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EDITORIAL

Dear Readers;

It is with great pleasure and pride that we present to you the fourth and final issue of our journal for 2023. This issue contains a total of fifteen articles, including twelve research articles and three case reports, examining various fields of veterinary medicine. The selection of publications for this issue is based on the chronological order of acceptance dates. As the editorial team, we endeavor to follow this order as much as we can. We need to be fair in presenting your valuable work to the scientific community. I would like to take this opportunity to express my sincere gratitude and appreciation to you for choosing our journal as a platform to share your scientific work.

In the last quarter, JIF values and quartile rankings of SCIE journals were published. In this classification made by Clarivate, our journal has found its place among the Q3 class journals. This can be considered an important and proud achievement for our Journal. On this occasion, I would like to express my gratitude to all our stakeholders, especially the Editorial Board of the Journal. Of course, while we were experiencing this successful period for our Journal, the natural disasters in Morocco and Libya deeply saddened us. We wish God's mercy to those who lost their lives in these disasters and a speedy recovery to the injured. We also hope that such disasters will never happen again.

Dear readers; I would like to extend my sincerest regards to all of you, hoping that the last issue of our journal will contribute to the world of science.

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

Determining the variables affecting the prices of animal products by the network analysis in Türkiye

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ABSTRACT

Recently, Türkiye has seen significant increases in animal products and input prices. These recent increases in prices have made it even more important to examine the prices of animal products and their associated factors. This study aimed to reveal the pattern network structure and characteristics of the prices of animal products and related variables for the 2010-2020 period in Türkiye. For this aim, a network analysis covered the prices of animal products, input prices used in producing these foods, and some economic variables. The study results revealed that Producer Price Index (PPI) and Agricultural PPI are key variables that are highly active in the network and act as a bridge between other unconnected variables. In addition, the results of the analysis suggest that the overall network consists of highly correlated variables and that the PPI and Agricultural PPI are the two most important variables. As a result, the pattern network structure shows that economic variables have a high impact on the prices of animal products. The network structure visualizes Türkiye's import-substitution model in animal production and foreign dependency on feed inputs, and the market structure affected by high exchange rates. In addition, as can be understood from the positive connections between feed inputs, the pattern network of the variables affecting the prices of animal products is largely shaped by feed prices and the internal dynamics of economic variables.

Introduction

Recently, significant increases have been seen in the prices of animal products and related variables in Türkiye. During the 2010-2020 period as analyzed in this study, some basic economic indicators increased significantly (PPI by 236.6%, Agricultural PPI by 195.06%, and Dollar Exchange Rate by 445.58%) (15). These increases have resulted in the following rising feed prices in the Turkish Lira: The price of dairy feed increased by 402.3%, the price of fattening feed by 372.1%, the price of broiler feed by 290.8%, feed prices of egg hens by 312.7% (42). On the other hand, the input (corn, barley, soybean, wheat, sunflower seed meal, and distillers dried grains with solubles-DDGS) prices increased between 306.7%-714.8% (42). The producer price of carcass meat and milk increased by 110.6%-138.7%, respectively (27). Consumer prices of animal products such as milk, beef, broiler meat and egg increased at the following rates 151.5%; 135.1%; 152.5%; 263.4%, respectively (43, 44). Such high increases make it even more important to examine the prices of animal products and their associated factors.

The price formations of animal products show an intricate structure on an international scale. It is known

that many factors have direct or indirect effects on this complex structure. Generally, the complexity of national and international trade structures prevents trade flows from being seen clearly, and as a result, it is often impossible to quickly resolve complex trade links (36). This also applies to the production of basic foodstuffs that are subject to national and international trade, especially animal products. Many inputs are involved in the production of animal products. In a free-market structure, strong and weak relationships can arise between the prices of these inputs and the prices of animal products. Depending on whether these relations are negative or positive, significant changes may occur in these products' supply and demand amounts. Various econometric models are used to examine the production amounts and prices of animal products, which have such a complex production structure. These models include different variables such as milk production quantity (3, 29), raw milk price (31, 34), consumer price of veal (5, 33), beef prices (4, 31), consumer prices index (4), broiler meat price (6), broiler feed prices (6), wheat price (1, 45), corn price (1, 10, 23), soybeans price (1) DDGS price (22). The network analysis method provides a different perspective for these recent studies' interpretation and visual expression. In addition, network analysis, which is widely used in social sciences, has, in recent years, been applied to data in the field of livestock, since it can visualize the relationship between variables (2, 35-37).

It is thought that such studies using network analysis can bring a new perspective to the relevant literature. In network analysis, networks provide a conceptual framework that can demonstrate the relationships among constituent elements (19 variables included in the study) (12). This analysis method allows identifying strong and weak connections in the network, determining the intensity of interaction in the network, revealing the roles of variables in the network, and visualising the animal products market through prices and rates. In other words, graph theory, which provides a rich analytical framework, can be used to examine the interactions between the prices of animal products and input prices (25), and where the connections between the constituents of these products have one direction (i.e., going from one node to another node), the network is represented by a directed graph (8).

Our aim and motivation are not to model or stochastically evaluate changes over time. Our main goal is to show all the path and process dynamics between the variables in a network. This study aimed to reveal the pattern network structure and characteristics of the prices of animal products and related variables for the 2010-2020 period in Türkiye. The present study aimed to apply a network analysis that included a total of 19 variables, consisting of the prices of animal products and inputs used in their production, as well as some economic indicators and ratios.

Materials and Methods

Dataset: The dataset of the study includes the prices of animal products and the variables associated with these foods: (producer and consumer price of milk, prices of dairy feed, corn, barley, soybean, wheat, sunflower seed meal and DDGS, dollar exchange rate, PPI, agricultural PPI, consumer price of beef, price of fattening feed, producer price of carcass meat, broiler feed price, price of feed for egg hens, consumer price of broiler meat, and egg). The dataset includes monthly changes in the prices and rates of variables for the 2010-2020 period. The variables that constitutes the dataset were obtained from the Turkish Statistical Institute, Turkish Feed Industrialists Association, General Directory of Meat and Milk Board, and Central Bank of the Republic of Türkiye (16, 27, 42-44).

Method of Analysis: This study aimed to reveal the pattern network structure and characteristics of prices of animal products and related variables for the 2010-2020 period in Türkiye. Network analysis was applied to 19 variables to define the strong and weak connections in the network, to determine the intensity of interaction in the network, and to reveal the roles of the variables in the network. JASP (Version 0.14) [Computer software] was used for the structural determination and visualization of the relationships between variables in the analysis (28).

Within the scope of the study, the position of 19 variables in the network was determined. To evaluate the connections, major centrality measures such as degree, betweenness, closeness, and influence centrality, as well as network density measures were used. Each of these centrality measures has a different assumption in finding the most efficient node (variable). Therefore, each has a different approach that makes any node effective or central in a network. The variables are positioned using the Fruchterman-Reingold layout algorithm, which organizes the network according to the strength of the connections between nodes (26). The Fruchterman-Reingold layout algorithm uses random numbers. With these criteria, it is tried to determine which variable is important, effective, and most known.

The sparsity measure, one of the most used measures of graph theory, is obtained by subtracting the ratio of all existing connections in the network to the maximum possible connections from one. The sparsity measure of a network takes a value between 0 and 1 (32). Nodes with a high degree of betweenness are referred to as nodes that act as bridges between two or more clusters of nodes that cannot communicate with each other, and they have the potential to control the network (14, 15, 38, 47). The

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degree of closeness shows how close a variable is to all other variables. The degree of closeness is defined as the inverse of farness, that is, the sum of the shortest distances between a node and all other nodes. This value indicates with which variable a variable will have a connection the fastest. It also measures the independence or effectiveness of the node (14, 15). Besides, a central node is quickly affected by changes in any part of the network with a high degree of closeness and can quickly affect changes in other parts of the network (13). In other words, the sphericity coefficient can also be defined as the statistical consistency level that measures the spherical density of the interconnected vertices in the network (24). In network analysis, the key member of the network is determined by degree centrality and betweenness centrality values (40).

Results

In the study, the relationship between the variables was visualized using network analysis (Figure 1). In Figure 1, the thickness of the lines indicates the severity of the relationship between the variables, the blue colors indicate the positive relationship between the variables, and the red colors indicate the negative relationship between the variables.

The graph consists of nodes (vertices) and edges (arcs, connections) connecting the nodes. The results of the analysis show that there are more connections between the variables indicating positive relationships. As can be seen in Figure 1, the total number of connections between the variables is 95. The maximum number of connections for this network is 171. Accordingly, the degree of sparsity is 0.44. This value indicates a low degree of sparsity and shows that there is a high level of density in the network. For a network with 19 variables, this sparsity rate is sufficient.

Based on this finding regarding the general structure of the network, it can be said that there is a relationship between the variables and that the variables interact with each other. In the study, four types of measures were used to determine the centrality levels of the products. They were degree, closeness, influence, and betweenness centrality measures. Table 1 presents the centrality measures in detail.

It can be said that nodes with a high degree of betweenness have a relatively more important position. Accordingly, it can also be said that among the variables, PPI and Agricultural PPI are the key variables that are highly active in the network (Table 1) and act as a bridge between other unconnected variables (Figure 1). As a result, the overall network consists of highly correlated variables, and PPI and Agricultural PPI variables are the two most important variables.

Accordingly, the variables with the highest degree of closeness are Dollar Exchange Rate and PPI, and the variables with the lowest degree of closeness are soybean and consumer price of milk. Since these variables can be reached in the easiest way, reaching these variables is more important than reaching other variables and when these variables are reached, other variables can be reached, too.

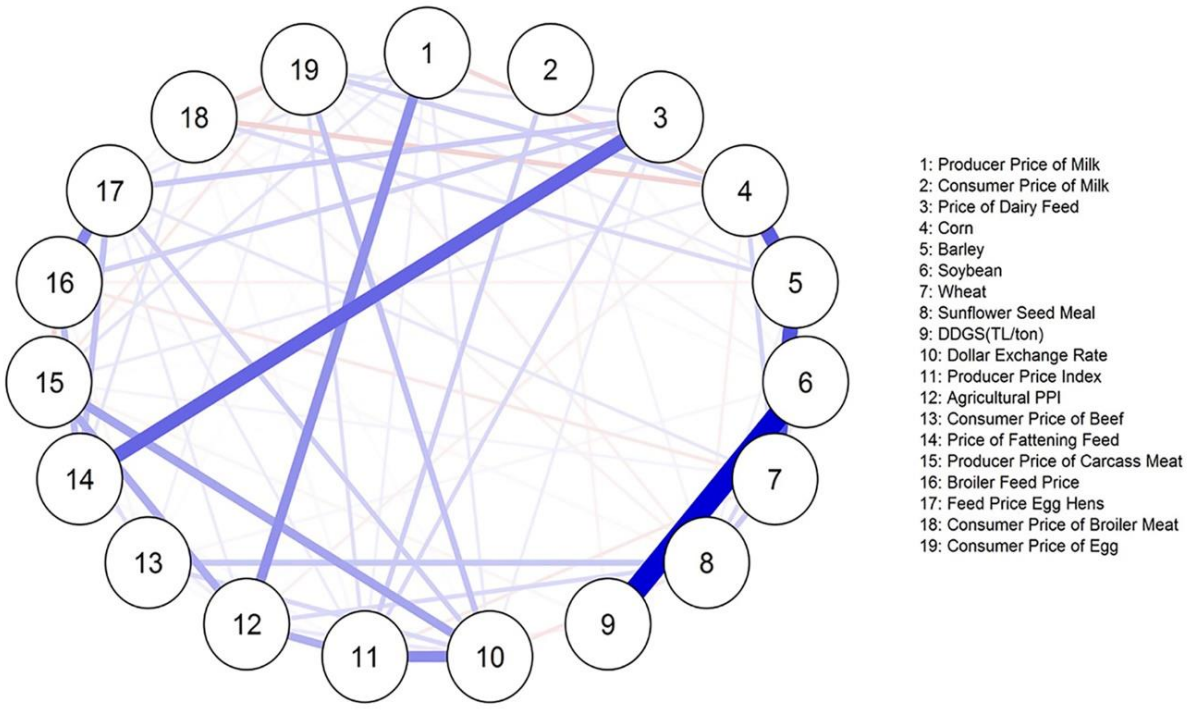

Figure 1. Network Structure Related to Variables.

Table 1. Centrality Values of the Network.

Variable	Betweenness	Closeness	Strength	Expected influence
Producer Price of Milk	-0.55	0.55	-0.75	-0.51
Consumer Price of Milk	-1.2	-1.07	-2.61	-1.6
Price of Dairy Feed	-0.37	-0.48	0.16	0.82
Corn	0.74	0.33	0.79	-0.69
Barley	0.55	0.19	1.24	1.39
Soybean	0.28	-1.82	0.49	0.79
Wheat	0.74	0.26	0.5	0.38
Sunflower Seed Meal	0.92	0.19	-0.38	-0.88
DDGS(TL/ton)	-1.11	-2	-0.14	-0.11
Dollar Exchange Rate	1.47	1.72	1.33	1.23
Producer Price Index	1.75	1.37	0.81	0.75
Agricultural PPI	1.66	1.22	0.7	0.97
Consumer Price of Beef	-1.2	-0.2	-1.55	-0.65
Price of Fattening Feed	-0.74	-0.8	0.16	0.82
Producer Price of Carcass Meat	-0.46	0.91	0.18	-0.38
Broiler Feed Price	-0.92	-0.25	0.17	-0.78
Price of Feed for Egg Hens	-0.37	0.02	0.63	1.17
Consumer Price of Broiler Meat	-1.2	-0.72	-1.35	-1.91
Consumer Price of Egg	0	0.57	-0.38	-0.81

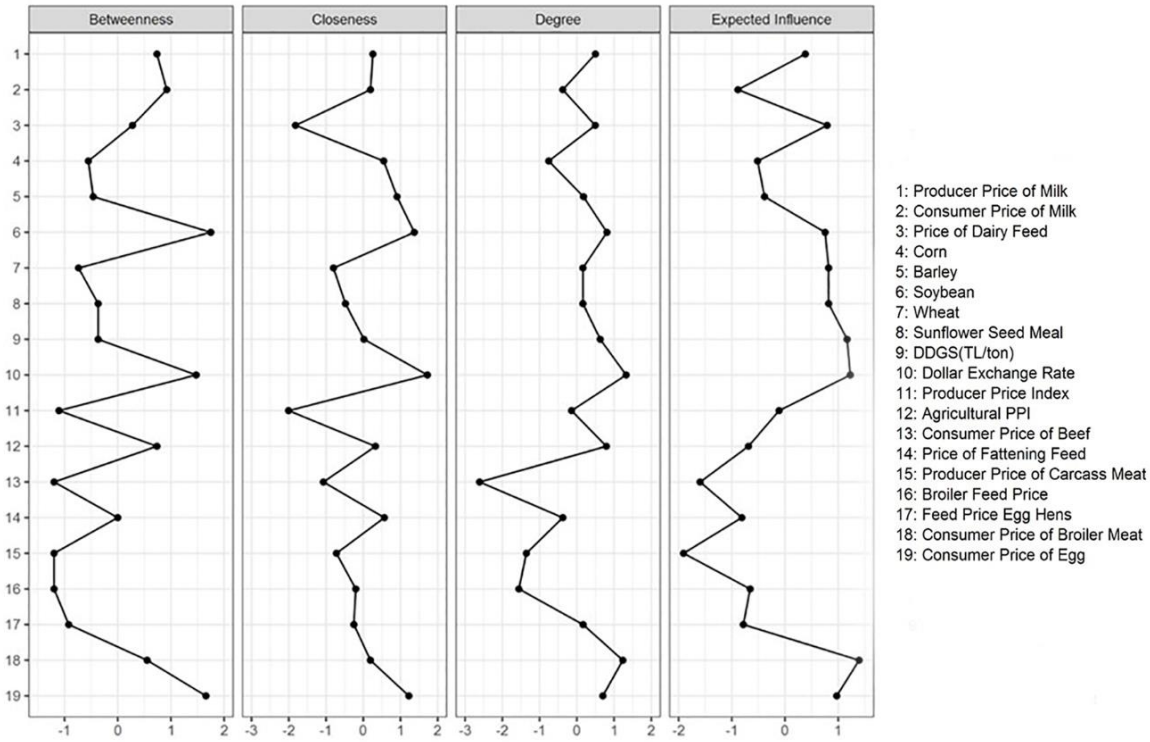

Figure 2. Centrality Graph for the Network.

According to the degree centrality, which calculates centrality over the number of connections, the Dollar Exchange Rate had the highest value with 1.33. On the other hand, the Consumer Price of Milk had the lowest value with -2.61.

As for the influence values, the variables with the highest degree of influence are Barley and Dollar Exchange Rate, respectively. They are followed by the Price of Feed for Egg Hens. In other words, Barley and Dollar Exchange Rate variables affect other variables in the pattern network. The graph made for these findings is given in Figure 2.

The clustering coefficient measures local cohesiveness and is defined as the fraction of connected

neighbours for any vertex. These coefficients reveal how strongly the variables are connected with their neighbours. Clustering coefficients for the variables are given in Table 2.

Figure 3 shows the graphs of clustering coefficients for the variables given in Table 2.

Clustering coefficients, which are used to measure the clustering tendency of the parameters in the network, are used to measure the frequency of the parameters in groups, that is, their tendency to cliques. This coefficient, which gives the frequency of connecting the parameters with which they are connected, also shows the importance of the parameter for the groups. So a high clustering coefficient indicates a high correlation of the variables, and a low one indicates less frequent connections.

Table 2. Clustering Coefficients for the Variables.

Variable	Barrat	Onnela	WS	Zhang
Agricultural PPI	0.58	1.3	0.53	-0.65
Barley	0.54	-0.19	-0.44	0.59
Broiler Feed Price	-1.05	0.35	-1.4	0.84
Consumer Price of Beef	0.75	-0.47	0.44	0.26
Consumer Price of Broiler Meat	0.27	-1.09	-0.39	0.41
Consumer Price of Egg	0.86	0.22	0.83	-0.15
Consumer Price of Milk	-0.33	-1.36	1.99	-0.55
Corn	0.64	0.4	1.14	0.92
DDGS(TL/ton)	-1.69	-1.42	-0.92	-1.71
Dollar Exchange Rate	1.21	1.12	0.51	-0.68
Price of Feed for Egg Hens	-1.16	-0.04	-0.65	0.59
Price of Dairy Feed	1.34	2.65	1.16	1.38
Price of Fattening Feed	0.54	0.9	0.33	1.63
Producer Price Index	-0.89	-0.42	-1.71	-0.9
Producer Price of Carcass Meat	-0.66	-0.27	-0.76	-0.69
Producer Price of Milk	1.23	0.06	0.83	0.56
Soybean	-1.73	-0.98	0.44	-1.81
Sunflower Seed Meal	-0.83	-0.65	-1.18	-1
Wheat	0.39	-0.12	-0.72	0.96

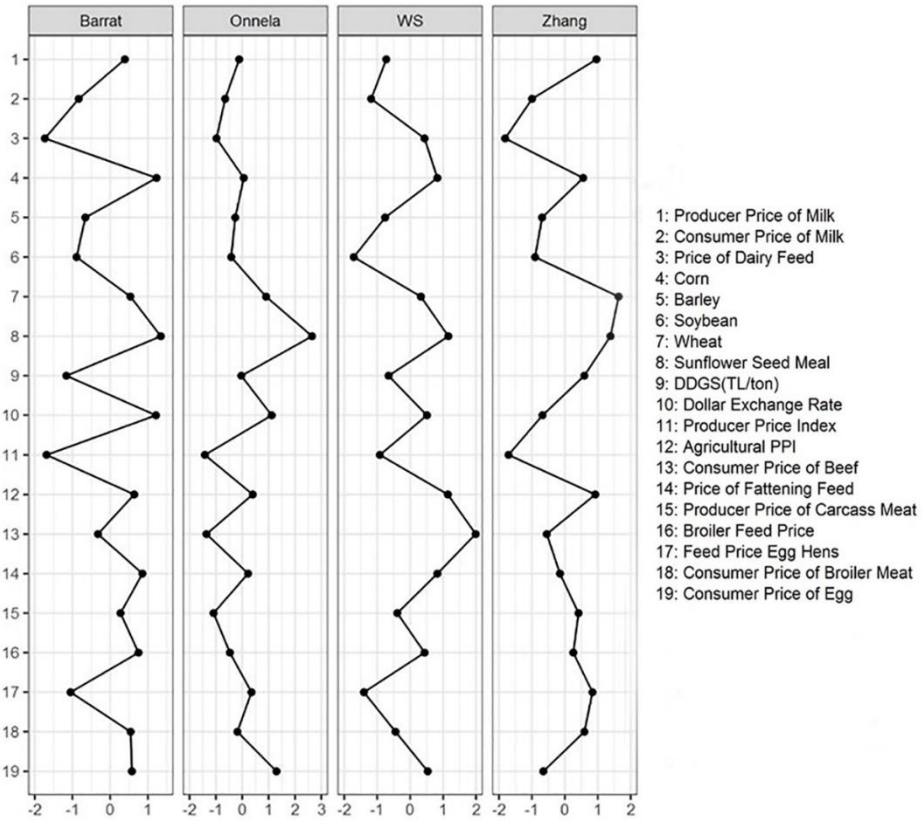

Figure 3. Graph of Clustering Coefficients for the Network.

Discussion and Conclusion

Animal products are one of the most basic needs of human beings. The increase in demand due to the increasing population limits the accessibility of these products due to both high prices and insufficient supply (7, 39). Nowadays in Türkiye, some basic economic parameters such as input prices and exchange rates are effective in the formation of the supply and demand amounts of these products. Due to the substitution effect of the products and the supply-demand relationship, the price of each product is interrelated, which, in turn, causes all products to be in a complex system as part of a whole (41). In this context, Figure 1 represents the pattern network structure of 19 variables determined for the prices of animal products and the severity and direction of the connections between them.

According to results, there is a positive and strong relationship between DDGS and soybean. The same positive relationship is seen between soybean and barley and between barley and corn. It is thought that these relations are based on foreign inputs-dependent production (7) of the Turkish poultry sector that has a production structure with vertical integration. The most important of these raw materials are soybean meal and corn (11). In Türkiye, 25-35% of corn and 90% of soybean are imported since production amounts cannot meet consumption (22). This structure leads to strong and positive relations between the prices of feed inputs used in many livestock sub-sectors, especially in the poultry sector. This structure in the poultry sector can be seen more clearly with the strong and positive connection that variables 9, 6, 5, and 4 given in Figure 1 have formed within themselves. As regards cattle breeding, the fact that feed costs constitute 60-70% of the total cost can be shown as the primary reason for the formation of the positive and strong connection between the price of fattening feed and the price of dairy feed (Figure 1). Based on Figure 1, most of the variables have a positive relationship and they are more pronounced and stronger than the variables with negative relationships. Similarly, it can be inferred from the same figure that the positive and relatively strong correlation of the number 10 variables (Dollar Exchange Rate) with the producer price index and the producer price of carcass meat is an important finding. This summarizes the general structure of the animal products market in Türkiye.

As a result of the network structure we have obtained, there are strong and positive relationships between feed inputs. To prevent increases in animal product prices, it is necessary to reduce input imports and support the production of feed inputs. On the other hand, the fact that producer prices are very effective on product prices requires livestock support at the input stage of the animal production process.

Indeed, the fact that the PPI, Agricultural PPI, and Dollar Exchange Rate nodes (Table 1) are nodes with a high degree of betweenness further reinforces the pattern network structure. Many studies have emphasized that economic variables such as inflation and dollar exchange rate interact with producer and consumer prices in Türkiye. According to the studies on this subject, there is a one-way causality relationship from exchange rate to PPI in Türkiye (9). Studies on feed inputs in Türkiye have revealed that wheat and sunflower seed prices interact with international reference prices (17). In another study, it was determined that the international prices of wheat interact with the domestic market prices, this interaction increases in crisis/drought periods, and the domestic prices of wheat move closer to the international prices with the depreciation of TL (30). According to another study, the change in the exchange rate is reflected in the cost of imports, and the increase in feed prices put more pressure on beef producer prices (20). It has been reported that a 10% increase in the price of soybean, which is the main imported feed inputs in Türkiye, causes an increase of 3.84% in chicken meat prices (18). This relationship has led to high feed prices for broilers, the import of most of the feed inputs, and a significant increase in broiler production costs. Because of this problem, Türkiye is at a disadvantage against competitor countries in terms of producer prices (46). This negative structure in feed costs has also become important for dairy cattle and fattening activities in recent years. International studies have also emphasized a similar situation, noting that both milk and feed prices have been so volatile in recent years that the profitability of dairy farms has been negatively affected (48). This price volatility in Türkiye is mainly a result of increases in exchange rates and costs. It has been found that there is a long-term positive effect between the uncertainty of agriculture and food prices and inflation in Türkiye (21) and that increases in agricultural price inflation are reflected in food price inflation and total CPI inflation in a statistically significant way (19). The degree of closeness measures the efficiency and independence of the node. Accordingly, the variables with the highest degree of closeness were found to be Dollar Exchange Rate and PPI (Table 1). Considering the position of both values in the network, the finding that they affect the network and exhibit an independent structure becomes clearer with their degree of closeness.

As a result of the network structure, the fact that the animal products market, especially the feed market, has an import-substitution production structure can be shown as the reason why these two variables have the highest degree of influence in the network. In this context, to minimize the impact of the activity experienced in the Dollar exchange rate in Türkiye on the animal products market,

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the state should consider subsidy policies for import substitute products (feed inputs) in this area.

Considering the four different coefficients of the clustering coefficients given in Table 2, the variables with the highest density around are the Price of Dairy Feed and the Producer Price of Milk. The variable with the least density is the Consumer Price of Broiler Meat. These results support the effect values given in Table 1 and show that these two variables have a high degree of influence on other variables.

In conclusion, this network analysis visualizes the situation of Türkiye, which adopts the import-substitution model in animal production, is foreign-dependent in feed inputs, and has a market structure affected by high exchange rates. Besides, as can be understood from the positive connections that feed inputs have formed among themselves, the pattern network of variables affecting prices of animal products is largely shaped by feed prices and the internal dynamics of economic variables. Revising this existing structure is necessary for sustainable animal production. In this regard, with the right policies and support tools, the supply and demand mechanism of animal products can be kept under control. As can be understood from the network, bringing both economic variables, inputs prices and production and consumption to optimum levels will benefit all stakeholders of the sector in the future. Otherwise, possible economic fluctuations may jeopardize the continuity and sustainability of production. An import-substitution production model in feed inputs, together with the upward movements in the exchange rates, may reduce the production in the medium and long term, disrupting the animal food supply.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ACA, MSA and MAT designed and planned the study. MP, BM and MBÇ collected data. ZÖ and MAT analyzes were performed. ACA, MBÇ and MSA contributed to the interpretation of the results. The ACA and MSA took the lead in writing the draft. All authors provided critical feedback and helped shape the research, analysis, and article.

Data Availability Statement

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

Ethical Statement

This study does not present any ethical concerns.

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The protective effect of caffeic acid phenethyl ester on cadmium-induced liver toxicity: A histopathological and biochemical study

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ABSTRACT

In this study, the changes caused by caffeic acid phenethyl ester (CAPE) in the histopathological and biochemical parameters in the oxidant / antioxidant balance in mice with experimental cadmium toxicity were investigated. A total of 40 female Swiss albino mice were used, with 10 mice in each group. The mice were divided into four groups (Group I - Control group, Group II - CAPE group, Group III - Cadmium group, Group IV - Cadmium + CAPE group). Plasma paraoxonase (PON) activity, high-density lipoprotein (HDL), low-density lipoprotein (LDL), total sialic acid (TSA), total antioxidant capacity (TAC), total oxidant capacity (TOC), and oxidative stress index (OSI) were analyzed on mice's blood samples. The results showed that cadmium intoxication triggered oxidative stress in the mice. It also lowered their PON activity alongside TAC and HDL levels ($P<0.001$, $P<0.01$, and $P<0.01$, respectively) and increased their TSA, LDL, TOC, and OSI levels ($P<0.05$, $P<0.01$, $P<0.01$, and $P<0.01$, respectively). The histopathological examination of the liver tissues revealed focal apoptotic regions, sinusoidal occlusion, and irregularity in the cadmium group and no significant change in the other groups. These results indicated that CAPE can significantly prevent biochemical and histopathological changes due to cadmium damage.

Introduction

The world's population has been rapidly growing and this, in turn, has led to environmental and soil pollution. Heavy metals are a leading cause of both environmental and soil pollution (7). Under normal conditions, heavy metals are found at low levels in nature. When their levels increase, they have toxic effects on living beings because they inhibit especially enzymes (54, 58). Cadmium (Cd) is a commonly found heavy metal in the ecosystem and is highly toxic to living organisms. It gets released into the environment through various sources, and then passes into plants, soil, and water – in turn adversely affecting human and animal health. Cadmium-induced toxicity in tissues and organs causes oxidative stress in the organism (4, 18). Oxidative stress occurs when there is a disruption of the balance between free radicals and the antioxidant system in an organism. Free radicals disrupt the structures of important organic compounds such as protein, DNA, carbohydrate, and lipid, in the cells of living organisms, thus resulting in lipid peroxidation (38, 40).

The paraoxonase (PON) enzyme – an antioxidant against lipid peroxidation in cell membranes – suffers a significant amount of activity loss during lipid peroxidation (55). PON is located in high-density lipoproteins (HDL) in serum and has a calcium-dependent glycoprotein structure (13, 52). It has several functions

such as participating in the detoxification of organophosphate compounds such as paraoxon and protecting low-density lipoproteins (LDL) from oxidation by hydrolyzing lipid peroxides (2, 59). For a long time, lipid peroxidation has been thought to be an important mechanism that plays a role in cadmium toxicity; therefore it is believed that antioxidant agents should be administered to protect and treat the organisms against oxidative stress caused by cadmium (43, 50). This has guided the related studies toward focusing on antioxidant molecules that can both strengthen the antioxidant defense system and prevent heavy metal poisoning. One of the molecules with antioxidant properties is caffeic acid phenethyl ester (CAPE). CAPE is a component of propolis, an extract collected from plants by bees. It has antimicrobial, anti-inflammatory, immunomodulatory, neuroprotective, and antioxidant properties (3, 6, 10, 37). Numerous studies have revealed that CAPE is a pharmacologically reliable molecule and increases the activity of antioxidant enzymes by suppressing lipid peroxidation (15, 21, 48). Sialic acid is a compound derived from neuraminic acid by N-acetylation and is one of the important structures of biological membranes (51, 56).

Natural antioxidants are popular remedies that have few side effects and are used by the majority of people (23). Experimental studies are powerful tools for investigating agents that induce and prevent oxidative damage and its role in carcinogenesis. As far as we know, the effect of CAPE on Cd-induced liver tissue impairment in ovariectomized mice has not yet been reported. This study, it was aimed to reveal the changes induced by CAPE, which is a powerful natural antioxidant, in the antioxidant/oxidant balance of the organism through histopathological and biochemical parameters against an experimental toxicity model created in mice by using cadmium, which is a major heavy metal.

Materials and Methods

Ethics committee approval was obtained from Kafkas University Animal Experiments Local Ethics Committee (2016-087) for this study. In the study, using G*Power (3.1.9.3) software, Type-1 (alpha) error rate = 0.05, power (1-beta) = 0.80, effect size = 0.30. Accordingly, a total of 40 female Swiss albino mice were used, with 10 mice in each group. They were fed with a standard mouse diet and water for 15 days to make them adapt. To make animals ready for the experiments, they were raised in cages at 25±2 °C and an average humidity of 50%±5. They were exposed to 12 hours of light, and 12 hours of darkness.

Lab animals: The mice were divided into four groups, as follows:

Group-I – Control group: 10 mice
Group-II – CAPE group: 10 mice
Group-III – Cadmium group: 10 mice
Group-IV – Cadmium + CAPE group: 10 mice

The ovaries of the mice in all four groups were surgically removed through an ovariectomy procedure. They were then left to heal for 20 days, after which point other experiments were conducted on them. Group-I was fed a normal diet. The mice in this group were given saline intraperitoneally throughout the experiment to eliminate any differences between groups that might arise from stress and to prevent the placebo effect – a possible result of injections. The mice in Group II were fed with a normal diet plus 10 µmol/kg intraperitoneal CAPE for 15 days (10). The mice in Group III were fed with a normal diet, plus 1 mg/kg/day subcutaneous cadmium-chloride for 15 days (32). The mice in Group IV were fed with a normal diet + 1 mg/kg/day subcutaneous cadmium-chloride + 10 µmol/kg intraperitoneal CAPE for 15 days.

Histopathological procedures: One day following the last administration, intracardiac blood samples were extracted from the mice and collected into heparinized tubes. Later, the mice were subjected to cervical dislocation. Tissue samples were taken from their systemic necropsies and fixed in a 10% formol solution. Next, sections were prepared on paraffin blocks following routine procedures. Last, they were stained with Hematoxylin-Eosin and then examined under a light microscope for any histopathological findings.

Biochemical parameters: Plasma was obtained by centrifuging the blood samples at 3000 rpm for 10 minutes and kept at -20 °C until the analysis began. Plasma total antioxidant capacity (TAC) and total oxidant capacity (TOC) levels in the blood samples were measured using a spectrophotometer (Epoch, Biotek, USA) with commercial kits (Rel Assay Diagnostics®, Gaziantep, Türkiye) based on Erel's method(s) (19, 20). Plasma paraoxonase activity was measured using Eckerson (16) and Gülcü's (25) methods. PON activity was determined by spectrophotometric measurement of absorbance at 25°C and 412 nm by color product yielded from 4-nitrophenol occurring as a result of enzymatic hydrolysis of O,O-Diethyl O-(4-nitrophenyl) phosphate (Sigma®, London, UK). For paraoxonase activity, the enzyme activity in 1 mL serum transforming 1 nmol paraoxonase into 4-nitrophenol in 1 min was identified as a unit and the results were given in U/L. Plasma total sialic acid (TSA) levels were measured using Sydow's method (53) in that sialic acid was separated by perchloric acid in the sample, and then it was boiled by Erlich reagent, and finally, the product was read at 525 nm. NANA (N-acetylneuraminic acid from *Escherichia coli*, Sigma®, London, UK) was used for the sialic acid calibration curve. Plasma LDL and HDL levels were measured with an automatic analyzer

(Huma Star 600, Germany) using a commercial kit (IBL®, Türkiye). The oxidative stress index (OSI) was calculated using the following formula: OSI (AU, arbitrary unit) = TOC*100/TAC.

Statistical analysis: SPSS (IBM SPSS Statistic 22) was used to analyze the parametric values, and then generate statistics. ANOVA-Duncan test was used to find out whether or not there was a difference between the study groups. Moreover, ANOVA was used to identify whether or not there was any difference caused by a particular group(s). The results were expressed in mean + standard deviation. The difference was deemed as significant at the level of P<0.05.

Results

Biochemical Results: Biochemical analysis revealed the biochemical values of the Control group and the CAPE group were at normal levels, while PON activity, HDL, and TAC levels dropped significantly in the Cadmium group and the Cadmium + CAPE group (P<0.001, P<0.01, P<0.01, respectively). Likewise, the biochemical values of the Control group and the CAPE group were again at normal levels, whereas LDL, TSA, TOC, and OSI levels had increased significantly in the Cadmium group and the Cadmium + CAPE group (P<0.01, P<0.05, P<0.01, P<0.01, respectively). These findings are presented in Figure 1 and Figure 2 (See below).

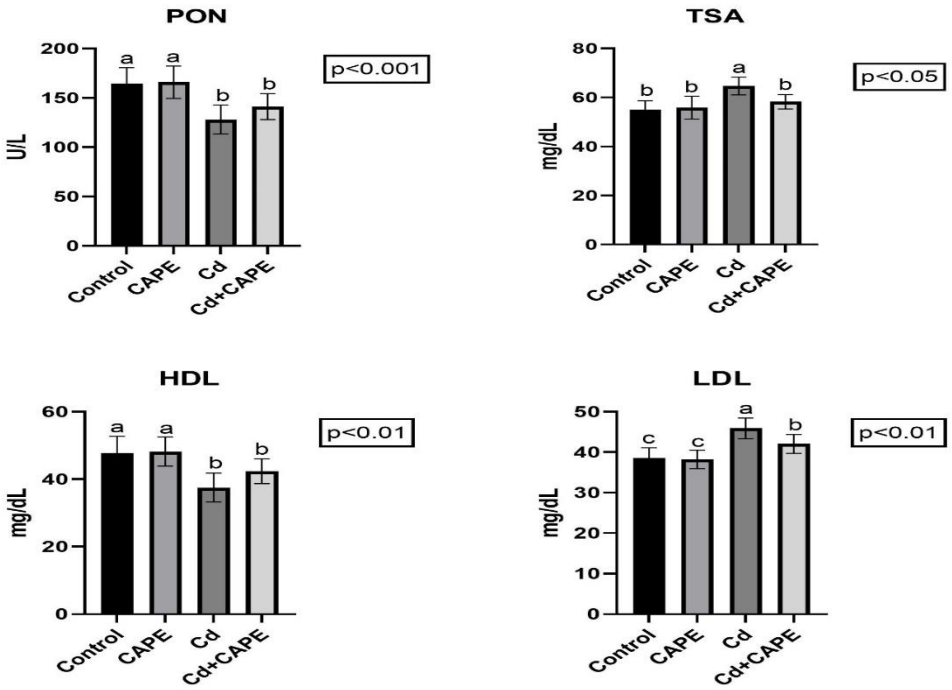

Figure 1. PON, TSA, HDL and LDL values obtained from all four groups.
Control: Control group, CAPE: caffeic acid phenethyl ester group, Cd: cadmium group, Cd + CAPE: cadmium + caffeic acid phenethyl ester group. PON: paraoxonase activity, TSA: total sialic acid, HDL: high-density lipoprotein, LDL: low-density lipoprotein. a, b, c: The difference between the group means shown with different letters in the same row is significant.

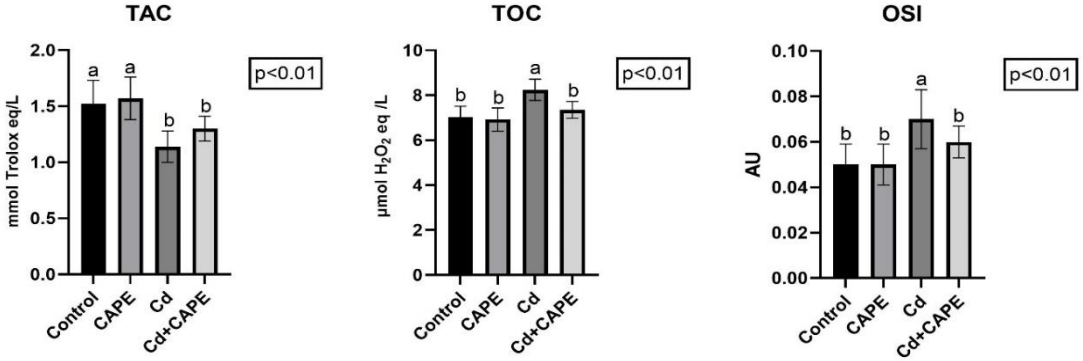

Figure 2. TAC, TOC and OSI values obtained from all four groups.
Control: Control group, CAPE: caffeic acid phenethyl ester group, Cd: cadmium group, Cd + CAPE: cadmium + caffeic acid phenethyl ester group. PON: paraoxonase activity, TAC: total antioxidant capacity, TOC: total oxidant capacity, OSI: oxidative stress index. a, b: The difference between the group means shown with different letters in the same row is significant.

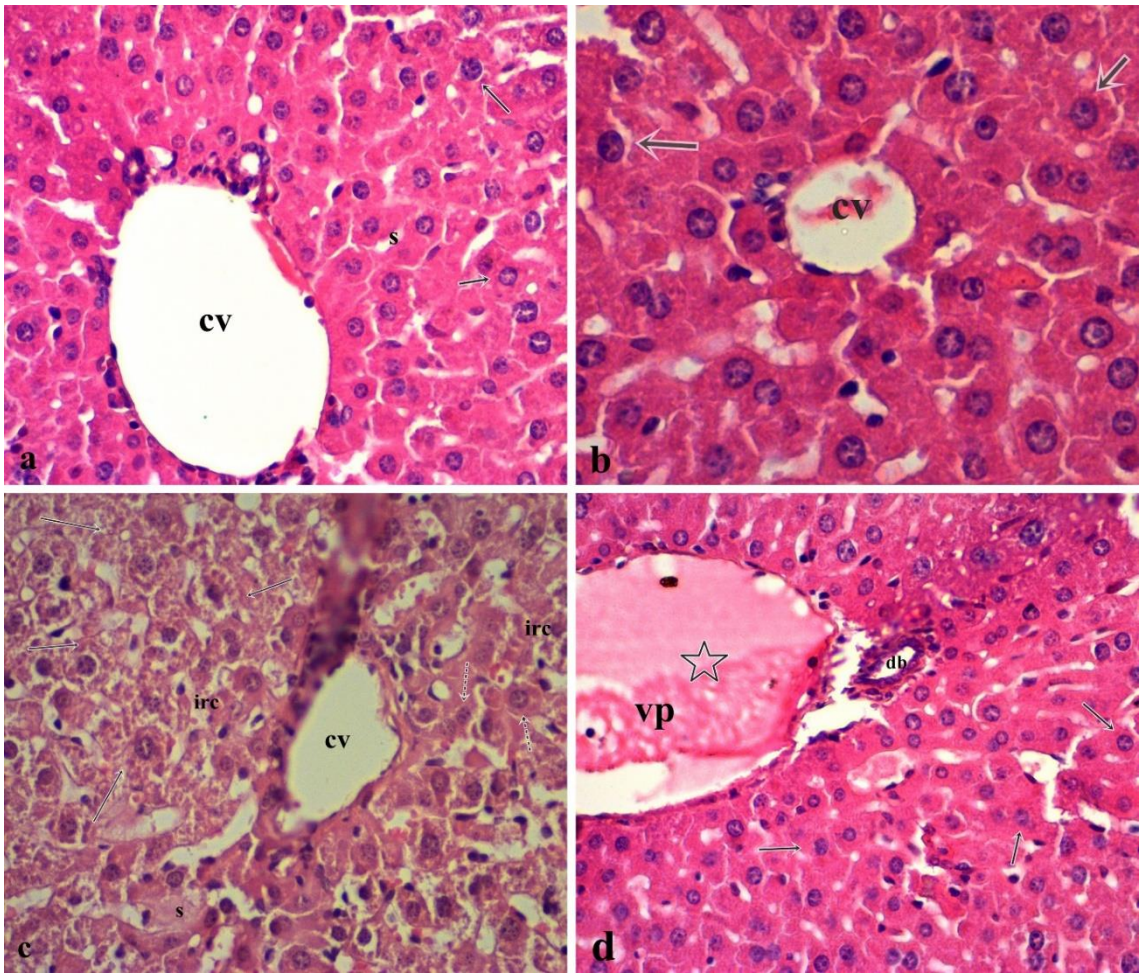

Figure 3. a. Liver tissue section view of Group I. Hepatocytes and sinusoidal structure had a normal appearance (cv: vena centralis, s: sinusoid, arrows: hepatocyte). **b.** Liver tissue section view of Group II. Hepatocytes and sinusoidal structure had a normal appearance (cv: vena centralis, s: sinusoid, arrows: hepatocyte). **c.** Liver tissue section view of Group III (arrows: focal apoptotic regions, dashed arrows: hepatocytes, cv: vena centralis, irc: irregular remark cords, s: sinusoidal congestion). **d.** Liver tissue section view of Group IV (star: vascular congestion, db: ductus biliferi, arrows: hepatocytes, vp: portal vena). Bar: 40 µm, Hematoxylin-Eosin.

Histopathological Results: Histopathological analysis revealed that the vena centralis and portal areas had a normal appearance and the hepatocyte sequence was regular in the Groups I and II. Congestion, focal necrosis, and degenerative areas were detected in the central and portal veins in the sections of Group III. In Group IV, a decrease was observed in both severity and frequency of the histopathological changes compared to Group III (Figure 3 a, b, c, d).

Discussion and Conclusion

Cadmium is easily absorbed by plants and can also get mixed into drinking water from soil thereby entering the food chain. Cadmium thus emerges as critical health and environmental problem. Heavy metals like cadmium pose serious health problems through contaminated water and food (30, 44). Living organisms do not have a mechanism to remove cadmium from their systems. It, therefore, accumulates in tissues and organs, especially the kidney and liver as well as the pancreas, bones, lung, and placenta. During chronic cadmium intoxications, organisms develop hepatotoxicity and nephrotoxicity (8, 31).

Both antioxidants and oxidants interact with one another in organisms. It is stated that the measurement of antioxidants separately in laboratories has many disadvantages such as time-consuming, intense workload, and costly complex techniques, and therefore TAC measurement can be used as a panel of antioxidant defence (5, 9, 57, 60). To determine the oxidative balance of an organism, it might be more useful to measure only TAC and TOC, rather than evaluating oxidants and antioxidants parameters one by one (19, 20). Some studies have suggested that an increased risk of disease due to cadmium exposure may result from systemic inflammation and induced oxidative stress (12, 41). Many animal model studies involving experimental cadmium toxicity have reported that cadmium causes oxidative stress, that results

in lipid peroxidation in cells and tissues, leading to a reduction in antioxidant enzymes of the organism and a rise in lipid peroxidation products (27, 29, 31, 34, 36, 45). Likewise, the present study revealed that the Cadmium group's plasma TAC levels decreased significantly and their TOC level and OSI increased. In the groups given CAPE, these values were closer to those of the control group. This may be because cadmium accumulation in major organs such as the liver causes oxidative stress in the organism by leading to an increment in free radicals and a reduction in antioxidant molecules.

Paraoxonase is a calcium-bound glycoprotein enzyme found in an organism's serum, liver, kidneys, and intestines. It functions to hydrolyze organophosphates. PON, which is bound to HDL in serum, exerts an antioxidant action through HDL and prevents lipid peroxidation (2, 59). Although the literature review did not reveal a study investigating the relationship between cadmium toxicity and PON activity in mice, many studies such as experimental studies in rats and mice have revealed the effects of various toxicants on PON activity. Also, paraoxonase activity has been associated with cadmium in some studies and inhibitory effects on PON activity have been demonstrated (1, 14, 28, 39). The findings of the present study demonstrated that plasma PON activity and HDL levels were lower and LDL levels were higher in mice treated with cadmium. The plasma PON activity, HDL and LDL levels of the CAPE group and the Cadmium + CAPE group got closer to the control group. The Cadmium group might have had low plasma PON activity and HDL level alongside high LDL level because oxidative stress caused by cadmium intoxication also induces lipid peroxidation; likewise, cadmium inhibits calcium-dependent paraoxonase activity.

Sialic acid is a derivative of N-acetyl neuraminic acid and is found in the structure of macromolecules and receptors. Any factor that causes malfunctions in living organisms also significantly increases total sialic acid levels (15, 51). Several studies have reported that endogenous and exogenous toxic agents may cause oxidative stress in tissues and cells and the release of sialic acid from oligosaccharides on the cell surface may begin with oxidative stress. That, in turn, causes lipid peroxidation and cell damage in cells and tissues (15, 17, 51). Karapehlivan et al. (33) reported that TSA levels in brain, kidney and liver tissue homogenates were higher in mice intoxicated with mercury (a major heavy metal, like cadmium) than those in control group. In the present study, plasma TSA levels in the Cadmium group were higher than those of the control group. It was also found that the plasma TSA levels in the CAPE group were closer to the plasma TSA levels in the control group. It was thought that lipid peroxidation caused by oxidative stress and subsequent tissue damage may cause a rise in TSA levels in the Cadmium group.

Various studies have stated that CAPE is highly effective in protecting core cytosolic proteins, membrane lipids, and DNA against oxidative damage because of its antioxidant and free radical scavenging properties (21, 46-48). A handful of studies have shown that CAPE is effective in protecting the oxidant / antioxidant balance of the organism, in favor of antioxidants because it prevents oxidative stress in cells caused by cadmium toxicity (11, 35). Cadmium is mainly known to target the liver. However, like other heavy metals, it can also accumulate in an organism's kidneys, lungs, duodenum, pancreas, bones, and testicular tissues, and even cause cytological and histopathological damage. Among the precursor organs, the liver and kidneys filter cadmium from the body (22). When Cadmium enters an organism, it forms a durable bond with metallothionein, a protein that plays an important role in the liver's metabolism. The kidneys excrete that the metallothionein-cadmium complex (62). In the liver, following Cadmium-induced oxidative stress, Kupffer cells activate tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) – both are pro-inflammatory mediators- that cause the release of adhesion molecules. This leads to a range of cellular and humoral activities that result in inflammation of hepatocytes and disrupt the cell membrane (61). Regardless of the route of exposure, Cadmium toxicity primarily causes liver necrosis and apoptosis of hepatocytes (26, 42). A study in which 5 mg/kg of cadmium was administered to rats for 28 days reported hydropic degeneration, degeneration around the central vein, infiltration foci, enlargement of sinusoids, and pyknotic nuclei (49). Another study involving 1 mg/kg cadmium reported both hydropic degeneration and infiltration foci in hepatocytes (24). The findings reported in all of the aforementioned studies support histopathological findings of the present study.

