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Research Article

Alterations of lipid profile in overweight and obese dogs

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This study was produced from a master thesis: Investigation of insulin resistance in obese dogs, 2023, Department of Internal Medicine, Institute of Health, Burdur Mehmet Akif Ersoy University, Burdur, Türkiye.

ABSTRACT

The purpose of the study was to determine the alterations of lipid profile in overweight and obese dogs. Obesity is defined as abnormal or excessive fat accumulation in the body. It is a chronic health problem for both human and animals. Dogs with restricted movements and fed high-energy diets are particularly at a high risk. In the study, 30 dogs of different ages, breeds and genders were used. Dogs were divided into 3 groups equally as ideal weight, overweight and obese according to the body condition score (BCS) chart. Serum samples were collected from all dogs and used to analyze lipid profile parameters using a biochemistry analyzer. In the study, significant increases were detected in total cholesterol (total-C, $p<0.01$), very low density lipoprotein (VLDL, $p<0.05$) and low density lipoprotein (LDL, $p<0.05$) in obese dogs compared to those of both ideal weight and overweight dogs. According to the cut-off values, the analyzed lipid profile parameters were found to increase in some dogs in both groups. However, the numbers of dogs with increases in lipid profile parameters were higher in obese dogs than in overweight dogs. Very strong positive correlations were also detected between some of lipid profile parameters. In conclusion, it has been revealed that there are significant alterations in lipid profile parameters in obese dogs. The levels of these changes are thought to be related to the severity of obesity in dogs. Presence of strong correlations between lipid profile parameters indicated that total-C, HDL, VLDL, LDL, total-C/LDL and Tg values can be used to evaluate severity of overweight and obesity in dogs.

INTRODUCTION

Obesity is a chronic health problem characterized by excessive fat accumulation in the body. It usually results from an imbalance between energy intake and use. This imbalance may develop due to many reasons such as age, gender, reproductive status, inactivity, nutrition, lifestyle, neutering, hypothyroidism, insulinoma, hyperadrenocorticism and corticosteriod use (Buishand and Kirpensteijn, 2023; Byers et al., 2011; Preet et al., 2021; Robertson, 2003; Ronja and Kölle, 2021). In dogs, an increase of 10-20% in ideal body weight is considered as overweight, while an increase above 30% is considered as obesity (Preet et al., 2021). Obesity rate in dogs has been increasing noticeably in recent years (Preet et al., 2021; Weir, 2024). Studies conducted in different countries revealed that approximately 22-66.1% of dogs are obese (Loftus and Wakshlag, 2015; Munoz-Prieto et al., 2018; Preet et al., 2021; Ronja and Kölle, 2021). It is well-known that obesity in dogs negatively affects the quality and duration of life (Preet et al., 2021; Shmalberg, 2013). High rates of cancer, diabetes mellitus (DM), heart diseases, hypertension, osteoarthritis, joint diseases, hormonal imbalances, skin diseases and urinary tract stones has been reported in obese dogs (Preet et al., 2021; Ramos and Castillo, 2020; Ronja and Kölle, 2021). It was found that 40 percent of obese dogs had at least one of the diseases such as diabetes, hypothyroidism and hyperadrenocorticism (Oh, 2011; Preet et al., 2021).

A number of metabolic changes have been shown to occur in obese dogs due to obesity. Increases in blood insulin, glucose, triglyceride, volatile fatty acids, cortisol, fructosamine, total cholesterol, leptin, glycogan-like protein-1 (GLP-1) levels and a decreases in high density lipoprotein (HDL) values have been reported in these dogs (De Marchi et al., 2020; Gonzlez-Villar and Perez-Bravo, 2022; Kennerman, 2006; Mori et al., 2013; Ramos and Castillo, 2020; Verkest et al., 2011). Type 2 DM due to insulin resistance has also been reported in obese dogs (Ramos and Castillo, 2020; Verkest et al., 2011; Zoran, 2010). However, the results of the studies in haematological and biochemical parameters of overweight or obese dogs were controversial. In some studies, increases in alkaline phosphatase (ALP), total protein, albumin, thyroxin, phosphorus, glucose, cortisol, insulin, insulin-like growth factor-1, LDL, leptin and type II cartilage, total-C and Tg values were obtained in overweight or obese dogs (De Marchi et al., 2020; Rafaj et al., 2016; Ramos and Castillo, 2020; Ricci et al., 2007; Usui et al., 2015). While decreases in creatinine, serum urea nitrogen (BUN) and C-reactive protein (CRP) were determined in obese dogs (Rafaj et al., 2016; Yamka et al., 2006). However, in some other studies, no changes were obtained in hema-

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tological and biochemical parameters including glucose, total protein, albumin, globulin, urea, bilirubin, ALT, AST ALP and GGT in overweight or obese dogs (Rafaj et al., 2016; Ricci et al., 2007). Lipid profile parameters have not been adequately investigated in overweight and obese dogs. Thus, the aim of the study was to investigate alterations in lipid profiles of overweight and obese dogs.

MATERIALS and METHODS

Animals and evaluation of obesity

A total of 30 owned dogs were used in the study to determine lipid profile parameters. Age, sex and breed characteristics were not considered in the selection of dogs. Body condition score in dogs was determined using the World Small Animal Veterinary Association (WSAVA), VCA Animal Hospitals Body Condition Scoring (BCS) system and existing studies (Chun et al., 2019; Williams and Buzhardt, 2022). Thus, the BCS in dogs was scored on a scale of 1-9 and dogs were equally divided into three groups: overweight, obese and ideal weight according to the BCS system. Ideal weight was considered for dogs with a BCS score of 4-5/9 and these animals were used as the ideal weight group (n=10). Animals with a BCS score of 6/9 were considered as the overweight group (n=10), and dogs with a BCS score of 7-9/9 were considered as the obese group (n=10). Animals with any systemic disease and undergoing surgery within 6 weeks were not included in the study.

Blood samples

Fasting (8-12 hours) peripheral blood samples were collected from each animal into plain tubes and then used to prepare serum samples by centrifuging at 4000 RMP for 20 minutes. Collected serum samples were then kept at -80°C until used to determine lipid profile parameters.

Biochemical analysis

In the study, total cholesterol (Total C), high density lipoprotein (HDL), very low density lipoprotein (VLDL) and triglyceride (Tg) levels in serum samples were measured by using a biochemistry device (Roche cobas integra 400 Plus, USA). Low density lipoprotein (LDL) concentrations in the serum samples were calculated with the following formula as described Ramos and Castillo (2020); LDLP= total cholesterol–(HDLP+Tg/5). Additionally, Total C/LDL and HDL/LDL were also calculated for each dogs.

Statistical analysis

The normality of the distributions of the data was analyzed with the Kolmogorov-Smirnov test. Statistical differences between the parameters obtained from the ideal weight group, overweight and obese groups were determined by One Way Anova (posthoc Duncan). In the presence of high variances in parameters, a nonparametric Kruskal Wallis H test was used to determine statistical significance between these groups. Furthermore, to determine the correlation between the data, the parameters with normal distribution were analyzed with Pearson's correlation coefficient (r), and the parameters with non-normal distribution were analyzed with Spearsman's rho. In correlation tests, negative (-) or positive (+) correlation values (r) were accepted as very weak (-,+ 0-0.19) weak (-,+ 0.2-0.39), moderate (-,+ 0.4-0.59), strong (-,+ 0.6-0.79) and very strong (-,+ 0.8-1.00) as described by Meghanathan (2016). All the values were expressed as mean and standard deviations of the mean (mean±SD), median, minimum maximum (min-max). The level of the significance was accepted as $p<0.05$.

In the study, the cut-off value for each parameter in overweight and obese dogs was determined by Reciver Operating Characteristic (ROC) analysis. The cut-off value of each parameter was used to determine individual increases or decreases within groups. Values higher or lower than the cut-off value were considered as an increase or decrease in the parameter of that animal.

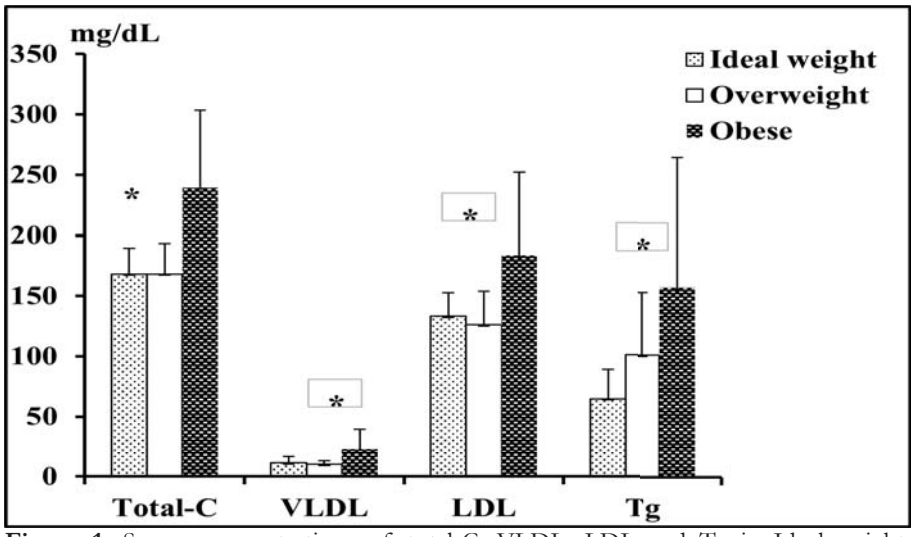

Figure 1. Serum concentrations of total-C, VLDL, LDL and Tg in Ideal weight, Overweight and obese dogs (mean±SD). Total-C: Total cholesterol, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein, Tg: triglyceride. *: $p<0.05$, **:$p<0.01$.

Table 1. Lipid profile of ideal weight, overweight and obese dogs (mean±SD).

Parameters	Ideal weight (n=10)	Overweight (n=10)	Obese (n=10)	Median (min-max)
Total-C (mg/dL)	168.22±21.32[b]	168.1±25.34[b]	239.33±64.06[a]	184.5 (132-391)
HDL (mg/dL)	111.37±9.86[a]	109±10.08[a]	127±27.96[a]	114.5 (88-190)
VLDL (mg/dL)	11.37±5.28[b]	10.1±2.84[b]	22.91±16.61[a]	11.5 (5-69)
LDL (mg/dL)	133.3±19.35[b]	126.06±27.63[b]	183.53±68.41[a]	139.2 (85.6-333.8)
Total-C/LDL	1.26±0.05[a]	1.34±0.15[a]	1.33±0.21[a]	1.28 (1.16-1.79)
HDL/LDL	0.84±0.07[ab]	0.87±0.19[a]	0.71±0.12[b]	0.79 (0.48-1.27)
Tg (mg/dL)	64.75±24.35[b]	101.2±51.75[ab]	157±107.51[a]	92 (32-380)

Total-C: Total cholesterol. HDL: High density lipoprotein. VLDL: Very low density lipoprotein. LDL: Low density lipoprotein. Tg: triglyceride. Different letters above the columns indicate significant difference between the groups. The significant level was accepted as $p<0.05$

As a result of ROC analysis, parameters were given as area under curve (AUC), sensitivity% (sns), specificity% (sps) and cut-off value.

SPSS software computer programme (version 27.0 for Windows, SPSS Inc, Chicago) was used to perform all the statistical analyses.

RESULTS

In the present study, the lipid profile was examined, and obesity related lipid metabolism changes were revealed. Significant increases were detected in total-C ($p<0.01$), VLDL ($p<0.05$), LDL ($p<0.05$) and Tg ($p<0.05$) values of obese dogs compared to those of ideal weigt group (Figure 1, Table 1). Additionally, serum concentrations of total-C ($p<0.01$),

Table 2. Cut-off values of the lipid profile parameters for overweight and obese dogs.

	AUC (Area)	Cut-off (95 confidence intervals%) (lower-upper bound)	p value	sensitivity%-specificity%
Total-C (mg/dL)	0.298	177.5 (0.114-0.483)	0.096	40.9-50
HDL (mg/dL)	0.452	113.5 (0.235-0.669)	0.69	45.5-50
VLDL (mg/dL)	0.344	11.5 (0.121-0.566)	0.197	50-50
LDL (mg/dL)	0.392	139.2 (0.192-0.592)	0.373	50-50
Total-C/LDL	0.409	1.26 (0.2-0.618)	0.453	40-62.5
HDL/LDL	0.556	0.81 (0.465-0.848)	0.97	63.6-37.5
Tg (mg/dL)	0.190	83.5 (0.029-0.351)	0.01	27.3-75

Total-C: Total cholesterol, HDL: High density lipoprotein, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein, Tg: triglyceride.

Table 3. Cut-off values of the lipid profile parameters for overweight and obese dogs.

Parameters	Cut-off	Overweight (n=10)	Obese (n=10)
Total-C (mg/dL)	177.5	4(40%)	9(90%)
HDL (mg/dL)	113.5	5(50%)	7(70%)
VLDL (mg/dL)	11.5	3(30%)	8(80%)
LDL (mg/dL)	139.2	3(30%)	8(80%)
Total-C/LDL	1.26	7(70%)	5(50%)
HDL/LDL	0.81	6(60%)	2(20%)
Tg (mg/dL)	83.5	6(60%)	9(90%)

Total-C: Total cholesterol, HDL: High density lipoprotein, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein, Tg: triglyceride.

VLDL ($p<0.05$) and LDL ($p<0.01$) were found to be significantly high in obese dogs compared to that of overweight dogs. The ratio of HDL/LDL in obese dogs were lower than that of overweight dogs ($p<0.05$, Table 1).

In the study, cut-off values were calculated for each parameter and used to determine the number of dogs showing an increase or decrease in these parameters (Table 2). The number and percentages of dogs with increases in parameters according to the calculated cut-off values are given in Table 3. In obese group, increase in total-C, HDL, VLDL, LDL and Tg values were obtained in 9(90%), 7(70%), 8(80%), 8(80%) and 9(90%) dogs, respectively. The numbers of obese dogs showing increases in these parameters were higher than overweight dogs. However, the numbers of obese dogs with increased in total-C/HDL and HDL/LDL were less than in overweight dogs (Table 3).

In the study, correlations between the parameters of the groups were analyzed and the results were given in table 4. In the obese group, very strong positive correlations were detected between total-C and HDL ($p<0.01$), VLDL ($p<0.01$), LDL ($p<0.01$). Furthermore, very strong positive correlations were also detected between HDL and VLDL ($p<0.01$), LDL ($p<0.01$), and between VLDL and LDL ($p<0.01$). Additionally, a very strong positive correlation was obtained between total-C/LDL and Tg in obese group (Table 4).

Table 4. Cut-off values of the lipid profile parameters for overweight and obese dogs.

Groups		Total-C	HDL	VLDL	LDL	Total-C / LDL	HDL/LDL	Tg
[2]Overweight	**Total-C**	1	0.570	0.697*	0.936**	-0.548	-0.785**	-0.162
	HDL		1	0.565	0.598	-0.449	-0.224	-0.396
	VLDL			1	0.663*	-0.531	-0.454	-0.297
	LDL				1	-0.803**	-0.889**	0.147
	Total-C/ LDL					1	0.811**	0.890**
	HDL/LDL						1	0.496
	Tg							1
[1]Obese	**Total-C**	1	0.834**	0.997**	0.958**	-0.279	-0.511	-0.286
	HDL		1	0.854**	0.840**	-0.473	-0.255	-0.445
	VLDL			1	0.926**	-0.474	-0.614*	-0.433
	LDL				1	-0.520	-0.605*	-0.546
	Total-C/ LDL					1	0.582*	0.946**
	HDL/LDL						1	0.468
	Tg							1

Total-C: Total cholesterol, HDL: High density lipoprotein, VLDL: Very low density lipoprotein, LDL: Low density lipoprotein, Tg: trygliceride. The significant level was accepted as $p<0.05$. 1: Spearsman's rho correlation test, *: $p<0.05$, **:$p<0.01$. 2: Pearson's correlation test: *: $p<0.05$, **:$p<0.01$.

DISCUSSION

Obesity emerges as a chronic problem in animals that are kept in home , movement is restricted, neutered and fed incorrectly (Preet et al., 2021; Robertson, 2003; Tunca, 2019). It is reported to be an increasing problem in dogs and the incidence rate is shown to be over 40-60% in dogs (Preet et al., 2021; Weir, 2024).

In studies conducted on obese and overweight dogs, conflicting results were obtained regarding lipid profile values (Kennerman, 2006; Rafaj et al., 2016; Usai et al., 2015). Rafaj et al. (2016) found that triglyceride concentrations were high only in overweight dogs, while no statistical difference was found in total cholesterol levels between overweight, obese and ideal weight dogs). In another study conducted by Kennerman (2006), an increase in total-C, LDL and Tg values, and decrease in HDL values were obtaned in obese dogs. Furthermore, Usui et al. (2015) reported that VLDL, Tg and Total-C values were higher in both overweight and obese dogs than in ideal weight dogs, LDL did not change, and HDL values increased only in obese dogs. However, in this study, no statistical differences were detected between overweight and obese dogs in lipid profile parameters. It has been suggested that the duration of obesity, severity of obesity, fasting time for sampling and age of the animals affected the lipid profile parameters in both overweight and obese dogs (Usai et al., 2015). In the present study, serum concentrations of total-C, VLDL, LDL and triglyceride values were significantly high in obese dogs compared to both overweight and ideal weight dogs. However, these values of overweight dogs were not statistically different from the ideal weight group. Although some of the results we obtained in the study were similar to the results obtained by Usai et al. (2015) and Kennerman (2006). On the other hand, the results of the current study are not compatible with the results obtained by Rafej et al. (2016). Although we tried to limit the fasting period to 8-12 hours in our study, there were differences in the ages and obesity periods of the animals used in the study. Therefore, possible reasons for this may be differences in the duration of obesity, duration of fasting, and age of the animals used in this study, as explained by Usui et al. (2015). According to the cut-off values, alterations in lipid profile were found to occur in some of dogs in both groups, but changes in these parameters were obtained in higher numbers of obese dogs than in overweight dogs, which may be related to the degree of obesity. Presence of strong correlations between lipid profile parameters indicated that total-C, HDL, VLDL, LDL, total-C/LDL and Tg values can be used to evaluate severity of obesity in dogs.

CONCLUSION

Changes in the lipid profile parameters do not occur in each obese or overweight dog. The levels of these changes are thought to be related to the severity of obesity in dogs. Strong and very strong correlations between parameters were determined in high number and in more parameters in obese dogs than in overweight dogs. It suggests that total-C, HDL, VLDL, LDL, total-C/LDL and Tg values can be used to evaluate severity of obesity in dogs. It is also conclusive that obesity is characterized as increases in total-C, VLDL, LDL and Tg concentrations in dogs.

DECLARATIONS

Ethics Approval

This study was approved by the Animal Ethics Committee (AEC), Burdur Mehmet Akif University, Türkiye (No:929/2022).

Conflict of Interest

Authors do not have any conflict of interests fort his study.

Consent for Publication

Consent on publication was confirmed with approval from the Republic of Türkiye Ministry of Agriculture and Forestry, Directorate of Burdur Provincial (No: E-69877819-325.04.02-5917267).

Author contribution

Idea, concept and design: HİG, EES

Data Collection and analysis: HİG, EES

Drafting of the manuscript: HİG, EES

Critical review: HİG, EES

Data Availability

Not applicable.

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Research Article

Antimicrobial susceptibilities of Aeromonas species isolated from medical leeches

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ABSTRACT

The aim of this study was to determine the antibiotic susceptibility of Aeromonas species isolated from leeches are grown for use in medical treatment. The water samples and two leeches which 2-10 g weight from a leech hatchery were brought to Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology. The samples were cultured on blood agar base added 7% defibrined sheep blood and MacConkey agar at 18-20 hours and 37°C. Besides the samples were incubated on two Sabourrauds' Dextrose agar supplemented chloramphenicol at 20°C and 37°C for 10 -14 days. Two different colonies on blood agar was detected to be Aeromonas spp. by conventional microbiological methods such as Gram staining, haemolysis, catalase, oxidase, triple tube methods. These colonies were identified to be Aeromonas hydrophila and Aeromonas veronii on species level by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry, too. According to antimicrobial susceptibiliy tests, while Aeromonas hydrophila and Aeromonas veronii isolates were found resistant to amoxicillin, ampicilin, gentamicin, erytromycin, oxacillin and penicillin, these isolates were susceptible to ceftriaxone, enrofloxacin, florfenicol and trimethoprim-sulphamethaxazole. Because of isolation Aeromonas hydrophila and Aeromonas veronii from medical leeches and environmental, and determine antimicrobial resistance to several antibiotics of Aeromonas species thought maybe a potential public health problem for humans of leeches.

