values before and after epidural injection.

Rectal temperature values decreased in both groups during 30 min of anesthesia. In the experimental group, a significant decrease of 2° was observed at 10 min, whereas a difference of more than 1° was recorded at the end of 30 min. In the control group, a steady and slow decrease was observed, and again, a difference of more than 1 ° between the last and first measurements was observed. The groups within the scope of the study did not show statistically significant difference in terms of the amount of change in rectal temperature at 0. min, 5. min, 10. min, 15. min, 20. min, 25. min and 30. min after epidural injection compared with the rectal temperatures before epidural injection (P>0.05) (Figure 6) (Table 4.).

The groups within the scope of this research did not show statistically significant differences in terms of the amount of change in the temperatures of 0. min, 5.min, 10.min, 15.min, 15.min, 20.min, 25.min and 30.min after epidural injection in all seven regions compared with pre-epidural temperatures (P>0.05) (Table 5., Table 6.).

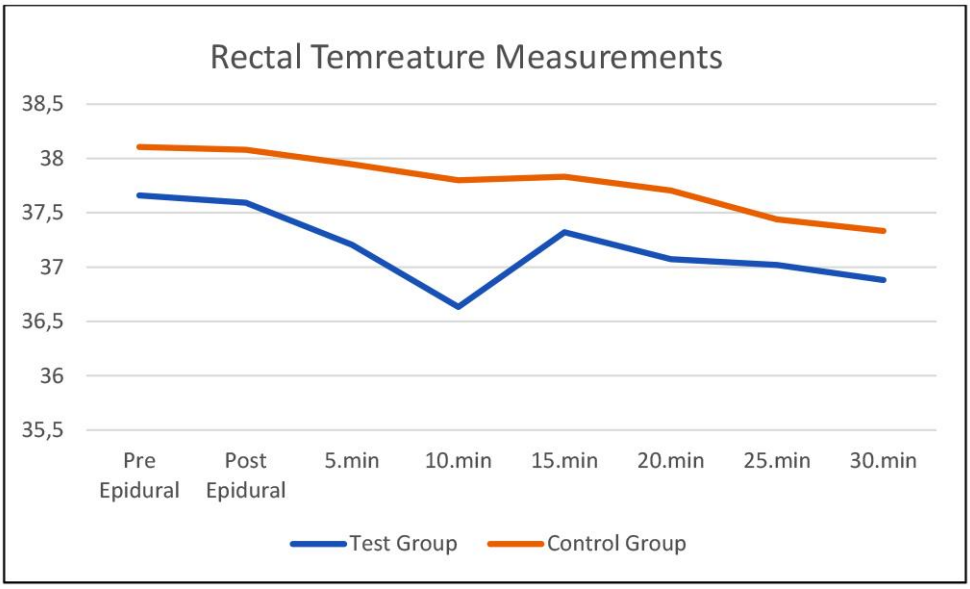

Figure 6. Graph of mean rectal temperature measurements.

Table 4. Examination of the amount of change in the rectal temperatures of the groups included in the study at 0. min, 5. min, 10. min, 15. min, 20. min, 25. min and 30. min after epidural compared to the rectal temperatures before epidural.

	Group	N	Average.±SS*/(Median(Min-Maks)**	t*/Z**	P
Post Epidural 0.min yd**	Test	10	0(-0.03-0)	-0.927	0.354
	Control	10	0(-0.01-0)		
Post Epidural 5.min yd*	Test	10	-0.02±0.01	-1.952	0.074*
	Control	10	-0,01±0,01		
Post Epidural 10.min yd**	Test	10	-0.02(-0.05-0.53)	-0.265	0.791
	Control	10	-0.01(-0.27-(-0.01))		
Post Epidural 15.min yd*	Test	10	-0.02±0.01	-1.323	0.202
	Control	10	-0.02±0.01		
Post Epidural 20.min yd**	Test	10	-0.02(-0.06-(-0.01))	-1.512	0.131
	Control	10	-0.02(-0.03(-0.01))		
Post Epidural 25.min yd*	Test	10	-0.03±0.02	-1.224	0.237
	Control	10	-0.02±0.01		
Post Epidural 30.min yd*	Test	10	-0.04±0.02	-1.369	0.188
	Control	10	-0.03±0.01		

*P<0.05. yd: Percentage Change, *Independent Sample t-Test, **Mann Whitney U Test*

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Table 5. To examine the amount of change in the temperatures of the groups within the scope of the research at 0. min, 5. min, 10. min, 15. min, 20. min, 25. min and 30. min after the 1st region epidural compared to the pre-epidural temperatures.

	Group	N	Mean ..SS	T	P
Post Epidural 0.min yd**	Test	10	0.04±0.06	0.271	0.789
	Control	10	0.04±0.03		
Post Epidural 5.min yd*	Test	10	0.06±0.06	-0.119	0.907
	Control	10	0.06±0.04		
Post Epidural 10.min yd**	Test	10	0.06±0.04	-0.161	0.874
	Control	10	0.06±0.06		
Post Epidural 15.min yd*	Test	10	0.07±0.04	-0.592	0.561
	Control	10	0.08±0.05		
Post Epidural 20.min yd**	Test	10	0.07±0.04	-0.247	0.808
	Control	10	0.07±0.06		
Post Epidural 25.min yd*	Test	10	0.07±0.05	-0.286	0.778
	Control	10	0.08±0.06		
Post Epidural 30.min yd*	Test	10	0.07±0.05	-0.549	0.589
	Control	10	0.08±0.05		

*P<0.05. yd: Percentage Change, *Independent Sample t-Test, **Mann Whitney U Test*

Table 6. To examine the amount of change in the temperatures of the groups within the scope of the research at 0. min, 5. min, 10. min, 15. min, 20. min, 25. min and 30. min after the 6th region epidural compared to the pre-epidural temperatures.

	Group	N	Mean.±SS	T	P
Post Epidural 0.min yd**	Test	10	0.02±0.06	-0.31	0.760
	Control	10	0.03±0.05		
Post Epidural 5.min yd*	Test	10	0.05±0.05	0.415	0.683
	Control	10	0.04±0.05		
Post Epidural 10.min yd**	Test	10	0.05±0.05	0.005	0.996
	Control	10	0.05±0.04		
Post Epidural 15.min yd*	Test	10	0.04±0.04	-1.941	0.068
	Control	10	0.08±0.04		
Post Epidural 20.min yd**	Test	10	0.05±0.04	-0.884	0.338
	Control	10	0.07±0.06		
Post Epidural 25.min yd*	Test	10	0.05±0.05	-0.257	0.800
	Control	10	0.06±0.08		
Post Epidural 30.min yd*	Test	10	0.05±0.05	-0.842	0.411
	Control	10	0.08±0.07		

P<0.05. pd: Percentage Change, Independent Sample t-Test

Discussion and Conclusion

It is recommended that bupivacaine be administered epidurally in cats and dogs at a dose of 1-1.65 mg/kg (15, 26, 27). It has also been reported that the duration of action of bupivacaine administered by epidural injection is 15-30 minutes (15, 26, 27). Di Filippo et al. (8) reported in their study on humans that 0.5% bupivacaine, 0.5% ropivacaine and 0.75% ropivacaine rapidly increased skin temperature after epidural injection, reaching a peak of 1 to 1.80 ° 30 minutes after the block. In this study, cats in the experimental group received bupivacaine at a dose of 1 mg/kg. In addition, measurements were continued for 30 min following epidural injection so that data could be collected during the onset of the duration of action.

In a similar study by Xu et al. (28) examining surface temperature changes in experimental mice after epidural injection, the change in thermography over time after epidural bupivacaine administration was significant for the lower extremities (RM-ANOVA, P<0.001), but not in the upper extremities (RM-ANOVA, P=0.78). After epidural saline administration, there was no significant effect on thermography of the extremities. Compared with bupivacaine, epidural saline was a significant between-subject variable (RM-ANOVA, P<0.001). After epidural bupivacaine, the mean (±SD) Emax for the lower extremities was +3.73 °C ± 1.56. After epidural saline, the mean Emax for the lower extremities was -0.88 °C ± 0.28. No significant effect of epidural saline was observed in

any of the regions. In contrast to the study by Xu et al. (28), there was no significant difference in infrared thermographic analysis of cats before and after epidural bupivacaine or saline administration. It was thought that the reason for this may be the difference in sensitivity of the infrared thermal cameras used in data collection.

Epidural administration of low-concentration local anesthetics in humans has been shown to cause significant changes in surface skin temperature, as measured by infrared thermography (2). Epidural administration of bupivacaine has been shown to cause a gradual increase in foot skin temperature, taking more than 15 minutes to reach a 50% maximal increase (12). Furthermore, epidural sympathetic blockade at the thoracic and lumbar levels with low concentrations of bupivacaine was found to cause changes in the skin temperature in the thorax and feet (16). Thoracic epidural analgesia with low concentrations of bupivacaine has been shown to induce thoracic and lumbar sympathetic blocks, supporting the effect of bupivacaine on skin temperature (10). However, in this study, no significant difference was found between the two groups before and after epidural in the 2 groups.

Available references indicate that epidural anesthesia may ffect skin temperature. Küls et al. (17) described and compared two blockade methods (epidural and sciatic) in dogs undergoing orthopedic surgery. They reported that only four out of 12 animals (33%) receiving epidural analgesia and one out of eight animals (12.5%) receiving sciatic block showed an increase in temperature in the plantar pad. Other studies have reported that hyperthermia is associated with prolonged epidural analgesia (23), while others have found that epidural anesthesia may cause a decrease rather than an increase in skin temperature (3). Di Filippo et al. (8) reported in their study in humans that epidural 0.5% bupivacaine rapidly increased skin temperature after injection, reaching a peak of 1–1.8C 30 min after the block. In these studies, there was no statistically significant difference between the group that received 0.5% bupivacaine injection into the epidural region and the two groups that received saline injection at 30 min.

Loughnan et al. (18) evaluated the effects of epidural bupivacaine 0.75% on epidural somatosensory evoked potential (SEP) after posterior tibial nerve stimulation and found that the most prominent change was the decrease in the amplitude of the second and third peaks with a decrease in the overall amplitude. Similar but less pronounced changes were found with 0.5% bupivacaine administration, as in our study.

The maximum conduction velocity of a nerve decreases with decreasing temperature (7, 14). A 1 °C decrease in skin temperature causes a 1.3-2.4 m/s decrease in motor and sensory nerve conduction velocities (1). Şirin et al. reported that a 1 °C change in temperature caused a decrease of 1.7-1.8 m/s (24). In this study, a decrease of 1 °in rectal temperature and an average increase of 1 ° in skin temperature were recorded at the end of the 30-minute period in the two groups. However, a decrease in potential duration was recorded only in the experimental group. The reason for this difference is thought to be the vasodilation effect of 0.5% bupivacaine applied from the lumbosacral region to the epidural space.

The infrared thermal camera could not confirm the success of epidural anesthesia. When the available data and the literature were examined, it was thought that SEP analysis performed before and after the application could confirm the success of epidural 0.5% bupivacaine application, albeit with a small difference. Although a slight clinical difference was noted in the experimental group, no statistically significant difference was found. The reason for the failure of our method to confirm the accuracy of epidural bupivacaine administration was thought to be that the thermal camera we used for recording was not sensitive enough, or that epidural administration of bupivacaine may not have a recordable effect on skin temperature in cats. Further studies on this subject are recommended.

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Ethical Statement

This study was conducted after the animal experiments were approved by the Burdur Mehmet Akif Ersoy University Local Ethics Committee (Decision number: 1182).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ÖŞŞ and MAK conceived and planned the experiments. MAK carried out the experiments. ÖŞŞ and MAK planned and carried out the simulations. MAK contributed to sample preparation. ÖŞŞ and MAK contributed to the interpretation of the results. MAK 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 the findings of this study are available from the corresponding author upon reasonable request.

Animal Welfare

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

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Investigation of the effects of electrical stimulation on BDNF and NGF levels in the sciatic nerve injury rat model

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ABSTRACT

The current study aimed to investigate the effects of electrical stimulation on brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels in rats with sciatic nerve injury. Twenty-eight rats were divided into four groups of sham (S, n=7), electrical stimulation (ES, n=7), sciatic nerve injury (SNI, n=7) and sciatic nerve injury+electrical stimulation (SNI+ES, n=7). An experimental nerve damage model was produced by applying a closing force to compress the sciatic nerve. Electrical stimulation was applied for twenty minutes at 200 µs, 2mA, and 20 Hz for fifteen days. Enzyme-linked immunosorbent assay analysis was used to evaluate the levels of NGF and BDNF. It was shown that the SNI group had higher brain BDNF levels than the other groups, while the S group had lower brain BDNF levels than the other groups (P<0.001). The ES and SNI groups had higher serum BDNF levels than the S and SNI+ES groups (P<0.01), while the SNI group had higher brain NGF levels than the S and SNI+ES groups (P<0.05). In comparison to the S and ES groups, the serum NGF levels in the SNI and SNI+ES groups were shown to be lower (P<0.01). Following the sciatic nerve damage, it was measured that the administration of electrical stimulation resulted in an increase in brain BDNF and a decrease in serum NGF. According to this research, electrical stimulation may have an impact on the release of NGF and BDNF after sciatic nerve damage.

Introduction

The rat sciatic nerve model is widely used to investigate functional, histological, and electrophysiological changes after different chirurgical repairs or pharmacological treatments, following peripheral nerve damage in experimental peripheral nerve studies (34). Many methods, including anastomosis, nerve conduits, non-neural tissue grafts, nerve grafts, combined grafts, and synthetic tubes, have been used in the treatments of peripheral nerve damages, and their possible effects on nerve healing have been revealed (8, 24, 28, 32). The importance of early physiotherapy applications following peripheral nerve injuries is emphasized, and it is known that methods that preserve or increase the muscle fiber diameter are effective in denervated muscle treatment. Electrical stimulation may also be useful to stimulate nerve regeneration in cases of nerve injuries (22). Electrical stimulation is used in orthopedic and neurological rehabilitation to create functional or therapeutic movements in upper motor neuron illnesses, including traumatic brain injury, spinal cord damage, cerebral palsy, stroke, or multiple sclerosis, by inducing muscle activation in paralyzed muscles to stand, walk, grip, and release (12, 17). Gordon et al. (7) reported in both human and animal studies that electrical stimulation may significantly accelerate axon growth and muscle reinnervation may occur significantly earlier. Electrical stimulation is not only a way to restore function; it is also used as a clinical efficacy motor function training tool (13).

The most well-characterized members of the neurotrophins are nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), and both of them play specific roles in the regeneration, protection, and tropism of axotomized peripheral nerve fibers (15, 16, 18,

31). BDNF, in combination with other factors or alone, has been shown to have a positive effect following neurorhaphy (10, 20, 35). Frostick et al. (5) reported that BDNF promotes the survival and differentiation of motor neurons *ex vivo*, and it organizes the development of neuromuscular synapses and the function of preventing neuronal death following nerve damage *in vivo*. Vögelin et al. (35) presented that by surgically removing a twenty-millimeter portion of the sciatic nerve of rats *in vivo*, local continuous release of BDNF not only stimulates the regeneration of peripheral nerve but also provides rapid axonal growth and significantly reduces neuropathic pain. Shakhbazau et al. (31), in their study investigating neurotrophin synthesis in a two-week period in the transvertebral model of bilateral and/or unilateral transections of the sciatic nerves in rats, reported that increased NGF concentrations in the nerve segment of bilateral transection compared to the uniletral transection. After nerve axotomy, it is mentioned that neurotrophins (especially BDNF) have a direct role in enhancing the viability of the damaged neurons (6). In addition, BDNF mRNA expression in damaged sciatic nerves is up-regulated three days following nerve damage, and the up-regulation, which is deemed necessary for axonal regeneration, takes several weeks (23). In the literature, it has been indicated that BDNF released in the dorsal root ganglia is carried to the primary sensory neuron, and also BDNF levels change rapidly in the dorsal root ganglia following peripheral inflammation and nerve damage (27, 37). To further augment the functional outcome of peripheral nerve repair, neurotrophins have been used experimentally, and it has been suggested that neurotrophins increase neuronal cell survival and regeneration (4, 19). Huang et al. (11) indicated the potential of using electrical stimulation as a useful method to enhance functional recovery for delayed repair of peripheral nerve lesions in rats with chronic axotomized motoneurons. In addition, they presented that when brief electrical stimulation was applied to the proximal nerve stumps in the delayed nerve lesions, the BDNF gene expression was significantly up-regulated in the motoneurons in the anterior horn of the spinal cord. Park et al. (29) indicated that electrical stimulation may improve neural plasticity by boosting BDNF production in sciatic nerves such as soleus and medial gastrocnemius muscles. In a study on electrical stimulation application following femoral nerve cutting in rats, it was indicated that the gene expression of motoneuronal BDNF, which is organized by electrical activity, correlates with nerve regeneration (1).

The observed developments in functional behavior induced by electrical stimulation therapy can be partly mediated by molecular plasticity in the brain. It is suggested that electrical stimulation of peripheral nerves leads to the rise of genes related to regeneration, including BDNF. By enhancing the expression of structural protection proteins and neurotrophins, electrical stimulation may be used therapeutically to reduce muscle atrophy and improve muscle reinnervation (25). However, neural plasticity due to electrical stimulation and the general mechanisms mediating healing remain unclear (12). Moreover, research suggested that electrical stimulation may accelerate the healing of body function after peripheral nerve injury, but electrical stimulation still possesses the following problem to be solved: what changes in brain activity are induced by electrical stimulation (25). In the current study, the evaluation of BDNF and NGF release after sciatic nerve injury and whether electrical stimulation had any effects on the release of relevant factors were investigated.

Materials and Methods

In the present study, twenty-eight male, 8-10 weeks-old Wistar Albino rats (250–300 g) were provided from the Experimental Research Application and Research Center at Hatay Mustafa Kemal University (HMKU), Turkey. The rats were housed in a ventilated environment with 12-hour cycles of light and darkness, a humidity of 45 ± 5%, and a temperature of 21-1°C. Food and water were available *ad libitum*. The experiments on animals were approved by the Local Ethical Committee of Experimental Animal Ethics of HMKU, Turkey (approval no. 2019/10-5, 17/12/2019).

Animals and Surgical Procedures: To determine the minimum required sample size before the study, power analysis was performed using the criteria of a type 1 error rate of 0.05, power of 0.90, and effect size of 1 (36). The minimum required sample size was determined to be n=5 per group, totaling 20. However, to allow for potential losses during the study, 7 animals per group were used. Animals were equally divided into four groups (n=7 each): the sham (S) group, the sciatic nerve injury (SNI) group, the electrical stimulation (ES) group, and the sciatic nerve injury + electrical stimulation (SNI+ES) group. The animals were anesthetized for the operation with a xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (50 mg/kg) cocktail. Surgical procedures were carried out aseptically. The sciatic nerve was dissected (Figure 1) meticulously by forming a longitudinal skin incision on the right extremity at the level of the trochanter major and preserving the perineurium without traction injury in the sciatic nerve injury groups (SNI and SNI + ES). The sciatic nerve injury rat model was created by compressing the sciatic nerve with a special temporary aneurysm clip (Yasargil FE693K, Aesculap AG, Germany) with a closing force (50 g/cm) for two minutes in SNI and SNI + ES groups

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(Figure 2) (30), the wound was closed, and then the skin was sutured. In addition, skin and muscle were opened, respectively, and instantly closed in layers by the sciatic nerves left intact on the right extremities in the S and ES groups.

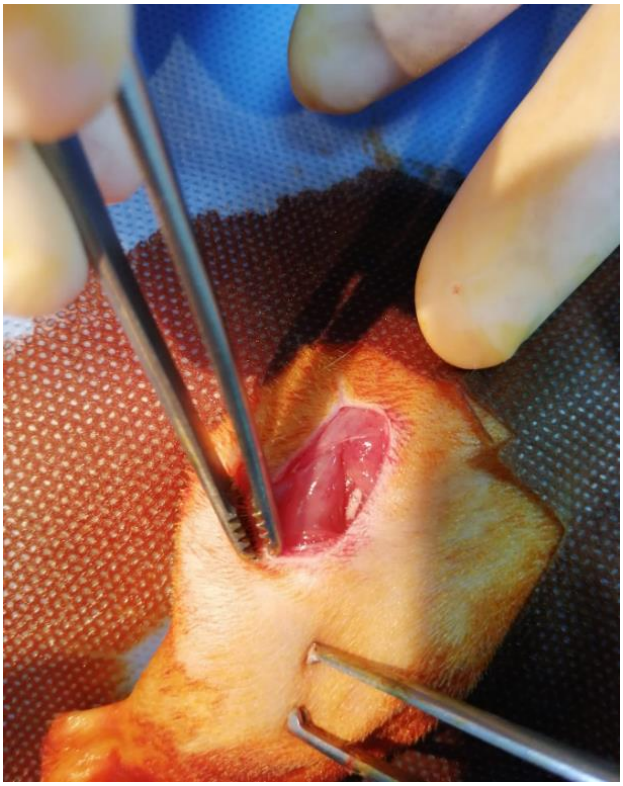

Figure 1. The exposure of the sciatic nerve.

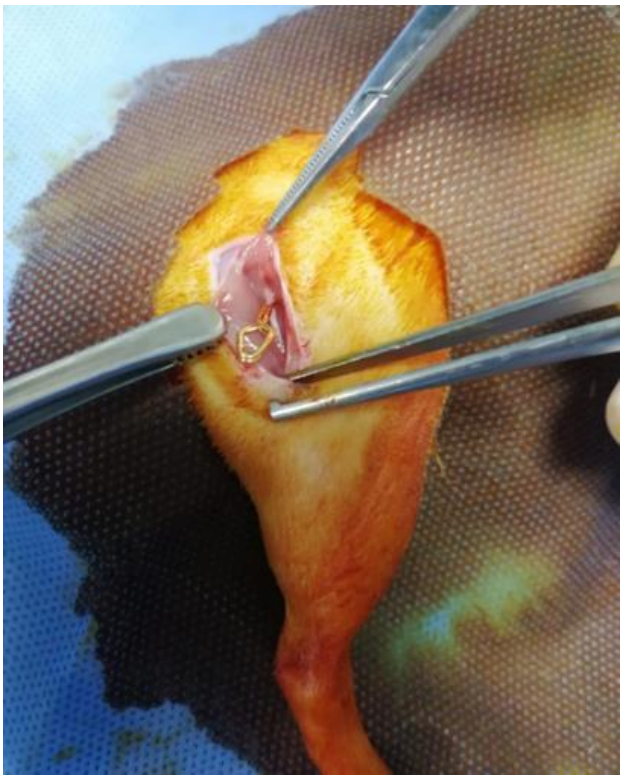

Figure 2. The sciatic nerve injury was made by compressing the nerve with a closing force.

Electrical Stimulation: The S and SNI groups received no treatment postoperatively. Electrical stimulation was commenced 3 hours following the operation. It was applied with two electrodes (Biomedical carbon film electrodes, Stimrodes); one of the electrodes was placed on quadriceps femoris muscle, especially 5 mm proximal to the injury site, and the other was placed on gastrocnemius muscle of the right extremity (Figure 3). Electrical stimulation was applied to rats in ES and SNI+ES groups at 20 Hz frequency, 200 µs current time, and 2 mA amplitude for 20 minutes parameters during 15 days with an electrical stimulation device (Chattanooga Intelect, Primera, England) (3, 22). In brief, the groups were formed as follows:

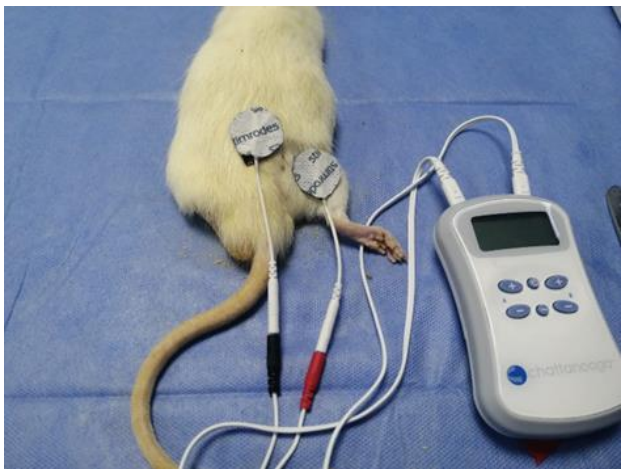

Figure 3. The application of electrical stimulation.

S Group: Only the tissues around the sciatic nerve were dissected from the dorsal surface of the right thigh of the rats, and the skin and subcutaneous tissue were closed properly without causing sciatic nerve injury.

ES Group: Only the tissues around the sciatic nerve were dissected from the dorsal surface of the right thigh of the rats, and the skin and subcutaneous tissue were closed properly without causing sciatic nerve injury. In addition, electrical stimulation was applied to the rats during fifteen days.

SNI Group: Right sciatic nerve injuries were experimentally induced in animals.

SNI + ES Group: Right sciatic nerve injuries were experimentally induced in animals, and then electrical stimulation was applied to the rats during fifteen days.

Sample Collection: On the 16th day of the study, the animals were anesthetized by using a mix comprising xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (100 mg/kg). Intracardiac blood was taken in serum clot activator tubes after the loss of righting, corneal, and withdrawal reflexes following anesthesia.

Rats were sacrificed with exsanguinations. Brain tissues were immediately harvested after the sacrification. The blood samples were centrifuged at 3000 rpm, +4 °C for 10 minutes, the serum was obtained, and then serum and brain tissues were kept at -80 °C until ELISA analyses.

Tissue Preparation: Brain tissues thawed, and the prefrontal cortex of the left hemisphere of the brain was used in the study. Approximately 100 mg of brain tissue sample was taken and homogenized by using an ultrasonic homogenizer (Bandelin Electronic UW 2070, Germany) in 1 ml phosphate buffer solution (pH: 7.4) in cooled tubes with ice (14). After centrifugation of the homogenates at 5,000 rpm, +4 °C for 30 minutes, the samples (supernatants) were obtained and kept at −20°C until enzyme-linked immunosorbent assay (ELISA) analyses.

Protein Analysis: Protein concentrations were measured spectrophotometrically (UV 2100 UV-VIS Recording Spectrophotometer, Shimadzu, Japan) according to Lowry's method (21). Brain protein levels were only determined to calculate neurotrophin levels. The method relies on the identification of cuprous ions, which are generated when proteins reduce cupric ions in an alkaline environment. Following a pre-treatment of the proteins with copper ions in an alkaline solution, the aromatic amino acids in the treated sample lowered the amount of phosphomolybdatephosphotungstic acid that was present in the Folin Reagent. This reaction resulted in a blue substance. By comparing the absorbance of the Folin reaction's final result at 700 nm to a standard curve of a standard (Bovine Serum Albumin) protein solution, the amount of protein in the sample was calculated.

BDNF and NGF Analysis: The levels of BDNF and NGF in the brain and serum samples were determined using the ELISA method. For this purpose, commercial ELISA test kits were used in the samples, and the procedure reported by the manufacturer was followed. Before beginning the ELISA analysis, the samples and solutions in the ELISA kits were brought to room temperature. The principles of the rat BDNF ELISA kit (201-11-0477, Sunred, China) and the rat NGF ELISA kit (201-11-0540, Sunred, China) are generally the same for each other. Briefly, the kits use a double-antibody sandwich ELISA to test the level of rat neurotrophin (NGF/BDNF) in samples. Neurotrophin was put in monoclonal antibodies. A rat neurotrophin monoclonal antibody has been used to pre-coat an enzyme well. The antibodies of neurotrophin were labeled with biotin and then combined with Streptavidin-HRP to constitute an immune complex. The choromogen solution A, the choromogen solution B, and the stop solutions were added, and the liquid color was ultimately changed to yellow. The color absorbance formed in the microplate was evaluated in the microplate reader (BioTek MQuant, USA), and the results were evaluated from the standard curve.

Statistical Analysis: The values obtained were evaluated by the Windows Statistical Package for the Social Sciences Program (IBM SPSS 22 version, Armonk, NY). Prior to the significance tests, all data were evaluated by the Shapiro–Wilk test for normality from the parametric test hypotheses and by the Levene test for the homogeneity of variances. The comparison of multiple groups was determined by analysis of variance (ANOVA) and post hoc Duncan's test. A difference of $P<0.05$ was considered significant. The values were expressed as mean ± standard error (SE).

Results

Brain-derived Neurotrophic Factor: Brain BDNF values were significantly ($P<0.001$) increased in the SNI group compared to other groups. Brain BDNF values in the S group were significantly ($P<0.001$) lower than in other groups. Brain BDNF values were found to be at almost the same values as the ES group (0.33 ± 0.008 ng/mg protein) and the SNI + ES (0.33 ± 0.006 ng/mg protein) (Table). However, serum BDNF levels in the ES group (1.62 ± 0.021 ng/ml) and in the SNI group (1.60 ± 0.024 ng/ml) were significantly ($P<0.01$) higher than the other groups. Serum BDNF values were determined to be at almost the same values as the S group (1.53 ± 0.007 ng/ml) and the SNI + ES (1.53 ± 0.024 ng/ml) (Table). Brain and serum BDNF values were significantly diminished in the SNI+ES group compared with the SNI group ($P<0.001$, $P<0.01$, respectively).

Table The BDNF and NGF levels of brain and serum (mean±SE).

Parameters	S (n=7)	ES (n=7)	SNI (n=7)	SNI+ES (n=7)	P
Brain BDNF (ng/mg protein)	0.29 ± 0.007^{c}	0.33 ± 0.008^{b}	0.36 ± 0.008^{a}	0.33 ± 0.006^{b}	<0.001
Serum BDNF (ng/ml)	1.53 ± 0.007^{b}	1.62 ± 0.021^{a}	1.60 ± 0.024^{a}	1.53 ± 0.024^{b}	<0.01
Brain NGF (ng/mg protein)	3.12 ± 0.13^{b}	3.57 ± 0.19^{ab}	3.77 ± 0.11^{a}	3.26 ± 0.14^{b}	<0.05
Serum NGF (ng/ml)	11.10 ± 0.30^{a}	11.23 ± 0.17^{a}	10.29 ± 0.24^{b}	10.26 ± 0.18^{b}	<0.05

[a-c]Different letters on the same column are statistically significant ($P<0.05$, $P<0.01$, $P<0.001$). NGF: Nerve growth factor, BDNF: Brain-derived neurotrophic factor, S:Sham, ES: Electrical stimulation, SN: Sciatic nerve injury, and SNI+ES: Sciatic nerve injury+electrical stimulation.

Nerve Growth Factor: Brain NGF values were elevated in the SNI group (3.77±0.11 ng/mg protein, P<0.05, Table) compared with the S group (3.12±0.13 ng/mg protein) and SNI + ES group (3.26±0.14 ng/mg protein). Meanwhile, brain NGF value increased in the ES group (3.57±0.19 ng/mg protein, Table) compared with the S group, but this increase was not significantly different (P>0.05). Brain NGF values were significantly diminished in the SNI+ES group compared to the SNI group (P<0.05). In addition, it was measured that the values of serum NGF in SNI and SNI + ES groups (10.29±0.24 ng/ml; 10.26±0.18 ng/ml, P<0.01, respectively) were lower than in S and ES groups (Table).

Discussion and Conclusion

It takes a long time for nerves to heal after injury. It is also known that neurotrophins such as NGF and BDNF possess a short half-life. Where the nerve is damaged, it is desirable to provide sustained secretion of these proteins *in vivo* for a long period (35). Various strategies have been developed to maintain controlled secretion of growth factors, including the implantation of mini-osmotic pumps, drug- or cell-containing polymers, viral vectors, agents conjugated with fibronectin receptors, gel foams, and films. However, although these systems have beneficial effects, it has been reported that each of them limits themselves significantly (10, 20, 35). Unlike the mentioned strategies, electrical stimulation was used in the present study to maintain an even longer-term release of neurotrophic factors. In the present study, the evaluation of BDNF and NGF release after sciatic nerve injury and whether electrical stimulation possessed any effects on the release of relevant factors were investigated.

It is stated that BDNF possesses a direct role in protecting the viability of damaged neurons after nerve axotomy (6). In addition, BDNF mRNA is up-regulated in damaged sciatic nerves three days following a nerve lesion, and upregulation takes several weeks, deemed necessary for axonal regeneration (23). It has been reported that BDNF synthesized in the dorsal root ganglia is transported to primary sensory neurons, and BDNF mRNA and protein rapidly change in the dorsal root ganglia following peripheral inflammation and nerve injury (27, 37). It is indicated that the differential regulation of BDNF in spinal motoneurons following sciatic nerve transection and ventral root avulsion increases in motoneurons to promote nerve regeneration following axotomy (9). In accordance with the literature, in the present study, it was determined that serum BDNF, brain BDNF, and brain NGF levels were significantly increased in the SNI group (1.05, 1.24, and 1.2-fold, respectively) compared to the S group. Thus, the present findings revealed that brain and serum BDNF and brain NGF levels increased in sciatic nerve damage.