Cadmium intoxication triggered oxidative stress in mice by lowering PON activity, TAC, and HDL levels and increasing TSA, LDL, TOC, and OSI levels. Histopathological analysis of all groups revealed that the Cadmium group developed focal apoptotic regions, sinusoidal congestion, and irregularity in the remark cords due to cadmium's toxicity; no significant changes were detected in any of the other three groups. Biochemical and histopathological parameters were found to be similar in the control group and the CAPE group. In conclusion, the findings of the present study showed that cadmium can cause significant changes in biochemical and histopathological parameters, due to oxidative damage. They also demonstrate that CAPE can significantly prevent those changes.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

HAD, GN, AK and MK conceived and planned the experiments. GN, AD, MK carried out the experiments. HAD and GN planned and carried out the simulations. GN, AK and AD contributed to sample preparation. HAD, GN and AK contributed to the interpretation of the results. HAD took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

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

Ethical Statement

All procedures performed with experimental animals comply with ethical standards and were approved by the Kafkas University Animal Experiments Local Ethics Committee (2016-087).

Animal Welfare

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

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Use of a bipolar vessel sealing device in canine orchiectomy

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ABSTRACT

Bipolar vessel sealing (BVS) devices are being used increasingly in veterinary medicine. The objective was to determine whether the use of a BVS device in prescrotal open orchiectomy of dogs reduced surgical time, postoperative pain and surgical site complications compared to ligation with suture. Fifty medium to large breed dogs admitted for elective castration were randomly assigned to either the ligation or BVS groups. Duration of surgery, pain score at postoperative 15 minutes, 1, 2, and 24 hours, and surgical site swelling and bruising scores at 24 hours were compared. Duration of surgery was shorter (P<0.001) in the BSV group (median 8.30; range 7.03 to 10.17 minutes) than the ligation group (median 10.18; range 7.47 to 12.33 minutes). There was a significant effect of age (r=0.458, P=0.021) and body weight (r=0.432, P=0.031) of the animal on the duration of surgery in the BVS group. Lower pain scores were observed in the BVS group at postoperative 15 minutes (P=0.001) and 1 hour (P=0.045). Pain scores were not different between groups at 2 hours and 24 hours (P>0.05). The surgical site swelling score was lower (P=0.034) in the BVS group (0.24 ± 0.09) compared to the ligation group (0.72 ± 0.17). A lower bruising score (P=0.015) was observed in the BVS group (0.44 ± 0.13) than in the ligation group (1.16 ± 0.22). The use of BVS method was associated with significantly shorter surgery times and lower postoperative pain and surgical site complications than traditional ligation technique in canine open orchiectomy.

Introduction

Canine castration is a common surgical procedure in small animal practice. Surgical castration provides contraception (38), decreases behaviour problems (26) and helps prevent and treat reproductive pathologies (3, 7). Many surgical castration techniques have been described in the dog. The open technique by using a prescrotal incision is commonly preferred in adult dogs, for it allows more reliable ligations by direct placement of the ligatures around the vascular pedicle (18).

Complications of canine castration include postoperative pain, scrotal swelling, haemorrhage, subcutaneous bruising, self-trauma to the surgical site and infection. Haemorrhage may be serious and may result in scrotal hematoma or intraabdominal haemorrhage (18). In the dog, surgical site swelling and bruising are frequently observed after open castration (4). The incidence of surgical site complications for prescrotal castration is high in adult dogs (22, 30) and complication rates of up to 61% have been reported (13).

Recent advances in minimal invasive techniques in surgery leading to reduced morbidity, less pain and faster patient recovery are driving forces behind efforts in veterinary practice to make procedures less invasive (28). Small animal practitioners may benefit from reducing the duration of common surgical procedures tremendously. Suture ligation, the standard haemostatic technique during open surgical castration of dogs, can be cumbersome and technically challenging (17). Recently, the use of bipolar electrosurgical forceps for haemostasis for canine castration was described. However, the use of electrocautery forceps did not reduce surgical time compared to ligation with sutures (37). Vessel-sealing devices offer a valuable alternative to mechanical ligation techniques to provide reliable haemostasis due to the short time needed and potential reduction in intraoperative

blood loss, surgical time and postoperative complications (24).

Electrothermal bipolar vessel sealing (BVS) technology depending on tissue response generators is one of the most recent advancements in electrosurgery (11). The efficacy and safety of BVS devices in many canine surgical procedures have been described and these devices are being used increasingly in veterinary surgery, especially during laparoscopic procedures (39, 40). Bipolar vessel sealing devices fuse vessels and form reliable seals by targeted, feedback-controlled delivery of electrical current and mechanical pressure (19). Denaturation of collagen and elastin combined with the mechanical pressure leads to coagulum formation (16). In comparison with monopolar coagulation that reliably seals small vessels with a diameter of 1-3 mm, BVS devices are able to provide hemostasis of vessels up to 7 mm in diameter depending on the instrument chosen (34).

The objective of this study was to compare bipolar vessel sealing method with routine ligation in prescrotal bilateral open orchiectomy of dogs in terms of surgical time, surgical site complications and postoperative pain scores.

Materials and Methods

Fifty medium and large-breed dogs admitted for elective castration were included. The health status of dogs was determined based on medical history, physical examination and complete blood count performed on the day of admission. Signed informed owner consent was obtained before random assignment of dogs to either ligation or BVS groups. All dogs were in generally good health. Dogs <6 months and >5 years of age and those requiring any additional surgery or having any evidence of scrotal or testicular disease were not included in the study. The study protocol was reviewed and approved (Approval no: 2017-4-30) by the Local Ethics Committee on Animal Experiments, Ankara University, Türkiye.

Preoperative evaluation: Food and water were withheld for approximately 6 hours before surgery. One hour before surgery, the animals received 0.2 mg/kg meloxicam intra-muscularly (im) as a pre-emptive analgesic and 15 mg/kg long-acting amoxicillin trihydrate im was used for prophylaxis. Dogs were premedicated with 0.01 mg/kg medetomidine im. Intravenous (iv) catheters were placed into a cephalic vein in all animals before induction of anaesthesia with propofol 6 mg/kg iv. The dogs were intubated and isoflurane in oxygen at a flow rate of 2% was used for anaesthesia maintenance. Electrocardiogram, heart rate, respiration rate and blood oxygen saturation were continuously monitored. Lactated Ringer's solution was administered iv at a rate of 5 mL/kg/hour during surgery.

Surgical technique: After the achievement of the surgical plane of anaesthesia, bilateral orchiectomy was performed using the standard open technique (15). Briefly, a prescrotal skin incision sufficient in length to allow exteriorization of the testicle was made with a scalpel blade. The testis was pushed cranially and the vaginal tunic was exposed and incised before exteriorization of the testicle. The tunics were separated from the remainder of the spermatic cord. In the ligation group, transfixation sutures were placed in the vaginal tunic and the spermatic cord separately by using 0.2-0 or 3-0 Polyglactin 910. The spermatic cord and tunic were transected distal to the ligatures and replaced into the incision after final bleeding control.

A BVS device (LigaSure™ 5 mm blunt tip 37 cm sealer) was used both for sealing and dissection of the vaginal tunic and the spermatic cord in the BVS group. Valleylab™ LS10 Generator energy platform was used as the energy source. The generator includes a feedback-controlled response system for automatic cessation of energy delivery once the seal cycle is complete. Standard settings according to the company instructions were maintained, with the instrument set at an intermediate power.

The procedure was then repeated for the other testis in both groups. The skin incision was closed with a 2-0 or 3-0 absorbable synthetic suture material (Poly lactic-co-glycolic acid, PLGA) in a simple interrupted subcutaneous and intra-dermal pattern. Duration of surgery, beginning with skin incision and ending with placement of the final skin suture, was recorded for each case. An aerosol antibiotic spray containing oxytetracycline hydrochloride was applied on the surgical site. The first author, experienced in canine castration, who had undergone basic 2-hour training in BVS performed all surgeries.

Postoperative care and follow up: After the surgical procedure, dogs received 0.01 mg/kg atipamezole im and were monitored until fully recovered from anaesthesia. An Elizabethan collar was placed and patients were followed up at least 24 hours. Postoperative pain and complications were evaluated by a veterinarian blinded to the treatment. The severity of postoperative pain was evaluated at 15 minutes, 1, 2 and 24 hours post-surgery using a pain score evaluation form (Table 1) as described (35). Ketoprofen 2 mg/kg im was planned to be administered as rescue analgesia in patients with pain scores greater than 4 at any time point. Swelling and bruising of the surgical sites were scored at 24 h using a previously described (35) scale (Table 2). Any surgical or anaesthesia related complication developed during or after surgery in the follow-up period was recorded as major complications requiring intervention or minor complications requiring observation only. The study

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protocol was terminated after a follow-up period of 24 h and the dogs were discharged from the hospital. The owners were asked to examine the surgical site and general status of their dogs and to represent them in case of suspected complications. Routine follow-up assessments were made 7 to 10 days after surgery.

Statistical analysis: All statistical comparisons were performed using SPSS (SPSS Statistics Version 23; IBM Corporation). Assumptions for homogeneity of variances and normality of the data were tested by Levene's test and Shapiro-Wilk's test, respectively. Student t-test was used in the comparison of normally distributed continuous variables between the study groups. Continuous variables that were not normally distributed were compared between groups by the Mann-Whitney U test.

The Friedman test was used to analyse the difference between times for pain scores and a Wilcoxon matched pairs test with Bonferroni correction was used for pairwise comparisons. Correlations between surgery duration, age, body weight, the severity of surgical site swelling and severity of surgical site bruising were assessed using Spearman's correlation coefficient. Results were presented as mean ± SEM when normally distributed and as median (range) otherwise. The significance level was set at $P<0.05$.

Table 1. Pain score evaluation form used (35).

Criteria	Score	Description
Comfort	0	Dog asleep or calm
	1	Mild-moderate agitation, awake, interested in surroundings
	2	Extremely agitated
Movement	0	No movement
	1	Frequent positional changes per min
	2	Continuous positional changes per min
Appearance	0	Eye normal or partially closed, ears flattened or normal in position
	1	Moderate changes, eyes glazed, unthrifty appearance
	2	Severe changes: eyes pale, pupils enlarged, guarding, legs in abnormal position or other abnormal facial expressions
Vocalization	0	No vocalization
	1	Crying, responds to calm voice and stroking
	2	Crying, does not respond to calm voice and stroking
Heart rate	0	<10% greater than preoperative value
	1	10-25% greater than preoperative value
	2	25% or more greater than preoperative value
Respiratory rate	0	<10% greater than preoperative value
	1	10-25% greater than preoperative value
	2	25% or more greater than preoperative value
Total (0–12)		

Table 2. Criteria used for surgical site evaluation at postoperative 24 hours (35).

Swelling evaluation	Score	Bruising evaluation	Score
No swelling	0	No bruising	0
Mild swelling	1	Mild bruising	1
Moderate swelling	2	Moderate bruising	2
Marked swelling	3	Marked bruising	3

Swelling score description	
0	No visible evidence of swelling noted
1	Swelling minor, raised less than 2-3 mm and extending less than 2-3 mm
2	Swelling obvious but not significantly irritated. Extending between 3 and 6 mm laterally or raised
3	Swelling clearly visible. Over 6 mm in width or height, obviously irritated

Bruising score description	
0	No visible evidence bruising
1	Minor bruising, color minimally changed, bruise extends less than 3 mm
2	More significant erythema or color change. Bruise obvious but between 3 and 6 mm in distance from incision
3	Bruise is obvious, over 6 mm in size darker in color, more pronounced erythema

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Results

The average age and body weight of the dogs and total surgical time in study groups are shown in Table 3. There were no significant differences between the age (P=0.168) and the body weight (P=0.678) of dogs in study groups. The median ages of the dogs were 9 months (range 6 months to 54 months) and 10 months (range 6 months to 36 months) in ligation and BVS groups, respectively. The median body weight of the ligation group dogs was 21.80 ± 1.34 kg and it was 22.72 ± 1.75 kg in the BVS group.

The median duration of surgery was 10.18 (7.47-12.33) and 8.30 (7.03-10.17) minutes in the ligation group and the BVS group, respectively (P<0.001). There was a significant effect of age (r=0.458, P=0.021) and body weight (r=0.432, P=0.031) of the animal on the duration of surgery in the BVS group.

Postoperative pain scores at 15 minutes, 1, 2 and 24 hours in ligation and BVS groups are given in Table 4. Pain score > 4 necessitating rescue analgesia was not observed at any time point. Pain scores were significantly reduced by time in both groups (P<0.001). Lower mean pain scores were observed in the BVS group at postoperative 15 min (P=0.001) and 1 h (P=0.045). Mean pain scores in ligation and BVS groups were not different at 2 and 24 hours (P>0.05).

None of the dogs had major surgical or anaesthetic complications. Surgical site swelling and bruising scores are shown in Table 5. The mean swelling score was lower in the BVS group compared to the ligation group (P=0.034). Nineteen dogs had no swelling and 6 dogs had minor swelling in the BVS group. No swelling was observed in 13 dogs in the ligation group. However, 6 dogs had minor and 6 dogs had moderate swelling.

Table 3. The average age, body weight of dogs and total surgical time in ligation and bipolar vessel sealing (BVS) groups.

Parameter	Group (n=25)	Mean ± SEM	Median (range)	P
Surgical time(minutes)	Ligation	9.81 ± 1.23[a]	10.18 (7.47-12.33)	<0.001
	BVS	8.50 ± 0.86[b]	8.30 (7.03-10.17)	
Age (months)	Ligation	14.20 ± 2.78	9 (6-54)	0.168
	Vessel sealing	13.92 ± 1.56	10 (6-36)	
Body Weight(kg)	Ligation	21.80 ± 1.34	20 (13-38)	0.678
	Vessel sealing	22.72 ± 1.75	24 (8-40)	

BSV, Bipolar vessel sealing; Kg, kilograms; SEM, standard error of mean.
[a,b] Within a column, means for same parameter without a common superscript differ (P<0.001).

Table 4. Postoperative pain scores in ligation and bipolar vessel sealing groups at different time points.

Postoperativepain score	Ligation group (n=25)		Bipolar vessel sealing group(n=25)		P
Time point	Mean ± SEM	Median (range)	Mean ± SEM	Median (range)	
15 minutes	2.80 ± 0.15[c]	3 (2-4)	2.04 ± 0.14[c]	2 (1-3)	0.001
1 hour	1.52 ± 0.17[b]	2 (0-3)	1.12 ± 0.12[b]	1 (0-2)	0.045
2 hours	0.72 ± 0.15[ab]	1 (0-2)	0.64 ± 0.13[ab]	1 (0-2)	0.765
24 hours	0.28 ± 0.14[a]	0 (0-2)	0.40 ± 0.10[a]	0 (0-1)	0.138
P	<0.001		<0.001		

SEM, Standard error of mean.
[a,b,c] Within a column, means without a common superscript differ (P<0.05).

Table 5. Surgical site swelling and bruising scores in ligation and bipolar vessel sealing groups at postoperative 24 hours.

Parameter	Group (n=25)	Mean ± SEM	Median (range)	P
Surgical site swelling score	Ligation	0.72 ± 0.17	0 (0-2)	0.034
	Bipolar vessel sealing	0.24 ± 0.09	0 (0-1)	
Surgical site bruising score	Ligation	1.16 ± 0.22	1 (0-3)	0.015
	Bipolar vessel sealing	0.44 ± 0.09	0 (0-2)	

SEM, Standard error of mean.

The surgical site bruising score at 24 h was significantly lower (P=0.015) in the BVS group (0.44 ± 0.13) compared to the ligation group (1.16 ± 0.22). There was no bruising in nine dogs in the ligation group. Seven, five and four dogs had mild, moderate and marked bruising on the surgical site in the ligation group, respectively. No marked bruising was observed in the BVS group. Sixteen dogs had no bruising on the surgical site. However, mild bruising was observed in seven dogs and moderate bruising was observed in two dogs. No interventions were made for surgical site complications, swelling and bruising resolved spontaneously in a couple of days.

Surgical site bruising was associated with surgical site swelling in ligation (r=0.769, P=0.001) and BVS group (r=0.783, P=0.001). Surgical site bruising and swelling were not associated with the duration of surgery, age or bodyweight of the animal in either group (P>0.05).

Discussion and Conclusion

The use of a BVS method in the current study enabled open prescrotal orchiectomy to be completed in a shorter time. This reduction in surgical time is due to the time saved by eliminating the need for tedious knot-tying. With the use of bipolar haemostasis devices, ligation techniques have become less preferred. In this way, bleeding control can be achieved more easily, especially in areas that are difficult to access, which shortens the operation time (28). Being one of the most common surgical procedures in veterinary practice, it is important to shorten the surgical time for orchiectomy, especially in intensive sterilization programs. The advantages of vessel-sealing devices in canine ovariectomy have been shown previously (8, 27), and the surgical time was reduced compared with ligation with suture (32). To the best of the authors' knowledge, this is the first study on the use of vessel sealing devices in canine orchiectomy.

The mean duration of surgery in both study groups is comparable to (31, 37) or shorter (21, 36) than previous reports. It is theoretically suggested that closed orchiectomy is easier and can be completed in a shorter time (4). However, in a study comparing open and closed orchiectomy, the mean surgical time was reported as 21.9 ± 11.5 min and 20.0 ± 9.5 min for open and closed orchiectomy, respectively (13). Although the open technique was used in the current study, relatively short surgical times were observed. This result has been associated with surgeon experience. Surgical time in canine orchiectomy strongly depends on surgeon experience (33), and repetition of the procedure helps improve surgical skills as measured by surgery time (10). Interestingly, increasing body weight and age were associated with a longer duration of surgery in the BVS group, but not in the ligation group. The change in relative anatomic size and tissue composition associated with body weight and age is a possible explanation for this finding. Large and dense tissues are hard to be positioned between the blades of the device and connective and fatty tissues necessitate multi-activation of the device. In addition, adherence of related tissue to the hot surface of the instrument, which is known as tissue sticking, intervenes with energy delivery that results in less effective and time-consuming sealing (6). In accordance with the results of the current study, significant effects of body weight and age of the animal on surgical time have been reported when bipolar electrosurgical forceps were used in canine orchiectomy (37).

The results have shown decreasing pain scores in both groups within 24 hours of surgery. In addition, the use of BVS device resulted in lower postoperative pain at 15 minutes and 1 hours of surgery. In dogs, the highest levels of postoperative pain are usually observed within the first 24 hours, and this time frame is evaluated in most clinical studies. It is known that the pain after surgical castration is high in the first hours and decreases afterward (21). It is suggested that shorter surgery time and decreased tissue trauma and handling with the BVS technique are responsible for the lower postoperative pain measured within 1 h of surgery.

Our results showing similar pain scores in study groups at 2 and 24 hours suggest that both ligation and BVS techniques caused comparable discomfort at these time points. Rescue analgesia was not required for any dogs in the current study. Reportedly, male dogs subjected to orchiectomy require less postoperative analgesia intervention than female dogs submitted to ovariohysterectomy (31). In agreement with our findings, no pain in 72.3% of dogs, mild discomfort in 25.0%, and mild to moderate pain in 2.7% of dogs were reported at 24 h after castration of 1066 dogs and rescue analgesia was not required (2). Similarly, rescue analgesia was not required for any of 34 dogs subjected to open castration as determined by the use of the Glasgow Composite Measure Pain Scale short form (13).

Post-surgical pain control in dogs is usually achieved by using opioids, non-steroidal anti-inflammatory drugs, local anaesthetics or their combinations. Non-steroidal anti-inflammatory drugs are effective for 12-24 hours and are commonly used in the clinical management of post-surgical pain and inflammation in dogs (20, 25). Meloxicam has been reported to provide adequate analgesia in dogs up to 72 hours after castration (23). In a study using 12 male dogs, pre-emptive meloxicam analgesia was found 100 % successful in terms of Modified Glasgow Pain Scale rescue analgesia

requirement within 24 hours of surgery (31). Our findings provide further evidence that pre-emptive analgesia with meloxicam is an effective method of controlling postoperative pain for 24 hours in dogs undergoing open castration.

One of the limitations of the present study is that the pain score evaluation form used relies on subjective assessments. Using a better objectivity-validated scale could contribute to improving the reliability of the results. Ease of application and less disturbance to dogs were the main reasons for the selection of pain score evaluation form used. In addition, the use of the Elizabethan collar posed the risk to intervention with expressions related to attention to the wound and response to touch.

Increased incidence of surgical site complications was previously associated with increased surgery time for canine orchiectomy (17). However, the current study showed no evidence of a relationship between the duration of surgery and surgical site bruising and swelling scores indicating that the reduction in the duration of surgery was not the reason for lower surgical site complications in the BVS group. Lower surgical site swelling and bruising scores in the BVS group were possibly due to better haemostasis and less tissue handling and damage. Haemorrhage following orchiectomy is usually related to bleeding from the tunic causing self-limiting incisional haemorrhage, subcutaneous bruising, and scrotal hematoma (1). In addition, poor pain management contributes to increased surgical site complications or result in self-inflicted trauma (2). By simultaneous application of sealer designed to grasp and cut the formed seal with a blade incorporated into the jaws of the instrument, it was possible to perform a less invasive procedure with no risk of ligature slippage and any foreign material left.

Safety against the lateral spread of thermal effects associated with the use of an electrothermal BVS devices has been investigated extensively. In general, the use of an electrothermal BVS device results in a minimal lateral spread of thermal effects. The energy delivered varies based on tissue density within the device forceps and precise amount of energy is delivered to the tissue, limiting collateral thermal damage to <2.5 mm (12, 14) Limited thermal spread and collateral tissue injury have been reported for the system used in this study. Person et al. (29) reported that the thermal spread of the LigaSure™ system is limited to an area less than 1.5 mm beyond the tissue bundle or vessel.

When using electrothermal BVS devices, maintaining a safe distance from important anatomic structures, avoiding continuous use of the device and cooling after prolonged application are suggested (9). These considerations did not cause any major problems in this study due to the relative simplicity of the procedure and ease in accessibility of anatomical structures. Furthermore, only a few instrument activations were needed in each case.

Another limitation of the present study is the short follow-up time. The discontinuation beyond 24 h was because of routine practices for privately-owned dogs. Although the owners were advised to examine their dogs and surgical wound and no complications were reported in any dog, a longer follow-up period would have been valuable. Besides, the use of prophylactic antibiotics and antimicrobial spraying of the surgical site may have affected the representation of surgical site complications. The use of antibiotics is a controversial area of elective castration in dogs. It is important to emphasize that no protocol used in this study should be evaluated as best practice. Elective orchiectomy is considered as a clean surgical procedure and antimicrobial use is not usually advised unless the surgery is prolonged or there is an obvious break in asepsis causing contamination of the wound (5).

In conclusion, the use of a vessel sealing device was associated with significantly shorter surgery times and lower postoperative pain and surgical site complications compared with the use of conventional ligation technique in canine open orchiectomy, possibly because of better haemostasis and less tissue handling and damage.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

CY and HK contributed to the design and implementation of the research and to the analysis of the results. HK wrote the manuscript in consultation with CY.

Data Availability Statement

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

Ethical Statement

The study protocol was reviewed and approved (Approval no: 2017-4-30) by the Local Ethics Committee on Animal Experiments, Ankara University, Türkiye.

Animal Welfare

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

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Expression and distribution of GPR55 and GPR119 during the development of rat testis

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ABSTRACT

G Protein-Coupled Receptors, GPR55 and GPR119 are widely distributed throughout the body and exert important biological functions. However, little is known about their roles in testis. This study aimed to examine the expression and distribution of GPR55 and GPR119 during the development of the rat testis. Sixty male Sprague Dawley rats (180–240 g) were divided into 10 groups as 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 postnatal days of age (PND) (six animals per group). The testicular expression of GPR55 and GPR119 has been investigated by immunohistochemistry, Western blot, and quantitative RT-PCR methods. We observed that GPR55 and GPR119 are expressed throughout the rat testis development from PND 7 to 70. However, no difference was observed between the groups in terms of expression levels, except for GPR55 mRNA expression in the group of PND 7. Immunohistochemistry analysis showed that GPR55 is expressed in spermatids and spermatocytes in the mid-term tubules and spermatocytes in the late-stage tubules in groups of PND 56, 63, and 70. For GPR119, very intense positivity was observed only in spermatids in the mid-term (stage VII-VIII) tubules in the groups of PND 56, 63, and 70. No significant difference was observed in the number of GPR55 and GPR119 positive cells in testes from PND 56 through PND 70. Taken together, both GPR55 and GPR119 receptors are expressed throughout the rat testis development (PND 7 to 70). These results suggest that GPR55 and GPR119 are involved in the modulation of male reproductive function.

Introduction

Endocannabinoid system (ECS) consists of two established cannabinoid receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) and some putative cannabinoid receptors including GPR55 and GPR119. Despite the low structural similarity to CB1 and CB2 receptors, the pharmacology of GPR55 and GPR119 displays an overlap with the established cannabinoid receptors (24). A wide variety of ligands, including phytocannabinoids and endocannabinoids, activate these established cannabinoid receptors, several of which activate putative cannabinoid receptors. GPR55 can be activated by cannabinoids such as Δ^9-Tetrahydrocannabinol (THC) and cannabidiol, endocannabinoids including N-Arachidonoylethanolamine (anandamide, AEA) and 2-Arachidonoylglycerol (2-AG), and endocannabinoid-like compounds including N-Palmitoylethanolamine (PEA) and N-Oleoylethanolamine (OEA) (31). GPR119 can be activated by several compounds including AEA, OEA and N-oleoyldopamine (13, 21). Numerous selective agonists and antagonists for GPR55 and GPR119 have also been synthesized and characterized for the targeted induction or inhibition of these receptors for research and therapy purposes (39). GPR55 is coupled to $G\alpha_{12/13}$ and $G\alpha_q$ proteins and mediates the activation of ras homolog gene family member A (RhoA) and Rho-associated protein kinase (ROCK). Then, ROCK can either stimulate phospholipase C (PLC) activity which leads to increased

intracellular Ca^{2+}, or activates mitogen-activated protein kinases (MAPKs) such as extracellular signal- regulated kinase (ERK) which regulates different transcription factors (18, 28, 40, 48). GPR55 expression has been observed in numerous tissues/organ systems, including the brain, lung, spleen, placenta, white adipose tissue, adrenal glands, gastrointestinal tract, liver, uterus, bladder, kidney, prostate, bones, testes and others (23, 27, 35, 37, 42, 47). GPR55 has been implicated in a wide range of pathophysiological conditions, including motor coordination, neural development (6), nervous system disorders (32), vascular function (19), platelet and endothelial cell function (25), bone metabolism (47), angiogenesis (50), immunity (7), cancer development (4), inflammatory and neuropathic pain (44).

GPR119 is coupled to the signal transducer Gαs whose activation is followed by adenylate cyclase action and results in a rise in intracellular cyclic adenosine monophosphate (cAMP) levels (20). GPR119 is primarily expressed in gastrointestinal tract (duodenum, stomach, jejunum, ileum and colon) (8, 34), and pancreatic β-cells (41). GPR119 expression has also been reported in testes, brain, skeletal muscles, cardiac muscles and liver (43). GPR119 has been associated with pathophysiological conditions including obesity (12), diabetes mellitus (36), cancer (22) and metabolic dysfunction associated with fatty liver disease (51). The wide distribution of GPR55 and GPR119 throughout the body suggests that they might be involved in diverse biological functions. Although GPR55 and GP119 expressions have been observed in normal rat testes (49), very little is known about their physiological roles in testes. THC and AEA, ligands for cannabinoid receptors, have previously been reported to reduce the plasma luteinizing hormone (LH) levels in male rats and mice, respectively (33, 46), which was accompanied by a fall in serum levels of testosterone produced by Leydig cells in the presence of LH in testes. Moreover, chronic exposure to THC caused a weight reduction in prostate, seminal vesicles and epididymis and the impairment of spermatogenesis in rats (11, 29). These findings suggest that induction of GPR55 and GPR119-mediated signaling may have a role in testis development. However, post-natal development of GPR55 and GPR119 expression in testis is unknown. This study aimed to examine the expression and distribution of GPR55 and GPR119 at the transcript (mRNA) and protein levels during the development of rat testes.

Materials and Methods

Animals: Male Sprague Dawley rats (180–240 g) ranging from postnatal day (PND) 7 to 70 were used in this study (Experimental Animals Production and Experimental Research Center). All animals were kept under a 12 h light/dark cycle at a constant temperature and humidity, with free access to food and water. All animal procedures were performed according to a protocol approved by Animal Experiments Local Ethics Board in Burdur Mehmet Akif Ersoy University (13.03.2019/501). Sixty male Wistar rats were divided into 10 groups of 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70 days of age (six animals per group). The rats were randomly assigned to each group and did not receive any treatment regimen. Prior to necropsy, clinical observations were recorded for each animal. Animals were euthanized by decapitation without anesthesia at PND 7 through PND 28 and by ketamine/xylazine injection at PND 35 and above. Testis tissues were removed and either fixed in buffered-formalin for histopathology and immunohistochemistry (right testis), or rapidly frozen in liquid nitrogen for Western blot and qRT-PCR analysis (left testis).

***Histopathology and Immunohistochemistry*:** For the histopathological examination, testicular tissues taken from each animal were fixed in Modified Davidson's solution for 24 h, then passed through graded alcohol series and embedded in paraffin after routine histological procedures. Then, 4-5 µm thick sections were taken from paraffin blocks with microtome (Leica RM 2125). The sections were deparaffinized in xylol (Xylene - Merck Millipore:108661), then dehydrated in alcohol series, and stained with Hematoxylin & Eosin. Finally, the tissue sections were examined under a light microscope for the presence of any lesions.

For immunohistochemical staining, 4-5 µm-thick sections were mounted on poly-L-lysine-coated slides (Menzel Polysine Slides: J2800AMNZ) and the sections were deparaffinized in xylol and dehydrated in alcohol series, then antigen retrieval was performed by autoclaving (15 min, 120 °C) in citrate buffer (pH 6). After a 20-30 min cooling step, slides were rinsed in phosphate-buffered saline (PBS) solution for 5 min. After antigen retrieval, staining was continued with ImmPRESS Excel Amplified HRP Polymer Staining Kit (Anti-Rabbit IgG) according to the manufacturer's instructions. Briefly, the tissue sections were incubated in BLOXALL block solution for 10 min to abolish endogenous peroxidase activity, followed by a 5 min wash with PBS. The sections were then incubated with normal horse serum (2.5%) for 20 min and excess serum was removed. Next, the slides were incubated overnight with either GPR55 (1:500 dilution, ab203663, Abcam) or GPR119 (1:250 dilution, NBP2-47661, Novus) antibodies at 4 °C. After incubation with primary antibodies, the slides were washed with PBS for 5 min and incubated with amplifier antibody solution. Following a 5-min-PBS wash, ImmPRESS Polymer Reagent was applied to the slides. After two PBS washes,

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slides were incubated with ImmPACT DAB EqV working solution and the staining status was observed under a light microscope. After staining was observed in the positive control, the slides were washed first with PBS, then with tap water. For counterstaining, the slides were kept in Mayer's hematoxylin stain (Merck) for 25 sec and then submerged in water. After air drying, the slides were covered using entellan, examined under a light microscope and images were captured during the evaluations.

Western Blot Analysis: Freshly collected tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until western blot was performed. The testicular tissue samples for each group were weighed and homogenized (TissueLyser LT, Qiagen) in radioimmunoprecipitation assay (RIPA) buffer (sc-24948, Santa Cruz Biotechnology). Tissue homogenates were centrifuged at 13,000 x g for 15 min at 4 °C and the resulting supernatants were transferred to tubes. Total protein concentration was measured by BCA assay (9300A, Takara). For each sample, 25 µg total protein extract was resolved using SDS-PAGE with 10% polyacrylamide gel and then protein bands were transferred to a polyvinylidene fluoride (PVDF) membrane (Immun-Blot PVDF, IPVH00010, Merck Millipore). After blocking the membrane with 5% skimmed milk for 2 h at room temperature (RT), the blots were incubated with primary antibodies for GPR55 (1:200, ab203663, Abcam), GPR119 (1:500, NBP2-47661, Novusbio), and β-actin monoclonal antibody (1:5000, 66009-1-Ig, Proteintech) overnight at 4 °C. The next day, the blots were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG: ab205718 and goat anti-mouse IgG: ab205719, 1:10,000 dilution, Abcam) for 1 h at RT, and then washed with Tris-buffered saline with Tween solution (TBST:50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20). Signals were detected by chemiluminescence substrate using Clarity Western ECL Substrate (#1705061, Bio-Rad) were quantified by the density of protein bands using Image J analysis program (Image J Version 1.52).

qRT-PCR Analysis: Freshly frozen rat testis tissues were weighed and maximum 100 mg tissue samples were excised. After finely mincing, tissues were homogenized in 1 ml Tri-reagent (Sigma, T9424) to obtain a single cell suspension. Then, samples were further processed according to the manufacturer's instructions to obtain total cell RNA. For each sample, 1 µg Dnase I (Thermo)-treated total RNA was converted to cDNA in a 20 µl reaction volume using iScript Reverse Transcription Supermix (Biorad). For qRT-PCR, following primer and probes were used to amplify target gene transcripts in a dublex PCR settings: rGPR55 forward primer TGTCTTCAC CATCTGCTTCATC, rGPR55 reverse primer TTCCAACCCATACCAGCATC, rGPR55 prob (5'-HEX, 3'-BHQ1) TCCTCGCCATCCAGTACCCTCTT; rGPR119 forward primer TGATACCTTGATTGGCG TGG, rGPR119 reverse primer CATGATCTGGAAGTAACGG AGG, rGPR119 prob (5'-HEX, 3'-BHQ1) AAGACCTT GTGTAGCCTTCGGATGG; rGAPDH forward primer AACCCATCACCATCTTCCAG, rGAPDH reverse primer CACGACATACTCAGCAC CAG, rGAPDH Prob (5'-FAM, 3'-BHQ1) ACCCCATTTGATGTTAGC GGGATCTC. PCR mix was prepared in a 10 µl volume containing iQ Multiplex Powermix (1X), 0.5 µl cDNA, 300 nM of each primer and 200 nM of each prob. Reactions were performed using CFX96 Touch (Biorad) thermal cycler with the following settings: 95°C for 2 min, then 40 cycles of 95°C for 10 sec and 60°C for 45 sec. During primer optimization process and each PCR run, cDNAs prepared from adult rat brain tissues were used as a positive control along with a no template control. Target and reference genes were run as single and duplex reactions and confirmed that their threshold cycles (Ct) were similar in both conditions.

For each sample, difference in the threshold cycles (dCt) was calculated by subtracting the Ct value of GAPDH from the Ct of GPR55 or GPR119. The calculated mean dCts from groups were first analyzed for normal distribution. Since the mean GPR55 dCt of each group displayed a normal distribution (Kolmogorov-Smirnov test, $P=0.258$) with unequal variances ($P<0.05$), the data were evaluated by Welch's ANOVA test. Mean dCts for GP119 followed a normal distribution (Kolmogorov-Smirnov test, $P=0.540$) with homogeneous group variance and were analyzed by Fischer's test. Using group 1 (PND 7) as calibrator, 2^ddCT indicating fold difference in gene expression was calculated according to Livak's method.

***Statistical Analysis*:** One-way ANOVA was used to compare the means of 10 independent groups. Tukey or Games-Howel (in cases of heterogeneous variance) tests were used as post-hoc tests. First, the normality of the data collected by RT-PCR and western blot methods were examined with tests (Anderson-Darling, Kolmogorov-Smirnov and Shapiro Wilk), scatter charts and quantile-quantile (Q-Q) plots. The homogeneity of variance, which is another important assumption of ANOVA, was also checked with Levene and Bartlett tests, and in cases of unequal variances, Welch test was used instead of Fischer's test. P values smaller than 0.05 were considered statistically significant. All statistical analyses were

performed using Jamovi program. Graphs were prepared in Graphpad Prism 9.

Results

Histopathology and Immunohistochemistry: Histopathologically, micrographs of H&E-stained testis sections were evaluated for the presence of germ cell loss, degeneration/apoptosis, germ cell exfoliation, macro/microtubular vacuolation and necrosis, and no pathological changes were observed. Strong positive staining for GPR55 was observed in spermatids (residual bodies of spermatids and round spermatids) and spermatocytes in mid-term tubules and spermatocytes (zygotene and pachytene) in late-stage tubules at the 8th, 9th, and 10th weeks. However, no GPR55-positive cells were found at the 7th week and before (Figure 1 and Figure 3). For GPR119, very intense positivity was observed only in spermatids (residual bodies of spermatids and round spermatids) in mid-term (stage VII-VIII) tubules at the 8th, 9th and 10th weeks. No positive signal was observed in the other weeks (Figure 2 and Figure 3). In addition, there was no significant difference in the number of GPR55 and GPR119 positive cells between weeks 8, 9 and 10.

Western Blot Analysis Findings: The protein levels of both GPR55 and GPR119 were determined in the rat testis using Western blot analysis. Both GPR proteins were detected in all testes samples and the blotting results confirmed the immunohistochemistry data. The relative expression levels of these GPRs in the developing testis are shown in Figure 4. For the statistical analysis of Western blot measurements, normality assumption was checked for both proteins; normality was achieved in both (K-S P=0.258 and P=0.110, respectively, QQ plots are normal); homogeneity of variance was provided for GPR55 (both tests P>0.05); not available for GPR119 (both tests P<0.01). As a result of the Fischer's test for GPR55, there was no difference between the groups (P=0.464). Although there was a difference between the groups (P=0.031) as a result of the Welch test for GPR119, no differing groups were found in the Post-hoc test.

RT-PCR Analysis: Both GPR55 and GPR119 mRNA transcripts were detected in testis tissues of all groups. However, according to their Ct values (Figure 5A and 5D), which is inversely correlated with the starting transcript levels, overall GPR119 expression (Ct>30) in each group was lower than GPR55 expression (Ct<30). Group 1 (PND 7) had the least GPR55 mRNA level (P<0.0001, compared to other groups except group 2, PND 14) (Figure 5B and 5C), then the transcript levels exhibited a gradual increase in the second and third weeks by 7-fold and 14-fold, respectively (Figure 5C). Despite minimal differences, GPR55 mRNA levels showed no significant change between weeks 3 and 10.

GPR119 was expressed in all developmental stages even though at a lower level than GPR55. The GPR119 expression level varied within and between groups (Figure 5E and 5F). Nonetheless, the Fischer's test analysis of the mean dCts for the groups showed that there was no significant difference between the groups (P=0.181).

Discussion and Conclusion

Spermatogenesis, the development of sperms from male germ cells, is a complex biological process including mitotic and meiotic divisions followed by differentiation stages. These events are modulated by endocrine hormones produced in the hypothalamus and pituitary and by a complex network of locally produced factors within the testis (17, 26). Endocannabinoid system is one of these factors and is known to regulate sperm development (16). AEA, an endocannabinoid, is synthesized by germ cells, Sertoli cells and spermatozoa in the testis (9, 45). Both GPR55 and GPR119 receptors can be activated by endocannabinoids including AEA and endocannabinoid-like compounds such as OEA (13, 21, 31). Cannabinoids and their endogenous counterparts bind to cannabinoid receptors, CB1 and CB2, and non-cannabinoid receptors, GPR55 and GPR119, of the ECS; thus regulate several aspects of male reproduction (31, 38). The functional importance of ECS in spermatogenesis has been shown by the ECS knockout mouse models. Detection of a higher number of motile sperms in the epididymis of CB1-deficient mice and impaired fertilizing ability of sperms in fatty acid amide hydrolase (FAAH)-knockout mice which have a high amount of AEA, indicated that depending on their expression level, ECS might have a negative regulatory role in sperm development. Another ECS receptor, vanilloid receptor TRPV1, can also react to AEA and inhibits acrosome reaction (30). Conversely, CB2 receptor signaling triggered by 2-AG promotes spermatogenic differentiation from spermatogonia (15). A previous study showed that endocannabinoid receptors and ligands, AEA and 2-AG, exist in both mRNA and protein levels in resting and capacitated mouse sperms. Besides, their concentrations further increased in capacitated sperms, suggesting an active role for endocannabinoids in the regulation of spermatogenesis and sperm functions (5). Altogether, ECS influences different stages of sperm development and function. Despite a growing number of studies on CB1, CB2 and TRPV1-mediated regulation of sperm development, the role of GPR55 and GPR119-mediated signaling in spermatogenesis has not been defined well.

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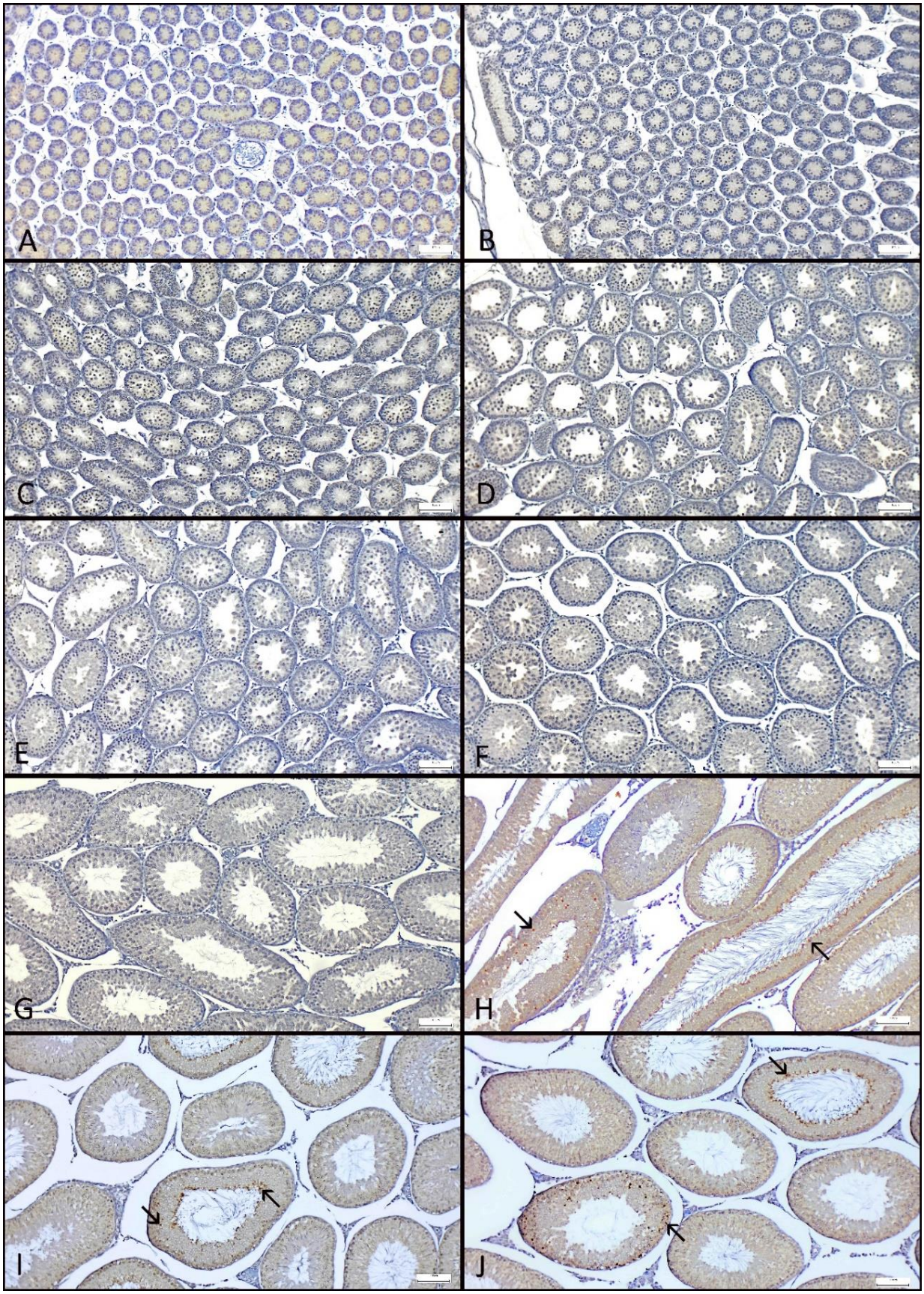

Figure 1. GPR55 immunohistochemistry. GPR55 negativity at weeks 1-7 (A-G). At week 8 (H), positivity of spermatids in the mid-stage (stage VII-VIII) tubule (right), and spermatocytes in the late-stage tubule (left) (arrows). At 9th week (I), positivity of spermatocytes and spermatids in mid-term (stage VII-VIII) tubule (arrows). At week 10 (J), positivity of spermatids in mid-stage (stage VII-VIII) tubule (right) and spermatocytes in late-stage tubules (left) (arrows). GPR55, DAB, Bar: 100 µm.

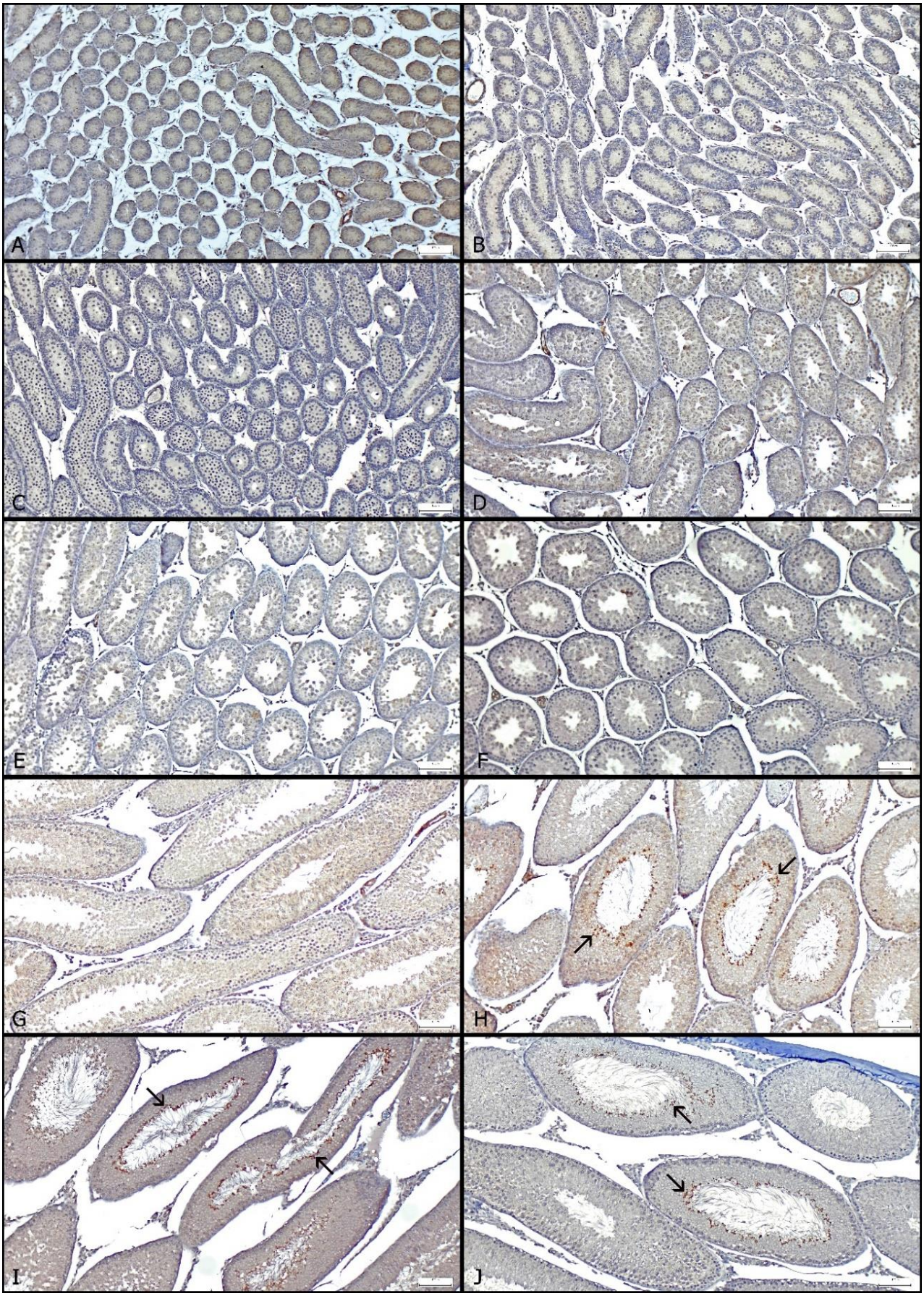

Figure 2. GPR119 immunohistochemistry. GPR119 negativity at weeks 1-7 (A-G). Intense positivity of spermatids in mid-term (stages VII-VIII) tubules at 8th, 9th and 10th weeks (H-J) (arrows). GPR119, DAB, Bar: 100 µm.