INTRODUCTION

Hirudotherapy, also known as leech therapy, has been used for the treatment of various diseases since ancient times. This traditional practice has gained renewed interest due to the discovery of numerous bioactive compounds in leech saliva. More than a hundred distinct compounds have been identified, and some of these exhibit anticoagulant, analgesic, bacteriostatic, and vasodilator properties (Unal et al., 2023). The leech's gut contains a predominantly *Aeromonas hydrophila* bacterial flora, which is crucial for the animal's ability to digest blood. Leeches do not produce digestive enzymes themselves; instead, they rely on various bacteria, such as *Aeromonas*, to help break down the blood they consume. Medicinal leeches are employed across a range of medical specialties, including traumatology, microvascular replantation, and plastic and reconstructive surgery. They have the ability to reduce edema and offer additional benefits, including preventing disorders of microcirculation, restoring the vascular permeability of injured organs and tissues, lowering blood pressure, boosting immunity, and alleviating pain. However, complications following leech therapy can include bleeding, anemia, allergic reactions, and infections such as local abscesses and cellulitis, as well as more severe conditions like myocarditis, peritonitis, and meningitis. *Aeromonas* species have been isolated from a wide range of aquatic environments. Many of these species have been shown to be opportunistic pathogens affecting not only humans but also various other animals, such as fish and amphibians. (Nonomora et al., 1996). Gram-negative bacteria, especially *Aeromonas* spp., have been isolated from leeches and their environments. Research has shown that *A. hydrophila* and *A. veronii* biovar *sobria* are the predominant symbiotic species in the leech digestive system. (Worthen et al., 2006; Litwinowicz and Blaszkowska, 2014). However, the primary pathogens responsible for patient infections are the symbiotic bacterial species *A. veronii* biovar *sobria* and *A. hydrophila*, which inhabit the digestive system of leeches. Consequently, prophylactic administration of antibiotics is recommended to mitigate the risk of bacterial infections before and during leech therapy. Additionally, there are notable variations in the antibiotic susceptibilities of *Aeromonas* spp. (Maetz et al., 2012; Jaberi et al., 2014; Çeviker et al., 2019; Korun et al., 2019). Although numerous studies have examined the antibiotic sensitivity of *Aeromonas* spp. isolated from food products, water, and fish, research on the antibiotic sensitivity of *Aeromonas* spp. isolated from leeches remains limited (Ozturk et al., 2007; Muş and Çetinkaya, 2013; Onuk et al., 2017; Ocak, 2018; Chen et al., 2019; Li et al., 2020; Silva et al., 2022).

The aim of this study was to assess the antibiotic susceptibility of *Aeromonas* species isolated from leeches and their environment.

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MATERIALS and METHODS

Samples

Two leeches (2-10 g weight) from a leech hatchery were brought to Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Microbiology. Inhalation anasthesia was applied to the leeches using isoflurane-USP (ADEKA, Samsun, TÜRKİYE) (Figure 1). The samples were also taken from water, skin and inside of leeches.

Figure 1. The leeches applied the inhalation anasthesia.

Isolation and Identification of Aeromonas spp.

The samples were taken from the skin and inside of leeches by the sterile swab and sterile pasteur pipets, respectively. The samples were cultured on blood agar base (Oxoid Ltd., Hampshire, UK) added 7% defibrined sheep blood and MacConkey agar (Oxoid Ltd., Hampshire, UK) at 18-20 hours and 37°C. Besides the samples were incubated on two Sabourrauds' Dextrose agar (SDA, Oxoid Ltd., Hampshire, UK) supplemented chloramphenicol (Oxoid Ltd., Hampshire, UK) at 20 °C and 37°C for 10 -14 days. Two different colonies were obtained in blood agar. The colonies were detected to be *Aeromonas* spp. by conventional microbiological methods such as Gram staining, haemolysis, catalase, oxidase, triple tube methods (Koneman et al., 1992). These colonies were identified to be *Aeromonas hydrophila* and *Aeromonas veronii* on species level by Matrix Assisted Laser Desorption Ionization- Time of Flight Mass Spectrometry (MALDI-TOF-MS) (Bruker Daltonics GmbH, Bremen, Almanya). There was no in SDA.

Antimicrobial susceptibility tests

The antimicrobial susceptibility of *A. hydrophila* and *A. veronii* were determined on Muller-Hinton agar (Oxoid Ltd., Hampshire, UK) for 14 antibiotics (amoxicillin (25µg, Bioanalyse, Türkiye), ampicillin (10µg, Bioanalyse, Türkiye), ceft-

Table 1. The antimicrobial susceptibility of A. hydrophila and A. veronii isolates to 14 antibiotics.

Antibiotics	*A. hydrophila*	*A. veronii*
Amoxycillin	R	R
Ampicillin	R	R
Ceftriaxone	S	S
Chloramphenicol	S	R
Enrofloxacin	S	S
Erythromicin	R	R
Florfenicol	S	S
Gentamicin	R	R
Nalidixic Acid	R	S
Oxacillin	R	R
Oxytetracycline	S	R
Penicillin	R	R
Tetracycline	S	R
Trimethoprim-sulphamethaxazole	S	S

R: resistance; S: susceptible

riaxone (30µg, Bioanalyse, Türkiye), chloramphenicol (30µg, Bioanalyse, Türkiye), gentamicin (10µg, Bioanalyse, Türkiye), enrofloxacin (5µg, Bioanalyse, Türkiye), erythromicin (15µg, Bioanalyse, Türkiye), florfenicol (30µg, Bioanalyse, Türkiye), nalidixic acid (30µg, Oxoid Ltd., Hampshire, UK), oxacillin (1µg, Bioanalyse, Türkiye), tetracycline (30µg, Bioanalyse, Türkiye), oxytetracycline (30µg, Bioanalyse, Türkiye), penicillin (10 units, Oxoid Ltd., Hampshire, UK), trimethoprim-sulphamethaxazole (25µg, Bioanalyse, Türkiye) by disc diffusion method (Ozturk et al., 2007).

RESULTS

In bacteriological cultures, two distinct colonies exhibiting beta-hemolysis were isolated from all leech samples. The microorganisms showed no growth on Sabouraud Dextrose Agar (SDA). The colonies were characterized as Gram-negative, motile, and positive for catalase and oxidase activities, and were identified as *Aeromonas* spp. Further confirmation of these colonies as *A. hydrophila* and *A. veronii* was achieved using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS), a technique that analyzes the protein profiles of microorganisms to provide precise identification.

In this study, *Aeromonas* isolates were found to be resistant to at least seven antibiotics. These isolates exhibited resistance to amoxicillin, ampicillin, gentamicin, erythromycin, oxacillin, and penicillin. Additionally, the *A. hydrophila* isolate was resistant to nalidixic acid, while the *A. veronii* isolate was susceptible to nalidixic acid. However, *A. veronii* isolates were resistant to chloramphenicol, oxytetracycline, and tetracycline. In contrast, the *A. hydrophila* isolate was susceptible to ceftriaxone, chloramphenicol, enrofloxacin, florfenicol, oxytetracycline, tetracycline, and trimethoprim-sulfamethoxazole. The *A. veronii* isolates were susceptible to ceftriaxone, enrofloxacin, florfenicol, nalidixic acid, and trimethoprim-sulfamethoxazole, but resistant to other antibiotics as well. (Table 1).

DISCUSSION

Medical leeches have been used for post-traumatic wounds to maximize tissue salvage in severed tissue replantation or flap surgery. Additionally, they can be employed to treat persistent wounds that do not heal, such as pressure ulcers, venous leg ulcers, and diabetic foot ulcers. In medical leech applications, several problems may be encountered, including long-term bleeding, wound infections, anemia, and allergic reactions (Al et al., 2011; Garça et al., 2011; Kaya et al., 2011; Maetz et al., 2012; Gönen et al., 2013; Savrun et al., 2015; Najjari et al., 2022; Sproll et al., 2022). Researchers (Nonomuro et al., 1996; Jaberi et al., 2014; Sproll et al., 2022) have reported that medical leeches can harbor opportunistic pathogens. Leech therapy can lead to several infections in humans due to pathogenic bacteria. (Maetz et al., 2012; Nelson and Graf 2012; Menteş et al., 2019; Sproll et al., 2022). After leech therapy, Gram-negative bacteria, particularly *Aeromonas* species, may cause several infections (Korun et al., 2019; Nonomura et al., 1996; Gönen et al., 2013). In the present study, the body surfaces of two medical leeches and their surrounding water were investigated for the presence of pathogenic bacteria. The results indicated that medical leeches may harbor *Aeromonas* spp., which are pathogenic to humans. In this study, two different Aeromonas spp. were isolated from all samples using bacteriological methods. These were identified as *A. hydrophila* and *A. veronii* by MALDI-TOF MS.

Aeromonas spp. isolated from fish, leeches, seawater, and other marine animals have been found to exhibit resistance to various antibiotics in several studies (Altanlar et al., 2003; Hatha et al., 2005; Ozturk et al., 2007; Odeyemi and Ahmad, 2017). Altanlar et al. (2003) reported that *Aeromonas* isolates from well water samples were sensitive to trimethoprim-sulfamethoxazole, ciprofloxacin, and cefixime, but 100% resistant to erythromycin and ampicillin. Hatha et al. (2005) found that *Aeromonas* spp. isolated from fish were 100% resistant to ampicillin, 40% resistant to oxytetracycline, 10% resistant to nalidixic acid, and sensitive to streptomycin. Odeyemi and Ahmad (2017) reported that 53 *Aeromonas* isolates obtained from sea cucumber, seawater, sediment, and bivalves were resistant to ampicillin, novobiocin, and trimethoprim-sulfamethoxazole, with 24 isolates resistant to nalidixic acid, 49 to penicillin, and 25 to trimethoprim-sulfamethoxazole, but sensitive to tetracycline. However, the isolates showed varying susceptibility to gentamicin and oxytetracycline. Karun et al. (2019) found that nine *Aeromonas* isolates were 100% resistant to ampicillin and 100% sensitive to streptomycin. These isolates were 77.7% sensitive to nalidixic acid and 66.6% sensitive to tetracycline, and 66.6% resistant to trimethoprim-sulfamethoxazole. Hermansdorfer et al. (1988) reported that 16 *A.hydrophila* isolates from leeches were resistant to ampicillin but sensitive to chloramphenicol, cefoperazone, cefotaxime, cefoxitin, gentamicin, tetracycline, and trimethoprim-sulfamethoxazole. Maetz et al. (2012) obtained two *A. veronii* isolates from patients after leech therapy. These isolates were resistant to amoxicillin and amoxicillin-clavulanic acid but sensitive to cefotaxime, gentamicin, and ciprofloxacin. One of the isolates was also resistant to cephalothin. Sun et al. (2016) reported two different *A. veronii* isolates from Gibel carp. One isolate was resistant to oxacillin, penicillin, and ciprofloxacin but sensitive to ampicillin, cefoperazone, cefotaxime, erythromycin, gentamicin, streptomycin, and chloramphenicol. The other *A. veronii* isolate was resistant to oxacillin, penicillin, ampicillin, ciprofloxacin, and vancomycin, but sensitive to cefoperazone, cefotaxime, erythromycin, gentamicin, streptomycin, and chloramphenicol. In another study, Chen et al. (2019) detected that *A. veronii* isolates from carp were resistant to oxacillin, ampicillin, penicillin, amoxicillin, enrofloxacin, ciprofloxacin, nalidixic acid, cefixime, cephalexin, erythromycin, and chloramphenicol but sensitive to cefoperazone, gentamicin, neomycin, kanamycin, and tetracycline. Sproll et al. (2022) found that *A. veronii* isolates from a patient and leeches were resistant to ampicillin but sensitive to amoxicillin, sulbactam-amoxicillin, cefuroxime, gentamicin, and ciprofloxacin.

In the present study, the research results showed that these two *Aeromonas* isolates were resistant to six antibiotic agents. Some researchers have reported that *Aeromonas* isolated from leeches exhibit multiple resistances (Korun et al., 2019). Both *A. hydrophila* and *A. veronii* were found resistant to ampicillin, which is consistent with the findings of other researchers

(Hermansdorfer et al., 1988; Hatha et al., 2005; Odeyemi and Ahmad, 2017; Chen et al., 2019; Karun et al., 2019; Sproll et al., 2022). In this study, *A. hydrophila* and *A. veronii* were found sensitive to trimethoprim-sulfamethoxazole. Hermansdorfer et al. (1988) also reported that *A. hydrophila* isolates were sensitive to trimethoprim-sulfamethoxazole. Karun et al. (2019) reported varying rates of resistance to trimethoprim-sulfamethoxazole among *Aeromonas* isolates.

CONCLUSION

In conclusion, leech, which contains many bioactive compounds in its saliva, has been used for therapeutic purposes since ancient times. However, Aeromonas species, which are dominant in the leech intestinal flora, are pathogenic for humans and animals. The pathogenic microorganisms associated with leeches used in hirudotherapy may cause infections following leech therapy in humans. Antibiotic susceptibility results of *Aeromanas* species isolated from leeches have the potential to guide the treatment of infections in humans.

DECLARATIONS

Ethics Approval

I hereby declare that Ethics Committee Approval is not required for the publication given below prepared by the study team.

Conflict of Interest

The authors declare that they have no conflict of interest.

Consent for Publication

No applicable.

Author contribution

Idea, concept and design: DÖ, SY, ADÖ

Data Collection and analysis: DÖ, SY, ADÖ

Drafting of the manuscript: DÖ, SY, ADÖ

Critical review: DÖ, SY, ADÖ

Data Availability

The author has provided the required data availability statement, and if applicable, included functional and accurate links to said data therein.

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Research Article

Evaluation of egg consumption and nutritional knowledge levels among pregnant women in Konya city centre

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ABSTRACT

This study evaluated the sociodemographic characteristics, health information, frequency of food group consumption, egg consumption and nutritional knowledge levels among pregnant women. The study sample included 449 pregnant women living in Meram, Selçuklu and Karatay, the central districts of Konya province. The mean age of the participants was 28.88 ± 5.99 years. When educational status was evaluated, it was found that 31.6% of the participants had a primary education, 55.2% were employed and the average age at marriage was 23.55 ± 3.01 years. In terms of body mass index during pregnancy, 21.2% were classified as obese, and the most commonly reported condition was diabetes. The higher educational level of pregnant women was associated with higher egg consumption. Moreover, the average scores for egg consumption among individuals with normal body mass index were higher than those who were overweight and obese. Thus, we determined that egg consumption among pregnant women significantly varied according to their nutritional knowledge levels ($p < 0.05$) and that higher nutritional knowledge levels were associated with increased egg consumption. The majority of the pregnant women had a moderate level of nutritional knowledge; however, a significant portion of the participants had inadequate to moderate levels of knowledge regarding egg consumption and their egg consumption scores were also low. Based on the main findings of the study, we conclude that increasing the nutritional knowledge levels of pregnant women and ensuring that an adequate amount of eggs are included in their dietary plans during pregnancy is important for maternal health.

INTRODUCTION

Pregnancy, which can be characterized as an allograft, involves numerous adaptive mechanisms from the mother. During this process, numerous physiological changes occur, such as the differentiation of nutritional requirements and metabolic activities (Haider and Bhutta, 2017). The optimisation of required energy and nutrient intake, increased nutrient absorption from the intestines, and decreased elimination of nutrients through the gastrointestinal system are among the primary adaptations observed during pregnancy. The primary purpose of all adaptations is to maintain physiological and anatomical balance, support fetal growth and prepare for lactation (Soma-Pillay et al., 2016). To achieve successful pregnancy, maternal diet should meet the nutritional needs of the developing fetus and the requirements of the lactation (Haider and Bhutta, 2017). It has been observed that pregnant women are among the most affected individuals facing inadequate and unbalanced nutrition-related problems globally. Clinical findings indicate that inadequate and unbalanced nutrition can have negative effects on maternal and fetal health (Mendez and Kogevinas, 2011).

Many factors, such as socioeconomic status, illnesses and physiological changes related to pregnancy, influence nutritional characteristics. Determining an individualised, appropriate nutrition plan not only facilitates favorable gestation but also helps protect the health of the mother and fetus. At this point, the consumption of animal-based foods, which are sources of protein, fats and certain vitamins and minerals, is recommended, during pregnancy. Eggs should be prioritised in the dietary programmes of pregnant women because of their high biological value protein content, their positive effects on maternal nutrition and intrauterine development and as a source of riboflavin, folate, vitamins B12, D and E, as well as growth factors (Iannotti et al., 2017; Gray, 2019).

Higher levels of nutritional knowledge among consumers is known to facilitate the adoption healthy eating habits, create adequate and balanced nutrition conditions and reduce the incidence of nutrition-related diseases. During pregnancy, it is particularly important for pregnant women to have a high level of nutritional knowledge to maintain nutrient reserves, support healthy fetal development and maintain the health of the mother and offspring after birth. The aim of this study was to evaluate the sociodemographic characteristics, health information, food group consumption frequency, egg consumption and nutritional knowledge levels among pregnant women living in Meram, Selçuklu and Karatay, the central districts of Konya province.

MATERIALS and METHODS

The study sample consisted of 450 pregnant women in different trimesters. Participants were selected using a simple

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random sampling method based on voluntary participation. In this context, descriptive research methods were used initially to explain the concepts related to the subject. Subsequently, a survey, as a data collection tool, was conducted to seek answers to the research problems. The findings obtained from the administered surveys were then analysed and evaluated. Upon evaluation of the data, one participant's survey was deemed invalid and the data from 449 participants were subjected to statistical analysis.

The survey implemented in the study consists of five sections. 'Sociodemographic Characteristics' and 'Health Information' of the participants were evaluated in the first two sections. The sections titled 'Food Group Consumption Frequencies', 'Nutritional Knowledge Levels', and 'Assessment of Egg Consumption', developed by the researcher using various sources of related literature, constituted the remaining sections of the survey.

Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences, IBM Corp., Armonk, NY, US) software version 25.0. To examine the sociodemographic characteristics, health information and egg consumption of the participants, the frequency and percentage distributions of the variables were analysed using descriptive statistical methods. To assess whether egg consumption varied according to the participants' descriptive characteristics, independent samples t-tests and one-way ANOVA tests were utilised. To determine the group that exhibited significant differences, a post-hoc (LSD) test was performed. A p value of <0.05 was accepted as statistically significant in all analyses.

RESULTS

Information regarding the participants' sociodemographic characteristics is presented (Table 1).

The participants' ages at marriage ranged from 19-32 years, with an average age of 23.55 ± 3.01 years. The mean body weights before and during pregnancy were 63.03 ± 11.25 and 68.60 ± 11.25 kg, respectively. Based on pre-pregnancy body mass index (BMI), 3.8% of the participants were underweight, 53.9% had a normal weight, 33.2% were overweight and 9.1% were obese. During pregnancy, the BMI distributions were 44.8%, 34.1% and 21.2% for normal weight, overweight and obese, respectively. Moreover, 6% and 90% of the participants had experienced miscarriage and had planned pregnancies, respectively. No smoking or alcohol consumption was reported during the pregnancy period among the participants. The rates of those who had never consumed tobacco and alcohol were 92.2% and 96.4%, respectively. However, 4.9% of the participants reported having previously smoked cigarettes, whereas 0.9% reported that they had consumed alcohol. Moreover, 2.9% stated that they quit smoking and 2.7% stated that they quit alcohol after the diagnosis of pregnancy. Among the participants 75.1% had no history of illness during their pregnancy. The most common condition observed was diabetes, with a prevalence of 9.4%. When examining pre-pregnancy health conditions, 81.3% of participants had no pre-existing health issues, whereas 8.2% reported a history of thyroid disease.

Information regarding participants' health check-ups and their knowledge level about pregnancy is presented (Table 2).

The most consumed foods according to consumption

Table 1. Sociodemographic characteristics of the participants.

Characteristics	Group	n	f (%)	$\bar{x} \pm SD$
Age	18–25	176	39.2	28.88 ± 5.99
	26–35	201	44.8	
	36–45	72	16.0	
Educational status	Illiterate	9	2.0	-
	Literate	43	9.6	
	Primary education	142	31.6	
	High School	130	29.0	
	University	113	25.2	
	Postgraduate	12	2.7	
Employment status	Employed	248	55.2	-
	Unemployed	201	44.8	
Social security status	Social Insurance Institution	254	56.6	-
	Green Card	53	11.8	
	Private Insurance	26	5.8	
	Pension Fund	81	18.0	
	No	35	7.8	
Number of family members	2	204	45.4	2.81 ± 0.88
	3	148	33.0	
	4	77	17.1	
	5	20	4.5	

n: number of people, f: frequency, $\bar{x}$: arithmetic mean, SD: standard deviation.

Table 2. Health check-ups of the participants and knowledge level about pregnancy.

Variable	Group	n	f (%)
Month of starting health check-ups	1st month	27	6.0
	2nd month	302	67.3
	3rd month	94	20.9
	4th month	26	5.8
Frequency of health check-ups	Once a week	18	4.0
	Every fifteen days	57	12.7
	Once a month	295	65.7
	Less frequent	79	17.6
Status of obtaining information about pregnancy	Yes	92	20.5
	No	357	79.5
Source of information about pregnancy	Doctor	14	3.1
	Dietitian	18	4.0
	Nurse-midwife	37	8.2
	Relatives, newspapers, radio or television, etc.	23	5.1

scores were as follows: fresh fruits excluding citrus and dried fruits (78.8%), fresh vegetables excluding leafy greens and potatoes (78.5%), yogurt and cheese (71.1%) and bread (70.4%). The least consumed foods were as follows: ready-made vegetable juices (0.3%), ready-made fruit juices (2.4%) and turkey meat (6.6%). Additionally, it was observed that the egg consumption score of the participants was 69.8%.

Information regarding the frequency of food consumption during pregnancy among participants is presented (Table 3).

When evaluating the results of the analysis conducted to determine the nutritional knowledge levels, majority of the participants (97.3%) claimed that the foods consumed during pregnancy affect the health of the baby, 86.1% of the participants stated that those of normal weight should increase their food intake during pregnancy and 66.6% stated that a weight gain ranging 9-14 kg is necessary. The foods considered to be the most risky for consumption were identified as raw meat and dairy products. Overall, 360 (80.2%), 52 (11.6%) and 37 (8.2%) participants had a moderate level, inadequate level, and good levels of nutritional knowledge.