Electrical stimulation application as a supporter of peripheral nerve regeneration was tested for the first time in 1982, and electrical stimulation of the regenerated nerve and its influence on motor recovery was found (26). Alrashdan et al. (2) indicated that the application of the electrical stimulation protocol at 100 μs, 20 Hz, and 2 mA for 30 minutes parameters instantly after the nerve crush injury accelerated axonal regeneration and increased BDNF mRNA levels in lumbal (L) 4, L5, and L6 dorsal root ganglion neurons at 5 days postoperation. It has been stated that when endogenous BDNF is inhibited by a functional blocking antibody applied within the first 3 days following damage, the enhancing influences of electrical stimulation on the regeneration of axons are eliminated and motor axonal regeneration is accelerated by BDNF with short low-frequency electrical stimulation (33). It has been reported that electrical stimulation exerts a supportive influence on the regeneration of nerves by elevating BDNF levels (1), while low-intensity electrical stimulation may enhance the regenerations of both sensory and motor neurons following injury (2). Park et al. (29) demonstrated that BDNF levels increase in the medial gastrocnemius and soleus muscles after the mentioned sciatic nerves are applied electrical stimulation with three distinct paradigms, including 1 ms / 40 Hz / 30 minutes, 1 ms / 40 Hz / 5 minutes, and 1 ms / 1 Hz / 30 minutes. Moreover, they suggested that electrical stimulation can enhance neuronal plasticity by boosting the production of BDNF, and all of the mentioned paradigms of the stimulus can modulate the amount of BDNF production. The findings from the present study are not far from previous reports in this area. In the present study, it was determined that serum BDNF and brain BDNF levels were significantly increased in the ES group (1.06 and 1.14-fold, respectively) compared to the S group. Similarly to the literature, it was revealed that electrical stimulation (at 200 μs, 20 Hz, 2 mA for 20 minutes during fifteen days) led to an increase in serum and brain BDNF values in the current study. Moreover, the mechanism by which electrical stimulation improves the nerve regeneration environment may be considered to be through the promotion of growth-related factors, especially BDNF.

When the denervated sciatic nerve was examined in terms of NGF with the intact sciatic nerve in the literature, it was reported that there was an increase in NGF levels in the denervated sciatic nerve (31). In the current study, it was revealed that while brain and serum BDNF levels and brain NGF levels increased, serum NGF levels decreased in sciatic nerve damage. Brain BDNF, serum BDNF, and brain NGF levels were significantly reduced in SNI+ES group compared with SNI group. It was observed that electrical stimulation led to an increase in brain and serum BDNF levels, but did not cause any changes in brain and serum NGF levels. Thus, it may not be accurate to say that

electrical stimulation enhances or deteriorates neurotrophin levels in the SNI+ES group when compared to the SNI group in the current study. Considering that it was revealed that electrical stimulation possesses an effect on the release of neurotrophins probably, the decrease in neurotrophin levels in the group that received electrical stimulation following sciatic nerve injury can be due to the fact that electrical stimulation accelerates the use of neurotrophin by metabolism to repair the damage. Shortly, in line with these data, it is thought that the release of BDNF and NGF and electrical stimulation have an influence on their release after sciatic nerve injury.

Since the period of nerve recovery after damage is lengthy and the half-life of NGF and BDNF proteins is short, a reliable prolonged sustained secret *in vivo*, precisely at the site of damage of the nerve, remains desirable (35). In this study, rat siatic nerve was injured and electrical stimulation was applied to provide a sustained secret of NGF and BDNF. In the present study, it was determined that serum BDNF, brain BDNF and brain NGF levels were significantly increased in the SNI group (1.05, 1.24, and 1.2- fold, respectively) compared to the S group, serum BDNF, brain BDNF and brain NGF levels were significantly decreased in the SNI+ES group (0.96, 1.09, and 0.87-fold, respectively) compared to the S group. It was thought that electrical stimulation reduced serum BDNF, brain BDNF and brain NGF levels in sciatic nerve damage. In addition, in the current study, it was found that the serum NGF levels of the SNI and SNI + ES groups decreased 0.93- fold compared to the serum NGF level of the S group. It is thought that this decrease is due to sciatic nerve damage. Because it was determined that the serum NGF levels of the SNI + ES group did not change compared to the serum NGF levels of the SNI group. Therefore, it was found that the electrical stimulation applied in this study had no effect on the serum NGF level in sciatic nerve damage.

The present study suggests that BDNF and NGF release after sciatic nerve injury and electrical stimulation possess an influence on the release. In the future, studies may be conducted to address the uncertainty regarding electrical stimulation, neural plasticity, and the mechanisms that mediate healing. It is predicted that the present study will contribute to scientific researches in the field of physiotherapy and neurochemistry.

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Ethical Statement

This study was performed after the animal experiment was approved by the Hatay Mustafa Kemal University Local Ethics Committee (Decision number: 2019/10-5, 17/12/2019).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

FK, EI, ZY and HA conceived, planned and carried out the experiments. FK and EI contributed to sample preparation. FK and EI contributed to the interpretation of the results. FK and EI 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 the findings this study are available from the corresponding author upon reasonable request.

Animal Welfare

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

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Blood macro (Ca, K, Mg, Na, P) and micro (Al, B, Mn, Mo, Sb, Se, Sn, Tl) element status and correlations in shelter dogs

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ABSTRACT

This study aimed at investigating the concentrations of macro (Ca, K, Mg, Na, and P), microelements (Al, B, Mn, Mo, Sb, Se, Sn, and Tl) and interaction between elements in dogs (n=125) whole blood samples that collected from clinical healthy individuals in the shelter. Depending on bioconcentration, elements are classified as macro and micro. Some are essential for biological processes but can be toxic above tolerable concentrations. In these cases, it is necessary to monitor concentrations. Also, the deficiency or excess of micro- and macroelements may diminish the effect of substances, such as other elements or drugs, or cause them to exert toxic effects. According to the results, the high to low micro- and macroelement concentrations (ng ml^{-1}) were found to be K (113255)>Ca (108257)>P (65381)>Mg (21538)>Na (4782) and Se (339.95)>Al (86.11)>Mn (32.92)>B (11.32)>Mo (8.41)>Sb (5.22)>Sn (0.50)>Tl (0.04) respectively. Statistically significant positive (Ca-Sn, Mg-Mn, Mg-Sb, Mg-Se, P-Al, P-Sb) and negative (K-Al, K-Mn, Na-Al, Na-Sb, Na-Sn) correlations were determined. The concentrations of the macroelements were within the reference range. Mn and Se concentrations were above the reference values and there is no reference value and study data on the concentrations and biological or toxic effects of B, Sb, Sn, and Tl in dogs. Concentration ranges should be identified for the early diagnosis of conditions induced by altered element concentrations and the health impacts of these changes should be thoroughly investigated.

Introduction

In mammals, elements are required for maintaining ideal health and growth via biological processes. These elements, which are found in different forms in nature, are required for various biological functions. These elements are classified as macro- or microelements depending on their concentration in the body. Microelements (trace) are present in living organisms in low concentrations. The macroelements include calcium (Ca), phosphorus (P), potassium (K), sodium (Na), chlorine (Cl), magnesium (Mg), and sulfur (S), whereas microelements (trace) include boron (B), iron (Fe), iodine (I), zinc (Zn), copper (Cu), manganese (Mn), cobalt (Co), molybdenum (Mb), selenium (Se), chromium (Cr), lithium (Li), vanadium (V), fluorine (F), silicon (Si), and nickel (Ni) (4, 29, 35, 44). These trace elements provide chemical and molecular stabilization in cell functions and control numerous vital biological processes at tolerable concentrations (32). Some trace elements, also known as potentially toxic elements (PTEs), such as Cu, Se, and Zn, are essential to maintain metabolism, but are toxic above tolerable concentrations (27). Other trace elements, such as aluminum (Al), antimony (Sb), cadmium (Cd), mercury (Hg), tin (Sn), lead (Pb), and thallium (Tl) are non-essential and not required for the metabolism of living organisms and have also toxic effects above tolerable concentrations (25, 26). The diseases and toxic effects due to possible deficiencies or accumulations of trace elements

can be determined by measuring their concentrations in the blood. Monitoring of blood concentrations is necessary when clinical symptoms triggered by increased or decreased element concentrations cannot be clearly identified (23, 25, 32). Recently, there has been an increase in the number of clinical studies that examine the changes in trace element concentrations in several diseases. The varying blood concentrations and metabolic distribution of these elements have an impact on different biochemical pathways and in cases of any imbalance, this poses a risk for numerous pathological diseases (23, 32). The metabolism of trace elements is associated with the amount of dietary intake, absorption, distribution, storage, biochemical activity, and excretion. Moreover, since trace elements can interact with each other, the deficiency or excess of one element can diminish the effect of the other element or cause toxic effects. For example, high Fe concentrations can cause Cu and Zn deficiency, and high concentrations of Zn can lead to Cu deficiency. It has been shown that excessive increases in Cu concentrations result in decreased Zn and Fe concentrations (32). A similar relationship exists between Al and Ca, Mo and S, and Tl and K, where the beneficial effects of essential elements are reversed once they are exposed to non-essential elements (15, 25, 36). There are limited studies on the measurement of macro- and microelement concentrations in animals. The reference values of some elements (Al, Mo, Sb, Tl, etc.) are either not available or differ among studies (13, 37, 39). Additionally, most reference values are based on serum concentrations (9, 13, 23, 28, 40). Only based on the serum concentration measurements, the evaluation of the elements that predominantly have intracellular functions (Cu, K, Mg, Se, Zn, etc.) can be inconclusive (22). The whole blood concentrations have been used in recent studies to determine and monitor the health status and to detect short- and long-term exposure (10, 20, 31, 33). It is crucial to improve understanding of the roles of macro- and microelements in the organism and to obtain data that can be clinically associated with physiological or pathological conditions. Accordingly, the present study aimed to determine the concentrations and correlations of macro- (Ca, K, Mg, Na, and P) and microelement (Al, B, Mn, Mo, Sb, Se, Sn, and Tl) in whole blood samples collected from dogs in Thrace region of Türkiye.

Materials and Methods

In the study, blood samples were collected from 125 (female n=73, male n=52) healthy dogs (10 months–11 years) according to no recent diagnosis of any disease in the anamnesis and clinical examination of body temperature, heart rate, respiratory rate, capillary refill time, physical and neurological conditions, weight condition, vision, hearing, urinating, defecating, appetite, etc. from shelters in the Thrace region of Turkey. For elemental analyses, 10 ml blood sample of each dog was collected from *V. cephalica antebrachium* into anticoagulant tubes, following the decision of the Local Ethics Committee (2017/02).

Standard solutions were prepared using stock solutions of the ICP multi-element standard and nitric acid (HNO_3) (Merck, Germany), and calibration curves were drawn. The blood samples were digested in the microwave system (CEM MARS, USA) in a 4-step program with 10 mL HNO_3 (70%) (600 W 100% power, 15 minutes at 210°C). Ca, K, Na, and P concentrations in the solutions were determined using an inductively coupled plasma-optical emission spectrometer (ICP-OES; Spectro Analytical Instruments, Germany) (plasma power [W]: 1400, pump speed [rpm]: 30, coolant flow [L/min]: 12, auxiliary flow and nebulizer flow [L/min]: 1). Al, B, Mg, Mn, Mo, Sb, Se, Sn, and Tl were measured using inductively coupled plasma-mass spectrometer (ICP-MS; Thermo Fisher Scientific, Germany) (plasma power [W]: 1300, pump speed [rpm]: 33 rpm, coolant flow [L/min] 13, auxiliary flow and nebulizer flow [L/min]: 0.8). The method validation was performed using the parameters of the limit of detection (LOD) (ng mL^{-1}; Ca: 48.76, K: 24.04, Mg: 2.23, Na: 4.39, P: 8.16, Al: 1.41, B: 0.37, Mn: 0.17, Mo: 0.09, Sb and Sn: 0.01, Se: 0.07, Tl: 0.06), recovery (%; 74.3-133.3), correlation coefficients (r^2) (>0.99), and relative standard deviation (rsd) (<20%). Non-parametric Spearman's test was performed to test the correlation between element concentrations after the normality test. P values of <0.05 (*) and <0.01 (**) indicated statistical significance.

Results

Arithmetic and geometric mean, median, standard error, minimum, and maximum values of macro and microelements are given in Table 1. The study findings showed that the whole blood concentrations of macro- (ng mL^{-1}) and microelements were ranked as K>Ca>P>Mg>Na and Se>Al>Mn>B>Mo>Sb>Sn>Tl. Ca and Mg mean concentrations were within the reference range, while K was lower, Na and P were higher. Ca concentrations were below the reference range in 30 samples (24%) and above in 39 samples (31.2%). The K concentrations were below (99.2%) the reference range except for 1 blood sample. Mg concentrations were below the reference range in 2 samples (1.6%) and above in 22 samples (17.6%). Na concentrations were below the reference range in 1 sample (0.8%) and above in 95 samples (76%). Na concentrations were below the reference range in 1 sample (0.8%) and above in 83 samples (66.4%). Among microelements, reference

ranges are given only for Mn, Se and the mean concentrations determined in the present study were above these. The correlations between Ca and K, Na, Sn; K and Na; Mg and Na, Mn, Sb, Se; P and Al, Sb; Al and Mn, Sn; B and Mo; Sb and Se, Sn, Tl; Sn and Tl were statistically significant positive, while between K and Al, Mn; Na and Al, Sb, Sn were significant negative (P>0.01, P>0.05) (Table 2).

Discussion and Conclusion

The varying blood concentrations of trace elements and their relationship with each other have a significant role in numerous key physiological, metabolic, and toxicological events; thus, they have gained attention in clinical practice as well as in the field of biochemistry (6, 23, 31, 32). Reference values of microelements, such as B, Co, Mb, Sn, Cr, Li, V, F, Si, and Ni, have not been specified in dogs (13, 18, 37, 39). In this respect, the results for B, Sb, Sn and Tl in the presented study, can be used for comparison in future studies.

Table 1. Macro and microelement concentrations of blood samples (ng ml^{-1}) (n=125) and reference concentrations (ng ml^{-1}) (13-15,19, 24)

	Macroelements					Microelements							
	Ca	K	Mg	Na	P	Al	B	Mn	Mo	Sb	Se	Sn	Tl
Arithmetic mean	108257	113255	21538	4782	65381	86.11	11.32	32.92	8.41	5.22	339.95	0.50	0.04
Standard error	2018.95	1604.87	259.85	37.41	992.98	4.69	1.07	0.92	0.55	0.46	8.22	0.04	0.02
Median	104300	111700	21160	4806	65600	75.47	9.53	31.81	7.87	4.06	336.30	0.42	0.03
Geometric Mean	105968	111904	21349	4763	6396	77.71	7.88	31.42	6.02	4.46	327.37	0.35	0.03
Minimum	44500	60610	13310	3211	6660	40.37	0.21	14.32	0.26	2.42	123.80	0.01	0.01
Maximum	189500	201870	31300	6064	97320	422.60	37.27	77.96	35.89	47.04	634.40	2.55	0.08
Reference Concentrations for Healthy Dogs	90000 - 117000	170867 - 209185	16000 - 24000	3266 - 4558	26000 - 62000			20			220		

Table 2. Correlations between macro and microelements.

	Macroelements					Microelements							
	Ca	**K**	**Mg**	**Na**	**P**	**Al**	**B**	**Mn**	**Mo**	**Sb**	**Se**	**Sn**	**Tl**
Ca	-												
K	0.25**	-											
Mg	-0.04	-0.09	-										
Na	0.49**	0.49**	0.17*	-									
P	0.07	0.00	-0.01	-0.10	-								
Al	0.08	-.176*	0.05	-0.27**	0.120*	-							
B	0.03	0.01	0.16	0.08	-0.01	0.06	-						
Mn	0.16	-.287**	0.30**	-0.08	0.07	0.20*	0.08	-					
Mo	0.01	0.06	0.03	0.13	0.12	0.01	0.29**	0.00	-				
Sb	-0.05	-0.08	0.21*	-0.17*	0.17*	0.16	0.04	0.12	0.06	-			
Se	0.00	0.03	0.43**	0.04	0.03	0.03	0.12	0.10	-0.16	0.19*	-		
Sn	0.21*	-0.07	0.02	-0.21*	0.15	0.47**	0.15	0.13	-0.08	0.27**	-0.02	-	
Tl	0.08	0.02	0.03	-0.01	-0.06	-0.02	0.09	0.01	-0.02	0.22**	0.04	0.19*	-

* (P<0.05), ** (P<0.01).

Although mostly serum samples have been used for the detection of element concentrations in the studies conducted at reference ranges (9, 23, 28, 39, 40) whole blood samples are preferred to detect the concentrations of a few elements (Mo, Pb, Sb) due to their biological mechanisms that include binding to erythrocytes (15, 25, 31). It has been suggested that measuring Pb, arsenic (As), mercury (Hg), Mo, Mn, and Se concentrations in whole blood and Cu, Zn, Fe, Mg, Ca, Na, and K concentrations in serum ensures more accurate results in terms of diagnosis (31). However, the measurement of intracellular elements, such as K, Zn, Mg, Cu, and Se, only in serum may not be sufficient (22). Trace element deficiencies in the body cause growth retardation reduced immunity, increased oxidative stress, glucose intolerance, delayed wound healing, and decreased bone density. Macro elements, such as Ca, P, and Mg are the structural elements that make up the bones in the skeletal system (3, 9, 10, 35). Na and K are the major cations of the intracellular and extracellular fluids. Na^+/K^+-ATPase activity is key to the homeostasis of osmotic pressure, the maintenance of acid-base balance, enzyme activation, and carbohydrate metabolism (35). The Ca and Na concentrations found in our study were within the normal range compared to the reference values (18, 37). In a study conducted with different dog breeds, serum Ca concentrations (ppm) were 73.21–98.59, Mg concentrations were 40.87–41.85, K concentrations were 4.72–5.18, and Na concentrations were 142.70–148.89 (23). Compared to this study, the Ca and K concentrations obtained in our study were higher and the Mg and Na concentrations were lower. There is limited data on the element concentrations of blood and other tissues in dogs and their extremely low blood concentrations make them difficult to detected. In addition, the reference values are not fully specified (10). The current methods of ICP-OES and -MS were used to measure the concentrations of trace elements in blood, plasma, and serum samples. Using these methods, toxic and trace elements in the blood can be accurately and precisely identified. However, it is likely that the devices and analyses are expensive (10, 35). Nevertheless, rapid, sensitive, and accurate detection of numerous elements by using these analysis methods, which have increasingly gained importance in human medicine, allows rapid intervention to the patient, and improves the prognosis (22). It is essential that these analysis methods become widespread in the veterinary field and that they are included in the clinical routine with the reference values of the elements to be determined for each species in serum and blood samples. Our study will increase awareness on this subject and contribute to more comprehensive studies to establish reference values for macro- and microelements in the whole blood of dogs. Trace element deficiencies and the disorders caused by them have been widely investigated in humans as compared with animals. Imbalances in trace elements may neither cause clinical signs nor be a specific sign of a disease. However, these imbalances may exacerbate various chronic and infectious diseases (10). Recent studies have suggested that trace element deficiency is associated with diseases, including heart, liver, and kidney failures and cancer (10, 41). Studies in dogs with epilepsy and primary hepatitis have also reported increased Mn concentrations (19, 39). In particular, the liver plays an important role in Mn metabolism, and most of the Mn absorbed from the gastrointestinal tract is metabolized by the liver. Although Mn is an essential element, it can cause toxicity (19). In a study, determine the reference ranges (ng L^{-1}) of serum essential trace elements in healthy dogs, Se was reported in the range of 154–447 and Mn was reported in the range of 0.14–7.44 (10). In another study, serum Se concentration (ng mL^{-1}) was reported as 300.50, Mn was 3.15 in dogs and not fully explain the differences between the studies and suggested fluctuations in blood or serum Mn concentrations as a potential factor (39). In a study of seven healthy dog breeds, serum Mn concentration was measured in the range of 0.006–0.020 ppm (23). Although the cause of low Mn concentrations has not been clearly stated, it has been reported that high concentrations of Mn could be neurotoxic. In human studies, it has been suggested that the measurement of hair concentrations of Mn is more reliable than that of blood or serum samples (14, 39). In a study, the Mn value (ng g^{-1}) in the blood of dogs living in three different regions in the range of 0.02–0.05, whereas hair concentrations were 0.02–0.15 (40). Considering this information, studies are needed to detect comparative Mn concentrations in different samples (hair, blood, serum, etc.) and to determine reference ranges in dogs. The reference concentration for blood Mn in animals is 20 µg L^{-1} (9). In the present study, the mean Mn (32.98 ng mL^{-1}) values were found to be high. Increased oxidative stress in patients may lead to the development of secondary diseases, such as tissue damage and organ failure. It has been stated that the concentrations of Se, an element with antioxidant properties, may decrease during these diseases (10, 41). It has been determined that low Se concentrations increased the incidence of neoplasms and allergies, whereas Se deficiency has been observed in dogs with diarrhea (10, 30). The reference range of serum Se in dogs has been reported to be 200–300 ng mL^{-1} (10, 39). In the present study, the mean Se (340.45 ng mL^{-1}) values were high, and the lowest blood Se concentration was 123.80 ng mL^{-1}. It is concluded that this may be due to the differences in the diets of the dogs or because of some nutrients on blood Se concentrations. In a study, the researchers have determined the concentration of Se in the range of 230–250 ng mL^{-1}; however, higher values (340.45 ng mL^{-1}) were detected in our study (40).

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In healthy dogs that were fed on a balanced diet, trace element concentrations are predicted to be at normal concentrations (10, 39). Although the dogs included in our study were fed with commercial food and remains, the dietary effect could not be revealed because the dietary element concentrations and feeding duration were not known. To determine the correlation between the dietary and blood concentrations of the elements, detailed studies where dogs are fed at certain time periods (short and long-term) with diets of known elements need to be carried out. Mo is known to be essential in animals, but there is limited information on its metabolism in dogs and its reference range has not been established (15, 39). Dietary sulfur (S) accompanied by low Mo intake is associated with chronic Cu intoxication (15, 36). Although the serum Mo concentration of 8.45 ng mL^{-1} in a study by Vitale et al. (39) was like the present study (8.44 ng mL^{-1}), the serum concentration of 0.006 ppb (5.48 ng L^{-1}) found in the study by Cedeño et al. (10) was quite low.

Recent studies regarding Al are notable, and it has been recently reported that Al, which was classified as having low toxicity in previous studies, has originated from urban and industrialized areas and is toxic to mammals and accumulates in tissues (37, 43). It plays a role in the etiology of neurological, hematologic, and respiratory diseases (8). In addition, it has been stated that Al causes cellular toxicity by replacing Ca in bone tissue (25). In a study, the serum Al concentration (1581 ng mL^{-1}) in dogs was higher than the concentration reported in our study (87.27 ng mL^{-1}) (37). In other studies, Al concentrations were reported as 136.67 and 215.14 mg kg^{-1} in hair samples of healthy dogs, whereas decreased concentrations (66.00 mg kg^{-1}) were detected in dogs with mammary adenocarcinoma (66.00 mg kg^{-1}) (7, 8). In a study conducted in three different age groups, hair Al concentrations of dogs were determined at the concentration of 84.10–110.17 mg kg^{-1} (21). In other studies, hair Al concentrations in healthy dogs have been found to be 23.1 µg g^{-1} and in the range of 297.8–937.5 µg g^{-1} (11, 34). These differences in values cannot be solely explained by the fact that the samples were obtained from different tissues, either blood or hair. Al tends to accumulate in keratinous tissues, so it is recommended to use hair tissue as a bioindicator to determine Al concentrations (8). On the contrary, since the growth rates and hair shedding time periods may vary in different breeds, hair Al concentrations have also been reported to vary in the study (11).

In animals, there is insufficient data on the biological functions, interactions with each other, and blood reference concentrations of elements, such as B, Sb, Sn, and TI, and studies on element concentrations in blood, plasma, or serum samples are limited (1, 2). B, which has been known to be involved in animal metabolism (carbohydrate, mineral, energy, enzyme activities, and embryonic development) for a limited period, produces toxic effects in high amounts (38). It has been demonstrated in a human study that blood concentrations in the control group were higher (30.00 ng g^{-1}) those that of the present study (12). Sb, a non-essential element, is generally known as toxic, but the mechanism of toxicity has not yet been understood (42). It has been reported that >95% of Sb^{3+} is found in red blood cells and approximately 90% of Sb^{5+} is found in plasma (25). In a study of pregnant women, the mean blood Sb concentration was reported to be lower (1.54 µg L^{-1}) than that reported in our results (17). In cases of chronic exposures to the non-essential element Sn, bone is the target organ where accumulation occurs. It has been reported that the mean blood concentration of Sn in erythrocytes in normal subjects was higher (0.14 mg L^{-1}) than that of the present study (25). Tl, whose biological function is not fully known, causes renal and hepatic dysfunction. It has been reported that Tl and K have common receptors, and that administration of K might be a useful intervention in response to Tl intoxication (25). Similar to our study, a study conducted with pregnant women have reported Tl concentration of <0.05 µg L^{-1} (17). In addition, although not statistically significant, a positive correlation was determined between them. The correlations between Ca and K, Na, Sn were statistically significantly positive.

Although they were not significant, there was a positive correlation between Ca and P and a negative correlation between Ca and Mg. According to the results of Tatara's study (35) on silver foxes, Ca and P concentrations were positively correlated, and both elements were positively correlated with Mg and K concentrations. In the same study, it was stated that there was a positive correlation between Se and Mn. Although it was not statistically significant, there was a positive correlation between Se and Mn in our study. A positive correlation was determined between Mg and Mn in the blood of healthy people, which is consistent with our study (16). Some studies show that excess Mn in the diet can lower Mg concentrations or support that it increases convulsions by triggering Mg deficiency (24, 26). The use of Al in the diet results in reduced Ca absorption, lower Mg concentrations, and adverse effects on P (26). When looking at the relationship between Al and these minerals in the current study, we could not find any data that supports this information. In a study, it was reported that no statistical difference was found between plasma and serum concentrations for Al and the choice of matrix (whole blood, serum, plasma) for measurements is important (5). While the correlations between Al and P,

Mn, Sn were statistically significant positive, the correlations between Al and K, Na were negative. In addition, statistically significant positive correlations (Sb-Se, -Sn, -Tl and Mo-B) and negative correlations (Na-Sb, Sn-Na, Al-K, Na) or no literature has been found to explain the relationship between these macro and microelements.

Detecting the disorders and clinical effects induced by trace elements is challenging. There is limited literature on these disorders in dogs and the determination of reference intervals. Even in recent human studies regarding trace elements, deficiency or excess states, relationships with each other, potential usage as a diagnostic marker, and detection methods have not been fully elucidated. Thus, further studies are needed to reveal the relationships between trace elements and physiology or disease in dogs and other species. Concentration ranges should be determined for the early identification of the alterations in the metabolism driven by trace elements, and associated health consequences should be extensively examined. Establishing a reference range in healthy as well as in various disease states enables us to use these elements as clinical markers and to reveal the risks associated with the disease due to their deficiency or excess. In addition, the reference sample type (whole blood, plasma, or serum), which will be used to determine the concentrations, should be selected based on differences in the mechanisms of action of the elements or it may be a more accurate approach to use whole blood to include elements involved in intracellular functions and structures. For this purpose, it would be easy to use blood samples that can be sampled by invasive and non-traumatic methods. However, there is a need for reference studies on a few elements (B, Sb, Sn and TI) whose biological functions and interactions with other elements are not fully understood. In this regard, it is recommended to monitor blood, serum, or plasma concentrations of elements that have a negative impact on health if they are deficient or above tolerable limits. In conclusion, our study has highlighted the importance of this topic and can contribute to future studies.

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Ethical Statement

Blood samples were collected following the decision of the Local Ethics Committee (numbered 2017/02).

Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

Conceptualization and design: FAY; Methodology: FAY, MY, NA; Sampling and laboratory analysis: FAY, MY, NA, NÖ; Statistical analysis and interpretation of the data: FAY, MY; Drafting: FAY; Reviewing and editing: FAY, MY, NA, NÖ.

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Forecasting the import and export values in Turkish livestock sector with ATA analysis

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ABSTRACT

Unlike traditional time-series methods, the ATA analysis method developed in recent years dynamically adjusts the smoothing parameters according to the sample size. This study aimed to estimate the export and import values of live animals and livestock products for the next five years (2024-2028) using the ATA method, based on the values from the previous years (2014-2023) in Türkiye. The study material consisted of export and import values (in dollars) of the "live animals," "meat/meat products," "milk/milk products + eggs" and "fish and other seafood" categories in Türkiye between 2014 and 2023. The Turkish Statistical Institute's records, divided into the aforementioned categories and presented in four annual quarters, provided the study data. The ATA method, which is a simple, highly accurate, and automatic forecasting method, was used for data modeling. According to the study findings, it was estimated that the import values of "live animals," "meat/meat products," "milk/milk products + eggs," and "fish and other seafood" may be a total of approximately $1.4 billion, $172 million, $157 million, and $300 million, respectively, whereas the export value of the same products may reach $113 million, $1.3 billion, $866 million, and $2 billion, respectively, in 2028. In conclusion, the ATA analysis method can be used to reveal a perspective in the coming years in terms of foreign trade in the livestock sector, but it should be updated constantly. It has been concluded that such studies can guide policymakers in the country in developing necessary strategies in foreign trade for the future.

Introduction

The livestock sector has an important role in people's healthy and balanced nutrition, meeting their basic biological needs, economic and social development, and increasing their level of welfare (19). The World Health Organization (WHO) recommends that animal proteins should account for at least 40% of a healthy person's daily protein intake per kilogram of body weight (16). Reports indicate that the average animal protein ratio is 64% in the USA, 58% in the EU, 39% globally, 36% in Turkey, and 23% in Africa (5, 11).

Türkiye is among the countries most suitable for animal husbandry because of the existence of pasture areas and fertile lands. The share of agriculture, forestry, and animal husbandry in the country's total GDP is approximately 6.5% (7). The number of cattle and sheep in Türkiye has generally increased by 15–35%, respectively, over the last decade, and this has also been reflected in production (6, 10). However, we should aim to increase the milk and carcass productivity per animal to the level of developed countries. For this purpose, it is crucial to select the appropriate breeds for the production

area, provide the breeds with appropriate care and feeding conditions, create markets, and raise the awareness of producers. Low levels of productivity per unit animal prevent sustainability in production and reduce profitability. Problems in livestock production cause difficulties in meeting the demand for livestock products, a decrease in exports of animals and livestock products, and a decrease in income. The inability to meet the total market demand due to low productivity per animal and the gradual increase in production costs in the domestic market will make imports attractive in the country and increase the foreign trade deficit. In recent years, factors such as climate change, drought in crop production that provides raw materials for animal husbandry, irregular rainfall regimes in some regions, and increases in exchange rates have caused both an increase in input costs in the sector and a decrease in competitiveness in international markets. However, Türkiye, like other developed countries, should decrease its imports of live animals and livestock products while increasing its exports. Increasing export values, as long as they meet domestic demand, can ensure economic growth for countries. Conversely, if import values exceed exports, it can lead to a foreign trade deficit and a contraction of the economy.

Given this information, the time-series analyses employed in the current study play a crucial role in forecasting the future, planning for the country's needs, and making informed investment decisions. Time-series analyses are a set of econometric analyses that enable the analysis of past and present data to reveal future data (6, 10). In contrast to traditional methods, the updated ATA method, a time-series analysis, dynamically adjusts the smoothing parameters according to the sample size and streamlines the initialization process by fine-tuning the parameters. However, the literature review found no studies using the ATA method in animal husbandry in Türkiye, despite its application in various fields (4, 15). Therefore, we conducted this study to estimate the export and import values of live animals and livestock products for the next five years (2024-2028) using the ATA method, a time series analysis, and the values from the previous years (2014-2023) in Türkiye.

Materials and Methods

The study material consisted of export and import values (in dollars) of the "live animals," "meat/meat products," "milk/milk products + eggs," and "fish and other seafood" categories in Türkiye between 2014 and 2023. We obtained the export and import values (in dollars) for the "live animals," "meat/meat products," "milk/milk products + eggs," and "fish and other seafood" categories from the records of the Turkish Statistical Institute (18) and grouped them into 4 annual quarters (1st quarter: January, February, March; 2nd quarter: April, May, June; 3rd quarter: July, August, September; 4th quarter: October, November, December) in Türkiye between 2014 and 2023. We modeled the official export and import values (in $) as time-series data, and obtained monthly forecasts for the next five years (2024-2028) by examining past behavior. We used the ATA method, a simple, highly accurate, and automatic forecasting method, in the modelling phase. The ATA method represents an innovative approach to forecasting that draws inspiration from exponential smoothing models. What distinguishes ATA is its adaptive nature, where smoothing parameters dynamically adjust based on the sample size. Unlike conventional methods, ATA fine-tunes parameters within a discrete space, simplifying the initialization process. Through simultaneous optimization and initialization, ATA minimizes the influence of initial values by rapidly converging toward zero weights, thereby ensuring robust forecasting outcomes. ATA's versatility across all-time series settings translates to superior forecasting performance, thanks to its inherent flexibility [1, 2, 3, 4]. The following paragraphs will explain the intricacies of the ATA method, including its formula and application nuances.