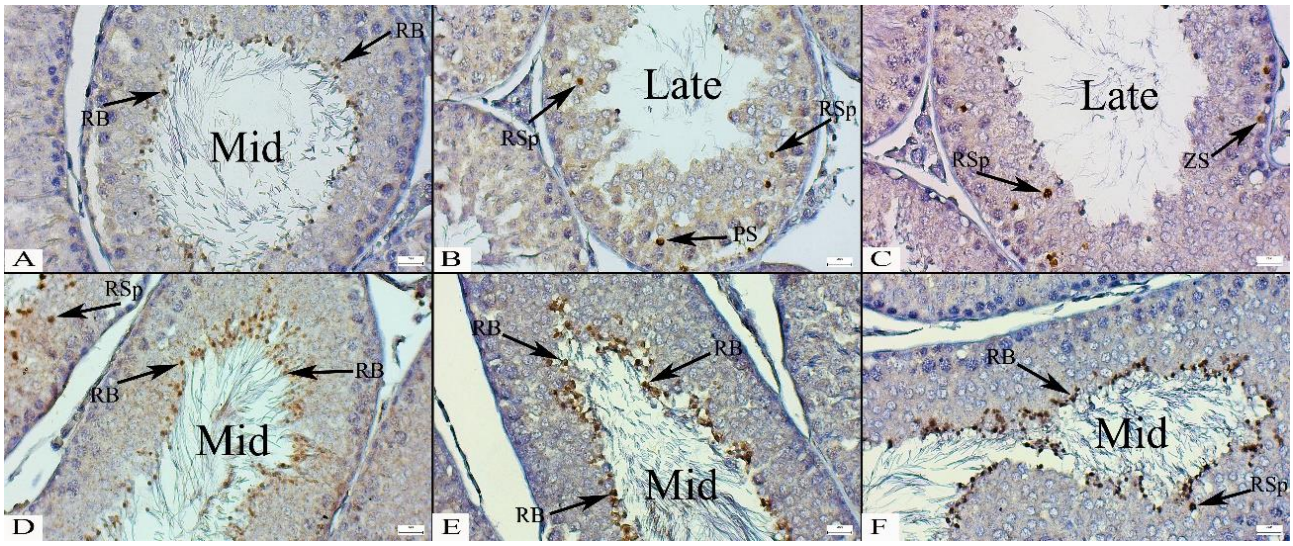

Figure 3. GPR55 and GPR119 immunohistochemistry. GPR55 positivity in residual bodies (RB) of spermatids in the mid-stage (stage VII-VIII) tubule (A) at 8th week, and pachytene spermatocytes (PS), round spermatids (RSp) and zygotene spermatocytes (ZS) in the late-stage tubules in 9th (B) and 10th (C) weeks. GPR55, DAB, Bar: 20 µm. GPR119 positivity of residual bodies of spermatids and round spermatids in mid-term (stages VII-VIII) tubules at 8th (D), 9th (E) and 10th (F) weeks. GPR119, DAB, Bar: 20 µm.

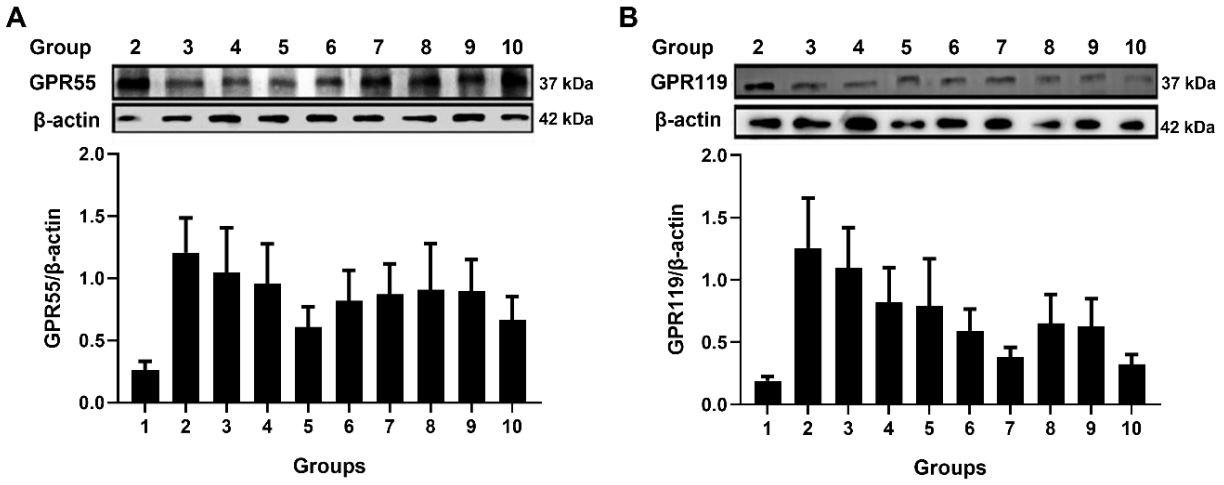

Figure 4. Expression levels of GPR55 and GPR119 proteins in rat testis tissues. Representative western blot images display A) GPR55 and B) GPR119 protein expression in one sample per group. For the quantification of protein abundance, blot images from separate experiments were analyzed by densitometry and the data was plotted as mean GPR55: β actin ratios ± SEM for each group (n = 6, 3 replicates/group).

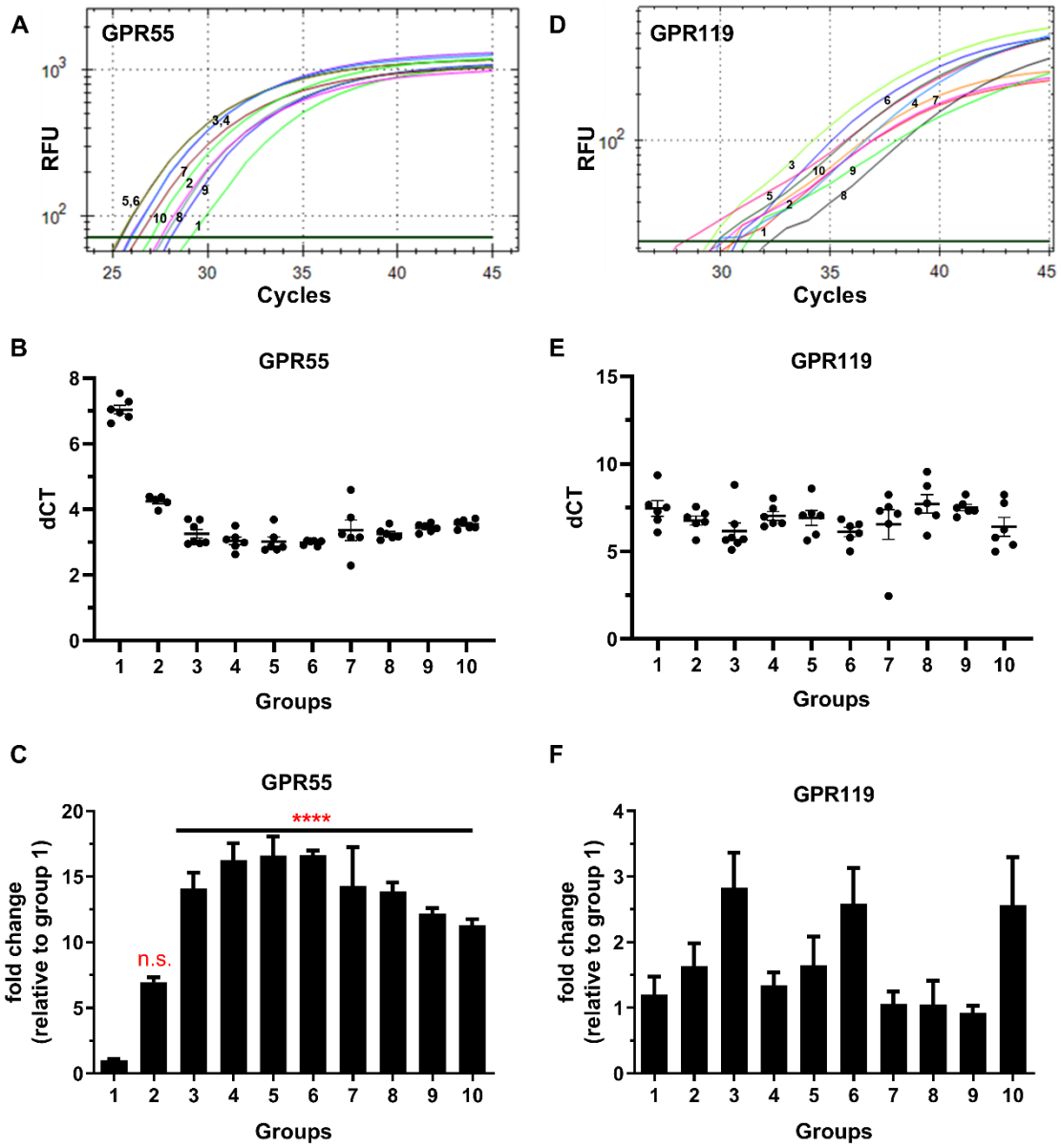

Figure 5. GPR55 and GPR119 mRNA levels at different developmental stages of rat testis. Representative amplification curves of A) GPR55 and D) GPR119 transcripts, labelled with their pertaining group numbers, show the detected florescence level in each reaction cycle. RFU means relative fluorescence unit. dCt (GPR55 Ct or GPR119 Ct- GAPDH Ct) values of each transcript plotted in B) and E) as the mean dCt of each group with SEM. n=6 for all, except n=7 for group 3 and n=5 for group 2 due to exclusion of an outlier in GPR55 data. Reactions were run as two technical replicates for GPR55 and three for GPR119. Welch's ANOVA test was applied. **** denotes P<0.0001, n.s. means not significant. The data were also shown as fold change (2^-ddCt) in gene expression C) and F) calculated by Livak method compared to group 1.

In the current study, we detected the expression of both GPR55 and GPR119 throughout the development of rat testis from PND 7 to 70 by two different methods, qRT-PCR and western blotting. The abundance of GPR55 transcript and protein was at the lowest levels at PND 7, then increases at PND 14 (not significant). From PND 14 and thereafter, GPR55 expression was easily detectable, but exist at variable amounts. Interestingly, immunohistochemical staining shows that GPR55 protein signal was detectable only in 56, 63 and 70 post-natal day rat tissues but undetectable in others. Similarly, GPR119 was expressed at the lowest amount at PND7 compared to other time points, then shows a shifting protein and mRNA abundance in the later weeks. In line with GPR55, positive signal for GPR119 protein was detectable only at PND 56, 63 and 70 but not before that. Even though both IHC and western blotting are used to detect protein targets, their detection sensitivity might differ by the abundance of target protein and steps of sample processing, which might the reason of negative IHC staining for GPR55 and GPR119 at earlier than PND56.

In rats, one spermatogenesis cycle takes about 8 weeks during which the sperm begins to develop and completes 8 different developmental stages until to become ready to be released into the tubular lumen (10). GPR119 immunoreactivity was only seen in spermatids (residual bodies of spermatids and round spermatids) in stage VII and VIII tubules which have mature spermatozoa ready to be released. However, GPR55 signal was observed both in spermatids as well as in spermatocytes at late stage tubules. Immunohistochemically, the presence of a strong positivity for both GPR55 and GPR199 at the 8th week suggests that both receptor signaling might be modulating the completion of spermatogenesis process. In line with our findings, a previous study from Amoako et al. indicated that GPR55 mRNA is present in human sperms and related with the normal sperm count, motility and morphology (1). Sperms with decreased motility have significantly lower GPR55 transcript levels than normal sperm samples (1). Furthermore, the engagement of GPR55 with a lipid ligand, palmitoylethanolamide (PEA), lead to increased mobility in human sperms (2). On the other hand, GPR119 transcripts were previously detected in normal human testis, but the role of GPR119 in testis and spermatogenesis still remains to be investigated (43). However, lower levels of OEA, a GPR119 receptor ligand, in human seminal plasma has been associated with abnormal sperm count, morphology and motility (3). Conversely, *in vitro* OEA-treatment of normal human sperms led to increased survival and motility, suggesting that these effects might be through GPR119 receptor (3). With findings from our study displaying an active GPR119 expression throughout the rat testis development, GPR119-mediated signaling might influence multiple stages of male gametogenesis.

Overall, CB1 and GPR55 receptors are extensively expressed in the central nervous system and the ECS is known to regulate gonadal function via the hypothalamic-pituitary-gonadal (HPG) axis (14). In addition to their endocrine regulatory effects, ECS components are expressed in the testis and might directly contribute to the sperm development and function. Unraveling the developmental regulation of the receptors and ligands of the ECS will enlighten both the molecular mechanisms of normal testis physiology and their involvement in male reproductive system pathologies such as infertility and cancer. Testicular expression of GPR55 and GPR119 receptors from early to late developmental stages shown by our study corroborates the possible role of these receptors in sperm development. Further investigation of GPR55 and GPR119 signaling through functional studies are necessary to clarify their role in key stages of spermatogenesis.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author Contributions

HT and VI conceived and planned the experiments. VI performed histopathology and immunohistochemistry experiments. MT carried out western blot analysis. HT, AK, and MMK performed RT-PCR experiment. HT, VI, and MMK contributed to preparation of testicular samples. HT, VI, MT, AK, and MMK contributed to the interpretation of the results. HT took the lead in writing the manuscript. All authors read 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.

Ethical Statement

All animal procedures were performed in accordance with the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Board (13.03.2019/501)'s regulations and approval.

Animal Welfare

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

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Molecular and serological investigation of *Lawsonia intracellularis* in weanling foals in Türkiye

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ABSTRACT

Equine proliferative enteropathy caused by *Lawsonia intracellularis* has been described in weanlings in many different countries including the USA, Western Europe, Israel, Korea and Brazil but not investigated in Türkiye and Eastern Europe. The objective of the study was to determine the seroprevalence and fecal shedding of *L. intracellularis* in weanling foals. A cross-sectional study was designed in randomly selected 97 weanling foals with or without clinical signs from 3 different provinces of Türkiye. Total protein and albumin levels in serum samples from 97 foals were measured using automated biochemistry analyser. Serum samples and fecal samples were also tested by bELISA and real-time PCR to detect *L. intracellularis*-specific antibodies and -DNA, respectively. Seropositivity was found to be 25.8% by bELISA. However, none of the fecal samples were positive by real-time PCR. Statistically, no significant associations were found between seropositivity and clinical signs or serum ALB/TP levels. Seropositivity indicated the exposure of weanling foal population to *L. intracellularis* for the first time in Türkiye. Further investigations are needed to better understand the epidemiology of the disease in the weanlings as well as adult horse populations and potential wildlife resorvoirs in the country/region.

Introduction

Lawsonia intracellularis (L. intracellularis), the causative agent of equine proliferative enteropathy (EPE), is an obligate Gram-negative, intracellular bacterium. EPE has been diagnosed increasingly in horse populations in the world (6, 8, 13). *L. intracellularis* mainly infects pigs and horses but a number of other animal species were also reported to be susceptible including rabbits, rodents, foxes, wild pigs, deer, ferrets, ostriches racoon dogs, Korean water deer, non human primates and domestic dogs (11). *L. intracellularis* infected fecal materials of wildlife reservoirs and subclinically infected horses are the sources of infection (7, 13, 15, 16).

L. intracellularis typically affects weanling age group under 1 year old of horses (3, 13, 15). The agent is mainly encountered in weanling foals possibly because of declined maternal antibodies and management changes. Seperation of a foal from the dam, transportation, introduction to a new herd, deworming therapies, vaccination schedules and training can cause stress and all of these could be predisposing factors (13). EPE was often diagnosed between August and January as this period is the weanling time for most foals in the northern hemisphere (12, 13).

Clinical cases of *L. intracellularis* infection generally manifests nonspecific signs such as lethargy, high rectal temperature (≥38.5 °C), peripheral edema, weight loss, colic and diarrhea. (1, 10, 15) Recovery period can take weeks to months before they regain the appearance of unaffected cohorts (13, 15). *L. intracellularis* infections are located in the intestinal enterocytes and clinical cases of EPE generally lack an intestinal inflammatory response; hypoproteinemia and hypoalbuminemia are the most consistent laboratory

findings associated with EPE (13, 15). Although the actual values depend on the laboratory analyser used, total protein (TP) and albumin (ALB) concentrations are generally less than 5.0 to 5.2 g/dl and less than 3.0 to 3.1 g/dl, repsectively (13).

Presumptive diagnosis of *L. intracellularis* infection is generally made based on the age of the affected animal, clinical signs, hypoproteinemia/hypoalbuminemia and presence of thickened small intestinal wall mainly in the jejenum and ileum in ultrasonography. Other ante mortem laboratory assays such as serological tests for the presence of antibodies and detection of the agent in feces by molecular assays were developed (8, 14), because *L. intracellularis* can only be grown in vitro cell culture and requires a specific atmosphere for growth (17).

To our knowledge, there is no report regarding the presence of the fecal shedding and seroprevalance of the disease in weanling foal populations in Eastern Europe and Türkiye. The goal of the present study was to determine the prevalence of *L. intracellularis* infection in weanling foal populations by means of fecal shedding of the agent and seropositivity in Türkiye for the first time.

Materials and Methods

Animals and sample collection: A cross sectional study was designed to detect the serological and molecular prevalence of *L. intracellularis* from weanling foals with or without showing any clinical signs by random sampling as some of the infected foals may not show any clinical signs. The present study was approved by the Ethics Committee of University of Istanbul-Cerrahpasa (Report no. 2019/51). Feces and blood samples were collected randomly from 97 weanling foals from İzmit province (n=17), Bursa province (n=59) and Thrace (Silivri) region (n=21) (Figure 1) between September and January. During sampling, clinical signs including high rectal temperature (≤38.5 °C), diarrhea, peripheral edema, weight loss and lethargy were also recorded. Fecal samples were collected directly from the rectum and put into sterile plastic containers for detection of *L. intracellularis* DNA. Blood samples were collected by jugular venipuncture using 10 ml sterile vacutainer serum tubes for serological determination of specific anti-*L. intracellularis* antibodies. After seperation of serum samples, TP and ALB levels were measured using automated biochemistry analyser. A cut off value of <5.0 g/dl for hipoproteinemia and <3.0 g/dl for hipoalbuminemia were used described as in the previous article (13). Fecal samples were frozen (-20 °C) until used for real time PCR analysis.

Serology (ELISA): Serum samples for antibodies against *L. intracellularis* were examined by using a commercially available blocking ELISA (bELISA) kit (Svanovir® *L. intracellularis*/ileitis-Ab, Art No: SV122275, Sweden) according to the manufacturer's instructions. In summary, serum samples were diluted 10-fold and incubated in microplate wells, precoated with whole cell *L. intracellularis* antigen, for 1 hr at 37 °C. The microplate wells were then washed three times with 300 µl washing buffer, and 100 µl diluted conjugate (Svanovir®, Sweden)

Figure 1. A represeantitive of TaqMan probe based real-time PCR analysis in fecal samples. All samples were negative and positive contol Ct value was around 23.

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(1/100) was added in each well. The wells were washed again three times with 300 µl wash buffer. 100 µl of substrate solution (Tetramethylbenzidine in substrate buffer containing H_2O_2) that included in the kit was added to each microplate well, microplate wells were incubated for 10 min in dark at 22 °C. Following incubation period, the reaction was stopped by adding 50 µl stop solution to each well and gently mixing the microplate wells. The reactions were read in an ELISA reader (AllSheng AMR-100 Microplate ELISA Reader, Republic of China) at 450 nm wavelength. Negative control and positive control serum were also included as reference provided by the manufacturer. Percent of inhibition (PI) was calculated according to the formula as in the manufacturer instructions. Samples with a PI <20 % were concluded as negative, samples between 20 %-30 % inconclusive and samples >30 % positive.

Real-time PCR detection of L. intracellularis from fecal samples: Nucleic acid isolation and real time PCR detection assays were carried out according to the previous study with minor modifications (16). Briefly, 2 ml of sterile isotonic saline solution were added to 1 g of fecal material in a sterile centrifuge tube. Afterwards, the mixture was vortexed for 5 minutes and then centrifuged at 12500 rpm for 1 min. 150 µl of supernatant fluid was used to purify DNA by using a commercial spin column DNA isolation kit according to the manufacturer's instructions (Patho Gene-spin™ DNA/RNA Extraction Kit, Intron Biotechnology, Korea).

To increase the sensitivity of the assay, the DNA samples underwent first a DNA precipitation and PCR preamplification step according to the previous study (16). Briefly, 50 µl of sample was precipitated using 3 µl of 5 M NaCl, 5 µl of (5 mg/ml) glycogen and 150 µl absolute ethanol. The mixture was inverted several times and stored at -20°C overnight. The sample was then centrifuged at 12500 rpm for 15 min. The supernatant was removed and 100 µl of 70 % ethanol was added to the sediment and resuspended. The last step was repeated and followed by drying the sediment for 10 min. The sediment was then resuspended in 20 µl of sterile DNase, RNase free water and stored at -20 °C until analysis. The pre-amplification step was carried out using *L. intracellularis* aspartate ammonia lyse (AAL) gene specific primer pairs (Table 1). The 20 µl PCR setup consisted of 10 µl Platinum™ II Hot-Start PCR Master Mix (2X), 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM), 4.2 µl sterile nuclease free water and 5 µl of template DNA. Cycling conditions were initial denaturation for 2 min at 94 °C and 35 cycles of denaturation step for 5 sec at 98°C and combined annealing/extension step for 15 sec at 60 °C.

After the preamplification step, real time TaqMan® PCR in real time PCR system (Applied Biosystems 7300 Real Time PCR, U.S.A) was performed in extracted DNAs for the presence of the AAL gene of *L. intracellularis* (16). The 20 µl master mix included 10 µl of 2X AmpliGene™ qPCR Probe Mix Hi-Rox, 0.8 µl of forward primer (10 µM), 0.8 µl of reverse primer (10 µM), 0.4 µl of probe (10 µM), 3 µl of sterile nuclease free water and 5 µl of template DNA. The cycling conditions were 1 cycle for 2 min at 95 °C and 40 cycles for 5 sec at 95 °C, 30 sec at 65 °C. All amplification reactions were performed duplicate with positive DNA extracted from cell culture infected with *L. intracellularis* (kindly provided from Professor Erdal Erol, University of Kentucky, U.S.A) and negative control.

Statistical Analysis: Kappa analysis was used to determine a significant relationship between bELISA positivity and presence of clinical signs related to *L. intracellularis* infection. Chi-square test was used to determine the significance of differences in the prevalence among regions and to determine if any significant relationship was yielded in TP/ALB value and bELISA positivity. *P* values of <0.05 were considered to be significant using SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0).

Table 1. Oligonucleotide sequences of PCR primers and probe used in the study (16).

Target Gene:	Assay:	Primer/Probe:	Oligonucleotide Sequences:
***L. intracellularis* aspartate ammonia lyse gene**	Preamplification/ PCR:	Forward primer	5'-AATTTGTTGTGGATTGTATTCAAGGA-3'
		Reverse primer	5'-CTTTCTCATGTCCCATAAGCTCAA-3'
	Real Time PCR:	Same primer pairs given above.	
		TaqMan® MGB™ Probe	5'-6-FAM-CAGGGACAAGTACAAATATGAATGCTAATGAAGCAA-MGB-3'

Table 2. Province residing, breed, age, clinical signs (h.r.t.: high rectal temperature, d.: diarrhea, w.l.: weight loss, leth.: lethargy, Tb: Thoroughbred.), serum TP-ALB value and bELISA -Percent of Inhibition (PI) value of seropositive foals. The data of serum TP, albumin and ELISA % inhibition values in foals with no clinical signs were shown in range (with minimum and maximum values).

Clinical status	ELISA seropositivity	Sample no.	Province	Breed	Clinical signs	Serum TP value	Serum ALB value	ELISA PI (%)
Clinically ill	ELISA-Positives	7	Silivri	Tb.	w.l.	5.1	3.2	40.73
		39	Karacabey	Tb.	w.l.; d.	5.9	3.2	51.71
	ELISA-Inconclusives or -Negatives	1	Silivri	Tb.	h.r.t.	5.9	3.8	14.56
		3	Silivri	Arab	w.l.	5.6	3.7	20.36
		6	Silivri	Arab	w.l.	5.3	3.5	19.83
		33	Karacabey	Tb.	d.	6.8	3.52	24.48
		34	Karacabey	Tb.	w.l.; d	6.0	3.4	6.33
		56	Izmit	Tb.	w.l.; leth.	6.2	3.3	16.03
		62	Izmit	Tb.	d.	6.0	3.0	23.95
		97	Izmit	Tb.	w.l.; d.	6.3	2.4	6.58
No clinical sign	ELISA-Positives	23 samples	Karacabey and Silivri	All Tb.	-	5.7-7.8	1.7-3.8	30.82-66.50
	ELISA-Inconclusives or -Negatives	Remaining 64 samples	All three provinces	Both breeds	-	5.3-7.7	2.7-4.0	2.38-29.97

Results

The mean age (± standart deviation) of the foals was 6.8 (±1.4) months ranging from 5 to 10 months old. Five (5.1 %) out of 97 foals were arabian and 92 (94.8 %) were Throughbred foals (Table 2). While a total of ten (10.3%) horses had one clinical sign (such as diarrhea, weight loss, lethargy or high rectal temperature), the remaining 87 foals did not show any clinical signs at the time of sampling (Table 2). None of the foals had peripheral edema. Mean TP and ALB values of the studied foals were 6.1 g/dl (± 0.5) and 3.4 g/dl (± 0.4), respectively.

ELISA results yielded that 25 foals were positive for *L. intracellularis* specific antibodies with a seroprevalence of 25.8 % (Table 2). Forty five (46.4%) and 27 (27.8%) of the foals' ELISAs resulted as inconclusive and negative, respectively. In the group of clinically ill foals, 2 of the foals were ELISA-positive while the remaining 8 foals were either ELISA-inconclusive or –negative (Table 2). In the group of foals with no clinical sign, 23 foals were ELISA positive; the remaining 64 foals were either ELISA inconclusive or negative (Table 2). Mean TP and ALB values in foals with a positive serology were 6.2 g/dl (± 0.5) and 3.3 g/dl (±0.5) respectively.

The distribution of the seropositivities according to the provinces were 6.2%, 1.0% and 18.5% in Silivri, Izmit and Karacabey region, respectively (Table 2). No significant difference was found between provinces for ELISA positivity (P= 0.117). No significant association was found between TP (P= 0.760) / ALB (P= 0.097) value and ELISA positivity. Statistical analysis yielded no significant relationship between presence of one or more than one clinical signs and ELISA positivity (κ= 0.108).

None of the fecal samples yielded positive results by real-time PCR (Figure 1).

Discussion and Conclusions

To the authors' knowledge, this is the first study that investigated the seropositivity and the status of fecal shedding of *L. intracellularis* by PCR in weanling foals in Eastern Europe and Türkiye. In this study, we targeted a weanling foal population (between 5 and 10 months old) as the most of the infections occur in animals under 1 year old (3, 4, 13, 15).

Different seropositivity rates were found in several previous studies. In the Netherlands, the seroprevalence was around 15 % in pre-weanling foals and seroprevalence increased significantly (to 23 %) in weanling (8). In the same study seropositivity was 89 % and 99 % in yearlings and older than 2 years old horses respectively. In Korea, seroprevalence of *L. intracelluaris* was determined to be 94%, (92 % in <2 years, 96 % in 2-5 years, 94 % 6-10 years and 93 % in >10 years) by bELISA while *L. intracellularis* DNA was found to be 18% in fecal samples. Contrary to serology, the younger age was associated with the PCR positivity; 3 % in <2 years, 25 % in 2-5 years, 13 % in 6-10 years and 8 % in >10 years (11). A recent study in Belgium also demonstrated 98.8% seropositivity by bELISA and

confirmed high seropositivity rates in adult horses (mean age 12.1 years) (9). It is evident that exposure of horses to *L. intracellularis* increases by age (11). In Brazil, 9.42 % of adult mares and weanling foals were found to be positive by immunoperoxidase technique (4). In the same study, fecal shedding was found to be 3.14 % by PCR. A recent study in Brazil demonstrated similar seropositivity (5.55 %) and fecal shedding (4.32 %) (2). A recent study conducted in Israel found close results to the present study with 30.5 % and 0.7 % horses positive by ELISA and PCR, respectively (18). The differences between the present study and others could be related to differences in geoghraphy, sample size and disease status of the horses.

In the present study, although serologically positive horses were detected, none of the fecal samples contained *L. intracellularis* DNA. This strongly suggests prior exposure of *L. intracelluris* in the horse populations in Türkiye. Low sample size (and only 10 of them with clinical signs) and possible intermittent shedding are likely the major factors for not detecting *L. intracellularis* DNA in fecal samples. Even though the analytical sensitivity of the PCR method used in our study was relatively high (as low as one *L. intracellularis* organism after DNA precipitation and PCR preamplification step) (16), detection of *L. intracellularis* could be limited due to the PCR inhibitors presented in fecal samples (13). As our assay did not contain internal control, PCR inhibition was not be able to be measured. This is in concordance with the the previous studies that determined high seropositivity and low fecal shedding of the bacteria (4, 11, 18). Seroconversion in horses has been documented to occur approximately 14 days after experimental challenges (17) and remain seropositive for more than 6 months (19) but fecal shedding of the microorganism starts between 12-18 days postinoculation and lasted for 11 to 21 days (17).

The same bELISA kit was also used in foals and adult horses in earlier studies (8, 9, 11). In a previous study, the authors compared the bELISA with immunoperoxidase monolayer assay (IPMA), as a reference test, for the detection of antibodies against *L. intracellularis* in horses. A total of 100 serum samples were tested and found 90 % sensitivity with 94 % specificity, 88 % sensitivity with 96 % specifity and 85 % sensitivity with 98 % specifity at 1/125, 1/150 and 1/175 dilutions, respectively, and concluded as an useful alternative for diagnosis of EPE (5).

In conclusion, the present study resulted in a 25.8 % seroprevalence against *L. intracellularis* in the weanling foals with or without clinical signs for the first time in Eastern Europe and Türkiye. However, no fecal shedders were found in fecal samples of the weanling foals by real-time PCR. The results of the study strongly suggest that the weanling foal population in Türkiye is exposed to *L. intracellularis* and therefore clinicians should be aware of this pathogen in differential diagnosis of enteric diseases. Further studies are needed for determination of fecal shedding in foals, adult horses and wildlife animals in order to better understand the status and epidemiology of *L. intracellularis* in Türkiye.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

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

Data Availability Statement

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

Ethical Statement

The present study was approved by the Ethics Committee of University of Istanbul-Cerrahpasa (Report No. 2019/51).

Animal Welfare

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

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Effects of short-term transportation and lairage time on meat quality, *CAST*, *CAPN1*, *CAPN3*, *HSPA1A* gene expression levels and myofibrillary structure of *M. longissimus dorsi* of Kıvırcık breed sheep

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ABSTRACT

The objective of this study was to examine the effect of short-term transportation and lairage time on meat quality, myofibrillary structure, calpastatin (*CAST*), μ-calpain (*CAPN1*), calpain 3 (*CAPN3*), and heat shock protein 70 (*HSPA1A*) expression levels of *M. longissimus dorsi* of Kıvırcık breed sheep (n=40). Meat quality was evaluated by muscle glycogen amounts, pH, temperature, cooking loss, water holding capacity, shear force, instrumental color, sarcomere length, and organoleptic properties. Cellular changes in the muscle during the aging process were scanned by electron microscopy and *CAST*, *CAPN1*, *CAPN3*, *HSPA1A* expression levels were measured to reveal the association on meat tenderness. As a result, a positive effect of long lairage time groups was observed in the pH, glycogen, SF values, and organoleptic evaluation. *CAST*, *CAPN1, CAPN3* expression showed no difference, however, *HSPA1A* showed a significant difference in the aging process. In conclusion, genotypic differences, their effect on gene expression, and protein level on meat quality should be further investigated.

Introduction

Animal welfare promotes living conditions that are free from diseases, injury, malnutrition, pain, distress, and hunger (44). Animal welfare includes not only the physical health and biological conditions of the animal but also the affective states of the animal that improve and standardize meat quality (34, 43). In animal husbandry, transportation is one of the most important factors affecting the welfare and health of the animals before slaughter (1).

According to animal welfare guidelines, it is recommended to choose the closest possible slaughterhouse location to the farm or a maximum of 8 h of transport called short term transportation (33). Schaefer et al. (39) stated that transportation that is not performed under appropriate conditions decreases meat quality. Animals should be rested at least 24 hours before slaughter to eliminate the negative effects of transportation (29). The lairage period has considerable attention for recovering from stress experienced during the transport (21). Mounier et al. (35) reported that lairage time could allow animals to recover muscle glycogen concentrations. Terlouw et al. (41) indicated that 24-hour lairage could be returned conditions before shipping.

Several studies regarding the effect of transport and lairage time on meat quality were carried out by Ekiz et al. (13), Diaz et al. (12), Najafi et al. (36) in lambs and Ferreira et al. (16), Gallo et al. (18), Maria et al. (34) in cattle. Consumer acceptance of meat depends on its quality characteristics including color, tenderness, flavor, and water holding capacity (WHC) can be affected by proteolytic degradation of the myofibrillar protein (31). In the proteolytic enzymes, calpastatin (*CAST*), μ-calpain (*CAPN1*), calpain 3 (*CAPN3*) and heat shock protein (*HSPA1A*) *g*enes are responsible for all biochemical reactions in the conversion of muscle to meat (5). However, the effects of *CAST*, *CAPN1*, *CAPN3*, *HSPA1A* expression levels on aging and quality have not been extensively investigated.

The aim of the study was to determine the effect of short-distance transport and lairage time on glycogen content and meat quality of Kıvırcık breed sheep and association with expression levels of *CAST*, *CAPN1*, *CAPN3*, and *HSPA1A g*enes. And also, the changes in the muscle cells during the aging, the sarcomere lengths, and contractile band shapes were examined, the histological structure of meat was scanned by scanning electron microscopy.

Material and Methods

Animals and experimental design: In the study, 40 male Kıvırcık breed yearling lambs were used. Pregnant sheep were taken to the birth chamber 2 weeks before the birth. After the birth, sheep and lambs were placed in individual compartments for three weeks. Lambs were weaned on day 75 and were fed with ad-libitum concentrate (16% HP) and roughage until slaughter time. Kıvırcık lambs were raised under the same breeding program until the age of about 1 year. The care and feeding of the lambs from birth to slaughter time were carried out in İstanbul University, Faculty of Veterinary Medicine, Training and Research Farm Sheep Unit (The research protocol of the study was approved by the Animal Experiment Local Ethics Committee of İstanbul University 2016/15). Yearling lambs were weighted around 46 ± 2 kg.

Pre-slaughter and slaughter process: Yearling lambs were transported from the Research Farm Sheep Unit to the slaughterhouse in a 1.90 m × 4 m truck body and a maximum of 20 animals were transported at a time. To eliminate the effect of large temperature variation during transport, the study was carried out at seasonal temperatures of 15-20°C. Transportation time was set at 3 hours. Yearling lambs were separated into 4 groups, consisting of 10 individuals. The first group was slaughtered immediately after transportation (LT_0), the second group was slaughtered after 12 hours lairage time (LT_{12}), and the third group was slaughtered after 24 hours lairage time (LT_{24}). The fourth group, kept as a control (C), was treated according to guidelines (21), where the animals were rested for 1 week at the slaughterhouse resting paddocks and then slaughtered.

Slaughtering was carried out in accordance with animal welfare in İstanbul University-Cerrahpaşa Faculty of Veterinary Medicine Education Research Slaughterhouse. Animals were electrically stunned at 220-250 voltage, 1.0–1.3 amps, and 1–3 second, stunning tongs applied on both sides of the head. The bleeding process was followed by skinning and removal of the internal organs, which took approximately 30 ± 5 minutes.

Carcass sampling and meat quality analysis: Samples, which were immediately taken after slaughtering (*M. longissimus dorsi* (LD) of the right side of carcasses) muscles, were used for biochemical, histological, and molecular analysis. The left side of carcasses were stored at the cold chain until 24 hours of postmortem and then used for meat quality (cooking loss, water holding capacity, shear force, sarcomere length, and color) and sensorial analyses. Samples were vacuum-packed in Cryovac barrier bags (Cryovac Sealed Air Corp., New Jersey, USA) and stored in cold storage (0-4°C) for 10 days. Molecular analysis of samples was carried out on days 0 and 2, biochemical and histological analysis were carried out on days 0, 2, 5, and 10. Meat quality and organoleptic analyzes were performed on days 2, 5, and 10.

Meat quality analysis: Muscle glycogen determination was performed using the Glycogen Assay Kit (Biovision, USA #K646-100) following the manufacturer's instructions for the colorimetric assay.

The temperature and pH were measured of the samples taken from the upper surface of the LD at the 1st hour of the postmortem stage and at different control times (1^{st}, 3^{rd}, 6^{th}, 12^{th}, 24^{th} hours) within the first day. A digital thermometer (Hanna HI 99163, USA) was used for

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temperature measurement. The electrodes of pH and temperature devices were placed near the center of three different points of the muscle and calculated by taking the average of the determined values (27).

Cooking loss (CL) analysis was carried out with 50 g meat samples, which were cooked in a mini water bath at 100°C for 10 minutes. After cooking, the meat was dried and weighed (45). The cooking loss was calculated by the following formula:

Cooking Loss: 100-100 × weight after cooking / weight of raw meat

The percentage of free liquid was evaluated as a measure of water holding capacity (WHC) by the filter press method described by Vural and Öztan (47). The outline area of the expressible juice and the meat film were traced, and two areas were measured using AUTOCAD 2008 (Apso Ltd, UK). The water retention capacity was calculated by the following formula:

Water Retention Capacity: Spread Area of Meat (cm^2) / Total Area (cm^2)

Shear force (SF) analysis of LD muscles was performed by Warner-Bratzler shear force texture analyzer (Instron, model 3343, UK). The meats taken on the relevant days of the enclosure were sliced 1 × 1 cm thick and then cooked in a water bath at 100°C for 10 minutes. For each sample, the highest force (kg / cm^2) and force x time graph of the Warner-Bratzler blade cutting sequence were recorded to a computer using the BlueHill 2 operating system (46). SF value was determined by calculating the arithmetic means of the multiple readings obtained from the samples.

Instrumental color measurements of the muscles at five different locations on each meat sample were averaged at each sampling day, immediately after opening each package in terms of CIE *L** (lightness), *a** (redness), and *b** (yellowness) values by measuring with a Colorflex HunterLab Spectrophotometer (Hunter Associates Laboratory Inc., Reston, VA, USA). Color values were evaluated using diffuse illumination (D65 2° observer) with an 8 mm viewing aperture and a 25 mm port size with the specular component excluded from the colorimeter (2).

Histological analysis and Scanning Electron Microscopy (SEM): To determine the sarcomere length (SL), 1 cm^3 of tissue was collected and fixed in 10% neutral buffered formalin. After following a routine histological process, the tissue was embedded in paraffin. Masson's Trichrome staining was performed on the sections and photographed by a microscope (Leica DM2500, Leica Microsystems GmbH, Wetzlar, Germany) with a digital camera (Leica DFC450, Leica Microsystems GmbH, Wetzlar, Germany). Each sample was evaluated by measuring 10 different sarcomere lengths (9).

For SEM analysis, the samples were rinsed with physiological saline then fixed in FAA solution (10% formaldehyde, 5% acetic acid, and 85% alcohol) solution. Following dehydration protocols and coated with hexamethyldisilazane (HMDS), they were placed in copper containers. Samples were sent to the Department of Histology and Embryology of the Faculty of Veterinary Medicine of Harran University and visualized under the scanning electron microscope (EVO50, Zeiss, Germany).

Gene expression analysis: Total RNA were isolated using 100-200gr pieces that were cut from the lateral ½ part of the LD muscle. Samples were homogenized with liquid nitrogen and mixed with PureZol (Biorad, US). An additional step was applied to remove the lipid in the meat, all samples were stored at -80°C overnight in PureZol solution (Biorad, US). On the second day, tissue residues were removed by 10 min centrifuge at 15000 rpm at +4°C. Then PureZol instructions were followed. Obtained RNAs were quantified spectrophotometrically, and DNase treated (5U/ul). Total cDNA were synthesized using 100 ng/ul RNA template by iScript™ Reverse Transcription Supermix **(**Biorad, US). Amplification program of the cDNA was 50°C for 2 min and 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers for target genes were designed specifically to the exon-exon junction points on the mRNA reference sequences using Primer3 website (Table 1) and GAPDH used as housekeeping gene. Real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Biorad, US) with 3 technical replicas by CFX Connect real-time PCR System (BioRad, US). The obtained cycle threshold values (Cycle Threshold, CT) were normalized according to housekeeping and differences between the groups were analyzed.

Sensory analysis: Organoleptic properties of LD samples were evaluated by a panel of ten trained participants of the staff of İstanbul University-Cerrahpaşa, Food Hygiene and Technology Department, who had previously participated in training sessions to become familiar with the sensory characteristics of meat (23, 25). The panelists were requested to score the sensory attributes (color intensity, color hue, odor intensity, tenderness, chewiness, juiciness, flavor intensity, flavor quality, and general appearance acceptability) using an unstructured 10-point line scale (0: extremely unacceptable, 1: unacceptable, 5: between acceptable, 10: extremely acceptable).

Table 1. Designed oligonucleotides sequences, amplicon length, annealing temperatures, and accession numbers.

	Oligonucleotides sequence	Amplicon length	Annealing temperature	Accession
CAST_F	CTGTGGCAGAGGATGTGCCT	109bp	62ºC	NM_001009788.1
CAST_R	GCCAAGGCTTCCACAGCATC			
HSPA1A_F	CCTGTTCGAGGGCATCGACT	193bp	60ºC	NM_001267874.1
HSPA1A_R	CAGCAGCTTCTGCACCTTGG			
GAPDH_F	GTCAGCAATGCCTCCTGCAC	138bp	60ºC	NM_001190390.1
GAPDH_R	GGAAGGGCCATCCACAGTCT			
CAPN1_F	AGTGGGTGGATGTGGTGGTG	75bp	60ºC	NM_001127267.1
CAPN1_R	CTTGGGCAGAGTGCACGAAC			
CAPN3_F	AAGCCTCCAGGCACCTCATC	183bp	60ºC	NM_001009212.1
CAPN3_R	CTGGCCTGTGGATAGCCGAA			

Table 2. Definition of the attributes used in the sensory analysis of meat samples.

Attribute	Definition
Color intensity	Clear, strong red color
Color hue	Deviation of color from yellow/red to red/green
Odor intensity	The intensity of sum of all odors related to sheep
Tenderness	The force required to bite through the sample
Chewiness	Time and numbers of chewing required to masticate the sample ready for swallowing
Juiciness	Perception of water content in the sample after 3-4 chewing
Flavor intensity	Level of the overall flavor in the mouth
Flavor quality	Flavor experienced prior to swallowing related with sheep
General appearance acceptability	Overall liking from the panelists

Primarily, the panelists were trained in three separate sessions for approximately 2 hours for the evaluation of each selected attribute (Table 2). Training sessions were conducted to acquaint panelists with the products and attributes to be evaluated and were followed by an open discussion session to familiarize panelists with the attributes and the scale to be used.

Following that a kitchen-type oven (Siemens, Germany) was used to cook meat samples at 200°C until the internal temperature reached 80°C. Samples were then served immediately to the panelists where they were seated in individual booths in a temperature and light-controlled room (fluorescent lighting of 2000 lx; Philips 40W Cool White). Each sample was labeled, at random, with a two-digit code number and sensory analysis was performed in duplicate in two sessions (24).

Statistical analysis: Descriptive statistics were calculated for all variables measured in the study and shown as "Arithmetic Mean ± Standard Error of the mean". Prior to the significance tests, the data were checked by Shapiro Wilk in terms of conformity to the normal distribution from the parametric test assumptions, and Levene's test in terms of homogeneity of variances. Two-way analysis of variance was applied by using the general linear modeling technique to analyze the effect of time and group on the variables obtained. In the established model, the basic effect of "Group" and "Time" and "Group x Time" interaction terms are included. In cases where interaction terms were found to be meaningful, simple effects analysis was performed by applying Bonferroni correction to analyze the interaction terms. In cases where the interaction terms were not significant, Bonferroni corrected pairwise comparisons were performed for any significant main effect term in the model to evaluate differences among the levels within each factor. Stata 16.1 was used for data analysis. P-value <0.05 criterion was taken into consideration in all statistical decisions. In gene expression analyzes, delta-delta Ct values obtained from the groups were evaluated with Student's *t*-test and obtained values from the comparisons between groups were examined. In the calculation of fold change, a previously proposed model was used (37). Each result represents the mean of 10 independent determinations.

Results

The glycogen values were found to be the lowest, especially in the groups with high lairage time (LT_{24}) and C, 4.33 and 4.21 respectively, whereas LT_0 group, the highest glycogen level was recorded with 5.62 (Table 3). The lowest pH values were determined in the LT_{24} and C groups, 7.20 and 6.99 respectively (P<0.001). Besides, in

correlation with the glycogen level, pH values continued to decrease with the longer storage time (P<0.001).

SF values were low in the LT_{24} and C groups (P<0.001), and decreased during the aging period with a similar deviation among groups (P<0.001). SF analyses resulted as 6.15, 5.34, and 4.14 on the days 2nd, 5th, and 10th days, respectively (Table 3).

The pH level at 24th hours storage time of LT_{24} and C groups was on average 5.67 whereas in LT_0 and LT_{12} groups pH level at 24th hours was on average 6.10 (Table 4).

Table 3. Mean values, standard errors, and significant interactions of biochemical, histological, and quality characteristics of sheep meats subjected to short-term transportation and lairage time.

Attribute	Time (day)	C	LT_0	LT_{12}	LT_{24}		Significance		
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	LS Mean ± SE	G	T	G x T
CL	2	39.14 ± 0.79	39.12 ± 0.93	39.15 ± 0.66	39.05 ± 0.55	**39.11 ± 0.39**	NS	NS	NS
	5	39.31 ± 0.85	39.47 ± 0.74	39.27 ± 0.94	39.37 ± 0.87	**39.35 ± 0.39**			
	10	39.57 ± 0.67	39.59 ± 0.79	39.61 ± 0.74	39.59 ± 0.69	**39.59 ± 0.39**			
	LS Mean ± SE	**39.34 ± 0.45**	**39.39 ± 0.45**	**39.35 ± 0.45**	**39.33 ± 0.45**				
WHC	2	36.42 ± 1.48	36.46 ± 1.65	36.44 ± 1.54	36.43 ± 0.77	**36.44 ± 0.69** [a]	NS	<0.001	NS
	5	28.73 ± 1.89	28.76 ± 1.75	28.75 ± 1.54	28.74 ± 1.29	**28.74 ± 0.69** [b]			
	10	22.75 ± 0.95	22.73 ± 1.20	22.71 ± 0.83	22.78 ± 1.13	**22.74 ± 0.69** [c]			
	LS Mean ± SE	**29.3 ± 0.8**	**29.32 ± 0.8**	**29.3 ± 0.8**	**29.3 ± 0.8**				
Glycogen value	2	2.37 ± 0.2	2.94 ± 0.36	2.77 ± 0.43	2.39 ± 0.22	**2.62 ± 0.18** [b]	0.005	<0.001	NS
	5	1.92 ± 0.26	2.45 ± 0.32	2.28 ± 0.45	1.93 ± 0.19	**2.15 ± 0.18** [bc]			
	10	1.52 ± 0.12	2.2 ± 0.34	1.93 ± 0.25	1.56 ± 0.16	**1.81 ± 0.18** [c]			
	LS Mean ± SE	**2.51 ± 0.18** [B]	**3.30 ± 0.18** [A]	**3.01 ± 0.18** [AB]	**2.55 ± 0.18** [B]				
*L**	2	21.25 ± 0.67	21.30 ± 0.43	21.33 ± 1.03	21.12 ± 0.42	**21.25± 0.42** [b]	NS	<0.001	NS
	5	28.68 ± 0.82	28.63 ± 0.47	27.74 ± 1.18	28.58 ± 0.48	**28.41 ± 0.42** [a]			
	10	27.92 ± 0.63	27.89 ± 0.89	27.89 ± 1.47	27.89 ± 0.87	**27.90 ± 0.42** [a]			
	LS Mean ± SE	**25.95 ± 0.49**	**25.84 ± 0.49**	**25.65 ± 0.49**	**25.86 ± 0.49**				
*a**	2	15.53 ± 0.49	15.51 ± 0.37	15.55 ± 0.35	15.58 ± 0.51	**15.54 ± 0.19** [b]	NS	<0.001	NS
	5	15.58 ± 0.23	15.65 ± 0.41	15.64 ± 0.35	15.6 ± 0.11	**15.62 ± 0.19** [b]			
	10	16.51 ± 0.21	16.53 ± 0.52	16.49 ± 0.47	16.55 ± 0.3	**16.52 ± 0.19** [a]			
	LS Mean ± SE	**15.86 ± 0.22**	**15.90 ± 0.22**	**15.89 ± 0.22**	**15.91 ± 0.22**				
*b**	2	6.29 ± 0.46	6.33 ± 0.34	6.32 ± 0.8	6.27 ± 0.33	**6.30 ± 0.33** [c]	NS	<0.001	NS
	5	10.1 ± 0.39	10.13 ± 0.84	10.1 ± 0.56	10.12 ± 0.48	**10.11 ± 0.33** [b]			
	10	11.4 ± 0.76	11.39 ± 1.15	11.39 ± 0.61	11.4 ± 0.72	**11.4 ± 0.33** [a]			
	LS Mean ± SE	**9.26 ± 0.38**	**9.29 ± 0.38**	**9.27 ± 0.38**	**9.26 ± 0.38**				
SF	2	5.09 ± 0.16	7.31 ± 0.19	6.62± 0.21	5.58 ± 0.2	**6.15 ± 0.12** [c]	<0.001	<0.001	NS
	5	4.55 ± 0.11	6.03 ± 0.32	5.87 ± 0.31	4.91 ± 0.25	**5.34 ± 0.12** [b]			
	10	3.74 ± 0.09	4.81 ± 0.28	4.2 ± 0.28	3.8 ± 0.27	**4.14 ± 0.12** [a]			
	LS Mean ± SE	**4.46 ± 0.14** [B]	**6.05 ± 0.14** [A]	**5.56 ± 0.14** [A]	**4.76 ± 0.14** [B]				
SL	2	1.37 ± 0.04	1.28 ± 0.05	1.3 ± 0.02	1.38 ± 0.07	**1.33 ± 0.02** [b]	NS	<0.001	NS
	5	1.55 ± 0.02	1.46 ± 0.03	1.49 ± 0.01	1.5 ± 0.01	**1.5 ± 0.02** [a]			
	10	1.51 ± 0.02	1.52 ± 0.03	1.51 ± 0.03	1.53 ± 0.02	**1.52 ± 0.02** [a]			
	LS Mean ± SE	**1.40 ± 0.02**	**1.35 ± 0.02**	**1.38 ± 0.02**	**1.39 ± 0.02**				

[a, b, c]: Different letters in the same column represent statistically significant difference (P<0.05).
[A, B, C]: Different letters in the same column represent statistically significant difference (P<0.05).
NS: Not significant.
C: Control (Animals slaughtered after 1 week of lairage).
LT_0: Immediate slaughter (0 lairage time).
LT_{12}: Animals slaughtered after 12 hours of lairage.
LT_{24}: Animals slaughtered after 24 hours of lairage.
G: Group, T: Time, G x T: Group x Time, LS: Least Square.
*L**: (lightness, *a**: redness, *b**: yellowness.