When evaluating the main findings of the analyses conducted on egg consumption, it was found that 82.2% of pregnant women consumed eggs. Moreover, the participants preferred standard natural eggs the most, with a preference rate of 21.6%. The most common time for egg consumption was during breakfast, with a preference rate of 66.8%. The reasons for consumption were reported as 37% for the nutritional value of eggs and 34.1% for being pregnant. During pregnancy, 48.1% of the participants increased their egg consumption, 14% decreased it and 20% had no change. The most commonly reported reason for not consuming eggs was 'disliking the smell'. Moreover, the participants predominantly consumed large-sized eggs (55.2%) and none preferred egg products, such as liquid or dried eggs, and the preferred place of purchase was identified as supermarkets, with a preference rate of 52.8%. Quail eggs were identified as the most commonly preferred alternative to chicken eggs, with a preference rate of 21.8%. When evaluating the findings related to eggs consumption and cholesterol levels, 6.2% of the participants stated that 'eggs raise cholesterol levels in healthy individuals and should be consumed in moderation', 50.1% indicated that 'individuals with elevated cholesterol should limit their egg consumption, whereas normal individuals do not need to restrict their intake', and 14.5% expressed the belief that 'eggs do not raise cholesterol levels'. Although 84% of the participants indicated that the presence of double yolk in eggs was not a reason for their preference, 77.7% did not consider the brand important. Moreover, 92% of the participants were unaware of the meanings of the codes on the eggs available for sale and 90.9% did not know the classifications for egg quality. It was determined that a significant number of the participants expressed the opinion that eggs should be washed before consumption and stored in the refrigerator without washing until they are consumed. Additionally, 80% of the participants expressed the opinion that 'hygiene measures are not implemented in places where eggs and egg products are produced and sold'. In response to the question, 'What is the shelf life of eggs?' majority of the participants (62.8%) answered, '28 days from the date of laying'. Overall, 36.3%, 30.5% and 33.2% of the participants had inadequate, moderate and good knowledge about egg consumption, respectively.

The results of the analysis on the differences in egg consumption among pregnant women based on age, education level, social security status, number of family members, BMI and nutritional knowledge level are presented (Table 4).

As observed in Table 4, the effects of variables, such as age, educational status, number of family members and current

Table 3. Participants' frequency of food consumption during pregnancy.

Food type	Consumption frequency																				CS
	Every day		2-3 times a day		Every other day		Once a week		2-3 times a week		Once every 15 days		Once a month		Once a year		Rarely		Never		
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	%
Milk	56	12.5	-	-	14	3.1	82	18.3	79	17.6	88	19.6	39	8.7	-	-	37	8.2	54	12.0	49.4
Yogurt	41	9.1	50	11.1	171	38.1	27	6	107	23.8	53	11.8	-	-	-	-	-	-	-	-	71.1
Cheese	41	9.1	50	11.1	171	38.1	27	6	107	23.8	53	11.8	-	-	-	-	-	-	-	-	71.1
Milk desserts	-	-	-	-	-	-	48	10.7	-	-	195	43.4	180	40.1	26	5.8	-	-	-	-	41.1
Red meat	-	-	-	-	68	15.1	195	43.4	132	29.4	27	6	27	6	-	-	-	-	-	-	61.7
Chicken meat	-	-	-	-	54	12	209	46.5	132	29.4	27	6	27	6	-	-	-	-	-	-	61.4
Fish meat	-	-	-	-	-	-	14	3.1	92	20.5	129	28.7	-	-	83	18.5	86	19.2	45	10	27.0
Turkey meat	-	-	-	-	-	-	-	-	-	-	-	-	18	4	36	8	142	31.6	253	56.3	6.6
Offal	-	-	-	-	-		28	6.2	-	-	28	6.2	41	9.1	52	11.6	46	10.2	254	56.6	13.7
Egg	59	13.1	-	-	110	24.5	136	30.3	126	28.1	18	4	-	-	-	-	-	-	-	-	69.8
Sausage, salami, pastrami	-	-	-	-	-	-	28	6.2	-	-	28	6.2	41	9.1	52	11.6	46	10.2	254	56.6	13.7
Legumes	-	-	-	-	-	-	123	27.4	41	9.1	204	45.4	81	18	-	-	-	-	-	-	49.5
Bread	154	34.3	83	18.5	94	20.9	14	3.1	-	-	-	-	-	-	-	-	54	12	50	11.1	70.4
Rice	-	-	-	-	-	-	124	27.6	102	22.7	120	26.7	-	-	37	8.2	52	11.6	14	3.1	46.0
Bulgur	-	-	14	3.1	-	-	124	27.6	102	22.7	120	26.7	-	-	37	8.2	52	11.6	-	-	48.8
Pasta	-	-	-	-	-	-	124	27.6	102	22.7	120	26.7	-	-	37	8.2	52	11.6	14	3.1	46.0
Pastries (cakes, cookies, pies, biscuit)	28	6.2	-	-	-	-	123	27.4	105	23.4	75	16.7	41	9.1	27	6	36	8	14	3.1	50.2

Table 3. (Continued) Participants' frequency of food consumption during pregnancy.

Food type	Consumption frequency																				CS
	Every day		2–3 times a day		Every other day		2-3 times a week		2-3 times a week		Once every 15 days		Once a month		Once a year		Rarely		Never		
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	%
Green leafy vegetables	54	12	106	23.6	68	15.1	52	11.6	52	11.6	54	12	36	8	-	-	27	6	-	-	67.6
Fresh vegetables	117	26.1	9	2	134	29.8	163	36.3	26	5.8	-	-	-	-	-	-	-	-	-	-	78.5
Potato	-	-	-	-	59	13.1	174	38.8	75	16.7	105	23.4	36	8	-	-	-	-	-	-	58.4
Freshly squeezed vegetable juices	14	3.1	-	-	-	-	41	9.1	39	8.7	-	-	27	6	27	6	135	30.1	166	37	20.7
Ready-made vegetable juices	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14	3.1	435	96.9	0.3
Citrus fruits	14	3.1	-	-	45	10	134	29.8	137	30.5	65	14.5	54	12	-	-	-	-	-	-	55.5
Fresh fruits	119	26.5	88	19.6	106	23.6	36	8	50	11.1	50	11.1	-	-	-	-	-	-	-	-	78.8
Dried fruits	52	11.6	53	11.8	67	14.9	95	21.2	45	10	27	6	-	-	-	-	70	15.6	40	8.9	57.8
Freshly squeezed fruit juices	14	3.1			36	8	89	19.8	45	10	-	-	-	-	-	-	188	41.9	77	17.1	31.7
Ready-made fruit juices	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	351	78.2	98	21.8	2.4
Margarine (liquid, solid, semi-solid)	-	-	-	-	9	2	95	21.2	-	-	92	20.5	119	26.5	-	-	106	23.6	28	6.2	36.2
Butter	123	27.4	14	3.1	92	20.5	53	11.8	41	9.1	63	14	-	-	-	-	36	8	27	6	66.2

n: number of people, CS: Consumption Score

Table 4. Analysis of the differences in egg consumption among pregnant women based on age, education level, social security status, number of family members, BMI, and nutritional knowledge level.

Variable	Group	n	x̄	SD	F	P
Age	18–25	176	18.41	10.718	5.726	0.004*
	26–35	201	19.60	10.312		
	36–45	72	23.19	7.841		
Educational status	Illiterate	9	10.00	.000	12.200	0.000*
	Literate	43	10.47	12.479		
	Primary education	142	21.80	10.112		
	High School	130	20.92	8.371		
	University	113	19.42	9.746		
	Postgraduate	12	25.00	7.385		
Social security status	Social Insurance Institution	254	19.04	9.108	35.106	0.000*
	Green Card	53	28.77	2.172		
	Private Insurance	26	27.50	2.550		
	Pension Fund	81	11.98	12.265		
	No	35	23.00	8.062		
Number of family members	2	204	16.99	11.452	14.472	0.000*
	3	148	21.72	9.326		
	4	77	24.29	6.108		
	5	20	15.00	5.130		
BMI (Current)	Normal	201	22.91	8.238	19.254	0.000*
	Overweight	153	17.19	11.193		
	Obese	95	17.00	10.604		
Nutrition knowledge level	Inadequate	52	1.46	0.670	24.187	0.000*
	Middle	360	1.97	0.834		
	Good.	37	2.65	0.484		

*P < 0.05. n: number of people, x̄: arithmetic mean, SD: standard deviation, F: ANOVA test value, P: significance value.

BMI, among pregnant women with respect to egg consumption were significant. Similarly, egg consumption differed statistically significantly according to the level of knowledge ($P < 0.05$). From post-hoc analysis, it was determined that the mean scores of pregnant women with high nutritional knowledge levels were higher than those with medium and inadequate nutritional knowledge levels and the mean scores of pregnant women with medium nutritional knowledge levels were higher than those with inadequate nutritional knowledge levels.

Egg consumption among pregnant women did not differ statistically according to their employment status ($P > 0.05$). From the post-hoc analysis, it was found that the mean scores of egg consumption of participants with higher food consumption frequency were statistically significantly higher than those with lower food consumption frequency. From the correlation analysis, it was found that egg consumption among pregnant women was most affected by cereal group food consumption followed by meat products and fat and milk products. However, egg consumption was least affected by food consumption by the fruit and vegetable groups.

DISCUSSION

The mean age of the participants was 28.88 ± 5.99 years and all were in their reproductive phase, which is considered as physiologically healthy. In fact, according to the Turkey Demographic and Health Survey, the age group with the highest fertility rate is 25-29 years. Advanced maternal age is defined as being a mother over the age of 35 years, and, in recent years, the number of mothers with advanced maternal age has been increasing especially in developed countries (Lean et al., 2017). Frederiksen et al. (2018) stated that chromosomal abnormalities, preterm birth and miscarriage are more likely in pregnant women over 40 years of age. Adolescent pregnancies, defined by the World Health Organization as those between the ages of 10 and 19 years, are known to cause serious public health problems in the short and long term (Sen and Kavlak, 2011). Askari et al. (2020) suggested that the risk of birth complications is high in pregnant women during adolescence as physiological development is not fully completed during this period.

There is an increasing trend towards obesity from 9.1% to 21.2% in terms of BMI during pregnancy. Above average

pre-pregnancy weight and excessive weight gain during pregnancy leads to difficult and related complications during parturition. Thus, BMI assessment in the prenatal follow-up of pregnant women and regulation of weight gain is of great importance in terms of pregnancy and intrauterine health. Considering that 6% of the pregnant women in the study had previously experienced miscarriage, it can be suggested that obesity may create a predisposition for recurrent miscarriage cases. As a matter of fact, the relationship between obesity and recurrent miscarriages is a topic that has been addressed. Cavalcante et al. (2019), in a meta-analysis aiming to reveal the relationship between obesity and recurrent miscarriage, revealed that the rate of miscarriage is higher in pregnant women with obesity. Ku et al. (2015) conducted a study consisting of 119 pregnant women between the 6th and 10th gestational weeks who presented to obstetricians with a miscarriage case. Researchers found that 25.2% of pregnant women had early pregnancy loss. Early pregnancy loss is one of the most common complications of pregnancy and du Fossé et al. (2020) stated that 10%–15% of clinically confirmed pregnancies result in miscarriage.

Similar to the findings of our study, Ozkoc (2013) found that majority of pregnant women had never smoked (96.7%) and drank alcohol (83.3%). It is known that tobacco use among pregnant women in European countries is ~20% (Candel et al., 2015). It has been scientifically demonstrated that smoking during pregnancy has serious negative health effects on the pregnant women and the developing fetus (Miguez et al., 2017). Mardby et al. (2017) tried to determine the alcohol consumption habits and the effects of alcohol consumption in 7905 pregnant and in women postpartum. The researchers found that 15.8% of the participants consumed alcohol regularly and 39% consumed alcohol once a month. The highest consumption by country was reported to be in the UK (28.5%), Russia (26.5%), Switzerland (20.9%), Poland (9.7%), Sweden (7.2%) and Norway (4.1%). Moreover, they suggested that higher education level and smoking were predictors of alcohol consumption during pregnancy. Similarly, Lanting et al. (2015) found that maternal age of mother, cigarette consumption and higher education level had a parallel relationship with alcohol consumption.

In the light of the data obtained in our study, it was determined that 24.9% of the participants had at least one illness during pregnancy, such as diabetes, hypertension, anemia, thyroid diseases, kidney diseases and bone and joint diseases according to the frequency of occurrence. When the pre-pregnancy disease history (18.7%) is considered, thyroid diseases, diabetes, liver diseases, gallbladder diseases, anemia, hypertension and kidney diseases were the most frequently reported diseases. The fact that pregnant women have a known chronic disease before pregnancy is important for the health of the mother and newborn. Chronic diseases may also be associated with the dietary characteristics and BMI of the pregnant mother. Gumus et al. (2010) found that the rates of gestational diabetes and hypertension were statistically significantly higher in individuals who were obese before pregnancy.

It was found that a significant proportion of pregnant women who participated in the study did not receive any information on nutrition during pregnancy and that the majority of those who received information received information from nurses-midwives. Aktac et al. (2018) aimed to evaluate the nutritional knowledge of pregnant women according to their sociodemographic characteristics. They found that 50.9% of the participants received nutrition information. Additionally, they identified the sources of information as doctors, nurses, internet, newspapers, magazines, television, and radio. Similar to the findings of the study, the researchers stated that the number of participants who consulted a dietitian for information on nutrition was low. Bryant et al. (2019) reported that 63% of women who applied to antenatal clinic in Australia received information on nutrition. Since adequate and balanced nutrition during pregnancy has a great impact on the psychological and physiological needs of pregnant woman before and after giving birth as well as on the mental and physical development of the fetus, it is important for pregnant women to receive information on nutrition during pregnancy. As a matter of fact, Blondin and LoGiudice (2018), in their study on increasing the nutritional knowledge level of pregnant women, provided nutrition education during the study. It has been reported that nutritional scores increased statistically significantly after training practices.

In the evaluations based on consumption scores, it is noteworthy that fresh fruits (fruits other than citrus fruits and dried fruits) and fresh vegetables (vegetables other than green leafy vegetables and potatoes) are the most consumed foods and the relatively high scores obtained for egg consumption are the main findings. It is known that the daily food consumption amounts required during pregnancy period should be 400-600 ml of milk and yogurt, 60 g of cheese, 3-4 portions (~100 g) of meat, chicken and fish, 1 portion of eggs and legumes, 5-6 portions of fresh vegetables and fruits, 6-10 slices of bread and none or 2-3 portions of rice, bulgur, pasta, etc. (Dibek, 2007). Nogay (2011) aimed to evaluate the nutritional status of pregnant participants aged 18-42 years. The researcher found that pregnant women did not change the amount of food consumption during the gestation period and the foods that they increased the most were fruit (47.1%) and milk and yogurt (42.9%). Pregnant women stated that citrus fruits (30.0%) were the food they most desired and chicken meat (22.9%) was the food they not prefer the least. Goksin Cihan et al. (2012) tried to reveal the level of knowledge among pregnant women about healthy pregnancy and nutrition. Pregnant women who participated in the study reported that the foods that should definitely be consuming during this period were milk and yogurt (75.2%), vegetables and fruits (57.4%) and meat and eggs (54.0%).

In the study, 77.5% of the participants stated that consumption of non-heat-treated meat and dairy products is risky during pregnancy. Contrary to the findings, Bryant et al. (2019) suggested that one-third of pregnant women were unaware of the foods that should be avoided during pregnancy. Considering that raw meat and its products are among the main foods responsible for toxoplasmosis, the tendency of the participants in this direction is thought to be conscious. Bieńkowski et al. (2022) found a statistically significant relationship between raw

meat consumption parameters and *Toxoplasma gondii* seropositivity in pregnant women.

It can be stated that egg consumption of pregnant women differed statistically significantly according to age ($P < 0.05$) and egg consumption of the participants increased with age. However, it is observed that egg consumption differed statistically significantly according to educational status ($P < 0.05$) and the mean scores of egg consumption increased with the increase in educational level in all groups. According to the social security status of the participants, egg consumption differed statistically significantly ($P < 0.05$) and as a result of the post-hoc analysis, it was determined that those who were members of the Retirement Fund had the lowest average, followed by Social Insurance Institution and those without social security. Moreover, it has observed that the number of individuals in the family has an effect on egg consumption, which was statistically significantly ($P < 0.05$). It was determined that those who had 4 members in their families had a statistically significantly higher mean than those who had 2-5 members. When the effect of the participants' BMI on egg consumption was evaluated, egg consumption differed statistically significantly according to BMI. Post-hoc analysis shows that those with normal BMI have higher mean scores on egg consumption than those who are overweight and obese. Additionally, those who are obese had the lowest mean score. It is important to note that a significant proportion of pregnant women (360 participants) had a moderate level of knowledge about nutrition during pregnancy and their egg consumption increases with the increase in their level of nutritional knowledge. Dibek (2007) found that 46.9%, 36.2%, 15.8% and 1.1% of pregnant women had very good, good, fair and poor nutritional knowledge, respectively.

It is important to note that pregnant women reported consuming eggs at a considerable level, 87.8% of the participants reported consuming eggs during pregnancy. Schnefke et al. (2019) stated that 50% of pregnant women in Kenya consumed eggs during pregnancy. Christian et al. (2006) reported that egg consumption during pregnancy is low in Nepal, resulting from religious reasons. Similarly, Hong et al. (2016) found that a small proportion of participants (5.0%) did not consume eggs during pregnancy in Zambia and suggested that religious beliefs were the main reason for not consuming eggs. Jardi et al. (2019) determined that the amount of egg consumed by pregnant women during pregnancy was not at the recommended level. Although the annual egg consumption per capita in our country is slightly above the global average, it remains well below the levels in developed countries (Mizrak et al., 2012). Therefore, eggs should be an important part of the diets of all consumer groups, especially pregnant women, because of their high nutritional value and easy availability.

In terms of the preferred egg types, it is seen that there is a ranking from most to least; standard natural eggs, organic eggs, village eggs and free-range chicken eggs. It was observed that a significant proportion of the participants (92.0%) did not know what the codes on egg stamps meant. Although it is known that cage-free eggs are the most consumed egg type globally, it is stated that the market for free-range and organic eggs is growing rapidly because of factors, such as increasing income and education levels. Derebasi (2019) aimed to examine egg consumption awareness and consumer behaviours. Similarly, the researcher found that 63.5% of the respondents consumed eggs primarily for breakfast, 45.0% of them bought eggs from the market, and 52.22% of them bought medium size eggs. The high quality protein contained in eggs contributes to the protein requirements of the body. Moreover, eggs are considered an ideal food recommended to be consumed during pregnancy as they have a high essential amino acid content. However, eggs are an important source of essential fatty acids. Egg consumption is reported to increase the positive evidence for maternal and intrauterine nutrition during pregnancy (Iannotti, 2017). In this context, it is noteworthy that 48.1% of the participants' egg consumption increased during pregnancy. Wallace and Fulgoni (2017) reported that adults who consume eggs have a higher healthy eating index than those who do not. However, they noted that adults who consumed eggs had approximately twice the choline intake compared to those who did not.

Mizrak et al. (2012) stated the reasons for not consuming eggs in Turkey as health problems (54.50%), dislike of eggs (31.80%) and high price of eggs (13.70%). Hillier and Olander (2017) systematically reviewed 898 scientific studies to determine the nutritional changes in women before and during pregnancy. In the light of the findings obtained, it was determined that the amount of eggs consumed by women before pregnancy was 22.2 g and the amount consumed during pregnancy was 11.1 g.

It is thought that all of the pregnant women who participated in the study reported that they did not consume egg products should be taken into consideration. The amount of eggs allocated for egg products in Turkey is 2.77% of the total amount of eggs produced. As egg products can be used as an alternative source for people who cannot consume eggs, it is thought that developing and expanding the use of egg products will increase egg consumption. Indeed, it is known that egg products such as frozen, dried and pasteurised liquid eggs have a large consumption volume, especially in developed countries For example, it is reported that ~25% of total egg production in the USA is used in the production of egg products (Dogruer et al., 2015).

It was found that about half of the pregnant women who participated in the study stated that 'individuals with elevated cholesterol should consume eggs in a limited way, normal individuals do not need to consume eggs in a limited way' in relation to eggs and cholesterol. Derebasi (2019) tried to determine the knowledge of consumers about the relationship between eggs and cholesterol and reported that 47.80% of the participants reported that eggs had no effect on blood cholesterol levels. The researcher stated that the consumer perception that eggs contain high levels of cholesterol and cause cardiovascular diseases by increasing blood cholesterol levels is the main reason why egg consumption amounts have not reached the desired levels. The relationship between dietary cholesterol and atherosclerotic cardiovascular disease remains controversial. Scientific efforts are ongoing to elucidate the

effects of dietary cholesterol on serum cholesterol concentrations and cardiovascular disease. Shin et al. (2013) investigated the effect of egg consumption on cardiovascular diseases and suggested that there was no significant relationship between egg consumption and cardiovascular diseases. Rosenson and Wen-Liang (2019) reported that egg consumption may alter the atherogenicity of triglyceride-rich lipoproteins such as low-density lipoprotein and that guidance on egg consumption should continue in dietary guidelines and recommendations.

There is a tendency that the places where eggs and egg products are produced and sold do not comply with hygienic standards. Similarly, Derebasi (2019) reported that 56.99% of consumers did not find the inspections in egg production sufficient. Mizrak et al. (2012) stated that 67.11% of consumers think that egg production facilities are not sufficiently inspected.

Through the ~10,000 pores in the eggshell, microorganisms can contaminate the interior of the egg and create microbial contamination. This can lead to numerous infections caused by pathogens and public health problems. Coronel-Reyes et al. (2018) reported that the maximum storage period of eggs at room temperature should be 14 days. Kosa et al. (2015) aimed to reveal the egg consumption behaviours of consumers and found that 61.8% of the participants checked the expiry dates before purchasing eggs. The Turkish Food Codex Communiqué on Eggs and Egg Products states that the expiry date of Class A eggs cannot be more than 28 days after the laying date.

It was determined that 36.3% of the pregnant women participating in the study had inadequate, 30.5% moderate and 33.2% good level of knowledge about egg consumption. Lutter et al. (2021) suggested that egg consumption should increase to reduce neonatal mortality. The researchers stated that trainings to increase the level of nutritional knowledge of pregnant women could be an effective strategy. Mizrak et al. (2012) conducted a study with 2241 families to determine egg consumption and consumer habits in Turkey and found that 24.67% of the participants were aware of the nutritional value of eggs. In addition, they argued that for a healthy society, consumers should be made aware of the nutritional value of eggs through effective promotions.