For a time series $\{y_1, \dots y_n\}$ ATA method can be given in additive form as below:

$$l_t = \left(\frac{p}{t}\right) y_t + \left(\frac{t-p}{t}\right)(l_{t-1} + \emptyset b_{t-1}),$$

$$b_t = \left(\frac{q}{t}\right)(l_t - l_{t-1}) + \left(\frac{t-q}{t}\right)(\emptyset b_{t-1}),$$

where p is the smoothing parameter for level, q is the smoothing parameter for trend, $\emptyset$ is the dampening parameter and $l_t = y_t$ for $t \leq p$, $b_t = y_t - y_{t-1}$ for $t \leq q$, $b_1 = 0$, $p \in \{1,2,\dots,n\}$, $q \in \{0,1,2,\dots,p\}$, $\emptyset \in (0,1]$. Then, the h step ahead forecasts can be obtained by:

$$\hat{y}_{t+h|t} = l_t + (\emptyset + \emptyset^2 + \cdots + \emptyset^h) b_t.$$

The method require three parameters we will distinguish between them by using the notation $ATA_{add}(p, q, \emptyset)$ for the additive form.

Notice that when $q = 0$ both forms of ATA are reduced to the simple form $ATA(p, 0, \emptyset)$ which can be written as:

$$l_t = \left(\frac{p}{t}\right) y_t + \left(\frac{t-p}{t}\right) l_{t-1},$$

where $p \in \{1,2,\dots,n\}$ and $l_t = y_t$ for $t \leq p$. Forecasts then can be obtained by $\hat{y}_{t+h|t} = l_t$.

When $q \neq 0$ and $\emptyset = 1$ the additive form of ATA are reduced to the trended versions $ATA_{add}(p, q, 1)$ which is given below respectively:

$$l_t = \left(\frac{p}{t}\right) y_t + \left(\frac{t-p}{t}\right)(l_{t-1} + b_{t-1}),$$

$$b_t = \left(\frac{q}{t}\right)(l_t - l_{t-1}) + \left(\frac{t-q}{t}\right)(b_{t-1}),$$

$$\hat{y}_{t+h|t} = l_t + h b_t ,$$

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To sum up, ATA can be given in 3 forms, namely the additive damped form $ATA_{add}(p,q,\emptyset)$ (equations (1.1)-(1.3)), simple form $ATA(p,0,\emptyset)$ (equation (2.1)), additive trend form $ATA_{add}(p,q,1)$ (equations ((3.1)-(3.3)) (20).

In this study, we implemented the holdout version of the ATA method. The holdout sample method allows for the fitting of models at one epoch, distinct from the epoch of assessment. The method divides the in-sample data into a training set and a validation set, also known as the holdout set, and optimizes the parameters using the training set. We then compute multi-step forecasts for the holdout sample period and evaluate the models based on the accuracy of these out-of-sample forecasts. We select and refit the model that best fits the holdout sample using all available data to obtain the final forecasting model. The ATA forecasting package facilitates the use of the holdout sample method for model selection, allowing independent control over the time range used for model fitting and evaluation. You can use automatic model selection to select the model that provides the most accurate out-of-sample forecasts for the holdout sample.

Results

Tables 1-4 and the time series graphs in Figures 1 and 2 provide the export and import values of live animals and livestock products used in the study for the years 2014–2023.

The import values of live animals and livestock products (meat/meat products, milk products + eggs, fish, and other seafood) showed a fluctuating (up-and-down) course in Türkiye between 2014 and 2023. An upward trend in live animal imports began again in 2023. The annual total import values of milk/milk products and eggs reached their peak in 2014, whereas the annual total import values of meat/meat products reached their peak in 2023. Imports of meat/meat products showed a decreasing trend in the last quarter of 2023, while imports of live animals, milk/milk products + eggs, fish, and other seafood showed an increasing trend. In general, during the examined period (2014-2023), total annual imports of live animals, meat/meat products, fish, and other seafood increased by 755%, 67.4%, and 34.8%, respectively, while imports of milk/milk products and eggs decreased by 24.1%.

The export values of live animals and livestock products (meat/meat products, milk/milk products + eggs, fish, and other seafood) in Türkiye between 2014 and 2023 showed a fluctuating trend, similar to the import values. In addition, the annual total export values of live animals, meat/meat products, milk/milk products + eggs, and fish and other seafood in 2023 increased by 142.2%, 14.5%, 1.7%, and 150.7%, respectively, compared to the total annual export values of 2014 (Table 1-4; Figure 1-2).

Table 1. Türkiye's live animal export and import values (000 $).

Live Animals	1st Quarter		2nd Quarter		3rd Quarter		4th Quarter		Total	
	Export	Import	Export	Import	Export	Import	Export	Import	Export	Import
2014	1.614	17.127	2.647	8.002	2.454	11.502	2.191	11.377	26.720	139.891
2015	3.037	10.782	2.011	17.101	3.263	31.426	3.180	51.247	34.473	322.768
2016	2.850	39.800	1.559	43.394	2.252	79.569	2.644	60.320	27.914	603.822
2017	2.475	57.821	2.940	124.986	2.182	78.051	3.961	143.162	34.673	1.212.194
2018	4.205	138.555	5.139	156.183	5.260	211.554	4.719	129.700	57.966	1.767.909
2019	6.012	58.538	6.288	50.316	9.509	99.714	6.395	60.576	84.612	700.574
2020	6.310	43.884	3.299	18.742	8.148	21.853	10.045	63.233	83.406	446.859
2021	6.876	41.585	9.031	17.253	8.606	42.193	10.862	20.554	106.121	310.235
2022	14.464	15.299	5.836	9.564	9.211	13.499	13.491	28.646	129.006	180.905
2023	7.122	55.503	5.178	70.360	3.859	128.717	5.414	156.184	64.719	1.195.977

Table 2. Türkiye's meat and meat products export and import values (000 $).

Meat and Meat Prod.	1st Quarter		2nd Quarter		3rd Quarter		4th Quarter		Total	
	Export	Import	Export	Import	Export	Import	Export	Import	Export	Import
2014	72.968	18.954	77.459	23.215	76.055	12.710	70.955	17.423	892.311	216.908
2015	57.031	4.866	35.884	8.862	42.933	15.560	37.678	20.053	520.578	148.023
2016	31.290	3.553	37.361	9.095	36.851	9.714	45.671	11.240	453.518	100.808
2017	45.722	9.274	49.727	4.505	61.096	6.408	62.626	33.012	657.512	159.597
2018	50.743	28.284	55.255	34.200	61.488	23.192	69.257	22.860	710.230	325.609
2019	53.950	9.985	63.241	8.896	62.177	4.255	60.439	6.658	719.421	89.384
2020	57.123	7.116	53.949	5.667	50.403	5.111	55.060	7.435	649.605	75.989
2021	54.978	4.543	81.512	5.769	89.838	8.313	105.310	7.618	994.912	78.726
2022	102.888	9.587	118.309	14.747	106.993	14.302	108.759	9.752	1.310.847	145.164
2023	76.348	10.329	81.598	17.115	89.070	50.194	93.698	43.372	1.022.142	363.031

Table 3. Türkiye's milk, milk products and eggs export and import values (000 $).

Milk, Milk Prod. and Egg	1st Quarter		2nd Quarter		3rd Quarter		4th Quarter		Total	
	Export	Import	Export	Import	Export	Import	Export	Import	Export	Import
2014	76.134	14.440	67.351	19.995	58.856	18.792	60.638	25.223	788.941	235.350
2015	57.843	12.892	45.038	13.292	43.080	11.230	47.506	18.737	580.402	168.452
2016	46.631	13.335	51.918	12.498	56.318	10.030	62.023	12.449	650.671	144.934
2017	64.228	6.578	57.063	10.259	63.833	14.659	62.759	19.609	743.649	153.313
2018	66.723	17.336	63.304	15.599	63.320	8.501	73.742	11.322	801.265	158.273
2019	70.940	9.110	59.089	12.211	50.956	11.278	54.467	18.842	706.353	154.324
2020	51.289	9.188	45.841	10.837	51.614	12.816	61.075	9.500	629.458	127.023
2021	59.479	9.747	81.138	8.567	70.175	8.688	83.397	11.937	882.565	116.820
2022	97.746	9.969	90.095	10.923	74.929	8.394	72.767	17.569	1.006.608	140.564
2023	63.122	12.495	66.802	13.138	62.736	11.802	74.867	22.160	802.579	178.783

Table 4. Türkiye's fish and other seafood export and import values (000 $).

Fish and Other Seafood	1st Quarter		2nd Quarter		3rd Quarter		4th Quarter		Total	
	Export	Import	Export	Import	Export	Import	Export	Import	Export	Import
2014	58.507	15.071	55.872	15.277	53.742	20.350	59.566	21.718	683.061	217.246
2015	60.533	20.151	57.956	18.873	52.346	21.489	61.400	27.171	696.706	263.050
2016	66.546	19.560	64.451	11.751	61.302	12.784	70.820	19.048	789.357	189.426
2017	68.074	15.332	68.227	16.016	68.993	18.930	79.826	28.744	855.358	237.065
2018	78.182	19.122	83.854	15.713	73.908	13.224	81.503	18.211	952.340	198.812
2019	92.273	12.054	81.742	13.743	80.986	17.520	84.137	23.081	1.017.413	199.194
2020	85.761	15.482	70.907	10.390	89.166	11.906	108.568	16.844	1.063.203	163.866
2021	104.102	17.158	115.077	12.966	114.052	18.275	125.107	25.774	1.375.012	222.519
2022	130.797	20.930	136.555	25.220	147.098	27.457	137.356	34.468	1.655.420	324.224
2023	142.410	25.878	144.691	21.857	147.931	21.973	135.835	27.930	1.712.599	292.917

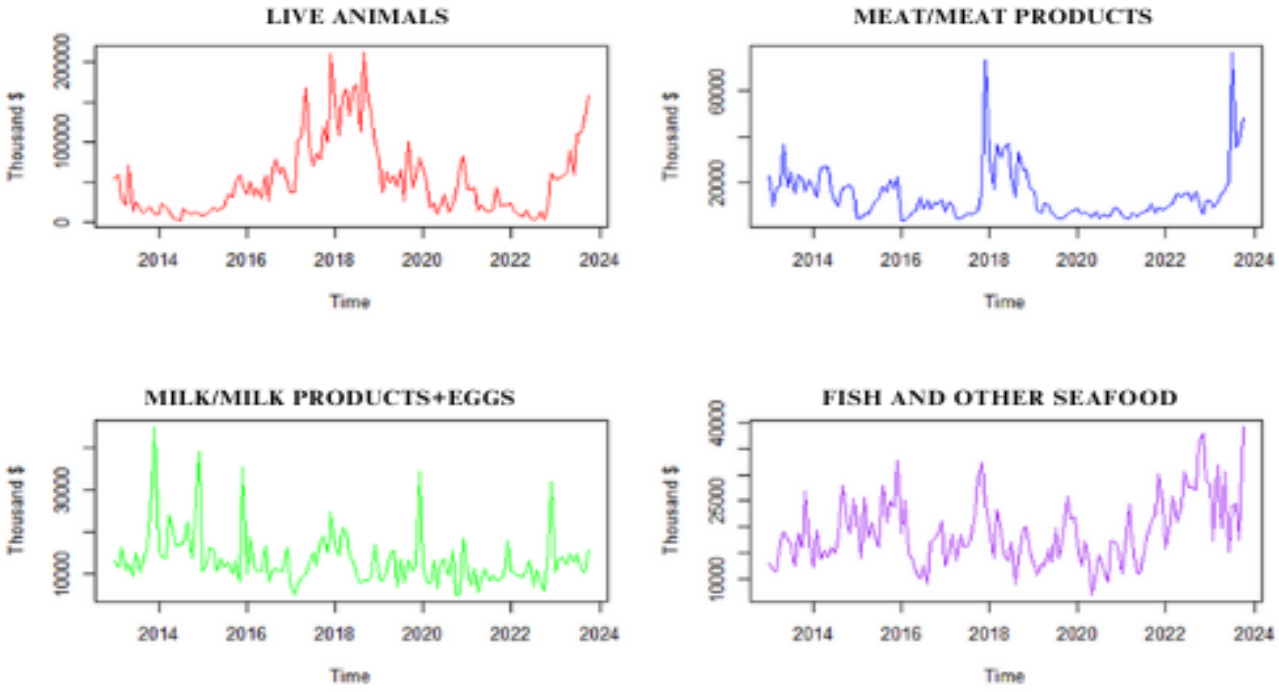

Figure 1. Graph of import values of live animals, meat/meat products, milk/milk products+eggs, fish and other seafood (2014-2023)

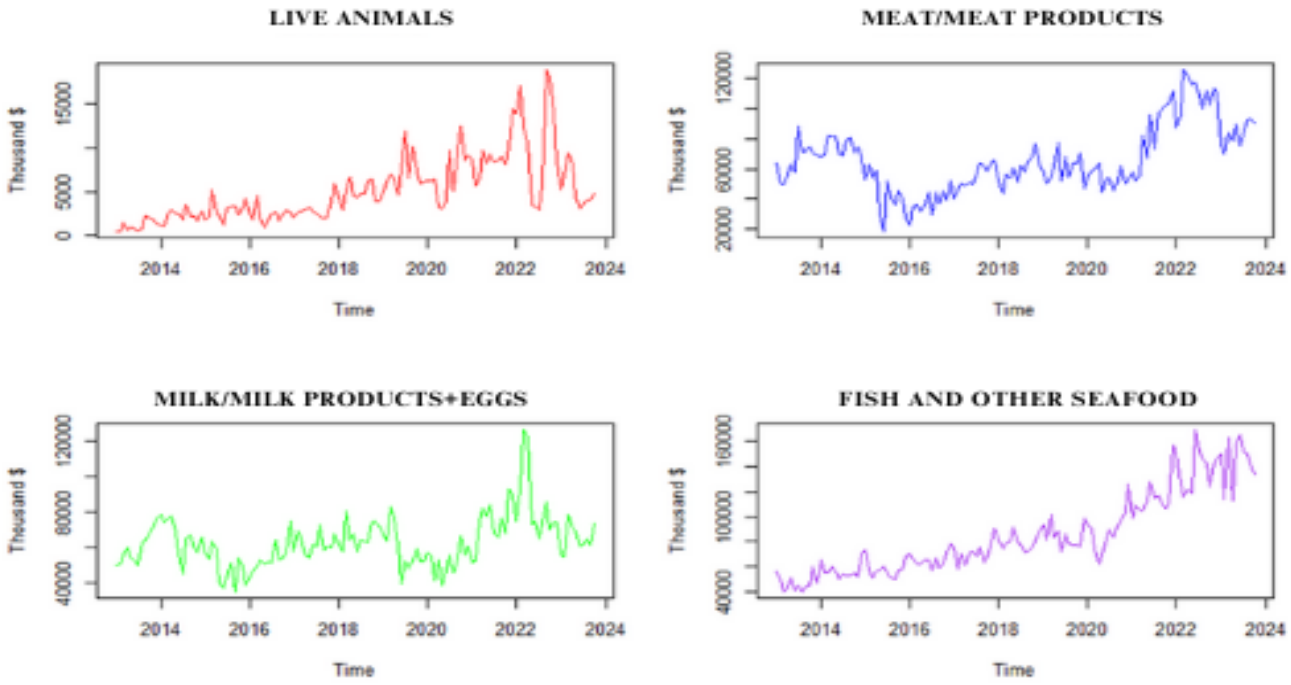

Figure 2. Graph of export values of live animals, meat/meat products, milk/milk products+eggs, fish and other seafood (2014-2023)

This study applied data sets of the ATA method to prospectively estimate the export and import values of live animals and livestock products. In the present study, the simple ATA model (Model 1), expressed as ATA (p,0,∅), and the linear trend model (Model 2), expressed as 〚 ATA_add^ (p,1,1), were used, and trend behavior was modeled additively. We utilized import and export data from eight distinct previous periods (2014-2023) pertaining to live animals and livestock products in these two ATA models (2), which excel at modeling the data set. Table 5 presents the accuracy measures of the models for the trained data.

Table 6 provides the sMAPE accuracy measure values of the test data.

Upon examination of the tables, we observed a consistent behavior between the accuracy measures of the trained data and the accuracy measures of the test data. This shows that there are no problems such as overfitting, and thus it is a good forecasting model (Tables 5, 6).

We obtained the forecasting values for the next 5 years (2024-2028) using the entire data set, which includes monthly (in 4 quarters) import and export data between 2014 and 2023, and estimated the model parameters at the holdout stage. We applied the forecasting combination, a method that significantly enhances model performance, to the forecast values derived from these two ATA models (3). To put it another way, we obtained the final values by taking the simple average of the 5-year forecasting values from both methods, as shown in Figure 3-4 and Table 7-10.

The present study, which analyzed data from the past 10 years in Türkiye and forecasted data for the next 5 years, estimated that live animal import values would reach approximately $1.4 billion in 2028, while export values would rise to $113 million. The study estimated a gradual increase in the foreign trade deficit in live animals over the next 5 years, with a potential difference of approximately $1.3 billion favoring imports over exports (Table 7).

We estimated that the import values of meat and meat products in Türkiye between 2024 and 2028 would show a decreasing trend in the next 5 years, potentially dropping to approximately $172 million in 2028. The export value of these products would reach approximately $1.3 billion, an increase of approximately 4.9%. (Table 8).

It was estimated that the import value of milk/milk products and eggs would be around $156-160 million in the next 5 years in Türkiye. The export values in this product group may exceed imports and reach levels of $820-866 million (Table 9).

Research indicates that in the next 5 years, export values in fish and other seafood, similar to the milk/milk products + egg group, will surpass imports. The total export data of fish and other seafood may increase to $2 billion and import data to approximately $300 million in 2028. We forecasted that there would be no foreign trade deficit in fish and other seafood, with export figures remaining above import figures for the next 5 years (Table 10).

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Table 5. Accuracy measures of trained data

	Data	Accuracy Measures	Model 1	Model 2
Import	**Live animals**	**MSE**	692,190,805	703,863,919
	Meat/meat products		144,884,775	155,754,727
	Milk/milk products+egg		20,788,670	22,218,132
	Fish and other seafood		32,070,514	45,302,164
Export	**Live animals**		5,435,202	7,041,579
	Meat/meat products		119,523,659	124,459,006
	Milk/milk products+egg		179,468,848	152,995,612
	Fish and other seafood		121,887,742	122,205,122
Import	**Live animals**	**MAE**	19,673	20,353
	Meat/meat products		7,693	7,733
	Milk/milk products+egg		3,647	3,762
	Fish and other seafood		4,437	5,662
Export	**Live animals**		1,604	1,876
	Meat/meat products		8,560	8,688
	Milk/milk products+egg		10,074	9,092
	Fish and other seafood		8,083	8,110
Import	**Live animals**	**sMAPE**	52.73	53.85
	Meat/meat products		50.66	54.59
	Milk/milk products+egg		27.83	28.64
	Fish and other seafood		23.50	29.29
Export	**Live animals**		32.24	39.72
	Meat/meat products		14.36	14.63
	Milk/milk products+egg		16.03	14.35
	Fish and other seafood		9.80	9.90
Import	**Live animals**	**MASE**	0.49	0.50
	Meat/meat products		0.69	0.70
	Milk/milk products+egg		0.62	0.63
	Fish and other seafood		0.61	0.78
Export	**Live animals**		0.67	0.78
	Meat/meat products		0.38	0.39
	Milk/milk products+egg		0.54	0.49
	Fish and other seafood		0.51	0.51

Table 6. sMAPE accuracy measure values of test data

Models	**Import**		**Export**	
	Model 1	**Model 2**	**Model 1**	**Model 2**
Live animals	81.2	82.6	37.8	42.6
Meat/meat products	50.4	99.9	33.1	34.9
Milk/milk products+egg	20.8	22.4	24.3	14.1
Fish and other seafood	31.4	21.7	29.5	19.6

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Table 7. Estimated values of live animals export and import in Türkiye (000 $).

Live Animals	1st Quarter		2nd Quarter		3rd Quarter		4th Quarter		Total	
	Export	Import	Export	Import	Export	Import	Export	Import	Export	Import
2024	7.914	100.273	6.948	101.673	8.017	104.136	8.712	114.615	94.776	1.262.090
2025	8.301	103.230	7.335	104.523	8.404	106.986	9.099	117.465	99.415	1.296.611
2026	8.688	106.080	7.721	107.373	8.791	109.836	9.485	120.315	104.055	1.330.811
2027	9.074	108.930	8.108	110.223	9.177	112.686	9.872	123.165	108.695	1.365.012
2028	9.461	111.780	8.495	113.073	9.564	115.536	10.259	126.015	113.335	1.399.212

Table 8. Estimated values of meat and meat products export and import in Türkiye (000 $).

Meat/Meat Prod.	1st Quarter		2nd Quarter		3rd Quarter		4th Quarter		Total	
	Export	Import	Export	Import	Export	Import	Export	Import	Export	Import
2024	92.910	13.271	99.114	15.193	104.378	16.094	107.398	18.783	1.211.401	190.025
2025	94.245	12.865	100.311	14.817	105.634	15.718	108.687	18.406	1.226.630	185.417
2026	95.373	12.488	101.508	14.440	106.890	15.341	109.976	18.030	1.241.239	180.897
2027	96.500	12.111	102.704	14.063	108.147	14.964	111.265	17.653	1.255.848	176.377
2028	97.628	11.735	103.901	13.687	109.403	14.588	112.554	17.277	1.270.457	171.858

Table 9. Estimated values of milk, milk products and eggs export and import in Türkiye (000 $).

Milk/milk Prod.+egg	1st Quarter		2nd Quarter		3rd Quarter		4th Quarter		Total	
	Export	Import	Export	Import	Export	Import	Export	Import	Export	Import
2024	70.215	11.663	67.388	12.885	64.824	11.881	71.093	17.117	820.561	160.641
2025	71.206	11.601	68.320	12.805	65.722	11.807	72.075	17.010	831.970	159.669
2026	72.183	11.529	69.253	12.724	66.621	11.732	73.057	16.902	843.337	158.662
2027	73.159	11.456	70.185	12.644	67.519	11.658	74.039	16.795	854.704	157.655
2028	74.136	11.383	71.118	12.563	68.417	11.583	75.020	16.687	866.071	156.648

Table 10. Estimated values of fish and other seafood export and import in Türkiye (000 $).

Fish and Other Seafood	1st Quarter		2nd Quarter		3rd Quarter		4th Quarter		Total	
	Export	Import	Export	Import	Export	Import	Export	Import	Export	Import
2024	151.113	21.799	149.370	19.780	148.754	22.157	154.700	27.796	1.811.815	274.596
2025	155.956	22.342	153.974	20.296	153.357	22.673	159.304	28.312	1.867.769	280.868
2026	160.559	22.858	158.577	20.812	157.960	23.189	163.907	28.828	1.923.007	287.059
2027	165.162	23.374	163.180	21.328	162.563	23.705	168.510	29.343	1.978.244	293.250
2028	169.765	23.890	167.783	21.844	167.166	24.221	173.113	29.859	2.033.482	299.441

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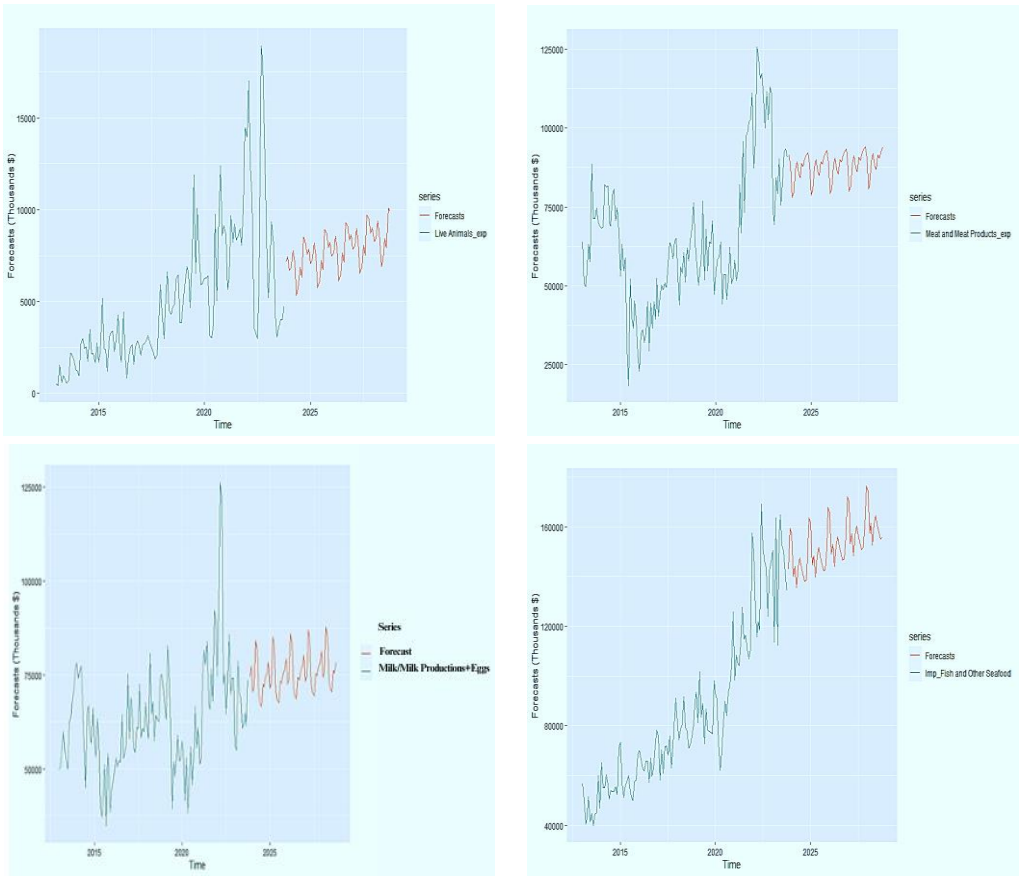

Figure 3. Estimated export values of live animals, meat/meat products, milk/milk products+eggs, fish and other seafood (2024-2028)

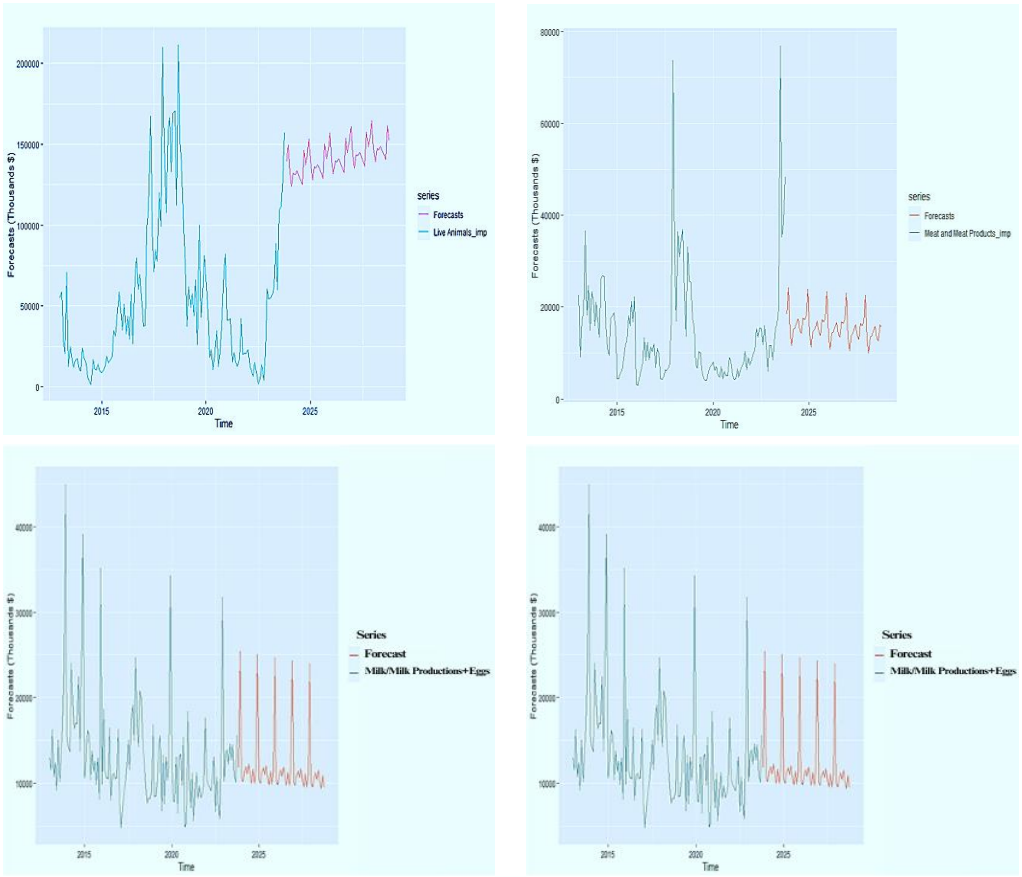

Figure 4. Estimated import values of live animals, meat/meat products, milk/milk products+eggs, fish and other seafood (2024-2028)

Discussion and Conclusion

Foreign trade has significant impacts on country economies through exports and imports. Exports generally have a positive effect (fund inflow) on country economies, whereas imports generally have a negative effect (foreign dependency, fund outflow, deterioration of the balance of payments, and increase in the foreign trade deficit) (1). However, with the right policy approaches, imports can also have positive effects on issues such as raw material supply, intermediate goods input, increasing productivity through technology transfer, and controlling inflation. In countries such as Türkiye, where there is a large population that earns their living from rural areas, the export and import amounts in the fields of agriculture and livestock significantly affect the country's economy. To be cautious and develop policies, all foreign trade relations that are experienced in this sector and are likely to be experienced in the future should be well known by sector representatives and country managers, especially producers.

Few studies have estimated the potential level of foreign trade in the agriculture-livestock sector using methods other than ATA analysis in previous years (6). However, we have not found any study that uses ATA analysis to make forward-looking forecasts of Turkey's foreign trade (exports and imports) in the livestock sector. Therefore, this study estimated possible import and export values for live animals and various livestock products in the next 5 years (2024-2028) using the ATA analysis method. Based on the current study's findings, we anticipate an increase in live animal imports over the next 5 years, while we anticipate a decrease in meat import values. It was observed that live animal imports, in particular, showed a decreasing trend after aggressive increases in every five-year period, as in the previous years, and it was estimated that they would increase in the next five years. The reasons for the general increase in live animal imports in the past decade can be listed as the population growth in the country, the increase in input costs in the domestic market, the restriction of animal movements within the country due to animal diseases and deaths (9, 13), and the emergence of inflation due to the lack of food supply as a result of inadequate meat supply (3). Estimates suggest that similar factors could sustain the imports of live animals, meat, and meat products in the upcoming years. However, the government's live animal import policies may result in a limited increase or even a slight decrease in the import of meat and meat products. We can interpret the import of cultured breeds as breeding animals to boost productivity, and this trend is likely to persist in the future, given the low productivity of local breeds. On the other hand, although no major change is expected in the exports of live animals and meat/meat products, it is anticipated that there will be a slight increase. Less appealing to the palate of ovine meat than bovine meat in Türkiye (14), preference for meat products produced in Türkiye by other countries, especially where Turkish citizens are present, good levels of poultry production in the country, and higher profits that producers can make per unit product in foreign sales (due to the depreciation of TL against the dollar) can be considered among the reasons for exports of meat and meat products despite the fact that there is a presence of demand for meat in the country. Reasons such as restrictions due to country policies, not being preferred by some countries due to the risk of transmission of diseases, high cost of animal transportation (transportation, protection, control, and quarantine costs, etc.), and live weight loss in animals due to transportation (17) may be effective in the emergence of the expectation that major changes will not occur.

Estimates suggest that milk/milk products and egg exports will not undergo major changes in the coming years, with export values not surpassing recent values, and only minor changes occurring. The reasons for this situation include the lack of a simultaneous increase in producer sales prices despite the recent increase in production costs in dairy cattle farming in the country (8), the decrease in the presence of dairy cattle in this process, and the occurrence of productivity losses (due to malnutrition). Furthermore, it's possible that raw milk, milk products, and eggs are susceptible to spoilage. Despite Türkiye's self-sufficiency in milk and milk products, the country likely imports these products in limited quantities to maintain product diversity (such as cheddar, mozzarella cheese, cream, and milk powder), with projections indicating continued imports in the near future.

Due to their abundance in Türkiye, which is surrounded by seas on three sides, export values of fish and other seafood are expected to exceed import values again. Despite the abundance of fish and other seafood in Türkiye, the primary reason for imports could be to offer a diverse range of products, such as Norwegian salmon.