Table 4. Mean values, standard errors, and significant interactions of pH and temperature changes of sheep meats subjected to short-term transportation and lairage time.

Attribute	Time (hour)	C Mean ± SE	LT_0 Mean ± SE	LT_{12} Mean ± SE	LT_{24} Mean ± SE	LS Mean ± SE	Significance G	T	G x T
pH	1	6.99 ± 0.08 $^{a\,C}$	7.46 ± 0.08 $^{a\,A}$	7.33 ± 0.05 $^{a\,AB}$	7.20 ± 0.03 $^{a\,B}$	**7.24 ± 0.03**	<0.001	<0.001	NS
	3	6.83 ± 0.07 $^{a\,b\,C}$	7.34 ± 0.06 $^{a\,A}$	7.31 ± 0.08 $^{a\,AB}$	7.05 ± 0.08 $^{a\,b\,B}$	**7.13 ± 0.03**			
	6	6.65 ± 0.03 $^{b\,B}$	7.08 ± 0.06 $^{b\,A}$	6.99 ± 0.02 $^{b\,A}$	6.92 ± 0.04 $^{b\,A}$	**6.91 ± 0.03**			
	12	6.09 ± 0.03 $^{c\,B}$	6.54 ± 0.02 $^{c\,A}$	6.32 ± 0.07 $^{c\,B}$	6.00 ± 0.03 $^{c\,B}$	**6.24 ± 0.03**			
	24	5.70 ± 0.03 $^{d\,B}$	6.14 ± 0.02 $^{d\,A}$	6.07 ± 0.03 $^{d\,A}$	5.64 ± 0.03 $^{b\,B}$	**5.89 ± 0.03**			
	LS Mean ± SE	**6.45 ± 0.02**	**6.91 ± 0.02**	**6.81 ± 0.02**	**6.56 ± 0.02**				
Temperature (°C)	1	37.11 ± 0.03	37.1 ± 0.03	37.08 ± 0.03	37.14 ± 0.05	**37.11 ± 0.3** ^{a}	NS	<0.001	NS
	3	26.68 ± 2.63	29.4 ± 0.26	29.39 ± 0.26	29.29 ± 0.27	**28.69 ± 0.3** ^{b}			
	6	21.07 ± 0.09	21.1 ± 0.18	21.11 ± 0.12	21.11 ± 0.17	**21.1 ± 0.3** ^{c}			
	12	11.74 ± 0.12	11.7 ± 0.1	11.73 ± 0.1	11.70 ± 0.07	**11.72 ± 0.3** ^{d}			
	24	1.6 ± 0.06	1.6 ± 0.06	1.6 ± 0.04	1.59 ± 0.07	**1.60 ± 0.3** ^{e}			
	LS Mean ± SE	**19.64 ± 0.27**	**20.18 ± 0.27**	**20.18 ± 0.27**	**20.17 ± 0.27**				

$^{a, b, c, d, e}$: Different letters in the same column represent statistically significant difference (P<0.05).
$^{A, B, C}$: Different letters in the same column represent statistically significant difference (P<0.05).
NS: Not significant, C: Control (Animals slaughtered after 1 week of lairage).
LT_0: Immediate slaughter (0 lairage time).
LT_{12}: Animals slaughtered after 12 hours of lairage.
LT_{24}: Animals slaughtered after 24 hours of lairage.
G: Group, T: Time, GxT: Group x Time, LS: Least Squares.

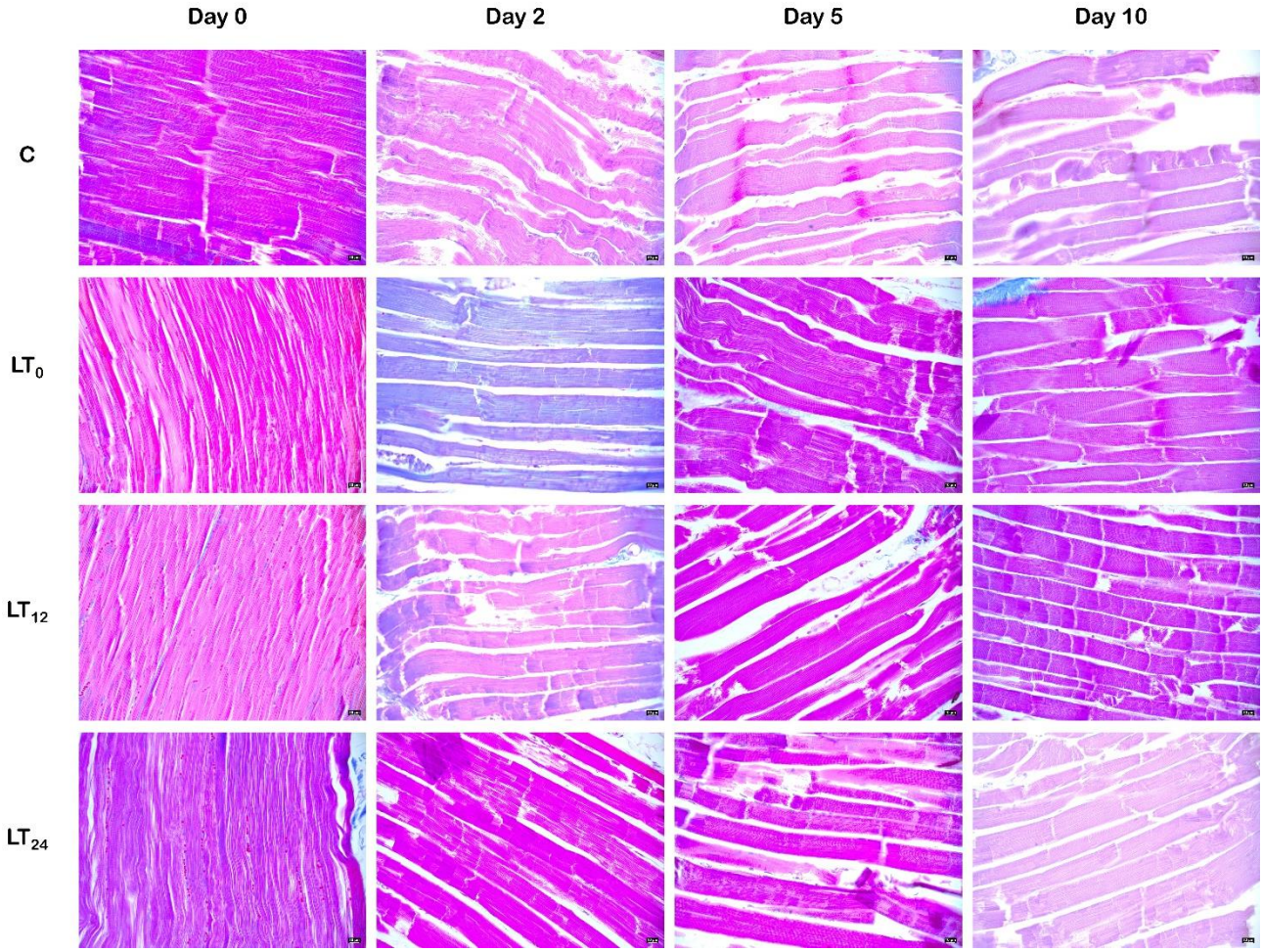

Figure 1. General view of *M. longissimus dorsi* tissue comparing sarcomere lengths. Masson's trichrome staining. Bar: 10 µm.

Conventional light microscopy results were revealed that intercellular space among myofibrils were getting larger towards the day 10 (Figure 1 and 2). Transverse and longitudinal sections of the fibra muscularis were also evaluated with a scanning microscope. According to these results, same as in light microscopy, no change was observed between groups on day 0. In all groups, it was found that the myofibrils homogeneously covered the entire sarcoplasm. On day 2 of maturation, it was noticed that in the cross-sections of the fibra muscularis, intercellular spaces were beginning to be visible among the cells (Figure 3). On day 5, the most remarkable finding was the increase in the number of fat droplets passing outside the cell sarcoplasm (Figure 4). In addition, it was noticed that the myofibrils have lost their homogeneous distribution and started to be separated from each other. As of day 10, it was determined that myofibrils was filled the entire sarcoplasm, and the homogeneous appearance of myofibrils were disappeared due to myofibrillar denaturation and shortening. In addition, the expansion of the intercellular space was confirmed by SEM (Figure 4).

Organoleptic evaluation of the meat samples by the panelists in terms of parameters examined, highest and statistically significant values were obtained for the groups LT_{24} and C (Table 5).

Measurable gene expression was determined for all genes examined in the groups on days 0 and 2nd (< 30Ct). But, due to the rapid degradation of RNA and samples on days 5 and 10, were not included in the molecular analyzes.

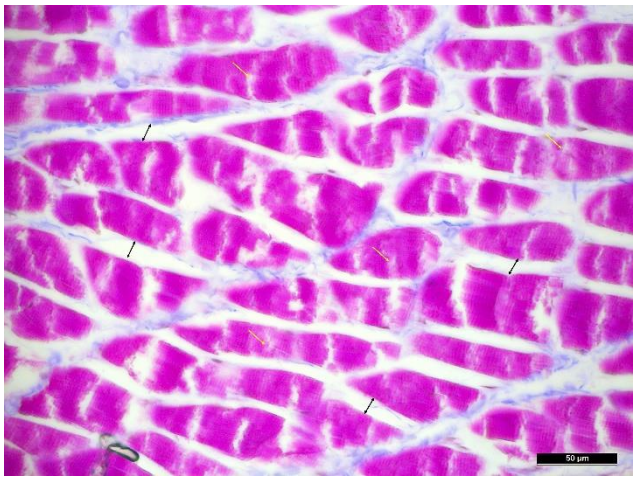

Figure 2. *M. longissimus dorsi* histomorphology on day 10. Increase in intercellular space (double headed arrow), Myofibril denaturation (arrows), Masson's trichrome staining, Bar: 50µm.

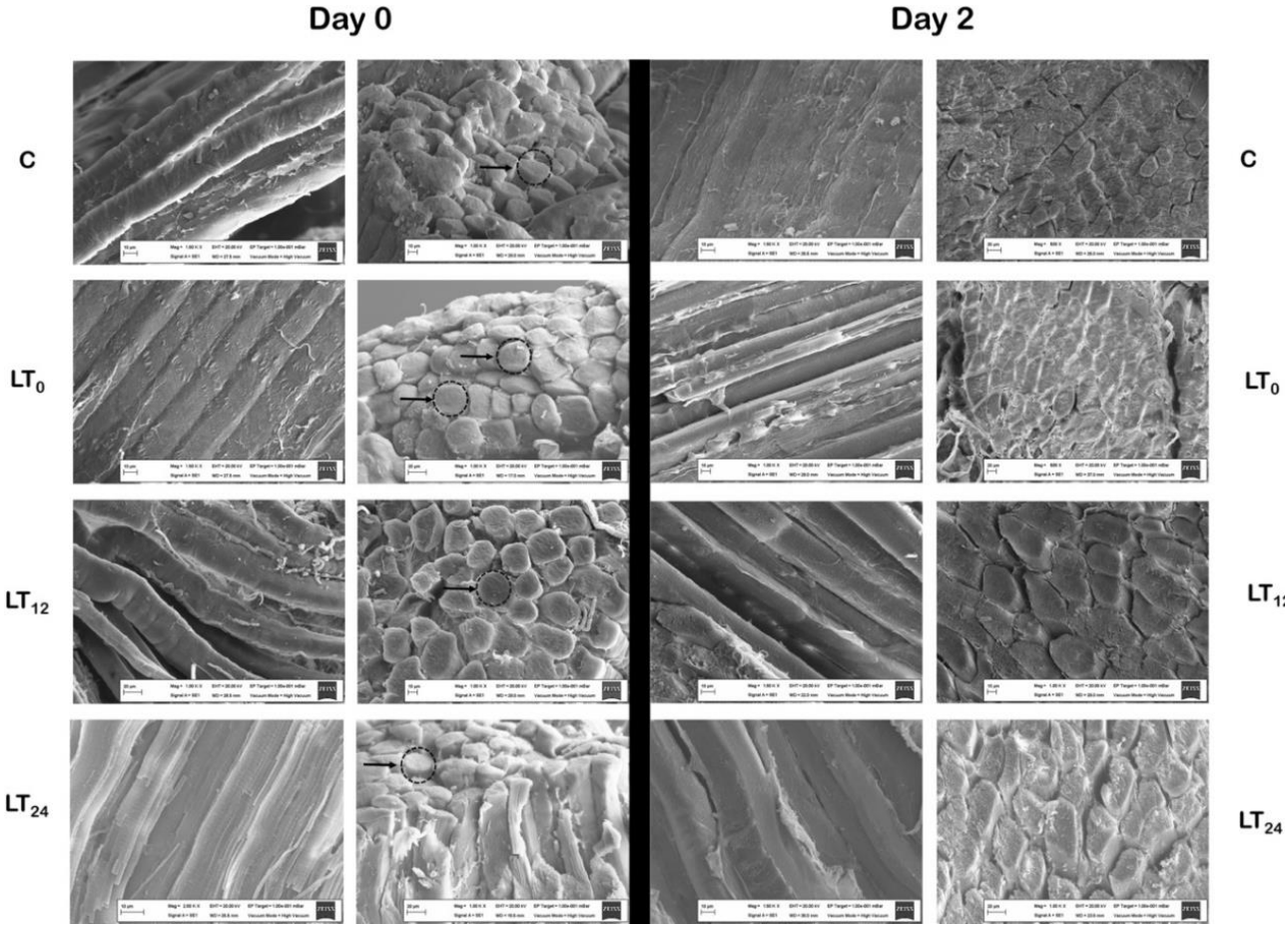

Figure 3. Scanning Electron Microscopy of myofibrils on days 0 and 2. Each circle indicates muscular fibrils, Bar: 10µm.

Table 5. Mean values, standard errors and significant interactions of sensorial characteristics (color intensity, color hue, odor intensity, tenderness, chewiness, juiciness, flavor intensity, flavor quality, and overall acceptability) of sheep meats subjected to short-term transportation and lairage time.

Attribute	Time (day)	C	LT_0	LT_{12}	LT_{24}		Significance		
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	LS Mean ± SE	G	T	G x T
Color intensity	2	5.61 ± 0.08	4.94 ± 0.19	6.7 ± 0.18	6.03 ± 0.12	**5.818 ± 0.11** $^{a\,b}$	**<0.001**	**0.001**	**0.144**
	5	5.58 ± 0.18	5.54 ± 0.36	6.75 ± 0.5	6.54 ± 0.17	**6.104 ± 0.11** ^{a}			
	10	5.4 ± 0.15	5.2 ± 0.06	5.8 ± 0.06	5.67 ± 0.12	**5.517 ± 0.11** ^{b}			
	LS Mean ± SE	**5.53 ± 0.127** ^{B}	**5.226 ± 0.127** ^{B}	**6.416 ± 0.127** ^{A}	**6.079 ± 0.127** ^{A}				
Color hue	2	5.97 ± 0.15	5.33 ± 0.03	6.97 ± 0.32	6.12 ± 0.2	**6.099 ± 0.133**	**<0.001**	**0.153**	**0.467**
	5	5.5 ± 0.24	5.43 ± 0.41	6.46 ± 0.48	6.13 ± 0.2	**5.877 ± 0.133**			
	10	5.13 ± 0.19	4.87 ± 0.19	6.74 ± 0.09	6.2 ± 0.32	**5.736 ± 0.133**			
	LS Mean ± SE	**5.536 ± 0.153** ^{B}	**5.208 ± 0.153** ^{B}	**6.723 ± 0.153** ^{A}	**6.149 ± 0.153** ^{A}				
Odor intensity	2	5.9 ± 0.08	5.76 ± 0.06	5.82 ± 0.05	5.97 ± 0.13	**5.861 ± 0.06** ^{c}	**0.013**	**<0.001**	**0.982**
	5	6.67 ± 0.23	6.46 ± 0.11	6.5 ± 0.13	6.79 ± 0.12	**6.606 ± 0.06** ^{a}			
	10	6.47 ± 0.03	6.13 ± 0.2	6.27 ± 0.08	6.47 ± 0.03	**6.333 ± 0.06** ^{b}			
	LS Mean ± SE	**6.346 ± 0.069** $^{A\,B}$	**6.117 ± 0.069** ^{B}	**6.196 ± 0.069** $^{A\,B}$	**6.408 ± 0.069** ^{A}				
Tenderness	2	7.06 ± 0.29 $^{a\,A}$	6.09 ± 0.23 $^{a\,B}$	6.76 ± 0.23 $^{a\,A}$	6.91 ± 0.07 $^{a\,A}$	**6.705 ± 0.079**	**<0.001**	**<0.001**	**0.016**
	5	6.17 ± 0.25 $^{b\,A}$	5.13 ± 0.03 $^{b\,B}$	5.29 ± 0.02 $^{b\,B}$	6.17 ± 0.06 $^{b\,A}$	**5.688 ± 0.079**			
	10	4.53 ± 0.13 $^{c\,A}$	4.26 ± 0.08 $^{c\,A}$	4.3 ± 0.09 $^{c\,A}$	4.4 ± 0.12 $^{c\,A}$	**4.373 ± 0.079**			
	LS Mean ± SE	**5.921 ± 0.091**	**5.158 ± 0.091**	**5.449 ± 0.091**	**5.826 ± 0.091**				
Chewiness	2	6.39 ± 0.13 $^{a\,A\,B}$	5.94 ± 0.03 $^{a\,B}$	6.76 ± 0.08 $^{a\,A}$	6.61 ± 0.06 $^{a\,A}$	**6.424 ± 0.076**	**<0.001**	**<0.001**	**0.04**
	5	5.25 ± 0.19 $^{b\,A}$	4.17 ± 0.22 $^{b\,B}$	4.88 ± 0.38 $^{b\,A}$	5.38 ± 0.02 $^{b\,A}$	**4.917 ± 0.076**			
	10	5.16 ± 0.05 $^{b\,A}$	4.02 ± 0.06 $^{b\,B}$	4.66 ± 0.09 $^{b\,B}$	5.24 ± 0.07 $^{b\,A}$	**4.77 ± 0.076**			
	LS Mean ± SE	**5.601 ± 0.087**	**4.709 ± 0.087**	**5.431 ± 0.087**	**5.74 ± 0.087**				
Juiciness	2	6.18 ± 0.3	5.55 ± 0.15	6.33 ± 0.04	6.76 ± 0.13	**6.204 ± 0.087** ^{a}	**<0.001**	**<0.001**	**0.701**
	5	5.63 ± 0.38	5 ± 0.06	5.42 ± 0.11	5.75 ± 0.06	**5.448 ± 0.087** ^{b}			
	10	5.53 ± 0.13	4.89 ± 0.03	5.33 ± 0.21	5.62 ± 0.09	**5.346 ± 0.087** ^{b}			
	LS Mean ± SE	**5.781 ± 0.101** ^{A}	**5.146 ± 0.101** ^{B}	**5.693 ± 0.101** ^{A}	**6.044 ± 0.101** ^{A}				
Flavor intensity	2	6.46 ± 0.28	6.08 ± 0.36	6.09 ± 0.23	6.94 ± 0.14	**6.393 ± 0.087** ^{a}	**<0.001**	**0.001**	**0.775**
	5	6.13 ± 0.11	5.64 ± 0.16	6.04 ± 0.12	6.58 ± 0.02	**6.097 ± 0.087** $^{a\,b}$			
	10	6 ± 0.06	5.43 ± 0.04	5.98 ± 0.15	6.31 ± 0.06	**5.931 ± 0.087** ^{b}			
	LS Mean ± SE	**6.194 ± 0.1** ^{B}	**5.718 ± 0.1** ^{C}	**6.038 ± 0.1** $^{B\,C}$	**6.611 ± 0.1** ^{A}				
Flavor quality	2	6.94 ± 0.1	5.94 ± 0.12	6.27 ± 0.07	6.67 ± 0.09	**6.454 ± 0.058** ^{a}	**<0.001**	**0.043**	**0.964**
	5	6.92 ± 0.1	5.96 ± 0.09	6.33 ± 0.21	6.54 ± 0.02	**6.438 ± 0.058** ^{ab}			
	10	6.73 ± 0.1	5.86 ± 0.11	6.12 ± 0.2	6.35 ± 0.04	**6.264 ± 0.058** ^{b}			
	LS Mean ± SE	**6.863 ± 0.067** ^{A}	**5.919 ± 0.067** ^{D}	**6.241 ± 0.067** ^{C}	**6.518 ± 0.067** ^{B}				
Overall acceptability	2	6.24 ± 0.21	5.79 ± 0.15	6.49 ± 0.06	7.06 ± 0.1	**6.393 ± 0.125** ^{a}	**<0.001**	**<0.001**	**0.827**
	5	5.38 ± 0.22	4.54 ± 0.16	5.21 ± 0.2	6.25 ± 0.18	**5.344 ± 0.125** ^{b}			
	10	5.53 ± 0.59	4.47 ± 0.07	5.33 ± 0.12	5.93 ± 0.38	**5.317 ± 0.125** ^{b}			
	LS Mean ± SE	**5.716 ± 0.144** ^{B}	**4.932 ± 0.144** ^{C}	**5.676 ± 0.144** ^{B}	**6.414 ± 0.144** ^{A}				

$^{a, b, c}$: Different letters in the same column represent statistically significant difference (P<0.05).
$^{A, B, C, D}$: Different letters in the same column represent statistically significant difference (P<0.05).
C: Control (Animals slaughtered after 1 week of lairage).
LT_0: immediate slaughter (0 lairage time).
LT_{12}: Animals slaughtered after 12 hours of lairage.

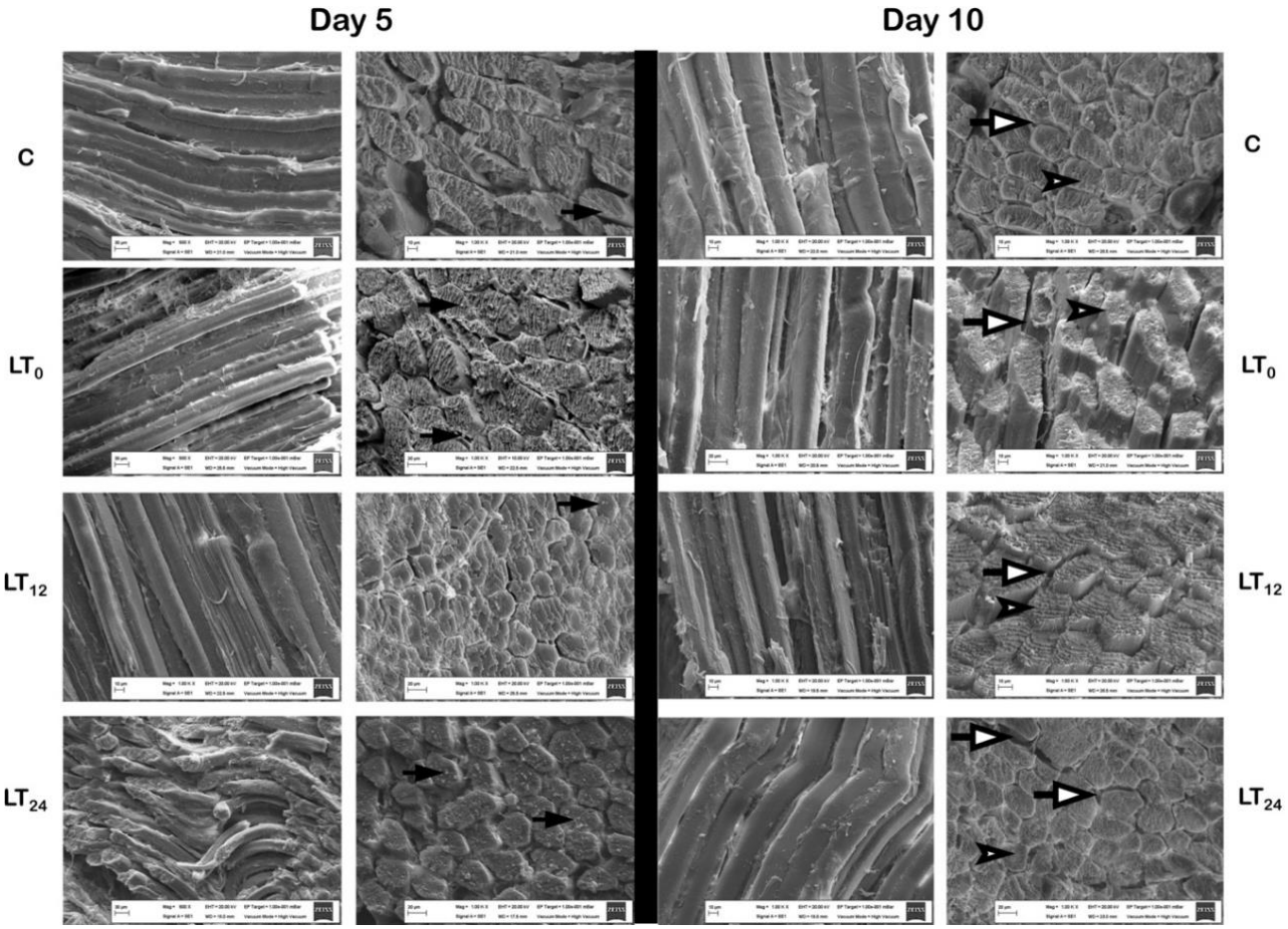

Figure 4. Scanning Electron Microscopy of myofibrils on days 5 and 10. Black arrow: Oil droplets, Arrowhead: Myofibrillar denaturation and myofibrillar shortening, White arrow: Expansion of the intercellular space, Bar: 10µm.

Discussion and Conclusion

The glycogen values difference between the groups were found to be statistically significant (P<0.001). A similar positive effect of lairage time was reported previously by Diaz et al. (12). Also, glycogen values decreased significantly during aging as expected (P<0.001).

The pH values were determined lowest in the longer storage time. These findings were similar to previous publications (18, 28, 35). Significant differences were found between measurements in different control times. The average pH level was different between groups. Similarly, Li et al. (32) and Tomovic et al. (42) reported pH deviations between 5.3-5.7. A recent article has reviewed the pH values and long-term aging positive effects on meat tenderness (38). Cold shortening is described as rapid chilling of the carcass to 10-16°C before pH reaches 6.2 (rigor mortis) (4). In the present study, optimum conditions were determined in L_{24} and C groups (pH 6.2, temperature above 11°C).

The lairage time had no impact on CL and WHC values. No statistically significant difference was found between groups (P>0.05). However, only WHC showed improvement during the aging period, especially on the 10^{th} day, and this was statistically significant (P<0.001). This is validated by earlier studies (7, 11, 30, 40). On the other hand, studies conducted on the effect of lairage time in Kıvırcık lambs revealed significant differences in WHC and CL values (13, 28). In this study, WHC (more water expelled) decreased with storage in all groups. This is due to the increase in drip loss during storage which resulted in free water available for expression.

SF values findings were in correlation with previous studies (13, 26, 36). On the contrary, Ferguson and Warner (14), Ferreira et al. (15), Maria et al. (34) have found no differences between the groups.

Initial color parameters were not affected by PS (P>0.05), however, color values improved in accordance with the aging time (P<0.001) in all groups. Similar to those was found by Kadim et al. (28). However, Ferreira et al. (15) reported that the a* values increased in the groups with high lairage time.

SL values were highest to lowest C, LT_{24}, LT_{12}, and LT_0 groups, respectively. Despite the relatively positive effect of lairage time on the SL, a significant increase in

SL was started with the maturation process of meat (Figure 1). However, the notable alterations at the microscopic level related to the maturation process of the meat started on day 5 and were completed on day 10. In other words, on days 5 and 10, myofibril denaturation and expansion of the intercellular space among the fibra muscularis were significantly ($P<0.001$) increased compared to day 0 (Figure 2).

Although SL and SF showed an inverse ratio, the time x group interaction of these values did not show any significance. A significant relationship, between SF values and SL values, is previously reported (20, 22) and as the SL increases, SF values decrease. Our results were similar to a study conducted on cattle (15), whereas in a sheep study higher SL values of longissimus thoracis muscle were determined in longer lairage time (28). Thus, we speculate that the different species and different muscle types, with additional applications to the expanded lairage time, could have different SF and SL values.

With the increased lairage time (LT_{12}, LT_{24}, and C) the sensation of consistency detected from the product also increased in the direct proportion. Similarly, an increase was observed in the appreciation values perceived by the panelist as a result of increased resting time in flavor intensity and flavor quality. As a result of all these evaluations, panelists determined the general acceptability ranking as LT_{24}> C> LT_{12}> LT_0. Although Ferguson and Warner (14) reported longer transportation time and stress effect on meat tenderness is an indistinct feature, our panelist reported a better sensation in the LT_{24} group than the C group. Similarly, other studies with different lairage time resulted with showed non-significant sensorial findings (36, 48, 49).

The calpain/calpastatin system; *CAPN1*, *CAPN3*, and *CAST* expression measurable for all genes examined in the groups on days 0 and 2nd (< 30Ct). However, on days 5 and 10 expression levels could not be measured. This was thought to be related to the rapid degradation of RNA. Contradictory studies on post-mortem RNA integrity are found in the literature (6, 17). However, studies have also proven that RNA can remain without degradation when preserved under appropriate storage conditions (16). In a study, ante and postmortem samples were compared and a series of transcriptional events that started with the death of the organism were determined. Also, the effects of postmortem gene expression changes of *CAST*, *CAPN1*, and *CAPN3* on meat quality in pigs could be examined (19). In pigs higher *CAST* and *CAPN3* expression was found in correlation with high SF ($P<0.2$) and *CAST* affected the activation time of calpain and drip loss (19). Higher *CAST* expression was associated with poor tenderization ($P=0.0563$) (3). However, in our study RT-PCR results showed that transportation and lairage time did not change expression levels of *CAPN1*, *CAPN3* and *CAST*.

In a study conducted on cattle, higher plasma *HSPA1A* was associated with an in long-time transportation process (8). The transportation duration is one of the most important stress factors for animals. Because the transportation time was set at 3 hours in all groups, the stress marker *HSPA1A* expression didn't show a significant difference between groups. However, *HSPA1A* expression showed significant differences between groups with passing time in the aging process ($P<0.5$). Different lairage times have different effects on *HSPA1A* levels in plasma; in a study, 18 hours lairage time had higher results than 24 hours lairage time (8). In correlation with these findings, a fivefold increase in expression was measured especially in the C and LT_{12} groups. *HSPA1A* protects the cell against destruction by preventing protein degeneration (10) but with our study results, it is not clear whether this increase is due to the deactivation of gene silencing mechanisms associated with cell death or due to the final protection effort of the cell due to the stress caused by cell damage. The high *HSPA1A* plasm level was correlated negatively with SF; positively with pH and meat temperature (8).

The results of this study showed that pH, glycogen, and SF values were found to be lower in groups with higher lairage time (LT_{24}) and control (C). It has been determined that the results obtained with organoleptic evaluations are consistent with the higher effect of lairage time. In contrast, there were no differences between the groups in terms of temperature, CL, color, WHC, and SL values. According to histological evaluations, SL was found to increase starting from day 0 depending on exposure time. However, it was observed that the period of maturation of the meat started with the fifth day. Furthermore, it was determined that the difference between *CAPN1*, *CAPN3*, and *CAST* expressions was not significant, while it was significant for *HSPA1A* expression. Considering that gene expression changes are also affected by variations in the DNA level, it is suggested to design a genotype/phenotype correlation study by larger samples including different strains with additional analyzes, such as determination of genotype, gene expression, and final product proteins, and measurement of enzyme activity at certain intervals.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

TK, AGB and NB conceived and planned the experiments. TK, AGB, NB, EA, EBB, FGE, AA, FYE and BÇK carried out the experiments. AC, İŞH and AGB planned and carried out the histological analysis. NB, BCK, AGB, TK, AC, DÖ, and ÖC contributed to the interpretation of the results. NB took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data Availability Statement

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

Ethical Statement

This study was approved by the İstanbul University Animal Experiments Local Ethics Committee (2016/15).

Animal Welfare

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

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Anatomical evaluation and preparation procedure of a cross-sectioned kidney plastination of a thoroughbred horse with local polyester resin

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ABSTRACT

This study aimed to develop a protocol for the thin section plastination of the kidney with local polyester resin and examine the anatomical details of this specimen. The sample was fixed with 10% formalin fixation solution and then sectioned into 3 mm slices. The four stages of the polyester plastination technique were used. Firstly, the samples were kept in 99.5% acetone baths at -25ºC for the dehydration. After this process, the sections were placed in polyester resin and the impregnation process was started at room temperature (20ºC) under vacuum. Following the forced impregnation, the curing chambers were constructed and the curing process was continued under ultraviolet light. The data of each applied stage were recorded carefully and the protocol for polyester plastination of kidney sections was successfully established. The specimens were observed from a different point of view with a thin cross-sectional appearance. The anatomical morphology and the structures of the sections of the kidney such as renal parenchyma and circulatory components were preserved well. The final products could be used as educational samples for cross-sectional anatomical training of kidneys.

Introduction

The P40 polyester plastination was first introduced in the mid-1990s (6). The P40 polyester technique follows similar classical steps of silicone plastination. However, when the processes are examined in detail, there are some important differences. The first main step of the silicone plastination technique is to remove the fixative solution applied to the specimens and replace it with some chemicals like acetone. This process is called the dehydration stage and is also involved in the polyester plastination technique. The impregnation stage that follows dehydration, is the replacement of acetone with a different polymer. This stage consists of replacing the acetone with polyester resin on the basis of polyester plastination. The last step is curing which includes polymerization of the polymer in the structure and obtaining a solid, partially flexible, and long-lasting structure. This stage varies according to the type of plastination techniques (1, 5, 6).

The polyester plastination technique is used for the durable preservation of sections of various structures. Especially due to the developing technology, improvement in imaging systems has highlighted the understanding and evaluation of cross-sectional images. As a result, the number of plastination studies has increased on cross-sectional anatomy (1, 2, 4, 6, 9, 10).

The objectives of this study were to produce cross sectional polyester plastinated kidney slices, to develop a polyester plastination protocol with local polyester resin for kidney specimens and examine the anatomical details of the plastinated horse kidney.

Materials and Methods

The present study was carried out in the Plastination Laboratory of of Ankara University Faculty of Veterinary Medicine Department of Anatomy. Also, additional support was received from TOBB University Faculty of Medicine Department of Anatomy. Ethical procedures were confirmed by Ankara University Animal Experiments Local Ethics Committee (Decision no. 2021-21-189, Ankara, Türkiye). Nomina Anatomica Veterinaria (12) was used for anatomical terms. All the processes were schematically given with photographs in Figure 1.

***Specimen Preparation**:* The exenteration of a right kidney from a thoroughbred horse was performed and used in this study. The sample was taken from one-week stayed cadaver after perfused with 10% formaldehyde. An incision was made into the renal capsule of the kidney and soaked in 10% formaldehyde for further 7 days to increase penetration of the fixative solution into the kidney.

After the fixation stage, the kidney was cut into thin slices with a deli slicer. The thickness of the sections was 2-3 mm. A total of eight sections were obtained and all serial sections were left in running water for one day to remove excess formaldehyde in the organ. All sections were then placed between square grids and stacked one after another. These grids were tied in a bundle to ensure that sections not to displace. This grid bundle was used in the plastination processes, respectively.

***Dehydration**:* The dehydration process is carried out in a cold environment (-25°C) to increase the quality of the final product (6). Sections were kept in three consecutive 99.5% acetone baths at -25°C. Throughout the dehydration process, acetone concentration was measured and monitored daily with an acetonometer. The bundle of the sections was stirred gently in acetone every day for 10 days. The duration of the first acetone bath took 5 days. At the end of the first acetone bath, the acetone value decreased to 92%. The second acetone bath took 3 days and the acetone value dropped to 97%. The final (third) acetone bath remained stable at 99.5% for 2 days. At this stage, the dehydration process was considered complete. Due to the texture of the kidney, no degreasing process was performed.

Impregnation: Forced impregnation of kidney sections was performed using local full-clear moulding polyester resin (Turkuaz Polyester, Türkiye) at room temperature (20-22ºC). No activator was used while preparing the polyester resin in the impregnation process. The grid bundle was placed in the prepared polymer and kept inactive for 12 hours (vacuum pump stopped) overnight. Then, the forced impregnation process was applied active for 12 hours (vacuum pump running continuously). During this procedure, the samples were carried out in a dark environment to prevent the reaction of any ultraviolet light. This stage took 3 days; during this 3 days the internal pressure was reduced from the initial measurement of 912 mbar to 20 mbar. At this point, it was observed that the bubbles, which are the indicator of acetone output, decreased on the surface of the polyester bath in which the samples were located. When this amount of pressure was reached, the forced impregnation stage was considered completed.

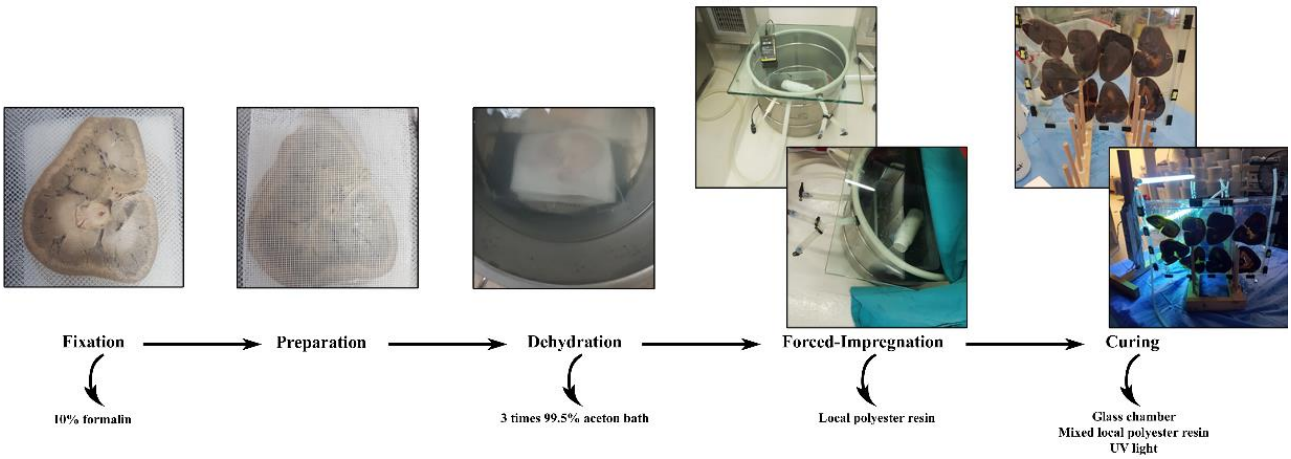

Figure 1. An overview of the polyester plastination procedure workflow from the fixation to the curing stage. Specimen observation in fixation and preparation phase. Three consecutive aseton bath with being started 99.5% concentration in dehydration phase. Local polyester resin being used with dark environment and vacuum in forced-impregnation phase. Vertical glass chamber with kidney sections being filled mixed local polyester resin and UV light application as a catalyst on the kidney slices in curing phase.

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Curing: After the forced impregnation process was completed, the kidney sections were taken from the polyester resin to the curing room for hardening. Every section was placed in a glass chamber. The glass chamber was prepared between the 5 mm thick glass plates on both sides. All sides, except the top, were wrapped with a gasket between two glass plates and fixed with clamps. Then the sections of the kidney were placed inside and filled with a new modified local full-clear moulding polyester resin solution (Polyester / 1% Cobalt / Methyl Ethyl Ketone Peroxide: 99.6% / 0.1% / 0.3%). In order to prevent any air bubbles from forming in the polyester, the opening of the upper edge was used for the exit of air bubbles. In the curing room, glass chambers were placed under a ultraviolet light (30 minutes on and 30 minutes off) placed from all directions were placed from all direction of the sample at a distance of approximately 30 cm. In addition, it was tried to provide temperature distribution and protection to high temperatures with the help of a fan. The hardening of the polyester resin in the sections was completed in about 12 hours.

After curing was finished, the surrounding glass plates were removed from the sections. The sample was wrapped with plastic wrap for protection.

Results

The protocol of section plastination prepared by using polyester resin was successfully applied to the kidney specimen. The local polyester plastinated sections were found to be durable and semi-transparent. These plastinated specimen is a good educational material in which anatomical features can be evaluated conveniently.

In the plastinated cross-sectional slices of the kidney, the anatomical details were preserved well and easily identified. In each layers of the kidney, renal cortex (cortex renalis), renal medulla (medulla renalis), and renal pelvis (pelvis renalis), were easily distinguished (Figures 2 and 3). On the plastinated sections the renal parenchyma consisted of two main parts, renal cortex and renal medulla. The outer zone of the renal medulla was darker than the inner zone of the renal medulla (Figure 2b and c). The radial striations belonging to the inner zone were visible and these lines extend to the renal sinus (Figure 3c and f). The endpoint of the medullary structure fused to form the renal crest (crista renalis) (Figure 3e). Papillary ducts were opened to the specific regions of the renal crest (Figure 3d, e, and f). It was seen that one of the large terminal recesses located at the end poles of the renal pelvis and many papillary ducts (ductus papillares) opened on this one to transfer the urine (Figure 3g).

Even though the kidney consists of the fused type of the cortical and medullary substances in the equine, it was determined that lobulation of the kidney can be seen due to the interlobar vessels when the parenchymal tissue was examined (Figure 2). The interlobar vessels and renal columns (columnae renales) were identified on the section image of the polyester plastination (Figure 2k).

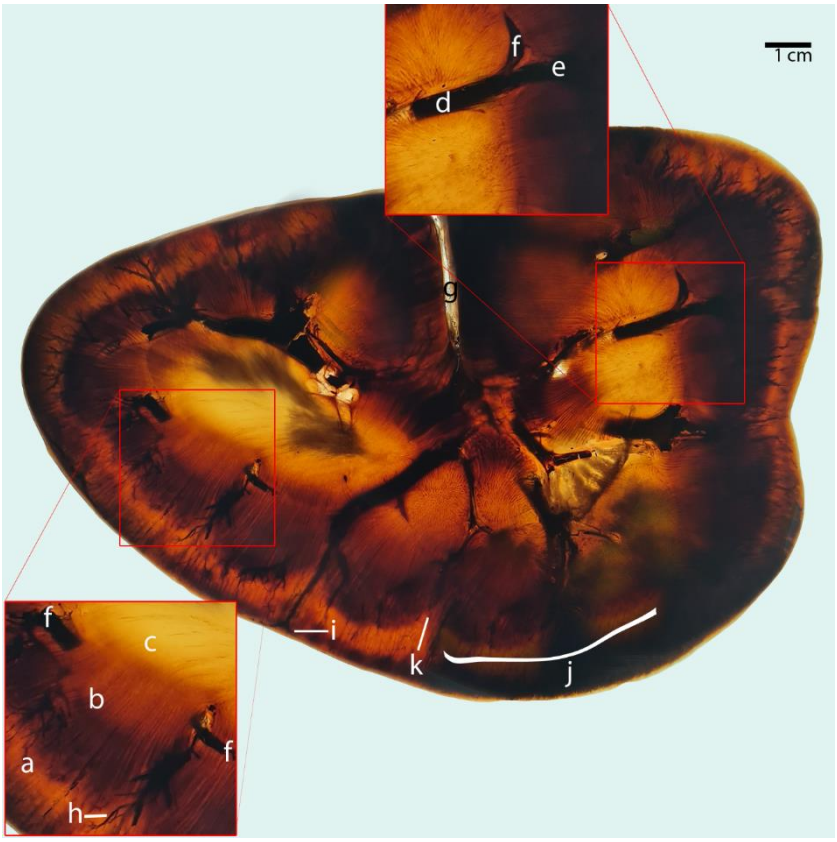

Figure 2. The polyester plastinated equine kidney slice.
a, renal cortex;
b, external zone of the renal medulla;
c, internal zone of the renal medulla;
d, interlobar vessels;
e, interlobular vessels at the crossing of the arcuate vessels;
f, arcuate vessels;
g, renal hilus;
h, efferent glomerular artery (arteriae glomerularis efferens);
i, stellate veins (venulae stellatae);
j, fused pyramidal shaped lobes;
k, renal column.

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Figure 3. The polyester plastinated equine kidney slice.
a, renal parenchyma;
b, renal cortex (cortex renis);
c, renal medulla (medulla renis);
d, renal pelvis (pelvis renalis);
e, renal crest (crista renalis);
f, papillary duct (ductus papillaris);
g, terminal recess (recessus terminalis);
* interlobular arteries (arteriae interlobulares).

Interlobar vessels were determined at the level of the renal hilus (hilus renalis) and it was observed that they proceed between the renal lobes (lobi renis) within the parenchymal tissue. Arcuate vessels were also detected in the corticomedullary region of the kidney and they were divided from the interlobar vessels. The part that kept on towards the renal cortex continues by taking the name of interlobular veins (Figure 2d, e, and f). These veins formed by the unity of the smaller efferent arterioles (arteriola glomerularis efferens) and capillary network (Figure 2h). Stellate veins (venulae stellatae), the subcapsular branches of the interlobular veins, were easily seen on the outer line of the renal cortex (Figure 2i).

Discussion and Conclusion

Sheet plastination is a type of plastination that is considered to be a vital tool in the enhancement and clarification of concepts of cross-sectional anatomy and relationships previously often difficult to appreciate (15). The introduction of sheet plastination has provided us an opportunity to combine modern cross-sectional imaging techniques with corresponding slices of humans and animal tissues. Cross-sectional plastination samples have been previously proven to be used for educational purposes. However, it may be limited due to the long processing time required to obtain plastination samples and the use of high-cost chemicals during the collection of samples (3, 8, 16). When the sections preserved in the cured local polyester resin were evaluated in terms of their use, it was observed that they were easy to handle, biosafe, and cost-efficient.

The plastination protocol applied to the kidney sections of the polyester resin in this study can be used both for the preservation of kidney sections and for the differentiation of different layers in the sectioned kidney. As mentioned before, polyester plastination can be applied not only to the brain sections but also to the different organ sections (1, 5). The result of this study is in line with the above findings. In a previous study, students confirmed the educational advantages of P40 plastinated specimens in practical lessons. It was pointed out that 60% of students preferred plastinated specimens in the anatomy lessons (13).

In our study, plastinated samples illustrated the anatomical features of the kidney section very well. It can be used for demonstration of cross sectional part of kidney for students in practical section and for museum exhibition. Although it was not among the aims of this study to conduct research on student opinions, our results demonstrated that local polyester plastinates were good examples for museum specimens as well as for anatomy education, in addition to their affordable cost. These can be exhibited as very interesting and attractive examples for students and visitors.

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A uniform kidney structure is observed in domestic mammals due to the fused type of cortical and medullary substances. However, when the parenchymal tissue is examined, lobulation of the kidney can be seen apertly with a determination of the interlobar vessels (11). This explanation is also similar to our study. Renal columns were identified on the section image of the plastinated specimens. The parenchymal tissue is divided into two part. These are renal cortex and renal medulla with their morphological differences. The endpoint of the renal medulla is fused in the non-lobulated type of kidney. This area is called the renal crest (7) and it was visible at the renal pelvis end of the medullary structure of polyester plastinated slices in kidney of the horse.

The kidney contains vessels to ensure blood flow. In this plastinated sectioned specimens, the branches of renal blood vessels were visible. These branches are distributed within parenchyma of the kidney. The main root divided into several interlobar vessels at the hilus of the kidney. These branches further divided into arcuate vessels between the different renal lobes in the corticomedullary region of the kidney. Other branches, interlobular vessels, raised from the renal medulla to the renal cortex, and there interlobular vessels gave branches to support the renal corpuscles. These veins formed by the unity of the smaller efferent arterioles (arteriola glomerularis efferens) and capillary network. Blood vessels distribution and branching through paranchyma of kidney in our finding was incorresponding with studies conducted previously (7, 11).

One of the stages required for the preparation of a plastinated specimen is the dehydration phase. Aceton was used for dehydration of the slices since it is the best intermediate solvent for dehydration. Studies have shown that the acetone level should be at least 99% at the end of the acetone baths and it is recommended to be applied under the cold chain in order to prevent both biosecurity and tissue shrinkage (5, 14). In a previous study, it was revealed that the dehydration process carried out under the cold chain had a less shrinkage ratio (14). The same procedure is used for this study; 99.5% acetone in each bath and they were kept under the cold chain. In our study, the dehydration process was completed by passing the samples through three 99.5% acetone baths. The last value was estimated as 99.5% before proceeding to the next step. Degreasing may or may not be applied in order to remove the adipose tissues in the structure between the dehydration and impregnation stages (1, 6). In one of the previous study, the degreasing process was evaluated on the pig kidneys of the S10 plastination. It was stated that dehydrated and degreased kidney slices of the silicone plastination were shown distinct differentiation of the renal structures due to the presence of fat (16). In this study, the degreasing was not applied both by taking into account the amount of the fat tissue and to prevent any shrinkage that may occur. The distinction of the layers of the kidney were shown clearly in the polyester plastinated specimens.