When the egg consumption of pregnant women according to their educational status was analysed, it was determined that the egg consumption of the participants differed statistically significantly. It is observed that as the education level of pregnant women increases, their egg consumption also increases. Similarly, Sarı et al. (2015) tried to determine the level of knowledge of pregnant women about nutrition during pregnancy. The researchers found that the level of nutritional knowledge of pregnant women with high school education and above was higher.

CONCLUSION

It is thought that it is important to increase the nutritional knowledge levels of pregnant women by preparing educational programmes. Pregnant women should be directed to nutritionists in health institutions where they go for regular follow-up during pregnancy and they should be helped to access the right information. Pregnant women should be made aware of the benefits of egg consumption during pregnancy and activities should be organised to increase egg consumption. Pregnant women who cannot consume eggs for some reason should be encouraged to consume egg products.

DECLARATIONS

Ethics Approval

Selcuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Center Ethics Committee (SUVDAMEK)

Written informed consent to participate and publish was obtained from all individual participants included in the study.

Meeting date: 14.11.2019; Meeting number: 2019/13; Decision number: 2019/91

Conflict of Interest

The authors declare that they have no conflict of interest.

Consent for Publication

We hereby provide consent for the publication of the manuscript detailed above. We confirm that this information will be freely available online, and accessible.

Author contribution

Idea, concept and design: NT, SK

Data collection and analysis: SK

Drafting of the manuscript: NT, SK

Critical review: NT

Data Availability

The data is available from the corresponding author on reasonable request.

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Research Article

Sensitivity of pure cultures of some Gram-positive and Gram-negative rumen bacteria to sigla storax (*Liquidambar orientalis*)

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ABSTRACT

Extracted from the wounded bark of the *Liquidambar orientalis* tree, sigla storax is a semi-viscous, balsamic resin. This study aimed to evaluate the effects of sigla storax on the growth of pure cultures of select Gram-positive and Gram-negative rumen bacteria, thereby elucidating its potential mode of action on rumen metabolism as an alternative antibiotic feed additive. Under strictly anaerobic conditions, the antimicrobial activity of sigla storax was assessed using the broth microdilution method. With the exception of *Streptococcus bovis*, storax demonstrated potential antimicrobial activity on all bacteria at doses starting from 1-2 mg/ml ($P<0.05$). The most susceptible bacterium was *Ruminococcus flavefaciens*, which was completely inhibited at 4 mg/ml sigla, while the most resistant was *S. bovis*, which showed no complete inhibition. For other Gram-positive bacteria, the minimum inhibitory concentration (MIC) varied: 16 mg/ml for *Butyrivibrio fibrisolvens*, and 32 mg/ml for *Ruminococcus albus*, *Eubacterium ruminantium*, and *Methanobacterium formicicum*. Interestingly, at lower doses, sigla storax exhibited a growth-stimulating effect on *E. ruminantium* (0.06-0.125 mg/ml) and *S. bovis* (0.125-2 mg/ml) ($P<0.05$). The Gram-negative *Megasphaera elsdenii* also showed a slight stimulatory response to sigla storax at concentrations of 0.06-0.5 mg/ml ($P<0.05$). However, at 32 mg/ml, sigla storax completely inhibited both Gram-negative bacteria tested: *M. elsdenii* and *Fibrobacter succinogenes*. While Gram-positive bacteria generally exhibited higher sensitivity to sigla storax compared to Gram-negative bacteria, the study concluded that its mechanism of action differs from typical antibiotic feed additives. This distinction is due to sigla storax's antibacterial activity against Gram-negative bacteria and its stimulatory effects on certain Gram-positive bacteria.

INTRODUCTION

The rumen hosts one of the most diverse and densely populated microbial ecosystems known. A symbiotic relationship has evolved between ruminant animals and their ruminal microbes. In this mutually beneficial arrangement, the ruminant provides a hospitable environment within the rumen, while the microorganisms supply the animal with essential energy and nitrogen sources in the form of volatile fatty acids and microbial protein. Approximately half of the rumen's microbial population consists of bacteria (Genzebu and Tesfay, 2015).

Over recent decades, enhancing rumen fermentation efficiency through bacterial modification has been a primary research focus. This emphasis stems from the fact that Gram-positive rumen bacteria produce higher quantities of hydrogen, formate, lactate, and ammonia compared to their Gram-negative counterparts, resulting in energy and protein losses (Castillejos et al., 2007). Hydrogen released by acetate and butyrate-producing Gram-positive bacteria is used for methane synthesis by methanogenic archaea. On the other hand, propionate, which is produced by mostly Gram-negative bacteria, consumes reducing equivalents for methanogenesis (Bharanidharan et al., 2021; Knapp et al. 2014). Methane is ultimately eliminated by belching, which is widely reported to represent a loss of feed energy of up to 2-15% (Martin et al., 2021). Enteric methane is also a greenhouse gas responsible for 15-24% of global warming (Steinfeld et al., 2006). Ionophore antibiotics such as monensin specifically target Gram-positive hydrogen producers and alter the microbiota in favour of Gram-negative propionate producers (Callaway et al. 2003). In cattle treated with monensin, methane production is reduced by up to 30% as a result of a shift in fermentation towards propionate production (Callaway et al., 2003; Wedegaertner and Johnson, 1983). Monensin also limits lactate production by inhibiting lactate-producing bacteria such as Gram-positive *Streptococcus bovis*, which is often involved in the development of rumen acidosis. Accordingly, cattle fed monensin had lower lactate concentrations and higher ruminal pH (Russell and Strobel, 1989). Reducing nitrogen losses by restraining deamination and proteolysis by Gram-positive ammonia producers and protozoa is another favourable effect of monensin on rumen fermentation (Russell and Strobel, 1989). Thus, ionophoric antibiotics have been employed as feed additives for years to modify rumen microbiota and rumen fermentation to improve animal productivity (Thompson et al., 2021). However, following the prohibition of antibiotics as feed additives (OJEU 2003), researchers have intensively explored the use of natural, plant-derived antibacterial agents to modulate rumen microorganisms and fermentation processes (Stefańska et al., 2021). This shift in focus represents an ongoing effort to optimize rumen function while adhering to current regulatory

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standards and addressing concerns about antibiotic resistance. Neverthless, the non-specific, broad-spectrum effect of most phytochemicals on rumen bacteria, which may depress rumen fermentation, is one of the major challenges in rumen studies (Bodas et al., 2012; Demirtas et al., 2021).

Commonly known as "sigla oil," sigla storax (Styrax liquidus) is a semi-liquid, balsam-like resin extracted from the wounded bark of the *Liquidambar orientalis* tree (Kılınç et al., 2020). This endemic member of the Hamamelidaceae family is native to Turkey's southwestern regions, particularly around Köyceğiz, Ula, Marmaris, and Fethiye. In Anatolian traditional medicine, sigla storax finds diverse applications. It is used to treat peptic ulcers, stomach aches, burns, wounds, cracked lips, bronchitis, and both parasitic and fungal infections (Atmaca et al., 2022). Recent research has explored its potential beyond traditional uses. Studies have investigated its cytotoxic effects on human cancer cells (Atmaca et al., 2022; Çetinkaya et al. 2022) and its neuroprotective properties (Zhang et al. 2019; Zhou et al. 2022). Sigla storax has demonstrated a broad spectrum of antimicrobial activity, particularly against various Gram-positive bacteria (Aşkun et al. 2021; Sağdıç et al. 2005; Keyvan and Savas 2021). Notably, surgical silk sutures coated with sigla storax exhibited the most potent anti-adhesion activity against the oral Gram-positive pathogen *S. aureus* (Kılınç et al. 2020). In addition, storax-loaded polymeric scaffolds demonstrated antibacterial and anti-biofilm properties against *S. aureus*, which is known to cause infections in chronic wounds (Demir et al., 2022). Given its pronounced antimicrobial effects, especially against Gram-positive bacteria, sigla storax shows promise as a potential antibiotic alternative for modifying rumen microorganism composition and activity. Supporting this potential, a recent study reported that sigla storax reduced rumen methane production without adversely affecting short-chain fatty acid (SCFA) production or feed digestibility. Importantly, it did not substantially alter the microbiota compared to monensin, a common antibiotic feed additive (Demirtas et al., 2023). However, to date, the literature lacks data on sigla storax's effects on pure cultures of rumen bacteria. An assessment of sigla storax's potential inhibitory or stimulatory impacts on specific rumen bacterial species would significantly contribute to elucidating its mechanism of action on rumen metabolism. Therefore, the present study aimed to investigate the effects of sigla storax on the growth of pure cultures of select Gram-positive and Gram-negative rumen bacteria.

MATERIALS and METHODS

Sigla storax

Sigla storax was sourced from a local supplier in Köyceğiz, Muğla, Turkey in August 2021, under the supervision of the Köyceğiz Forestry Management, which operates under the General Directorate of Forestry, Ministry of Environment and Forestry, Turkey. Until its use, the storax was stored at +4ºC. The traditional and producer-verified process for obtaining sigla storax is as follows: In early spring (around April), the trunks of *L. orientalis* trees are deliberately damaged, prompting the inner bark to absorb an exudate. By early July, the outer bark is shaved using specialized knives, and the balsam, along with the inner bark, is boiled in water for 10 to 30 minutes. As the mixture boils, the storax rises to the surface and is skimmed off. Afterward, the remaining bark is pressed to separate any remaining storax (Sağdıç et al., 2005). The resulting sigla storax in balsam form was used in the experiments.

Bacterial strains and anaerobic medium

The study examined several Gram-positive bacterial strains. These included *Ruminococcus albus* (ATCC 27210) and *Ruminococcus flavefaciens* Sijpesteijn C97 (ATCC 49949), both known for producing hydrogen and formate. Butyrate producers *Butyri-*

Table 1. Composition of the anaerobic medium (for 100 ml) (Orpin 1976)

Component*	
Mineral solution 1**	15
Mineral solution 2***	15
Clarified rumen fluid****	15
$NaHCO_3$ (Sigma S5761)	0.6
Yeast extract (Sigma Y1625)	0.25
Trypticase peptone (BD 211921 Bacto™)	1
Resazurin (%0.1, v/v) (Sigma R7017)	1
Cysteine HCl (Sigma C7880)	0.1
Cellobiose (Sigma 22150)	0.5
Deionized water	55

*Units are ml for liquid components and g for solid components.
**Mineral solution 1: 3 g/l K_2HPO_4 (Sigma P3786)
***Mineral solution 2: 3 g/l KH_2PO_4 (Sigma P9791), 6 g/l $(NH_4)_2SO_4$ (Sigma A4915), 6 g/l NaCl (Sigma S7653), 0.6 g/l $MgSO_4 \cdot 7H_2O$ (Sigma 230391) and 0.6 g/l $CaCl_2$ (Sigma C1016)
****The ruminal fluid collected from the slaughterhouse was mixed and filtered through three layers of cheesecloth to seperate into liquid and solid (digesta) fractions. The liquid fraction was centrifuged at 15,000 rpm and, the clear supernatant was used as a component of the anaerobic medium.

vibrio fibrisolvens D1 (ATCC 19171) and *Eubacterium ruminantium* GA 195 (ATCC 17233) were also studied, along with the lactate producer *Streptococcus bovis* (ATCC 33317). *Methanobacterium formicicum* (ATCC 33274), a mesophilic methanogen, served as the methane producer. Additionally, two Gram-negative bacteria were included: *Megasphaera elsdenii* LC1 (ATCC 25940) and *Fibrobacter succinogenes* S85 (ATCC 19169), both known for producing succinate and propionate. To maintain anaerobiosis, anaerobic medium was prepared under CO_2 following Orpin's (1976) method. Table 1 details the chemical composition of the anaerobic media. The medium was heated to 60ºC in a hot water bath while being bubbled with CO_2 to eliminate oxygen. Resazurin (0.1%, v/v), an indicator of redox potential, was used to monitor oxygen elimination, with a color change from bluish-purple to dull yellow signifying successful oxygen removal. After preparation, the flask containing the medium was stoppered and autoclaved. Anaerobic bacteria were cultivated for 24 to 72 hours in an anaerobic cabinet (Whitley DG250, Don Whitley, West Yorkshire, UK) maintained at 37ºC with a N_2-CO_2-H_2 (80:10:10) atmosphere.

Antimicrobial susceptibility assay

To assess the impact of sigla storax on rumen bacterial growth, a broth dilution technique was employed in an anaerobic cabinet, adhering to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2016). The process began with the preparation of a sigla storax stock solution (350 mg/ml) by dissolving sigla in ethanol (99.8% purity, Sigma 32221). This stock was then serially diluted two-fold in anaerobic medium to achieve concentrations of 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 mg/ml. Using a flat-bottom 96-well plate (Corning 3599), 200 µl of each dilution was pipetted into designated wells. Each well was then inoculated with 20 µL of bacterial suspension (4×10^{10} cells/ml). For reliability, triplicate wells were used for each concentration. The experimental design included negative control wells (pure medium without sigla storax solution) and medium control wells (without bacteria) for each set. Following preparation, the plates were incubated in an anaerobic chamber at 37ºC for 24 hours. Microbial growth was subsequently assessed using a microplate reader (Epoch, BioTek, USA) set at 600 nm. Interpretation of results followed established guidelines: The minimum inhibitory concentration (MIC) was defined as the lowest concentration yielding an OD600 ≤ 0.1 (Kang et al., 2008). OD600 values significantly lower than the control dose (0 mg/ml) were considered indicative of potential antimicrobial activity (Ko et al., 2018), while significantly higher OD600 values were interpreted as evidence of stimulatory activity (Das et al., 2015).

Statistical analyses

Each well of the 96-well plate served as an experimental unit. Each sample has three replicates. Data of observed optical density (OD600) were statistically analysed by one-way ANOVA followed by Dunnet's test. A *P*-value of ≤0.05 was considered statistically significant.

RESULTS

Table 2 summarizes the MIC values of sigla storax, while Figures 1 and 2 illustrate its effects on Gram-positive and Gram-negative rumen bacteria, respectively. Among the tested species, *R. flavefaciens* exhibited the highest sensitivity, with growth inhibition occurring at 4 mg/ml of sigla storax. *R. albus* showed complete inhibition at 32 mg/ml, but potential antimicrobial effects were observed at 2-16 mg/ml ($P<0.05$). For *B. fibrisolvens*, potential antimicrobial activity was noted from a concentration of 1 mg/ml ($P<0.05$), with complete inhibition at 16 mg/ml. Sigla storax showed potential antimicrobial activity against both *E. ruminantium* and *M. formicicum* at 1-16 mg/ml ($P<0.05$) and complete inhibition at 32 mg/ml. Interestingly, sigla storax stimulated growth of *E. ruminantium* and *S. bovis* at lower concentrations: 0.06-0.125 mg/ml and 0.125-2 mg/ml, respectively ($P<0.05$). *S. bovis* emerged as the most resistant species. While no complete inhibition was observed, potential antimicrobial effects were noted at 8-32 mg/ml ($P<0.05$). Regarding Gram-negative bacteria, sigla storax exhibited a slight stimulatory effect on *M. elsdenii* at 0.06-0.5 mg/ml concentrations ($P<0.05$). Sigla storax showed potential antimicrobial activity against both *M. elsdenii* and *F. succinogenes* starting from 2 mg/ml ($P<0.05$), with complete inhibition at 32 mg/ml.

Table 2. Minimum inhibitory concentration (MIC) values of sigla storax on rumen bacteria

Rumen bacteria	MIC values (mg/ml)
Gram-positives	
R. albus	32
R. flavefaciens	4
B. fibrisolvens	16
E. ruminantium	32
S. bovis	-
M. formicicum	32
Gram-negatives	
F. succinogenes	32
M. elsdenii	32

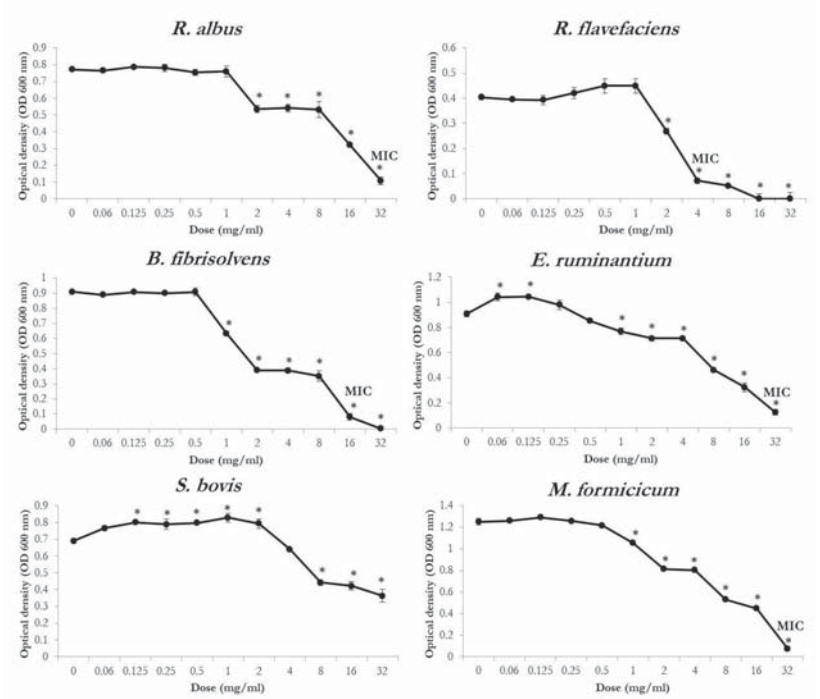

Figure 1. Effects of sigla storax on Gram-positive rumen bacteria by the broth microdilution method. The results represent the mean ± standard error. *P<0.05, difference of sigla storax-treated culture compared with the control. Control level was 0 mg/ml of the storax. MIC: Minimum inhibitory concentration.

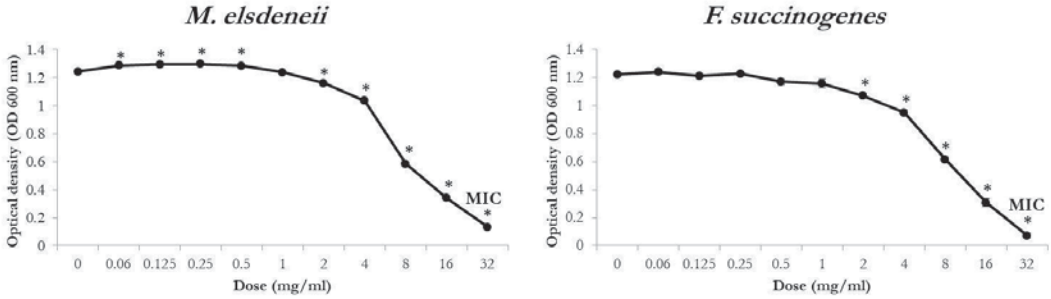

Figure 2. Effects of sigla storax on Gram-negative rumen bacteria by the broth microdilution method. The results represent the mean ± standard error. *P<0.05, difference of sigla storax-treated culture compared with the control. Control level was 0 mg/ml of the storax. MIC: Minimum inhibitory concentration.

DISCUSSION

Phytochemicals have been extensively studied as potential alternatives to antibiotics for modifying rumen microorganisms and fermentation (Kholif and Olafadehan, 2021). The desired effect of these agents is antibacterial activity, particularly against Gram-positive rumen bacteria that produce acetate and butyrate. These bacteria release more hydrogen, which is subsequently utilized in methane synthesis (Demirtas et al., 2018). In this study, sigla storax demonstrated inhibitory effects on representative species of acetate and butyrate-producing bacteria. Specifically, *R. flavefaciens* and *B. fibrisolvens* were inhibited at doses of 4 and 16 mg/ml, respectively. For other members of these groups, namely *R. albus* and *E. ruminantium*, the MIC was 32 mg/ml. A previous study using the same storax (Demirtas et al., 2023) employed an *in vitro* semi-continuous rumen microbial culture system (rumen simulation technique; Rusitec). With 0.5 mg/ml sigla storax supplementation, this study observed a reduction in the abundance of acetate-producing bacteria from the Ruminococcaceae family, including genera *Ruminococcus* 2 and *Ruminococcaceae* UCG-013, and uncultured *Ruminococcaceae* NK4A214 species. Additionally, some butyrate producers such as *Lachnospiraceae* UCG-002 and *Lachnospiraceae* UCG-006 were also suppressed. Notably, the same study reported suppression of the methane-producing genus *Candidatus Methanomethylophilus* and an uncultured genus from Methanobacteriaceae. These findings align with the observed mitigating effect of sigla on methane production. Likewise, in this study, sigla exhibited potential antimicrobial activity at concentrations ranging from 1 to 16 mg/ml, while demonstrating inhibitory effects on the methane-producing *M. formicicum* at 32 mg/ml. The discrepancies in effective sigla dosages across studies may be attributed to variations in bacterial species and the possible transformation of sigla within

the Rusitec's mixed culture environment, potentially yielding derivatives with enhanced antibacterial properties. The antimicrobial impact of sigla storax on methanogenic archaea and certain Gram-positive bacteria has been linked to cinnamic acid esters. Demirtas et al. (2023) identified two primary components in the essential oil composition of storax: *E*-cinnamyl cinnamate (38.8%) and 3-phenylpropanyl cinnamate (38.1%). As secondary plant metabolites, essential oils are renowned for their wide-ranging antimicrobial effects (Seow et al., 2014). In line with this, cinnamic acid derivatives have been reported to possess antibacterial, antiviral, and antifungal properties (Sova, 2012; Tawata et al., 1996).