As a result, the current study has shed light on the livestock sector's foreign trade outlook for the coming years. To prevent or reduce the foreign trade deficit in Türkiye, the country must lower the costs of producer inputs, enabling producers to produce more profitably, boost domestic production, transition to exports after satisfying domestic demand, and ultimately boost the country's foreign exchange income. This requires the implementation of correct and consistent policies for producer support. However, politicians must conduct a cost-benefit analysis and evaluate import decisions, taking into account the country's interests, to mitigate potential negative effects from imports. These include the emergence of diseases not found in the country, the

decline in local races, the shift away from production, and the export of the country's economic resources.

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Ethical Statement
Ethical committee approval is not required.

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

Author Contributions
MK, EA, designed the research, supervision, writing, and editing. CİZ, SD, investigation, collecting the data. SS, writing, and review. MAA, HTS, analysis, writing.

Data Availability Statement
The data and materials of this study are available from the corresponding author.

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Thyme oil and argan oil's effects on horse welfare

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ABSTRACT

This study investigates both the effectiveness of thyme oil and argan oil as potential treatments for leg edema in horses, as well as their possible side effects, such as rash, itchiness, and increased temperature, to provide a comprehensive evaluation of their therapeutic potential and safety. Swollen legs, which are often a result of infections, injuries, allergies, and underlying health conditions, can significantly affect a horse's mobility and overall health. Traditional treatments include antibiotics, anti-inflammatory drugs, cold therapy, compression bandaging, and rest, but there is growing interest in natural remedies. For this purpose, argan and thyme oil were applied to 22 horses of various ages and breeds at different times at Mersin Doğa Horse Farm. Before and after the study, the health status and various behaviors of the horses were examined and recorded. These examinations and records were made separately for the administration of both argan oil and thyme oil. Positive effects on animal welfare have been observed due to the relief of pain and edema. In addition, there was a significant association between oils and the side effects of rash, itchiness, temperature increase, excessive drinking, and excessive urination ($P<0.05$). This study demonstrated that herbal ointments containing thyme oil, argan oil, paraffin, and Vaseline® effectively reduced leg edema and alleviated fatigue in horses compared with alternative formulations. By improving circulation, reducing inflammation, and protecting the skin, this ointment has the potential to enhance equine performance and recovery, especially after strenuous activity. The control formulation further supports the unique benefits of thyme and argan oils. Future trials involving direct application to horses are essential to validate these findings and optimize the formulation for wider equine therapeutic use.

Introduction

Horses can develop leg edema for a number of reasons. Edema can occur in only one leg, only in the hind legs, or in all four legs. Typically, swollen legs occur in the hind legs, in addition to being called filling, this condition may also be known as "stocking up" and is quite common. It is usually the area between the fetlocks and coronary bands that is affected. As a result of gravity, fluid can build up due to the leakage of fluid from blood vessels and tissues. This condition, called edema, can be caused by minor injuries like cuts or scratches or more serious conditions like cellulitis or lymphangitis. The accumulation of fluid in body tissues, commonly seen in the legs but also in other parts of the body, is referred to as edema in horses. Numerous factors, such as infections, wounds, allergies, heart illness, kidney issues, and liver abnormalities, can cause this syndrome (4). Horses' legs can be affected by far more serious conditions like cellulitis and lymphoma. Cellulitis is an inflammation of the skin and soft tissues directly beneath the skin (13). Lymphangitis develops when inflammation spreads to deeper tissues and vessels that move lymph throughout the body. Both can be caused by bacterial infections that enter a horse's skin through an open wound or scratch, and both require prompt veterinary care. The symptoms that horses may experience due to this condition are typically observed by the owner. Symptoms of edema include swollen leg joints in one leg, swollen leg joints in more than one leg, painful limbs, trauma to the

limbs, cuts or scratches on the lower legs, loss of appetite, fever, warmness of the legs to the touch, and lethargy (2).

Controlling inflammation in the affected area and lowering the risk of additional injury should be the goals of first aid for leg edema. A more routine approach, such as using a leg brace, bandaging, or rubbing the legs with liniment or oil, is recommended for mild episodes of edema. Other treatment methods include cold therapy, abscess treatment, and antibiotics and anti-inflammatory medications. Cold therapy is the first-line treatment for edema. Cold therapy helps constrict damaged blood vessels, which will reduce ongoing internal bleeding and fluid accumulation in the injured tissue and providing some pain relief by numbing nerve endings (4).

In mild cases of edema-relieving gel, such as RAPIGEL®, the gel can be applied twice daily for the first few days after the injury to soothe the legs and help reduce tissue swelling (22). Thyme oil is obtained from the thyme plant (Thymus vulgaris) through the process of steam distillation. It has a long history of use in traditional medicine because of its diverse therapeutic properties. Thyme oil contains active compounds such as thymol, carvacrol, and terpinene, which play a role in its medicinal effects (15). It has been acknowledged for its potential in reducing edema as it exhibits anti-inflammatory and diuretic properties while also stimulating the lymphatic system. For topical application, thyme oil can be diluted with a carrier oil and used in massage therapy (6, 15).

Argan oil is a natural oil derived from the kernels of the argan tree (Argania spinosa), which is native to Morocco. It is rich in essential fatty acids, antioxidants, and various beneficial compounds (10). While argan oil is primarily known for its cosmetic and skincare applications, it may also have potential effects on pain relief. Although its effects on edema have not been extensively studied, argan oil possesses properties that may contribute to reducing edema, such as anti-inflammatory, moisturizing, nourishing, and antioxidant properties. In addition, argan oil may help stimulate lymphatic circulation. The lymphatic system plays a crucial role in fluid balance and waste product removal from tissues. By enhancing lymphatic flow, argan oil may reduce edema and promote fluid drainage (10, 16). The ability of an animal to adjust to its surroundings painlessly and to maintain both physical and mental health is known as animal welfare. Massage treatments, including vegetable oils, have been shown to ease stiff joints and muscles, increase blood flow, and lessen edema. Animals experience less pain, and their welfare level increases as a result of improved blood circulation and decreased edema (18). To our knowledge, no studies have investigated the application of argan or thyme oil for the treatment of edema in horses. The effectiveness of argan oil and thyme in horses with leg edema was assessed in this study.

Materials and Methods

Ethical Statement: The study was approved by the Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (Decision No. 2024/051, Date: 28.03.2024).

The animal material of the study consisted of 22 horses of various ages (ranging from 2 to 16 years, with a mean age of 9 years) and different breeds housed at “Doğa Horse Farm” in Mersin. The research horses were randomly selected from the facility’s population. All horses were housed in individual stalls, provided with adequate shelter, and provided access to clean drinking water at all times. A control group was not used in this study because the efficacy of the base ingredients—Vaseline® and paraffin—has been well documented in prior studies and clinical applications. Retrospective data from past experience consistently demonstrate the moisturizing and protective properties of these substances, making them a reliable foundation for topical treatments. Similarly, thyme and argan oils have shown strong anti-inflammatory and skin-repairing effects in various animal care practices. Given this well-established baseline, we focused on evaluating the combined effects of these ingredients at different concentrations, building on their known benefits, and optimizing their application for horse leg health. Thus, while a control group could provide further comparative insights, the decision to omit it in this study was based on these solid, previously established data points. Feeding is done in the morning, noon, and evening, and the daily feed consists of 4 kg of oats, 2 kg of barley, 1 kg of ready-made feed with a high vitamin, mineral, and protein ratio, 6 carrots, 6 apples, and 10 kg of hay. Thyme and argan oils were applied to the horses at different times. The horses’ performance before and after treatment, as well as the psychological and physiological changes induced by the oils, were observed. The age, gender, breed, and coat color of the horses were obtained from pedigree records kept by the Turkish Equestrian Federation. Table 1 lists the horse information used in the study.

Preparation of Thyme Oil and Argan Oil Mixes: Vaseline®, paraffin, thyme oil, and argan oil were all bought from stores. Analytical grade reagents were all used exactly as supplied. Vaseline’s exceptional ability to create a protective barrier on the skin makes it a prominent ingredient in ointments. Like vaseline, paraffin is a waxy material that is frequently used to ointments because of its ability to build a barrier against moisture. First, an ointment mixture was prepared at room temperature using equal amounts of paraffin and Vaseline®. Thyme oil and argan oil were then added to the mixture at a ratio of one part oil to two parts paraffin/Vaseline®. To ensure comprehensive results and assess the efficacy of the different formulations, tests were conducted using varying amounts of thyme and argan oil. These adjustments included

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Table 1. Numbers, ages, genders, colors, and breeds of the horses used in the study.

Horse Number	Age	Gender	HorseCoat Color	Breed
1	6	Female	Flea-Bitten Gray	Belgian Draught
2	9	Female	Chestnut	Hannover
3	9	Male	Dapple Gray	Hannover
4	15	Male	Bay	Belgian Draught
5	7	Male	Bay	Hannover
6	16	Female	Chesnut Bay	Hannover
7	9	Male	Bay	Hannover
8	2	Female	Chesnut Bay	Hannover
9	2	Male	Chesnut	Hannover
10	11	Male	Bay	Thoroughbred
11	10	Male	Black	Thoroughbred
12	6	Female	Chesnut	Thoroughbred
13	6	Male	Bay	Thoroughbred
14	8	Male	Chesnut	Thoroughbred
15	1	Male	Bay	Thoroughbred
16	7	Female	Chesnut	Thoroughbred
17	8	Female	Chesnut	Thoroughbred
18	4	Male	Chesnut	Arabian
19	7	Female	Dapple Gray	Arabian
20	11	Female	Chesnut	Pony
21	6	Male	Dapple Gray	Pony
22	7	Male	Mixed Color	Pony

different ratios of oil to paraffin/Vaseline® base (e.g., 1:1, 1:2, and 2:1). This allowed for the evaluation of which concentration in the trial had the most beneficial effect on lowering fatigue and leg edema.

The Application of Thyme and Argan Oils: For two minutes, each produced oil mixture was administered to each horse's leg. For each leg, five milliliters of oil were administered. It was applied to the suspected pain or swollen areas for 5 min. In addition to the legs, it was used for friction or massage to target troublesome muscle groups. During the application, no bandage was used to stop absorption. Within an hour of training, the horses were observed to record the required data. After that, soap and cold water were used to cleanse the oily regions.

Throughout the three days, the research horses received thyme oil 10 min before training. After 1 week and for 3 more days, argan oil was administered 10 min prior to exercise. From the beginning of the hoof capsule to the higher part of the carpal joint in the front legs and from the end of the hoof capsule to the upper portion of the tarsal joint in the rear legs, oils were applied to all four of the horses' legs. The farm's trainer, veterinarian, and horse caretaker reviewed the gathered information. Until the legs were cleaned, data were continuously recorded during and after exercise. After washing, the legs were observed for 10 minutes every 2 hours.

Evaluation of Psychological and Physiological Parameters: There are two types of edema. Peripheral edema, characterized by swelling in the legs, can vary depending on the body position. The skin over the swollen area is tight and shiny. Pressure applied to this region creates an indentation, known as peripheral or pitting edema. The regions without visible swelling and where no indentation forms upon pressure are considered non-edematous. Pulmonary edema, another form of edema, was not examined in this study.

When an area of pain is squeezed with fingers, the horse may react by trying to bite, kick, or escape. If the horse displays these discomfort behaviors when body parts like joints are flexed, this indicates the presence of pain. Horses that do not exhibit these behaviors are considered pain-free.

Horses that exhibit itching behavior have been observed scratching the itchy area with the tip of their nose or by using a surface.

In the evaluation of redness, any color changes close to red in the area were considered positive.

Horses that behave differently towards their caregivers and trainers in the stable or paddock (such as biting, kicking, trying to escape, or trying to throw the rider) are considered aggressive.

Horses with more feces and urine than usual in their stalls or those frequently observed defecating and urinating around people are considered positive for excessive defecation and urination. It is noted that horses typically hesitate to defecate and urinate in the presence of unfamiliar individuals.

Horses that do not sufficiently rest while standing or do not sleep lying down are observed to be tired during training and exhibit a decrease in performance.

Problematic areas typically have higher temperatures than similar regions. Areas with detected temperature increases during manual examination are considered positive.

Restless horses tend to scrape the ground and lick painful or itchy body parts; these behaviors are recorded as positive.

Hair loss or scaly skin is considered an irritation and side effect in areas where oil is applied.

Statistical Analysis: Chi-square analysis was conducted to evaluate the side effects of argan and thyme oil in horses. Descriptive statistics are presented as numbers and percentages. Results were deemed significant when the P-value was less than 0.05. The data were analyzed using SPSS version 21.

Results

In this study, the psychological and physiological changes in horses induced by the oils evaluated were as follow; rash, burning, pain, smell, itchiness, temperature increase, restlessness, swelling, decrease in edema, hair loss, sleep disturbance, loss of appetite, struggling, irritation, scratching the ground, kicking, aggression, leg/hoof licking, lack of water intake, drinking too much water, excessive urination, excessive defecation, and increase or decrease in performance. Figure 1 shows the effects observed after the application of thyme and argan oil.

As shown in Figure 2, the application of thyme oil caused rashes in most of the horses. Primary irritation occurs rapidly the first time an essential oil is used, presenting as red wheal or burn. This reaction is more

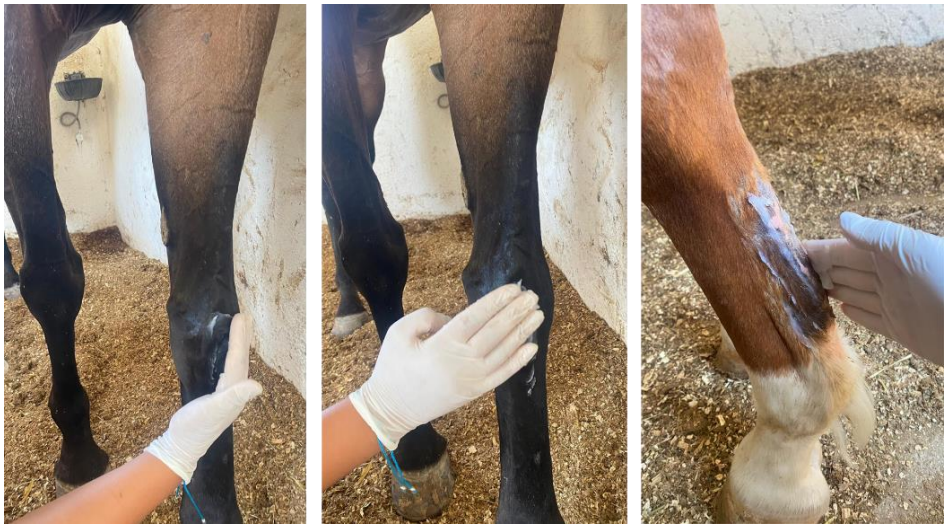

Figure 1. Oils applications to areas of concern in horses.

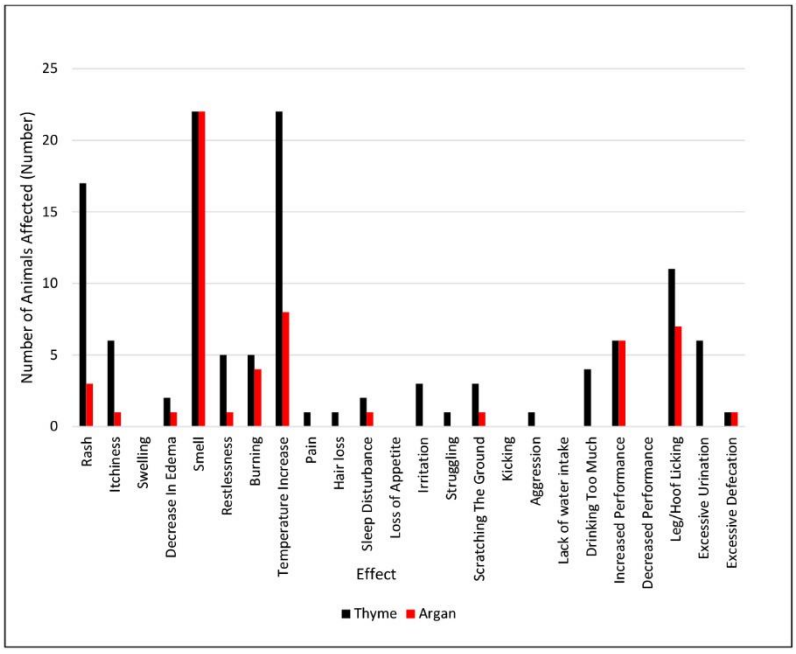

Figure 2. Side effects observed after the application of thyme and argan oil.

common when essential oils contain high concentrations of compounds such as thymol (found in thyme). The skin reaction is typically confined to the area where essential oil is applied (5).

In addition, thyme oil has caused a rise in temperature within the regions of interest for all horses. Since thyme oil is well known for its aromatic properties, all the horses' regions of interest had a strong smell. Regarding reducing edema, thyme oil did not exhibit any beneficial effects except in the cases of horse number 1 and 2. The use of thyme oil did not have any significant effect on symptoms such as swelling, loss of appetite, or kicking, and it did not decrease the performance of the horses. In contrast, the performance of horse number 1, 2, 3, 6, 10, and 16 improved.

In this study, following the administration of thyme oil, horse number 1 experienced a reduction in fetlock edema, while horse number 2 and 3 demonstrated a decrease in swelling and pain. Additionally, horse number 20 initially experienced muscle pain in the right shoulder, but after the application of thyme oil, the pain decreased. Table 2 lists some observations made before and after the application of thyme oil.

Argan oil did not cause any rashes except for horse number 3, 19, and 21. Did not cause any itchiness, restlessness, burning, sleep disturbance or excessive defecation. Argan oil did not exhibit any noticeable effect on symptoms like swelling, pain, hair loss, appetite loss, kicking, abnormal drinking behavior (either excessive or insufficient water intake), excessive urination, or overall performance of the horses. In contrast, the performance of horse number 1, 3, 6, 7, 10, and 18 improved.

Although argan oil did not reduce edema in horse number 1, the horse exhibited improved comfort while using the foot affected by tendonitis. Horse number 2 did not experience a decrease in swelling, whereas horse number 3 exhibited a reduction in swelling. In contrast to thyme oil, the application of argan oil did not alleviate the pain experienced by horse number 20. Argan oil is all of the horses regions of interest had a strong smell. Table 2 shows observations made before and after the application of argan oil.

A comparison of the side effects observed when using thyme oil and argan oil was performed using Chi-square analysis (Table 3).

When comparing the effects of thyme oil and argan oil, significant differences were observed in several side effects, while others showed no notable differences. For rash, argan oil had a markedly better side effect profile, with only 3 cases of rash out of 22 compared with 17 cases for thyme oil. This difference was statistically significant with a P-value of $p<0.001$, indicating that argan oil had fewer rash side effects. Similarly, for itchiness, argan oil performed better, with only 1 case compared to 6 cases for thyme oil (P-value of 0.039), showing a significant reduction in itchiness as a side effect with argan oil. Regarding restlessness, there was a trend suggesting that it might be more common with thyme oil (5 cases) than with argan oil (1 case), although this difference was not statistically significant (P-value of 0.079). Both oils had similar burning sensation profiles, with no significant difference (P-value of 0.709). Temperature increases were significantly more common with thyme oil, occurring in 22 cases compared with 8 cases with argan oil (P-value of 0.000). Other side effects such as pain, hair loss, sleep disturbance, and ground scratching were not significantly different between the oils. A slight reduction in edema was observed more frequently with thyme oil (2 cases) than with argan oil (1 case), but the difference was not statistically significant (P-value of 0.555). Irritation was slightly more common with thyme oil (3 cases) than with argan oil, with a P-value of 0.073, in the cating a trend but not statistical significance. Thyme oil also exhibited a higher incidence of excessive urination (6 cases compared to none for argan oil), with a, significant P-value of 0.008. Both oils showed similar results for other side effects, such as excessive defecation and leg/hoof licking, with no significant differences. "Drinking too much" was more commonly absent with argan oil (P-value of 0.036). Since both oils did not cause symptoms such as swelling, loss of appetite, kicking, lack of water intake, or performance reduction, and both resulted in a noticeable smell, they are considered equivalent regarding these particular side effects.

Table 2. Observations noted before and after the application of thyme oil and argan oil.

Horse No.	Before Application	After Application of Thyme Oil	After Application of Argan Oil
1	The horse's posterior fetlock joints were edematous. It had left anterior tendonitis.	The horse was comfortable during training, and the edema in the fetlocks decreased.	The edema was not reduced; the horse used the foot with tendonitis more comfortably.
2	Right carpal joint swollen due to bone growth.	The horse was comfortable during training, and the swelling decreased.	The horse was comfortable during training, and the swelling did not decrease.
3	Anterior fetlock joint swelling due to capsular enlargement and anterior right chronic tendinitis.	Swelling has not decreased, but tendon pain has decreased.	The swelling decreased and the horse used his tendinitis foot more comfortably.
4	-	-	-
5	Bone growth at the posterior left phalanx two	The horse has no complaints before or after the application.	The horse has no complaints before or after the application.
6	Two front fetlock joints broken part	The horse was comfortable during training.	The horse used its forelegs better during training.
7	Inflammation of the right navicular bone	The horse has no complaints before or after the application. The horse was comfortable during training.	The horse has no complaints before or after the application. The horse was comfortable during training.
8	The two posterior hocks have beaks	The horse has no complaints before or after the application. The horse was comfortable during training.	The horse has no complaints before or after the application. The horse was comfortable during training.
9	-	-	-
10	The fragment of the left anterior fetlock joint has undergone stippling.	The horse was comfortable during training.	The horse was comfortable during training.
11	-	-	-
12	-	-	-
13	-	-	-
14	-	-	-
15	-	-	-
16	An old crack in the left phalanx, stumbling.	Horse stumbling decreased during training.	The stumbling continued.
17	-	-	-
18	The horse had a sore shin and underwent cold cauterization.	The horse has no complaints before or after the application.	The horse has no complaints before or after the application. The horse was comfortable during training.
19	-	-	-
20	Muscle pain in the right shoulder	Muscle pain decreased.	Muscle pain did not subside.
21	-	-	-
22	No problem, the horse is blind in both eyes.	The horse has no complaints before or after the application.	The horse has no complaints before or after the application.

- : The horse had no pre- or post-treatment problems

Table 3. Comparison of side effects observed in subjects treated with thyme oil and argan oil, including their absence or presence and the associated P-values indicating statistical significance.

Side Effect		Oil		P
		Thyme Oil n (%)	Argan Oil n (%)	
Rash	Absent	5 (22.7)	19 (86.4)	0.000***
	Presence	17 (77.3)	3 (13.6)	
Itchiness	Absent	16 (72.7)	21 (95.5)	0.039*
	Presence	6 (27.3)	1 (4.5)	
Decrease in edema	Absent	20 (90.9)	21 (95.5)	0.555
	Presence	2 (9.1)	1 (4.5)	
Restlessness	Absent	17 (77.3)	21 (95.5)	0.079
	Presence	5 (22.7)	1 (4.5)	
Burning	Absent	17 (77.3)	18 (81.8)	0.709
	Presence	5 (22.7)	4 (18.2)	
Temperature increase	Absent	0 (0.0)	14 (63.6)	0.000***
	Presence	22 (100.0)	8 (36.4)	
Pain	Absent	21 (95.5)	22 (100.0)	0.312
	Presence	1 (4.5)	0 (0.0)	
Hair loss	Absent	21 (95.5)	22 (100.0)	0.312
	Presence	1 (4.5)	0 (0.0)	
Sleep disturbance	Absent	20 (90.9)	21 (95.5)	0.550
	Presence	2 (9.1)	1 (4.5)	
Irritation	Absent	19 (86.4)	22 (100.0)	0.073
	Presence	3 (13.6)	0 (0.0)	
Struggling	Absent	21 (95.5)	22 (100.0)	0.312
	Presence	1 (4.5)	0 (0.0)	
Scratching the ground	Absent	19 (86.4)	21 (95.5)	0.294
	Presence	3 (13.6)	1 (4.5)	
Aggression	Absent	21 (95.5)	22 (100.0)	0.312
	Presence	1 (4.5)	0 (0.0)	
Drinking too much	Absent	18 (81.8)	22 (100.0)	0.036*
	Presence	4 (18.2)	0 (0.0)	
Increased performance	Absent	16 (72.7)	16 (72.7)	0.999
	Presence	6 (27.3)	6 (27.3)	
Leg/Hoof licking	Absent	11 (50.0)	15 (68.2)	0.220
	Presence	11 (50.0)	7 (31.8)	
Excessive urination	Absent	16 (72.7)	22 (100.0)	0.008**
	Presence	6 (27.3)	0 (0.0)	
Excessive defecation	Absent	21 (95.5)	21 (95.5)	0.999
	Presence	1 (4.5)	1 (4.5)	

*: P<0.05, **: P<0.01, ***: P<0.001.

Discussion and Conclusion

Edema in horses is characterized by an abnormal buildup of fluid in the body tissues, resulting in swelling. Although it predominantly occurs in the legs, it can also manifest in other parts of the body. Thyme is widely used in traditional medicine and phytotherapy (24). Essential oils possess a great variety of biological properties, including antibacterial (21, 25), antifungal (12), anthelmintic (11), insect repellent (7), antioxidant (6), and anticancer (20, 25) effects, which are often attributed to their high yield of phenolic compounds, i.e., thymol and carvacrol. Up to now, little is known about the anti-inflammatory effects of thyme oil (14) though there is strong evidence that the antioxidant potential of phenols such as thymol is associated with their inhibitory effects on inflammatory processes (3).

Fachini-Queiroz et al. (9) investigated the effects of thyme oil on the inflammatory response and edema

reduction in a mouse ear model (9). To induce cutaneous inflammation, 5% croton oil (10 µL) in acetone was applied to the inner surface of the right ear of each mouse. Thymol (10 mg per ear) was topically administered to the right ear. Contrary to expectations, thymol did not reduce the development of edema but instead caused an irritative response, likely due to the release of histamine and prostanoids. However, the study findings suggest that thymol's anti-inflammatory effect is associated with its ability to inhibit inflammatory edema and the migration of leukocytes.

Abdelli et al. (1) investigated the composition of essential oils extracted from the leaves of Thymus vulgaris in two regions of Northwestern Algeria, Mostaganem and Tlemcen. The researchers also aimed to assess the acute oral toxicity and anti-inflammatory properties of essential oils using the carrageenan-induced paw edema test in vivo (1). Carrageenan-induced paw edema is a commonly used experimental model of inflammation characterized by biphasic development of edema (22). The administration of different doses of Thyme oil to mice resulted in a significant and highly noteworthy reduction in edema compared with the control group, starting at 4 hours post-treatment ($P<0.001$). These findings are consistent with a prior investigation into commercially available thyme oil (17).

The anti-inflammatory effects of thyme oil can be primarily attributed to its main component, thymol. Previous studies have provided evidence of its anti-inflammatory properties of thymol (8, 19). This study clearly demonstrated that thyme oil exhibits substantial anti-inflammatory activity even at doses well below toxic levels.

There is limited scientific research investigating the effects of argan oil on edema. Argan oil is primarily known for its cosmetic and culinary uses, rather than its medicinal properties. Although argan oil contains various bioactive compounds that may have potential anti-inflammatory effects, there is currently insufficient evidence to support its effectiveness in reducing edema. Makbal et al. (16) assessed acute and subacute toxicity, as well as the anti-inflammatory and antioxidant effects of argan extract (16). The results showed that argan extract significantly inhibited xylene-induced ear edema in mice. In addition, the extract significantly reduced paw edema in mice following carrageenan injection. These findings suggest that argan extract has promising anti-inflammatory and antioxidant properties.

Although studies conducted on mice have demonstrated the anti-inflammatory effects of thyme and argan oils, no clinical field study has directly applied these findings to horses. This lack of research highlights the uncertainty regarding whether results obtained from mice can be directly translated to equine applications. Given the high economic value of racehorses, particularly in terms of performance and breeding, it is crucial to conduct studies directly on these horses to ensure more reliable and species-specific data. Testing treatments for leg edema and fatigue prevention in horses is essential to safeguard their health and optimize their performance. Therefore, moving beyond mouse findings to clinical trials in horses is significant.

During the course of the study, several noteworthy observations were made. Horse number 22, who was blind, displayed fear in response to the application of the oils. An interesting observation was made in horse number 1 and 3, both of whom received irritant medication for treating tendon inflammation, as they exhibited reactions similar to those observed in horse 18. Another observation pertained to hair reduction and thinning of skin on the metacarpus 3 bones of horse 18 due to cold cauterization, which indicated heightened reactions towards the oils. To address shoulder muscle pain experienced by horse number 20, larger volumes of oil were generously applied to the affected area. It was observed that both the benefits and side effects increased with increasing oil content. Additionally, it was noted that smaller breeds such as ponies and horses, with gray frost coloration had a higher incidence of side effects. Thyme oil, known for its stronger aroma and greater effectiveness in relieving pain and reducing swelling, has exhibited more side effects (8,19). The study results are consistent with the literature and showed that thyme oil has anti-inflammatory effects in vivo. Thymol has an irritative effect on the other hand, argan oil demonstrated properties such as improved blood circulation, skin soothing, and preventing irritation. Experiments involving the combined use of both oils were conducted, which resulted in greater overall benefits with fewer side effects. These findings suggest the potential utility of such studies in the future.

Leg edema in horses is the accumulation of fluid and subsequent swelling in the legs, which is primarily observed in the lower limbs. It can manifest in one leg or affect multiple legs, with a higher prevalence in the hind limbs. The condition can arise from various causes, including infections, injuries, allergies, heart disease, kidney problems, liver disorders, and underlying health issues.

Treatment for leg edema in horses aims to address the root cause while managing swelling and providing relief. Veterinary care is typically necessary, and treatment may involve the administration of medications such as diuretics, anti-inflammatory drugs, or antibiotics, depending on the specific underlying condition. Supportive measures may include cold therapy, bandaging, massage, controlled exercise and ensuring a balanced diet. Thyme oil, derived from the thyme plant, has anti-inflammatory and diuretic properties and can

stimulate the lymphatic system. Argan oil, extracted from the argan tree, is known for its moisturizing, and antioxidant qualities and may enhance lymphatic circulation. Although research on the specific use of these oils for treating horse edema is limited, this study aimed to investigate their efficacy in addressing this condition. Thyme oil, renowned for its potent scent and notable efficacy in pain and swelling relief, was associated with a higher incidence of side effects. Conversely, argan oil has attributes such as enhanced blood circulation, skin soothing, and irritation prevention. Experimental studies investigating the combined application of both oils yielded superior overall advantages while minimizing adverse reactions. These findings indicate the potential value of further research in this area.

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Ethical Statement

The study was approved by Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (Decision no: 2024/051, Date: 28.03.2024).

Conflict of Interests

The authors declare that they have no conflict of interests.

Author Contributions

Conceptualization, Y.P.; writing-original draft preparation, Y.P. and N.K.; data analysis, M.Ç.G.; writing-review and editing, Y.P.; N.K. and M.Ç.G. All authors reviewed and approved the final version of the manuscript.

Data Availability Statement

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

Animal Welfare

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

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Impact of melamine exposure on apoptotic proteins and oxidative stress markers in mouse hepatic tissue

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ABSTRACT

Most melamine studies have focused on renal toxicity and its effects on the liver are still not well known. We investigated the apoptotic and oxidative effects of melamine on the liver using thirty BALB/c mice, divided into three groups. The control group received saline, while the low-dose melamine (LDM) group was given 400 mg/kg (1/8 LD50) and the high-dose melamine (HDM) group received 1600 mg/kg (1/2 LD50) intragastrically (0.25 ml) for 5 consecutive days. Liver Bcl-2 and caspase-3 expressions were analyzed at the protein level by immunohistochemistry and ELISA, and also at the gene level by quantitative Real-Time PCR. In addition, total antioxidant (TAS), total oxidant (TOS), and oxidative stress index (OSI) levels in liver tissues were measured spectrophotometrically. The immunohistochemical expression of caspase-3 was higher in the LDM and HDM groups compared to the control group ($p = 0.002$). TOS and OSI levels were increased significantly ($P < 0.05$) in the HDM group as compared to controls. Bcl-2 ELISA levels in the HDM group increased significantly compared to the control ($P = 0.0024$). Caspase-3 values increased significantly in the HDM group compared to the control ($P < 0.0001$) and LDM ($P = 0.0016$) groups. This study provides evidence that exposure to melamine induces oxidative stress and increases apoptosis in the liver. In conclusion, we suggest that both apoptotic and anti-apoptotic mechanisms may be disrupted at high melamine exposures, which has not been reported extensively in previous publications.

Introduction

Melamine is a nitrogen-containing organic compound, also known as 2,4,6-triamino-1,3,5-triazine. It is widely used in plastics, coatings, adhesives, and fire retardants. However, melamine can also be present as a contaminant in human and pet foods (8, 15, 20). Low melamine levels (3.0-5.12 mg/kg) in food do not cause acute symptoms, so melamine is an easily overlooked environmental toxin (6).