The impregnation step was carried out in a dark environment to preserve the color of the kidney sections and to prevent undesired curing. In this way, any possible polymerization can be avoided as UV light acts as a catalyst of such polymers (6). In our study, the forced impregnation was applied at room temperature, and finished when the pressure reached 20 mbar (15 mmHg) at the end of the third day. In a study conducted by Guerrero et al. (5) on brain sections, 20 mmHg pressure is recorded at the end of the first day. Although the measurement result was in parallel with our study, the appropriate value was reached on the 3rd day. This situation is thought to be related to the applied passive pressure time. Since these types of polymers contain styrene, the applied pressure should be controlled frequently (6). In this study, the pressure was continuously monitored electronically to avoid the loss of styrene in the polymer at the forced impregnation stage.

In our study, fixation and dehydration stages were applied adequately and the values of the dehydration solution were checked every day. For this reason, local polyester resin was applied without using any catalyst. Another positive aspect of this is that polyester will thus be usable for a long time. It is thought that the high cost of plastination methods, which is the most discussed part, can be avoided with such approaches. It is known that samples with adequate fixation can be applied without using catalyst in the impregnation stage. The most important disadvantage of the use of impregnation solutions with a catalyst is the shortening of the polymer usage time (6). In addition, in a previous study, it was stated that the polyester bath not mixed with the activator could be used for a long time (1). In impregnation stage of our study, acceptable results were obtained.

In previous studies, it has been stated that no problems were encountered with the horizontal position of the samples in the curing process. However, in the same studies, it was stated that the specimens could be displaced vertically or curved curing process. For this reason, it has been suggested that attention should be paid to such problems (1, 6). The procedure in which the organ and polymer are placed in a vertical chamber is called the "vertical chamber method" (1). In this study, the curing process was applied with the vertical chamber method with a 15º inclination without any moving of the samples. No displacement of the samples in polyester was encountered.

The findings of this study showed that the polyester plastination method can be used to evaluate anatomical structures in kidney sections. It was observed that

polyester plastinated specimens can be used in thin sections obtained from various organs other than brain sections. One of the most important conclusion of this study is that using local full-clear moulding polyester for the preparation of plastinated slices of kidney can be used for demonstration of cross-sectional part of kidney for students in practical section and for museum exhibitions with an affordable cost.

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Conflict of interest

The authors declare no conflict of interest.

Author Contributions

CB conceived and planned the experiments. CB, HAY, BB and ST carried out the experiments. CB and HAY planned and carried out the simulations. CB, HAY, BB and ST contributed to sample preparation. CB, BB and OE contributed to the interpretation of the results. CB and HAY took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

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

Ethical Statement

The present study was approved by the Ankara University Animal Experiments Local Ethics Committee (Decision No. 2021-21-189, Ankara, Türkiye).

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Hepatoprotective effects of *Nigella Sativa* oil against acrylamide-induced liver injury in rats

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ABSTRACT

Acrylamide (AA) is a heat-induced carcinogen founds in some foods due to cooking or other thermal processes. This study was designed to investigate the possible protective efficacy of *Nigella sativa* essential oil against acrylamide-induced liver toxicity. Four groups of 32 male Wistar albino rats were formed. The control group was fed ad libitum. Acrylamide was administered to the rats in Group AA at a daily dose of 40 mg/kg by gavage. *Nigella sativa* essential oil was administered intraperitoneally at a dose of 10mg/kg to the rats in Group NS. In Group AA+NS, daily applications of acrylamide and *Nigella sativa* essential oil were applied simultaneously as mentioned above. The trial period lasted 15 days. While acrylamide statistically increased MDA levels in liver tissue ($P<0.05$), (146.13 %) and decreased GSH and SOD activity statistically ($P<0.05$), (55.28% and 40.46%). In addition, liver proinflammatory cytokine levels increased due to acrylamide administration ($P<0.05$), [TNF-α (78.17%), IL-6 (76.08%)], anti-inflammatory cytokine levels ($P<0.05$), [IL-10 (61.16%)] decreased. Co-administration of *Nigella sativa* essential oil with acrylamide significantly reduced oxidative stress and pro-inflammatory cytokine levels in liver tissues ($P<0.05$). In conclusion, our findings highlighted the potential therapeutic role of *Nigella sativa* essential oil in preventing acrylamide-induced hepatotoxicity.

Introduction

Acrylamide (AA) is a white solid powder, easily soluble in water, ethanol, and other solvents. It is widely used in the water treatment industry, textile industry, and cosmetics production (46). Consumption of high-temperature-cooked carbohydrate-rich foods is recognized as another major route of AA exposure (29). The level of AA found in fried and baked foods such as french fries (500 µg/kg) and potato chips (750 µg/kg) is reported to be more than 500 times the maximum allowable limit in drinking water determined by the World Health Organization (WHO) (38). Researchers note that the AA level is quite high in bread, biscuits, and coffee (46). Although the ways of exposure to AA in daily life are diverse, the main sources are food and drinking water. AA enters the body through the skin, digestive system, and respiration, and can be rapidly distributed to all tissues and blood in the body, as it has strong tissue permeability (34,37).

AA decreases glutathione (GSH) and level superoxide dismutase (SOD) activity and significantly increases malondialdehyde (MDA) levels (33, 39). This imbalance between the increase in reactive oxygen species (ROS) and the antioxidant defense system increases oxidative stress in various tissues, especially in the liver, brain, and kidney (24). Increased oxidative stress leads to, genotoxicity reproductive system toxicity, neurotoxicity, carcinogenicity, and hepatotoxicity (17, 45). Also, it was reported that the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) increase after AA intoxication (41). It was reported that AA intoxication

may have effects on the liver (9). AA is catalyzed in the liver via cytochrome P450 2E1 (CYP2E1) and metabolized to glycidamide (GA), a highly toxic substance for the organism (19). Besides, AA conjugates with GSH to be excreted in the urine as an alternative route in the liver (15). Although the metabolism of AA in the liver has been well defined, its hepatotoxicity has not been adequately clarified. There is also a growing concern about its effects on human and animal health since exposure to AA is both relatively easy in daily life and can be rapidly distributed throughout the organism.

Using in vitro and in vivo models, the researchers focused on finding protective compounds to reduce the toxic effects of AA. Therefore, some well-known antioxidants such as curcumin, crocin, linalool, and chrysin have been reported to be beneficial against the toxic effects of AA (20). *Nigella sativa* essential oil, (NS) is widely performed as a preservative and food additive as well as various diseases all over the world (26). NS, generally known as black seed or black cumin, has traditionally been used as a food preservative, additive, or applied as spice (6). According to the findings obtained from the studies carried out NS has antioxidative, anti-inflammatory, antifungal, anticonvulsant, immunomodulator, antinociceptive, antiallergic, antibacterial, antiviral, antidiabetic, antitumor, anti-hepato-nephrotoxic and neuroprotective effects (7, 43, 48).

In this context, the antioxidant and immunomodulatory effects of NS, which is claimed to have positive effects on many different diseases and is widely utilised as a spice, on the liver of rats with AA toxicity were investigated.

Materials and Methods

Chemicals and other reagents: AA was obtained from Sigma Chemical Company (St. Louis, Mo, USA). *Nigealla sativa* L. was supplied by Botalife (Isparta, Türkiye). Malondialdehyde (MDA), glutathione (GSH, Cat. No: E1101Ra), superoxide dismutase (SOD) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Bioassay Technology Laboratory (Shangai, China). Tumour necrosis factor-alpha (TNF-α, Cat. No: ERA57RB), interleukin-1 beta (IL-1β, Cat. No: BMS630), interleukin-6 (IL-6, Cat. No: ERA32RB) and intrleukin 10 (IL-10, Cat. No: ERA24RB) ELISA kits were obtained from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA).

Animals: The Selçuk University Experimental Medicine Research and Application Center Ethics Committee approved the animal handling and study protocol as ethical (Approval No. 2020-29). Thirty-two adults male Wistar Albino rats weighing 240 ± 20 g were obtained from Selçuk University Experimental Medicine Research and Application Center. All rats were kept in a well-ventilated room with 12 hours of light/12 hours of natural light/dark cycle under standardized housing conditions at constant temperature (25 ±2 °C) and humidity (45±5%). During the study, commercial pellet diet and water were given *ad libitum*.

Animal groups and treatment schedule: In Control group, rats were exposed to free access to food and water; AA group rats were subjected to AA was dissolved in distilled water and animals gavaged 40 mg/kg /bw. one a day for fifteen consecutive days, according to Tabeshpour et al. (43); Rats including Ns group were administrated NS 10mg/kg/bw. one a day for fifteen consecutive days, according to Abduallah et al. (4); and AA+NS group, rats co-administrated with AA and NS one a day for fifteen consecutive days. AA was administered orally by gavage, while NS administration was performed intraperitoneally. Body weight (bw) was measured baseline and after treatment weekly. All animals were sacrificed by servical dislocations under thiopental sodium anesthesia (40 mg/kg, i.p.) and tissue samples were taken after 24 h from the last application. Tissue samples were stored at -80 °C until spectrophotometric analysis. Some of the tissues were stored in Baker's formaldehyde-calcium solution until histopathological analysis.

Tissue sampling and homogenates: The liver was quickly removed, weighed, and cleaned with a cold saline solution. Tissues were chopped and homogenized (10% w/v) separately in a Potter–Elvehjem homogenizer in ice-cold sodium, potassium phosphate buffer (0.01 M, pH 7.4) containing 1.15 percent KCl. Tissue homogenates were then centrifuged at 5000 rpm for 10 minutes at 4 °C, and aliquots of supernatant were separated and quickly stored at 20 °C until further usage to test lipid peroxidation, antioxidant, and cytokine parameters (21). All reagents used were of the highest commercially available quality.

Evaluation of oxidative/antioxidant indices: Tissue oxidative stress and antioxidant indicators were assessed. As Ohkawa et al. (36), described, we measured the malondialdehyde (MDA, nmol/mg) level in liver homogenates to determine lipid peroxidation (36). Reduced glutathione (GSH, nmol/mg), an antioxidant marker, was examined as previously reported Breutler (11). The activities of antioxidant enzymes, superoxide dismutase (SOD, nmol/mg), was evaluated as stated by Nishikimi et al. (35).

Evaluation of cytokines levels: In tissue samples, pro-inflammatory (TNF-α, IL-6, IL-1β) and anti-inflammatory (IL-10) cytokines were quantified using commercial

ELISA kits. TNF-α, IL-6, IL-1β, and IL-10 (Pg/ml) levels were measured with anti-rat ELISA kits according to the manufacturer's instructions as mentioned above (47).

Histopathological investigation: Liver tissue samples were fixed in Baker's formaldehyde-calcium solution at +4 ºC in the dark for at least 16 h. From each fixed liver sample, to estimate percentage volume of fat in the liver cells, 12-micrometer cross-sections were cut and stained with oil Red O. The sections were examined under the light microscope (Leica DM2500, Switzerland). According to the degree of hepatic lipidosis was classified as none (%0), mild (< 20%), moderate (20-40%) and severe (> 40%) (13).

Statistical Analysis: The SPSS 20.00 package program was used to conduct a statistical analysis of the data gathered at the study's end and determine the significance of the differences between groups. Analytical methods were preferred to analyze variables for normal distribution. All variables were reported as means standard error. The groups were compared using a one-way ANOVA test. Following the determination of variance homogeneity, in cases where the p-value was less than 0.05, pairwise post hoc comparisons (Tukey) were employed to test the significance of the groups, and Duncan's Multiple Range test was utilized in the analysis of variance.

Results

Oxidative/antioxidant indices following AA and/or NS treatment: AA exposure led to a significant increase in MDA content (P<0.05) and a significant decrease in GSH and SOD activities (P<0.05) in liver samples compared to the control group. Meanwhile, the AA+NS group showed a significant increase in GSH and SOD activities compared to the AA group (P<0.05), while the MDA level demonstrated a statistically significant decrease (P<0.05). Besides, NS-treated rats had non-significant and similar values in oxidative stress/antioxidant parameters compared to the control group (P>0.05) (Figure 1).

Cytokine levels indices following AA and/or NS treatment: AA-induced a series of inflammatory changes that mediated liver injury in this study. TNF-α, IL-1β, and IL-6 levels were significantly higher in the AA group compared to the control group (P<0.05). However, overproduction the levels of IL-1β in the AA+NS group significantly inhibited compared with the AA-induced group (P<0.05), but there was no significant difference in TNF-α and IL-6 levels (P>0.05). While AA administration caused a significant decrease in IL-10 level differing from the control group. (P<0.05), the level of this cytokine in the AA+NS group showed a statistically significant increase compared to the AA group (P<0.05). Also, rats treated with NS had similar values in cytokine parameters compared to the control group (P>0.05) (Figure 2).

Steatosis findings following AA and/or NS treatment: Histopathologically, no signs of steatosis were found in oil red-o staining performed to determine the fatty liver caused by acrylamide. Similar findings were also observed in the C, NS, and AA+NS groups (Figure 3).

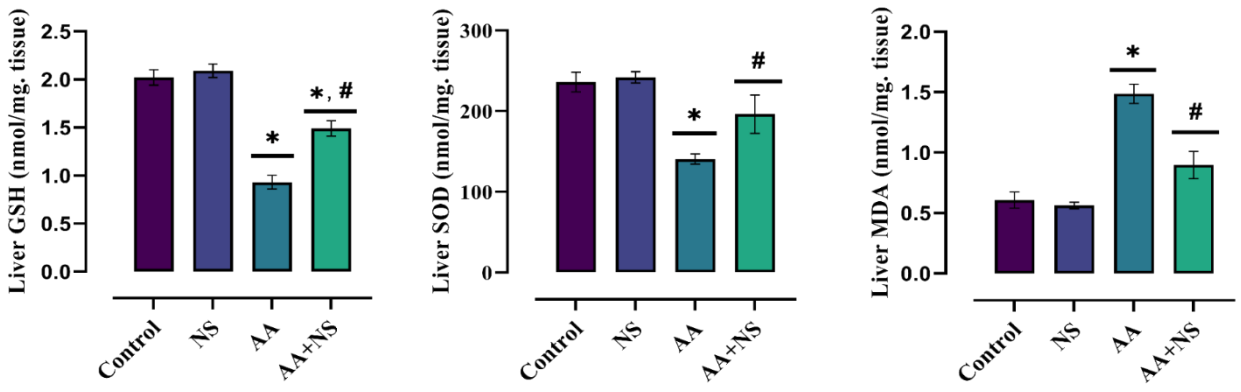

Figure 1. Results of Nigella sativa on AA-induced liver oxidative stress and antioxidant levels (Values are expressed as mean ± SEM). Different symbols in the columns show a statistical difference (* indicates significant difference (P<0.05) when compared with the control group, # indicates significant difference (P<0.05) when compared with the acrylamide group). (NS; *Nigella sativa* group; AA; Acrylamide group; AA+NS; Acrimide+*Nigella sativa* group).

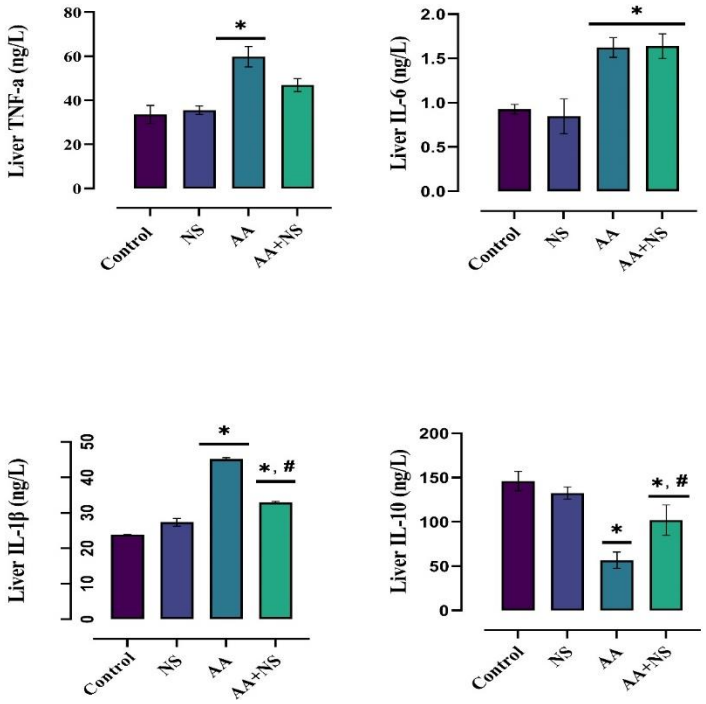

Figure 2. Results of Nigella sativa on AA-induced liver cytokine levels (Values are expressed as mean ± SEM).
Different symbols in the columns show a statistical difference (* indicates significant difference (P<0.05) when compared with the control group, # indicates significant difference (P<0.05) when compared with the acrylamide group). (NS; *Nigella sativa* group; AA; Acrylamide group; AA+NS; Acrimide+*Nigella sativa* group).

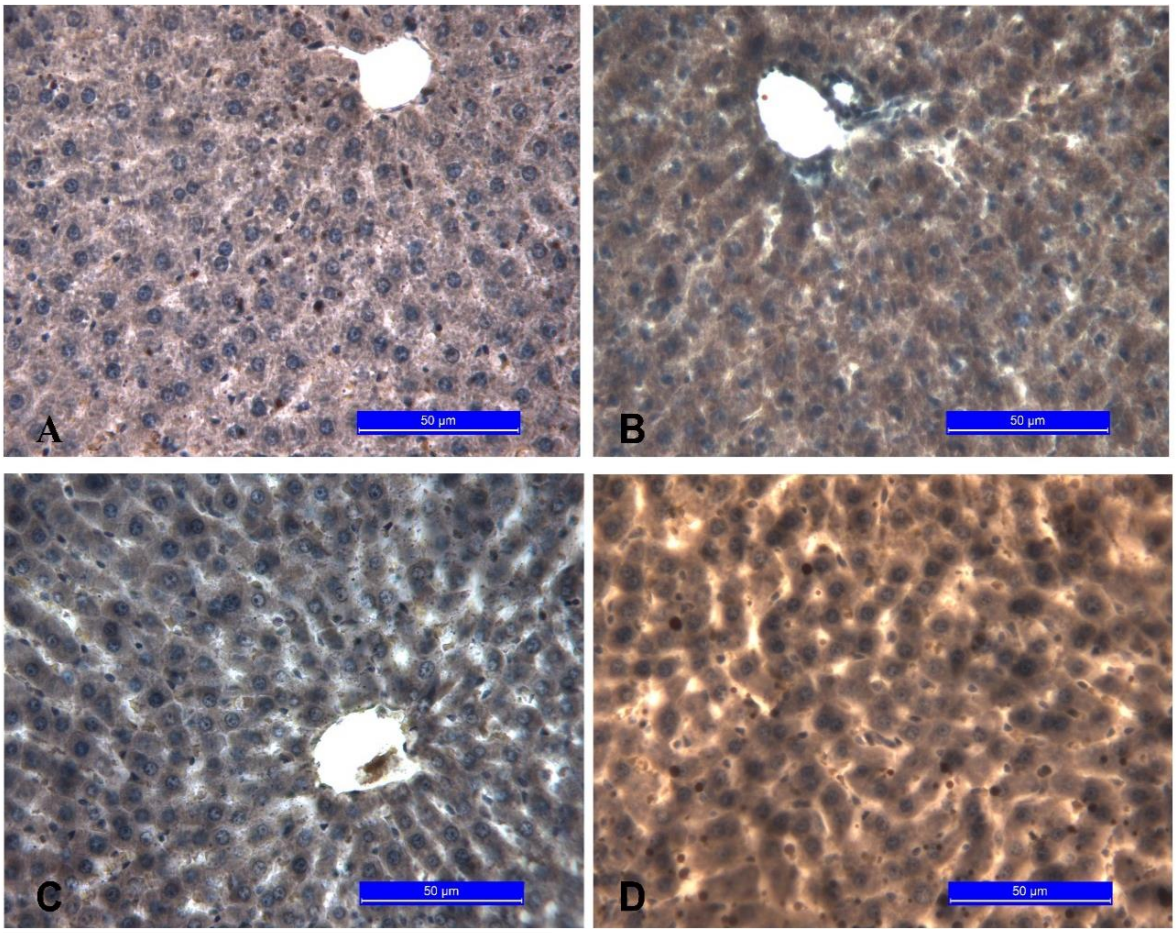

Figure 3. Results of *Nigella Sativa* on AA-induced liver steatosis findings.
A. Control group, B. NS group, C. AA group, D. AA+NS group.

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Discussion and Conclusion

AA, classified as Group 2A (possibly carcinogenic to humans) by the International Agency for Research on Cancer, is a process contaminant formed as a result of heat treatment, especially in foods high in reducing sugar and asparagine (23). This contaminant, which is detected at different levels in foods that are frequently consumed in the daily diet, has been the focus of attention of many food and health authorities due to its known harmful potential health effects. Both the US National Toxicology Program and the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives JECFA consider AA as a human health problem. AA has a highly toxic effect as it can spread easily to tissues after ingestion (10).

ROS, which is produced as a physiological product of cellular metabolism, leads to oxidative stress, which is considered pathological, in abnormal conditions such as toxicity (25). Exposure of animals or humans to a xenobiotic or hazardous substance such as AA gives rise to an imbalance in ROS production and neutralization (1).

As shown in Figure 1, while AA significantly increased MDA, a lipid peroxidation product, in liver tissues, it led to a decrease in antioxidant capacities such as GSH and SOD. This change in MDA, GSH, and SOD levels has been accepted as an indicator of the occurrence of oxidative stress induction and cell membrane lipid damage resulting in AA-induced hepatocyte necrosis. The findings we obtained are similar to previous studies (5, 12). It was reported that AA causes the formation of free radicals, disrupts the antioxidant defense system, and ultimately leads to oxidative stress and carcinogenesis (40). Non-physiological changes found in the levels of oxidative stress and antioxidant defense system are accepted as an indicator of AA-induced hepatotoxicity (5). Biotransformation to glycidamide (GA) (44), which causes mutagenicity and carcinogenicity in the liver via CYP2E1 of AA, is considered a key process in the carcinogenesis of this toxic substance (41). Both AA and GA are covalently bound to GSH for subsequent urinary excretion and are capable of forming AA-GSH and GA-GSH conjugates. GSH conjugates are an important detoxification pathway against ROS resulting from the toxic effect of AA (32). After the GSH pools are depleted, ROSs react with lipids in the cell membrane (42). As a result, these reactive molecules, which cause lipid peroxidation, leading to an increase in lipid peroxidation products such as MDA (36). The body's enzymatic (such as SOD, CAT, and GPx) or non-enzymatic (such as GSH, selenium, vitamin C, vitamin E) antioxidant systems are responsible for scavenging these ROS and protecting DNA and macromolecules from degradation (8). In conclusion, we thought that AA toxication caused liver damage by decreasing the amount of GSH and SOD and inducing the synthesizing of MDA.

NS, which shows biological activity through its active ingredient thymoquinone, has a radical scavenging (anti-oxidative) effect (16). In this study, it was concluded that NS could protect against AA-induced toxicity by significantly reducing the level of lipid peroxidation product (MDA) and increasing antioxidant enzyme levels (GSH, SOD) (Figure 1). These findings comply with previous studies showing the hepatoprotective efficacy of NS as a result of exposure to different toxic substances (1). As a matter of fact, Abdel-Daim and Ghazy (2) reported that significant changes in serum biochemical markers, tissue antioxidant, and lipid peroxidation products in animals treated with oxytetracycline significantly inhibited hepato-lipid peroxidation when treated with NS (2). Similarly, Abdel-Wahab (3) stated that thymoquinone (TQ) administration greatly normalized suppressed enzymatic and non-enzymatic antioxidant levels and reduced hepatic biomarkers and lipid peroxidation (3). Besides, studies have revealed that thymohydroquinone, the reduced form of TQ, shows strong antioxidant properties by returning electrons to hydroxyl radicals (OH) and superoxide radicals, which attack polyunsaturated fatty acids in the cell membrane (27). Based on these findings, we concluded that NS plays a key role in cellular protective mechanisms and exerts hepatoprotective effects by preventing or reducing tissue destruction caused by AA-induced ROS, increasing the level of GSH and SOD, and preventing the formation of lipid peroxidation products.

Cells in the liver can produce cytokines, which act paracrine or autocrine, leading to more cytokine production and amplification of the inflammatory response (30). However, the main source of this inflammatory response in the liver is believed to be Kupffer cells (14). Toxic damage by xenobiotics leads to the secretion of different mediators, including cytokines (TNF-α, IL-1β, and IL-6). The inability of the organism to compensate for the over-release of reactive oxygen species (depletion of enzymatic and non-enzymatic antioxidants) forms the basis of oxidative stress that gives rise to liver damage (30). The present study shows that AA, an industrial xenobiotic, increases the levels of liver IL-6, IL-1β, and TNF-α proinflammatory cytokines, and decreases the anti-inflammatory cytokine IL-10 (Figure 2). The data we obtained comply with previous studies stating that this toxic substance stimulates inflammatory cells and causes an increase in inflammatory response by releasing cytokines (12, 30). In the current study, we concluded that the increase in TNF-α, IL1β, and IL-6 levels in the liver as a result of AA toxicity may be associated with severe oxidative damage due to decreased

levels of enzymatic and non-enzymatic antioxidant defense system members (GSH and SOD) and increased MDA level.

Concomitant NS treatment with AA significantly decreased liver IL-1β levels, while this decrease in TNF-α level, although not significant, tended to decrease (Figure 2). The reason for this is thought to suppress the inflammatory process by neutralizing the oxidative stress that causes liver damage, thanks to the antioxidative and anti-inflammatory effects of NS (48). Our findings are also consistent with another study stating that thymiconicone, the main component of NS, decreased the activities of inflammatory markers such as TNF-α and IL-1β in rats exposed to experimental AA toxicity (1). Also, the increase in IL-10 levels caused by NS treatment was remarkable. (Figure 2) The positive changes in pro-inflammatory and anti-inflammatory cytokine levels as a result of NS treatment reveal that this herbal agent is a good immunomodulator. Indeed, the use of NS has been shown to have immunomodulatory effects in various inflammatory diseases (48).

The liver is the primary organ for drug and environmental agent metabolism. Increased fatty acid production, mitochondrial dysfunction, modification of nuclear receptor activation, insulin resistance, and reduced lipid excretion have all been linked to the development of hepatic steatosis after exposure to some of these drugs and enviromental agents (22). Many industrial chemicals, including halogenated hydrocarbons, volatile organic mixtures, pesticides, and certain nitro organic compounds, have been related to toxicant-associated fatty liver disease and toxicant-associated steatohepatitis (22). Chronic amiodarone exposure has been associated with hepatic steatosis in individuals due to mitochondrial dysfunction and lipid oxidation inhibition (28). Long-term usage of the antiepileptic medication valproic acid has also been linked to fatty liver disease (18). Other drugs, such as tamoxifen, tetracycline, methotrexate, and corticosteroids, have also resulted in human steatosis has been associated with steatosis in the liver (31). No evidence of hepatic steatosis was found between the groups in the present study (Figure 3). Hepatic steatosis, which is closely related to chronic exposure to toxic and pharmaceutical substances, was not thought to occur due to the short duration of the current study.

In conclusion, this study revealed that AA intoxication leads to lipid peroxidation, oxidative stress, and liver damage through a severe inflammatory response. Besides, the present study also showed that intraperitoneally administered NS has a hepatoprotective effect by protecting the liver from AA-induced oxidative stress and pathological cytokine synthesis.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

DH, MÖ and ND conccived and planned the experiments. DH and MÖ carried out the experiments. DH, MÖ and HHD contributed to sample preparation. DH, ND and HHD contributed to the interpretation of the results. DH took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

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

Ethical Statement

This study was approved by the Selçuk University Experimental Medicine Research and Application Center Ethics Committee (2020-29).

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 the combined effects of Turkish mad honey and 5-Fluorouracil in colon cancer model in rats

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ABSTRACT

It was aimed to evaluate the regressive effect of grayanotoxin-rich Turkish mad honey and 5-fluorouracil (5-FU), separately and together by using the N-methyl-N-nitrosourea (MNU)-induced colon cancer modelling in rats. Study groups were designed as control group (CG), cancer control group (CCG), 5-Flourouracil group (FUG), Turkish mad honey group (HG), Turkish mad honey and 5-FU combined group (FU-HG). White blood cell (WBC), lymphocyte, eosinophil, basophil, serum lactate dehydrogenase (LDH), total oxidant status (TOS), and total protein values of the rats in the CCG were significantly lower than the values of the rats in the CG, whereas serum Bcl-2 and survivin levels were significantly higher in the rates belonged to the CCG in comparison to those in the CG. The presence of anaplastic epithelial cells, vascularization, precancerous changes, and inflammatory infiltration detected in the colon and small intestine of the rats in FU-HG, FUG, HG were less intense ($P<0.05$) compared to the findings in the rats in CCG. In conclusion, mad honey and 5-FU reduced anaplastic cell growth and oxidative stress via suppressed anti-apoptotic activity. Considering the histopathological findings in the liver and kidney, no toxicity occurred related to mad honey and 5-FU metabolization. Therefore, the combined use of these two substances may be an alternative method in the treatment of colon cancer.

Introduction

The histopathologic changes in the colorectal cancer (CRC) consist of precancerous changes such as the formation of abnormal crypt foci and mucosal cell aggregations, as well as cellular changes ranging from polyps and adenomas to adenocarcinomas (20).

5-Fluorouracil (5-FU) is an effective and frequently used therapeutic agent in the developmental stages of CRC, when it acquires malignancy characteristics (10). It is suggested that the use of specific antioxidants together with chemotherapeutics in cancer treatment can reduce the incidence of side effects related to cancer drugs and increase the anti-carcinogenic effects of these drugs (19). Antioxidants can effectively protect cells against damage, and therefore they are of vital importance for the homeostasis of cells and tissues (5, 25). A large number of phenolic compounds in the content of Turkish mad honey give the honey its antioxidant properties. These substances defend the cells against the attacks of free radicals and inhibit metabolic action processes (15, 16, 30).

In the CRC model ted with MNU, a directly proportional relationship was determined between inflammation and degenerative changes in the colonic mucosa and oxidative stress (5, 16). Also the oxidative stress index (OSI) value expressed by the total oxidant status (TOS)/total antioxidant status (TAS) ratio is an important parameter in the evaluation of oxidative stress (37). In the evaluation of oxidative stress with PON-1, which is an indicator of oxidation in tissues and PON-1 levels changes due to the changes in the redox status (6, 10).

In order to increase the quality of life in cancer patients, it is very important to investigate the effects of antioxidant substances contained in mad honey against the chemotherapeutic activity and side effects of 5-FU (1, 34). Also the antioxidant properties and cytotoxic activities of Turkish mad honey and Rhododendron ponticum extracts have been evaluated in many studies *in vivo* and *in vitro* (7-9, 14, 29, 39).

For this purpose, it was aimed to evaluate the effects of 5-FU and Turkish mad honey, which are used in the treatment of experimental CRC induced by MNU, on the histopathological changes, oxidative stress and apoptotic mechanisms in cancerous tissues.

Materials and Methods

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (Approval no: 2020-7-55). 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 first group of 6 rats constituted the control group (CG), which did not receive any treatment. The solution, obtained by dissolving 2 mg/rat N- MNU in 0.5 mL of water, was administered rectally to the remaining 24 rats 3 times a week for 5 weeks to induce colon cancer. These 24 rats were then randomly divided into 4 groups (n=6/each group). The second group, consisting of 6 rats, was the cancer control group (CCG), which did not receive any treatment. The third group, consisting of 6 rats, constituted the honey group (HG), in which Turkish mad honey was administered orally at a dose of 0.3 mg/g BW 3 times a week for 4 weeks. The fourth group, consisting of 6 rats, constituted the 5-FU group (FUG), in which 5-FU was administered intraperitoneally at a dose of 12.5 mg/kg BW 3 times a week for 4 weeks. The fifth group consisted of 6 rats (FU-HG) in which 12.5 mg/kg BW of 5-FU was administered 3 times a week for 4 weeks and 0.3 mg/g BW of Turkish mad honey 3 times a day was administered orally by gavage for 4 weeks.

Sampling: Honey samples collected from the Black Sea region in Türkiye and brought in glass jars right after the honey harvest were stored at -80°C until the study was conducted. Before the study, the honey samples were taken out of the freezer and left until they reached room temperature, and these samples were then diluted 1 to 3 with deionized water. In order to provide a good mixture, the mixing process was carried out in a vortex (Heidolph, Reax 2000) device and made ready for study (32).

A total of 7 mL of blood, 5 mL for each tube without anticoagulant and 2 mL for each tube with EDTA, was collected from the rats. Serum samples were removed within 3 hours of the collection of blood samples and these serum samples were stored at -80 °C until analysis.

Blood Analysis: The hematological analyses were performed using an automatic blood count device (Mindray BC 5000) within 3 hours of blood collection. In the serum samples, CRP, total protein, albumin, LDH, ALT, AST, urea, and creatinine levels were measured with an automated biochemistry analyzer (Mindray BS300). Serum Bcl-2, epidermal growth factor, colon cancer specific antigen, matrix metalloproteinase-7, and survivin levels were measured spectrophotometrically, using the ELISA method and related ELISA kits (Sun Red Biotechnology Company, No: 201-11-0038 for Bcl2, No: 201-11-0153 for epidermal growth factor, No: 201-11-6117 for colon cancer-specific antigen-3, No: 201-11-0320 for matrix metalloproteinase-7, and No: 201-11-0192 for survivin).

Histopathologic Analysis: After the rats were sacrificed, necropsy was performed. Possible cancer developing areas and pathological changes in the liver and kidneys as well as other tissues and organs were evaluated according to general macroscopic definition criteria. Samples were fixed in 10% formalin solution for 48 hours. Then, for histopathological examination, the tissues were followed-up at ethanol 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). All tissue sections were stained according to the standart 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 fields under a digital light microscope (Olympus BX51) at 400× magnification. Malignant colonic epitheliums and gland cells as well as proportions in tissue distribution were taken into attention. The results of the evaluations scored according to their numerical equivalents were scores from 1 to 6 (1 =0-4%; 2=5-19%; 3 = 20-39%; 4 = 40-59%; 5 = 60-79%; 6 = 80-100%). The scoring were done on the basis of intensity of nuclear hyperchromasia in malignant cells and proportion as proposed in Quickscore (QS) method modified by Detre et al. 1995 (12). Differences in the numbers of the groups were evaluated statistically. Eligible sites chosen under microscopy were visualized.

Statistical Analysis: The data were initially summarized with descriptive statistics and checked for whether assumptions were met. Results were evaluated by using Shapiro-Wilk test and Q-Q plot for normality and the Levene test for homogeneity of variances. The WBC, lymphocyte, CRP, total protein, LDH, AST, Urea,

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Albumin, rat EGF elisa kit, Rat bcl-2 elisa kit, PON-1 and MMP-7 parameters were found to be normally and heteroscedasticity distributed thus One-Way Anova was used. Monocyte, Creatinine, Rat CCAG3 elisa kit, Rat Survivin elisa kit, TAS parameters' variances were not homogen, consequently, Welch's ANOVA was used. Because of violating both normal and heteroscedasticity distrubition, neutrofil, ezonofil, basophil, ALT, TOS and OSI parameters were compared with Kruskal-Wallis test. Furthermore, histopathological examinations were compared using and One-way ANOVA (GraphPad, USA). In case of statistically significant difference between groups, Tukey, Games-Howell or DSCF multiple comparation analysis were used appropriately. The data were analysed using IBM SPSS Statistics 26.0 (SPSS®, IL, USA) statistical software. The results were analyzed and expressed as mean ± standard error (M±SE). A P value of <0.05 was considered to be statistically significant for all analyses.

Results

Hematological Results: The WBC, lymphocyte, monocyte, neutrophil, eosinophil, and basophil values of the rats in the CCG, FU-HG, FUG, HG, and CG groups are shown in Table 1.

Biochemical Findings: The blood serum CRP, total protein, albumin, LDH, ALT, AST, urea, and creatinine values of the rats in the CCG, FU-HG, FUG, HG, and CG are shown in Table 1.

The blood serum CCSAG3, TAS, TOS, OSI, and PON-1 and tissue EGF, survivin, Bcl-2, MMP-7, values of the rats in the CCG, FU-HG, FUG, HG, and CG groups are shown in Table 1.

Histopathological Findings: Small intestine and colon: Anaplastic glandular and crypt epitheliums were evaded into propria mucosa. While capillarization and hyperemia in the capillaries and medium-sized vessels were observed in the large intestine, these changes were not observed in the small intestine. In both the small intestine and colon, inflammatory changes, usually moderate, in the form of mononuclear cell infiltrations were detected. There were precancerous areas, more intense in the CCG. In these areas, more proliferating dysplastic cells were observed. These cells did not meet the anaplastic criteria and were similar to each other in terms of uniformity.

Table 1. Hematologic-Biochemical parameters according to experimental groups.

	Hematologic-Biochemical Parameters					
Groups	**CCG (n=6)**	**FU-HG (n=5)**	**FUG (n=6)**	**HG (n=6)**	**CG (n=6)**	**P**
WBC (10^9/L)	5.84±0.62^{b}	8.89±0.32^{a}	5.97±0.6^{b}	6.96±0.25^{ab}	8.19±0.31^{a}	*P<0.001*
Lymphocyte (10^9/L)	4.02±0.44^{b}	6.49±0.42^{a}	4.42±0.41^{b}	5.08±0.2^{ab}	6.04±0.24^{a}	*P<0.001*
Monocyte (10^9/L)	0.36±0.04^{a}	0.38±0.04^{a}	0.32±0.03^{ab}	0.24±0.02^{b}	0.33±0.06^{ab}	*P=0.028*
Neutrophil(10^9/L)	1.36±0.22^{ab}	1.88±0.09^{a}	1.28±0.16^{b}	1.46±0.1^{a}	1.57±0.08^{a}	*P=0.049*
Eosinophil (10^9/L)	0.07±0^{b}	0.1±0^{ab}	0.08±0.01^{b}	0.15±0.01^{a}	0.18±0.04^{a}	*P<0.001*
Basophil (10^9/L)	0.03±0^{b}	0.05±0^{b}	0.11±0.06^{a}	0.03±0^{b}	0.18±0.04^{a}	*P=0.003*
CRP (mg/L)	0.45±0.02^{a}	0.22±0.06^{b}	0.35±0.04^{ab}	0.43±0.04^{a}	0.05±0^{b}	*P=0.008*
TP (g/dL)	6.01±0.17^{a}	7.69±0.44^{ab}	7.48±0.54^{ab}	7.93±0.46^{a}	0.11±0.06^{a}	*P=0.018*
Alb (g/dL)	3.91±0.09	3.86±0.18	4.11±0.21	4.42±0.22	0.03±0^{b}	
LDH (U/L)	2447±225.06^{c}	3418±371.29^{bc}	2940.33±433.24^{bc}	5868.83±252.72^{a}	0.07±0.01^{a}	*P<0.001*
ALT(U/L)	100±7.63	65.4±6.3	143±47.87	74.67±5.96	86.83±7.89	
AST(U/L)	180.33±16.86	183.4±27.19	185.67±13.04	241±20.57	201±8.61	
Urea (mg/dL)	60.67±2.43	58.4±2.4	63.83±3.15	63.83±1.9	66.17±2.76	
Creatinine (mg/dL)	1.26±0.05^{a}	0.94±0.03^{b}	1.11±0.11^{ab}	1.08±0.05^{ab}	1.11±0.05^{ab}	*P=0.003*
CCSAG3 (ng/mL)	90.06±2.92	89.46±7.72	86.85±3.05	86.02±5.27	82.08±6.2	
EGF (ng/L)	90.75±1.24	60.84±5	66.89±2.29	66.63±1.95	66.46±4.79	
Survivin (pg/mL)	58.75±4.05^{a}	17.6±1.42^{b}	15.01±1.83^{b}	15.55±2.2^{b}	7.43±0.44^{b}	*P<0.001*
Bcl-2 (ng/mL)	18.47±0.69^{a}	12.15±0.79^{b}	13.83±0.72^{b}	12.32±1.43^{b}	12.88±1.17^{b}	*P<0.001*
MMP-7 (µg/L)	0.22±0.02	0.17±0.01	0.18±0.01	0.2±0.02	0.15±0.01	
TAS (mmol/L)	1.53±0.1	1.66±0.11	2.19±0.38	1.91±0.11	1.89±0.13	
TOS (µmol/L)	2.78±0.54^{b}	17.68±2.94^{a}	14.08±1.47^{a}	19.61±1.59^{a}	18.79±2.56^{a}	*P=0.002*
OSI	1,77±0,62^{b}	10,93±4,56^{a}	7,66±3,96^{ab}	10,72±3,91^{a}	10,11±3,47^{a}	*P=<0.001*
PON-1 (U/L)	488.17±32.01^{bc}	220.6±32.7^{c}	446.5±70.04^{c}	884±63.59^{a}	722.67±99.77^{ab}	*P <0.001*

Values in the table are given as arithmetic mean ± standard deviation (M±SD).
*a,b indicate differences between groups in the same column.

Atypic cells in the small intestine in the CCG animals were statistically significantly higher than the values determined in the rats in the CG (respectively P=0.049, P=0.014, P=0.016, P=0.014, P=0.012, P=0.015, P=0.023). In addition, a statistically significant decrease was determined malignancy, vascularization, and precancerous images seen in the FU-HG, unlike the findings in the CCG (respectively P=0.049, P=0.014, P=0.043, P=0.024, P=0.012, P=0.015). The aforementioned findings determined in the HG were significantly different to the findings in the CCG (P= 0.045, P=0.012, P=0.015, respectively). In addition, a statistically significant increase was found in the pleomorphism and inflammatory infiltration levels determined in the HG rats, compared to the CG rats (respectively P=0.015, P=0.019). It was noted that the inflammatory infiltration status determined in the rats in the FUG was different from the findings in the HG, CG, and CCG rats (P=0.031, P=0.023, P=0.023, respectively).

The previous findings as well as inflammatory infiltration values detected in the colons of rats in the CCG showed a statistically significant increase compared to the values determined in the rats in the CG (respectively P=0.015, P=0.014, P=0.014, P=0.012, P=0.008, P=0.015, P=0.016). In addition, the malignancy findings in the FU-HG were also statistically significantly lower than the findings in the rats in the CCG (P=0.002, P=0.014, P=0.023, P=0.022, P=0.008, P=0.015, respectively). Such findings in the HG and FUG rats were significantly decreased compared to the data in the CCG rats (P=0.048, P=0.045, P=0.008, P=0.015, respectively). The inflammatory infiltration values determined in the HG, FUG, and FU-HG rats were significantly different from those determined in the CG rats (P=0.014, P=0.014, P=0.017, respectively).

Liver: Areas of predominantly vacuolar degeneration and less necrosis started at the periphery of the central vein parts of the lobes and increased at the periphery at varying degrees according to the groups. The vessels and sinusoids in the central vein and portal regions were filled with erythrocytes at different densities. There was no inflammatory cell infiltration as a reaction. Kupffer cells were hypertrophic and hyperplastic.

A significant difference was found between the CG and CCG rats in terms of degeneration, vascular changes, and increases in Kupffer cell activation (P=0.012, P=0.012, P=0.008, respectively). The degeneration detected in the FU-HG, FUG, and HG rats was significantly less severe than that in the CCG rats (P=0.012, P=0.03, P=0.019, respectively). A significant increase was found in the vascularization determined in the rats in the CCG, compared to the results of the evaluations made in the other groups of rats (P=0.016, P=0.028, P=0.015, respectively).

Kidney: Areas showing degeneration were observed in the tubular epithelium of the cortical and medullary regions, especially in the cortical tubular epithelial cells, with varying degrees according to the groups. Tubular degeneration and hypercellularized glomeruli as well as hyperemia in capillariese and medium-sized vessels were noted according to the groups.

Significant increases were found in the data for degeneration, vascular changes, and mesangial cell and podocyte activation in the CCG rats compared to the data in the CG rats (P=0.012, P=0.012, P=0.008, respectively). In the evaluations made in terms of degeneration, the data determined in the HG, FUG, and FU-HG rats were significantly lower than the value in the CCG rats (P=0.012, P=0.021, P=0.012, respectively). The changes in the tissues are shown in Fig. 1-5.

Discussion and Conclusion

The main reason for the frequent occurrence of cancer in the gastrointestinal tract is the occurrence of cell signal pathways that change depending on genetic and environmental factors in the cells in this region (11, 24, 28).

It has been reported that there is a direct correlation between the oxidative stress and inflammatory response in the colon and small intestinal tissue after MNU application and the severity of carcinogenesis (28). Akcilar et al. (3) examined ischemia- reperfusion (IR) damage in the rat intestine, and they determined an increase in serum TAS level and a decrease in TOS level. On the other hand, Dogan et al. (13) reported that there were no significant differences between the groups in terms of serum TOS and OSI levels in IR model rats. Jisha et al. (26) reported a positive correlation between the severity of oxidative stress and inflammation and the developmental rate and severity of the pathogenesis of the tumor in the colon cancer model. In addition, they found significant differences in the serum CRP levels between the studied groups. In our study, we interpreted the fact that the serum CRP value determined in the FU-HGG rats was significantly lower than the value determined in the CCG rats as evidence of the anti-inflammatory effect of the combination of 5-FU and mad honey. In addition, we found that serum TOS and OSI value determined in the CCG rats was significantly lower than the values in the other groups. We attributed the low serum TOS and OSI values to the occurrence of antiapoptotic and proliferative activities metabolits. We also thought that the molecular mechanism and pathyways could demonstrate the exact mechanism. The fact that the serum PON-1 value determined in the HGG rats was significantly lower than the value determined in the CCG rats, was attributed to the anti-inflammatory and antioxidative effects of the bioactive substances in Turkish mad honey. Ahmed et al. (2) stated that mad honey, which contains bioactive substances such as polyphenols and flavonoids with strong antioxidant properties, is used to prevent and eliminate pathological changes in the body due to its free radical scavenging effect.

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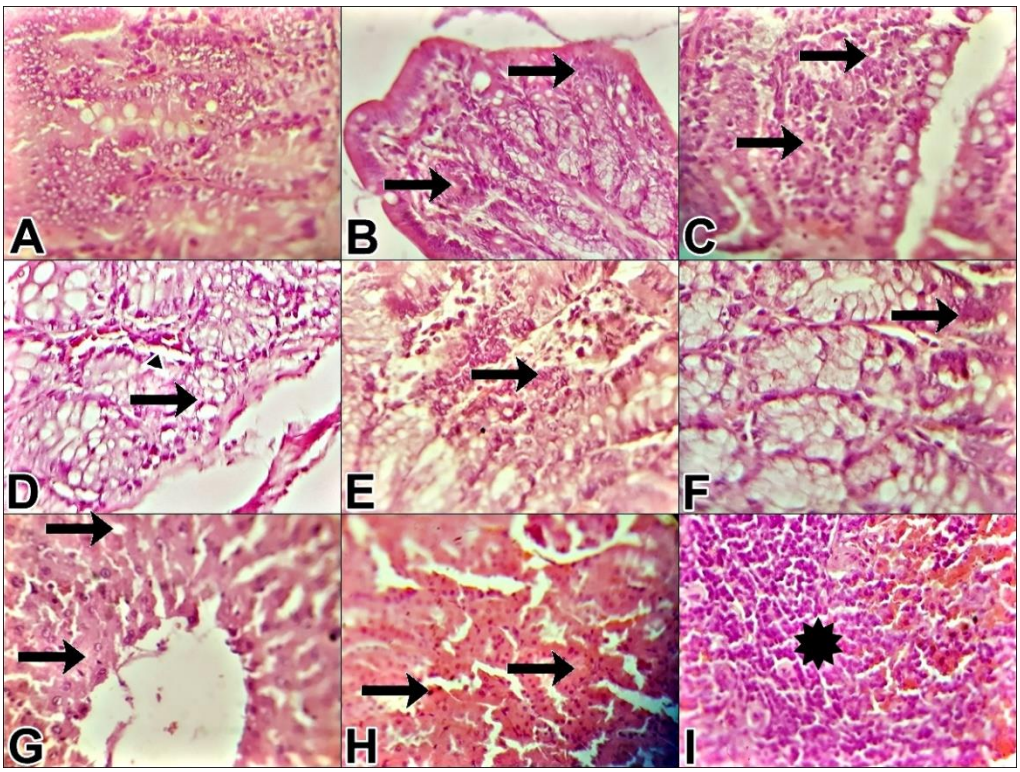

Figure 1. Histopathological findings in the CCG.
(A) Precancerous and cancerous changes in glands in the lamina propria of the intestine, x100, (B) Proliferation of atypical glandular epithelial cells (arrows), x200, (C), Mononuclear cell infiltrations (arrows) in the lamina propria, x400, (D-E) Proliferation of atypical cells in the colon gland epithelium (arrow) and vascularization (arrowhead) in the interstitium, x200, and (F), x400, (G) Congestion in the central vein in the liver and vacuolar degeneration (arrows) in hepatocytes (G), x200, (H) Acute cell swelling and several vacuolar degenerations (arrows) in cortical tubular epithelium of the kidney, (H), x200, (I) Hyperplastic lymphoid follicle in spleen (star), x400, H&E staining.