Interestingly, at low concentrations, sigla storax exhibited a mild growth-promoting effect on certain bacteria. This included Gram-positive species like *E. ruminantium* and *S. bovis*, as well as the Gram-negative *M. elsdenii*. Demirtas et al. (2023) reported that supplementing Rusitec with 0.5 mg/ml sigla increased the abundance of some butyrate-producing Gram-positive bacteria and propionate-producing Gram-negative bacteria. The used storax has been found to have high phenolic content, measuring 79.2 mg/g in catechin equivalents or 57.9 mg/g in gallic acid equivalents (Demirtas et al., 2023). Phenolic substances, including phenolic acids, flavonoids, and tannins, are phytochemicals or secondary plant metabolites that can interact both positively and negatively with microorganisms (Broudiscou et al., 2000). Many polyphenols, such as tannic acid, gallic acid, catechol, and catechin, can be hydrolyzed by rumen bacteria and used as carbon and energy sources for growth (Bhat et al., 1998). Thus, the phenolic compounds in sigla storax might be responsible for stimulating the growth of some of the studied bacteria. However, at higher doses, sigla demonstrated antibacterial activity against both Gram-negative bacteria. This dual effect is reminiscent of saponins, another type of secondary plant metabolite, which have been shown to promote the growth of certain rumen bacteria *in vitro* at low doses while potentially inhibiting bacterial growth at high doses (Patra et al., 2012).

One of the phytochemicals contained in sigla storax used in our study was the essential oil, dominated by *E*-cinnamyl cinnamate and 3-phenylpropanyl cinnamate. Essential oils, due to their hydrophobic nature, have a high affinity for the lipids of bacterial cell membranes. Their antibacterial properties appear to be linked to their lipophilic nature (Benchaar et al., 2008). The interaction with the cell membrane induces conformational alterations, leading to ion leakage across the membrane. This disruption causes a loss of the transmembrane ionic gradient, which results in energy depletion and ultimately leads to microbial cell death (Griffin et al., 1999). This mode of action enhances the effectiveness of essential oils against Gram-positive bacteria, as their cell membranes can directly interact with the hydrophobic components of the oils. In contrast, Gram-negative bacteria have a hydrophilic outer layer around their cell membrane that serves as a barrier to the penetration of hydrophobic compounds (Patterson et al., 2019). However, essential oils with small molecular weight such as thymol and carvacrol can penetrate the outer hydrophilic cell wall of Gram-negative bacteria and act on them (Calsamiglia et al., 2007). It has also been reported that this mechanism reduces the selectivity of essential oils against certain bacterial populations in the rumen (Calsamiglia et al., 2007). The efficacy of sigla storax against Gram-negative as well as Gram-positive bacteria in this study may be attributed to a similar mechanism of action of sigla storax essential oil. Thus, considering the antibacterial activity of sigla storax against Gram-negative bacteria along with stimulatory effect on certain Gram-positive bacteria, the impact of sigla on rumen bacteria appears to be less selective than that of antibiotic feed additives.

CONCLUSION

In this study, sigla storax demonstrated inhibitory effects against Gram-positive rumen bacteria at concentrations ranging from 4 to 32 mg/ml, while Gram-negative bacteria were inhibited at 32 mg/ml. Although Gram-positive bacteria generally showed higher sensitivity to sigla storax compared to Gram-negative bacteria, the findings suggest that its mechanism of action differs from that of typical antibiotic feed additives. This conclusion is based on two key observations: first, sigla storax's antibacterial activity extended to Gram-negative bacteria, and second, it exhibited growth-promoting effects on certain Gram-positive bacteria. These dual actions indicate a more complex interaction with rumen microbiota than traditional antibiotics. To fully elucidate sigla storax's mode of action and its potential for modifying rumen metabolism, further research is needed. Specifically, investigating its effects on a broader range of bacterial species within the rumen ecosystem would provide valuable insights.

DECLARATIONS

Ethics Approval

Not applicable.

Conflict of Interest

None declare.

Consent for Publication

Publication is appropriate.

Author contribution

Idea, concept and design: AD

Data collection and analysis: AD

Drafting of the manuscript: AD

Critical review: AD

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknoledgements

Not applicable.

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Research Article

Brain damage from lung ischemia-reperfusion injury, its potential link to Alzheimer's disease, and the protective role of riociguat

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ABSTRACT

Lung ischemia-reperfusion (LIR) injury can cause widespread systemic and neurological damage, potentially increasing susceptibility to Alzheimer's disease (AD). Riociguat (RIO) has shown potential in reducing damage from ischemia-reperfusion injuries. This study examines whether RIO could help slow AD progression by decreasing brain injury, neuroinflammation, and beta-amyloid (Aβ) accumulation resulting from LIR. In this experiment, forty male Wistar Albino rats were randomly divided into four groups: sham, LIR, LIR+RIO, and RIO. LIR was induced by clamping the hilus for 60 minutes, followed by 60 minutes of reperfusion. RIO was administered 30 minutes before ischemia. Brain tissues were then analyzed through histopathological and immunohistochemical techniques. Histopathology in the LIR group revealed significant hyperemia, degenerative changes, neuronal loss, gliosis, and inflammatory cell infiltration. Immunohistochemical analysis showed elevated levels of Aβ, Caspase-3, and TNF-α. RIO treatment effectively reversed these changes, indicating its potential to reduce brain damage, neuroinflammation, and Aβ accumulation. These findings suggest that lung ischemia-reperfusion injury in this rat model may increase vulnerability to Alzheimer's disease, but that RIO could play a protective role.

INTRODUCTION

In ischemia-reperfusion events, blood flow to organs is temporarily reduced and then restored during reperfusion (Zhang et al., 2024; Kalogeris et al., 2016). Arteriosclerosis, which is often aggravated by chronic conditions such as diabetes and hypertension, as well as by environmental factors like stress and smoking, can lead to endothelial damage and aneurysmal ruptures, making it one of the most significant causes of ischemia (Gusev and Sarapultsev, 2023; Singh et al., 2020). Similar to atherosclerosis, the main contributor to hypoxia-induced damage in distal tissues is the inability to produce nitric oxide, which normally promotes vasodilation. This issue is compounded by lipid accumulation in the subendothelial space and platelet plug formation, which narrows arterial diameter and impairs tissue repair (Badimon et al., 2016; Kubota et al., 2016). Ischemic conditions or other causes that compromise the lung—the primary organ responsible for oxygenation—can lead to similar dysfunctions (Sakar et al., 2017; Ferrari et al., 2015). Research suggests that lung disease, reduced lung function, or poor lung health may be associated with an increased risk of dementia or cognitive decline (Lutsey et al., 2019; Dodd, 2015; Vidal et al., 2013; Rusanen et al., 2013; Pathan et al., 2011; Hozawa et al., 2006).

Prooxidant and proinflammatory molecules formed in hypoxic conditions, such as in lung ischemia-reperfusion (LIR), bind to receptors in distal tissues, triggering cellular damage and activating several intracellular damage pathways (Ferrari et al., 2015; Lahousse et al., 2015). Inflammation and apoptosis are key mechanisms underlying this cellular damage. These prooxidant and proinflammatory molecules can also disseminate through the bloodstream, potentially causing injury to distant organs (Kalogeris et al., 2012). In particular, damage to the blood-brain barrier (BBB) increases its permeability, leading to brain tissue injury and various neurological disorders, depending on the affected regions. Studies indicate that some of these injuries may contribute to the development of neurodegenerative diseases, such as Alzheimer's and Parkinson's (Archie et al., 2021).

In Alzheimer's disease, β-amyloid peptide (Aβ) accumulation, often accompanied by inflammation in specific brain regions, serves as a diagnostic hallmark. Impaired clearance of Aβ due to a compromised BBB and microglial dysfunction from neuroinflammation are contributing factors. According to recent literature, studies focused on reducing inflammation and apoptosis, both of which are critical to AD progression, have also reported reduced Aβ accumulation (Chen et al., 2023).

Riociguat (RIO), a soluble guanylate cyclase (sGC) stimulant,

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is approved for treating chronic thromboembolic pulmonary hypertension and pulmonary arterial hypertension in adults. It promotes vasodilation either independently or by enhancing the effects of nitric oxide (NO) (Mihalek et al., 2022; Lian et al., 2017). A recent study suggests that RIO protects neonatal rats from pulmonary hypertension and hyperoxia-induced lung damage without hindering long bone growth (Donda et al., 2018). Although RIO has limited penetration into the central nervous system, it may still help reduce hypoxia-induced damage through its effects on lung tissue (Frey et al., 2018).

Several pathways, particularly those involving systemic inflammation, oxidative stress, physiological stress, and damage to small vessels from prolonged hypoxemia, are thought to increase the risk of dementia and cognitive impairment (Dodd, 2015; Maclay & MacNee, 2013). However, little is known about how lung ischemia affects the brain, and the mechanisms remain unclear.

This study aims to examine brain pathology resulting from LIR and assess whether RIO, known for its antiproliferative, antihypertensive, antifibrotic, and anti-inflammatory properties, can reduce brain damage and Aβ accumulation associated with LIR.

MATERIALS and METHODS

Animals and ethical approval

All animal experiments in this study adhered to the Animal Research: Reporting in Vivo Experiments (ARRIVE) 2.0 guidelines. The experimental protocol was approved by the Suleyman Demirel University local animal ethics committee on June 6, 2024, under permission number 304. This research was funded by the Scientific Research Projects Coordination Unit of Suleyman Demirel University, with the project code TSG-2023-9010.

Forty male Wistar Albino rats, each weighing between 250 and 350 g, were obtained from the Suleyman Demirel University Experimental Animals Laboratory. Based on relevant parameters (α=0.05, 1-β=0.90, effect size=0.40) and calculated using GPower 3.1.9.7 software, the study was designed with four groups of 8 rats (total n=32). The rats were kept in a controlled environment with 12-hour light-dark cycles, at a constant temperature (21–22 °C) and humidity (55%). They were fed ad libitum and housed in Euro type-2 cages with wood shavings as bedding.

Experimental Procedure

To minimize potential confounding factors, all experimental procedures were conducted in a standardized manner within the same group and sequence. The rats were randomly assigned to four groups, each consisting of eight rats:

Sham Group: The hilus was visualized, and a thoracotomy was performed without inducing ischemia.

LIR Group: Following a left thoracotomy, a nontraumatic vascular clamp was placed on the hilus, and ischemia was induced for 60 minutes, followed by 60 minutes of reperfusion (Abu-Amara et al., 2010).

LIR+RIO Group: This group underwent the same procedure as the LIR group, but 10 mg/kg of Riociguat (RIO) was administered orally 30 minutes prior to the induction of ischemia (Seker et al., 2022).

RIO Group: Rats in this group received only an oral dose of 10 mg/kg RIO.

After a 12-hour fasting period, the thoracic area of each rat was shaved, and a left thoracotomy was performed under intraperitoneal anesthesia using 90 mg/kg Ketamine (Bioveta, Czech Republic) and 10 mg/kg Xylazine (Doğa İlaç, Türkiye). The left lung hilus was identified through visualization of the trachea, and a nontraumatic vascular clamp was applied for 60 minutes (in the LIR and LIR+RIO groups), followed by 60 minutes of reperfusion. Once blood flow was restored, the animals were sacrificed.

Blood was collected from the inferior vena cava through an abdominal incision to perform surgical exsanguination. After sacrifice, brain tissues were carefully extracted and preserved in a 10% formaldehyde solution for subsequent immunohistochemical and histological analyses.

Histopathological analysis

During necropsy, tissues from the brain, cerebellum, and hippocampal regions were carefully collected and preserved in 10% buffered formalin for histological analysis. The tissues were processed using an automated tissue processor according to standard protocols. A rotary microtome was utilized to section the paraffin blocks into 5 µm thick slices.

Following sectioning, the tissue samples underwent deparaffinization and were cleaned with xylene. They were then stained using hematoxylin-eosin (HE) and rehydrated with ethanol. After staining, the sections were mounted with coverslips for examination.

Histological alterations were evaluated under a light microscope. Various brain regions were semiquantitatively assessed for histopathological lesions, including the degree of gliosis, hemorrhage, hyperemia, and neuronal damage. Each type of damage was graded on a severity scale from 0 to 3, as outlined in Table 1 (Unlu et al., 2024).

Table 1. Histopathological scoring criterion for brain.

0	No lesions
1	Lesions in fewer than 20% of the fields
2	Lesions in 20% to 60% of the fields
3	Lesions in every field

Immunohistochemical analysis

For the immunohistochemical analysis, antibodies against beta-amyloid [Beta amyloid Recombinant Antibody [EPR16630] (ab205529)], Caspase-3 [Recombinant Anti-Caspase-3 p12 antibody [EPR16888] (ab179517)], and TNF-α [Anti-TNF alpha recombinant antibody [RM1005] (ab307164)] were utilized, all

sourced from Abcam, Cambridge, UK. The tissue slices were mounted on poly-L-lysine slides for the immunohistochemical staining process, which employed the streptavidin-biotin-peroxidase technique.

Each primary antibody was diluted to 1:100 and applied to the sections, which were then incubated overnight. Following this incubation, sections were stained using biotinylated secondary antibodies and a streptavidin-alkaline phosphatase conjugate. A ready-to-use commercial kit, the EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436) from Abcam, provided the necessary secondary antibody and chromogen. For negative controls, the primary antiserum was replaced with the antibody dilution solution.

Blinded samples were employed to eliminate bias, and each analysis was conducted by a trained histopathologist from a different university. The percentage of cells positively immunostained for each marker was calculated in ten distinct fields on each slide for each group, using an objective magnification of X40. The images were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD, version 1.48), and microphotographs were captured using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Co., Tokyo, Japan).

Statistical Analysis

Statistical analysis of the histopathological scores and the number of immunohistochemically positive cells was performed using GraphPad Prism software. The Shapiro-Wilk test was initially applied to assess the normality of data distribution. Since the data demonstrated a normal distribution ($P > 0.05$), ANOVA was utilized to compare the groups. Pairwise differences between the groups were determined using the post hoc Duncan test. A significance threshold of $P < 0.05$ was established, and results are presented as means ± standard deviation.

RESULTS

No significant macroscopic findings were observed in the brains of any group, except for the LIR group, which exhibited marked hyperemia and edema.

During histopathological evaluation, both the control group and the RIO group displayed normal findings without abnormalities. In contrast, the LIR group showed significant hyperemia, edema, mild degeneration, neuronal death, and slight gliosis within the brain. Additionally, edema and infiltration of inflammatory cells were noted in the meninges of this group. Following treatment with RIO in the RIO+LIR group, notable improvements in these lesions were observed, as depicted in Figure 1.

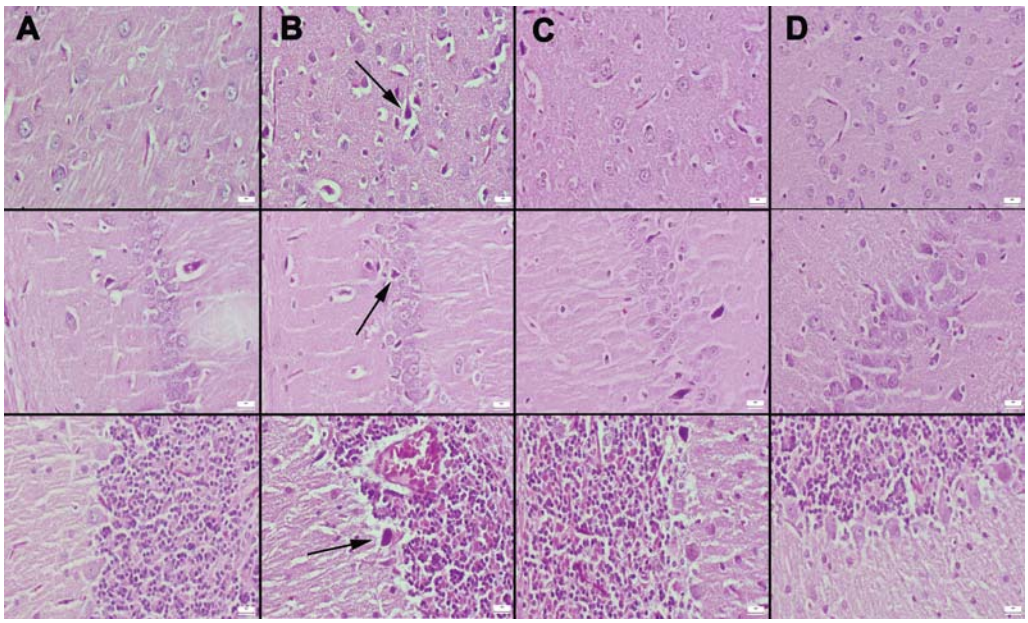

Figure 1. Histopathological differences between groups in the brain (top row), hippocampus (middle row), and cerebellum (bottom row) tissues. (A) Normal tissue histology in the control group, (B) Significant hyperemia and edema (arrows) in the LIR group, (C) Reduced histopathological lesions in the LIR+RIO group, (D) Normal tissue histology in the RIO group. HE staining, scale bars = 20 μm.

The application of LIR resulted in a significant increase in the expression levels of Aβ, Caspase-3 (Cas-3), and TNF-α within the neurons of the central nervous system. Immunohistochemical analysis revealed predominantly negative to very slight expressions of these three proteins in brain, hippocampal, and cerebellar tissues. However, following RIO therapy, the expressions of Aβ, Cas-3, and TNF-α were reduced in the brain, cerebellum, and hippocampal regions of the LIR+RIO group. Minimal to negligible expression of Aβ, Cas-3, and TNF-α was observed in both the RIO and control groups (Figures 2-8). The results of the statistical analysis are also presented in the figures. The possible pathogenetic mechanism of brain damage caused by LIR is illustrated in Figure 9.

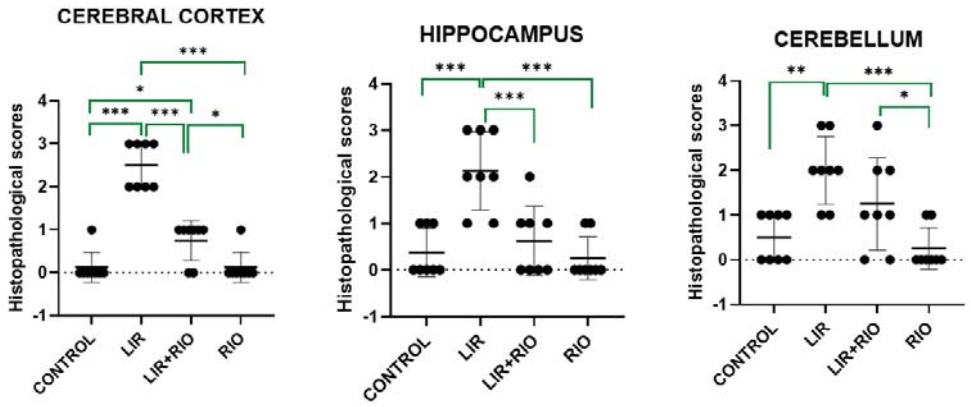

Figure 2. Statistical analysis results of histopathological scores.

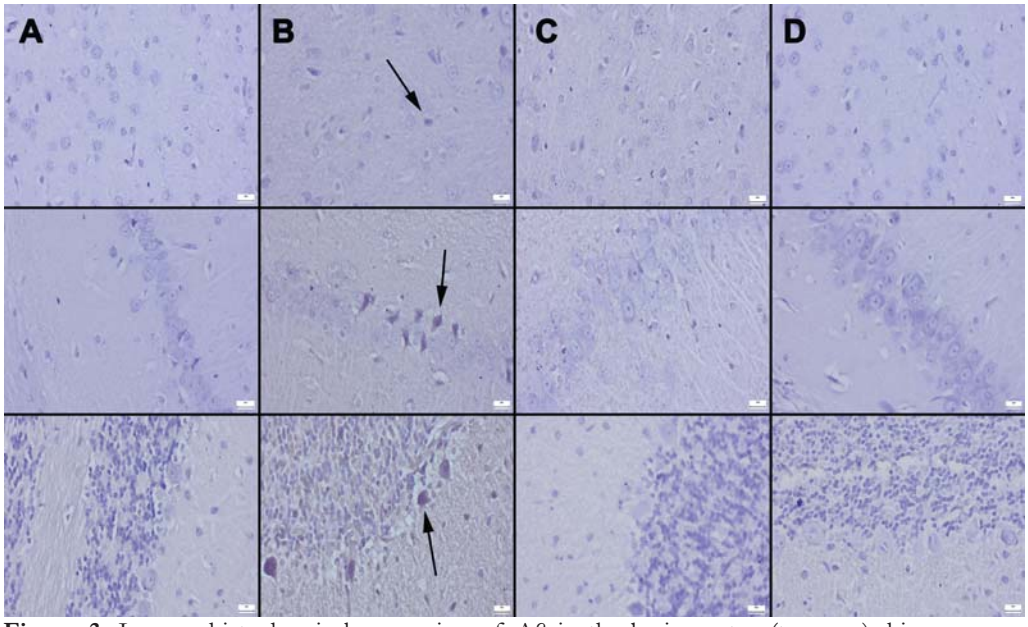

Figure 3. Immunohistochemical expression of Aβ in the brain cortex (top row), hippocampus (middle row), and cerebellum (bottom row) across groups. (A) No expression in the control group, (B) Increased expression (arrows) in the LIR group, (C) Decreased expression in the LIR+RIO group, (D) Negative expression in the RIO group. Streptavidin-biotin peroxidase method, scale bars = 20 μm.