Melamine toxicity studies have focused mainly on renal pathological changes. So far, the toxic effects on kidney tissue have been reported in detail (11). Although the liver is the largest organ involved in the neutralization and detoxification of various harmful substances and chemicals, it is easily damaged by toxic compounds, and the reports on the effect of melamine on the liver remain restricted (18). A few studies have suggested hepatotoxic potential and reported that melamine caused notable changes in liver histopathology and the induction of oxidative stress, inflammation, and apoptosis (1, 7). Although melamine-induced oxidative stress on the total antioxidant (TAS), total oxidant (TOS), and oxidative stress index (OSI) levels analyzed in this study have not

yet been reported in the literature, Habotta et al. (19) reported that melamine-increased oxidative stress via the formation of malondialdehyde (MDA) and nitric oxide (NO) and decreased the levels of reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) activities.

Caspase-3 is recognized as a crucial mediator of apoptosis, triggered within apoptotic cells through both extrinsic and intrinsic pathways. This enzyme is a pivotal zymogen in cellular apoptosis, remaining inactive until cleaved by initiator caspases during the apoptotic process (5, 28). Also, the Bcl-2 protein has important actions in organizing cell death through the regulation of apoptosis (27, 31). It is the most important anti-apoptotic protein, whose overexpression may influence chemo-resistance, DNA repair, cell proliferation, and tumorigenesis. High levels of Bcl-2 protein expression were reported in many tumors, including liver cancers (10, 32).

Exposure to toxic agents promotes the degradation of nucleic acids and disruption of apoptotic mechanisms by increasing oxidative stress products in the liver (2). Presently, our understanding of melamine's hepatic toxicity remains limited. This study aimed to delve deeper into the toxic effects of melamine on the liver in mice and evaluate the hepatotoxic, apoptotic/anti-apoptotic, and harmfully oxidative potentials of melamine on liver tissue.

Materials and Methods

Experimental design and treatment of animals: The experimental design was approved by the Ethics Committee of Animal Experiments (Meeting/Decision no. 2018-18.09/47). Thirty 10 weeks old, male BALB/c mice weighing 33.43 ± 2.63 g were equally divided into 3 groups: the negative control group given saline (control, group 1), the group receiving low dose melamine (LDM, group 2), and the group receiving high dose melamine (HDM, group 3). The specifications of the experimental environment, feeding, and care of the mice were designed as previously reported (13). Routine health checks and weighing were made after the adaptation period. Applications (0.25 ml) were made intragastrically once daily for 5 consecutive days at 10 a.m. (20). Dose calculations were based on the median lethal dose (LD50, 3200 mg/kg) of melamine (M2659, Sigma Aldrich®, USA) and the mice received 400 mg/kg (1/8 LD50) once daily in the LDM group and 1600 mg/kg (1/2 LD50) in the HDM group for 5 consecutive days (35). During the experimental period, subjects were provided with free access to food and water ad libitum. Throughout the experiment, the animals were monitored daily for body weight, behavioral changes (irritability), and signs of poisoning (hematuria).

On the 35th day after the first administration, mice were euthanized by gradually increasing concentrations of carbon dioxide inhalation (9). The livers were removed, weighed, and allocated for histopathology, biochemistry, immunohistochemistry, and Real-Time PCR analyses.

Histopathological and immunohistochemical evaluation: Liver tissues were fixed in 10 % formalin for 24 hours and embedded in paraffin wax. Subsequently, they were cut as 5 µm sections using a microtome (Leica, DE). Prepared tissue sections were stained with hematoxylin and eosin (H&E). These were examined and evaluated for apoptosis in each group. The apoptotic cells seemed to have dark eosinophilic cytoplasm and dense nuclear chromatin. They were in single-cell formation. Apparent apoptotic cell death in the liver parenchyma was determined microscopically (7).

For immunohistochemistry tissue samples embedded in paraffin wax were sectioned at 5 µm thickness and placed on Poly-L-Lysine-coated positively charged slides. They were stained on the automated immunohistochemistry staining device (Ventana Benchmark XT, Roche, Basel, Switzerland) using Bcl-2 (Rabbit polyclonal, NB100-56098, Novus Biologicals, USA, 1/100 dilution) and caspase-3 (Rabbit polyclonal, NB100-56/113, Novus Biologicals, USA, 1/200 dilution) primary antibodies. Benign tonsil tissue was used as a positive immunohistochemical control for both proteins. H&E and immunohistochemistry sections were examined under a light microscope (Nikon Eclipse Ni, Japan) by two pathologists.

The immunoreactivity of Bcl-2 was based on the stained liver sinusoidal endothelial cells (LSECs) and caspase-3 was evaluated from cytoplasmic staining of hepatocytes. Immunostaining was scored semi-quantitatively by the presence of positively stained LSECs: 0 (negative), and 1 (positive) for Bcl-2 (36). Caspase-3 immunostaining results were evaluated by modifying the method reported by Ramalho et al. (25) using the percentage of positively cytoplasmic stained cells: 0 (0-50%), +1 (51-100%). The rate of reactivity in the hepatic tissue parenchyma was evaluated at 400 final magnification using grids of 100 squares and 10 adjacent fields that together constitute an area of 0.025 mm^2.

Oxidative stress analysis: Livers were washed with saline (+4 °C) and dried on blotting paper. Afterward, they were placed in the 1 ml storage vials and stored in a deep freezer (–80 °C) until analyses were performed. Prior to analyses 140 mM KCl solution at +4 °C was added (10%, w/v) to samples and homogenized using a homogenizer (Stuart, SHM1/EURO, UK) (26). Homogenized samples were centrifuged (Nüve, NF1200R, TR) at 3000 rpm for 5 minutes at +4 °C, and supernatants were used for determining the levels of Total Antioxidant Status (TAS, mmol Trolox equiv./lt) and Total Oxidant Status (TOS,

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μmol H_2O_2 equiv./lt) in liver tissue homogenates. Assays were performed spectrophotometrically (MultiskanGO, Thermo, USA) using the methods described in the kits (Rel Assay Kit Diagnostics, TR). The oxidative stress index (OSI) was calculated as a percentage of the ratio of TOS levels to TAS levels and the results were expressed in the arbitrary unit (AU) (26).

ELISA analysis: ELISA tests were performed in uncentrifuged liver tissue homogenates described in detail above. Bcl-2 and caspase-3 ELISA assays were performed spectrophotometrically (Multiskan GO, Thermo, USA) using commercial kits (SunRed Biotechnology, CN) according to the manufacturer's instructions.

Quantitative Real-Time PCR: RNA was isolated from liver samples using an RNA isolation kit (74106, QIAGEN, DE). Total RNA samples were measured at 260 and 280 nm using a nanodrop spectrophotometer (MultiskanGO, Thermo, USA) and calculated by A260/A280 ratio prior to cDNA synthesis (K1671, Thermo, USA). Synthesized samples were quantified to 200 ng and used for quantitative real-time PCR (qPCR) reactions. qPCR analyses were performed using a Real-Time PCR detection system (Light-Cycler® 480 II, Roche, CH) and the suitable kit (SYBR Green I Master Kit, Roche, CH). Detected Bcl-2 and caspase-3 expressions were normalized to GAPDH as the housekeeping gene (Table 1). The obtained crossing point (Cp) values were utilized to determine the relative expressions using the equation of $2^{-\Delta\Delta Ct}$ (33).

Table 1. Oligo-primer sequences used in the qPCR analyses of the present study.

Gene	Primers (5' to 3')	Reference
Bcl-2	R: AGCCAGGAGAAATCAAACAGAGG F: CCTGTGGATGACTGAGTACCTG	*(34)* *(21)*
Caspase-3	R: GCAGTAGTCGCCTCTGAAGA F: CCTCAGAGAGACATTCATGG	*(22)* *(17)*
GAPDH	R: CTGGGATGGAAATTGTGAGG F: TGGCCTCCAAGGAGTAAGAA	*(14)* *(30)*

Statistical analysis: The data collected underwent separate statistical analyses using the Statistical Package for the Social Sciences (SPSS 20.0, IBM, USA). Significance was assessed through the Kruskal-Wallis test, followed by the examination of group differences using the Mann-Whitney U test. A significance level of $P < 0.05$ with a 95% confidence interval was employed.

Results

Clinical signs, body and organ weights: Throughout the entire experimental duration, no instances of mouse mortality were observed. Animals showed no statistically significant differences in body weight throughout the experiment. They did not exhibit behavioral changes or signs of intoxication.

Histopathological and Immunohistochemical evaluation: There were no significant differences in the liver weights among the groups. The formation of apoptotic bodies in H&E stained sections in the control, LDM, and HDM groups were evaluated. No histopathologically significant difference was observed between the liver tissues of the control, LDM, and HDM groups in terms of mononuclear cell infiltration, congestion, and focal bleeding. Scattered single-cell hepatocyte necrosis was observed in the liver tissues in the LDM (10 %) and HDM (90 %) groups. This situation was not detected in the control group (Figure 1).

No positive LSEC immunostaining was detected for Bcl-2 in the control group. There was 30 % staining in the LDM group and 60 % staining in the HDM group. We found diffuse caspase-3 staining in only one case (10 %) in the control group, and this rate was higher for the LDM (80 %) and HDM (90 %) groups.

Using the Kruskal-Wallis test, significant differences were found between the groups in terms of apoptosis ($P < 0.0001$) as well as the expression of Bcl-2 ($P = 0.016$) and caspase-3 ($P = 0.002$) immunohistochemistry. When the groups were evaluated by the Mann-Whitney U test, there was a significant increase in the presence of apoptosis in the HDM group compared to the control and LDM groups ($P < 0.0001$, $P = 0.002$ respectively). Bcl-2 expression was higher in HDM groups compared to the control groups ($P = 0.004$). Although there was no statistically significant difference ($P = 0.067$), Bcl-2 expression was higher in the LDM group compared to the control group. Caspase-3 expressions were higher in the LDM and HDM groups compared to the control groups ($p = 0.002$).

Oxidative stress analyses: TAS levels did not alter significantly between the study groups. However, TOS and OSI levels were increased significantly ($p < 0.05$) in the HDM group as compared to controls (Figure 2).

ELISA analyses: Bcl-2 levels increased significantly ($P = 0.0024$) in the HDM group compared to the controls. Caspase-3 values increased significantly in the HDM group compared to the control ($P < 0.0001$) and LDM ($P = 0.0016$) groups. The alterations of Bcl-2 and caspase-3 proteins determined by ELISA are shown in Figure 3.

qPCR analyses: mRNA expression levels of Bcl-2 and caspase-3 genes in the liver did not exhibit significant differences between the study groups (Figure 4).

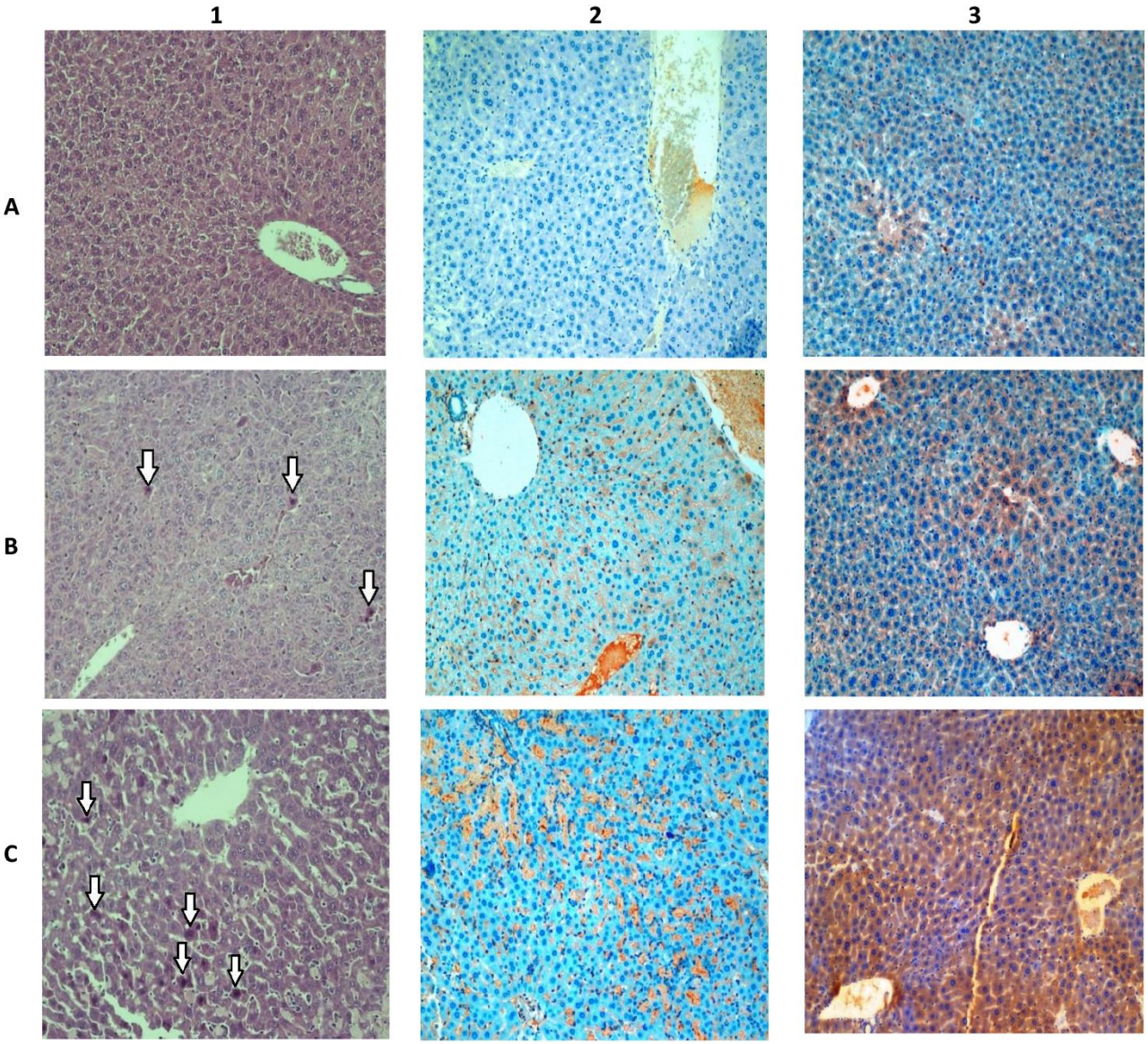

Figure 1. Microscopic images (*x200*): H&E staining (column 1), Bcl-2 immunohistochemistry (column 2), and caspase-3 immunohistochemistry (column 3) of the study groups (**A**: Control, **B**: Low dose melamine, and **C**: High dose melamine). Arrows indicate apoptotic hepatocytes.

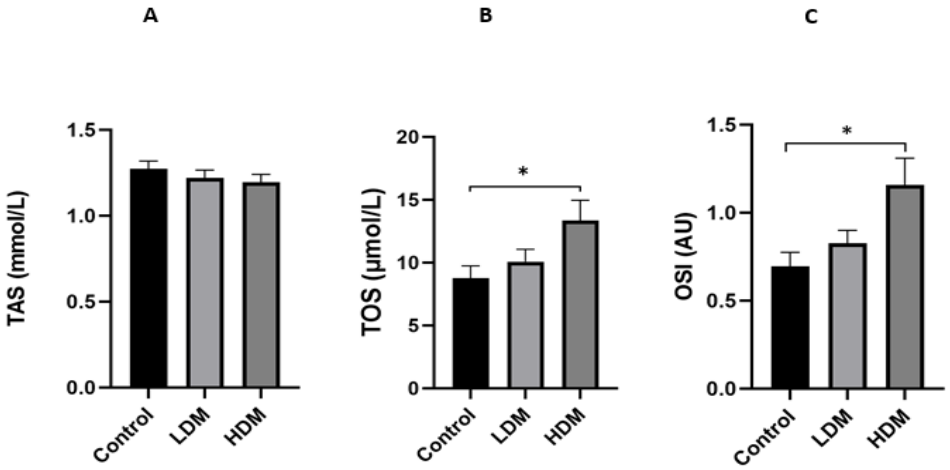

Figure 2. Levels of the oxidative stress markers (**A**: TAS, **B**: TOS, **C**: OSI) in liver tissues of control, low (LDM), and high dose (HDM) melamine applied study groups. Asterisks indicate significant (*: P <0.05) differences between the groups. Data are presented as the mean±standard error (SE).

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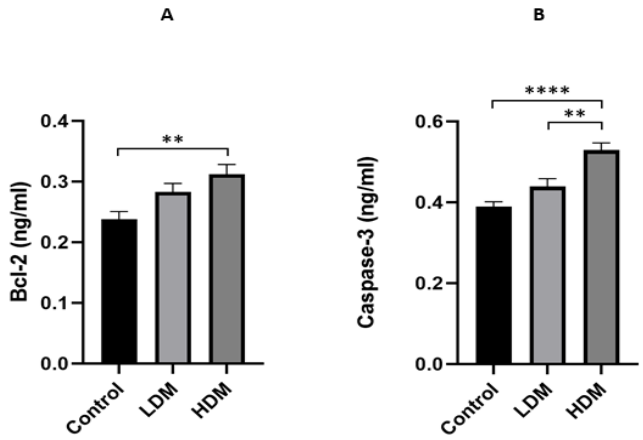

Figure 3. Results of the ELISA analyses of apoptotic markers (**A**: Bcl-2, **B**: Caspase-3) in liver tissues of control, low (LDM), and high dose melamine (HDM) applied study groups. Asterisks indicate significant (**: P <0.005, ****: P <0.0001) differences between the groups. Data are presented as the mean±standard error (SE).

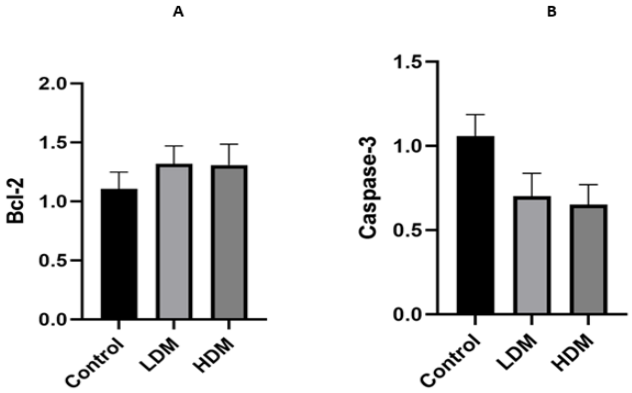

Figure 4. Quantitative Real-Time PCR (qPCR) results of the apoptotic genes (**A**: Bcl-2, **B**: Caspase-3) in liver tissues of control, low (LDM) and high dose melamine (HDM) applied study groups. Data are presented as the fold changes (mean±standard error).

Discussion and Conclusion

The histopathological examination of the present study disclosed increased levels of apoptotic cells in a dose-dependent manner in the liver tissues of all exposed groups, demonstrating that melamine could cause liver damage. Chang et al. (7) reported the pathological damage of the liver caused by orally administered 25, 50, and 100 mg/kg/day melamine. Similar to our results, they reported significantly increased levels of apoptosis even with 100 mg/kg/day melamine administration. Apoptosis, a main pathological indicator of liver injury, was also noticed in previous studies reporting cell injury induced by melamine exposure (1, 16).

As an anti-apoptotic protein, Bcl-2 supports cell survival by blocking the mitochondrial membrane pore formation (12, 27). We determined high protein values of Bcl-2 in the HDM group confirmed by ELISA and immunohistochemistry. Chang et al., (7) reported alterations in Bcl-2 levels that did not correlate with melamine doses with low (25 mg/kg) and high (50 mg/kg) melamine doses. However, they determined a Bcl-2 decrease in the melamine-treated groups. In addition, the fact that the given dose range was very low as compared to our study may be the reason for the different results obtained. Unphosphorylated Bcl-2 inhibits apoptosis, and Bax homodimers normally induce apoptosis. Bax can bind to and inhibit unphosphorylated Bcl-2, promoting apoptosis (24, 29). In our study, apoptosis in the liver increased with a rising dose of melamine, but the level of Bcl-2, which is known as the anti-apoptotic marker, also increased. Probably this was due to the binding effect of Bax, which is involved in apoptosis, to unphosphorylated Bcl-2, hence promoting apoptosis by inhibiting its anti-apoptotic effect. It is also known that post-translational mechanisms may regulate the function of Bcl-2 (23, 24). It is thought that melamine may exert an apoptotic effect by phosphorylating Bcl-2 and/or mediating Bax.

In their study investigating the protective effects of starch-stabilized selenium nanoparticles on melamine-induced hepato-renal toxicity in albino rats, Ahmed et al. (1) detected high caspase-3 protein levels at 300 mg/kg melamine-applied groups, consistent with this study. Likewise, An et al. (4) found increased caspase-3 values in melamine-applied groups as the indicator of the

progression of neuronal damage. In addition, Chang et al. (7) reported increased caspase-3 levels for the dose of 100 mg/kg/day melamine administration. A similar significant caspase-3 elevation determined by ELISA was also found in liver tissue in our study indicating liver damage.

Oxidative stress has a pivotal function in melamine-induced pathogenesis. Significantly increased levels of oxidative stress were reported by various studies inducing toxicity with oral melamine administration ≥ 100 mg/kg/day (1, 7). In the study evaluating whether Nootkatonea, naturally occurring sesquiterpenoid had a regulatory effect on oxidative stress and inflammatory liver damage triggered by 700 mg/kg melamine, it was reported that the mentioned melamine dose increased oxidative stress via the formation of MDA and NO) and decreased the levels of reduced GSH, CAT and SOD activities. In the present study, we determined high TOS and OSI values in melamine-administered groups, especially in the HDM group. Mechanisms that underlie melamine-induced liver damage are yet unclear; however, melamine exposure could evoke oxidative stress in liver parenchyma with subsequent damage and stimulating apoptosis-related proteins.

The present study assessed the pathobiochemical effects of melamine on the liver and proposed that high doses of melamine could lead to the disruption of the apoptotic mechanisms mediated by oxidative stress in liver tissue. Contrary to the reported decreasing effect of the level of 100 mg/kg, this study showed that high doses of melamine provide an increase in Bcl-2 levels, which means its anti-apoptotic effect.

In conclusion, our findings suggested that melamine toxication could cause oxidative stress-induced apoptosis in the hepatic tissue. With this study, we present evidence that both apoptotic and anti-apoptotic mechanisms may be impaired at high melamine exposures, which has not been studied extensively in previous publications, and that melamine exposure increases apoptosis in the liver by inducing oxidative stress.

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Ethical Statement

This study was carried out after the animal experiment was approved by Kırıkkale University Local Ethics Committee (Decision number: 2018-18.09/47).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Concept and Design: AKD, TD, HE and AS; Data Collection or Processing: AKD, TD, HE, MS, AS, NK and MS; Analysis or Interpretation: AKD, TD, HE, MS, AS, NK and MS; Literature Search: AKD, TD; Writing: AKD, TD.

Data Availability Statement

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

Animal Welfare

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

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The combined effects of 5-fluorouracil and Turkish propolis extract on EPGF and KRAS expressions and apoptotic cascade changing in rat colon cancer model

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ABSTRACT

This study aimed to investigate the effectiveness of 5-fluorouracil (5-FU) and Turkish propolis extract on the colon cancer model induced by azoxymethane (AOM) in Wistar rats. Study groups were designed as cancer group (AOM), control group, AOM+5-FU group, AOM+propolis group, AOM+Propolis+5-FU group. The findings showed that there was a significant decrease in WBC, and lymphocyte levels in the treatment groups AOM+5FU and AOM+Propolis+5FU compared to the AOM group ($P<0.0001$, $P<0.005$, respectively). The increase in TAS level and TOS level were found to be significant in the AOM+Propolis+5FU group compared to the AOM group ($P<0.0001$). Compared to the the AOM group, caspase-3, caspase-8 and bax levels were significantly increased and Bcl-2 evel was significantly lower in the 5 AOM+Propolis+5FU group ($P<0.0001$, $P<0.05$, $P<0.0001$, $P<0.0001$, respectively). In the 5-FU and propolis used-therapy groups, decreased anaplasia in colonic cells. In conclusion, we strongly believe that as a chemotherapeutic, 5-FU, and propolis might have a stopper effect on cellular anaplasia over extrinsic apoptotical pathway. We also believe that this antiproliferative effect on cellular anaplasia can appear if the combined therapy is preferred.

Introduction

Colorectal cancers (CRCs) have been reported to be amongst the health problems in developed and developing countries most associated with a positive socioeconomical status (40). In the pathogenesis of CRCs, there are complex interactions between genes, signaling cascade systems, specific molecules and receptors. One of these factors is genetic mutation. Some specific genes play an pivotal role during critical events in carcinogenesis. In other words, activations / inhibitions of genes and molecules assist in triggering cancer cell proliferation (15, 45).

Epidermal growth factor (EPGF) is a transmembrane protein that include a receptor (EPGR) amongst members of the epidermal growth factor family (EGF family). In epithelial origined cancers, it is released plenty of this transmembran protein after activated of EPG receptor. Kirsten rat sarcoma (KRAS) oncogene produce KRAS protein and is a member of RAS/MAPK signaling cascade. It is a fact that KRAS is an oncogene stimulated after activation by several cancer cascade molecules. EPGR-KRAS receptor are indeed homolog signaling receptor which playing role are among the key factors in the pathogenesis (9, 30, 45, 54). This leads, firstly, to an

upstream at receptor tyrosine kinase (RTK) and secondly at mitogen-activated protein kinases (MAPKs) / extracellular signal regulated kinases (ERKs) (MAPKs/ERK) signal transduction pathway. After intracellular tyrosine kinase receptor undergoes stimulation, it is then activated by extracellular ligand to the transmembrane receptor of EPGF. In such, cellular growth and proliferation are provided under control of chief oncogenes in colon cells. Also The KRAS gene is a member of the rat sarcoma viral oncogene family (RAS). In CRC cells eventually die and then go under the regulation of apoptosis through activation RAS protein as well as Protein kinase B (PKB/AKT)-phosphoinositide 3-kinases (PI3K) pathway. Firstly, RAS activates PI3K, which results in AKT activation. After AKT-PI3K pathway triggered, proapoptotic proteins and anti-apoptotic proteins are indirectly activated (30, 35).

One of the mechanisms of apoptosis, caspase -3,-8 and 9, is inhibited by AKT upregulation (3). On this point, RAS can also interact with anti-apoptotic bcl-2 protein (37). Cell death cannot happen alone when Bcl-2 protein is knock downed and bax and bak proteins influence growth factors (28).

5-FU, which has been a widely preferred chemotherapeutic agent against colorectal cancer malignancies to date, continues to be used effectively and frequently against the developmental stages of colorectal cancers that have acquired malignant properties through genetic mutations such as the KRAS oncogene (7, 18, 36).

On the other side, there have been many natural supplements and complementary therapeutics for cancer therapies nowadays. Herein, propolis is thought providing crucially role for clinical applications of anti-cancer treatments and lessen side effects of adjuvant therapy to main cancer chemotherapeutics. Its chemical composition is made up of polyphenols-flavonoids and phenolic acids (27, 33, 49). Also it has potential to be both chemotherapeutic and chemopreventive for pre-cancerous changes and cancer progressions (2, 17, 34). Its anti-cancer effectivities have been shown both in-vivo and in-vitro (12, 14, 25).

During cancer progression in the body, continuously exposure of oxidative stress on cells can prone to proliferation by disturbing cellular and microenvironmental homeostasis. For this purpose, important data regarding oxidative stress and oxidant/antioxidant balance in the body are obtained by revealing the total oxidant status (TOS) and total antioxidant status (TAS) of the body (8, 20).

The hypothesis of this study was based on investigating whether the separate and combined use of 5-FU, a chemotherapeutic agent, and propolis would cause mutations and apoptosis-related changes in cancer tissue and antiproliferative effects on cellular anaplasia in an experimentally created colon cancer model in rats.

In order to decide on the propositions in this hypothesis, it was aimed to investigate the changes in hemogram and biochemical parameters (various protein and enzyme concentrations) and histopathological results with the presence of chemotherapeutic 5-FU and propolis extract in AOM-induced CRC modeling in rats. It was also planned to evaluate how the expressions of KRAS and EGFR in colon cancer cells and their levels in apoptotic state (caspases, pro-apoptotic Bax, and anti-apoptotic Bcl-2 proteins) could be affected.

Materials and Methods

The study was conducted upon permission given from the ethics committee decision numbered 2022/05/23 of Kırıkkale University Animal Experiments Local Ethics Committee, Türkiye. In the study, 6 weeks old (average weight of 150–180 g), 30 male Wistar albino rats were used. They were given standard feed and water ad libitum throughout the study and maintained an air-conditioned animal facility under constant temperature and humidity with a 12 h day-night cycle.

The 30 rats used in the study were randomly divided into 5 groups of 6 each. The first group was the group in which cancer was induced by giving AOM. The second group was the healthy control group to which no application was made. The third group was the group in which AOM and 5-FU were given. The fourth group was the group in which AOM and propolis were given. The fifth group was the group in which AOM and 5-FU and propolis were given. The applications made in these experimental groups are explained below. In first group (AOM group) (n=6), Azoxymethane (AOM) in 0.5 ml physiological saline solution (PSS) were dissolved and administered at 15 mg/kg (Body Weight-BW) intraperitoneally once a week for three weeks. Additionally, this group was administered orally distilled water three times a week for four weeks. In the second group of the study (Control group) (n=6), a PSS was administered intraperitoneally once a week for three weeks. Additionally, this group was administered distilled water orally three times a week for four weeks. In the third group of the study (AOM+5-FU treatment) (n=6), AOM was administrated at the same dosage and duration to be in AOM group. Additionally, 12.5 mg/kg/BW 5-fluorouracil (5-FU group) given intraperitoneally three times a week for four weeks. In the fourth group (AOM+propolis administration group) (n=6), AOM and PSS were administered as they were in the previous groups. In addition, 0.3 mg/kg/BW propolis dissolved in distilled water was administered orally by gavage to this group, occurring three times a week for four weeks (12). In the last group (AOM +propolis+5-FU group) (n=6), AOM, 5-FU and propolis were administered the same dosage

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Table 1. Experimental design in groups.

Experimental Groups	Physiological saline solution (once /every week during 3 weeks)	Azoxymethane (cancer induction) (once /every week during 3 weeks)	5-Flourouracil Chemotherapy (three times / every week during 4 weeks)	Propolis Additive therapy (three times /every week during 4 weeks)
AOM		✓		
Control	✓			
AOM+5-FU		✓	✓	
AOM+propolis		✓		✓
AOM+5FU+propolis		✓	✓	✓

and duration as in the previous treatment groups. Study design described in Table 1.

At the end of the experiment, the rats were anesthetized by intraperitoneally injections of xylazine (10 mg/kg) and ketamine (90 mg/kg). The blood (tube without anticoagulant and tube with EDTA) was taken from the V. Cava Caudalis of anesthetized animals. After blood was taken from the animals, the animals were sacrificed, and tissues were taken. Serum samples were removed from blood samples and stored at -80 °C until analysis.

Propolis samples were collected after honey harvest in beekeeping areas in the Black Sea region of Türkiye. Obtained from herbalist and beekeeping commercial products from sales points, the propolis was stored at -4°C until the samples were studied. Before the study, the samples were removed from the cold and allowed to reach room temperature. 5 g of propolis the solution obtained by adding 100 mL of 70% ethanol and shaken regularly placed in ultrasonic device (Bandelin electronic device, Sonorex D12207, Berlin Germany) at 220 W and 40 kHz for 30 min. The extract was filtered to a volume of 100 ml to obtain the extract for further analysis. After ultrasound extraction, the mixtures were centrifuged for 10 min at 1,600 g. Supernatants of different extracts were placed in an orbital shaker (Stuard, orbital incubator S1500, Bibby Scientific, Staffordshire, UK) 24 h at 40°C. The extract was filtered to a volume of 100 ml to obtain the extract for further analysis.

The hematological analyses were performed using an automatic blood count device (Mindray BC 5000, China) within three hours of blood collection.

TAS and TOS, which are oxidative stress parameters, were determined in the colon samples collected from the all the study groups. Total antioxidant activity was calculated based on the free radical scavenging effects of *1,1-diphenyl-2-picrylhydrazyl* (DPPH) in propolis using the indirect method of the prepared extracts. Using ascorbic acid as a reference, the measurement was carried out in a spectrophotometer at 520 nm. (29, 41, 50).

Antioxidant activity was expressed as the percentage of inhibition of free radical DPPH and calculated using the formula specified above.

B-cell lymphoma 2 (Bcl-2) protein, b-cell lymphoma-extra-large (Bcl-xl) protein, caspase 3, 8, 9 (Bioassay Technology Laboratory, Bcl-2 Cat No: AP00861, Bcl-xL Cat No: E3340Ra, caspase-3 Cat No: E1648Ra, caspase-8 Cat No: E1370Ra, caspase-9 Cat No: E1898Ra, Zhenjiang, China) levels were measured spectrophotometrically (Sunrise RS-232, Tecan, Grödig, Austria).