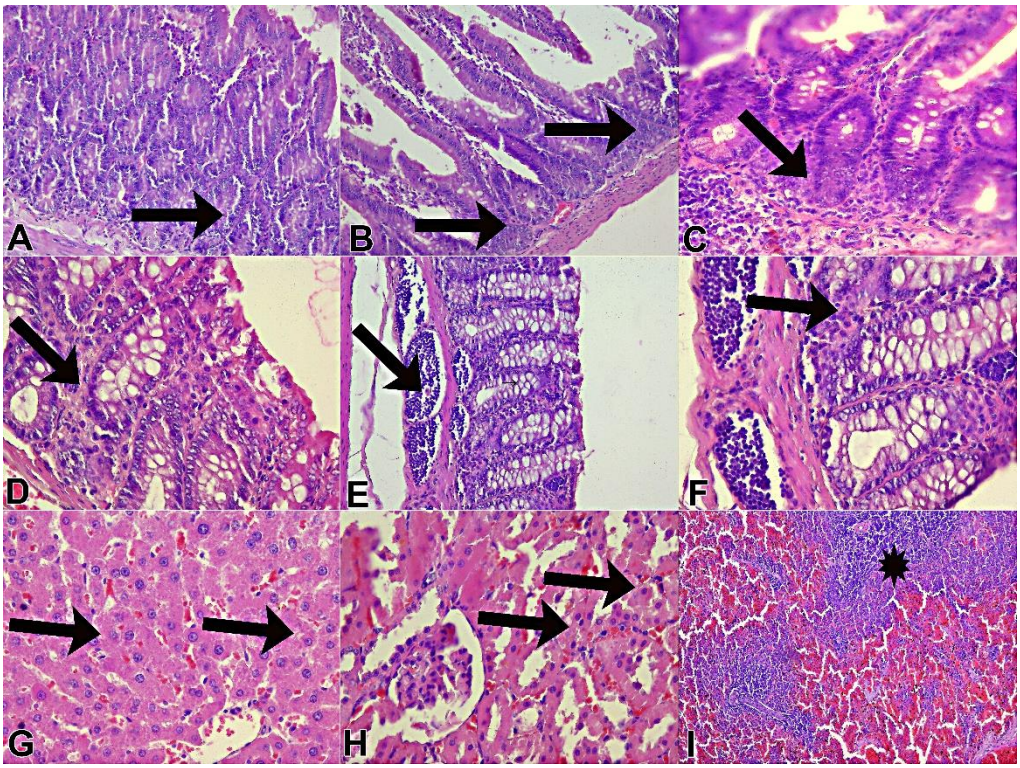

Figure 2. Histopathological findings in the HG.
(A) Atypical proliferation (arrow) in intestinal crypt and glandular epitheliums, x40, (B) Atypical proliferation in glandular epithelial cells (arrows), x40, Mononuclear cell infiltrations in the lamina propria (arrow), x100 and (C), x200, (D-E) Atypical cells in the colon gland epithelium proliferation and mononuclear cell infiltrations (arrow) in the lamina propria, x100, and (F), x200, (G) Congestion of sinusoids in the liver, vacuolar degeneration (arrow) in a few hepatocytes, x200, (H) Congestion of interstitial vessels and acute cell swelling in a few cortical tubular epithels (arrows) in the kidney, x200 (I) Hyperplastic lymphoid follicle (star) in spleen, x40, H&E staining.

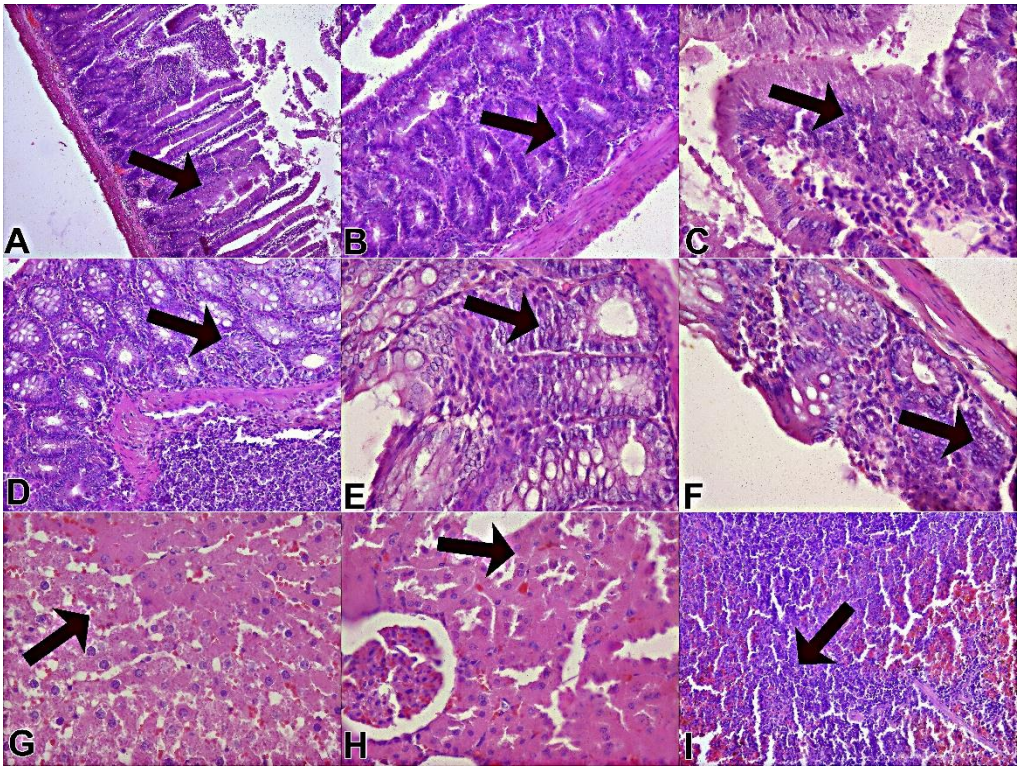

Figure 3. Histopathological findings in the FUG.
(A) Atypical proliferation in intestinal crypt and glandular epitheliums, x40, (B) Atypical proliferation in glandular epithelial cells (arrows), x100, (C) Proliferation in epithelial cell (arrow) and mononuclear cell infiltrations in the lamina propria, x200, (D-F) Proliferation of atypical cells in glandular epithelium (arrow) and mononuclear cell infiltrations in lamina propria of colon x40 and x100. (G) Vacuolar degeneration of hepatocytes (arrow) in liver, x200. (H) Acute cell swelling and several vacuolar degenerations (arrow) in epithelial cells of cortical tubules (arrow), x200, (I) Hyperplastic change in lymphoid follicle in spleen (arrow), x200, H&E staining.

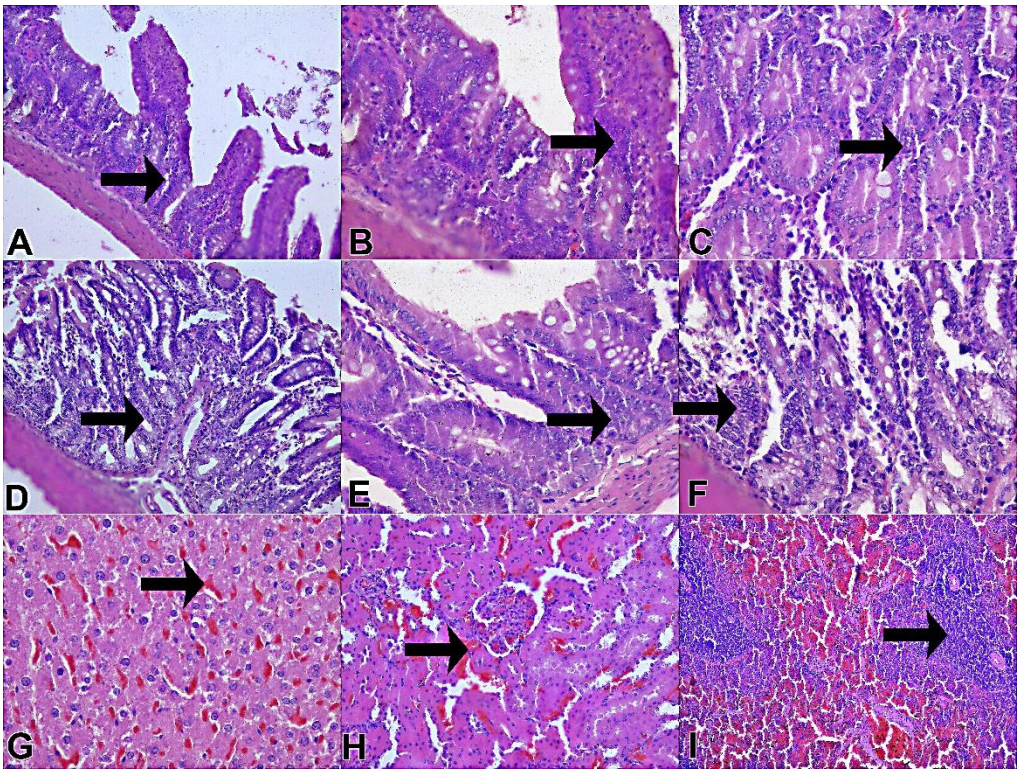

Figure 4. Histopathological findings in the FU-HG.
(A) Proliferation of atypical cells (arrow) in intestine, x40, (B-C), x200, (D-E) Proliferation of atypical cells in a few areas in glandular epithelium (arrow) in colon, x100, and (F), x200, (G) Congestion in sinusoids (arrow) in liver, x200, (H) Interstitial vessels (arrow) in kidney congestion, (H), 200×, (I) Lymphoid follicles (arrow) in spleen, x40, H&E staining.

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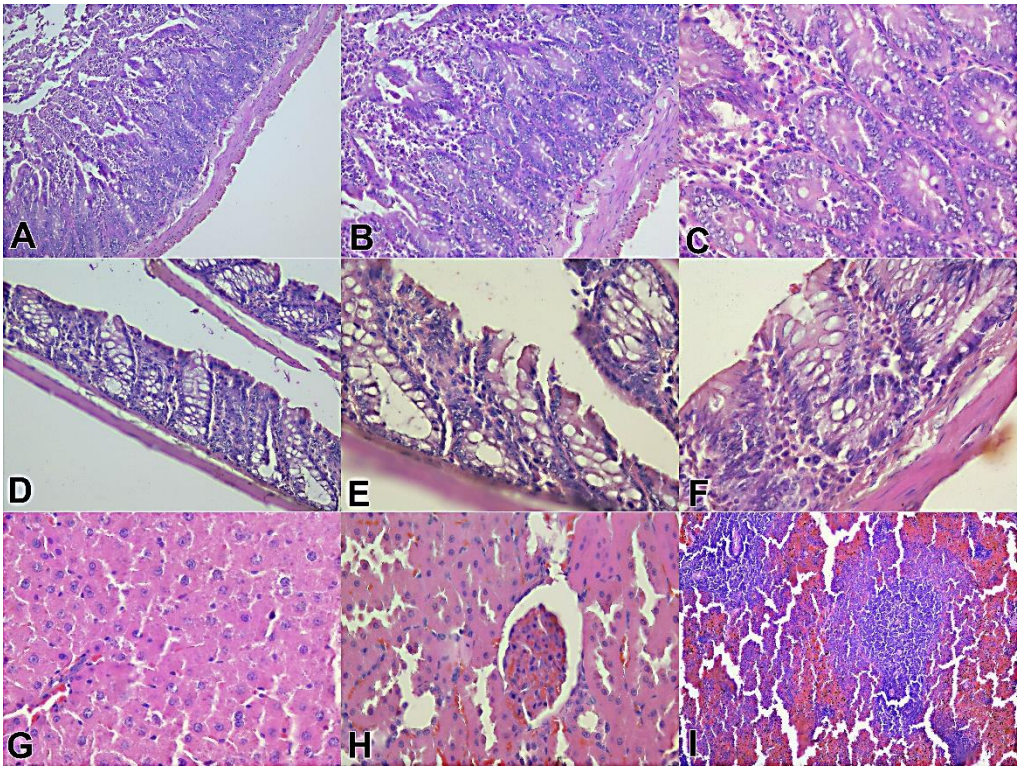

Figure 5. Histopathological views in the CG.
No finding, (A-B) Intestines, x40, and (C), x200, (D) Colon epithelium x40, (E-F), x100, (E-F), x200, (G) Liver, x200, (H) Kidney, x200, (I) Spleen, x40, H&E staining.

In studies conducted in the colon cancer model created with MNU, serum CCSA-3, anti-proliferative effect (TGF, EGF), pro-apoptotic survivin, Bcl2, anti-metastasis MMP-7, and anti-inflammatory CRP levels were evaluated (17, 18, 35). Iliemene and Atawodi (23), determined that the administration of Brachystegia eurycoma (Harms) was effective against serum CEA level, oxidative stress, and inflammatory responses, and ultimately procarcinogenic formations. Rehman et al. (33), determined that piperine application had positive effects on CEA, oxidative stress, and inflammation markers. Hamza et al. (19) attributed the decrease in serum transforming growth factor-beta (TGF-β), carcinoembryonic antigen (CEA), matrix metalloproteinase-7 (MMP-7), and colon cancer specific antigen-4 (CCSA-4) levels to the curative effects of fluorouracil and turmeric applications against inflammation, proliferation, and apoptosis in a colon cancer model. Ahmed et al. (2) determined that Punica granatum caused a decrease in CAE and CCSA levels (which are colon cancer markers), TGF-β and EGF levels (which are antiproliferative effect markers), survivin and Bcl2 levels (which are pro-apoptotic potential markers), and MMP-7 levels from the antimetastasis maker. In our study, it was noted that serum survivin and Bcl-2 values determined in the CCG rats were significantly higher than the values in the other groups. Similar to the results reported by Ahmed et al. (2), we noted that the serum CCSA-3 value, which is one of the colon-specific antigens determined in the rats in the CCG, was higher than the values in the other groups. We interpreted the fact that serum survivin and Bcl-2 levels, which are inflammation and proapoptotic markers determined in FU-HG rats, were significantly lower than the value determined in CCG rats, as the positive effect of the combination of 5-FU and Turkish mad honey.

In the MNU-induced colorectal cancer models, Al-Hassan and Atawodi (4), found a significant difference between the groups in terms of neutrophil/lymphocyte ratio values, while Osowole et al. (31) found no significant difference in the hematological parameters between the groups. Hazilawati et al. (21), found a significant increase in the total leukocyte and lymphocyte levels determined in the groups in which MNU was applied, compared to the values in the control group, in the leukemia model they created by applying MNU in rats. Consistent with reports from various investigators (4, 21, 31). WBC, lymphocyte, eosinophil, and basophil values thought to be related to MNU administration were significantly lower in CCG rats than those determined in CG rats. The fact that WBC and lymphocyte values in FU-HG rats were significantly higher than the values determined in CCG rats was attributed to the combined effect of Turkish mad honey and 5-FU. In the necropsy of the rats in our study, the detection of hyperplastic changes in lymphoid follicles in the spleen tissue and the presence of free erythrocytes in some follicles were interpreted as the degenerative effect of MNU application, in accordance with the literature data (15, 21, 31).

Similar to the reports of researchers (27, 36, 38). pointing out that there are significant changes in LDH value in the colon cancer model in rats, in our study, it was determined that the serum LDH value determined in CG rats differed significantly ($P<0.001$) from the values determined in CCG rats and HG rats. In our study, as a result of the evaluation of serum AST, ALT, total protein, albumin, urea, creatinine levels and histopathological examination results, we determined that there were no significant changes in liver and kidney functions in the process of cancer formation in rats using MNU or during the short-term treatment with Turkish wild honey and 5-FU.

Studies in which tissues were evaluated in colon cancer model studies created with MNU in rats were also carried out. Ahmed et al. (2), stated that they observed the therapeutic effect of punica peel extract at the tissue level in their histopathological evaluations of colon tissue. Huang et al. (22), reported that the efficacy of omega-3 PUFA in colon cancer tissue can be demonstrated by histopathological and protein-level evaluations. In our study, in the evaluation of nuclear atypia, mitotic figures, pleomorphism, polychromasia, vascularization, precancerous changes, and inflammatory infiltration, it was found that the increases in the values in the CCG rats were significantly different from those in the CG rats. In addition, we noted that nuclear atypia, mitotic figures, pleomorphism, polychromasia, vascularization, and precancerous changes were significantly decreased in the FU-HG rats, unlike the values in the CCG rats.

As a result, in the experimental colon cancer model created using MNU in rats, the use of Turkish mad honey and 5-FU separately and in combination reduced the development of anaplastic cells. It was observed that it decreased oxidative stress by reducing and suppressing anti-apoptotic activity, increased inflammatory activity in the intestine, and developed a response to anaplastic development. In addition, it was determined that there was no toxicity in the metabolism of Turkish mad honey or 5-FU due to the rare occurrence of liver and kidney damage in these groups. Therefore, it is thought that their combined use may be an alternative treatment in colon cancer. In addition, it is thought that the present study will guide the research on value-added and research and development products to be used in colon cancer.

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Conflicts of interest

The authors declare no conflict of interest.

Author Contributions

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

Data Availability Statement

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

Ethical Statement

Permission to conduct the study was granted by the ethics committee decision numbered 2020-7-55 of Ankara University Animal Experiments Local Ethics Committee, Türkiye.

Animal Welfare

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

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Determination of *in vitro* digestibility and some quality characteristics of fermented sucuk foods produced for dogs

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ABSTRACT

The aim of current study was to determine *in vitro* digestibility, some microbiological properties and shelf life of fermented sucuk foods consisting mixture of animal and vegetable natural foods produced for dogs. Grain-inclusive and grain-free (GF) formulations were prepared. Grain-inclusive group was subgrouped as cooked (CG) and uncooked grain (UCG). *In vitro* digestibility, nutrient composition, pH and thiobarbituric acid reactive substances (TBARS) values of 3 groups of sucuk foods were determined at 0, 1, 3, and 6 months after production. Microbiological characteristics (Aerobic colony number, *E. coli*, coagulase positive Staphylococcus, coliform bacteria, yeast mold, Salmonella spp.) and lactic acid levels of sucuks were determined at the end of 1, 3, and 6-months of storage (+4°C). There were differences in nutrient compositions of groups and storage times within groups ($P<0.05$). All sucuk foods were negative for *E. coli* and Salmonella spp. Count of aerobic colonies were $5.8x10^7$, $3.0x10^7$ and $3.1x10^9$ CFU/g in CG, UCG and GF, respectively. Total yeast-mold counts were between $5.5x10^3$-$9.6x10^4$ CFU/g. The highest pH drop (5.38 to 4.25) and *in vitro* organic matter digestibility (92.02%) were determined in CG sucuk ($P<0.05$). TBARS value of UCG group was the highest at the end of the 6-months storage ($P<0.05$). Lactic acid levels were not different between storage times and groups ($P>0.05$). As a result, healthy and highly digestible sucuk foods were obtained for dogs, which contain sufficient and balanced nutrients and have a long shelf life.

Introduction

Additives and preservatives that claimed to be harmful to health are used in production of commercial dog foods and affect intake, digestibility, appearance, consistency and shelf life (16). The choice of dog food is similar to the choice of food for people's family members (40). Therefore, natural foods have become popular in nutrition of dogs as well as humans (14). Usage of raw meat-containing products in dog nutrition has increased in recent years among pet owners in many countries. A survey conducted in the USA in 2016 revealed that 17% of dog owners feed their dogs with raw or cooked human food (6). However, there is a little information on the evaluation of dog diets prepared as homemade, organic and with products for human consumption.

As a result of chemical preservatives and usage of poor-quality raw materials in commercial dog foods, people want to prepare diets for their dogs on their own. Meat-based natural feeding is preferred for reasons such as using quality natural foods, imitating feeding of dogs in nature, belief of advantages on health, and avoiding the processes applied in commercial food production (23). Such raw diets may be constructed from recipes that do not have nutritional expertise and feeding studies (38). Therefore, homemade diets are susceptible to nutritional imbalances and deficiencies. Inadequate and unbalanced nutrition of dogs is inevitable as a result of the difficulty of preparing a balanced homemade diets (54).

Good quality ingredients can be used in formulation of homemade diets and artificial additives and preservatives could be avoided. This type of diets provide opportunity to preserve natural enzymes and use of herbal sources and whole ingredients which, may provide health benefits that the individual fractionated ingredients or single nutrients cannot provide (14, 35). Properly formulated homemade diets offer pet owners a good

alternative. Ingredients and nutrient composition can be changed according to the physiological state of the animal. They can also be used effectively in dogs showing allergic reactions to commercial foods (46).

Natural raw diets can be beneficial for animals but are risky for both animals and their owners as they can be contaminated with the zoonotic pathogens (Campylobacter, Salmonella and Yersinia). Dogs expel too much bacteria in their feces, live in the same environment with humans, and are likely to be carriers of pathogenic microorganisms. Therefore, even such diets do not cause disease in healthy animals, they have potential to affect human health (37). Limited data known about the prevalence of these pathogens in dog diets (24). Studies on the natural diet of pet animals based on raw meat have generally focused on microorganism contamination (41).

Food fermentation has beneficial effects such as low-cost preservation, improving digestibility, shelf life, nutritional quality, eliminating toxic components, harmful microorganisms, and protecting against infection (52). Allergic reactions caused by excessive use of animal proteins in dog feeding could be resolved by appropriate fermentation. This could be achieved thanks to pre-prandial proteolysis occurring in fermented foods changing the allergen presentation or cleaving the allergenic protein epitopes (18). The appearance, palatability and texture of foods are also improved by fermentation (18). However, it has been determined that fermented foods are less palatable for dogs due to their acidic odor and taste (59). But in a study, fermented chicken meat did not show a negative effect on intake and body weight in dogs (36).

No scientific studies have been conducted on diets in which animal and vegetable products are fermented together for dogs. Therefore, the objectives of the present study were to obtain natural and nutritionally balanced dog food in the form of fermented round sucuk (turkish sausage) with a relatively long shelf life, highly digestible, microbiologically safe and preservative-free.

Materials and Methods

Preparation of sucuk foods: In the study, two different sucuk formulas were prepared as grain-inclusive and grain free. Formulations were prepared in the diet program designed with the Microsoft Office Excel Package Program for dogs. Formulas have been adjusted to meet the nutrient needs of a healthy adult dog according to FEDIAF (21). Rice and barley were ground in mill (Retsch SM100, Germany) using a 0.5 mm diameter sieve and added to sucuk mixture without cooking in one group (uncooked grain-inclusive). Same grains were added to mixture of other group after cooked for 20-30 minutes for gelatinization and dried for at 55°C 48h before grinding (cooked grain-inclusive). Peas, potatoes and carrots were used instead of grains in grain-free sucuk formula (Table 1).

Table 1. Ingredient and chemical compositions of grain-inclusive and grain-free sucuk foods.

Ingredients, %	Cooked grain	Uncoked grain	Grain-free
Beef, 5-10% fat, raw	12	12	12.37
Liver, chicken	7	7	7
Chicken, breast meat, raw	22	22	20
Beef lung, raw	0.05	0.05	1
Beef tripe, raw	11.5	11.5	11
Eggshell	0.4	0.4	0.4
Barley	19.5	19.5	
Peas, green, raw			7
Carrots, raw			10
Potatoes			30
Rice, white, raw	22.88	22.88	
Garlic	0.1	0.1	0.1
Bone meal	0.7	0.7	
Potassium chloride	0.15	0.15	
Iodized salt	0.07	0.07	0.075
Vit-Min. (dog)[a]	0.15	0.15	0.055
Sunflower oil	3.5	3.5	1
Calculated chemical composition			
Crude protein, % DM	25.24	25.24	25.96
Ether extract, % DM	10.94	10.94	13.05
Carbohydrate*	59.84	59.84	29.67
Crude fiber,% DM	4.92	4.92	5.77
ME (kcal/kg DM)**	3912	3912	4045
Calcium, %	0.67	0.67	0.66
Phosphorus, %	0.48	0.48	0.49
Arginine, %	0.9	0.9	0.68
Histidine, %	0.41	0.41	0.31
Isoleucine, %	0.67	0.67	0.52
Methionine, %	0.35	0.35	0.26
Leucine, %	1.08	1.08	0.79
Lysine, %	1.01	1.01	0.85
Phenylalanine, %	0.64	0.64	0.42
Threonine, %	0.52	0.52	0.39
Tryptophan, %	0.17	0.17	0.12
Taurine, %	0.05	0.05	0.06
Linoleic acid, %	3.77	3.77	2.87
Arachidonic acid, mg/kg $BW^{0.75}$	41.69	41.69	94.11
Vitamin A, IU/kg $BW^{0.75}$	1911.32	1911.32	5603.22
Vitamin D, IU/kg $BW^{0.75}$	32.78	32.78	36.79
Vitamin E, IU/kg $BW^{0.75}$	5.68	5.68	4.62

[a] Premix for dogs. Added per kg of food: iron, 120 mg; copper, 15 mg; magnesium, 75 mg; zinc, 150 mg; iodine,2 mg; selenium, 0.3 mg; vitamin A, 18,000 IU; vitamin D3, 1000 IU; vitamin E, 100 IU; vitamin K, 2 mg; biotin, 0.6 mg; thiamine, 20 mg; riboflavin, 10 mg; pantothenic acid, 50 mg; niacin, 75 mg; vitamin B6, 6 mg; folic acid, 4 mg; vitamin B12, 0.1 mg.

*Carbohydrate (NFE), % = 100 - (% crude protein + % ether extract + % crude fibre +% moisture + % ash).

** Metabolizable energy was calculated with NRC (2006) equations.

Chicken breast meat and liver, beef meat, cattle lung and tripe were minced using a grinder (Fakir, minso plus, Türkiye) through a 3 mm plate. Vegetable sources (peas, carrots, potatoes, rice, barley) were dried for 48h at 55°C and ground before mixing and turned into sucuk mixture. All ingredients were mixed homogeneously using a hand type mixer (44). The samples were taken from each mixture and 3000 g mixture was prepared for each three group. Mixtures containing 54-55% dry matter were kept in the refrigerator overnight. High dry matter level was desired. Because sucuk food rounds with high dry matter content were in good shape (hard, not floppy). The mixtures were stuffed into intestine casings by using a hydraulic filling machine (Emir sausage filling machine, Türkiye). Machine was cleaned and sanitized after filling each group. Ten rounds of sucuk food weighing approximately 300 g were prepared from each group. Sucuks were kept for ripening in laboratory environment at 23-25°C and at 75-95% relative humidity (9). They were placed in vacuum bags when the pH was between 4.7-5.4. After this process, all sucuk foods were stored in the refrigerator (+4°C).

***Determination of nutrient composition and pH*:** Samples were taken from all sucuks after 0, 1, 3 and 6-months storage and pH levels were measured (56). Nutrient analyzes were performed on 3 rounds of sucuks (2 parallel each) at the 0, 1st, 3rd and 6th months of storage. After the intestinal casings was stripped, dry matter levels were determined (VWR-Venti-line, USA) at 55°C for 48h. Dried samples were ground with a chopper (Kenwood CH250, England). Dry matter (DM), ash, crude protein (CP), ether extract (EE), crude fiber (CF) and starch analyzes were performed (5). The metabolizable energy (ME) levels of the sucuk foods were calculated using the following 4-step-calculation formula according to NRC (43):

I. Gross energy (GE): GE (kcal) = (5.7 × CP%) + (9.4× EE%) + [4.1× (NFE%+ CF%)]
   Nitrogen-free extract, NFE (%) = DM% – (EE% + CP% + ash% + CF%)
II. Energy digestibility (%) = 91.2 – (1.43 × CF%)
III. Digestible energy: kcal DE = (kcal GE × energy digestibility)/100
IV. Metabolizable energy: ME (kcal) = kcal DE – (1.04 × CP%)

Determination of thiobarbituric acid reactive substances (TBARS) value and lactic acid level: In order to monitor lipid oxidation, TBARS analysis was performed on the sucuks by using the method of Kilic and Richards (32) at the 0, 1, 3 and 6 months after storage. A sample of 20 g was homogenized with 100 ml of 1:1 20% trichloroacetic acid (TCA) (w/v) in 2M phosphoric acid and distilled water. The slurry was then filtered through the Whatman No. 1 filter paper and the volume was completed to 100 ml. After that, 5 ml of the filtrate was mixed with 5 ml of TBA (0.02M) in a test tube. A blind solution was prepared using 1:1 TCA:distilled water. The tubes were incubated at 80°C for 35 min. Finally, the absorbance was measured using a spectrophotometer (Shimadzu, mini-1240, Japan) at 532 nm. The TBARS value was calculated by multiplying the absorbance by 5.2 to express the concentration as mg malonaldehyde/kg samples. Spectrophotometric method was used to determine the lactic acid levels (10).

Microbiological analysis: Total mesophilic aerobic colony, *E. coli* (12), coagulase positive Staphylococcus, coliform bacteria (4, 56) and yeast-mold count (20) on the 1st, 3rd and 6th months of storage in all sucuk food samples was performed. Among the pathogenic bacteria, Salmonella spp. presence was investigated following AOAC (2000) method (4).

Determination of digestibility by in vitro enzymatic method: To determine dry matter (IVDMD) and organic matter digestibility (IVOMD) of sucuk foods in the 0, 1st, 3rd and 6th months of the storage, a 3-phase *in vitro* enzymatic method was followed by using a Daisy^{II} incubator (Ankom Technology Co., Fairport, NY, USA) (Table 2) (39). Six bags were weighed for each sucuk food for each storage time. Each of the digestion jars, which rotates in the Daisy^{II} incubator at constant temperature, were filled with the enzymatic solution. Filter bags (F57, Ankom Technology Corp. Country) used for sample and blank were soaked in pure acetone (99%) to remove substances that could clog pores and inhibit enzyme activity, and then dried prior to use. At the end of the 6h incubation, the bags were removed from the jars and rinsed thoroughly under tap water until clear and dried in oven (VWR VENTI-Line, Germany) at 65°C overnight. After subtracting the blind bag weight change from the sample bag weights, the *in vitro* digestibility of sucuk foods were calculated with the following formula:

In vitro Digestibility (%) = [(initial DM – final DM) / initial DM)] x 100

Table 2. Determination *in vitro* digestibilities of sucuk foods using a Daisy^{II} incubator.

Phases	
	0.5 ± 0.01 g sample was placed in the bags.
1-Gastric digestion	1440 ml pepsin-lipase-HCl solution (HCl 0.075N; pepsin 2g/L; gastric lipase 1g/L) 39˚C, 2 hours
2-Small intestine digestion	1440 ml -pancreatin-bile salt-phosphate buffer solution (10g/L pancreatin 25g/L; bile salt) pH 7.5, 39˚C, 4 hours
3-Collection of undigested sample	F57 bags were washed, dried overnight at 65˚C Ash and dry matter analyzes were performed

Statistical analysis: Statistical analysis was performed using the Statistical Package of SPSS version 22.0 (SPSS, Chicago, IL, USA). The experimental data were subjected to Levene's test to detect the variance homogeneity. The multivariate analyses were implemented for homogeneous variances by General Linear Model procedures to compare the means of nutrient composition, starch, ME, TBA, lactic acid, pH, *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVOMD) values for all groups. Data were analysed using a randomised complete design with sucuk type, storage time and sucuk types × storage time interactions. Storage time differences within each group were also evaluated with same tests. Values were expressed as arithmetic means ± standard deviation. Data were analysed based on the statistical model:

$$Y_{ijk} = \mu + E_i + D_j + ED_{ij} + e_{ijk}.$$

Where, Y_{ijk} = dependent variable; μ = overall mean; E = effect of storage time on the parameters; D = effect of food types on the parameters; ED interaction between the sucuk types and storage time; e*ijk* = the standard error term.

Tukey HSD test was used as a post hoc test for multiple comparison and the level of significance used in all of tests was $P<0.05$.

Results

According to nutrient analysis performed at the 0, 1, 3, and 6 months after storage of sucuks, nutrient levels generally increased in most values in all groups when compared to the day 0. DM, CA and CP levels of GF sucuk group were higher than others. But CG group had the highest level of starch and EE in average evaluation ($P<0.05$). (Table 3).

Table 3. Determined nutrient composition (%DM) and metabolic energy (kcal/kg DM) levels of sucuks at 0, 1, 3 and 6-months of storage (Mean±SD).

Sucuk type	ST	N	DM	Ash	EE	CF	CP	Starch	ME
CG	0	6	54.22±0.05^{d}	2.98±0.07^{b}	13.65±0.13^{b}	7.36±0.16^{a}	24.79±0.42^{b}	38.59±0.44^{c}	3845±20.52^{c}
	1	6	57.12±0.05^{c}	2.72±0.14^{c}	13.83±0.08^{b}	7.04±0.20^{b}	26.69±0.36^{a}	41.53±0.34^{a}	3905±23.29^{b}
	3	6	60.44±0.11^{a}	3.25±0.09^{a}	13.99±0.11^{b}	6.26±0.14^{d}	26.33±0.14^{a}	39.83±0.12^{b}	3969±24.81^{a}
	6	6	57.40±0.14^{b}	2.52±0.09^{d}	16.23±0.45^{a}	6.63±0.19^{c}	26.30±0.18^{a}	41.21±0.73^{a}	4011±52.21^{a}
Tukey HSD									
SEM			0.46	0.06	0.22	0.10	0.17	0.27	18.94
P values			**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
UCG	0	6	55.45±0.17^{b}	3.43±0.16^{a}	13.07±0.06^{b}	6.73±0.15^{a}	25.21±0.15^{d}	35.49±0.37^{c}	3858±26.46
	1	6	64.33±0.28^{a}	3.28±0.13^{b}	13.51±0.16^{a}	6.74±0.14^{a}	26.49±0.16^{c}	37.04±0.17^{b}	3884±24.43
	3	6	54.41±0.30^{c}	3.29±0.08^{b}	13.03±0.15^{b}	6.35±0.17^{b}	28.56±0.11^{b}	39.13±0.21^{a}	3878±21.57
	6	6	56.94±0.10^{b}	2.95±0.11^{c}	13.06±0.19^{b}	6.34±0.16^{b}	28.96±0.12^{a}	37.97±0.20^{ab}	3855±30.68
Tukey HSD									
SEM			0.81	0.04	0.05	0.05	0.31	0.28	5.60
P values			**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.163
GF	0	6	55.29±0.32^{c}	5.04±0.09^{b}	12.02±0.16^{a}	10.12±0.19^{a}	30.96±0.08^{d}	26.72±0.36^{a}	3801±28.59^{c}
	1	6	55.62±0.27^{c}	5.23±0.11^{b}	11.77±0.17^{b}	9.48±0.15^{b}	31.02±0.09^{c}	25.27±0.33^{b}	3854±28.60^{b}
	3	6	58.77±0.28^{a}	5.70±0.09^{a}	10.59±0.19^{c}	8.48±0.17^{c}	32.70±0.38^{a}	24.11±0.21^{c}	3843±20.40^{b}
	6	6	56.65±0.42^{b}	5.75±0.10^{a}	12.09±0.27^{a}	7.82±0.15^{d}	31.91±0.25^{b}	25.26±0.21^{b}	3963±20.17^{a}
Tukey HSD									
SEM			0.65	0.101	0.43	0.367	0.41	0.284	20.16
P values			**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**
CG(average)		24	57.30^{b}	2.87^{c}	14.42^{a}	6.82^{b}	26.04^{c}	40.29^{a}	3933^{a}
UCG(average)		24	57.78^{a}	3.24^{b}	13.17^{b}	6.54^{c}	27.31^{b}	37.40^{b}	3869^{b}
GF(average)		24	56.58^{c}	5.43^{a}	11.62^{c}	8.97^{a}	31.65^{a}	25.34^{c}	3865^{b}
SEM			0.049	0.023	0.042	0.034	0.049	0.071	5.73
ST(average)	0	18	54.98±0.59^{d}	3.82±0.91^{b}	12.92±0.70^{b}	8.07±0.15^{a}	26.99±2.90^{c}	33.60±5.18^{c}	3834±171.93^{c}
	1	18	59.02±3.91^{a}	3.74±1.11^{b}	13.04±0.93^{b}	7.75±0.14^{b}	28.07±2.16^{b}	34.61±7.06^{ab}	3881±177.36^{b}
	3	18	57.87±2.67^{b}	4.08±1.18^{a}	12.54±1.47^{c}	7.03±0.16^{c}	29.20±2.72^{a}	34.36±7.46^{b}	3897±189.86^{b}
	6	18	56.99±0.40^{c}	3.74±1.47^{b}	13.80±1.84^{a}	6.93±0.16^{c}	29.06±2.36^{a}	34.82±7.09^{a}	3943±150.82^{a}
SEM			0.08	0.04	0.06	0.06	0.07	0.10	8.11
P values									
type			**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
ST			**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
type*ST			**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

DM = Dry matter; EE = Ether extraction; CF = Crude fiber; CP = Crude protein; ME = metabolizable energy as the amount of kcal/kg in DM, ST= storage time, month; CG= cooked grain-inclusive sucuk food; UCG = uncooked grain-inclusive sucuk food; GF = grain-free sucuk dog food, SEM = standard error of the mean.

[a,b,c] Values in the same column that are not sharing a common superscript differ significantly ($P<0.05$).

In vitro dry matter digestibility (IVDMD) and organic matter digestibility (IVOMD) values of sucuk foods were given in Table 4. CG group had the highest coefficients of IVDMD (89.73%) and IVOMD (92.02%), UCG group had the lowest IVDMD and IVOMD ($P<0.05$).

Microbiological characteristics (Aerobic colony number, *E. coli*, coagulase positive Staphylococcus, coliform bacteria, yeast mold, Salmonella spp.) are shown in Table 5.

pH decreases were statistically significant for all sucuk groups determined in 6-months of storage (Figure 1). The highest pH drop was determined in CG group of sucuk foods (5.38 to 4.25) after 6-month storage. It was 5.55 to 5.30 in UCG group and 5.45 to 4.84 in GF group. The highest thiobarbituric acid reactive substances (TBARS) values were determined in UCG sucuk group in the 1, 3 and 6-months of storage ($P<0.05$). TBARS level differences between in-group storage times were insignificant ($P>0.05$) (Figure 2). There was no difference of time and type effects between storage times and main groups in terms of lactic acid ($P>0.05$) (Figure 3).

Table 4. *In vitro* dry matter and organic matter digestibilities of sucuk foods (Mean±SD).

	N	ST	IVDMD	IVOMD
CG	6	0	89.12±0.29^{b}	91.44±0.24^{c}
	6	1	90.32±0.28^{a}	92.91±0.07^{a}
	6	3	90.04±0.13^{a}	92.51±0.22^{b}
	6	6	89.45±0.18^{b}	91.21±0.26^{c}
SEM			0.11	0.15
P			**<0.001**	**<0.001**
UCG	6	0	76.05±0.02^{d}	78.15±0.08^{d}
	6	1	78.16±0.03^{c}	81.03±0.10^{c}
	6	3	81.5±0.07^{a}	84.30±0.11^{a}
	6	6	80.16±0.56^{b}	81.71±0.16^{b}
SEM			0.43	0.45
P			**<0.001**	**<0.001**
GF	6	0	84.96±0.11^{c}	86.08±0.17^{c}
	6	1	87.59±0.12^{b}	89.79±0.30^{b}
	6	3	87.62±0.22^{b}	89.42±0.16^{b}
	6	6	89.48±0.26^{a}	91.26±2.11^{a}
SEM			0.33	0.43
P			**<0.001**	**<0.001**
CG(average)	24		89.73^{a}	92.02^{a}
UCG(average)	24		78.98^{c}	81.29^{c}
GF(average)	24		87.41^{b}	89.26^{b}
SEM			0.053	0.051
ST(average)	18	0	83.37±5.61^{c}	85.22±5.62^{c}
	18	1	85.35±5.36^{b}	87.91±5.18^{b}
	18	3	86.40±3.68^{a}	88.74±3.48^{a}
	18	6	86.37±4.52^{a}	88.23±4.76^{a}
SEM			0.06	0.05
P values				
type			**<0.001**	**<0.001**
ST			**<0.001**	**<0.001**
type*ST			**<0.001**	**<0.001**

^{a,b,c} Values within a row with different superscripts differ significantly at $P<0.05$; IVDMD = *In vitro* dry matter digestibility; IVOMD = *In vitro* organic matter digestibility; SEM = Standard error of the mean.
ST = storage time (month); CG = cooked grain-inclusive sucuk food; UCG = uncooked grain-inclusive sucuk food; GF = grain-free sucuk dog food.

Table 5. Microbiological analysis results of sucuk foods at the end of 1, 3 and 6-months of storage.

Microbiological Analysis	**Group**	**1 month**	**3 month**	**6 month**	
Number of aerobic colonies	Cooked Grain	4.2×10^{8}	7.0×10^{7}	5.8×10^{7}	cfu/g
	Uncooked Grain	1.2×10^{2}	5.0×10^{8}	3.0×10^{7}	
	Grain free	8.0×10^{8}	9.5×10^{8}	3.1×10^{9}	
E. coli	Cooked Grain	<3	<3	<3	EMS/g
	Uncooked Grain	<3	<3	<3	
	Grain free	<3	<3	<3	
Coagulase (+) Staphylococcus	Cooked Grain	<10	<10	<10	cfu/g
	Uncooked Grain	<10	<10	<10	
	Grain free	<10	<10	<10	
Coliform bacteria	Cooked Grain	1.2×10^{2}	<10	<10	cfu/g
	Uncooked Grain	1.5×10^{2}	<10	<10	
	Grain free	7.2×10^{2}	<10	<10	
Number of total yeast and mold	Cooked Grain	2.0×10^{4}	6.0×10^{3}	5.5×10^{3}	cfu/g
	Uncooked Grain	<10	1.1×10^{4}	9.6×10^{4}	
	Grain free	1.7×10^{5}	7.0×10^{4}	6.0×10^{4}	
Salmonella spp.	Cooked Grain	Negative	Negative	Negative	/25 gr
	Uncooked Grain	Negative	Negative	Negative	/25 gr
	Grain free	Negative	Negative	Negative	/25 gr

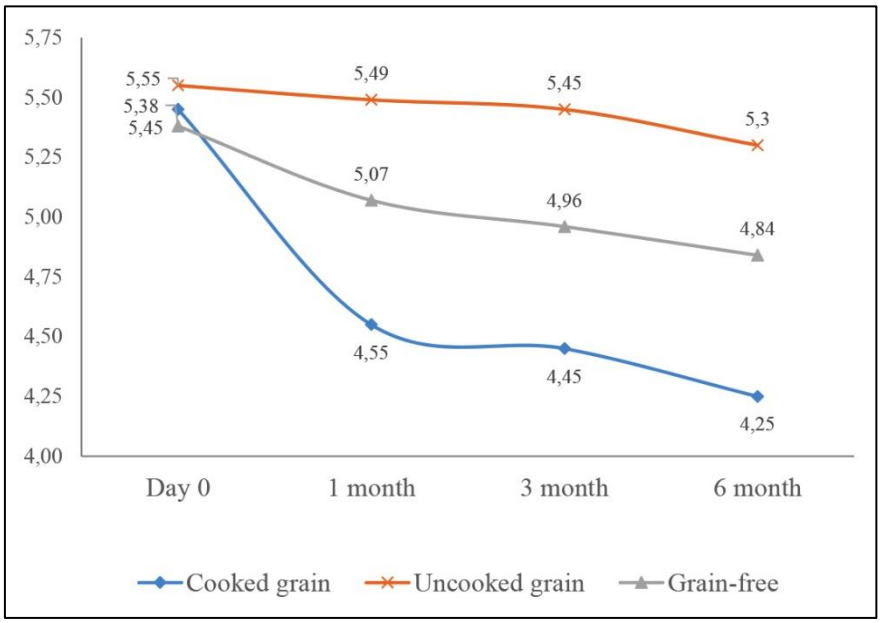

Figure 1. pH changes of sucuk foods in different storage times.

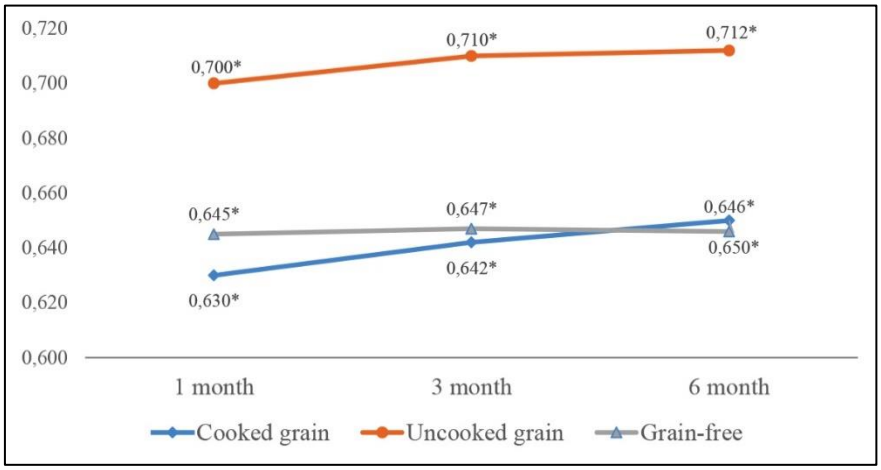

Figure 2. TBARS values of sucuk foods in different storage times (mg malonaldehyde/kg).

*: The values between the groups differ significantly (P<0.05).

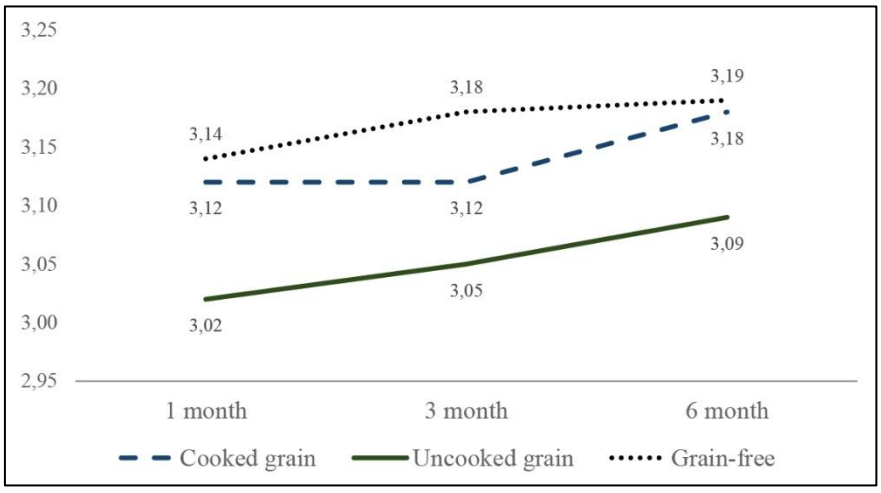

Figure 3. Lactic acid values of sucuk foods in different storage times (% DM).

Discussion and Conclusion

According to nutrient analysis results of the 1, 3, and 6-months after storage of sucuk foods, there were some changes compared with the results of the analysis performed on the first day (day 0). However, despite a storage period of 6 months, all sucuk foods had sufficient nutrients for adult dogs. In a study, there was no nutrient loss or gain as a result of 24-hour fermentation of chicken meats with 9.7% corn starch inoculated with *Pediococcus* spp. as a snack for dogs (36). In this study, nutrient losses were not determined in sucuk foods left to spontaneous fermentation without using starter culture. This is an indication of proper storage conditions without any contamination. The reasons for the increase in nutrients in some sucuk foods in the later stages of storage were that bacteria and yeast breaking down compounds during fermentation and grinding of vegetable ingredients with a diameter of 0.5 mm before mixed into sucuk mixture. By the grinding, the cellulotic structures surrounding the protein and carbohydrate-rich endosperm were physically broken down and nutrients were released (51). In addition, bacteria, yeast and molds also took part in the degradation of the cellulotic structure (26). That was the reason of decreased CF levels at 6th month.

Raw meat diets even have a higher risk of foodborne pathogens are gaining popularity among dog owners. Raw meat has a potential to produce harmful bacteria such as *E. coli*, Salmonella, Neospora, Campylobacter (50). Although healthy adult dogs are sometimes resistant to these pathogens, they can be fatal in puppies with immune system problems (53). Pathogen bacteria like Salmonella and *E. coli* were not detected in sucuk foods analyzed after 1, 3, and 6 months of storage. Sucuks were given nutritional properties and physical, biochemical and microbial changes prevented the growth of various pathogenic microorganisms with fermentation (33). Sucuk foods were also safe for coagulase positive Staphylococcus bacteria. Staphylococcus bacteria are likely to be found in fermented meat products with a pH above 4.2 (58). Since the pH values of the sucuk foods were between 4.2 and 5.5, it is possible that coagulase positive Staphylococcus bacteria were determined at a level of <10 CFU/g in this study. Also, appropriate lactic acid levels help to protect sucuk foods against harmful microorganisms by decreasing pH. The presence of coliform group microorganisms above a certain level is an indication that the sucuks are not fully matured, inadequate hygienic conditions and contamination during production (47). Coliform group bacteria were detected in the sucuk foods prepared without heat treatment in the 1st month after production (1.2-7.2 x 10^2 CFU/g). However, in the next counts (3 and 6-months), this group of bacteria, which is an indicator for sucuk quality, was not detected. Possible reasons for this were decreased water activity during ripening, low pH, competitive flora and bacteriocins (15).