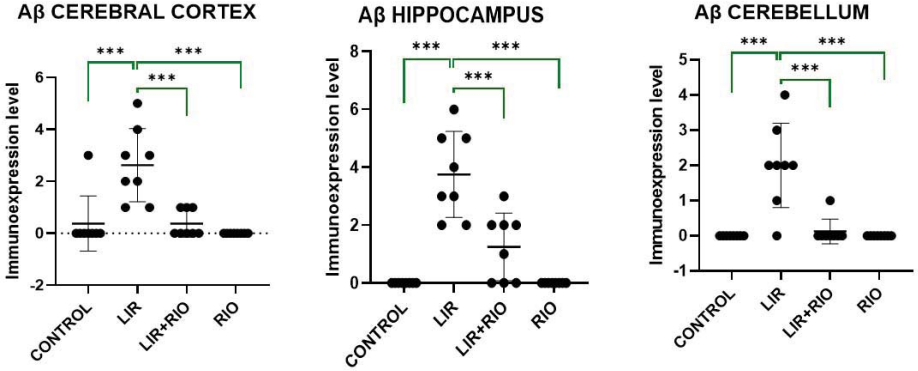

Figure 4. Statistical analysis results of Aβ expression scores

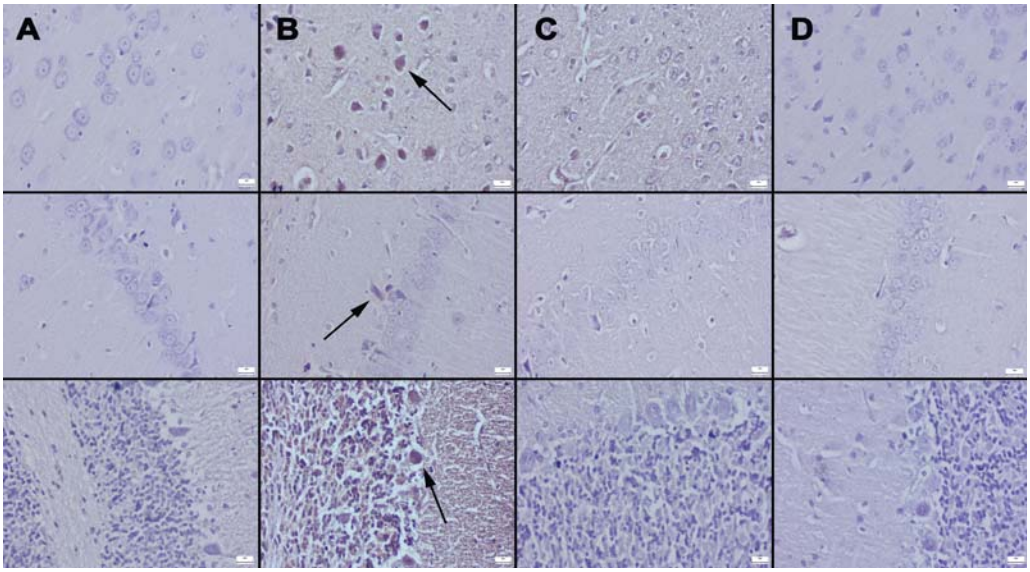

Figure 5. Cas-3 expression in the brain cortex (top row), hippocampus (middle row), and cerebellum (bottom row) across groups.(A) Negative expression in the control group, (B) Increased expression (arrows) in the LIR group, (C) Decreased expression in the LIR+RIO group, (D) Negligible to slight expression in the RIO group. Streptavidin-biotin peroxidase method, scale bars = 20 μm.

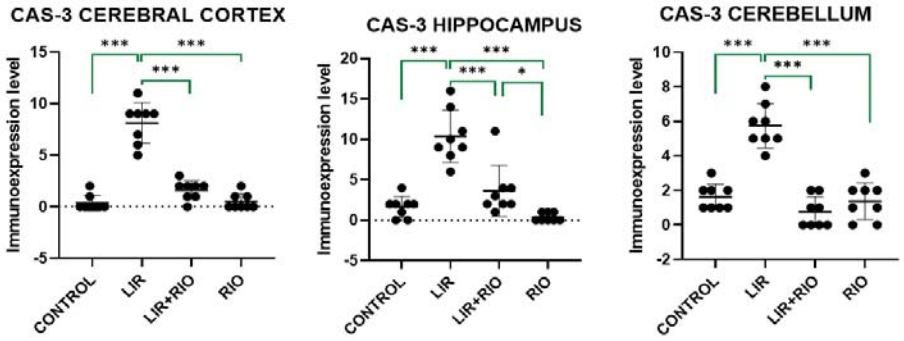

Figure 6. Statistical analysis results of Cas-3 expression scores

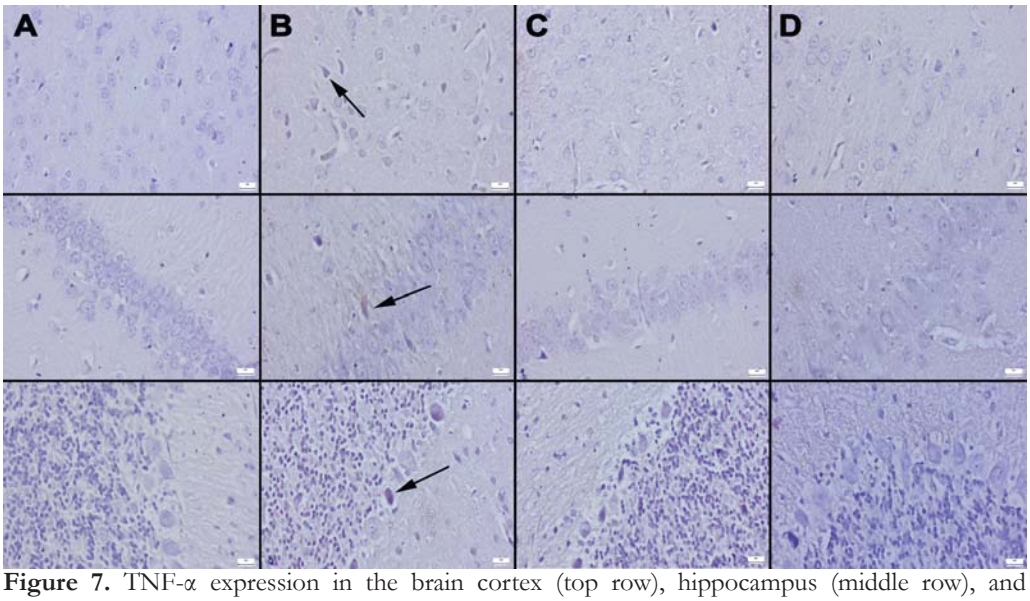

Figure 7. TNF-α expression in the brain cortex (top row), hippocampus (middle row), and cerebellum (bottom row) across groups. (A) No expression in the control group, (B) Increased expression (arrows) in the LIR group, (C) Decreased expression in the LIR+RIO group, (D) Negative expression in the RIO group. Streptavidin-biotin peroxidase method, scale bars = 20 μm.

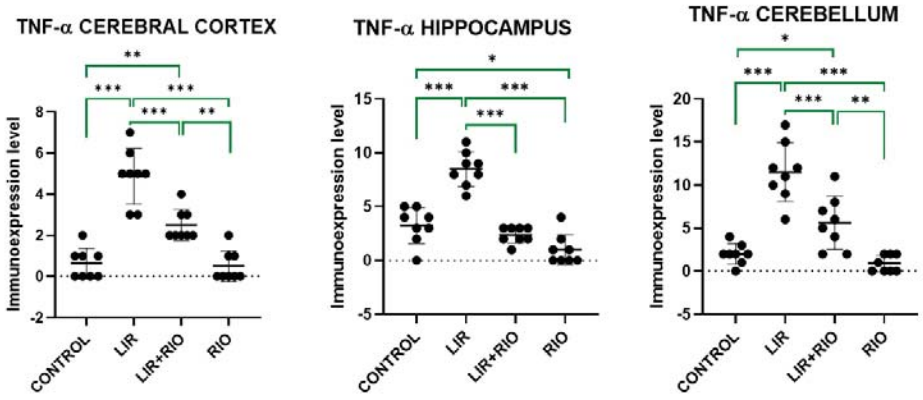

Figure 8. Statistical analysis results of TNF-α expression scores

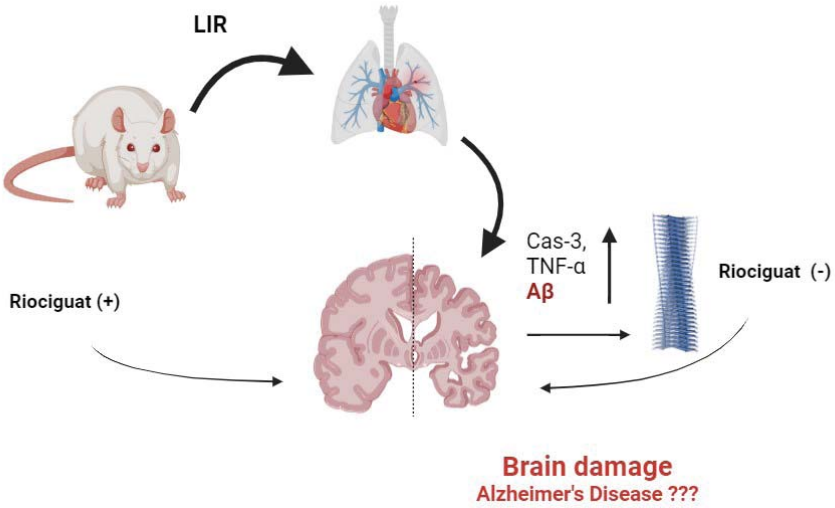

Figure 9. Possible mechanisms of RIO on LIR.

DISCUSSION

Lung ischemia-reperfusion injury is a significant clinical challenge, often leading to severe neurological and systemic side effects, including the exacerbation of neurodegenerative diseases such as Alzheimer's disease (AD). This study highlights the neuroinflammatory response in brain tissue following LIR, which contributes to the accumulation of amyloid beta (Aβ) plaques, a hallmark of AD. The findings suggest that Riociguat (RIO) could serve as a protective agent against the risk and progression of Alzheimer's disease by mitigating brain damage, reducing neuroinflammation, and decreasing Aβ accumulation as a consequence of LIR.

The lungs, being one of the most vascularized organs in the body, can be influenced by pathological events occurring elsewhere. Damage to lung tissue can have detrimental effects on multiple organ systems (Zhang et al., 2024; Mammoto and Mammoto, 2019; Kalogeris et al., 2016). Inflammatory responses triggered by conditions such as interstitial lung injury, trauma, or ischemic damage can lead to the release of inflammatory cytokines into the bloodstream due to increased vascular permeability in the lung. These cytokines can then affect distant organs, exacerbating the injury (Elma et al., 2022; Kalogeris et al., 2012). Notably, the disruption of the blood-brain barrier during inflammation allows these cytokines to reach brain tissue, initiating neuroinflammation and potentially leading to neuronal cell death through apoptotic mechanisms. Literature supports that protecting lung tissue, which plays a crucial role in these processes, could reverse the pathological changes occurring in brain tissue (Sun et al., 2022; Yang et al., 2022).

Histopathological findings from this study reveal marked hyperemia, edema, and gliosis in the cortex, along with edema and inflammatory cell infiltration in the meninges of brain tissues in the pulmonary ischemia group. These observations indicate an inflammatory response, while the documented neuronal cell death suggests apoptosis is also occurring. Furthermore, the presence of similar pathological changes in both hippocampal and cerebellar tissues indicates a more generalized brain injury. The application of RIO, with its potential vasodilatory effects as a soluble guanylate cyclase (sGC) activator, appears to reduce lung tissue damage, leading to a propor-

tional decrease in brain injury due to the reduced systemic release of inflammatory cytokines. This is further supported by the results demonstrating that the LIR+RIO group exhibited less severe pathological changes compared to the LIR group.

The protective effects of Riociguat (RIO) may indeed play a crucial role in preventing balance disorders associated with cerebellar dysfunction and mitigating cognitive impairments related to hippocampal damage. This study highlights the significance of maintaining lung health as a strategy to preserve neurological function and decrease the risk of neurodegenerative diseases following ischemic injuries.

Previous research indicates that Aβ plays a central role in synaptic damage via pathways activated by local caspases (Park et al., 2020). Elevated levels of the pro-inflammatory cytokine TNF-α have been observed in the brains of individuals with Alzheimer's disease. Given that TNF-α-converting enzyme (TACE) releases TNF-α from cell membranes, inhibiting TACE may reduce the deleterious effects of TNF-α in Alzheimer's patients (Kim et al., 2008). Notably, our study found that expressions of TNF-α and Aβ significantly increased 60 minutes post-LIR, suggesting a rapid neuroinflammatory response that could exacerbate neuronal damage.

Alzheimer's disease is characterized by progressive neurodegeneration and inflammation (Archie et al., 2021). Memory impairments resulting from neuronal loss, particularly within the cholinergic system, severely impact patients' daily lives (Park et al., 2020). The increased synthesis of Aβ during neuroinflammatory states—often associated with inadequate microglial responses—can lead to its accumulation in various brain regions, which is critical for diagnosing AD. Studies suggest that the severity of clinical progression in Alzheimer's disease may correlate with the extent of Aβ aggregation (Chen et al., 2023).

In this context, the lack of Aβ accumulation in the RIO-treated groups aligns with the histopathological findings, suggesting that RIO may prevent significant neurodegenerative changes linked to Alzheimer's disease. The reduction of LIR-induced Aβ accumulation in the cerebral cortex, hippocampus, and cerebellum through RIO treatment may thus protect cognitive functions and alleviate neurological and balance disorders associated with ischemic brain injury. Overall, these findings emphasize the potential of RIO as a therapeutic intervention in mitigating neurodegenerative processes triggered by inflammatory responses following lung ischemia-reperfusion injury.

The correlation between TNF-α expressions—an acute phase reactant associated with inflammatory diseases—and the histopathological findings of inflammation underscores the extent of brain damage secondary to LIR. The observation that elevated TNF-α levels across all three examined tissues could be reversed by RIO suggests its potential effectiveness in protecting brain tissue from inflammatory damage. Although RIO is known to have limited permeability through the blood-brain barrier, it may exert protective effects by either utilizing the increased permeability associated with inflammation or by mitigating peripheral lung tissue damage, which subsequently reduces inflammatory signaling to the brain.

The relationship between inflammation and apoptosis is well-documented in physiopathological mechanisms. Inflammatory processes can activate various intracellular pathways leading to apoptosis, while apoptosis does not inherently trigger inflammation due to the preservation of cellular membranes (Zhang et al., 2018). The elevated expression of Caspase-3 observed in our immunostaining results aligns with TNF-α levels and the histopathological evidence of neuronal cell death. Notably, one of the most significant findings of this study is that RIO treatment downregulates Cas-3 expression across all examined tissues, indicating that neuronal protection may prevent the neuronal death associated with the pathogenesis of Alzheimer's disease.

Furthermore, it has been documented that anti-inflammatory mechanisms are activated during the clearance of apoptotic cells, which may contribute to a reduction in inflammation (Szondy et al., 2017; Wan et al., 2013). Our study demonstrated that, in a rat model, brain expressions of Aβ, Cas-3, and TNF-α significantly increased 60 minutes following lung ischemia and subsequent reperfusion. Given the challenges associated with examining brain tissue during this critical period in human patients—who are often under anesthesia—animal models serve as a valuable alternative to provide insights into these processes.

However, this study has notable limitations. Being a preliminary investigation, it did not allow for the examination of molecular changes in the brain during the ischemic and reperfusion phases. Future studies should aim to assess the molecular alterations occurring in the brain during this time frame and investigate whether these changes are reversible. Such research would enhance our understanding of the protective mechanisms of RIO and its potential therapeutic applications in the context of neurodegenerative diseases following ischemic events.

CONCLUSION

As a result, expressions of TNF-α and Caspase-3, which are indicators of damage in brain tissue, along with Aβ expressions that are crucial to the onset of Alzheimer's disease, were found to be elevated in the cerebral cortex, hippocampus, and cerebellum following lung ischemia-reperfusion (LIR) injury. Treatment with Riociguat (RIO) reduced both histopathological and immunohistochemical findings, thereby preserving brain tissue. These results highlight the need for further studies incorporating more detailed molecular investigations.

DECLARATIONS

Ethics Approval

The experimental protocol received approval from the local animal experiments ethics committee of Suleyman Demirel University date 06.06.2024 and approval number 304.

Conflict of Interest

The authors have no conflict of interest with any person, institution or organization.

Consent for Publication

Publication is appropriate

Author contribution

Idea, concept and design: OO, HA

Data collection and analysis: OO, AM, HA

Drafting of the manuscript: OO, HA

Critical review: OO, AM, HA

Data Availability

The data is available from the corresponding author on reasonable request.

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Research Article

Comparative analysis of the effects of monochromatic LED light on jejunum morphology in Japanese quails

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ABSTRACT

It is known that environmental influences such as different light sources have effects on the digestive systems of avians. This study examined the effects of different light sources (blue, white, and green) on the jejunal structure of Japanese quails. Twenty-one male quails were exposed to LED lights emitting specific wavelengths from hatching until they reached 42 days of age. Histological analyses of jejunal tissues focused on villus length, crypt depth, and the villus length-to-crypt depth ratio (VL/CD index). The results revealed that green light exposure had the longest villi, while blue light exposure had in the shallowest crypts. Furthermore, these results showed that the VL/CD index was significantly higher under green light compared to blue and white light. These findings indicate that manipulating the light spectrum could enhance intestinal health and improve nutrient absorption efficiency in quail, offering potential benefits for optimising poultry production.

INTRODUCTION

The quail (*Coturnix coturnix*) is a migratory poultry species widely distributed across Eurasia and Africa. Its distinct flavour, rapid reproductive capacity, and potential for quick financial returns have led to the development of new hybrid breeds through selective breeding initiatives. Quail meat and eggs have broad market appeal; however, managing quail presents several challenges. These include management issues in large-scale or intensive production systems, as well as factors such as lighting requirements, housing density, and other welfare considerations (El-Sabry et al., 2022).

In recent years, the demand for poultry products, including chicken meat and eggs, has increased significantly. Chicken meat production, currently at 117 million metric tonnes, is projected to reach 132 million metric tonnes by 2026 (Van Boeckel et al., 2015). This growing demand has driven technological advancements aimed at increasing output, including a transition from traditional lighting sources to LEDs. According to Xie et al. (2008), this transition can substantially reduce energy consumption, enhance animal well-being, improve growth efficiency, and reduce stress. Poultry possess highly developed visual systems and exhibit strong light sensitivity (Bian et al., 2019). Light, as a key environmental stimulus, can disrupt the circadian rhythm of chickens, affecting endocrine pathways, oxidative stress levels, and metabolic activities (Yang et al., 2020). Research also highlights the influence of coloured light sources on chick growth and development. For example, green light exposure may accelerate cell division and T lymphocyte growth, while blue light promotes small intestine development and the maintenance of its protective lining (Liu et al., 2010; Guo et al., 2017; Zhang et al., 2022). However, studies have shown that chicks raised under red light exhibit poor growth performance, higher oxidative stress levels, and lower growth hormone levels (Li et al., 2015).

The relationship between intestinal health and the length of villi and crypt depth is well established (Pluske, 1996). An increased villus height-to-crypt depth ratio may reduce maintenance requirements and improve growth efficiency by decreasing intestinal mucosa turnover (Van Nevel et al., 2005).

Recent research highlights strong correlations between gut microbiota and various physiological factors, including metabolite balance (Xie et al., 2008), immune system health (Yang et al., 2020), growth and development processes (Yeoman et al., 2012), and overall well-being (Zhang et al., 2022). However, data on the effects of monochromatic light on jejunal microbiota composition and function are limited. Most studies investigating the influence of light on gut microbiota focus on chicks, specifically their caeca (Zhang et al., 2022).

In line with the increasing demands of the poultry industry,

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this study aimed to investigate the effects of blue, white, and green light on the jejunum, one of the crucial part of the digestive system, in Japanese quails.

MATERIALS and METHODS

Animals and Experimental Design

This study examined the jejunum of 21 male Japanese quails, which were exposed to LED light for a period of 42 days from hatching. At the end of the experiment, the quails were 42 days old, with an average weight of 211.3 ± 29.97 g (range: 177.4–266.0 g). The birds were divided into three groups of seven and housed under LED lights emitting wavelengths of 480 nm (blue), 400–770 nm (white), and 560 nm (green) throughout the entire experimental period. The research was approved by the Aydın Adnan Menderes University Animal Experiments Local Ethics Committee (No. 645583101/2023/37).

Histological Procedures

Jejunal tissues were fixed in 10% formaldehyde solution. Following fixation, the tissues were processed by embedding them in paraffin wax after treatment with a series of alcohol solutions of increasing concentrations (70%, 80%, 90%, 96%, and 100%) and successive applications of xylene. Thin sections (4–5 µm) were cut using a microtome (Leica RM 2135), mounted on slides, and stained with haematoxylin and eosin (HE) following Luna's method (1968). The slides were examined under an Olympus BX51 light microscope.

Measurements

Villus length: Measured from the base to the tip (Figure 1). Measurements were taken from well-oriented, intact villi to ensure accuracy.

Villus width: Measured at the midpoint (Figure 1). Only fully visible and undamaged villi were included.

Crypt depth: Measured parallel to the villus length (Figure 1). Crypts were selected based on clear structural visibility and intact epithelium.

VU/CD: Calculated by dividing villus length by crypt depth.

Statistical Analysis

Statistical analyses were performed using SPSS 22.0 (Inc., Chicago, IL, USA). First, the normal distribution of the data was checked with the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was employed to compare jejunal data between light groups. In the case of non-parametric data, the Kruskal-Wallis test was applied. The homogeneity of variances was checked with the Levene test. Since the variances were homogeneous, Oneway-ANOVA test results and posthoc-LSD test results were used in all comparisons.

RESULTS

Image analysis revealed that villus length and width was greatest in quails exposed to green light. Crypt depth was shallowest in quails exposed to blue light (Figure 2). The VC/CD index was highest under green light compared to white and blue light, with statistically significant differences ($p = 0.031$) (Figure 3 and Table 1).