In the macroscopic and histopathological examinations the rats were humanely euthanasied according to general protocol for rats, necropsies were performed. Possible cancer developing areas in colon and intestines, and pathological changes in the livers and kidneys as well as other tissues and organs were evaluated according to general macroscopic definition criteria. Samples were fixed in a 10%-neutral buffered formalin (NBF, Merck, Germany) solution for 48 hours. Then, for histopathological examination, the tissues were followed-up at alcohol series and xylene in an automatic vacuum tissue processor (TP1020, Leica, Germany) and embedded in paraffin in a paraffin dispenser (Leica, EG1150H, Germany). Next, 5-µm-thick sections were taken from the paraffin blocks (Shandon AS320, Germany). All tissue sections were stained according to the standard hematoxylin–eosin (H&E) staining method. Sections were covered with the cover slip using the mounting medium Entellan® (Merck). Tissue sections were evaluated semiquantitatively by counting 10 high power fields (HPFs) under a digital optical light microscope (Olympus BX51, Germany) at 400× magnification. Differences in the numbers of the groups were evaluated statistically. Eligible areas chosen under microscopy were photographed (Olympus DP5 camera attachment, Germany). Additionally, colon epithelial and glandular cells were scored in terms of anaplastic criteria (including chromatin density, nuclei/cytoplasm proportion and mitotic activity) and other preneoplastic changes (such as dysplasia, metaplasia, hyperplasia etc.) according to Quickscore (QS) method modified by Detre et al. 1995 (11).

In EPGF and KRAS expressions in colon tissue avidin-biotin complex peroxidase (ABC-P) method were preferred according to the manual instruction of the immunoperoxidase detection kit (Novocastra- RE7110-K, Leica, Germany). EPGF and KRAS primary antibodies were utilized for detection of precancerous and cancerous proliferation in colon. For this purpose, tissue sections were taken 5-micron thick on positive charged slides. The slides were deparaffinized in xylenes and dehydrated by passing through ethanol series. Then, they were placed in a citrate buffer (pH=6.0, 10x, Bioptica, Italy) solution and kept in a microwave oven at 800-Watt power for 25 minutes to reveal the antigen in the tissue. To eliminate endogenous peroxidase activity, the slides were kept in 3% hydrogen peroxide-methanol mixture at room temperature for five minutes, then taken into a humid chamber, where one drop of normal blocking serum was added. They were kept in the oven at 37°C for 25 minutes. In the following stage, primary serums containing commercially available antibodies were dropped onto the sections and incubated in an oven at 37°C for 60 minutes. Then, appropriate secondary antibodies labeled with biotinylated horse radish peroxidase (HRP) were used and incubated in the oven at the specified temperature and time. For the reaction to become visible, *3,3'-Diaminobenzidine* (DAB) chromogen was dropped onto the sections and given five minutes to set in. Finally, Gill's hematoxylin was used for ground staining. The slides were passed through degraded ethanol series and xylene and covered with a coverslip using Entellan (Merck, Germany). The findings were evaluated under a digital light microscope (Olympus BX51, Germany) as in other histopathological examinations, and the results were scored according to Quickscore (QS) method modified by Detre et al. 1995 (11).

Statistical Analysis: In the study, the normality test was used to check whether the groups were distributed parametrically or not. The results were expressed as mean ± standard error (M±SE). A P value of <0.05 was criteria was considered statistically significant for all analyses. Data showing parametric distribution (TAS, TOS, Caspase3, caspase9, bax, Bcl-2, caspase8, neutrophil, eosinophil, monocyte, PCT and lower values) One-Way ANOVA, groups Duncan test (post hoc) was performed to check the significance of the difference between the biochemical data were analyzed using the SPSS statistical software program (PASW Statistics for Windows, ver. 18.0. Chicago, USA). Caspase-8, creatine, and lymphocyte values showed nonparametric distribution; Kruskal-Wallis test followed by Mann-Whitney *U* (post-hoc, Bonferroni correction; P<0.005) test was performed to check the significance of the difference between groups. One-Way ANOVA was applied for histopathological changes, with Tukey (post-hoc) to check significance between groups.

Results

The hematological parameters table for the study groups is given in Table 2. In the hemogram profile, WBC levels in the AOM group were significantly higher than AOM+5-FU and AOM+Propolis+5-FU groups (P<0.0001). Lymphocyte levels decreased significantly in the AOM+5FU and the AOM+5FU+propolis groups compared to the AOM group (P<0.005).

In the oxidative stress parameters, the increase in TAS level and TOS level were found to be significant in the AOM+Propolis+5-FU group compared to the AOM group (P<0.001). The oxidative stress parameters (TAS and TOS) of colon tissue in the study groups are given in Table 2.

Table 2. Hemogram profile, TAS and TOS levels, Apoptosis status (M±SE) in all experimental groups.

Groups	AOM	Control	AOM+5FU	AOM+Propolis	AOM+Propolis+5-FU	P value
Hemogram profile						
WBC	8.32±0.30^{ab}	7.42±0.67^{bc}	5.66±0.42^{d}	8.97±0.54^{a}	6.56±0.15^{cd}	**P<0.0001**
Neutrophil	1.80±0.12^{ab}	1.37±0.18^{b}	2.56±0.38^{a}	2.31±0.42^{a}	1.39±0.06^{b}	**P<0.05**
Lymphocyte	6.00±0.32^{a}	5.52±0.47^{ad}	2.42±0.14^{c}	5.99±0.11^{a}	4.75±0.11^{bd}	**P<0.005**
Monocyte	0.37±0.03	0.34±0.05	0.36±0.02	0.35±0.07	0.27±0.04	P>0.05
Eosinophil	0.11±0.01	0.14±0.04	0.13±0.02	0.16±0.02	0.13±0.02	P>0.05
PCT	2.58±0.13	2.17±0.24	2.00±0.36	2.62±0.15	2.51±0.25	P>0.05
TAS and TOS levels						
TAS	1.60±0.03^{b}	1.52±0.05^{b}	1.59±0.02^{b}	1.55±0.03^{b}	1.86±0.06^{a}	**P<0.0001**
TOS	15,15±0.23^{b}	14.78±0.44^{b}	15.31±0.19^{b}	15.46±0.28^{b}	17.81±0.55^{a}	**P<0.0001**
Apoptosis status						
Caspase3	101.11±0.58^{d}	84.66±0.58^{c}	121.72±1.12^{b}	96.94±0.66^{d}	136.90±3.00^{a}	**P<0.0001**
BCL2 protein	6.35±0.09^{a}	7.83±0.08^{c}	5.99±0.09^{d}	6.64±0.10^{e}	5.32±0.11^{b}	**P<0.0001**
Caspase9	5.35±0.25	5.30±0.07	5.03±0.07	5.44±0.19	5.54±0.03	P>0.05
Bax protein	6.72±0.03^{d}	6.45±0.05^{c}	7.36±0.02^{a}	7.37±0.11^{a}	7.67±0.02^{b}	**P<0.0001**
Caspase8	138.45±0.79^{bd}	129.68±0.64^{bc}	133.97±2.12^{b}	134.33±0.52^{bcd}	157.22±2.58^{a}	**P<0.005**

$^{(a,b,c,d)}$ **P<0.05, P<0.005, P<0.0001** were statistically significant between different captions.

In this study, compared to the AOM group, caspase 3, and bax levels were significantly increased, and bcl-2 levels were significantly lower in the AOM+5FU and AOM+propolis+5-FU groups (P<0.0001). The caspase-8 levels increased significantly in the AOM+propolis+5-FU group compared to the AOM group (P<0.05). Caspase-3, -8, -9 and bcl-2, bax protein levels of colon tissue in the study groups are given in Table 2.

In the AOM group, there was anaplastic proliferation having high mitosis in the epithelial cells lining the colon surface and in the glandular epithelial cells in all cases. Some hyperplastic and mild to moderate degenerative-necrotic changes were detected in the cells. There were moderate inflammatory changes in the propria mucosa. In control group, tissue morphologies were intact and normal epithelial cell proliferation were just seen in almost all cases. In some areas, degeneration and necrosis were also seen. In AOM+5-FU, although anaplasic changes and mitosis ranged from mild to severe, mild to moderate hyperplasia as well as higher inflammatory cell infiltration in the propria mucosa, were observed as seen in the AOM group. Degeneration and necrosis, unlike the previous control group, there were moderate to severe alterations in only two cases and moderate in the remaining cases. In AOM+propolis, the general view was similar to AOM. However, anaplasia was generally moderate to severe and did not have a mitotic index as low as AOM+5-FU. Although the general view of AOM+ propolis+ 5-FU group was similar to AOM+5-FU, anaplasia was mild or mild-moderate and degenerative-necrotic activities were again more mild-moderate. The results of the histopathological examinations of the study groups are presented in Table 3, while the histopathological changes observed in the colon tissues are illustrated in Figure 1.

Liver, kidney, and heart were evaluated in terms of degeneration, necrosis, vascular changes, inflammation, and fibrosis as related to acute and chronic histomorphological changes. In the liver, degeneration, and necrosis were found higher in the AOM group when compared to mainly control and the others. AOM+5-FU and AOM+propolis findings were relatively higher than AOM+ propolis+5-FU and similar to AOM group. Vascular changes were elevated in all groups excepting negative control cases. Other findings were not observed. In the kidney, the general view was similar to liver histopathology obtained from all groups. However, in the heart, degeneration, and necrosis in AOM+5-FU group were elevated when compared to control and AOM+proopolis+5FU groups. These findings of control and AOM+propolis groups were again decreased relatively according to other groups. In heart, vascular changes including hyperemia were found relatively at low level when compared to vascular changes in liver and kidney. The illustrations showing findings were given in Figure 2.

EPGF expressions were more elevated in AOM group when compared to healthy Control group. In treatment groups including AOM+propolis and AOM+ propolis+ 5-FU, expressions were decreased when compared to AOM group. KRAS expressions were more elevated in AOM group. The expressions in other groups were found similar. However, AOM+5-FU and AOM+propolis group expressions were slightly increased when compared to AOM and AOM+ propolis+ 5-FU. No statistical difference was found in the EPGF and KRAS expression results between the study groups. The KRAS and EPGF expression results are given in Graphic 1. The EPGF and KRAS expressions in the colon surface and gland epithelium of the study groups are given in Figure 3 and Figure 4.

Table 3. Histopathological scoring of groups.

Group	AOM	Control	AOM+5FU	AOM+Propolis	AOM+Propolis+5FU	P value
Inflammation	4.17 ± 0.60^{b}	1.50 ± 0.22^{a}	4.50 ± 0.50^{b}	2.83 ± 0.17^{ab}	3.17 ± 0.40^{ab}	P<0.001
Degeneration	2.83 ± 0.31^{a}	0.33 ± 0.33^{c}	4.33 ± 0.21^{b}	2.67 ± 0.58^{a}	2.50 ± 0.22^{a}	P<0.001
Necrosis	1.00 ± 0.00^{a}	0.33 ± 0.33^{c}	3.00 ± 0.26^{b}	2.17 ± 0.48^{ab}	2.17 ± 0.60^{ab}	P<0.001
Hyperplasia	2.00 ± 0.00^{b}	2.00 ± 0.00^{b}	2.83 ± 0.17^{a}	3.00 ± 0.00^{a}	2.83 ± 0.17^{a}	P<0.001
Anaplasia	4.17 ± 0.31^{b}	0.00 ± 0.00^{c}	3.33 ± 0.33^{ab}	3.33 ± 0.49^{ab}	2.50 ± 0.22^{a}	P<0.001
Mitosis	3.17 ± 0.31^{b}	0.00 ± 0.00^{c}	1.00 ± 0.00^{a}	1.00 ± 0.37^{a}	1.00 ± 0.00^{a}	P<0.001

$^{(a,b,c)}$P<0.0001 were statistically significant between different captions.

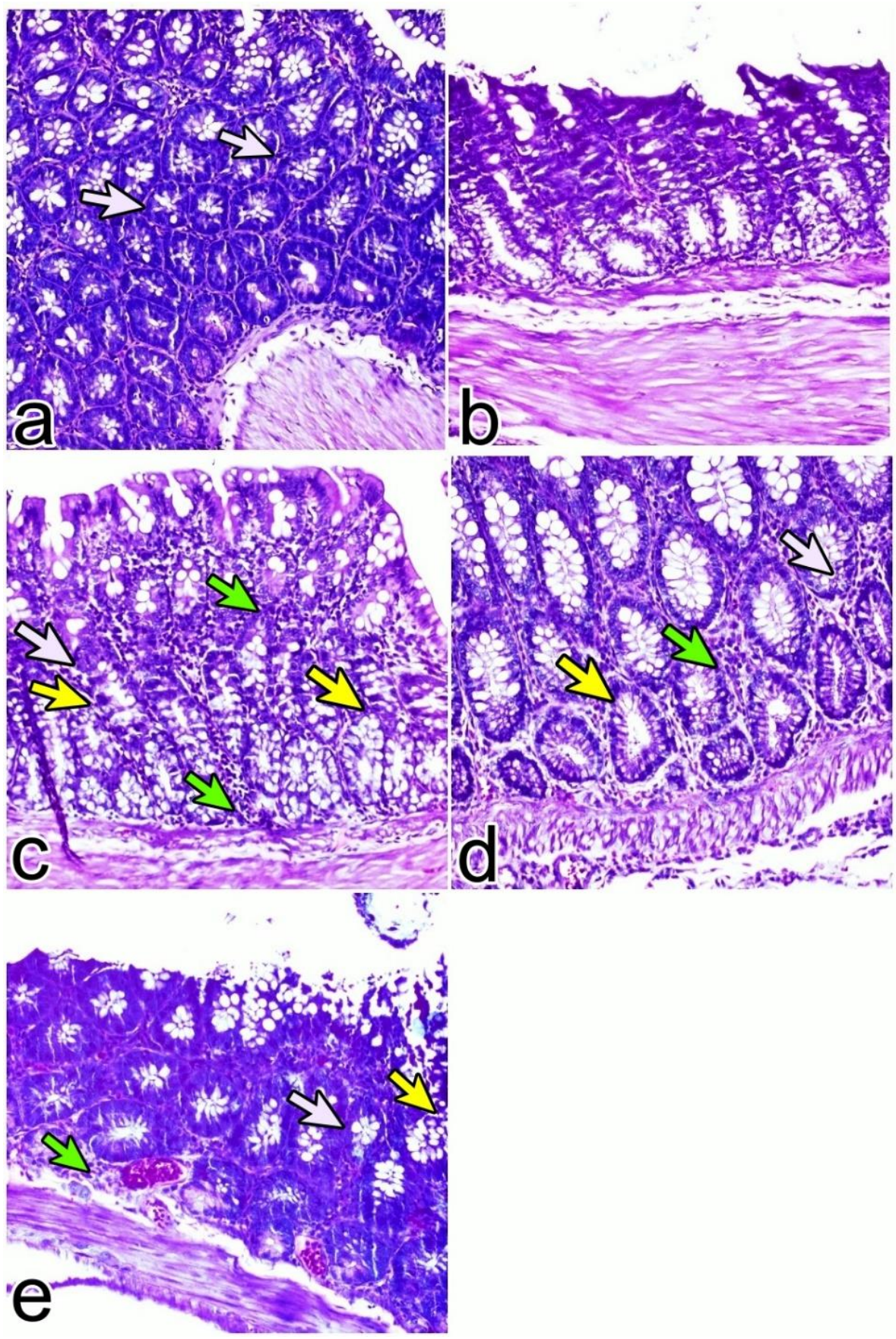

Figure 1. Histopathological findings in the experimental groups.

Anaplasic colon epitheliums (white arrows), degenerated cells (yellow arrows), inflammation (green arrows), group numbers consequtively corresponding to captions: AOM(a), Control(b), AOM+5-FU (c), AOM+propolis (d), AOM+ propolis +5-FU (e), x200, H&E staining).

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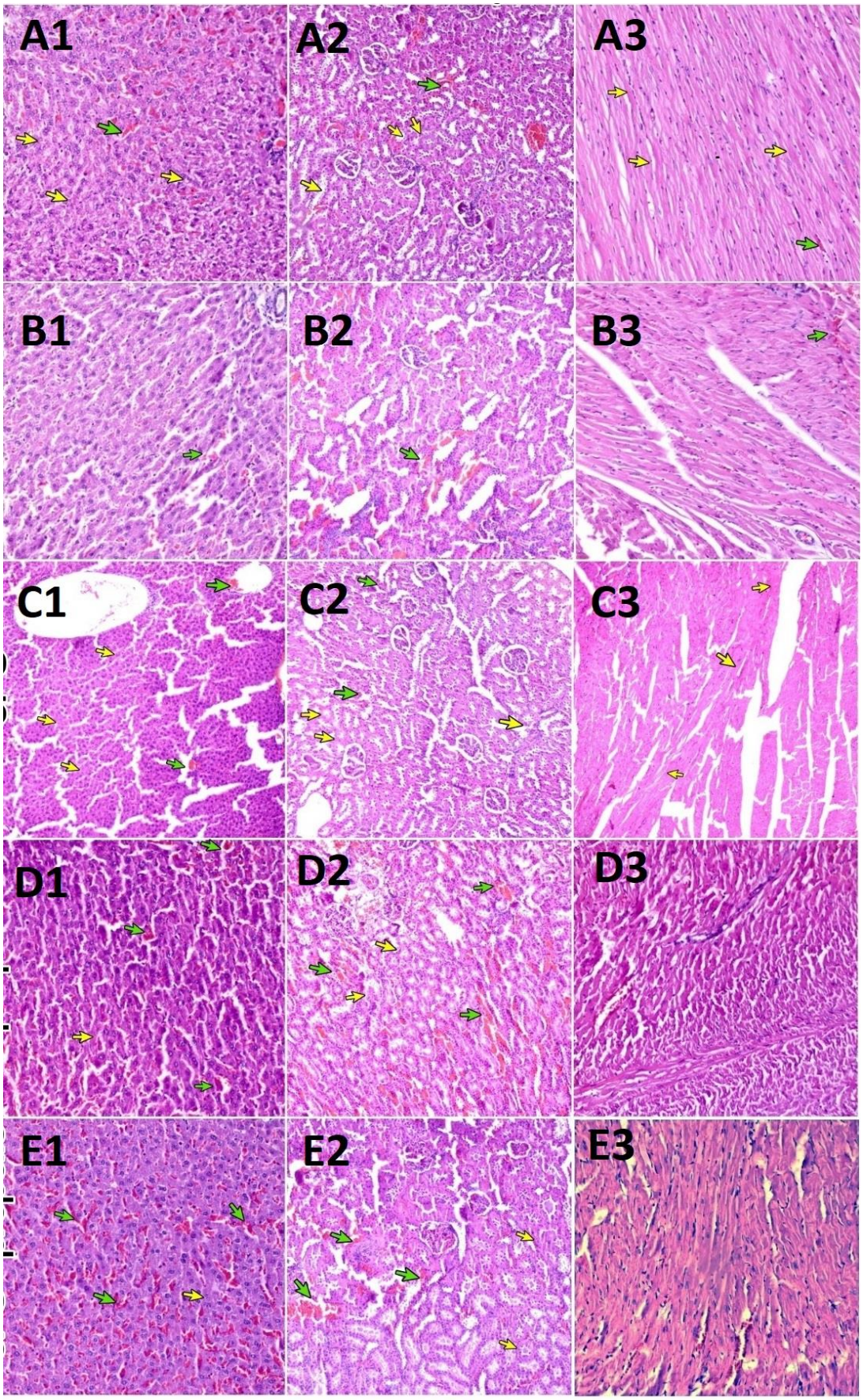

Figure 2. Histopathological findings in other organs including liver, kidney and heart.

Cell swelling and vacuolar degeneration (yellow arrows) and vascular changes including hyperemia (green arrows) in the experimental groups (numbered in vertical axis), x200, H&E staining.

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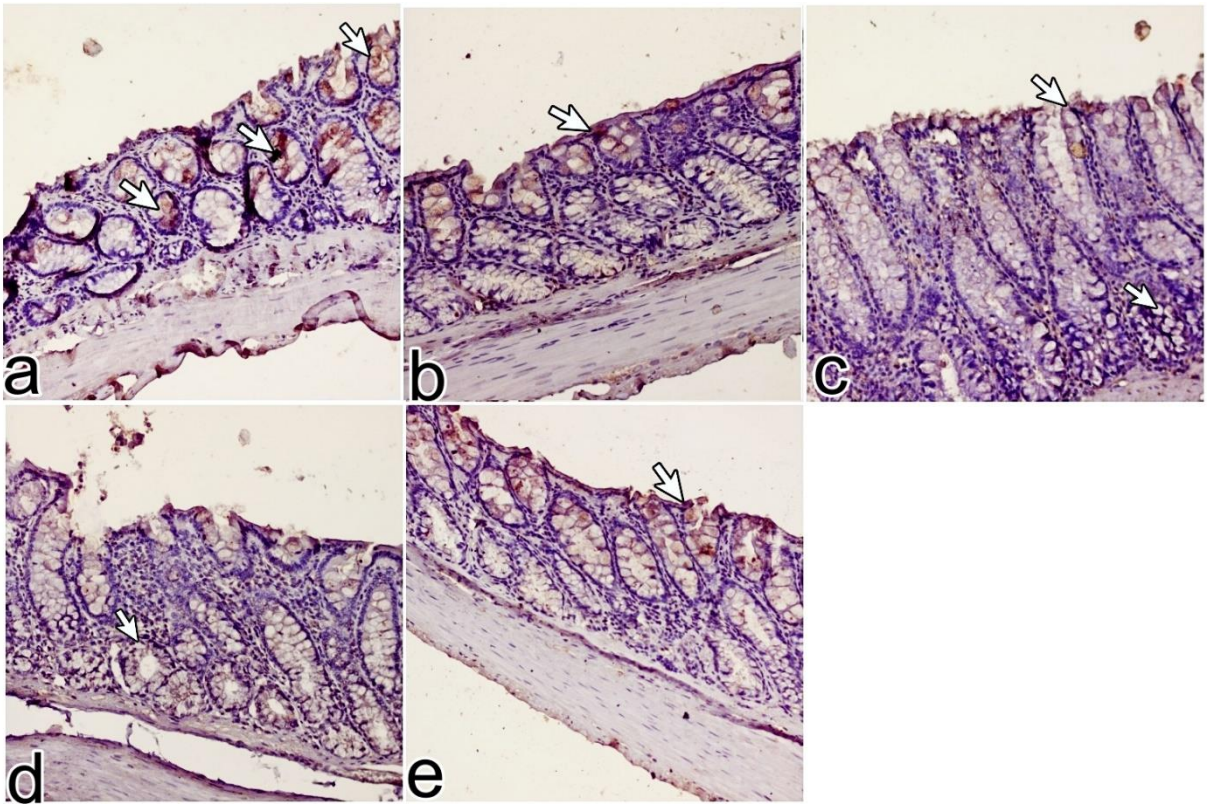

Figure 3. EPGFexpressions in colon surface and gland epitheliums (arrows).

AOM(a), control (b), AOM+5-FU(c), AOM+propolis(d) and AOM+ propolis+ 5-FU groups, ABC-Peroxidase staining, Gill's hematoxyline counterstain and DAB chromogene, x200.

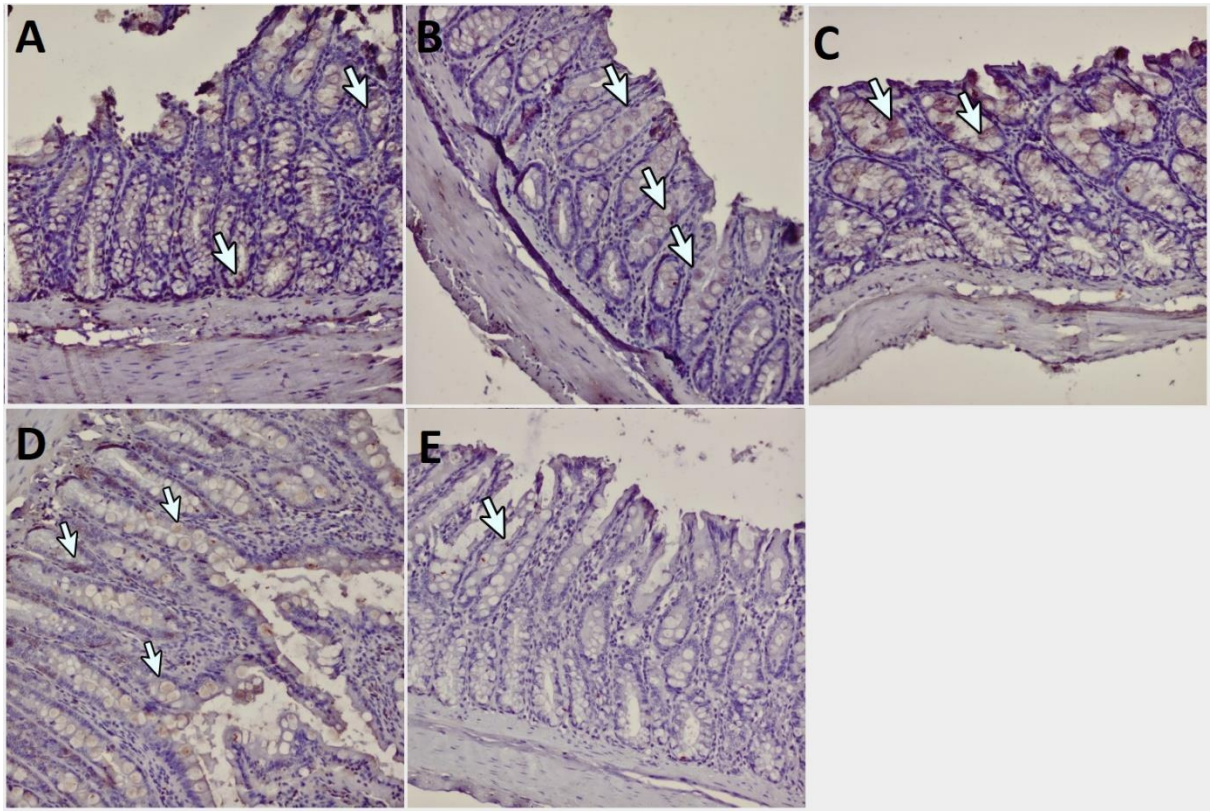

Figure 4. KRAS expressions in colon surface and gland epitheliums (arrows).

AOM(A), control (B), AOM+5-FU(C), AOM+propolis(D) and AOM+ propolis+ 5-FU groups (E), ABC-Peroxidase staining, Gill's hematoxyline counterstain and DAB chromogene, x200.

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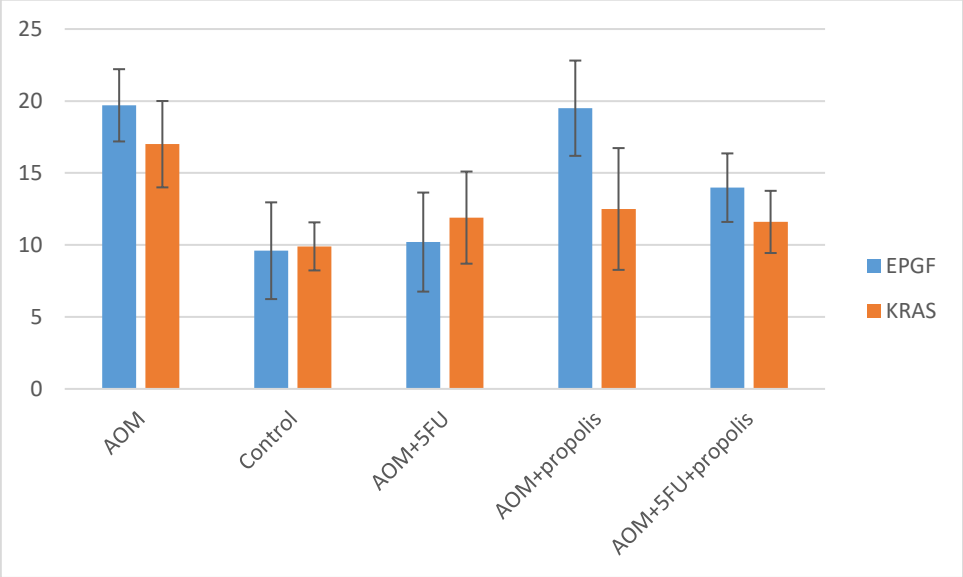
Graph 1. KRAS and EPGFexpressions.



Discussion and Conclusion

The study found that (1) AOM triggers anaplastic changes and mitotic activity in colon surface and gland epitheliums. This is why AOM is considered a useful model chemical for CRC in rats; (2) treatments with both 5-FU and propolis might be effective individually and co-effective in preventing cancer cell proliferation in the colon (3) 5-FU, used both alone and in the combined treatment, led to much more suppression in expressions due to the chemicals increasing TOS levels in the cellular microenvironment. Therefore, cancer cells with low TOS levels and high mutation and receptor activity were exposed to much more apoptosis in the cancer group compared to the treatment groups (5-FU, propolis, propolis+5-FU); (4) The chemicals using treatment of CRC direct the cells to programmed death over apoptosis pathway. 5-FU and propolis can be more successful fighting cancer cell progression in colon.

Colon cancer is one of most common types of malignancies. It is responsible for a high mortality rate around the world. In the pathogenesis of colon cancer, the formation of abnormal crypt foci, mucosal and gland cell clusters constituting adenocarcinoma are evident (24, 26, 42).

Colon and other gastrointestinal cancer types may progress rapidly because of speedy mitosis and epithelial cell cycle. Some genetic and cell signaling pathways as well as growing factors take control the cycle during the development of CRC (19, 23, 51). Due to its higher malignancy and rapid cell cycle, prognosis is unfortunately negative. Metasis can be easily developed in the gastrointestinal system and other organs. For this reason, remedies continue to seek out the best effective chemical or combined therapy.

It is worth noting that 5-fluorouracil (5-FU) has been used frequently in the treatment of colon cancer for the last 50 years. 5-fluorouracil is considered an important therapeutic agent in various stages of colon cancer (6, 53). However, there is evidence of toxic effects on all rapidly proliferating cells as well as normal tissue. Thus, its combined treatment with antioxidants can be potent in fighting cancer cells and relieving side effects. Quercetin, genistein, and geraniol have been frequently studied in recent years as agents with the potential to eliminate the side effects of cancer drugs without reducing their effectiveness (13, 47). Propolis obtained from honey bees (nom. Apis mellifera) is one of these anti-oxidants. It has a phytochemical composition, which includes polyphenols-flavonoids and phenolic acids (33). By powerful antioxidant effects, propolis provides bioactivation and prevention in cancer cells. The presence of Bcl-2 and surviving antiapoptotic protein properties revealed their excessive production in cancer cells (21, 31). In the evaluation of apoptosis, caspase-3 has regulatory properties in the proliferation and survival of tumor cells. It has also been stated that caspase-3 has an important role in recurrence and the effectiveness of radiotherapy and chemotherapy (38). In cancer, apoptotic expressions such as bax, bax-xl proteins and caspase-8 and -9 levels can be changed. Consequently, cancer cells drift preferring mitochondria-dependent apoptosis go to death through the upregulation of bax and caspase-3 and caspase 8 (39). In our study, we thought that propolis and 5-FU would be prone to the cells to death. Accordingly, the levels of mimic and effector caspases (caspase-8 and -3) changed much more than caspase-9 and bax protein. The changing levels for caspase-8 and -3 among cancer and treatment groups vary according to the levels of pro-

apoptotic bax and anti-apoptotic Bcl-2 proteins between the groups. Therefore, the results showed us that imitator to effector caspase signaling cascade gave a more sensitive reaction. The cells undergoing apoptosis used the extrinsic pathway. This did not change between treatment groups including 5-FU and propolis alone.

Some studies have reported direct relationships between oxidative stress and changes in cell structures after AOM application and the severity of carcinogenesis (22, 48). In the colon cancer model created with AOM, a significant increase was determined in the TAS levels in the treatment group compared to the TAS level in the AOM applied group (22). In their colon cancer model using AOM, Thirupurasundri et al. (43) emphasize that treatment applications significantly eliminate the effects of oxidative stress. In addition, they suggest that the treatment protocol they applied prevents the development of malignant morphology of AOM-induced cancer and the emergence of ultrastructural changes by producing apoptosis-like changes. Thus, the applied treatment method induces the antioxidant defense system, as well as inhibiting neoplastic transformation by inducing apoptotic-like changes. As a result, the anti-cancer role of this treatment method becomes apparent. Pallem et al. (32) determined a correlation between the decrease in hydroxyl radicals in the reactive oxygen radicals formed and the frequency of KRAS mutation in their evaluation of neoplastic lesions caused by AOM in rats. Akcakavak and Ozdemir (1), in the colon cancer model they created with AOM in rats, determined that the KRAS mutation detected in the treatment group was lower than the KRAS mutation determined in the AOM applied group. Fichera et al. (16) and Akcakavak and Ozdemir (1) reported that the increase in EGFR signals in rats treated with AOM triggered KRAS. We attest that the decrease in KRAS levels in the group to which we administered propolis and 5-FU in our study is due to the positive effect of propolis and 5-FU on the formation of EGFR signals. Additionally, we found that the TAS and TOS levels in the group to which we applied propolis and 5-FU were significantly higher than the values determined in the AOM applied group. As a result, it was suggested that the application of 5-FU together with propolis may have positive effects on the development of carcinogenesis in the treatment of the colon cancer model we created with AOM in rats.