The contribution of yeast to flavor is due to their strong proteolytic activity (48). In fermented sausages, yeasts are preferentially present internally, while molds are present on the surfaces due to the presence of oxygen (49). Different levels of yeast and mold were determined in the sucuk foods in this study. Molds have the ability to produce lipase and protease. It also facilitates dehydration by forming micropores in the intestinal envelope. In the first days of maturation, the number of mold and yeast increases rapidly depending on environmental conditions (11). Yeast and mold growth were detected in UCG sucuk food group. Since no preservatives and additives were used in the study, yeast and mold growth was expected. In some studies yeast and mold detected in fermented sucuks and sausages produced for human consumption (17, 48).

Sausages offered for human consumption consist of almost 100% meat products. Salami-like products for dogs usually contain 80-95% meat and 5-10% rice. In this study, the rate of meat products in sucuk foods prepared for dogs was at the level of 53-56%. The rest consists of vegetable products and vitamin mineral additives. It is thought that the low rate of meat is an important factor in the absence of harmful bacteria. No comparison was made as there were no other studies about a product where meat and vegetable sources were fermented together for dogs. High starch from vegetable products contributed positively to the fermentation of sucuk foods produced without the addition of starter culture (57).

Feeding and digestibility studies of homemade diets in dogs are negligible. In addition, no study was found that reported digestibility of sucuk, sausage and salami type dog foods. A few studies have been conducted about raw meat-based BARF (Biologically Appropriate Raw Diet) diets for dogs. The advantages of BARF diets are based on a few popular publications (13). The sucuk foods produced in this study also consist of high percentage of raw meat, but unlike BARF, sucuks are fermented products. Kara (30) determined the IVOMD values of premium quality lamb meat dry dog foods (n=9) as 76.3-87.9%. IVOMD values of the sucuk foods produced in this study ranged from 78.15% to 92.91%. The DM and OM digestibility rates of the sucuk foods appear to be higher than those of premium quality commercial dry dog foods. However, it should be noted that the methods for determining digestibility and research designs affect the results. However, it has been reported in previous studies that fresh homemade diets are more digestible in dogs (22, 54). Felix et al. (22) determined the *in vivo* DM and OM digestibility values of the homemade diet as 86.8% and 90.1%, respectively. The diet of these researchers consisted of 56.7% puffed rice and 29% fresh meat. In a study, DM digestibility value of wet dog food containing

35.7% CP, 30.3% EE and 19.3% starch was determined as 84.8% with an *in vitro* method similar to this study (8). A positive effect of fermentation on *in vitro* pepsin nitrogen digestibility in dogs has been reported (36). Determination of higher digestibility after 1 month in CG, 3 months in UCG and 6 months in GF groups compared to the day 0 supported this effect. The highest digestibility was determined in CG sucuk foods. Dogs eat and digest diets that high in heat-treated starch (29, 2). It has been determined in previous studies that the OM digestibility rate of cooked starch in dogs is very high (7, 39). The high digestibility of diets with heat-treated starch in dogs has also been confirmed for sucuk foods. Tanprasertsuk et al. (55) found nearly 90% DM digestibility rate of homemade diets containing 30% fresh meat. *In vitro* digestibility coefficients of sucuk foods consisting 53-56% meat was also high in this study.

Lipid peroxidation is considered one of the most important causes of quality deterioration in meat products (44). TBARS changes of the sucuk foods in this study were insignificant at the end of 1, 3 and 6-months of storage and were at the levels of 0.6-0.7 (mg malonaldehyde/kg). Increased TBA values in sucuk foods which produced without nitrate and antioxidant addition were expected to reach the highest levels at the end of 1 month. Because TBARS value increases at a high rate during the fermentation of sucuks due to intense lipid oxidation in ripening period (44). The increase in TBARS value continues at a slower rate during storage with the decomposition of the TBARS into volatile compounds. In a study conducted with pork sausages, a continuous increase in TBARS values was determined in products stored at 4°C for 1 month (60). However, it has been emphasized in a previous study that the TBA increase is lower in sausages that kept in 4°C (42). Karsloğlu et al (31) found that TBARS values between 0.2-0.4 mg malonaldehyde/kg in fermented Turkish sucuks.

The pH decreases of the sucuk foods were significant in all groups during the 6-month storage period. Spontaneous fermentation in meat products is characterized by the presence of lactic acid bacteria. The results of the lactic acid determination analyzes performed on sucuk foods confirmed this. Kurt (34) added carbohydrates at the level of 0.6% to sausages offered for human consumption, and determined that lactic acid levels increased from 4.14% to 14.49% at the end of 9 days. On the other hand, Acton et al. (1) added 1% carbohydrate (dexrose, sucrose) to sausages and determined the lactic acid levels to be similar to the control group. In this study, lactic acid levels were found to be between 3.02-3.19 g/kg. Sucuk foods which have a very high carbohydrate source were prepared without the addition of starter culture had lower lactic acid levels than those offered for human consumption. The lactic acid level of the sucuk foods reached the highest level after 1 month. When starter culture is not used in sausages, the highest lactic acid level is usually reached in 8-10 days (3). The reactions involving carbohydrates (glycolysis), proteins (proteolysis) and lipids (lipolysis) are effective in the formation of taste, aroma, color and texture in fermented sausage products. Gökoğlu et al. (25) determined that the pH value of vacuum-packed beef meat decreased from 5.58 to 4.90. In this study, the pH of CG group of sucuk foods decreased from 5.45 to 4.25, and in the UCG group 5.55 to 5.30. Since the CG group contains the highest percentage of starch, the highest pH decrease was determined in this group. Slow pH decreases in sucuk foods produced without addition of starter culture was also an expected situation (28).

According to the results of this study, it is possible to obtain fermented grain-free and grain-inclusive diets with high digestibility, long shelf life, healthy and consisting of animal and vegetable sources for dogs. Storage of sucuk foods at refrigerator temperature (+4°C) is recommended to avoid bacterial spoilage and reduce lipid peroxidation. Results of this study also indicate that utilization of different source of foods together by fermenting is suitable for dog nutrition. Since homemade diets are difficult to prepare and store, the use of sucuk foods would be practical. The effects of such diets on animal health, stool characteristics and digestibility need to be determined by *in vivo* methods. Such products would be a balanced natural feeding method for dogs when properly prepared and stored under the necessary hygiene conditions. For this reason, it is essential to conduct nutritional trials to determine the intake, palatability, preference and effects on health in future studies.

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Conflict of Interest

The authors declared that there is no conflict of interest. Authors are responsible for all content and writing of this paper.

Author Contributions

OK, Fİ, NG and MSA conceived and presented the idea. Fİ provided to sucuk food's ingredient and chemical formulations. OK and MSA contributed to dog food preparations, laboratory analysis, in vitro digestibility trials and carried out the experiments. Fİ and NG contributed to the interpretation of the results and supervised the work. OK took the lead in writing the manuscript with input from all authors. All authors discussed the results, provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

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

Ethical Statement

This work was approved by the Selçuk University, Faculty of Veterinary Medicine Experimental Animals Production and Research Center Ethics Committee under protocol number 2020/17.

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Effects of algae derived pure β–Glucan on *In vitro* rumen fermentation

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ABSTRACT

The major purpose of this study was to determine how varying doses of algae-derived pure β–glucan affected *in vitro* gas generation, volatile fatty acid (VFA) concentrations, methane production, and protozoa populations. Different doses of β–glucan [i.e., 0, 50, 100, 150, and 200 mg/kg feed (DM basis)] were applied to corn silage as experimental treatments. After 6–96 hours of incubation, the dose of 200 mg/kg of DM β–glucan reduced total gas production compared to control (P<0.01). The concentration of total VFA decreased quadratically (P<0.01) as the amount of β–glucan inclusion decreased (except for 200 mg/kg DM) when compared to the control group. The total VFA concentration was found to be the lowest (P<0.01) at 50, 100, and 150 mg/kg DM β–glucan than the other doses. Propionate and butyrate concentrations increased linearly (P<0.01) in the β–glucan supplemented groups, except for the 50 mg/kg DM dosage. When compared to the control group, all doses of β–glucans lowered acetate and the acetate: propionate ratio linearly and quadratically (P<0.01). The addition of β–glucans reduced the number of protozoa linearly (except at the lowest dose) and reduced the methane generation linearly and quadratically (P<0.01). The concentration of NH_3-N did not differ (Linear, P=0.12; Quadratic, P=0.19) between treatments. The key findings were that β–glucan acted as a rumen modulator, and levels of more than 50 mg/kg of feed DM functioned as a potential methane regulator in the rumen due to reduced acetate and acetate to propionate ratio.

Introduction

Antibiotics have been widely used in animal production as low-dose feed additives with effects on growth and feed conversion efficiency, as well as to reduce infections. Antibiotics have been restricted in the majority of countries due to concerns about the spread of resistant bacteria (1, 11). As a result, nonantibiotic feed additives have emerged as an important research topic. β–glucans are seen as a new generation of promising feed supplements (7, 9, 19, 20, 27). β–glucans contain natural polysaccharides with glucose as their basic elements that are connected by beta-linkages. These polysaccharides not only engage easily with immune cells, but they can effectively bind bacteria, preventing pathogen adhesion and colonization in the intestinal system (27). Plants, yeasts, fungi, algae, protozoa, and bacteria all contain β–glucans (12). Previous research has primarily focused on the impact of nutritional β–glucan administration on poultry and pig growth parameters or immunity (7, 9, 20, 32). Specific findings in studies examining the starter concentrate of β–glucan supplementation revealed immune system stimulation (33), an increase in *Lactobacillus* and fibrolytic bacteria, and a decrease in archaea (16). The addition of yeast β–glucan to milk replacer improved nutrient digestibility, villous height to crypt depth ratio, and immune system performance in calves (19). According to Grove et al. (14), the benefits of barley β–glucans inclusion in beef cattle include altering the rumen bacteria to speed up feed digestion. Ma et al. (19) found that supplementing pre-ruminant calves with yeast β–glucan at 75 mg/kg of feed increased nutrient digestion, strengthened immunity by increasing antibody

concentration and had no negative impacts on metabolism. The addition of yeast glucan to the diet of ewes at a rate of 3 g/kg increased milk production, milk fat percentage, and protein percentage by 13.5–14%, 15–30%, and 11%, respectively (35). A recent study (15) found that feeding yeast β–glucans to lambs improves feed utilization. However, most of the commercially available β–glucans have been derived from yeasts and cereals. β–glucan polysaccharides derived from algae are found in red, green, and brown algae seaweed in the forms of starch and cellulose, laminarin, fibers, and foridean starch, respectively, and are considered superior bioactive molecules. When compared to yeast-derived products, algal-sourced β–glucan has various advantages, including being a more concentrated source with over 95 percent β–glucan, compared to 60-80 percent in purified yeast products. Algal-sourced glucan is predominantly β–1,3 glucan, whereas yeast-based products are a combination of 1,3 and 1,6 glucan, which affects bioavailability. Linear β–1,3 glucan and the small particle size interact more directly with the immune cells (10). The role of pure β–glucan produced from algae in modifying ruminal fermentation has not been adequately investigated. The mechanisms of algae-derived pure β–glucan actions on ruminal metabolic activities, rumen pH, volatile fatty acid (VFA) concentrations, NH_3-N amounts, methane generation, and protozoa counts in cattle are yet unknown. As a result, additional research is required. In this study, we utilized algae-derived pure β–glucan and investigated the effectiveness of different doses on the *in vitro* rumen fermentation pattern and methane production.

Materials and Methods

To monitor rumen fermentation parameters over time, an *in vitro* ruminal fermentation trial was carried out. Rumen fluid was obtained before morning feeding from ruminally cannulated 600 kg Holstein non-lactating dairy cows (n=2). To ensure that the rumen fluid had a balanced cellulolytic and amylolytic activity, the donor cows were fed a 50/50 mix of corn silage and concentrate. Rumen fluid was immediately transferred to the laboratory after being placed in a warm Thermos flask (39 °C). Rumen fluid was squeezed through cheesecloth and placed in an erlenmeyer flask (39 °C). Menke and Steingass (23) method was used to make a buffer combination (comprising micro-and macro-elements, a reducing agent, and a resazurin reduction indicator) for *in vitro* rumen fermentation. Particle-free rumen fluid (15 mL) and buffer medium (25 mL) were mixed in a warmed bottle (39 °C) and gassed with CO_2 on a continual basis. As incubation vessels, glass syringes (Fortuna®, Häberle Labortechnik, Germany) with a calibrated volume of 100 ml were employed. In each syringe, a total of 40 mL of rumen fluid–buffer medium mixture was provided. Approximately 300 mg of dry feed sample was contained in each syringe. Five doses of algae-derived pure β–glucan (Sigma-Aldrich - 89,862–5G-F), i.e. 0, 50, 100, 150, and 200 mg/kg feed (DM basis), were added to corn silage as experimental treatments. Corn silage was used as the rumen fermentation substrate. The nutrient composition of corn silage (Table 1) was performed according to AOAC (2). Metabolizable energy (ME) and net energy of lactation (NEL) values in corn silage were calculated using the equations of Menke and Steingass (22). ME (MJ/kg DM) = 0.136 GP + 0.0057 CP + 0.000286 EE2 + 2.20 and NEL (MJ/kg DM) = 0.096 GP + 0.0038 CP + 0.000173 EE2 + 0.54, where GP is 24-h net gas production (mL 300 mg/ DM), CP and EE, are crude protein and ether extract (% DM), respectively. *In vitro* incubation of the samples was carried out in triplicate. Triplicates of bottles with no substrate were used as blanks. The volume of total gas produced at 3, 6, 12, 24, 48, and 96-hour periods were recorded. The produced gas quantities were calculated according to the model developed by Ørskov and McDonald (25) in the Neway computer program.

Table 1. Nutrient composition of substrate (corn silage) used for incubation (% of DM).

Variable	Corn Silage	SE Mean	StDev
OM	94.38	1.19	2.06
CP	7.18	0.01	0.03
CA	5.64	0.05	0.08
Fat	4.21	0.08	0.14
NDF	40.33	0.36	0.63
ADF	26.31	0.50	0.86
ADL	2.27	0.15	0.25
HSEL	14.02	0.84	1.45
OMD (%)	50.16	2.03	3.51
ME (MJ/kg DM)	7.20	0.31	0.53
NEL (MJ/kg DM)	4.08	0.22	0.37

SEM – standard error of the mean; OM – organic matter; CP, crude protein; CA, crude ash; NDF – neutral detergent fiber; ADF – acid detergent fiber; ADL – acid detergent lignin; HSEL –hemicellulose; OMD– organic matter digestibilty; ME – metabolizable energy, NEL – net energy of lactation.

96 hours of gas production were used in 100 mL glass syringes to enable CH_4 measurement and analysis, as described by Kinley et al. (17). After 96 hours of incubation, rumen fluid samples were collected to be analyzed for pH, NH_3-N, VFA, and protozoa. A pH meter (Sartorius PB-20, Germany) was used to measure the pH of the rumen fluid. For NH_3-N and volatile fatty acids (VFA) analysis, each syringe's incubation residue was transferred to a 20-ml centrifugation tube and centrifuged for 15 minutes at 15 000 g at 4°C. Ammonia-N was

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determined according to AOAC (2) using a Kjeltech auto-analyzer (Gerhardt, Bonn, Germany) without a digestion step. The VFA analysis was done in centrifuged media after adding 1 mL supernatant to 0.25 mL meta-phosphoric acid (25 percent, v/v) with an Acclaim 4x250 mm organic acid column using HPLC (ICS 3000, Dionex Corporation, San Francisco, CA). For counting protozoa; one ml of rumen fluid was mixed with 49 ml of rumen protozoa counting solution (2.02 % formalin and 15.15 % glycerol) to determine the counts of rumen protozoa. Diluted ruminal fluid samples were used for counting cells with the help of the Fuchs Rosenthal counting chamber by the method of Boyne et al. (6).

The data were subjected to SAS's GLM analytical processes (Statistical Analysis System, Version 9.1, 2003). To assess the linear and quadratic effects of supplementary algae-derived β–glucan, orthogonal contrast was used. When $P<0.05$, differences between treatments were regarded as significant, and when $0.05<P<0.10$, they were considered tended to be significant.

Results

To evaluate the ruminal fermentation pattern, pH value, and *in vitro* gas production at various hours (Table 2, Figure 1) and quantities of total VFA production and VFA profile, NH_3-N, methane production, and protozoa counts in rumen fluid at 96 h were monitored (Table 3). Rumen pH ranged from 6.82 to 6.93 (Table 2). The highest ($P<0.01$) rumen pH was found at 100 and 200 mg/kg DM β–glucan when compared to the control group. The effects of other doses on rumen pH were found to be insignificant ($P>0.05$). The β–glucan treatment did not affect gas production at 3 hours ($P>0.05$). After 48-96 hours of incubation, the dose of 200 mg/kg DM β–glucan reduced total gas production more than the other doses ($P<0.01$, Table 2, Figure 1). At 6, 24, and 72 hours, 50, 100, and 150 mg/kg DM β–glucan doses did not affect gas production ($P>0.05$) when compared to the control group. However, at 12 and 48 hours, all β–glucan dosages reduced gas production linearly ($P<0.01$). The total VFA concentration decreased quadratically ($P<0.01$) in the β–glucan supplemented groups, while the highest dose (200 mg/kg DM) had no effect. The total VFA concentration at 200 mg/kg DM β–glucan was higher ($P<0.01$) than only 50 mg/kg DM β–glucan. Propionate and butyrate concentrations were linearly elevated ($P<0.01$) in the β–glucan supplemented groups while no effect was observed at the lowest dose (50 mg/kg DM). All doses of β–glucan linearly increased ($P<0.01$) isobutyrate, valerate, and isovalerate concentrations (Table 3). All doses of β–glucans reduced the acetate and the acetate: propionate ratio linearly and quadratically ($P<0.01$) as compared to the control group. The concentration of NH_3-N did not differ (Linear, $P=0.12$; Quadratic, $P=0.19$) between treatments (Table 3). The addition of β–glucans, reduced the number of protozoa linearly (except at the lowest dose) and methane production decreased linearly and quadratically ($P<0.01$, Table 3).

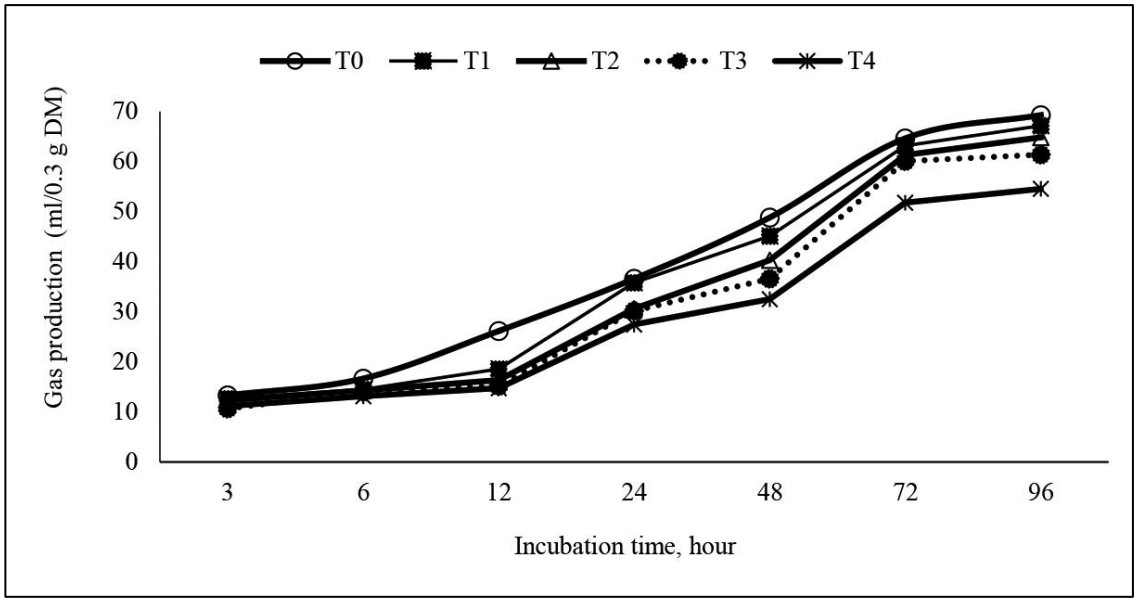

Figure 1. The total gas production (ml/0.3 g DM) from *in vitro* fermentation of corn silage with various β–glucan dosages [T0, T1, T2, T3 and T4 represent the doses of 0, 50, 100, 150, and 200 (mg/kg DM basis), respectively].

Table 2. Effects of different β–glucan levels on *in vitro* ruminal pH and total gas production (ml/0.3 g DM).

							P value	
Variables	T0	T1	T2	T3	T4	StDev	Linear	Quadratic
pH	6.82b	6.85b	6.93a	6.88ab	6.93a	0.03	*	0.30
3 h	13.33	12.67	12.33	10.67	11.17	1.64	0.32	0.77
6 h	16.67a	14.50ab	14.33ab	14.00ab	13.17b	1.10	*	0.26
12 h	26.17a	18.61b	16.34b	15.17b	14.78b	1.58	*	*
24 h	36.60a	35.83a	30.61ab	30.17ab	27.50b	2.63	*	0.81
48 h	48.84a	45.17b	40.35c	36.60d	32.56e	1.09	*	0.89
72 h	64.65a	63.17a	61.33a	60.06a	51.83b	1.98	*	*
96 h	69.30a	67.17a	64.84ab	61.40b	54.56c	1.96	*	*

T0, T1, T2, T3 and T4 represent the doses of 0, 50, 100, 150, and 200 (mg/kg DM basis), respectively.
The differences between the averages indicated with different letters on the same row are significant (P<0.05).

Table 3. Effects of different β–glucan levels on *in vitro* ruminal VFA, NH_3-N concentration, methane production, and protozoa count.

							P value	
Variables	T0	T1	T2	T3	T4	StDev	Linear	Quadratic
Total VFA (mM)	113.74a	105.19c	107.43bc	108.83bc	110.69ab	1.55	0.70	*
Acetate (A, mM)	63.55a	53.53b	51.17bc	52.68bc	50.79c	0.92	*	*
Propionate (P, mM)	35.33c	36.01c	38.99b	38.72b	41.18a	0.80	*	0.89
Butyrate (mM)	9.80c	10.31bc	11.34ab	11.62a	12.42a	0.44	*	0.87
Isobutyrate (mM)	3.24d	3.41c	3.80b	3.72b	4.03a	0.04	*	0.42
Valerate (mM)	0.51d	0.53c	0.59b	0.58b	0.63a	0.01	*	0.42
Isovalerate (mM)	1.32d	1.39c	1.54b	1.51b	1.64a	0.02	*	0.42
A/P	1.80a	1.49b	1.31cd	1.36c	1.23d	0.03	*	*
NH_3-N (mM)	5.37	5.78	5.90	5.89	5.85	0.37	0.12	0.19
Methane (mM)	42.23a	39.00b	39.15b	38.28b	38.12b	0.56	*	*
Protozoa (log 10/mL)	5.65a	5.66a	5.54b	5.56b	5.52c	0.06	*	0.75

T0, T1, T2, T3 and T4 represent the doses of 0, 50, 100, 150, and 200 (mg/kg DM basis), respectively.
The differences between the averages indicated with different letters on the same row are significant (P<0.05).

Discussion and Conclusions

The current study shows that algae-derived β–glucan influences ruminal fermentation, which has previously been demonstrated *in vivo* with yeast-derived β–glucan (8, 16, 19). In this study, the dose level had a substantial impact on gas generation from 3 to 96 hours following the addition of the β–glucan. Although β–glucan doses of 50 and 100 mg/kg did not affect gas generation (except at 12 and 48 hours), the 150 and 200 mg/kg doses significantly reduced overall gas production in the rumen in 12, 48, and 96 hours. Gas release, which results from microbial degradation of the substrate and buffering of acids produced during ruminal fermentation, gives us a brief evaluation of gluconeogenesis (23, 34). The amount of gas produced is proportional to the amount of VFA formed. The majority of the gas is produced during the fermentation of substrate to acetate and butyrate. Propionate production is associated with relatively lower gas production because substrate fermentation to propionate only produces gas from acid buffering (34). Indeed, the current study found that β–glucan supplementation reduced acetate and increased propionate concentrations (Table 3), resulting in a reduction in gas production (Table 2). The increased rate of rumen passage and the kind of VFA produced both contribute to the rate of CH_4 generation by ruminants. Some dietary additives may promote rumen fermentation and digestion while decreasing rumen CH_4 production (5). β–glucans could be predicted to modify and assist rumen microorganisms to expedite fiber digestion and hence increase the generation of volatile fatty acids (14). In contrast to the previous research, the reduction of total VFA production at 50, 100, and 150 mg/kg doses of β–glucan in the current study may be linked to decreased nutrient digestibility and changes in the composition of ruminal microbiota (30). This decrease in total VFA may be unintended or a side effect of feed additives. This warrants further investigation. The primary gases produced during the fermentation inside the

rumen are methane, CO_2, and H_2 (3, 21). The reduction of gas production and decreased proportional CH_4 production in the current study show that β–glucan addition holds promise in reducing CH_4 production and, thus, CH_4 emissions in ruminants. Polyorach et al. (26) discovered that supplementing *Saccharomyces cerevisiae* reduced *in vitro* CH_4 generation, which applies to the present observations. Methanogenic microorganisms are known on the outer layer of rumen ciliate protozoa (31) and as symbionts within the ciliates (13). Methanogens associated with ciliate protozoa were estimated to be responsible for 9 to 25% of methanogenesis in rumen fluid by Newbold et al. (24), and protozoa removal from the rumen (defaunation) has been linked to lower methane generation (29). In the current study, β–glucan supplementation decreased the number of protozoa, which resulted in a decrease in CH_4 production, which agrees with the above statement.

β–glucan doses of 100 and 200 mg/kg DM increased rumen pH, while doses of 100, 150, and 200 mg/kg DM reduced protozoa count to levels acceptable for rumen microbial feed digesting activity but had no effect on ruminal ammonia concentrations (Table 2 and 3). Rumen pH is primarily determined by diet characteristics (chemical and physical) and can range from 5.6 to 7.5 in response to different feedstuffs (18). In contrast to our findings, Cherdthong et al. (8) found that yeast β–glucan supplementation did not affect rumen pH but increased protozoa population at 4 hours of ingestion, with 4.7 g of β–glucan resulting in the largest population. These effects appear to be diet-dependent, with higher responses on high-concentrate diets compared to high-forage diets (28) and differences in derivation source. Similar to our findings, Cherdthong et al. (8) discovered that yeast β–glucan supplementation did not affect ruminal ammonia concentrations.

After 96 hours of *in vitro* rumen fermentation, the addition of algae-derived β–glucan to corn silage significantly impacted total gas production, acetic, propionic, and butyric acid content, the A:P ratio, and CH_4 generation. The addition of β–glucan did not affect the concentration of NH_3-N. The addition of β–glucan dramatically lowered acetate concentrations compared to the control, while significantly increasing propionate, butyrate, isobutyrate, valerate, and isovalerate relative content and lowering the A:P ratio.

The research indicates that β–glucan is performed as a rumen modulator and that levels more than 50 mg/kg feed DM served as a possible methane regulator in the rumen due to lower acetate and acetate to propionate ratios. These findings may also benefit animal performance. Propionate and acetate are in a delicate balance, with butyrate formation playing a key role in methanogenic archaea's H_2 availability. The redirection of metabolic hydrogen to propionate was thought to be a CH_4-blocking strategy (4). However, more research is needed to determine the ideal *in vivo* dose in units of the algae-derived β–glucan, to address the potential impacts on rumen fermentation, animal health, and product quality (milk and meat), and to demonstrate advantages in animal performance and methane emissions.

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Conflict of Interest

The authors are declared that there is no conflict of interest.

Author Contributions

ES conceived and planned the experiments. ES carried out the experiments. FAS helped to identify rumen protozoa. ES and FAS contributed to the interpretation of the results. ES took the lead in writing the manuscript. FAS helped with manuscript editing and proofreading. All authors provided constructive feedback and contributed to the development of the research, analysis, and manuscript.

Data Availability Statement

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

Ethical Statement

This study was carried out after the animal experiment was approved by Bursa Uludağ University Local Ethics Committee (Decision number: 2021-15/04, Approval date: 30.11.2021).

Animal Welfare

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

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An evaluation on research and publication ethics policies of journals publishing in the field of veterinary sciences in Türkiye

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ABSTRACT

The research quality and publication of journals in any field are crucial because they show the development in the field. This study aimed to examine the research and publication ethics policies of the professional journals that continue to publish in the field of veterinary sciences and reveal the current situation. In this study, journals publishing in the field of veterinary sciences were searched through DergiPark, Web of Science database, and their websites. The features of these journals, the indexes in which they are indexed, statements about research-publication ethics, and the national and international academic institutions were gathered under appropriate headings. The obtained data were analyzed using descriptive statistical techniques such as frequency and percentage. Moreover, 11.5% (*n* = 3) of the veterinary journals in Türkiye were indexed in SCI-E, 3.8% (*n* = 1) were indexed in E-SCI, 57.7% (*n* = 15) were university journals, 42.3% (*n* = 11) published two issues per year, and 57.7% (*n* = 15) of journals had publication languages as Turkish and English. Therefore, the number and quality of publications in journals in veterinary sciences have increased in recent years. They comply with "national peer-reviewed journal" criteria and the COPE and ICMJE criteria, wherein the TR index, one of the most comprehensive scanning platforms in Türkiye, has also adopted their principles in several parameters. However, there is no common standard for journals.

Introduction

Science is an indispensable element for society's progress. It aims to reach the scientific knowledge, present the knowledge produced and developed to the scientific world, ensure that it is widely spread and used for the benefit of humanity, and open it for questioning and criticism as much as possible. Research and publication facilitate in spreading knowledge of science (2, 8).

The transmission of information obtained from scientific research to the relevant audience is known as the homestretch of the scientific method (37). When research is completed, either new scientific information is obtained, or the correctness of its assumptions is borne out, and the research is converted into publication and shared with the scientific world (27, 53). It is the necessity of the age to share the scientific information produced through appropriate tools such as scientific journals to reach large masses (38). Scientific publishing is crucial for the proliferation of scientific knowledge and opening it to discussion, and making new scientific contributions (1). The most effective way to spread scientific knowledge to the scientific world is by publishing it in scientific journals (54). Journals play a crucial role in exchanging scientific information by converting scientific knowledge into literature. Scientific periodicals, the most crucial means of publication in sharing scientific knowledge, are making rapid progress in Türkiye (41, 44). As it facilitates access to academic information produced in Türkiye, the visibility of the journal increases. There is an "index" accepted by the Council of Higher Education (CoHE) and the Inter-university Council (IUC) for appointments and promotions known as TR Index, where journals want to be included (52).

As a conciliatory activity with broad socio-political implications, science is open to ethical issues and opposing ideas (42). Science and ethics are evaluated as a whole. Ethical criteria are crucial in presenting research and publications made in the scientific world with integrity to the public (2). It is extremely crucial to comply with the principles of scientific research and publication ethics at all stages of the process from the design of the study to the accuracy and completeness of the information in the publication, production, and development of information for reporting, publishing, and reaching to the reader in a scientific publication (27). In this study, researchers, editors, editorial boards, and referees have crucial responsibilities (1, 2, 27). Scientific research is evaluated together with publication ethics. It is essential to protect academic values such as academic integrity, adherence to professional standards, impartiality, clarity, reliability, and transparency in the writing, presentation, and sharing of research results with the public (27) for a scientific publication to be considered ethically reliable. Moreover, it is necessary to adhere to scientific values during the writing process (37).

The most accurate indicator of the development of a profession is journal values (45). After veterinary schools were started, professional organizations were established to solve the professional and other problems of veterinarians who graduated from these schools (46). To be benefitted from these established associations, journals began to be published as publication organs in the mid-19th century for the scientific and social development of the profession. The first periodical in veterinary medicine in Türkiye was the journal "Vasıta-i Servet," which included articles on agriculture and began its publication life in 1880. The first scientific professional journal in veterinary medicine is known as "Mecmua-i Fünûn-i Baytariye," which was started in 1908 (17, 20).

Since the 1970s, there has been a considerable increase in the number of academic journals in all fields of science in Türkiye (28). Similarly, the number of academic journals increased in the veterinary sciences, and along with several institutions, organizations, and nongovernmental organizations operating in the veterinary field, faculties, vocational schools, and institutes have started to publish research to spread their studies to wider audiences. By the 2000s, dozens of journals were reported to be published in veterinary medicine (40).

The study aimed to examine the research and publication ethics policies of the journals that continue to publish in the field of veterinary sciences in Türkiye as of 2021 and present the current situation regarding these journals.

Materials and Methods

The study's material included veterinary sciences journals that published in Türkiye as of June 2021 and for at least one year in the field of veterinary sciences. The journals to be included in the study were searched using the keywords "veterinary," "animal," "livestock," and "agriculture" through DergiPark and the Web of Science (WoS) databases. The journals not available in the DergiPark and WoS databases were accessed through the internet search engine using the "veterinary journal" keyword, and the relevant information was obtained through their websites. A total of 26 journals were obtained using the mentioned keywords through the DergiPark and WoS databases and the internet search engine.

The journals that are actively publishing in the field of veterinary sciences, the characteristics of these journals, the indexicals in which they are indexed, the statements about research and publication ethics that they include in their publication policy, and the national and international academic institutions of which they are members were identified. Data on research and publication ethics were obtained from DergiPark for the journals that are included in their system, and for those journals that are not included in the DergiPark system, their websites were used by examining "Ethical Principles and Publication Policy, Ethics Policy, Publication Policy, Copyright Transfer Agreement, Conflict of Interest Statement, About the Journal, Authorship Rules, Author's Responsibilities, Information for the Authors, and Ethical Statement in Research with Animals" sections.

The content analysis method was used for the obtained data (58). In addition, the data were analyzed using descriptive statistical techniques such as frequency and percentage. The statements of journals on research and publication ethics were collected under 27 headings, and the national and international academic institutions/databases considered by the veterinary sciences journals examined were collected under 11 headings and presented in detail in tables in the findings section. The works of Yılmaz Gören and Yalım (59) and Aydın (6) were also used for making tables.

Results

In this study, a total of 26 journals that continue to publish as of June 2021 and in the field of veterinary sciences have been identified. Of these journals, 15 were accessed through DergiPark, three through WoS, and five from both DergiPark and WoS databases; three journals that were not available in the DergiPark and WoS databases were accessed through their websites. The findings regarding the veterinary sciences journals examined in this study are presented in Table 1. Moreover, all the journals examined were open access.

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Table 1. Veterinary sciences journals reviewed within the scope of the research.

Journal Name	Beginning Date of Publication **	Publisher	Publication Language	Number of Publications (Year)	Accessed Database
Journal of the Turkish Veterinary Medical Society	1930	Association	Turkish-English	2 Issues	DergiPark
Ankara Universitesi Veteriner Fakultesi Dergisi	1954	University	English	4 Issues	DergiPark-WoS
Livestock Studies*	1959	Institution	English	2 Issues	DergiPark
Journal of Etlik Veterinary Microbiology	1960	Institution	Turkish-English	2 Issues	DergiPark
Journal of Research in Veterinary Medicine*	1981	University	Turkish-English	2 Issues	DergiPark
Eurasian Journal of Veterinary Science*	1985	University	Turkish-English	4 Issues	WoS
Van Veterinary Journal	1990	University	Turkish-English	3 Issues	DergiPark
Journal of the Faculty of Veterinary Medicine, Kafkas University	1995	University	English	6 Issues	WoS
Turkish Journal of Veterinary and Animal Sciences	1996	Institution	English	6 Issues	DergiPark-WoS
Acta Veterinaria Eurasia*	2002	University	English	3 Issues	WoS
Journal of Faculty of Veterinary Medicine Erciyes University	2004	University	Turkish	3 Issues	DergiPark-WoS
Atatürk University Journal of Veterinary Sciences	2006	University	Turkish-English	3 Issues	DergiPark
Journal of Istanbul Veterinary Sciences*	2007	Institution	English	3 Issues	DergiPark
Dicle University Journal of Faculty of Veterinary Medicine	2008	University	Turkish	2 Issues	DergiPark
Kocatepe Veterinary Journal	2008	University	Turkish-English	4 Issues	DergiPark
Fırat University Veterinary Journal of Health Sciences*	2009	University	Turkish-English	3 Issues	Website
Turkiye Klinikleri Journal of Veterinary Sciences	2010	Private	Turkish-English	2 Issues	Website
Bulletin of Veterinary Pharmacology and Toxicology Association	2010	Association	Turkish-English	3 Issues	DergiPark
Animal Health, Production and Hygiene	2012	University	English	2 Issues	DergiPark
Harran University Journal of the Faculty of Veterinary Medicine	2012	University	Turkish-English	2 Issues	DergiPark-WoS
Journal of Bahri Dagdas Animal Research	2014	Institution	Turkish-English	2 Issues	DergiPark
Journal of Advances in VetBio Science and Techniques	2016	Private	Turkish-English	3 Issues	DergiPark
Veterinary Journal of Mehmet Akif Ersoy University	2016	University	Turkish-English	3 Issues	DergiPark-WoS
Turkish Journal of Veterinary Research	2017	Private	English	2 Issues	DergiPark
International Journal of Veterinary and Animal Research	2018	Private	English	3 Issues	Website
Turkish Veterinary Journal	2019	University	Turkish-English	2 Issues	DergiPark

* These journals are given in the table with their current names, **Journals are listed in the table in chronological order.

Moreover, 57.7% (n = 15), a significant part of the journal, were of the faculty of veterinary medicine (university journals); 57.7% (n = 15) were published both in Turkish and English, and 42.3% (n = 11) published two issues per year (Table 1). Moreover, among the journals examined, 65.4% (n = 17) were indexed in TR Index, 50.0% (n = 13) in Türkiye Citation Index, 23.1% (n = 6) in Zoological record, 11.5% (n = 3) in Science Citation Index-Expanded (SCI-E), and 3.8% (n = 1) in Emergency SCI (E-SCI) (Table 2).

While evaluating the content of the research and publication ethics policies of veterinary sciences journals, the statements identified in research and publication ethics were discussed under 27 headings and presented in Table 3. National and international academic institutions/ databases are considered by the veterinary sciences journals examined within the scope of the research presented in Table 4.

Table 2. Indexes of veterinary sciences journals examined within the scope of the research.

	Indexes*	**N**	**%**
1.	TR Index	17	65.4
2.	Google Scholar	14	53.8
3.	Turkey Citation Index	13	50
4.	Cab Abstracts	10	38.5
5.	ResearchBib	10	38.5
6.	EBSCO/EBSCOhost	7	26.9
7.	COSMOS/COSMOS IF	6	23.1
8.	Index Copernicus	6	23.1
9.	Zoological Record	6	23.1
10.	ELSEVIER – Scopus	5	19.2
11.	CABI/CABI full text	5	19.2
12.	Eurasian Scientific Journal Index	5	19.2
13.	CrossRef	3	11.5
14.	DOAJ	3	11.5
15.	SCI-E	3	11.5
16.	Bielefeld Academic Search Engine (BASE)	2	7.7
17.	E-SCI	1	3.8

*In the examination made through the DergiPark and WoS database and the website of the journals that are not in this database, the data specified for each index were obtained by the journals' own declarations.

Table 3. Statements regarding research and publication ethics in veterinary sciences journals examined within the scope of the research.

	Statements Regarding Research and Publication Ethics	**N**	**%**
1.	Plagiarism	23	88.5
2.	Copyright and License	22	84.6
3.	Confidentiality	21	80.8
4.	Acknowledgment	20	76.1
5.	Reader Information	20	76.9
6.	Conflicts of Interest	20	76.9
7.	Responsibility	20	76.9
8.	Research Ethics Committee Approval	20	76.9
9.	Duplication	18	69.2
10.	Supporting Institutions and Organizations	17	65.4
11.	Submission to the Multiple Journals	17	65.4
12.	Originality of the Study	17	65.4
13.	Contribution Notice	17	65.4
14.	Fake Data (Fabrication)	12	46.2
15.	The Timeliness of the Data	11	42.3
16.	Copyright Declaration Form	11	42.3
17.	Similarity	11	42.3
18.	Informed Consent/Elucidating	9	34.6
19.	Animal Welfare and Protection	9	34.6
20.	Animal Rights	8	30.8
21.	Inappropriate Authorship (Gift/Ghost Authorship)	7	26.9
22.	Non-Returning of Unpublished Manuscripts to the Author	6	23.1
23.	Citation Manipulation	6	23.1
24.	Reliability	4	15.4
25.	Ministry Permission for Studies on Wildlife	2	7.7
26.	Ministry Permission for Notifiable Diseases	1	3.8
27.	Patent and Trademark Rights	1	3.8

Table 4. National and international academic institutions/databases are considered by the veterinary sciences journals examined within the scope of the research.

	Journal name	WAME	COPE	ICJME	CC	HELSINKI	CSE	EASE	NISO	ECoC	ORI	CIOMS
1.	Journal of the Turkish Veterinary Medical Society	+	+	+	+		+	+	+			
2.	Ankara Universitesi Veteriner Fakultesi Dergisi		+		+					+		
3.	Livestock Studies				+							
4.	Journal of Etlik Veterinary Microbiology		+		+		+				+	
5.	Journal of Research in Veterinary Medicine		+	+	+							
6.	Eurasian Journal of Veterinary Science			+	+							
7.	Van Veterinary Journal	+	+	+	+							
8.	Journal of the Faculty of Veterinary Medicine, Kafkas University		+		+							
9.	Turkish Journal of Veterinary and Animal Sciences			+	+							
10.	Acta Veterinaria Eurasia	+	+	+	+	+	+	+	+			
11.	Journal of Faculty of Veterinary Medicine Erciyes University				+							
12.	Atatürk University Journal of Veterinary Sciences				+							
13.	Journal of Istanbul Veterinary Sciences	+	+	+	+							
14.	Dicle University Journal of Faculty of Veterinary Medicine		+			+						
15.	Kocatepe Veterinary Journal											
16.	Fırat University Veterinary Journal of Health Sciences											
17.	Turkiye Klinikleri Journal of Veterinary Sciences	+	+	+	+		+	+				
18.	Bulletin of Veterinary Pharmacology and Toxicology Association		+		+							
19.	Animal Health, Production and Hygiene				+							+
20.	Harran University Journal of the Faculty of Veterinary Medicine		+		+							
21.	Journal of Bahri Dagdas Animal Research											
22.	Journal of Advances in VetBio Science and Techniques	+	+	+	+		+	+	+			
23.	Veterinary Journal of Mehmet Akif Ersoy University		+	+	+							
24.	Turkish Journal of Veterinary Research	+	+	+	+							
25.	International Journal of Veterinary and Animal Research		+		+							
26.	Turkish Veterinary Journal											

WAME: World Association of Medical Editors, COPE: Committee on Publication Ethics, ICJME: International Committee of Medical Journal Editors, CC: Creative Commons Attribution 4.0 International License, CSE: Council of Science Editors, EASE: European Association of Science Editors, NISO: National Information Standards Organization, ECoC: European Code of Conduct for Research Integrity, ORI: Office of Research Integrity, CIOMS: Council for International Organizations of Medical Science.

Discussion and Conclusion

In some cases, unethical issues may come up in scientific publications, such as plagiarism, fabrication, falsification, duplication, salami slicing, author rights issues, biased selection of references, biased publication, no acknowledgment, and conflict of interests (27).

In case of plagiarism, the authors quote without citing or present the reference as their study by not complying with the determined standards (19); citations that exceed acceptable limits are unethical, and the freedom to cite never means freedom to steal (27). Plagiarism in electronic publications is much more serious than in printed publications because of the ease of downloading, copying, and pasting (14). It is reported that 20% of the applications made to the "Office of Research Integrity" in the USA have allegations of plagiarism (55). In a study conducted to determine the opinions of the editors on publication ethics of the journals included in "The Scientific and Technological Research Council of Türkiye (TUBİTAK) Turkish Medical Index," the rate of

"publishing information, examples, cases or data belonging to others without permission and without being cited" is 20.6% (1). In a study conducted by Wager and Williams (56) on why journals rejected articles between 1988 and 2008, 16% (n = 312) of the articles were reported to be retractions because of plagiarism, and some journals even banned authors who made plagiarism or unnecessary publications. Therefore, this issue is an extremely crucial ethical flaw; 88.5% (n = 23) of the journals examined in this study also emphasize the issue of "plagiarism" (Table 3), and they try to both eliminate possible ethical flaws and have the necessary standards in terms of publication ethics.

Although there is a "recommendation" in the TR Index criteria as "using a plagiarism program and announcing it in the journal and/or web page," there is no clear statement regarding the plagiarism rate (51). Therefore, with the programs used to detect plagiarism, a total similarity rate (similarity index) of 20% according to some editors and 10% according to others is accepted as the limit of plagiarism (26). Articles 9 and 22 of the "Graduate Education and Training Regulation," which came into force after being published in the Official Gazette on April 20, 2016, included a provision regarding the plagiarism reports of master's and doctoral theses and a rate of 15% excluding citations and 30% including citations were accepted (35). Considering that scientific studies on a similar topic can be similar, particularly in the literature, it is objectionable to report plagiarism on studies that are similar to other studies by only looking at the results obtained from plagiarism programs (50). We found that 42.3% (n = 11) of the journals evaluated in this study focused on similarity. On reviewing for the main text of the study for "plagiarism policy, ethical issues, evaluation processes, and principles" of some journals, it was stated that the similarity rate should not exceed 15%-25% in general; the articles with a similarity rate between 15% and 25% are returned to the relevant author or requested to be corrected by the author, and those exceeding 25% are rejected for being weak in terms of originality and contribution to the field. Sometimes, the same title is sent again without making changes, and thus, similarity exceeding 50% may cause the author to be banned from the journal, and future submissions of the authors may not be considered for publication. In addition, inconsistent with the statement of Toplu (50), a clear distinction should be made between the two as the similarity criteria are different from plagiarism, and journals should set the standard limits, such as the percentages determined for master's and doctoral theses and this determined similarity rate should not be considered plagiarism.

The "Committee on Publication Ethics" (COPE), "Directory of Open Access Journals" (DOAJ), and "World Association of Medical Editors" (WAME) are academic organizations that are experiencing an increase in membership applications and a wide variety in quality. Compliance with the "Best Practice Guidelines" (The Best Practice Guidelines for Journal Editors) of journals is not required. These principles are mostly recommendations developed as guides in complex ethical situations upon requests from editors (15, 18). COPE advises editors and publishers on all aspects of publication ethics and specifically on the handling of research and publication abuse cases. In addition, COPE provides a forum for its members to discuss individual cases. Simultaneously, DOAJ aims to establish, maintain, and develop a reliable source of information about open-access scientific journals on the web (18). The "International Committee of Medical Journal Editors" (ICMJE) determines the standards required for articles submitted to biomedical journals (23). In Türkiye, TR Index has journal evaluation criteria for scientific articles sent to journals to consider the ICMJE recommendations and COPE's "International Standards for Editors and Authors." In addition, in the TR Index, detailed information on open access regarding the journal publication process and policy should be included under the title of "publication policy" (51). When the current status of open access of journals examined was evaluated, we found that all articles of these journals are open access, and 12% (n = 3) of them are indexed in DOAJ (Table 2). In addition, when the COPE (n = 16), WAME (n = 7), and ICMJE (n = 11) memberships of the journals were evaluated (Table 4), journals that are not members of these organizations need to make more efforts, and this number should increase within the scope of "Transparency and Best Practice Principles in Scientific Publishing."

In the "Law on Intellectual and Artistic Works" (LIAW), the improper use of a citation without the written consent of the right holders is considered an "unlawful act" and the penalties and liabilities to be applied are mentioned in Article 71 of the Law (30). Creative Commons Licenses provide a standard way to provide everyone, from individual creators to large institutions, permission to use their creative work under the copyright law. It allows the user to learn the terms of using the work without contacting the copyright owner (9). Copyright regulations must be complied with for the intellectual and artistic works used (51). However, the "copyright" problem is more serious, particularly in e-publications than in printed journals (14). In a study on the research and publication ethics policies of national nursing journals, 26.6% (n = 4) of the journals refer to the issue of "citation permission" in their publication policies (59). In a study examining the opinions of academicians on research and publication ethics, 50.4% (n = 137) of the participants believed that ethical faults are committed on issues such as "inappropriate citation" (39). A study revealed that

80.8% (n = 21) of the journals provided content information for "CC License," 84.6% (n = 22) for "Copyright and License," and 23.1% (n = 6) for "Citation Manipulation" (Table 3); therefore, the journals that publish in the field of veterinary sciences in Türkiye emphasize on "CC License" and "Copyright and License" by their publication policies. In addition, it is necessary to give more place to "Citation Manipulation" in publication ethics policies by showing the same sensitivity for complying with the TR Index journal evaluation criteria and LIAW, aiming to protect the rights of the authors of the cited sources and to be a solution to the copyright problem that can be seen more frequently in e-publications.

According to the ICMJE, when submitting an article with multiple authors, the corresponding author should clearly state the order of the authorship (23). COPE recommends adopting policies that allow transparency about the extent to which authors contribute to the study (10). According to TR Index, the contribution rate and conflict statements of researchers should be included at the end of the article (51). On the other hand, the "Turkish Academy of Sciences" (TÜBA) states in its report that clearly stating possible contributions to the project in experimental studies will minimize the ethical problems that may arise regarding the order of the author (53). In an international study by Broome et al. (7), in which academicians refereeing in journals published in the field of nursing participated, 23% of the participants were concerned about a conflict of interest at least once, and 92% of them reported this to the editor. In a study in which, the opinions of research assistants about the difficulties they encounter regarding research and publication ethics and their self-efficacy were investigated, it was found that the participants had difficulties in expressing themselves while determining the order of authors in the article (29). On the basis of this, 76.9% (n = 20) of the journals considered in this study mentioned the issue of "conflict of interest" (Table 3), which should not be missed in the research data as it is a crucial step and is necessary to prevent wrong evaluations. In addition, this situation is considered and meets the ICMJE, COPE, TÜBA, and TR Index criteria.