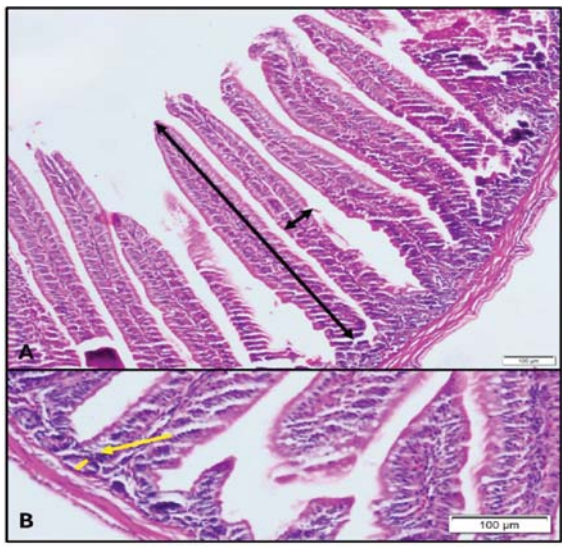

Figure 1. Presentation of measurement locations. Big black two-pointed arrow; villus length, small black two-pointed arrow; villus width (A), yellow line; on the leading edge of the yellow arrow crypt depth (B).

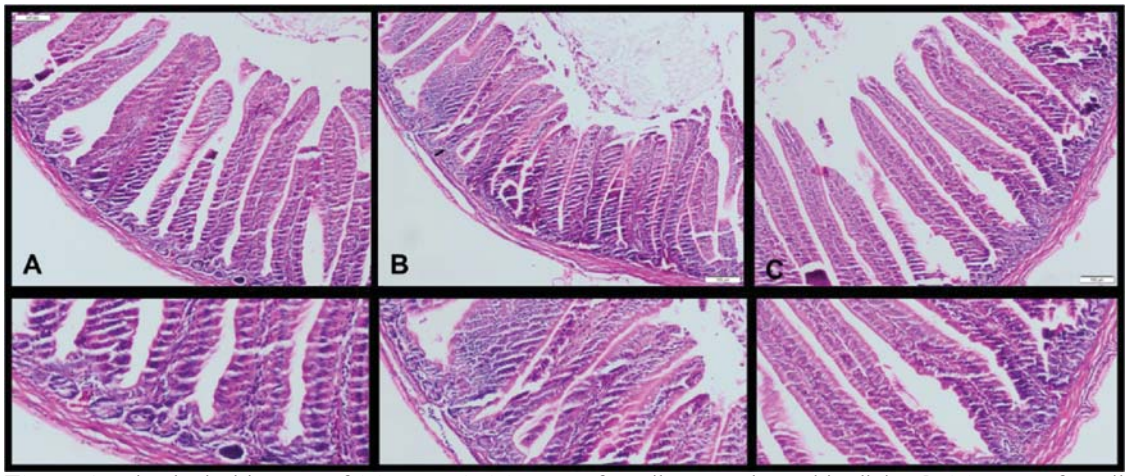

Figure 2. Histological images of jejunum. A; jejunum of quail exposed to white light, B; jejunum of quail exposed to blue light, C; jejunum of quail exposed to green light.

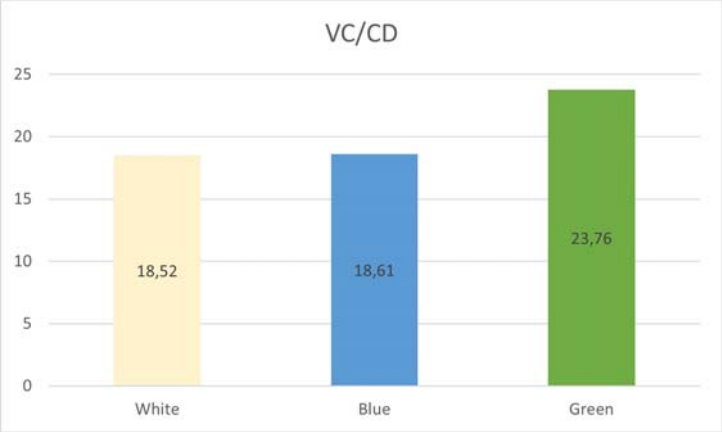

Figure 3. Graphical representation of villus length / crypt depth.

Table 1. Statistical the comparison of morphometric data for the jejunum was performed. The mean ± standard deviation values for villus length, crypt depth, villus width, and villus length/crypt depth (VL/CD) index were obtained from quails exposed to white, blue, and green light, along with their 95% confidence intervals.

	White *(n=7)* Mean±Std (95% CI	Blue *(n=7)* Mean±Std (95% CI)	Green *(n=7)* Mean±Std (95% CI)	P
Villus length (µm)	491.26±160.33 (402.13-592.44)	430.19±99.71 (376.37-48685)	541.47±138.01 (460.23-619.65)	0,171
Crypt depth (µm)	26.29±4.45 (24.02-29.09)	23.03±2.55 (21.69-24.54)	23.09±3.19 (21.30-24.87)	0,061
Villus width (µm)	92.60±15.06 (83.85-100.99)	89.80±12.41 (83.16-96.87)	102.52±14.88 (94.62-112.43)	0,103
VL/CD	18.52±4.31 [a] (15.88-20.98)	18.61±3.70 [a] (16.58-20.80)	23.76±6.62 [b] (19.82-27.92)	0,031

DISCUSSION

In terms of intestinal health, the thickness and composition of the mucus layer vary along the intestine, playing a crucial role in protecting the intestines from mechanical, enzymatic, and chemical factors, as well as facilitating lubrication (Sharma and Schumacher, 1995). Long villi are generally associated with optimal intestinal health, high absorption efficiency, and a healthier intestinal system in poultry (Alfaro et al., 2007). The villus height-to-crypt depth ratio (VH:CD) reflects the superior digestion and absorption capacity of the avian small intestine, owing to its larger absorptive surface area, which enhances nutrient uptake (Van Nevel et al., 2005).

The histological findings of our study are both consistent with and, in certain respects, divergent from existing literature. For example, studies by Liu et al. (2010) and Guo et al. (2017) demonstrated that green light enhances mitotic activity and T lymphocyte proliferation in chickens. Our study also supports the beneficial effects of green light on intestinal health. Additionally, Zhang et al. (2022) reported that green light promotes intestinal development and mucosal integrity. In line with these findings, we observed that green light increased villus length and reduced crypt depth.

However, studies on the effects of blue light have produced conflicting results. For instance, research by Yang et al. (2020) and Zhang et al. (2022) suggested that blue light may have beneficial effects on the intestinal microbiota and metabolic functions. In contrast, our findings showed that blue light did not increase villus length or reduce crypt depth; instead, it decreased the villus length-to-crypt depth ratio. These results suggest that the effects of blue light on intestinal health may vary between species or depend on specific experimental conditions.

Reviewing other studies, Simsek et al. (2020) reported that green light increased villus length, while blue light resulted in shorter crypt depth in quails. Similarly, Xie et al. (2011) observed increased villus height in chickens exposed to green and blue light compared to the white light group. They noted that chicks raised under 560 nm green light exhibited increased mitotic activity in crypt cells and higher proliferation activity in T lymphocytes, with these beneficial effects being most pronounced under 480 nm blue light in later stages (Zhang et al., 2022).

The results of our study offer valuable insights for optimizing lighting strategies in poultry production. Specifically, the use of green light may enhance growth performance and overall health by supporting intestinal health. These findings align with Pluske's (1996) research on intestinal health and growth

efficiency. A higher villus length-to-crypt depth ratio results in a slower turnover of the intestinal mucosa, leading to greater growth efficiency (Van Nevel et al., 2005).

This study examined the effects of different coloured light sources on the jejunal structure of Japanese quail. The findings highlight the significant impact of light colour on intestinal health and microscopic structure. Specifically, green light may find to increase villus length and decrease crypt depth, suggesting potential positive effects on quail intestinal health. Additionally, blue light resulted in the shortest crypt depth and the lowest villus length-to-crypt depth ratio. These results demonstrate the distinct effects of various light colours on intestinal morphology.

This study is limited to the effects of specific LED wavelengths (blue, white, and green), excluding the potential influences of other light spectra. Furthermore, the experimental duration was restricted to 42 days, preventing an assessment of long-term effects.

CONCLUSION

This study demonstrates the significant effects of monochromatic light on the jejunal structure of Japanese quails. Green light positively influences intestinal health, providing insights for optimising lighting strategies in poultry production. Future research should explore the long-term effects of various light spectra and intensities on intestinal health to achieve optimal welfare and performance.

DECLARATIONS

Ethics Approval

The study was conducted with the decision and permission of Aydın Adnan Menderes University Animal Experiments Local Ethics Committee dated 09.03.2023 and numbered 64583101/2023/37.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for Publication

Not applicable.

Author contributions

Idea, concept and design: FTY

Data collection and analysis: FTY, UC, ANA

Drafting of the manuscript: FTY, UC

Critical review: FTY, ANA

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Research Article

Morphometric study of the intraorbital muscles (musculi bulbi) in chinchilla (chinchilla lanigera)

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ABSTRACT

The intraorbital muscles (musculi bulbi) are critical for the movement and stabilization of the eyeball, enabling essential ocular functions such as rotation, convergence, and visual tracking. While much is known about the ocular musculature of larger mammals, little research has been conducted on the musculi bulbi in small rodents, particularly the chinchilla (Chinchilla lanigera), a species with specialized nocturnal adaptations. This study presents a detailed morphometric analysis of the intraorbital muscles in adult chinchillas, examining the size, shape, and structural variations of these muscles. Using dissection and digital caliper measurements, we analyzed 12 chinchillas (6 males and 6 females), focusing on muscle length, thickness, width, and attachment points. Key findings include the identification of an L-shaped curve in the m. obliquus dorsalis and a tendinous termination at its end, as well as the observation that the m. obliquus dorsalis is the longest muscle in females. These results highlight both common and unique features of ocular muscle structure, including the straight-line attachment of the rectus muscles and the oblique alignment of the m. obliquus ventralis. The study also reveals potential sex-based differences in ocular muscle morphology, which may have implications for eye movement and visual acuity. This work contributes to the understanding of the functional adaptations of ocular musculature in rodents, offering a comparative basis for further studies on the evolution and biomechanics of eye movement in mammals.

INTRODUCTION

The intraorbital muscles, or musculi bulbi, are a group of specialized skeletal muscles responsible for the movement and stabilization of the eyeball within the orbit. These muscles play a crucial role in various ocular functions, including eye rotation, convergence, and stabilization during visual tracking, which are vital for optimal visual performance and spatial orientation. While the anatomy and functional significance of these muscles have been well-documented in larger mammals, there is a notable lack of comprehensive studies on the musculi bulbi in small rodents, particularly species like the chinchilla (*Chinchilla lanigera*), which have unique adaptations for nocturnal and crepuscular lifestyles (Chesney & Nogueira, 1999; Nowak, 1999).

Chinchillas, native to the Andes Mountains, possess large, highly specialized eyes that are well-adapted to their low-light habitats, allowing them to detect predators and navigate their environment at dusk or dawn. These adaptations necessitate precise eye movements and control, a function facilitated by the intraorbital muscles (Koenig, 2003). However, despite the significance of ocular muscles in maintaining visual function, little is known about the morphometry and detailed structure of the musculi bulbi in *Chinchilla lanigera*. Existing studies on ocular musculature in rodents generally focus on species like rats and mice (Karten & Hodos, 1967; Haring et al., 2007), leaving a gap in our understanding of how these muscles may vary in other rodent species, especially those with unique visual systems. In the literature, the morphometry of intraorbital muscles in rabbits has been analyzed; however, no morphometric studies on chinchillas have been reported (Gultiken et al., 2006).

This study aims to conduct a detailed morphometric analysis of the intraorbital muscles in *Chinchilla lanigera*, focusing on their size, shape, and variations in structure. Through a combination of qualitative and quantitative methods, we seek to characterize these muscles' anatomical features and examine potential adaptations related to the chinchilla's specialized visual ecology. In addition to contributing to the existing literature on ocular anatomy in small mammals, the findings of this study will offer valuable insights into the evolutionary adaptations of the chinchilla's visual system, as well as provide a comparative basis for understanding eye movements and motor coordination in other rodent species. Ultimately, this research aims to expand the knowledge base on the functional and structural diversity of intraorbital musculature across mammals, shedding light on the intricate relationship between anatomy and behavior in visually adapted species.

MATERIALS and METHODS

In this study, the right and left eyes of 6 adult female and 6 adult male chinchillas were used. The eyes carefully were removed from the orbit along with the muscles. After the ocular muscles were made prominent through dissection, the length, thickness, width, and the distances from the sclera attachment points to the limbus cornea of all muscles, except for the m. retractor bulbi, were measured. The measurements were made

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Table 1. Morphometric measurements (mm, mean ± SD) of intraorbital muscles in the chinchilla.

	Female Right	Female Left	Male Right	Male Left	Total
M. rectus dorsalis length	13,9733±0,33584	14,3517±1,03225	14,5700±1,02927	13,6733±0,42688	14,1421±0,80626
M. rectus dorsalis width	3,7867±0,16801	4,0183±0,27682	3,6933±0,23500	3,7200±0,28580	3,8046±0,24819
M. rectus dorsalis thickness	0,50000±0,073485	0,65667±0,255786	0,64833±0,034881	0,55400±0,038262	0,58975±0,143101
M. rectus dorsalis distance	2,2717±0,35969	2,1650 ±0,44711	2,0483±0,14838	2,2817±0,34114	2,1917±0,33316
M. rectus ventralis length	12,9150±0,71436	14,3517±0,52500	14,2267±0,53504	12,7550±1,02150	13,2858±1,05250
M. rectus ventralis width	3,0083±0,38902	2,9283±0,35897	3,3783±0,33385	3,1750±0,09670	3,1225±0,34381
M. rectus ventralis thickness	0,9067± 0,30111	0,9617±0,18681	0,8317±0,13790	0,8683±0,09109	0,8921±0,18882
M. rectus ventralis distance	2,1117±0,82715	1,7633±0,32934	1,7650±0,14625	1,8167± 0,56740	1,8642±0,51840
M. rectus lateralis length	9,4433± 1,12313	9,4433±1,12313	9,3900±0,83876	9,8300±0,44860	9,5267±0,88183
M. rectus lateralis width	2,7650±0,27682	2,7650 ±0,27682	2,4983±0,24750	2,6950 ±0,49136	2,6808± 0,33400
M. rectus lateralis thickness	0,9083 ±0,10167	0,9083±0,10167	0,64833±0,15471	0,9683±0,14511	0,9108±0,12594
M. rectus lateralis distance	4,8017±0,41053	4,8017±0,41053	4,4533±0,44194	4,2467±0,50808	4,5758±0,48025
M. rectus medialis length	9,6100±0,53669	9,6100±0,53669	8,8250±0,53504	10,1850±0,74363	9,5575±0,74284
M. rectus medialis width	3,0950±0,33804	3,0950±0,33804	2,5783±0,25309	2,8367±0,47260	2,9013±0,40005
M. rectus medialis thickness	0,6433±0,12469	0,6433±0,12469	0,5517± 0,09988	0,5900±0,16432	0,6071±0,12791
M. rectus medialis distance	5,3150±0,62478	5,3150±0,62478	4,5183±0,58663	4,2483±0,41431	4,8492±0,71958
M. obliguus dorsalis length	15,2983±1,33309	15,2983±1,33309	13,5667±1,28813	13,4233±0,37195	14,3967 ±1,41933
M. obliguus dorsalis width	2,5433±0, ,24196	2,5433±0, ,24196	2,4467±0,42917	2,3100± 0,20100	2,4608± 0,28951
M. obliguus dorsalis thickness	0,5517±0,11089	0,5517±0,11089	0,6083±0,04792	0,5383±0,04262	0,5625±0,08368
M. obliguus dorsalis distance	2,5033± 0,53921	2,5033± 0,53921	2,4500±0,33184	2,8933± 0,99875	2,5875±0,63264
M. obliguus ventralis length	11,2450±0,94847	11,2450±0,94847	11,7267± 0,58483	11,0950±1,07964	11,3279± 0,88210
M. obliguus ventralis width	2,8650±0,42241	2,8650±0,42241	2,9433±0,46603	3,2367±0,28147	2,9775±0,40798
M. obliguus ventralis thickness	0,4717±0,12703	0,4717±0,12703	0,5833±0,17694	0,4283±0,07521	0,4888±0,13598
M. obliguus ventralis distance	2,5083±0,74352	2,5083±0,74352	1,5950±0,38868	2,2133±0,47848	2,2063±0,68415
M. retractor bulbi cranialis daimeter	1,7133±0,42547	2,1833±0,22580	1,8167±0,14404	2,2117±0,35975	1,9813±0,36521
M. retractor bulbi caudalis daimeter	7,4200±1,13275	7,8717±1,17598	8,6350±0,31002	8,1217±0,42673	8,0121±0,91707
M. retractor bulbi distance	6,9733±0,39338	7,4967± 0,35280	7,6433±0,62292	7,6217±0,55127	7,4338±0,53677

Figure 1. Ventro-lateral view of the right eye. a. musculus obliquus ventralis, b. musculus rectus ventralis, c. musculus rectus lateralis, d. musculus retractor bulbi.

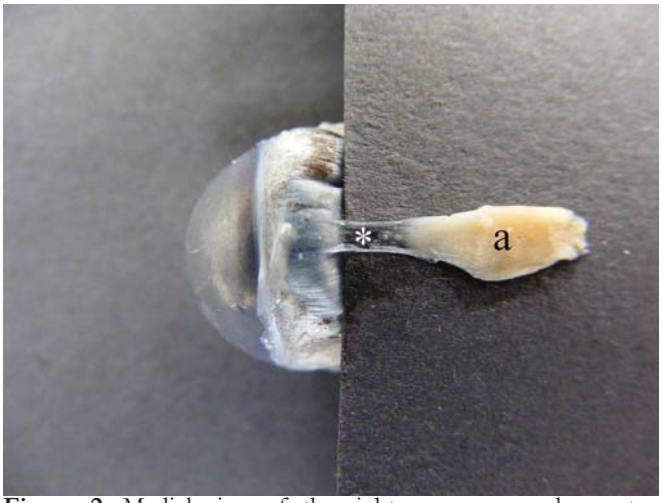

Figure 2. Medial view of the right eye. a. musculus rectus medialis, white star. Attachment of the muscle to the sclera with a tendinous character.

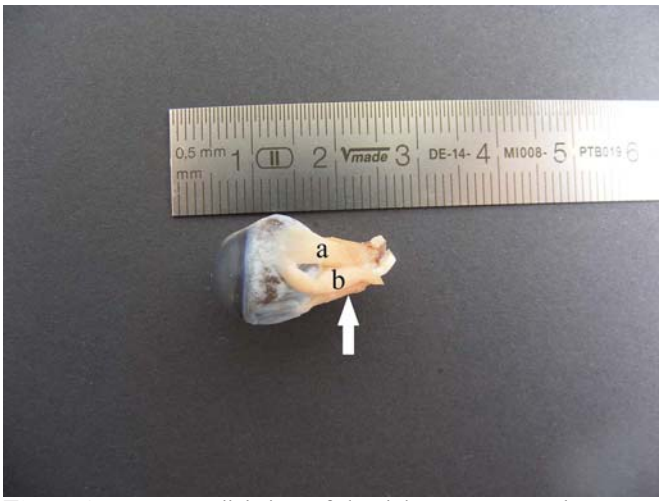

Figure 3. Dorso-medial view of the right eye. a. musculus rectus dorsalis, b. musculus obliquus dorsalis, white arrow. musculus rectus medialis.

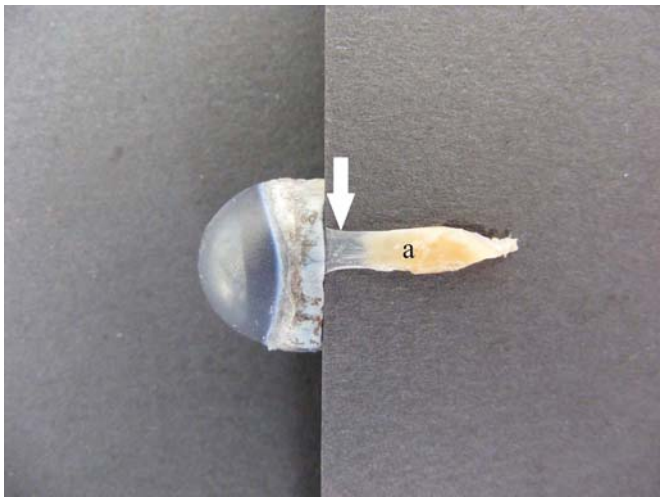

Figure 4. Lateral view of the right eye. a. musculus rectus lateralis, white arrow. Attachment of the muscle to the sclera with a tendinous character.

using a Mitutoyo Digital Caliper. Statistical analyses were performed using the SPSS 19.0 program. The dissected eyes were photographed using a Fujifilm Finepix S5700.

RESULTS

The m. rectus dorsalis was observed to attach to the sclera in a straight line just above the m. obliquus dorsalis, consistent with typical muscular descriptions. It was determined that the m. obliquus dorsalis extends cranially between the m. rectus medialis and the m. rectus dorsalis. Just in front of the termination line of the m. retractor bulbi, a L-shaped curve was observed extending toward the m. rectus dorsalis, and it was found to terminate ventrally beneath the m. rectus dorsalis. The terminal end of the m. obliquus dorsalis was found to be tendinous in nature.

It was determined that the m. rectus medialis and m. rectus lateralis attach to the sclera in a straight line with a tendinous character. The m. rectus ventralis was observed to terminate on the sclera as muscular describes.