In some cancer reports over the last decade, propolis extracts have been claimed to be an advantageous therapy option to complement classic chemotherapeutics. Its increasing apoptotic activity in cancer cells was documented as Egyptian propolis as additive therapeutic (13, 38) in several cancer cells, Algerian propolis (48) as additive in lung cancers, Philippine propolis (10) in gastric cancer, Brazilian propolis (12) in CRC, Portuguese propolis in several cancer cells (5), Iranian propolis (3) in gastric cancer, and Omani propolis (46) in CRC. Turan et al. (44) reported that Turkish propolis has potent cytotoxic effects on human cancer cell lines, liver, colon, breast, prostate, and cervical cancers thanks to its higher polyphenolic and flavonoid contents, antioxidant properties, and cytotoxicity. We also found effective it to be in co-treatment against CRC even though propolis obtained from the Black Sea region in Turkiye is not very effective on its own by scoring method. We inferred from results that chemical content in association with polyphenols in propolis can be effective on cancer cells and more effective on cancer cells of different origins. The antioxidants and some biological substance ratios contained in propolis samples produced in different parts of the world and in our country show partial differences according to the regions where they are collected. Medically, these substances contained in propolis are supportive substances that positively affect body health.

Various studies have been conducted in the world and in our country on the local and systemic effects of propolis against experimental or natural tumoral disorders that occur in different parts of the body and their results have been evaluated (44, 52). These studies did not aim to investigate whether propolis has a curative therapeutic effect like an antitumoral chemotherapeutic drug. However, it was aimed to investigate whether propolis can reduce the severity of the pathological lesion in the patient and cause the lesion to regress by increasing body resistance. For this reason, researchers did not find it meaningful to categorize the use of propolis samples produced in different parts of the world for supportive treatment purposes in tumoral disorders in terms of superiority in effectiveness.

In this study, a propolis sample produced in the Black Sea region of our country, which has similar basic content characteristics to propolis samples produced in different countries or regions, was collected. It was investigated whether this substance has an indirect slowing and/or regressive effect on pathological lesions in experimentally induced colorectal cancer, and it was concluded that propolis, a product of the Black Sea region, is a substance that can suppress the severity of local lesions in colorectal cancer cases and support general health status.

In conclusion, anaplastic changes and higher mitotic index in CRC cells are progressed by high receptor activation in epithelial growth factor and genetic mutation. Amongst several treatment choice, 5-FU and propolis might be co-effective more when compared to administration alone against cancer cell fighting. By implementing both 5-FU and propolis treatment, cancer cells can undergo cell death and preserve morphology in cells by higher TAS capacity. Without any chemical induction, TOS capacity could not stop proliferation of CRC cells. In contrast to what has been suggested, we think that apoptotic reactions are more connected to the

extrinsic pathway (caspase-8 and -3) in AOM+propolis+5-FU group. Cellular toxicity by 5-FU can be more effective in extrinsic pathway drifting because high toxic content is accumulated in cell membrane. According to the resultswe believe that propolis can be investigated under co-administration with 5-FU, trying different doses.

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Ethical Statement

This study was carried out after the animal experiment was approved by Kırıkkale University Local Ethics Committee (Decision number: 2022/05/23).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

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

Data Availability Statement

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

Animal Welfare

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

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Evaluation of blink parameters obtained by electrical stimulation in general anaesthetised dogs

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ABSTRACT

The aim of this study was to evaluate the electrophysiological relationship between blink parameters R_1, R_2, R_3 and R_C and general anaesthesia in dogs. The study included 16 dogs that were brought for castration or ovariohysterectomy, did not show any cranial neurological signs, no signs of ocular disease, and had not recently used analgesic or sedative drugs. The end tidal minimal alveolar concentration (ETMAC) value was kept constant at 2.9 in dogs that were anaesthetically maintained with sevoflurane for the surgical procedure. After the procedure, supramaximal electrical stimulation was applied to the supraorbital nerve at each 0.1 ETMAC decrease starting from 2.9 ETMAC and blink parameters (R_1, R_2, R_3, R_C) were recorded and evaluated from both orbicularis oculi muscles. In the evaluation, R_1 parameter was obtained at 1.1-1.4 ETMAC values in all cases, R_2 parameter was most commonly obtained at 1.0-1.1 ETMAC values in 14 cases, R_3 parameter was most commonly obtained at 1.1 ETMAC value in 14 cases, R_C parameter was most commonly obtained at 0.9 ETMAC value in 7 cases. As a result, it was revealed at which ETMAC values the blink parameters were obtained under sevoflurane anaesthesia.

Introduction

The blink reflex is an eyelid closure in response to an exteroceptive-nociceptive stimulus (5). The blink reflex is generally considered a trigemino-facial reflex (9). In clinical practice, the blink reflex is usually elicited by mechanical stimulation of the cornea or eyelashes, electrical stimulation of the supraorbital branch of the trigeminal nerve, or touching the glabellar region (16). Recording the electromyographic (EMG) activity from the orbicularis oculi, however, provides quantitative information about the refex circuit. The most commonly used sensory stimulus for eliciting the blink reflex is a brief electrical stimulation applied to the supraorbital nerve (8). Stimulation of the supraorbital nerve (6) elicits one ipsilateral early response (R_1) and two late responses, one ipsilateral (R_2) and one contralateral (R_C) (1). The R_1 is seen only on the side of stimulation and is a simple pontine reflex. The R_2 and R_C are observed on both sides after a unilateral stimulus, and the responses occur synchronously. The R_2 and R_C are relayed through a more complex pathway that involves the pons and lateral medulla oblongata (3). The R_3 response was not noticed until 20 years after the first electrical stimulation study (16). The neuronal synapses of the R_3 response are less well known, but they are believed to be mediated by a polysynaptic neuronal circuit in the medulla oblongata or rostral portions of the cervical spinal cord (15).

General anesthesia provides a reversible loss of consciousness, immobility, muscle relaxation, and loss of sensation in the whole body with the administration of one or more anesthetic agents (17). It provides controlled elimination of sensation by reversibly depressing the central nervous system. Motor responses and sensitivity of animals under general anesthesia to external harmful stimuli are reduced (18). The concept of the minimum alveolar concentration (MAC), which is defined as the volumetric concentration of an inhaled anesthetic that prevents movement in response to a noxious stimulus in

50% of subjects, remains the most used parameter to guide anesthetic depth during inhalational anesthesia (14). In practice, it is a measure of anesthetic effect that determines the level of inhibition of all painful stimuli and muscle movements (2).

Animal and human studies have shown that an electrically evoked blink reflex is suppressed during sedation and anesthesia (12, 13). Therefore, measuring the blink reflex may reflect the depression of reflex arcs induced by anaesthetics (13).

The aim of this study was to evaluate the electrophysiological relationship between blink parameters R_1, R_2, R_3 and R_C and general anesthesia in dogs and to evaluate the relationship between ETMAC and responses affected by the level of consciousness.

Materials and Methods

Animals: This study was conducted with the approval of Burdur Mehmet Akif Ersoy University Experimental Animals Ethics Committee (Decision no: 401). Sixteen dogs of various breeds, ages, sex and body weights brought to Burdur Mehmet Akif Ersoy University Animal Hospital were included in this study. Cranial nerve examinations were conducted, and the animals brought in were found to have no neurologic clinical problems. They also showed no signs of eye disease and reported no use of analgesics or sedatives. These dogs underwent castration or ovariohysterectomy procedures and were classified as ASA (American Society of Anesthesiologists) status I.

Anesthesia: Latency, amplitude, duration and differential latency data of R_1, R_2, R_3 and Rc parameters were collected while the patient was under general anesthesia. For antibiotherapy, cefazolin (20 mg/kg, IV) was administered intravenously to each anesthetized patient. Propofol was administered intravenously for induction until the jaw tone disappeared. The animal was then orotracheally intubated and connected to an anesthesia device (Dräger, Primus, Lübeck, Germany). After connection, the ETMAC value was adjusted to allow the patient to enter a sufficient depth of anesthesia until the end of surgery, and anesthesia was maintained with sevoflurane. A balanced electrolytic solution infusion at a dose of 5 ml/kg/h was started in all patients. Immediately afterwards, meloxicam (0.2 mg/kg, SC) was administered as an analgesic.

Electromyography: Electromyographic stimulations and recordings were performed with a five-channel EMG System (Medelec Synergy, Oxford Instruments, UK) with a total scan time of 100 ms, a sensitivity of 500 mV, and a sampling rate of 10 kHz. The blink reflex test was performed by stimulating the supraorbital nerve with silver needle electrodes (2-2.5 mm). To stimulate the eye, the cathode was placed along the supraorbita of the frontal bone, 1 cm dorsally to the medial canthus of the eye, where the supraorbital nerve exits the orbital space, and the anode was used as a reference electrode. Needle electrodes were placed on the lateral parts of the right and left ventral eyelids to obtain recordings from the orbicularis oculi muscle. The needle electrode placed on the nose was used as a ground (Figure 1).

Figure 1. Placement of needle electrodes during electromyography recording includes 1) cathode stimulating electrode, 2) ipsilateral recording electrode, 3) contralateral recording electrode, 4) ground electrode.

After the vaporizer was turned off at the end of the operation, supramaximal stimuli in the form of 0.1 millisecond square waves were given for each 0.1 ETMAC value decrease, starting from 2.9 ETMAC, while the patient was under deep anesthesia. Each stimulus was applied three times, depending on the changing ETMAC value, and the stimuli were administered at 15-second intervals to prevent habituation of the response. In each patient, the stimulus was given only from the right supraorbital nerve and recordings were taken from the right and left orbicularis oculi. Recordings were taken until the R_C value was revealed. In addition, the ETMAC values at which the blink parameters appeared were also recorded.

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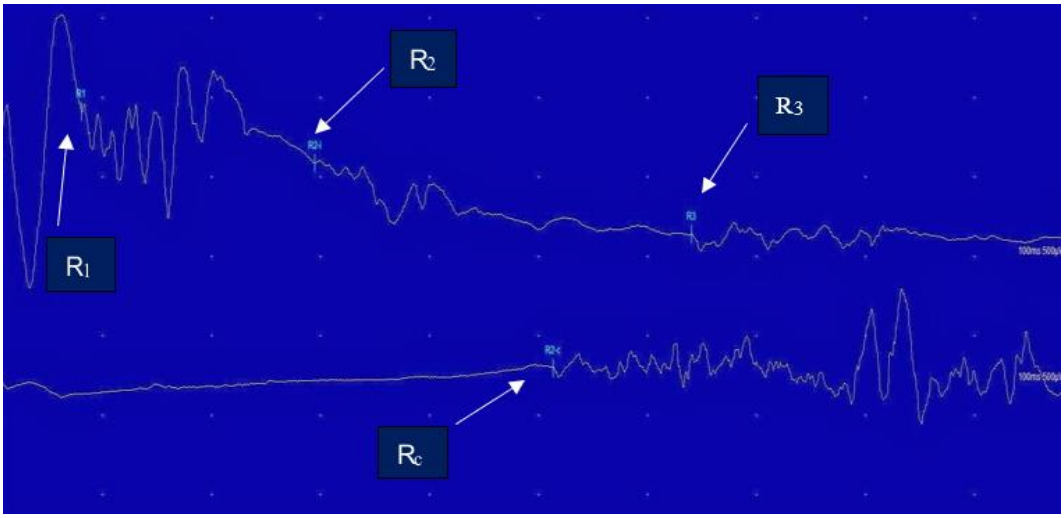

Figure 2. Blink reflex recording of case 9, in which parameters with a MAC value of 0.9 were obtained.

Latency, amplitude, duration and differential latency values of R_1, R_2, R_3, R_C parameters were measured (Figure 2). Latency values were measured from the stimulus artefact to the onset of the reflex components. Amplitude was measured as the distance between parallel lines drawn at the base and the peak of the trace. To measure the duration, the vertical lines drawn at the beginning and end of the tracing were measured. The differential latency value was calculated by taking the difference of the R_2 and R_c values.

Statistical analysis: Descriptive statistics were made on the data, and "Arithmetic Mean + Standart Deviation" for continuous variables and "n, %n" for categorical variables. The correlations between the variables obtained and ETMAC values were analyzed by Spearman's correlation analysis. In all statistical evaluations, values with $p<0.05$ were considered statistically significant. The SPSS 14.01 package programme was used for statistical analysis.

Results

Among cases, all mixed breed, 11 were female (68.80%) and 5 were male (31.30%). The body weights of the cases ranged from 13-22 kg (21.31±1.41), while their ages fell within the 2-3 years range (3.13±0.24).

The latency of the R_1 parameter displayed a polyphasic waveform and demonstrated remarkable stability. In contrast, the latency of the R_2 parameter also exhibited a polyphasic waveform but with greater variability and less stability compared to R_1. The R_C parameter exhibited a polyphasic waveform and appeared after R_2. The R_3 parameter exhibited a polyphasic waveform with a highly variable delay time (Table 1).

The R_1 component exhibited a higher amplitude value compared to the other parameters. The mean amplitude values of the R_2 and R_C components were found to be lower than the mean amplitude value of the R_1 component. The R_3 component had a small amplitude value (Table 2). R_c had the longest duration, R_1 and R_2 times were close to each other (Table 3).

Table 1. Average latency values and differential latency values of parameters R_1, R_2, R_3 and Rc in milliseconds (msec).

Variables (msec)	n	Arithmetic mean	Standard deviation
Latency R_1	303	10.98	0.16
Latency R_2	92	32.68	0.88
Latency Rc	17	48.96	3.12
Latency R_3	71	64.86	0.91
Differential Latency	15	12.56	3.11

n: Number of traces obtained from all cases.

Table 2. Average amplitude values of parameters R_1, R_2, R_3 and R_C in millivolt (mV).

Variables (mV)	n	Arithmetic mean	Standard deviation
Amplitude R_1	303	523.32	24.02
Amplitude R_2	92	197.99	14.87
Amplitude Rc	17	353.79	56.52
Amplitude R_3	71	315.26	22.02

n: Number of traces obtained from all cases.

Table 3. Duration values of parameters R_1, R_2 and Rc in milliseconds (msec).

Variables (msec)	n	Arithmetic mean	Standard deviation
Duration R_1	303	10.40	0.29
Duration R_2	92	10.10	0.52
Duration R_C	17	23.03	4.24

n: Number of traces obtained from all cases.

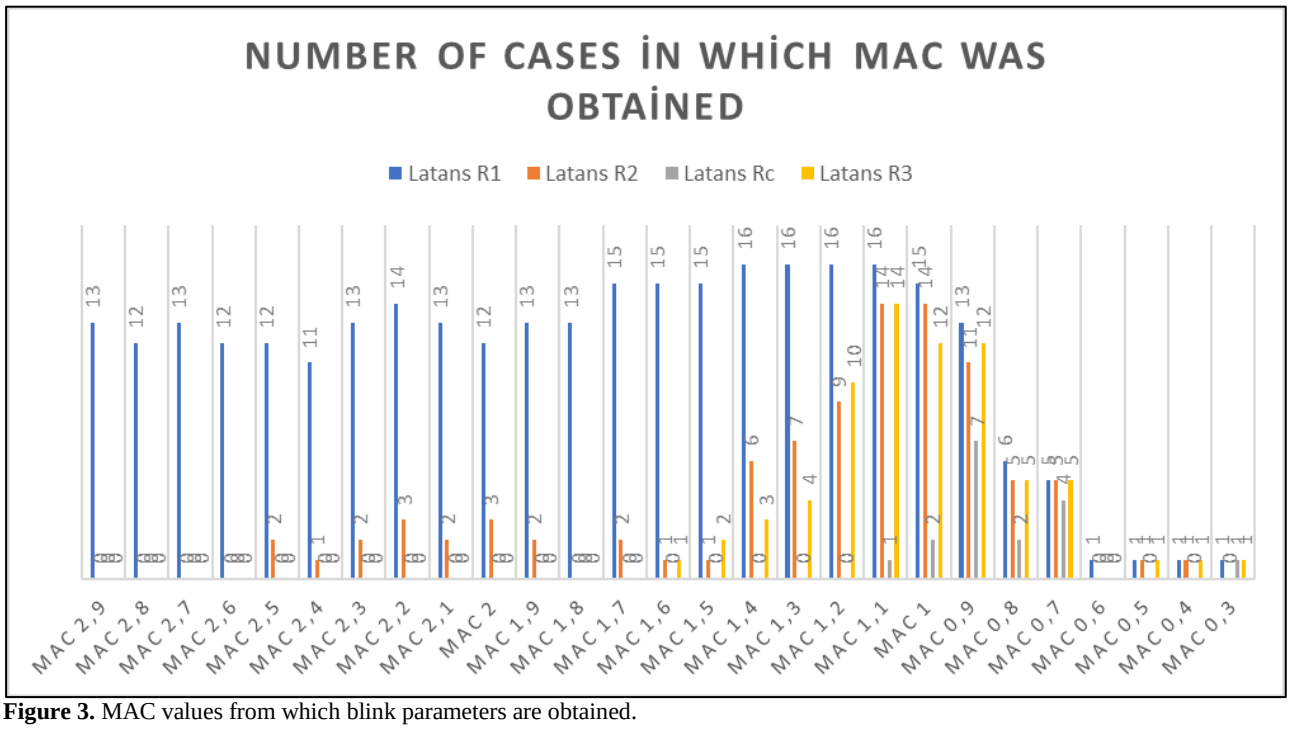

Figure 3. MAC values from which blink parameters are obtained.

The R_1 parameter was obtained in 13 cases at an ETMAC concentration of 2.9, which is accepted as an indicator of deep anesthesia (surgical anesthesia) for dogs, but it was obtained at later ETMAC values in 3 cases. The R_1 parameter was obtained between 1.5 and 1.7 ETMAC in 15 cases (93.80%) and between 1.1 and 1.4 ETMAC in all cases (100%). The R_2 parameter was initially obtained at a ETMAC concentration of 2.5 in only two cases (12.50%). It was most common in 14 cases (87.50%) at 1.0 to 1.1 ETMAC concentrations. The R_3 parameter was initially obtained at a ETMAC concentration of 1.6 in only one case (6.30%). The R_3 parameter was obtained at 1.1 ETMAC concentration in 14 cases (87.50%) at most. The R_C parameter was initially obtained at a ETMAC value of 1.1 in only one case (6.30%). The R_C parameter was obtained in 7 cases (43.80%) at 0.9 ETMAC concentration. The mean ETMAC value for the R_C value was 0.88 (Figure 3).

A significant relationship was found between R_1 latency ($p<0.001$), R_2 latency ($p<0.001$), and ETMAC. No significant relationship was found between R_C latency ($p=0.208$), R_3 latency ($p=0.538$), and ETMAC. A statistically significant relationship was observed between the amplitudes of the R_1 ($p<0.001$), R_2 ($p<0.001$), R_C ($p=0.038$), and R_3 ($p=0.035$) parameters and the ETMAC. A statistically significant relationship was observed between the durations of the R_1 ($p<0.001$) and R_2 ($p<0.001$) parameters and ETMAC. There was a statistically insignificant relationship between the durations of the R_C ($p=0.329$) parameters and ETMAC.

Discussion and Conclusion

R_1 is stable and reproducible, usually in a two- or three-phase form. On the other hand, R_2 has a polyphasic shape; it tends to change and become habitual after repeated stimulation (19). Late reflexes are observed on both sides (ipsialteral R_2 and contralateral R_C) after unilateral stimulation and occur synchronously, with a delay of R_C delay slightly longer than R_2. R_3 has also been found to have a highly variable polyphasic muscle potential but a longer latency than R_2 (3). In present study, the R1, R2, RC and R3 parameters were found to be equivalent to previous human studies and the dog study of Anor et al. (3).

The amplitude of R_1 and R_2 is metrically low due to variability and signal noise (4). Other researchers have reported that amplitude and duration have no value due to the large standard deviation and large variability (19). In the study, the R_1 component had a larger amplitude value than the other parameters. The R_3 component had a small amplitude value. The mean amplitude values of the R_2 and R_C components were found to be lower than the mean amplitude value of the R_1 component. Amplitude values were found to be close to the study of Anor et al. (3) in beagle dogs, but it did not indicate an intersubject value since it had a high standard deviation.

In a study, it was revealed that the most resistant component to propofol was R_1, the first component of the blink reflex, R_2, the second component of the blink reflex, was suppressed more than R_1, and R_3 was deeply suppressed. It was found that the R_3 component was more sensitive than the R_2 component and the most resistant

component was R_1 (13). Late responses (R_2 and R_C) are highly affected by the level of consciousness, and R_C disappears under general anaesthesia due to suppression of the polysynaptic reflex pathway to the contralateral facial motor nucleus (3). In the study, it was observed that blink parameters were suppressed during anesthesia. Anesthetic agents suppress reflex pathways by acting on GABA and glycine receptors, which is thought to be the cause of the suppression of the parameters with the depth of anesthesia. Obtaining the R_1 parameter at a concentration of 2.9 MAC in 13 cases showed that R_1 was the most resistant parameter to anesthesia. The R_2 parameter obtained at a concentration of 2.5 MAC showed that R_2 was less resistant than R_1, but more resistant than R_3, which was initially obtained at a concentration of 1.6 MAC. The least resistant parameter was recorded as the R_C parameter, which occurs when the animal regains consciousness. At the same time, the MAC value at which the R_C value was obtained was 0.88, which can be considered the average MAC value for dogs at which the effect of general anesthesia disappears. The dogs could not tolerate any other stimulus once the R_C value appeared. The R_2 value was most commonly obtained between 1.1 and 1.0 MAC, but the dogs were not responding to any stimulus when R_2 was obtained. But since prior research has shown that consciousness level influences R_2 values, it was assumed that surgical anesthesia might have vanished in the MAC ranges where this value was measured.

Marelli and Hillel (10), stated that no parameters would appear during surgical anaesthesia in patients anesthetized with isoflurane and halothane inhalation anaesthetics. At the same time, Moller and Jannetta (11) stated that blink reflex parameters cannot be revealed during surgical anaesthesia using modern inhalation anesthetics in humans. Once a short train of stimuli was used, it became the standard for eliciting motor evoked potentials (MEPs) under general anesthesia. The discovery that a short train of stimuli can elicit an R_1 component of the blink reflex in a patient under general anesthesia, when a single stimulus cannot in most patients, is very similar to the history of MEPs. The efficacy of a short train of stimuli to overcome the inhibitory action of anesthetics is very likely due to temporal summation and building-up of excitatory postsynaptic potentials in the interneuronal chain involved in the blink reflex in the brainstem (7). In the study obtained, the R_1 value at the end tidal MAC concentration was 2.9, which is accepted as an indicator of deep anaesthesia (surgical anaesthesia) for dogs, in 13 cases, and was obtained later in 3 cases. This suggests that the R_1 parameter may occur during surgical anesthesia in dogs anesthetized with sevoflurane. The values of R_2, R_C, and R_3 could not be obtained at first, but were obtained later. This suggests that blink parameters can be obtained during sevoflurane anesthesia with a short train stimulus.

As a result, the mean latency, amplitude, duration and differential latency values of the blink parameters R_1, R_2, R_3 and R_C obtained under sevoflurane anesthesia were presented in the study. At the same time, the suppressive effect of anesthesia on the blink reflex, at which MAC values it disappears and at which MAC value which blink parameter is obtained were revealed. The study was conducted on mixed breeds and it is thought that species-specific studies can also be conducted.

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Ethical Statement

This study was conducted with the approval of Burdur Mehmet Akif Ersoy University Experimental Animals Ethics Committee (Decision no: 401).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

MNÇ and YSŞ conceived and planned the experiments. MNÇ and YSŞ carried out the experiments. MNÇ and YSŞ contributed to the interpretation of the results. MNÇ 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.

Animal Welfare

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

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Molecular characterization of *Hepatozoon* spp. in naturally infected dogs in Aydın province

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ABSTRACT

Canine hepatozoonosis, a disease caused by the protozoan *Hepatozoon canis* and *Hepatozoon americanum*, represents a significant tick-borne disease affecting domestic and wild carnivores. The objective of this study was to detect *Hepatozoon* species in randomly selected dogs from Aydın by PCR and to elucidate their molecular characterization and phylogenetic differences through sequence analysis. In total, 100 blood samples collected from dogs were analyzed, and the prevalence of *Hepatozoon* DNA was determined to be 3%, with only three samples testing positive. Partial sequences of the 18S rRNA gene exhibited 100% similarity with corresponding *H. canis* isolates. Phylogenetic analysis of the 18S rRNA gene region revealed the formation of two primary clusters, one consisting of *H. canis* isolates and the other comprising different *Hepatozoon* species. While *H. canis* isolates formed distinct subclusters, they were all grouped separately from other *Hepatozoon* species. Furthermore, phylogenetic analysis highlighted the presence of multiple *H. canis* haplotypes in Türkiye, with intraspecific nucleotide differences ranging from 0.0% to 2.9%. The nucleotide differences among the isolates identified in this study ranged from 0.0% to 1.6%. All sequences obtained in this study have been submitted to GenBank and assigned accession numbers PQ669652, PQ671331 and PQ669658. These findings underscore the need for further investigations into *Hepatozoon* infections among cats and wild animals in the region. Additionally, the detection of the parasite in vector ticks could offer valuable insights into the genetic diversity and distribution of circulating *Hepatozoon* species.

Introduction

Canine vector-borne diseases (CVBDs) have become a global phenomenon, driven by a combination of factors such as cross-country canine migration, close human-dog interactions, animal transportation, and the growing accessibility of international travel. Additionally, global warming, which affects all aspects of life, plays a crucial role in this context. It may alter the epidemiology of diseases by influencing the presence and mobility of vectors (23). As a result, tick-borne diseases have emerged as significant veterinary and public health concerns, particularly in regions where environmental conditions favor the breeding and survival of vector tick species. Moreover, tick-borne pathogens, which cause both clinical and subclinical infections in domestic and wild animals, are distributed worldwide and can lead to significant morbidity and mortality (28). Among wild carnivores, foxes also may play an important role in the transmission of tick-borne diseases due to their role in the transmission of some infectious diseases in humans and dogs, their suitability as reservoirs of pathogens and also as hosts of vector ticks (25).

Ticks transmit several significant canine infectious diseases, including babesiosis, hepatozoonosis, anaplasmosis, and ehrlichiosis (26). Furthermore, co-infections transmitted by vectors are commonly observed

in dogs, especially in regions with a high prevalence of vector arthropods. In such endemic areas, certain arthropods can carry more than one pathogen. Consequently, dogs are often exposed to vectors infected with different pathogens, which increases the likelihood of co-infections. Moreover, the abundance of arthropods in a specific area plays a critical role in determining the diversity of vector-borne pathogens affecting dogs. For instance, in regions with high arthropod populations, dogs may be infected with multiple pathogens, such as *Ehrlichia canis*, *Anaplasma platys*, *A. phagocytophilum*, *Hepatozoon canis*, *Leishmania infantum*, *Babesia canis vogeli*, *Dirofilaria repens*, and *D. immitis* (12, 26).

Canine hepatozoonosis, caused by the protozoan *H. canis* and *H. americanum* represent a significant tick-borne disease affecting domestic and wild carnivores. *Hepatozoon* species are transmitted by ingestion of ticks. *H. canis* and *H. americanum*, which cause hepatozoonosis, are transmitted by hard ticks (Ixodidae). *H. americanum*, which is more restricted in distribution than *H. canis* but more pathogenic to its hosts, has only been reported from the United States, whereas *H. canis* has been reported from Europe, Asia, Africa, and even the America (8). Although canine hepatozoonosis is typically regarded as a subclinical infection in dogs, it has the potential to manifest a spectrum of clinical findings, ranging from mild to moderately severe on occasion. In severe cases, symptoms may include elevated body temperature, loss of appetite, fatigue, wasting, elevated globulin levels, significant weight loss, and, in extreme instances, anemia (8, 25). The diagnosis of *Hepatozoon* species often involves a combination of clinical signs, laboratory tests, serological tests, and, in some cases, molecular techniques. However, clinical observations may lack sufficient specificity, as the symptoms can overlap with those of other diseases. While microscopic examination is a straightforward and cost-effective method, it has limitations. These include the difficulty of detecting the agent at a microscopic level and the inability to identify species, particularly in infections with low parasitemia. These challenges underscore the importance of molecular methods (9). PCR and sequence analysis provide the opportunity for phylogenetic characterization of *Hepatozoon* sp. isolates. These molecular techniques play a pivotal role in comprehending the detection, distribution, prevalence, and interrelationships of the species, thereby contributing valuable insights to the broader understanding of these infections. In Türkiye, studies on *Hepatozoon* have utilized a range of methodologies in areas with diverse ecological and geographical characteristics (2-5, 10, 13, 24, 25). Unlike other pathogen detection studies on *Hepatozoon* spp. in dogs and ticks, a previous study showed the presence of *Hepatozoon canis* in foxes in Türkiye. This indicates that both domestic and wild carnivores living in similar habitats are infected and underlines the necessity to examine the disease distribution by considering ecological factors (25). Despite this, the study region lacks information about the phylogenetic characterization of the species detected. Of the three studies conducted in the region, two are case reports, while the third employs both serological and molecular methods (17, 31, 36).

The objective of this study was to detect *Hepatozoon* species in dogs randomly selected from the study area by PCR and to uncover their molecular characterization and phylogenetic differences through sequence analysis.

Materials and Methods

Sample Collection and Microscopic Examination: Blood samples were collected from 100 dogs taken to Aydın Adnan Menderes University Faculty of Veterinary Medicine Animal Hospital clinics for treatment between 2018-2019. Five ml of blood was collected from the *vena cephalica antebrachii* of each dog into tubes coated with ethylenediaminetetraacetic acid (EDTA). For microscopic examination, blood was taken from the dog's ear tips. Prepared smears were dried, fixed in methanol for five minutes, stained with 5% May-Grünwald Giemsa for 30 minutes, and examined under a microscope (21). Additionally, anticoagulated blood samples were stored at -20°C until DNA extraction.

Genomic DNA Extraction and PCR: Genomic DNA was extracted from blood samples using the GeneJET™ Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific Cat. No K0781, USA) following to the manufacturer's instructions. *Hepatozoon* spp. was diagnosed by PCR using the primers Hep-F (5'-ATACATATGAGCAAAATCTCAAC-3') and Hep-R (5'-CTTTATTATTCCATGCTGCAG-3'), which amplify a 666 bp conserved region of the lineage-specific 18S rRNA gene. (16).

DNA Sequencing and Phylogenetic Analysis: To confirm the PCR results, *Hepatozoon* positive PCR products were sequenced using Sanger sequencing by a commercial company. Subsequently, the obtained sequences were edited according to sequence chromatograms and aligned using Geneious 11.0.2 software, to generate the final sequences. In addition, the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST) was used to compare isolates and to make species identifications, and to create a quality data set. Moreover, the isolates were compared with *Hepatozoon* isolates from different geographical regions of Türkiye and the world, as registered in GenBank. To reveal intraspecific and interspecific nucleotide differences among isolates, the Kimura 2-parameter model (18) was performed using the MEGA X bioinformatics program (19). Additionally, the Maximum Likelihood (ML) method was applied within

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the same program to determine phylogenetic relationships (19). At this stage, MEGA X was also employed to identify the most suitable DNA evolution model for sequence analysis, based on Akaike Information Criterion. Thus, the Hasegawa-Kishino-Yano + Gamma distribution (HKY+G) model was determined to construct the phylogenetic tree (15, 19). Finally, *Babesia canis canis* (KF499115) was included as an outgroup.

Results

All dogs included in this study had been regularly treated for ectoparasites, and no ticks were detected during the clinical examinations. Additionally, none of the 100 blood smears tested positive for *Hepatozoon* gametocytes. However, PCR analysis revealed that only three out of 100 samples (3%) were positive for *Hepatozoon*. The positive samples were subsequently confirmed through sequence comparisons. Notably, all three positive dogs had a history of residing in outdoor environments, such as streets, shelters, and gardens.

The 666-bp-long sequences of the 18S rRNA gene region from the isolates identified in this study were successfully obtained. BLASTn analysis confirmed that these isolates belonged to *H. canis*, which were designated as ADU-HC1, ADU-HC2, and ADU-HC3. Additionally, the sequences were submitted to GenBank (NCBI), and accession numbers PQ669652, PQ671331, and PQ669658 were assigned.