In the "Higher Education Institutions Scientific Research and Publication Ethics Directive," the "republishing" conditions are handled meticulously (48). Moreover, COPE disapproves "over-publishing (or double, duplication)," which means reprinting a publication that has been published via print or electronic media and coincides with its other edition (10). It is stated that the relevant author must sign the "Copyright Form" to ensure that the article is an original work, has not been published before, and is not intended to be published elsewhere in its final form in print or electronic form (21). In a study conducted to determine the opinions of journal editors on publication ethics, the rate of "publishing a publication in more than one journal" is 19.6%, and the rate of "publishing a study in both Turkish and a foreign language" is 8% (1). In a study examining the views of nursing doctoral students on research ethics, the rate of "writing more than one article using the same data" behavior was 22.8%, and the rate of witnessing this behavior was 56.1% (22). In this study, the theme of "duplication (republishing)" was 69.2% (n = 18), and the proportion of journals that included the themes of "originality of the study" and "submission to more than one journal" was 65.4% (n = 17) (Table 3). This indicates that the journals publishing in the field of veterinary sciences largely complies with the framework rules determined by the CoHE and COPE and show sensitivity to preventing duplication.

In the Scientific Research and Publication Ethics Directive of CoHE, the expression "not complying with ethical rules in research on humans and animals" is included as action against scientific research and publication ethics (48). According to TR Index journal evaluation criteria, approval from the ethics committee should be obtained for studies conducted in all scientific fields that require it, and this approval should be stated and documented in the article (51). Knowing the special requirements of using experimental animals, respecting them, and complying with universal ethical principles require a scientific and ethical approach; therefore, the experimental animals and research methods to be used in medical research and education must be inspected by ethics committees of animal experiments (13). Moreover, 76.9% (n = 20) of the journals examined in this study requested "research ethics committee approval" (Table 3). On the basis of this, it can be stated that the journals provide a considerable place for the Scientific Research and Publication Ethics Directive of CoHE and TR Index journal evaluation criteria in their publication policies. However, all journals should include the requirement of obtaining ethics committee permission, which is extremely crucial for scientific studies.

The three Rs rule (replacement, reduction, refinement) regarding the use of animals in experiments also forms the basis of today's bioethics rules (43). Moreover, the research and veterinary faculty ethics committees, established in 1998 in Türkiye, protected the ethical rights of animals before the "Animal Protection Law" is enacted, and the protection of the rights of animals has gained rapid momentum with the spread of ethical committees (57). Article 9 of the "Animal Protection Law" numbered 5199 states that "In institutions and organizations that conduct animal experiments, these experiments are allowed through ethical committees established and to be established within their structure"

(31). The "Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees" contains the relevant provisions (34). In recent years, philosophical and legal views on animal research have been changing, and more emphasis has been placed on animal welfare. In the USA, all proposed research projects that will use animals for experiments should be examined and approved by the "Institutional Animal Care and Use Committee" (IACUC) in terms of three basic ethical principles (3Rs) (11). Moreover, 76.9% (n = 20) of the journals examined in this study and included "research ethics committee approval" in their publication policy (Table 3) can be evaluated as the journals publishing in the field of veterinary sciences largely comply with the criteria determined by the legislation. While 30.8% (n = 8) of them included "animal rights" and 34.6% (n = 9) of them included "animal welfare and protection" in their publication policy (Table 3), it can be stated that ethical sensitivity is shown for the protection of animals to be used in experiments through ethical committees. Moreover, these journals in the field of veterinary sciences, including animals, should include the concepts of "rights" and "welfare" as a separate section in their publication policies in more detail.

The effect of wildlife research on the environment is cited as one of the reasons why wildlife research is so morally complex. Similarly, research that will cause great harm to animals and does not provide significant gains and information that is not worth the harm are not allowed by the IACUCs (12). The 40th article of "Regulation on the Protection of Game and Wild Animals and Their Habitats, and the Principles and Procedures of Combating Pests" (32), the fifth article of "Regulation on Animal Diseases that are Obligatory to Report and Notification" (33), and the Scientific Research and Publication Ethics Directive of CoHE in Türkiye contain the relevant provisions (48). In this study, the data on "Ministry's permission for studies on wildlife" and "Ministry's permission for notifiable diseases" were 7.7% (n = 2) and 3.8% (n = 1), respectively (Table 3). Therefore, journals should include more place for the above-mentioned regulations and the criteria determined by CoHE in their publication policies. In addition, the inclusion of information on studies on wild animal species which need permission from the Ministry and notifiable diseases that need to be mentioned in the publication policies of the journals will raise awareness about the direction of the authors. This will contribute to the country's policy in the context of protecting wild animal species and combating notifiable diseases.

In the context of "Academic Promotion and Appointment Criteria" determined by CoHE, the importance of publications in peer-reviewed academic journals and even in indexes such as Science Citation Index (SCI), SCI-E, Social Sciences Citation Index (SSCI), and Arts and Humanities Citation Index (AHCI) have become essential tools for academic promotion and appointment criteria (36, 49). Journals that are not scanned by crucial indexes find it difficult to get quality articles. This situation has become very evident, particularly in our country (4). In Türkiye, it is essential to publish in journals within the scope of SCI-E, SSCI, and AHCI, which are the indexes of Thomson Reuters, for associate professorship applications in the fields of health, science, and engineering (24). Moreover, these indexes scan journals not only in the field of health and life sciences but also in almost all scientific disciplines, and it is considered prestigious for a journal to be in these indexes. In addition, the rate of reading and/or the possibility of citation of the contents of the journal worldwide increases if included in these indexes (4). SCI, SCI-E, SSCI, and AHCI are indexes used by the "Institute for Scientific Information" (ISI), headquartered in the USA. In brief, ISI is a system that regularly scans numerous scientific journals and announces its content to its readers. The importance of ISI has increased even more with the effective use of the internet. Since 2001, after the obligation to publish articles in journals scanned by these indexes in academic promotions in Türkiye, these indexes have become more recognized in the academy. Timely publication of the journal; proper use of English for bibliographic information; publications by the journal's editor, advisory board members, and article writers in journals within the scope of ISI; and citations to them are very crucial criteria in the selection of journals by ISI (3, 4). Aydın (6), in his study on veterinary sciences-themed scientific journals in Türkiye, reports that three journals are within the scope of SCI-E. In consistent with Aydın's research data (6), a study revealed that 11.5% (n = 3) and 3.8% (n = 1) were within the scope of SCI-E and E-SCI, respectively (Table 2). The increase in the number of journals published in the field of veterinary sciences since 2016 and the increase in the number of journals scanned in national and international indexes such as TR Index (65,4%, n = 17) and Zoological Record (23,1%, n = 6) can be associated with the improvement of publication quality. Although the number of journals scanned in SCI-E does not change, the inclusion of a journal within the scope of E-SCI is crucial; however, other journals have made improvements in publication ethics (Table 3) even if they are not in the international index. Although particularly the journals in the international indexes include the statements regarding the research and publication principles (Table 3) in their publication ethics policies, it can be stated that the indexes in which they are included are a factor affecting the ethical policy of the journals.

TR Index makes considerable contributions to the development of scientific journal standards in Türkiye by putting forth binding provisions in the "Criteria for

journals to be included in TR Index." The indexing criteria include the concern of "complying with the standards" of scientific journal publishing (5). Since 2016, the journals indexed in the TR Index have been included in the evaluation by the IUC as per "article published in national peer-reviewed journal scanned by ULAKBİM" in the "National Article" section of the Associate Professor Application Conditions (24). Journals that have editors and an advisory group consisting of faculty members from at least five different universities publish original scientific/artistic research articles, which are published at least twice per year, have been regularly published and distributed in the last five years, and are accessible in university libraries and accepted as "national peer-reviewed journal" (25). The DergiPark Project, which has been operating under TUBİTAK ULAKBİM since September 2013, aims to ensure that journals are published in Türkiye with high quality and by certain standards (to be scanned in the ISI indexes and other international indexes, etc.) and to build a data path between universities and TUBİTAK (47). DergiPark is a free infrastructure service that enables journals to be managed electronically, and it is not an index. All journals from Türkiye that declare to be academic and peer-reviewed can participate in DergiPark. However, journals that do not meet the requirements for an academic journal are removed from DergiPark. For a journal application that will start its publication life, the publisher needs to be "University or Public." If the publisher of journals with at least one year of the archive is "University, Public, Association, Foundation, and Professional Chamber," they can apply. It is mandatory that the archives of the journals are up-to-date and completed in accordance with the published frequency. Refereed academic journals that fulfill the criteria and are published in Türkiye can participate in DergiPark if they are open access (16). In this study, 76.9% (n = 20) of the journals were included in the DergiPark database (Table 1), 57.7% (n = 15) of the journals related to research were university journals, 42.3% (n = 11) of the journals were published twice per year, and 57.7% (n = 15) of the journals published three to six issues per year (Table 1), which is consistent with the data above. In addition, the focus has been on publishing two or more issues in veterinary sciences journals; therefore, considering the annual publication frequency, these journals comply with the criteria of a "national peer-reviewed journal." Moreover, these journals have publication policies to continue to be included in the TR Index and DergiPark database and to comply with the associate professorship application criteria determined by CoHE.

Therefore, the number of international veterinary sciences journals originating from Türkiye has been increasing in recent years. Considering the annual publication frequency of the journals in the field of veterinary sciences in Türkiye, journals are compatible with the criteria of "national peer-reviewed journals," and they comply with the criteria of COPE in several parameters such as "plagiarism, duplication, originality of the study, and ethics committee approval." However, the journals do not have a common standard and provide insufficient information on some issues. Particularly, the journals that include explanations on "animal rights" and "animal welfare and protection" in their publication policy are not at the desired level. In the publication policies of the journals, the criteria regarding the Ministry's permission for studies on wildlife and notifiable diseases, determined by CoHE, are insufficient. Therefore, efforts should be made to increase the quality of scientific journals publishing in the field of veterinary sciences regarding some parameters (fake data, citation manipulation, Ministry's permission for studies on wildlife, Ministry's permission for notifiable diseases, animal rights, and animal welfare and protection) and to increase the number of journals included in the international index such as ISI.

In addition, this study revealed that TR Index and DergiPark are extremely crucial for researchers and journals to establish a common standard. However, for scientific articles sent to journals, TR Index journal evaluation criteria should be considered to be consistent with ICMJE recommendations and the standards set by COPE. In terms of journal policies, professional organizations such as ICMJE, COPE, and WAME are also very crucial in terms of publication ethics, publication quality, and compliance with international standards.

It has been determined that some issues are not addressed by journals in their publication ethics policies regarding research and publication ethics principles, and some deficiencies have been observed in standards, such as ICMJE, COPE, WAME, and CC License, which are not members and/or do not adopt principles. Moreover, the indexes of the journals are an essential factor affecting their ethical policies. Particularly, a basis was formed for readers and young authors to better understand the standards and general concepts (TR Index, DergiPark, etc.) associated with research and publication ethics and to establish their relationships with each other.

Future studies can be warranted on the basis of the following: the extent to which the journals adopt standards such as ICMJE, COPE, and WAME and the criteria determined by CoHE in research and publication ethics; what motivates journals to adopt ethical principles and policies; and to what extent are they aware of the principles which they include in their policies. Moreover, academic studies in the future are recommended to follow the development processes of the veterinary sciences journals, examine the policies to increase their

searchability in international indexes, and bring different policies to the agenda for the applicability of the standards to be considered. In addition, regulating whether Google Scholar is an index and/or database will be beneficial to eliminate the uncertainty in this direction.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

GA conceived and planned the study. GA and EÇ designed the study. All authors conducted literature review and writing the manuscript. All authors 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.

Ethical Statement

This study does not present any ethical concerns.

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First molecular detection of *Neospora caninum* in red fox (*Vulpes vulpes*) brain sample in Türkiye

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ABSTRACT

The red fox is the wild carnivore with the widest distribution in the world. Thus, this animal acts as intermediate and final host for many parasite species. *Neospora caninum* is one of the most important protozoan agents causing abortion in cattle, sheep and goats in the world. The final hosts of *N. caninum* are domestic dogs and wild canids such as wolves and coyotes, while its intermediate hosts are domestic ruminants and many warm-blooded animals, including red foxes. The aim of this study was to research *N. caninum* in brain samples of three red foxes obtained from wildlife in Türkiye by using PCR. At the end of the study *N. caninum* DNA was detected in one of three brain samples. To the best of our knowledge, with this study, *N. caninum* was detected for the first time in a red fox brain sample in Türkiye.

The red fox is the wild carnivore with the broadest range both in the world and in Türkiye (9). They are final and intermediate hosts of many parasites and travel long distances due to their feeding and nest-seeking behaviors. They spread parasites to animal and human settlements. Therefore, it is very important to determine which parasites red foxes act as both intermediate and final hosts (11, 12, 15).

Neospora caninum is a coccidian protozoan in the Toxoplasmatidae family. The final host of *N. caninum* is canids (dog, wolf, coyote) and its intermediate hosts are warm-blooded animals such as cattle, small rodents, red foxes, and poultry (6). It is transmitted to active final hosts with consumption of infected intermediate host tissues. Infection of intermediate hosts occurs horizontally by oral ingestion of sporulated oocysts, or vertically in pregnant animals by transplacental transmission of tachyzoites to the offspring (6, 7).

Serological (ELISA, IFAT, agglutination test and immunoblotting) and molecular (PCR, real-time PCR) methods are used for the diagnosis of *N. caninum* in intermediate hosts (6). Although serological diagnostic methods are used in large-scale epidemiological studies, they may cause detection of false positives due to cross-reaction with some agents such as *Hammondia heydorni* (7). Molecular-based methods, on the other hand, have higher sensitivity and specificity than serological diagnostic methods. Thus, molecular-based methods are preferred in many studies examining *N. caninum* in intermediate hosts (1, 2, 6, 17).

Neospora caninum was detected in red foxes in Ireland (16), Spain (1), Belgium (3), Czechia (10), and Romania (17). However, there is no data on the presence and prevalence of *N. caninum* in red foxes in Türkiye. Purpose of this study was to investigate *N. caninum* in red fox brains obtained from wildlife by PCR and to

contribute to the literature by determining the state of the parasite in wildlife in Türkiye, where there is very little data.

The study material was composed with three red fox brains, two from Yozgat and one from Ankara. Total brain samples from red foxes were extracted as described by Munson et al. (13) and stored in a deep freezer at -20°C until use. Brains were examined for the presence of *N. caninum* with the digestion method described by Dubey et al. (6) with little modifications.

Brain samples were placed in the blender and mixed by adding 200 mL of Acid-Pepsin solution (6). The Blender was run at the highest speed for 30 seconds and the brain samples were homogenized. Homogenized samples were transferred to a beaker containing acid-pepsin solution at 37°C and digested in a heated magnetic stirrer for 5-6 hours until it completely dissolved. Undigested tissue pieces were removed by filtration through 250 micron sieves. The resulting mixture was left to sedimentation. At the end of the process, the upper liquid part was removed. The underlying sediment was mixed with PBS (pH: 7.4, Sigma Aldrich®, Germany) and transferred into 50 mL falcon tubes. The sedimentation process was repeated at least 5 times in falcon tubes to deport the acid-pepsin solution. Each time, the liquid above the sediment was removed and the remaining sediment was diluted with PBS and vortexed. The obtained sediment was stored in a deep freezer at -20°C until used for DNA isolation.

Genomic DNA was extracted from 200 µl sediment by using QIAamp® DNA Mini Kit (Qiagen®, Germany) according to the manufacturer's instructions and stored at -20°C until used in PCR. The primer pairs Np21 (5'-GTGCGTCCAATCCTGTAAC-3') and Np6 (5'-CAGTCAACCTACGTCTTCT-3'), which amplify the Nc-5 region of *N. caninum* at a size of 328 bp, were used in this study (18). PCR master mix with total volume of 25 µL was prepared with 14.375 µL DNase, RNase-free sterile distilled water, 2.5 µL 10× Taq Buffer with KCl, 2.5 µL 25 mM MgCl2, 1 µL dNTP mix (10 mM), 1 µL of each primers (10pmol/µL) 0.125 µL Taq DNA Polymerase (5 U/µL, ThermoScientific) and 2.5 µL template DNA. PCR was carried out as described by Gondim et al. (8).

The 9 µL of PCR product was mixed with 1 µL of 10× Blue Juice TM Gel Loading Buffer (Invitrogen®, Lithuania) and loaded onto a gel (1.5%) stained with SYBR™ Safe DNA Gel Stain (Invitrogen, USA). Electrophoresis was performed in a gel tank with 0.5×TBE buffer solution at 90 volts for 40 minutes, then the agarose gel was checked for the presence of specific bands in a UV transilluminator (3UV Benchtop Transilluminator, UVP®, Canada) and photographed.

A 328 bp product which is compatible with *N. caninum* was detected in one of the samples obtained from Ankara (Figure-1).

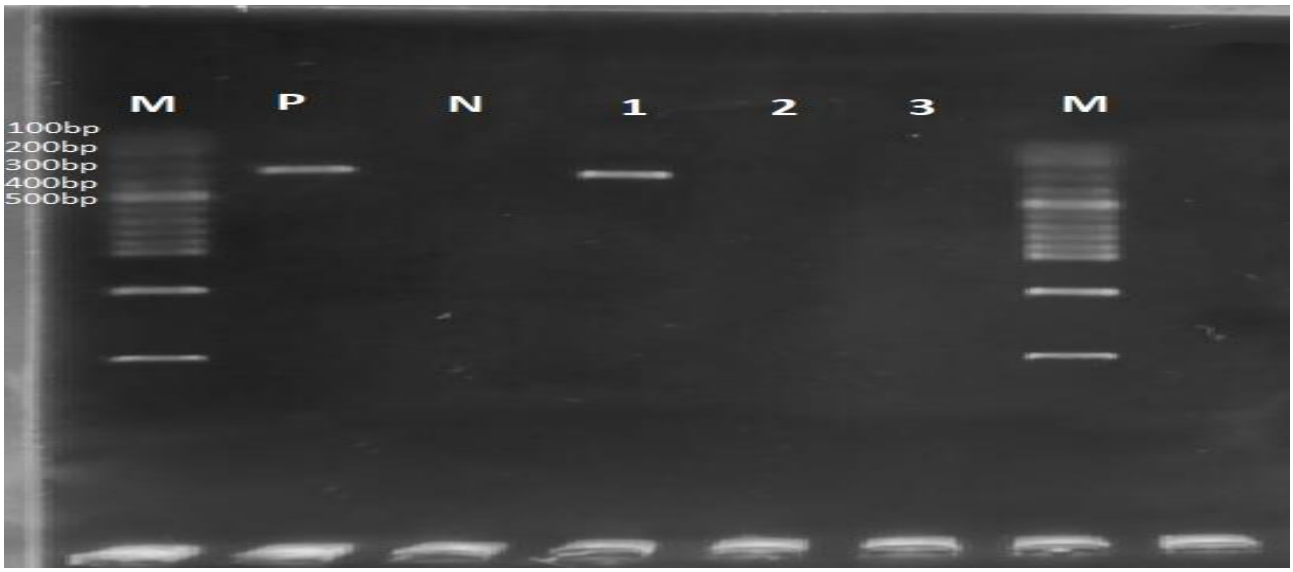

Figure 1. PCR results of *Neospora caninum* NC-5 region.
M: Marker
P: Positive Control
N: Negative Control
1: Positive Sample
2-3: Negative Samples.

Molecular-based methods (PCR and modifications) are important diagnostic tools for the identification of *N. caninum* in wild animals (6, 7). Molecular prevalence of *N. caninum* in the brain of red foxes was detected as 10.7% (13/122) in Spain (1), 5.96% (9/151) in Ireland (16), 4.8% (4/83) in Great Britain (2), 6.6% (20/304) in Belgium (3), 4.61% (7/152) in Czech Republic (10), and 0.54% (1/182) in Romania (17). In this study, we detected the *N. caninum* DNA in one of the three red fox brain samples which were coming from Yozgat and Ankara provinces, in Central Anatolia, Türkiye.

Neospora caninum is one of the most important protozoan agents that can cause abortion in farm animals such as cattle, sheep and goats and fatal neurological infections in canids (6, 7). The annual economic losses caused by the agent in cattle farms are 1.298 billion dollars globally (14) and it is estimated to be 40.5 million dollars in Türkiye (4). There are two separate cycles in the life cycle of *N. caninum*. One of them is the domestic cycle between domestic dogs and farm animals while the other is the sylvatic cycle that develops between wild canids (wolves, coyotes) and warm-blooded animals (deer, antelope, red fox and small rodents) (6). The presence of *N. caninum* in red foxes obtained from wild life in Türkiye was investigated for the first time by PCR in this study.

Red foxes are important intermediate hosts of *N. caninum* in the wildlife. Nowadays the population size of these animals is increasing especially in European countries due to the oral rabies vaccination program (9). With this increase, the parasites carried by the red foxes reach the wider geographical areas which are in contact with farm animals. Therefore, the determination of parasites in foxes is becoming a more important issue to prevent economic losses in farm animals and to protect public health (5, 9).

In conclusion, with this study, to best of our knowledge we detected the *N. caninum* in a red fox brain for the first time in Türkiye. We think that it is so important to detect the wild intermediate and final hosts of *N. caninum* for clear understanding the epidemiology and the control of the disease.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

UE, ED and AEU conceived and planned the experiments, UE and ED prepared all samples, carried out laboratory experiments, UE, ED and AEU contributed to the interpretation of the results, UE 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.

Ethics statement

Permission to examine parasitic diseases in fox samples was obtained from Veterinary Control Central Research Institute Local Ethics Committee with the decision numbered 2017/02.

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Isolation of *Citrobacter freundii* from a dermal lesion of a captive Green iguana (*Iguana iguana*)

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ABSTRACT

A 1-year-old Green iguana (*Iguana iguana*) was brought to the clinic with a complaint of a slowly growing mass on the left mandible and symptoms of weakness and loss of appetite for several weeks. The clinical examination noted that the mass was quite firm, invasive to the region, and had a necrotic appearance. It was removed by surgical operation and sent to the laboratory for investigation. In the microbiological evaluation, *Citrobacter freundii* was isolated and identified very intensely. According to the laboratory results, enrofloxacin was chosen as the broad-spectrum antibiotic and used for 1 week.

The reptilian class, which includes many animal groups such as turtles, lizards, iguanas, and snakes, differs from the common pet animals in terms of microbiota. In the modern world, the feeding of these animals in homes as pets has increased the risk of many diseases not only in themselves but also in humans. They are considered to be the source of many gram-positive and negative bacterial infections that can be transmitted especially through urine saliva, blood, wounds and breathing to humans. In particular, immunocompromised individuals and children are in the risk group (2). *Citrobacter freundii* is a species of facultative anaerobic Gram-negative bacteria of the family Enterobacteriaceae. It can be isolated from reptiles: tortoises, iguanas, lizards, chelonians, snakes (1, 3, 8). Considering that the bacterium is zoonotic, this resistance can also pose a serious danger to humans (6). In this case report, it has been revealed the presence of an opportunistic bacteria found in normal flora of a captive iguana skin lesion and successful treatment was performed. It has been underlined that unsuitable environmental conditions (temperature, humidity) may be effective in the disease.

A one-year-old, female green iguana (*Iguana iguana*) was brought to the clinic with complaints of appetite and lethargy. On clinical examination, a solid, necrotic mass was detected in her left jaw (Figure 1). According to the anamnesis, it was learned that the animal had a terrarium, but spent most of its time at home. Medetomidine HCl (0.1 mg/kg) was administered intramuscularly for preanesthesia. After 20 minutes, general anesthesia was induced with ketamine HCl (5 mg/kg, intramuscular). Approximately 30 minutes after ketamine injection, iguana was intubated and anesthesia was maintained with Isoflurane (100% oxygen) (Figure 1). The mass was removed totally and the wound was sutured with 4/0 non-absorbable monofilament suture

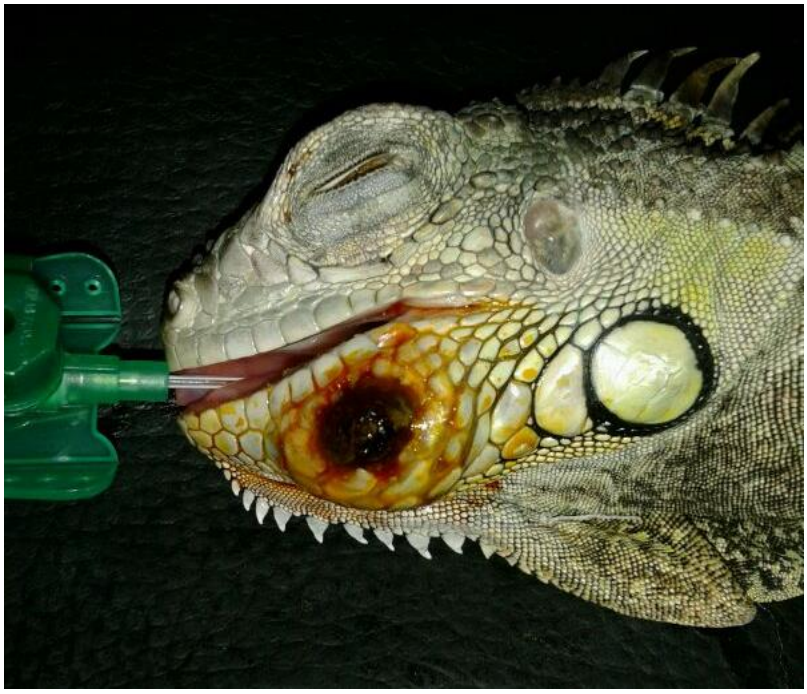

Figure 1. A solid, necrotic mass on the green iguana's left jaw.

material. The material was sent to the laboratory as soon as it is taken. Carprofen (2 mg/kg, intramuscular) was administered for 3 days for analgesia.

Inoculations were made on blood agar and MacConkey agar for bacterial identification. After incubation under aerobic and microaerobic conditions, hemolyzed colonies grew on blood agar and lactose-fermenting colonies on MacConkey agar. Gram-negative rod-shaped bacteria were detected after Gram staining of the colonies. According to biochemical tests, the causative agent was determined to be *Citrobacter freundii*. Confirmation of the isolated and identified strain was performed by MALDI-TOF-MS (Matrix-assisted laser desorption/ionization-time of flight mass spectrometry).

Antibiotic susceptibility testing of the isolate was performed according to the Kirby-Bauer disc diffusion method recommended by NCCLS (NCCLS, 2013). For this purpose, gentamicin (10 ug), oxytetracycline (30 ug), enrofloxacin (5 ug), amoxicillin (10 ug), chloramphenicol (30 ug), tetracycline (30 ug), erythromycin (15 ug), sulphamethoxazole/trimethoprim (1.25 µg/23.75 µg), lincomycin (2 µg) and ceftiofur (30 µg) containing commercial antibiotic discs were used. A small number of colonies taken by the inoculation loop was passed into Nutrient Broth (NB) and distributed homogeneously. NB's were incubated at 37 °C for 24 hours and adjusted to McFarland 0.5 (108 microorganisms/ml) after turbidity. The sample taken from that suspension with the help of a sterile swab was inoculated onto the Mueller-Hinton agar surface. Subsequently, discs containing different antibiotics were placed on the agar surface with the help of sterile forceps and incubated at 37 °C for 24 hours. Inhibition zones formed after incubation were measured. After the measurement, it was determined that the agent was sensitive to gentamicin, oxytetracycline, enrofloxacin, chloramphenicol, tetracycline, sulphamethoxazole /trimethoprim and resistant to other antibiotics. According to the laboratory results, enrofloxacin was chosen as the broad-spectrum antibiotic and given orally for 1 week after the operation.

The choanae, cloacae and conjunctival bacterial flora of healthy captive green iguanas may consist of *Acinetobacter spp*., *Stenotrophomonas maltophilia*, *Pseudomonas spp*., *Citrobacter spp*., *Proteus vulgaris*, *Enterobacter spp*., *Serratia spp.*, and *Salmonella spp*. These are the opportunistic pathogens in iguanas and many reptiles (8, 9). *Citrobacter spp*. is associated with ulcerative skin diseases, stomatitis, cloacitis, abscesses and respiratory infections (4). *Citrobacter freundii* was identified in the lesion of this case. There has been no report that characterized opportunistic bacteria in the gastrointestinal flora of green iguanas cause disease alone.

Poor husbandry may result in pathogen infections such as *Citrobacter spp*. in reptiles. In cases where terrarium cleaning is done adequately, soil and water create a suitable environment for the proliferation of such bacteria. Adverse conditions caused disease in reptiles, as well as horizontal transmission (5). Since the green iguana in this case report spends most of its time outside the terrarium, it causes contamination of the same environment that it shares with its owner. Conditions, where environmental hygiene is not provided adequately, can create a contaminated environment, such as the soil effect in the terrarium. This kind of condition will increase the exposure of the iguana to an opportunistic pathogen and reduce its antimicrobial activity severely.

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Temperature plays an important role in reptiles' bacterial activity. Poor thermal acclimation changes their blood antimicrobial activity and disrupts the immune system, behavioural and physiological features. Also, reptiles increase body temperature in response to an infection like mammals; however, because they are ectotherms, they try to use warmer environmental conditions to raise their body temperature (10, 11). The fact that the green iguana reported in this case does not spend its time in the terrarium means exposure to the heat of the home environment. This unfamiliar condition affects the immune system of the green iguana after a while and increases the ability of opportunistic microorganisms to cause disease. However, the pathology showed up as a skin lesion in this animal.

Many bacterial isolates are potential zoonoses such as *Citrobacter spp*. *Citrobacter freundii* is a turtle pathogen and can also develop many bacterial zoonoses in immunosuppressed turtles. Especially children, immunosuppressed, elderly or debilitated people are at high risk for reptile-derived *Citrobacter spp. Citrobacter freundii* can cause infections such as urinary tract infections, diarrhoea, pneumonia or meningitis in humans (5).

The treatment is provided by choosing the appropriate antibiotic according to the antibiogram test results. However, due to the widespread use of broad-spectrum antibiotics, *Citrobacter freundii* has become increasingly resistant (7). In this case report, the antibiotic was selected according to the antibiogram test results. It was noted that there was a rapid improvement in the general condition of the patient after the operation.

In conclusion, little information is available regarding bacterial diseases in green iguanas. They have an adaptive immune response. However, climate and environmental changes may cause complete disruption of the function of bacterial flora. Additional research is necessary to determine the effects of normal flora on infectious diseases in iguanas. Until then, we have clear data on dangerous zoonotic agents for humans. Preventive medicine is vital. Veterinarians should inform owners of the risks of hygienic conditions for reptiles.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

BB, KGC and IE conceived and planned the report. IE took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

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

Ethical Statement

This study does not present any ethical concerns.

Animal Welfare

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

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A case of Monkeypox in a baby monkey

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ABSTRACT

Despite having a low fatality rate in monkeys, monkeypox remains an important disease because of its zoonotic potential. The aim of this study is to describe the clinical, pathological, ultrastructural and virological findings of the first monkeypox case diagnosed in a baby monkey in Türkiye with unknown transmission. A monkeypox infection was detected in a 1-month-old, female, baby monkey born in Antalya Zoo and died with skin lesions. It was reported that the baby's mother did not care for and caregivers took care of the cub, and skin lesions on the hands of the caregivers and responsible veterinarian were reported. Necropsy, histopathology, electron microscopy and chorioallantoic membrane (CAM) test were performed. Typical cutaneous and pulmonary poxvirus finding in a baby monkey was described in this case. CAM results firstly reported in a monkeypox case. Because of the zoonotic potential of illness, monkey colonies, particularly in zoos, must be controlled with vigilance. This is the first monkeypox report in a baby monkey in Türkiye.

Monkeypox is an important disease because of its zoonotic potential, despite the fact that it has a low mortality rate in monkeys. A virus that produces smallpox-like infection in humans causes the disease and belongs to the orthopoxvirus. The virus is relatively large, having a diameter of 200-250 nanometers with a collapsed center. Although they translate mRNA on host ribosomes, their genomes include all of the required transcription, replication, egress, and assembly proteins (1).

The first natural case of monkeypox in monkeys was reported in 1959 in a cynomolgus macaque colony. It was characterized by typical poxvirus lesions forming on the skin, and no deaths were documented (9). Immediately following that, in 1960, a spontaneous case of monkeypox in both rhesus and cynomolgus macaques was reported (8). Only juvenile death was reported in cynomolgus macaques in that case, and ulcerative mucosal lesions, hemorrhagic skin lesions, facial edema, and generalized lymphadenopathy were described in dead monkeys. The histopathological examination of the skin lesions revealed that they were extremely comparable to human smallpox lesions (2).

The disease is spread through skin sores, body fluids or excretions of affected animals' respiratory system, and direct or indirect contact with contaminated materials. Although human-to-human transmission is feasible, disease transmission between humans is extremely infrequent (1, 3). Viral isolation or PCR can be used to confirm a conclusive diagnosis of monkeypox infection. Electron microscopy imaging and immunohistochemical staining for orthopoxvirus antigens can also be conducted (7).

The first case of monkeypox in humans was reported in Türkiye on June 30, 2022 and eleven cases of monkeypox have been documented so far. The purpose of this study is to present the clinical, pathological, ultrastructural, and virological findings of the first monkeypox case diagnosed in a baby monkey in Antalya Zoo in 2019. Because monkeypox has recently spread rapidly in humans, the goal is to assess the data and call the attention of those who have contact with animals, particularly veterinarians.

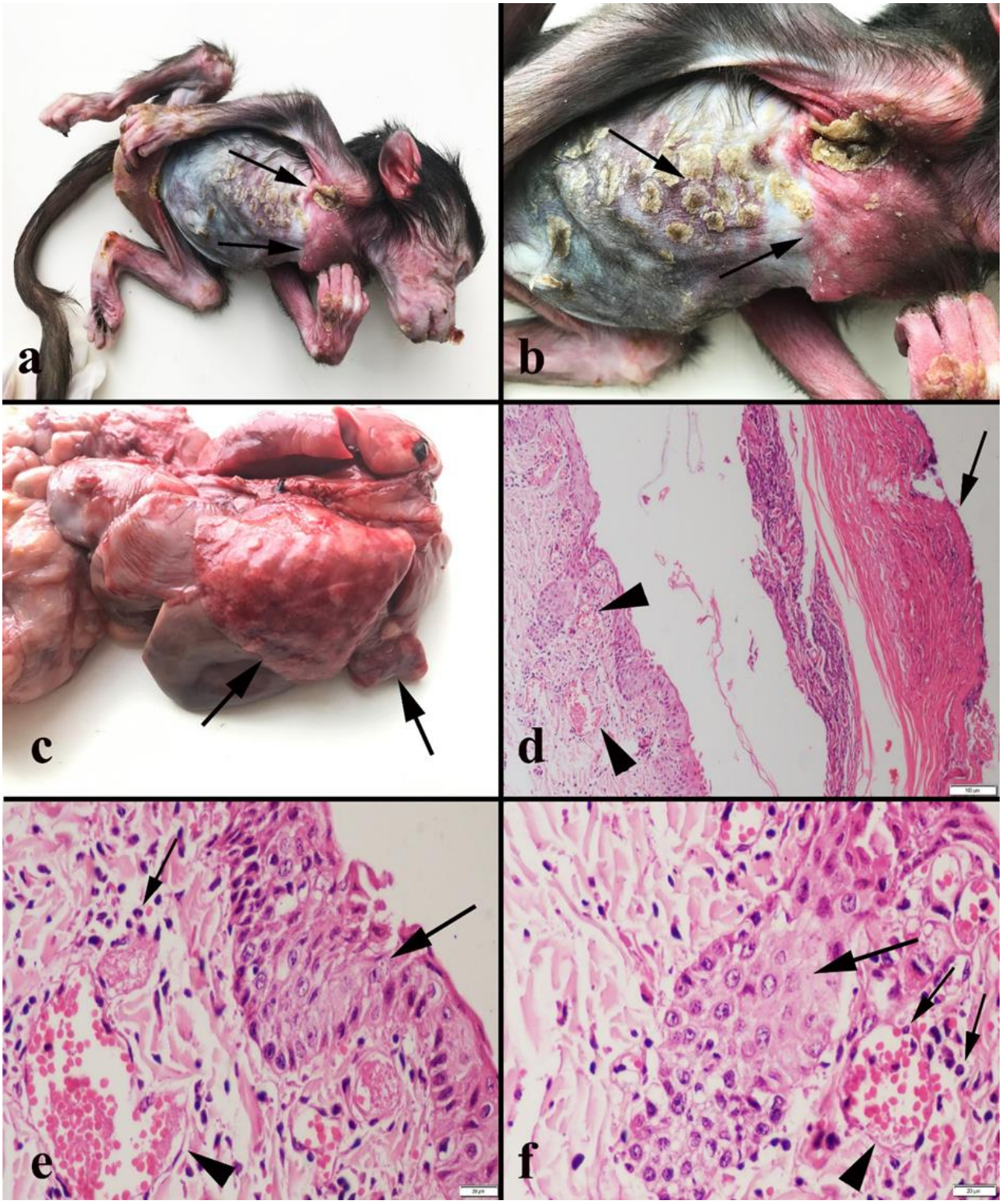

Figure 1. Necropsy findings of the baby monkey died from the monkeypox.
a. Macroscopical appearance of the skin lesions initial hyperemic lesion and crusted papules (arrows).
b. Closer appearance of the lesions (arrows).
c. Histopathological appearance of the skin lesions, thick necrotic crust (arrow) on the epidermis and hyperemic dermal vessels (arrow heads), HE, Scale bar=100µm.
d. Marked hyperemia (arrow heads) and inflammatory cell infiltrations in dermis and and intracytoplasmic inclusion bodies (thick arrow) in epidermal cells, HE, Scale bar=100µm.
e. Hyperemia (arrow heads) and inflammation and intracytoplasmic inclusion bodies (thick arrows), HE, Scale bar=100µm.
f. Marked hyperemia (arrow heads) and inflammatory cell infiltration and intracytoplasmic inclusion bodies (thick arrows), HE, Scale bar=100µm.

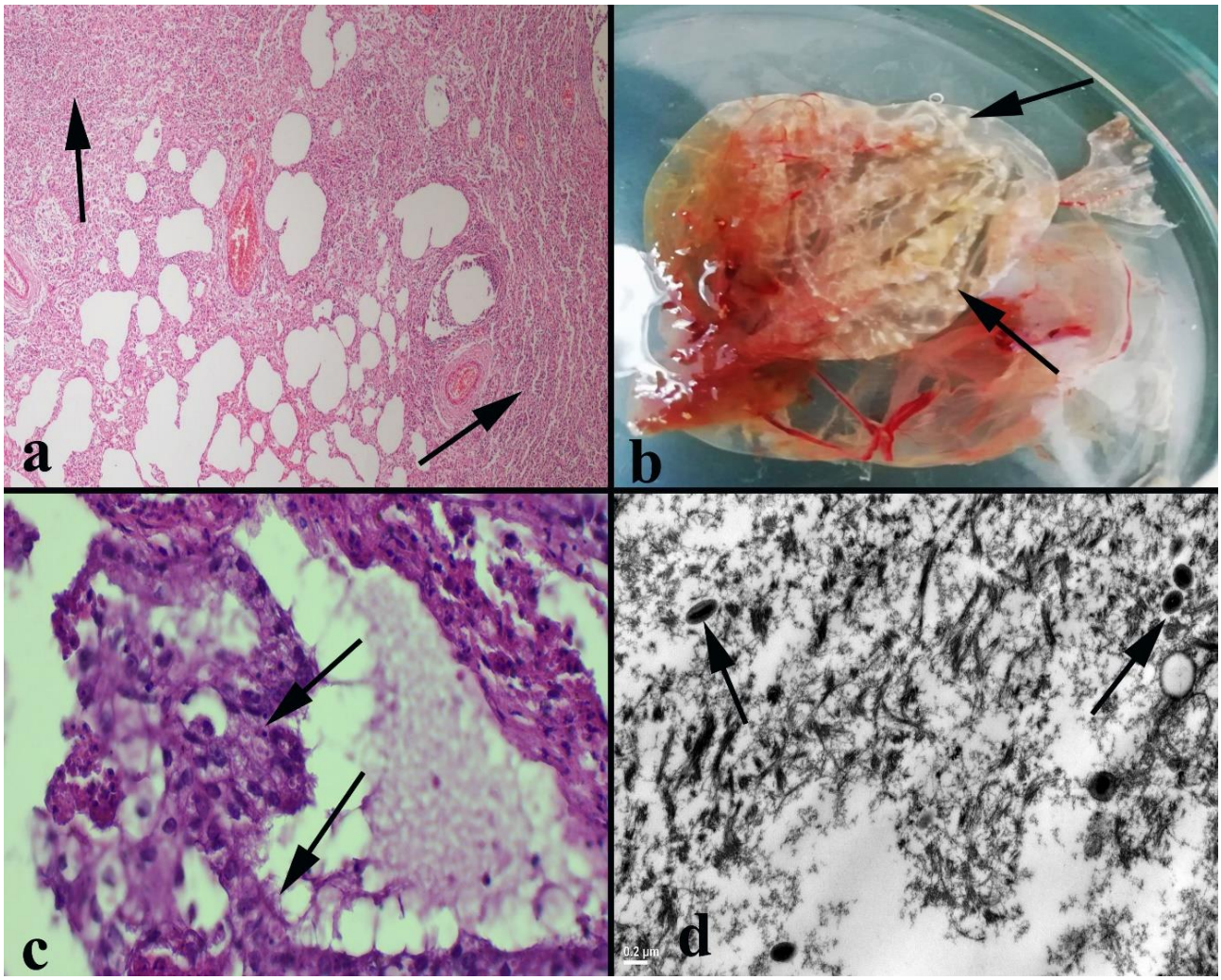

Figure 2. a. Histopathological appearance of the pneumonic lung, hyperemia, emphysema and marked inflammatory reaction (arrows), HE. **b.** Typical pockformations (arrows) on the CAM after inoculation. **c.** Microscopical appearance and intracytoplasmic inclusions (arrows) in the CAM cells. HE. **d.** Ultrastructural view of the lesions, typical centrally collapsed pox viral particles (arrows) in the epidermal cell cytoplasm, Scale bar= 0.2µm.

A female monkey imported from Africa to Antalya Zoo gave birth to a female baby monkey at the end of the regular gestation time. However, skin lesions were reported to emerge within a few weeks. Because the mother did not care, the baby was fed by the caregivers, and then respiratory system issues arose, and the baby died when it was around one month old.

The baby monkey was taken to the Department of Pathology at Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine for the diagnosis of the disease. She was thoroughly examined, and tissue samples were taken from lesioned skin and all internal organs. During necropsy, tissue samples were fixed in 10% neutral formalin solution (Formaldehyde solution, Cat. No: 1.04002, Merck, Missouri, USA) for histopathological evaluation and 2.5% glutaraldehyde solution (Glutardialdehyde solution 25%, Cat. No: 111-30-8, Merck, Missouri, USA) for ultrastructural study. The remaining tissue samples were stored at -20 °C for CAM inoculation.

At necropsy, crusted and papular lesions were more visible, especially on the face, armpit, abdomen, chest, and extremities. The skin was covered with markedly hyperemic plaque-like lesions. Initial skin lesions often grow over the first four days becoming deeply localized, firm, and 5 to 20 mm in size before crusts form (Figure 1a-b). Except for severe pleuropneumonia, no macroscopic findings in visceral organs were noted (Figure 1c). The other organs were not histopathologically examined due to the autolytic changes.

Histopathological examinations revealed marked hyperemia of the cutaneous vessels. The epidermis was densely thickened, and the crusts formed as necrotic masses on the epidermis (Figure 1d). Hydropic degeneration and necrosis were noticed, especially in stratum spinosum cells. Ulcers were also found in several

areas. Some keratinocytes were found to have inclusion bodies (Figure 1e-f). Inflammatory cell infiltrates were seen, with the dermis showing the most. The skin lesions resembled those seen in human smallpox. Hyperemia of the lungs' arteries, thickening of the alveolar septal tissue, and inflammatory cell infiltrations were observed. Desquamation of the epithelial cells of the alveolus, bronchioles, and bronchus was also seen (Figure 2a).

Inoculation of an embryonated chicken egg (11-12 day-old) with an air sac to CAM following homogenized skin samples indicated thickening and typical pox lesions (Figure 2b). Histopathological examination of the CAM revealed epithelial cell proliferation and numerous intracytoplasmic inclusion bodies (Figure 2c). Furthermore, lesioned skin and CAM samples were examined ultrastructurally. On electron microscopy, typical pox virus particles were found.

In the cytoplasm of the keratinocytes, naked viroplasm, sequential stages of virus particle development, and mature virions were recognized by transmission electron microscope TEM. Thin electron microscopical sections of skin revealed oval and brick-shaped monkeypox virions within the cytoplasm of epidermal cells. The virions had a typical ovoid to round shape, lateral bodies, and a central core (Figure 2d).

Monkeypox is a viral zoonotic disease that is clinically comparable to smallpox but has milder clinical symptoms. With the end of smallpox vaccination in humans in 1980 due to the smallpox eradication, monkey pox emerged as the most important orthopox virus. The disease is found in Central and West Africa, particularly prevalent in the Democratic Republic of the Congo. The virus has also been found in animals imported from Africa in the United States. Similarly, cases of monkeypox in people have been reported following African-related trips (4). Monkeypox spread fast throughout the world, with countless cases reported from various regions. Although there have only been eleven human cases of monkeypox in Türkiye, no monkeypox virus has been reported in monkeys.

In the experimental model of monkeypox in prairie dogs, the virus multiplies soon after entering the body via the oropharynx, nasopharynx, or intradermal routes, and then spread to the adjacent local lymph nodes. Lymphadenopathy and fever are common signs. The initial lesions appear in the oropharyngeal mucosa, followed by skin eruptions. The rash is accompanied by typical skin lesions, and the disease is characterized by typical papule formation (5). In this case, anamnesis led to the development of skin lesions one week after the onset of the initial clinical signs, suggesting possible pathogenesis in monkeys.

Both virus isolation and serological tests revealed that the most likely viral reservoir is wild squirrels (6). The fact that this was a single case, the mother monkey showed no clinical signs, and the presence of squirrels and other rodents in the environment suggested that these might be reservoirs. The mother, on the other hand, could be subclinically sick or a carrier. Therefore, the source of infection in this case could not be determined.

Dermatitis is the most common and distinguishing feature of the disease in monkeys. It can appear as single small papules at times, but it can also appear as large lesions, mainly in the face, abdomen, inguinal, and thoracic regions. Lesions are less common on the palmar surfaces of the hands and plantar surfaces of the feet. The spread of the lesions increases as the disease progresses. While the early lesions appear on the skin as hyperemic areas, characteristic papules and crusts grow with time (10). In this case, similar typical findings were observed. Within a week, the lesions had spread throughout the body. This rapid spread could be attributed to the monkey's age. The lesions were mostly localized on the face, abdomen, armpits, and hands. While the lesions initially appeared hyperemic, they eventually took on a typical papular appearance. Crusting and crust shedding were also observed in several areas.

Pneumonia is the leading cause of death in this condition. A common consequence is secondary bacterial septicemia. The lungs become heavy, hard, and dark red in color. The presence of lobular, edematous, and atelectatic regions is common. Although the pleura exhibits an inflammatory reaction, adhesions are rarely found. Hydropericardium may be seen at necropsy (10). In this case, pneumonia was determined to be the cause of death for the animal. Lungs had already described in her findings. Pleuritis occurred, although no adhesions or hydropericardium were found.

In the histopathological examination, epithelial hyperplasia is typical in skin lesions, but necrosis is also seen. Swelling and degeneration of cells in the stratum spinosum are particularly common. It is usual to see intra-epithelial vesicopustules with swollen epithelial cells, neutrophils, eosinophilic fluid, and fibrin. It is possible to see intracytoplasmic inclusion bodies in epithelial cells (10). The findings in this case were congruent with those found in the literature. However, necrosis and crusting were more visible, most likely due to the pup's thin skin. Typical inclusion bodies were also frequently encountered.

The respiratory system is the most susceptible system other than the skin. In general, all monkeys who succumb to the disease develop fibrino-necrotic bronchopneumonia. Histopathological findings include diffuse necrotic changes in the bronchial and bronchiolar epithelium, inflammatory cell infiltrations, edema, and fibrin accumulation (10). In this case, the most significant extracutaneous lesion was bronchopneumonia, and the findings were consistent with previous research.

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Because of the presence of autolytic findings, the lesions of the other organs were not evaluated. Electron microscopy revealed characteristic collapsed viral particles in both skin lesions and CAM. The animal caregivers and veterinarian caring for the monkey developed typical skin lesions, but these were not examined. The diagnosis of monkeypox in this baby monkey was made based on characteristic macroscopic, histopathological, ultrastructural, and CAM findings.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ÖÖ, MK, Vİ and HSS conceived and planned the study. ÖÖ and MK carried out the study. ÖÖ, MK, Vİ and HSS contributed to sample preparation, laboratory examinations and drafting. ÖÖ and MK contributed to the interpretation of the results. ÖÖ and MK took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

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

Ethical Statement

There is ethical approval for this study.

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Instruction to Authors

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   Bahk J, Marth EH (1990): Listeriosis and Listeria monocytogenes. 248-256. In: DO Cliver (Ed), Foodborne Diseases. Academic Press, San Diego.
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