The analysis of the dataset revealed that the ADU-HC isolates showed 0–1.6% nucleotide differences among themselves. The ADU-HC1 and ADU-HC2 isolates found in the study showed 100% similarity to isolates from dogs in Ankara (accession number: MG254603-20; KX588232) and the isolate from red fox in Türkiye (accession number: MG077087). ADU-HC2 isolate was also 100% similar to a dog isolate from Kayseri (accession number: KJ513193) and another dog isolate (accession number: JQ867390). In terms of intraspecies variation, the nucleotide differences among *H. canis* isolates from different hosts in Türkiye, included in the dataset, were determined to range between 0.0% and 2.9%.

In the phylogenetic tree of the 18S rRNA gene region, two main clusters were formed, including *H. canis* and other species. Although *H. canis* isolates formed different cluster among themselves, all *H. canis* isolates grouped separately from other *Hepatozoon* species (Figure 1). Specifically, the ADU-HC1 and ADU-HC2 isolates were grouped with *H. canis* isolates previously reported from Ankara and Konya, whereas the ADU-HC3 isolate clustered with isolates from the Aegean region.

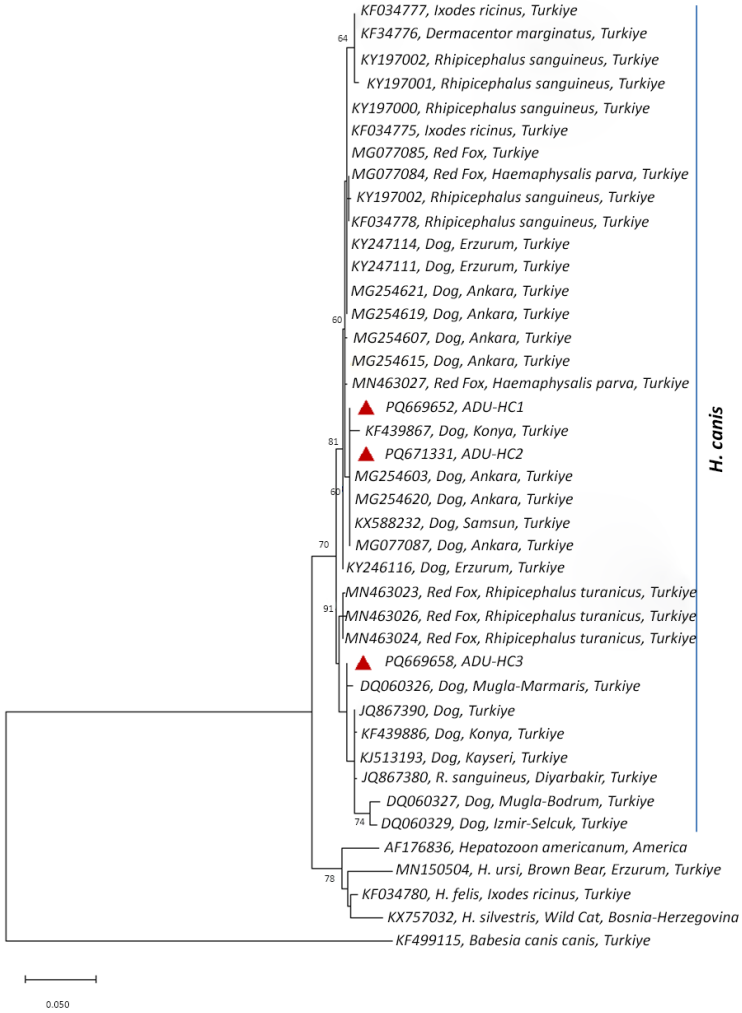

Figure 1. Phylogenetic relationships among *Hepatozoon* isolates. The Maximum-Likelihood (ML) analysis method based on the HKY+G model was used to analyze the 18S rRNA dataset of *Hepatozoon* species. *Babesia canis* (KF499115) was used as an outgroup. Isolates are listed with their GenBank accession numbers, hosts, and locations. Isolates detected in this study are marked with a triangle symbol. Scale bar represents nucleotide substitutions per position.

Discussion and Conclusion

Hepatozoonosis is one of the important tick-borne diseases affecting the health of domestic and wild canids worldwide. It can present in a variety of forms, ranging from subclinical to severe (8). In dogs with suspected *H. canis* infection, the presence of the parasite in Giemsa stains of peripheral blood slides within neutrophils and rarely in monocytes is considered sufficient for diagnosis (8). However, the absence of detectable parasites in animals with low parasitemia does not necessarily indicate the absence of the disease. In such cases, microscopic examination may still be effective when samples are prepared from the buffy coat layer (11, 27). Serological tests, such as indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA), are also employed for disease detection. Molecular methods such as polymerase chain reaction (PCR), real-time PCR and sequence analysis are widely used to detect the disease in dogs (17, 27).

The first study on Hepatozoonosis in Türkiye was conducted by Tüzdil (32) in 1933, followed by Voyvoda et al. (36), who presented a comprehensive clinical description of the disease in 2004. Since then, numerous contributions to the scientific literature have been made using various diagnostic methods in different geographical regions of Türkiye (3, 5, 10 13, 14, 17, 24, 25, 31). Despite the high infection rates found in most previous studies in Türkiye (3, 13, 17, 24), a very low infection rate (3%) was found in the present study, similar to that of studies conducted in Aydın et al. (5) and Bolukbas et al. (10). The fact that one of the highest infection rates detected in dogs in Türkiye to date was found in Aydın by Karagenç et al (17) and that, despite the prominence of the study area in relation to tick-borne diseases (6, 7), one of the lowest infection rates detected in Türkiye in this study was found in Aydın. This may be due to several reasons, such as the successful use of ectoparasiticides in the region over time, the season during which samples were collected, host immune status, vector presence/density (27), small sample size, and the fact that samples were collected from a single site. Moreover, when analyzing discrepancies in infection prevalence, it is essential to consider multiple factors that influence regional differences. These factors include the DNA extraction methodology, the primers used, and the targeted gene region, all of which may exhibit varying sensitivities. Host-related variables, such as age and breed, and, most importantly, tick infestations (34) are also critical contributors. Additionally, the discrepancy between PCR-positive and microscopically negative results observed in three dogs may be explained by the chronic nature of the infection or the inherent limitations of microscopic examination. Although the positivity rate found in the study was low, the possibility that street dogs are exposed to ticks and therefore to tick-borne diseases should not be ignored.

As illustrated in the phylogenetic tree, the *H. canis* isolates identified in red foxes were grouped within the same or analogous clusters as those recovered from dogs and ticks. In a previous study, *H. canis* isolates obtained from dogs, wolves, and jackals were identified as belonging to the same group in the phylogenetic tree (22). Additionally, it was emphasized that wolves and jackals may play a significant role in the dissemination and distribution of this agent (20). The intraspecific nucleotide differences of the *H. canis* isolates obtained from disparate hosts in Türkiye were determined to range from 0.0 to 2.9%. A review of the phylogenetic tree of the 18S rRNA gene region reveals the presence of numerous *H. canis* haplotypes in Türkiye. In addition to *H. canis*, two new species/genotypes (*Hepatozoon* sp. MF) were identified in another study (5) conducted in Türkiye and isolates identical to these new cryptic species were reported in other studies conducted in different province (25). In a previous study conducted in Pakistan, it was revealed that only one species, *H. canis*, is present, but that 15 different genotypes exist. It was also stated that genetic diversity among *H. canis* isolates is quite high (1). Additionally, the isolates identified in this study exhibited similarities with those from different geographical regions and were classified within the same group. It has also been proposed that the genetic diversity of this parasite is likely due to the widespread presence of dogs in diverse geographical regions, countries, and continents, which is facilitated by human migration (22, 33, 35) Finally, it is underscored that this situation may contribute significantly to the dissemination of vectors and vector-borne diseases, highlighting the need for continued vigilance (29, 30).

This study represents the molecular characterization of *Hepatozoon canis* in dogs in the Aydın province of Türkiye and the positivity rate of a relatively small sample group in the study area. Isolates obtained from positive dogs in the study exhibited high similarity with those obtained from different regions. Additionally, although a low positivity rate (3%) was observed in dogs in this study, high positivity rates were reported in previous studies conducted in the study area. This discrepancy highlights the need for further investigation into *Hepatozoon* infections, particularly among cats and wild animals other than dogs in the region. Furthermore, the detection of the parasite in vector ticks within the study area could offer valuable insights into the genetic diversity and distribution of circulating *Hepatozoon* species understanding of the current situation.

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Ethical Statement

Ethical approval for this study was obtained from the Aydın Adnan Menderes University Local Ethics Committee (Decision number 64583101/2018/069).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

NA, MVD, AGO and MP conceived and planned the experiments. MVD, MP, HK, HBA and ES carried out the study, NA, ES, MP, MVD contributed to the interpretation of the results. MP, ES, NA, HBA took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The accession numbers given by NCBI for the isolates identified in this study (https://www.ncbi.nlm.nih.gov/) are PQ669652, PQ671331, and PQ669658.

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

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A clinical case of feline crown restoration with monolithic zirconia

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ABSTRACT

Direct pulp exposure necessitates immediate extraction or root canal treatment in pets. Monolithic zirconia is used as a crown material in dentistry. In the current study, a 4-year-old female Ankara cat had a monolithic zirconia crown restored following root canal therapy. The tooth underwent root canal therapy, and preparation was done for a fixed monolithic zirconia prosthetic full crown restoration. Monolithic zirconia crowns are new, significant restorative materials that have been used rarely and little studied in veterinary dentistry despite having important material properties. Consequently, this is the first reported case of monolithic zirconia crown restoration from Türkiye, and the use of this material is strongly suggested to clinicians.

Direct pulp exposure from a fractured tooth occurs in 10% of pet animals, and it necessitates immediate extraction or root canal treatment (RCT) (1). Patients who have been exposed to dentine because of trauma from falling, being hit by cars, or fighting should have a radiograph taken to check for signs of infection, inflammation, or loss of tooth vitality (3, 9). Bacterial invasion through the dentinal tubules may result in pulpitis. The only appropriate clinical solution is immediate RCT and restoration in one visit (13). Monoblock yttrium oxide stabilized tetragonal zirconia polycrystals are widely used by clinicians in fixed crown restorations due to their biomechanical advantages, such as high mechanical properties, dimensional and chemical stability, fracture resistance, and non-toxic properties (4,5). Monolithic zirconia (MZ) ceramic is the restoration of only zirconia ceramic in one piece (6). Since it does not contain a superstructure, it has high fracture resistance (8). The aim of this study is to present the endodontic treatment of pulp exposed after trauma in pet animals and its restoration with MZ crowns.

A complicated left maxillary canine tooth fracture due to falling on a 4-year-old female cat was diagnosed. An oral and dental examination confirmed the tooth as suffering from a significant crown fracture with pulp exposure under sedation (Figure 1a). Enamel tissue loss necessitated a full crown restoration and RCT. A standard RCT was performed with complete isolation using a rubber dam under general anesthesia. The tooth was ultrasonically scaled prior to the RCT. Handheld endodontic files and rotary devices were used to clean, disinfect, and shape the root canal before sealing the pulp cavity. Sodium hypochlorite and EDTA solutions were used as lubricants and endodontic irrigants to disinfect the canal. Several files were used to remove the exposed pulp through the coronal fracture site (Figure 1b). Before and immediately after the treatment, digital radiographic images of the affected tooth were taken using the bisecting angle technique in order to determine the working length and check the final obturation (Figure 1c). The root canals were dressed after being dried with paper points. A lentulo

spiral filler was used to introduce endodontic sealer. At the 12,5 mm working length, calibrated gutta-percha points were positioned and compressed vertically without heating the compacting tool. The canal was filled consistently. Self-adhesive resin cement was used to lute the glass fiber post. The core was constructed from composite resin. The tooth was prepared following polymerization (Figure 1d). With the putty wash technique, impressions were made. A three-dimensional computer-aided design technology was used to produce the plaster model. The restoration was made from a MZ semi-sintered ceramic block using computer-aided manufacturing. Examining and making the necessary adjustments to the internal adaptation of the crown on the model, occlusal contacts, marginal fit, harmony of the external contours, and approximate surfaces. Afterwards, external B1 color staining and glaze were done. In the patient's mouth, the restoration was evaluated. The crown was cemented with self-adhesive resin cement. The restoration was exposed to the curing light for 20 seconds. Overflowing cement residues were cleaned immediately (Figure 1e). Approximately one month after the procedure, the patient returned to the referring veterinarian for a postoperative check-up. The gingiva in the area was in good health and showed no signs of inflammation. The MZ crown fit perfectly. The patient had resumed regular chewing patterns and was eating normally. About six months after the initial treatment, the patient went back to the referring vet for a conscious oral examination. The tooth and its surrounding structure healed properly. Palpation revealed no signs of pain, and the tooth remained functional. There was no swelling, movement, or fluctuation during the intraoral inspection. The marginal adaptation of crown restoration was well done. On probing, there were no signs of caries. Moreover, the restoration still has its original hue. No gingival recession occurred. The teeth of the antagonist have no wear (Figure 1f).

Canine teeth commonly encounter dentin and enamel breaking off since they are the longest and most front teeth (10). RCT is frequently used on pets to avoid tooth loss (11, 12). In this case, a complicated crown fracture was determined, and a single-session RCT was applied. Prosthodontic crowns have been recorded in client-owned animals during the past 45 years as a technique to support endodontic therapy, replace missing tooth structure, safeguard the tooth from future harm, and finally restore the patient's normal function (12, 13). Despite the fact that full metal crowns are commonly used in pets, there is limited research in the literature using MZ crowns (9, 14).

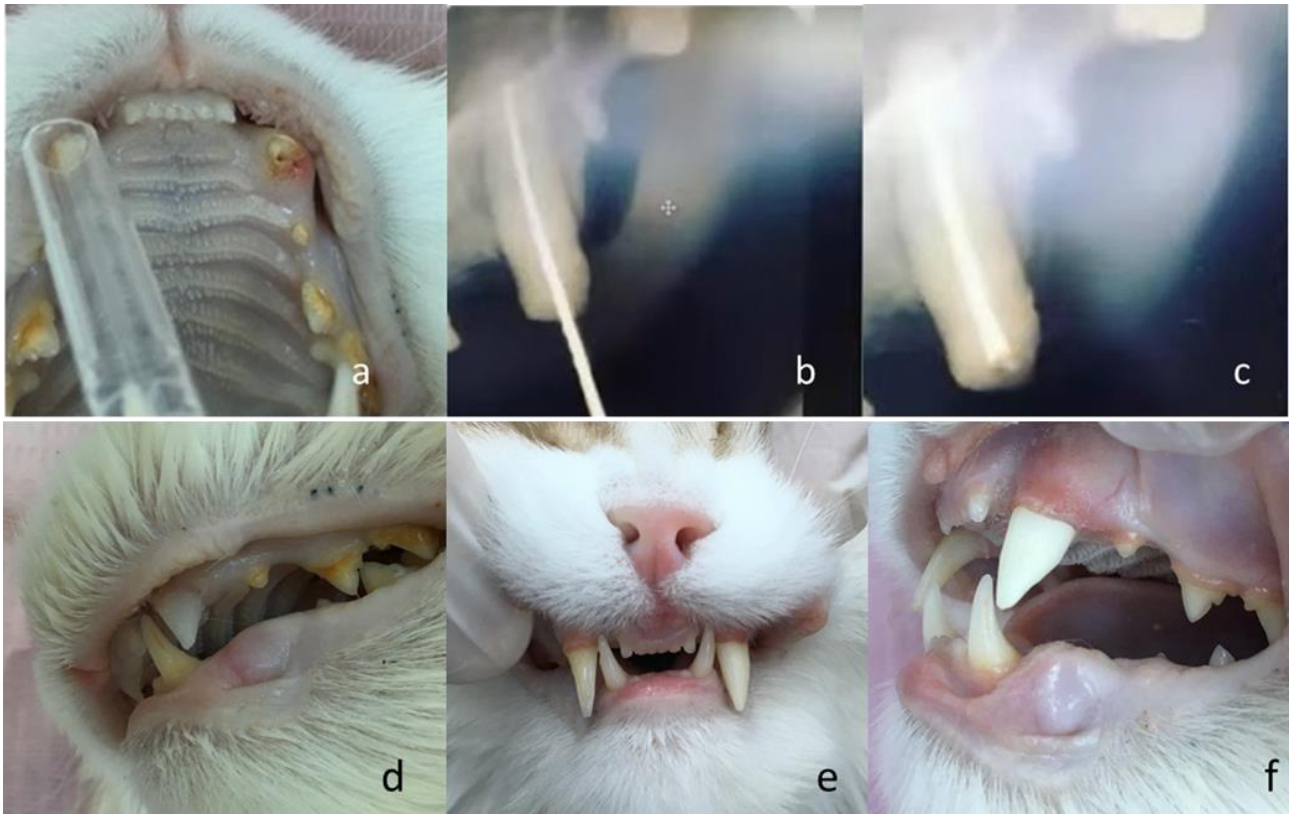

Figure 1. a: Pulp exposure of left maxillary canine tooth b: The initial file for the tooth. c: The final obturation. d: Tooth preparation for monolithic zirconia crown. e: Monolithic zirconia crown. f: Clinical appearance of six months follow-up.

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Coffman et al. (5) mentioned zirconia crowns as an as an alternative to porcelain or ceramic restorations. Ahmed et al. (2) applied two different crowns and found that apoptosis occurred less with zirconia crowns. Similar to this case, Fink et al. (7) reported two cases of tooth fractures restorated with zirconia crowns after RCT to protect teeth from further damage. After being informed of their options for care, the owners chose to proceed with a RCT and subsequent prosthetic repair. The findings of this case study lead us to the conclusion that MZ crown restorations following RCT demonstrated adequate strength and could serve as a replacement for complete metal crown restorations in animal dentistry. Further studies are needed to assess long-term efficacy and treatment success in other animal species.

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Ethical Statement

This clinical case report does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Both SC and ÇC conceived and planned the study. SC and ÇC did clinical and radiographic examintion, patient's follow-up. All authors contributed to the interpretation of the results. SC 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.

Animal Welfare

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

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Treatment of hemihydranencephaly with ventriculoperitoneal shunt in a cat

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ABSTRACT

Hemihydranencephaly is extremely rare in cats. In this case report, the aim is to share data on the diagnosis and treatment of a hemihydranencephaly in a cat. This case report is for an 1 year old, 3 kg, male, Scottish fold cat. The cat was brought in by its owner with behavioural changes and decreased activity since birth. Cranial nerve examination revealed that threat reflex and lateral palpebral reflex were decreased and corneal reflex was delayed. Spinal reflex examination revealed a decreased patellar reflex and proprioceptive deficit in all extremities in the postural reaction test. As a result of the tests, a cranial lesion was suspected. Magnetic resonance imaging revealed that the right cerebral hemisphere and ependyma were absent and instead were filled with fluid. In light of the findings, the patient was diagnosed with a hemihydranencephaly. Furosemide, and prednisone were used for medical treatment. However, despite a week of medical treatment, no improvement was observed. Ventriculoperitoneal shunt placement was preferred as the surgical method. Although all of the patient's neurological findings did not disappear in the postoperative period, they improved. A cat with haemihydraocephaly was successfully treated with ventriculoperitoneal shunt for the first time.

Hydranencephaly is a rare congenital abnormality characterized by absence and replacement of the cerebral hemispheres by a large cerebrospinal fluid pool (3). Hydranencephaly contiguous with the lateral ventricles but not completely lined by ependyma; the cavity does not communicate with the subarachnoid space (2, 6) Hemi-hydranencephaly is a rare brain anomaly characterised by unilateral complete or near complete absence of the cerebral hemisphere (7). There are three case reports associated with hydranencephaly in cats (1, 2, 6). Hemihydranencephaly was also described in a cat (5).

This case report, it is aimed to present the findings related to the diagnosis and treatment of a case of hemihydranencephaly in a cat.

A 1-year-old, 3-kg male Scottish fold cat was brought to Burdur Mehmet Akif Ersoy University Faculty of Veterinary Medicine Animal Hospital with a history of behavioural changes and decreased activity since birth. Cranial nerve examination revealed that menace reflex and lateral palpebral reflex were decreased and corneal reflex was delayed. Spinal reflex examination revealed a decreased patellar reflex and proprioceptive deficit in all extremities in the postural reaction test. The complete blood count and serum chemistry profile were within the normal ranges for all parameters. The serologically evaluated feline immunodeficiency virus, feline leukemia virus tests, toxoplasma and feline infectious peritonitis tests were negative. As a result of the tests, a cranial lesion was suspected. Under general anaesthesia, magnetic resonance images (MRI) of the brain were obtained with the patient in the prone position using a 1.5 T MRI scanner (Magnetom Avanto, Sıemens). Sedation was provided with xylazine (1mg/kg intramuscular (IM)) and induction with ketamine (15mg/kg, IM). T1 (TR: 599, TE: 12) and T2 weighted (TR: 6180, TE: 105), sagittal, and axial images of the brain were obtained on MRI. The original MRI data were transferred to the MicroDicom DICOM Viewer programme for image analysis. On T1-weighted

sequence, it was determined that the right cerebral hemisphere and ependyma were absent, and hypointense fluid was present instead. On T2-weighted sequence, it was determined that the right cerebral hemisphere and ependyma were absent and hyperintense fluid was present instead. Based on these data, the patient was diagnosed with hemihydranencephaly (Figure 1). First, the patient received medical treatment. Furosemide (2 mg/kg IV, q 12h) as diuretic and prednisone (0.5 mg/kg, q 24h) were administered as medical treatment. However, despite treatment, no improvement was observed, so surgery was decided.

Ventriculoperitoneal shunt placement was preferred as the surgical method. Cefazolin (22 mg/kg, intravenous (IV)) was used half an hour before the operation for premedication, and butorphanol (0.1 mg/kg, IV) was used for analgesia. Diazepam (0.2 mg/kg, IV) was used for premedication in anesthesia, then propofol (6 mg/kg, IV) was used for induction and maintenance with sevoflurane. The patient was placed in a sternoabdominal lying position with a neutral angle to the head. The pelvic extremities were oriented laterally, towards the surgeon. The patient's skin area from the level of the medial canthus rostrally to the cranial side of the second cervical vertebra caudally and the entire lateral surface up to the level of the tuber coxae were shaved and prepared according to routine antisepsis rules. An incision was made on the caudodorsal region of the os parietale, then two holes were opened in the os parietale with the help of a dental drill, one for the shunt and the other for the stabilizing stitch. The meninges over the area where the shunt would enter were incised with a size 11 scalpel, and the previously

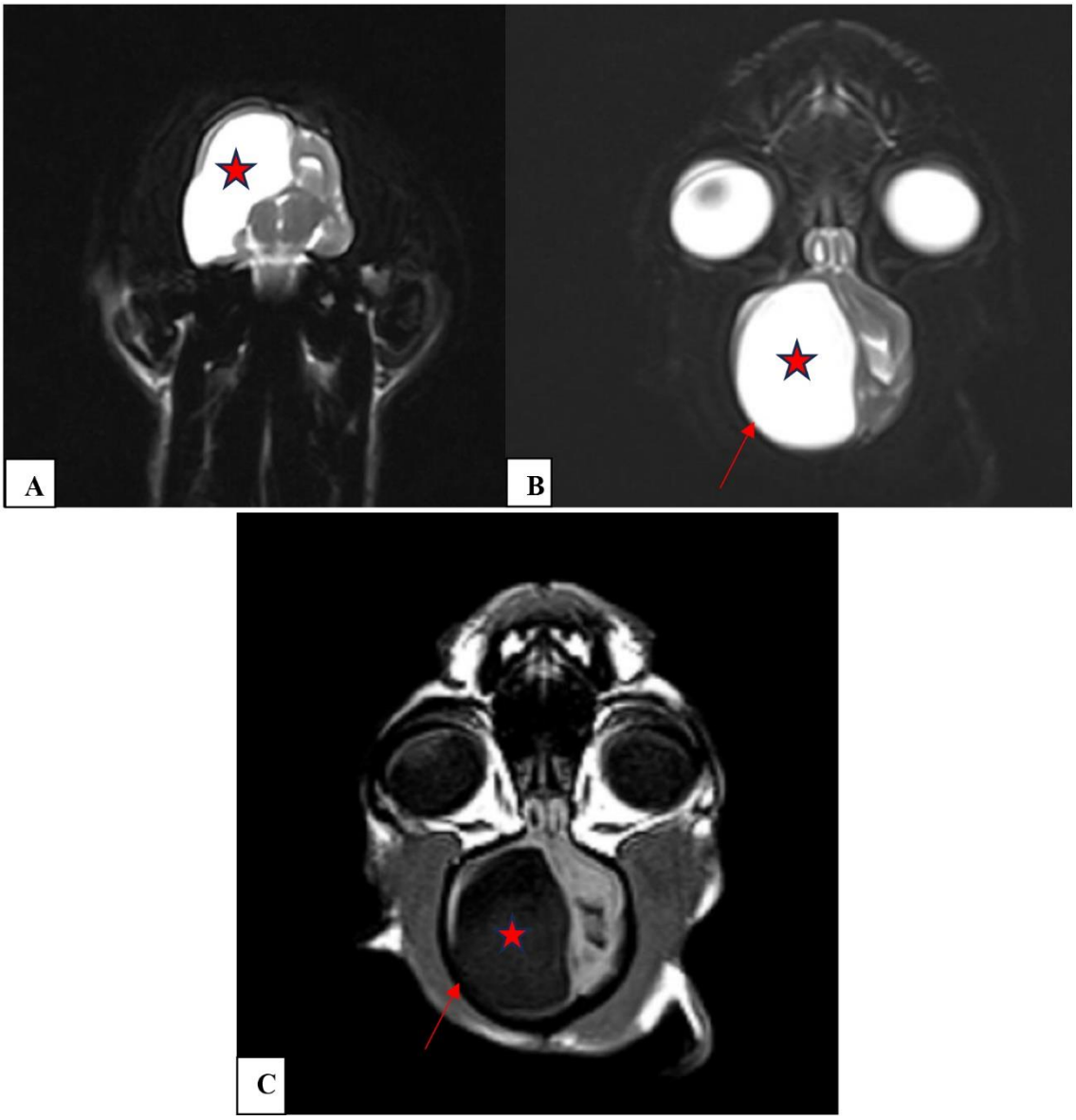

Figure 1. Hyperintense fluid (star), absence of cerebral hemisphere and ependyma (arrow) on coronal and axial T2-weighted sequences (A, B), On axial T1-weighted sequence, hypointense fluid (star), absence of cerebral hemisphere and ependyma (arrow) (C).

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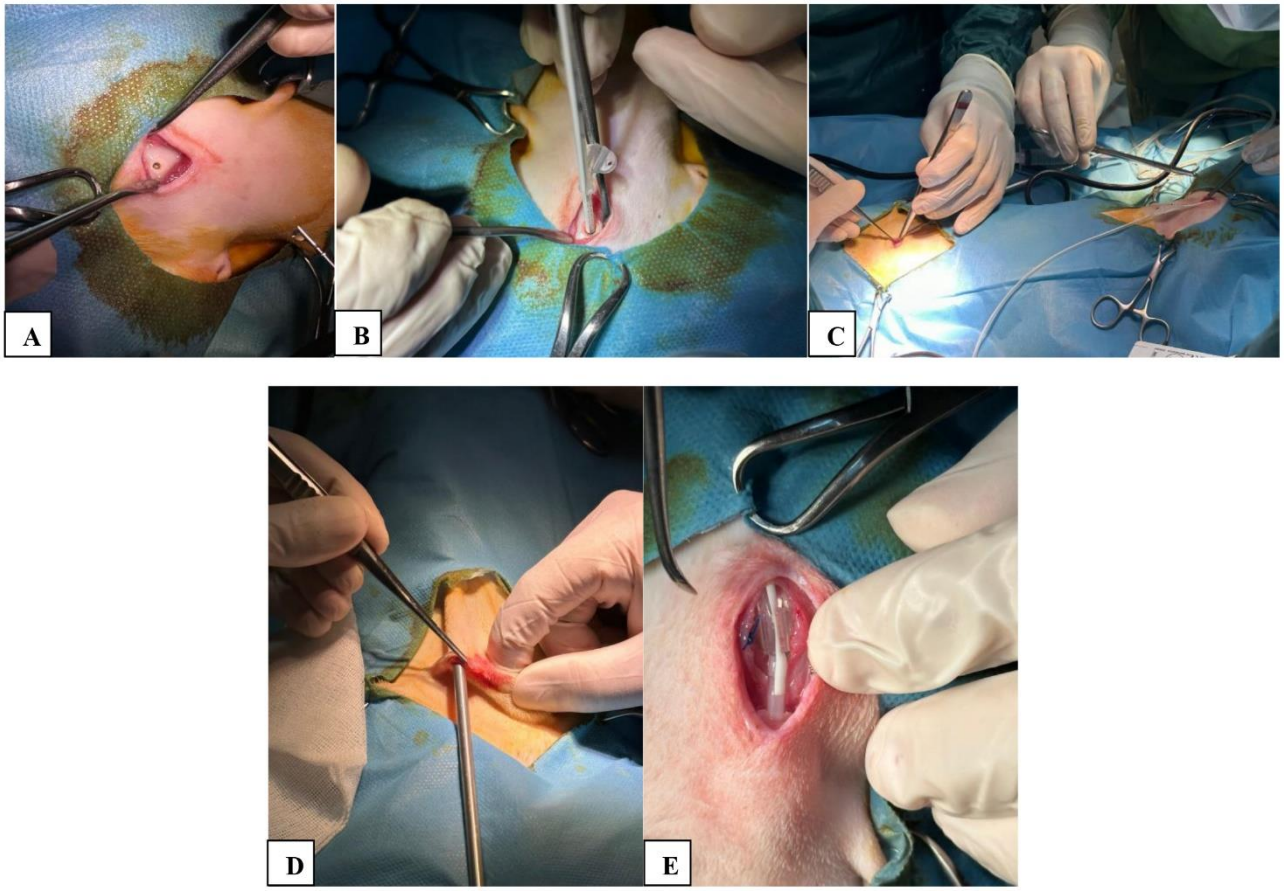

Figure 2. Drilling a hole in the os pariatale with a dental motor (A), placing the shunt in the opened hole (B), incision made at the level of the last rib to place the shunt into the abdomen (C), advancing the shunt subcutaneously towards the last rib with a shunt advancer (D), fixing the shunt (E).

measured shunt was placed into the dorsal side of the lateral ventricle. After the rostral end of the shunt was placed in the lateral ventricle, it was fixed to the fixation hole using 3-0 prolene thread and a grid approach was made to the peritoneal cavity. The distal end of the shunt was tunneled from the cranial incision to the caudal incision using a long Doyen forceps, and the distal end of the shunt tube was placed into the peritoneal cavity. While the muscle layers were closed with separate sutures using appropriate 3-0 PDS thread, the skin layer was closed with separate sutures using 3-0 PDS thread (Figure 2). The patient was awakened from general anesthesia and placed in a softly filled cage. Tramadol hydrochloride (1 mg/kg, subcutan (SC), q 12h) was used postoperatively for analgesia. Postoperative cefazolin (22 mg/kg, IV, q 8h) was used as antibiotic therapy. Postoperative neurological examination revealed that the delay in the menace reflex continued and the corneal reflex, patellar reflex and proprioceptive deficit improved. In the feedback received from the patient owner, it was reported that there was an increase in activity.

The defect is the result of a destructive process that occurs in utero, usually during midgestation, the most common cause being an intrauterine viral infectio. Numerous viruses are known to cause hydranencephaly as well as central nervous system malformations (6). It is thought to occur in humans as a result of blockage of the internal carotid arteries (3). In three case reports, hydranencephaly in cats was associated with parvovirus (1, 2, 6), while in one case, hydranencephaly in a cat could not be associated with any virüs (5). In the case report, hydranencephaly could not be associated with any cause or virus. This is because many viruses can cause this disease.

In a study, it was reported that there was no ependyma in the MRI images obtained and the cerebral hemisphere was filled with fluid (6). In another study, it was reported that cerebral hemispheres were absent (5). In the present study, both the absence of ependyma and the absence of the right cerebral hemisphere were found to be compatible with other studies.

There is no standard treatment for hydranencephaly. Treatment is symptomatic and supportive (4). However, in a human case, hemihydranencephaly was treated by placing a ventriculoperitoneal shunt and good results were obtained (7). The cat with haemihydranencephaly did not

receive any treatment and neurological symptoms persisted (5). In the case report, hemihydranencephaly was treated with the ventriculoperitoneal shunt method and successful results were obtained. Although not all neurological symptoms have disappeared, the patient has improved.

Haemihydranencephaly, which is extremely rare in cats, has been successfully treated in a cat for the first time using the ventriculoperitoneal shunt technique. It has been demonstrated that the ventriculoperitoneal shunt technique can be used in cases of hemihydranencephaly in cats, and successful results can be obtained.

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Ethical Statement

Patient consent form was received from the patient owner.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

MNÇ and YSŞ conceived and planned the experiments. MNÇ and YSŞ carried out the experiments. MNÇ, YSŞ, MYŞ and BN contributed to the interpretation of the results. MNÇ 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.

Animal Welfare

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

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