The m. obliquus ventralis was seen to travel over the terminal end of the m. rectus ventralis toward the m. rectus lateralis. It was observed that the terminal portions of the m. rectus ventralis and m. rectus lateralis terminate on the sclera as muscular describes. The connection line of the m. obliquus ventralis to the sclera was found to be oblique relative to the limbus cornea.

It was observed that the m. obliquus dorsalis has the longest structure among the ocular muscles in females.

DISCUSSION

The present morphometric study of the intraorbital muscles (musculi bulbi) in *Chinchilla lanigera* provides new insights into the anatomical arrangement and positions of the ocular muscles in this species. The findings confirm several features described in other mammals, while also highlighting some species-specific differences, especially with regard to the structure and attachment of the extraocular muscles.

The *m. rectus dorsalis* was observed to attach to the sclera in a straight line just above the *m. obliquus dorsalis*, which is consistent with typical descriptions of ocular muscle attachment patterns in other rodents and mammals (Gacek, 1985; Aoki et al., 2011). This muscle's arrangement aligns with its role in controlling vertical eye movements, confirming its expected orientation and attachment. Additionally, the *m. obliquus dorsalis* was found to extend cranially between the *m. rectus medialis* and *m. rectus dorsalis*, which corresponds to its usual anatomical positioning in other species (Meyer, 1983).

One noteworthy observation was the presence of a distinct L-shaped curve just in front of the termination line of the *m. retractor bulbi*, which extends toward the *m. rectus dorsalis* and terminates ventrally beneath it. This finding is significant as it highlights a unique morphological feature that may play a role in the stabilization or functional integration of the extraocular muscles, potentially contributing to the precise control of eye movement (Cushing & Haring, 1984). The tendinous nature of the terminal portion of the *m. obliquus dorsalis* is another notable finding, as tendinous terminations are less commonly observed in ocular muscles, which generally end in more muscular attachments (Hering, 2010). This tendinous termination could confer additional stability to the muscle attachment, potentially aiding in the fine-tuning of eye movements.

The *m. rectus medialis* and *m. rectus lateralis* were found to attach to the sclera along a straight line with tendinous character, a configuration that aligns with their typical roles in mediating horizontal eye movements (Leigh & Zee, 2006). The straight-line attachment of these muscles to the sclera has been described in various species (Berman et al., 2012), and our findings reinforce this general pattern of ocular muscle attachment.

The *m. rectus ventralis* exhibited a terminal attachment to the sclera as described in previous anatomical studies, where it contributes primarily to downward eye movement (Jampel,

1993). Similarly, the *m. obliquus ventralis* was observed to travel over the terminal end of the *m. rectus ventralis* toward the *m. rectus lateralis*, with its attachment line to the sclera being oblique relative to the limbus cornea. This oblique attachment of the *m. obliquus ventralis* is consistent with its role in controlling rotational and vertical eye movements, as previously noted by Cummings (2005).

A particularly striking result from this study was the observation that the *m. obliquus dorsalis* was the longest ocular muscle in female *Chinchilla lanigera*. This finding suggests a potential sex-based morphological difference that could be associated with functional variations in eye movement. Such sexual dimorphism in ocular muscle structure has been observed in other species, where it is thought to relate to differences in visual acuity, behavioral roles, or the biomechanics of eye movements (Kikuchi et al., 2012). The longer *m. obliquus dorsalis* in females could potentially reflect a greater need for complex or precise vertical eye movements, although further studies are required to understand the functional implications of this difference.

CONCLUSION

The morphometric analysis of the intraorbital muscles in *Chinchilla lanigera* has provided a detailed description of the attachment and structure of these muscles, reinforcing some established anatomical features while also uncovering novel observations, particularly in the arrangement of the *m. obliquus dorsalis*. These findings offer a foundation for future studies on ocular motor control in this species and provide comparative insights into the evolutionary and functional adaptations of the ocular musculature in mammals.

DECLARATIONS

Ethics Approval

The experiments were conducted in strict compliance with the ethical guidelines of Burdur Mehmet Akif Ersoy University (protocol 18.0.2019-536).

Conflict of Interest

The authors declare that there have no conflict of interests.

Consent for Publication

No applicable.

Author Contributions

Idea, concept and design: ÖG, EK.

Data Collection and analysis: ÖG, EK.

Drafting of the manuscript: ÖGD.

Critical review: ÖGD, EK.

Data Availability

No applicable.

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Research Article

Influence of fetal sex and litter size on pregnancy-associated glycoprotein concentration at the end of the embryonic period in Hasak ewes

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ABSTRACT

Reproductive efficiency is crucial for the economic profitability of sheep flocks and is strongly influenced by effective reproductive management. Accurate pregnancy diagnosis and fetal counts help breeders make informed decisions regarding the nutrition and care of pregnant ewes. This study aimed to determine whether plasma concentrations of pregnancy-associated glycoproteins (PAG) on day 35 of gestation can predict the number and sex of fetuses in Hasak ewes. Seventy-five pregnant Hasak ewes (2-7 years old), maintained under uniform conditions, were randomly selected. Pregnancy and the number of fetuses were determined on day 35 post-mating using transrectal ultrasound with a 7.5 MHz linear probe. Immediately after pregnancy diagnosis, blood samples were taken from the jugular vein of all selected ewes, and the plasma samples were separated. At lambing, the ewes were categorized into five groups according to the number and sex of lambs born: Ewes with a single male lamb (SM group, n=27), ewes with a single female lamb (SF group, n=27), ewes with twin male lambs (TM group, n=8), ewes with twin female lambs (TF group, n=7), ewes with one male and one female lamb (TMF group, n=6). Plasma PAG concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit. Differences in PAG concentrations between groups were analyzed using independent samples t-tests and one-way ANOVA. The results indicated that neither the number nor the sex of the fetuses significantly influenced the PAG concentrations in the plasma of Hasak ewes on day 35 of gestation ($p>0.05$). In conclusion, plasma PAG concentration on day 35 of pregnancy is not a reliable predictor of the number or sex of fetuses in Hasak ewes.

INTRODUCTION

Sheep are adaptable to diverse breeding conditions and have a relatively short gestation period. Their high prolificacy makes them suitable for various production goals, including meat, milk, and wool (Goldansaz et al., 2022). Due to these characteristics, sheep farming remains a significant industry within the global livestock sector, providing an indispensable contribution to human life with its diverse production outputs and the industrial products derived from them (Aydın et al., 2024). Flock-based sheep farming is often influenced by socio-economic factors, with profitability largely depending on production volume and efficiency (Simões et al., 2021). Effective herd management requires a balance between production and reproduction, with reproductive efficiency playing a central role in this process. In intensive sheep farming, reproductive efficiency is an important profitability factor, ensuring flock sustainability and influencing overall meat and milk yields (Theodoridis et al., 2018).

Reproductive efficiency refers to the number of lambs born per ewe exposed to a ram during a breeding season. Various factors such as breed, prolificacy, nutrition, conception rate, and viability of the embryo and fetus, influence this efficiency. As a result of reproductive flock management, reproductive efficiency varies considerably between sheep flocks. Reproductive flock management consists of various components, and determining the number of pregnant ewes and the number of lambs they carry as early as possible in a breeding season is shown as the most fundamental component for success. If pregnancy is detected as early as possible after mating during the breeding season, non-pregnant ewes can be rebred, removed from the flock, and unnecessary feed costs avoided, thereby increasing the pregnancy rate of the flock (Goldansaz et al., 2022). The main objectives in the management of pregnant ewes are to complete the pregnancy with a successful birth and to obtain live and healthy lambs with optimal birth and weaning weights (Fthenakis et al., 2012). During pregnancy, maternal nutrition is a crucial factor that directly affects fertility, lamb survival, and growth performance. Knowing the number of fetuses carried by pregnant ewes allows the implementation of a nutritional plan compatible with their nutritional requirements. Feeding pregnant ewes according to the number of fetuses they are carrying can help reduce the incidence of metabolic disorders such as pregnancy toxemia and hypocalcemia, as well as dystocia associated with high birth weight in singleton pregnancies. It can also help to optimize lamb birth weight, increase survival rates and reduce production costs (Jones and Reed, 2017; De Carolis et al., 2020). Furthermore, production costs can be reduced by ration optimi-

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sation according to the nutritional requirements of pregnant animals. In meat-breed sheep, multiple births are generally undesirable due to the lower birth weight of the lambs and the lower milk production capacity of the dams. Multiple births can lead to increased lamb mortality and negatively affect lamb meat production (Trabzon and Öztürk, 2019; Alataş, 2021). Predicting the number and sex of lambs of pregnant ewes in advance facilitates more accurate flock management decisions, such as flock renewal and flock expansion. It also enables the creation of markets for breeding animals based on the number and sex of lambs they carry and supports the development of future projections (Fthenakis et al., 2012). In summary, the development of a cost-effective, easy-to-use, and reliable method for determining the number of embryos/fetuses of pregnant ewes can significantly improve sheep health, lamb survival rates, animal welfare, and farm sustainability (Pickworth et al., 2020).

Under field conditions, transabdominal ultrasound is the most preferred technique for pregnancy diagnosis and determination of the number of fetuses in small ruminants, and it is generally recommended to be performed after 35 days of pregnancy (Barbagianni et al., 2017). However, the effectiveness of ultrasonography, especially in determining the number of fetuses, can be limited by several factors such as the experience of the operator, the type of probe used, the stage of pregnancy, and the facilities of the farm (availability of veterinarians, access to services, labor and time management) (Bretzlaff and Ramano, 2001; Sharkey et al., 2001). Gestation length is a genetically encoded reproductive trait in farm animals, and it is approximately 150 days in ewes (Jainudeen and Hafez, 2000). Most offspring losses during pregnancy occur in the embryonic period, when there is a delicate balance and reciprocal interactions between the embryo and the dam in the establishment and maintenance of pregnancy, especially prior to implantation. The rate of pregnancy loss decreases significantly as the conceptus develops and grows (Rickard et al., 2017; Chundekkad et al., 2020). Considering that pregnancy losses after day 30 of pregnancy are only 1-5%, it can be concluded that the pregnancy will most likely continue after the embryonic period is completed (O'Connell et al., 2016; Rickard et al., 2017). All these data make the 35th day of pregnancy, which is the end of the embryonic period, an important reason for preference in terms of pregnancy diagnosis, health evaluation, and determination of the number of fetuses in ewes (Tekin and Köse, 2022). This situation provides initial data for the development of alternative methods to ultrasonography.

Currently, pregnancy-associated glycoproteins (PAG) are among the primary biomarkers investigated for pregnancy diagnosis, monitoring pregnancy health, and determining the litter size in ruminants (Barbato et al., 2022). The main source of these molecules is binuclear trophoblast cells that migrate during the implantation process, connect to the epithelial cells of the endometrium, and are involved in the formation of cellular plaques called syncytium. Pregnancy-associated glycoproteins are products of gene homologs that are well conserved throughout evolution in species of the Cetartiodactyla order, including cows and sheep, and can be detected in maternal peripheral blood from the 3rd week of pregnancy in ewes (Haugejorden et al., 2006). Studies have confirmed that PAGs are molecules secreted by trophoblast cells and have shown that their concentration in maternal blood increases with the increase in functional placental mass as pregnancy progresses. This increase is particularly marked in early pregnancy (Uçar et al., 2018; Barbato et al., 2022). Indeed, the detection of PAG using commercially available enzyme-linked immunosorbent assay (ELISA) kits enhances their applicability under field conditions and highlights their potential as reliable diagnostic tools in veterinary practice (Friedrich and Holtz, 2010; de Miranda et al., 2017). In addition to the usefulness of these molecules for pregnancy diagnosis, it is also emphasized that they may play crucial roles in the regulation of the maternal immune system during early pregnancy, embryo implantation, and blastogenesis, which are essential for pregnancy maintenance and health (Pohler et al., 2013; Pohler et al., 2016). In ruminants, the decrease or lack of differentiation and development of binuclear cells has been shown to be a potential factor leading to pregnancy loss (Reese et al., 2019; Barbato et al., 2022; Wooding, 2022). In addition, studies have summarized that in ewes, which have a better capacity for multiple births than other species, peripheral blood PAG concentrations are influenced by several breeding-related factors, including breed, litter size, fetal sex, and gestational age (Haugejorden et al., 2006).

The Hasak sheep is a meat breed developed through crossbreeding studies using Akkaraman as the maternal line and Hampshire and German Blackheaded Mutton sheep as the paternal lines (Köse et al., 2012). Several studies have been conducted on the anatomical characteristics, blood parameters, and the growth and fattening performance of lambs of this breed (Tekin et al., 2005; Şimşek et al., 2015; Teke et al., 2017). However, the reproductive traits of this breed, which are crucial for the continuity and spread of the breed, have not been sufficiently researched. Limited studies on the fertility of this breed have found that reproductive performance indicators are not as expected, with low lambing rates per ewe and a low twin rate (Köse et al., 2012; Trabzon and Öztürk, 2019). The aim of this study was to contribute to the knowledge of reproductive physiology by determining plasma PAG concentrations on day 35 of pregnancy in Hasak ewes carrying male or female single or twin fetuses and evaluating the effects of the number and sex of fetuses on PAG concentrations during pregnancy.

MATERIALS and METHODS

Animals

The study was conducted on pregnant Hasak ewes kept in the Small Ruminant Breeding Department of the Bahri Dağdaş International Agricultural Research Institute located in Karatay/Konya/Turkey. All animals included in the study were clinically healthy. The animals were 2 to 7 years old, with body condition scores (BCS) ranging from 2.50 to 4.00 on a 1-5 scale (Russel et al., 1969).

Housing and feeding system

All ewes were housed under uniform conditions in a se-

mi-open barn and fed a balanced ration prepared at the institute's feed unit, following NRC (2007) guidelines. The animals had ad libitum access to water. All routine health checks, vaccinations, and parasite treatments were carried out regularly before the breeding season.

Estrus detection, breeding, and pregnancy diagnosis

During the breeding season, estrus was detected in ewes by mating acceptance behavior with teaser rams. Ewes exhibiting estrus were mated with fertile, same-breed rams that had previously passed fertility testing. The details of the mating were recorded and the day of mating was considered day 0 in the study. On day 35 after mating, pregnancy examinations were performed via a transrectal approach using a B-mode real-time ultrasound device (Scanner 480 Vet, Esaote Pie Medical, Maastricht, The Netherlands) equipped with a 7.5 MHz probe. Among the ewes whose pregnancy was diagnosed by observing the embryonic heartbeat, 75 Hasak ewes were randomly selected using a blind sampling method and included in the study.

Blood collection and plasma samples

Immediately after pregnancy diagnosis (on day 35 of pregnancy), approximately 10 mL of venous blood was collected from the jugular vein of the 75 ewes included in the study in Na-EDTA-containing vacuum tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). To separate the plasma, the collected samples were centrifuged at 3000 rpm for 20 minutes without delay. The plasma samples were aspirated using an automatic pipette and transferred to Eppendorf tubes. The prepared samples were immediately frozen and stored at -20°C until the PAG analysis was performed.

Experimental design

Nutritional and health monitoring of the ewes in the study was carried out regularly from the 35th day of pregnancy until the postpartum period. The lambing records of the ewes were examined and information including the lambing date, type of birth, and number and sex of lambs were obtained from the flock birth register. The results of the ultrasonographic pregnancy diagnosis performed on the 35th day of pregnancy were confirmed. Based on these data, the following five groups were formed: Ewes with a single male lamb (SM group, n=27), ewes with a single female lamb (SF group, n=27), ewes with twin male lambs (TM group, n=8), ewes with twin female lambs (TF group, n=7), ewes with one male and one female lamb (TMF group, n=6).

Determination of plasma PAG levels

The plasma PAG concentrations were determined by analysis at the Research Laboratory of the Dicle University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, and the Laboratory of the Dicle University Health Sciences Application and Research Center. The plasma PAG concentrations were measured using a commercial ELISA kit (Bovine Pregnancy-Associated Glycoproteins ELISA Kit, BT LAB, Zhejiang, China). The measurement procedures were performed according to the manufacturer's instructions. The manufacturer reported the kit's sensitivity as 0.1 µg/mL, the standard curve range as 0.05–20 ng/mL, the intra-assay coefficient of variation as <8%, and the inter-assay coefficient of variation as <10%.

Statistical analysis

The statistical analysis of the study was performed using SPSS 24.0 package program (SPSS, IBM SPSS Statistics, Chicago, IL, USA). Data were presented as mean ± SEM and $p<0.05$ was considered statistically significant. The distributions of the data were tested using the Shapiro-Wilk test. The analysis of the data concerning the groups was performed using the one-way ANOVA test. The mean values of the groups were compared using the independent t-test.

RESULTS

Table 1 shows the mean plasma PAG concentration, total lamb weight, mean lamb weight, and gestation length for the groups in the study. No statistically significant differences were found between the groups in terms of plasma PAG concentration and gestation length ($p>0.05$). The mean plasma PAG concentration was also similar in ewes with a single lamb and twin lambs, regardless of the sex of the lambs ($p>0.05$). However, total lamb weight was significantly higher in the TM group than in the other groups, and it was also higher in the TF and TMF groups than in the SM and SF groups ($p<0.05$). In addition, the average weight of lambs was significantly higher in the SM group than in the TF and TMF groups, while it was higher only in the SF group than in the TMF group ($p<0.05$).

Table 1. The relationship between litter size and sex at day 35 of pregnancy with plasma PAG levels, lamb birth weight, and gestation length in Hasak ewes.

Groups (n)	PAG-I (ng/mL)	PAG-II (ng/mL)	Total lamb weight (kg)	Average lamb weight (kg)	Gestation length (day)
SM (27)	3.20±0.37	3.38±0.26	4.95±0.20^{C}	4.95±0.20^{A}	148.89±3.46
SF (27)	3.55±0.37		4.70±0.16^{C}	4.70±0.16^{AB}	148.96±2.10
TM (8)	4.01±0.67		9.10±0.42^{A}	4.55±0.21^{ABC}	149.88±1.36
TF (7)	2.78±0.72	3.24±0.42	7.78±0.58^{B}	3.89±0.29^{C}	148.86±1.77
TMF (6)	2.76±0.78		7.94±0.61^{B}	3.97±0.30^{BC}	148.67±2.80

SM: Ewes with a single male lamb, SF: Ewes with a single female lamb, TM: Ewes with twin male lambs, TF: Ewes with twin female lambs, TMF: Ewes with one male and one female lamb, PAG-I: PAG concentration based on the number of lambs and sex, PAG-II: PAG concentration based on the number of lambs, A, B, C: Different superscripts in the same row indicate a significant difference between the columns at the $p<0.05$ level.

DISCUSSION

In sheep farming, breeders generally prefer breeds with high lamb yield per ewe. Within sheep breeds, certain breeds such as Sakiz and Romanov are particularly known for their high prolificacy. However, in meat-breed sheep, the increased numbers of fetuses during pregnancy can lead to nutritional deficiencies in the dams, especially during the late gestation period and early neonatal period due to the rapid fetal growth. This nutritional imbalance contributes to reduced lamb viability and higher neonatal lamb mortality, making multiple births an undesirable trait in some meat breed sheep. The Hasak sheep is also a meat-breed sheep. In a previous study, it was found that the number of lambs born per ewe in Hasak ewes was 113%, the survival rate of lambs at day 90 was 86.7% and the survival rate of twin lambs at day 90 was significantly lower compared to singleton lambs. It was also found that the effect of birth type on survival rate could be related to the pregnancy of the ewe, its ability to care for the lamb after birth, and its ability to produce sufficient milk, as well as the birth weight of the lamb (Trabzon and Öztürk, 2019). Therefore, estimating the number of fetuses during pregnancy is of great importance in meat sheep production, both to ensure healthy lamb births and to increase lamb meat production. In this study, it was aimed to investigate the effect of litter size (singleton or twin births) and the sex of fetuses on maternal circulating PAG concentrations on day 35 of gestation, which marks the end of the embryonic period, in Hasak ewes.

The ability to predict ewes with multiple fetuses in a flock enables the implementation of better management strategies and the maximization of productivity while improving animal welfare (Llanes et al., 2019). Currently, ultrasonography is the most commonly used method for both pregnancy diagnosis and litter size determination in small ruminant reproductive management. However, it is well known that ultrasound-based pregnancy diagnosis and especially litter size determination are subject to various limitations (Bretzlaff and Romano, 2001; Sharkey et al., 2001). Therefore, performing laboratory analysis to determine PAG concentrations in blood and body secretions, especially during early pregnancy, could open up new applications not only for pregnancy diagnosis but also for litter size determination. This approach could offer breeders an alternative to ultrasound examination and thus enable more informed management decisions (Llanes et al., 2019).

Studies have been conducted to determine litter size during pregnancy by measuring PAG concentrations using radioimmunoassay (RIA) or ELISA techniques in various ruminant species and breeds, including cattle, buffalo, goats, and sheep. In studies in ewes, Karen et al. (2006) reported that PAG concentrations were higher in ewes pregnant with twin or multiple fetuses than in ewes pregnant with a single fetus when measured on days 43-56 of pregnancy using a homologous RIA assay. Similarly, Pickworth et al. (2020) and Çebi & Akköse (2024) found increased PAG concentrations in ewes carrying multiple fetuses when measured using ELISA on days 46 and week 7 of pregnancy, respectively. Barbato et al. (2009) and El Amiri et al. (2015) reported significant differences in PAG concentrations between ewes carrying one lamb and those carrying multiple lambs as early as day 18 of pregnancy using a homologous RIA test. However, it has been noted that this difference may not be clearly detectable until day 28, depending on the specificity of the antisera used (El Amiri et al., 2015). These results suggest that the RIA technique could be used to discriminate between singleton and multiple pregnancies in ewes based on PAG levels during early gestation. However, it has been emphasized that differences in the sensitivity of antisera to different epitopes may lead to discrepancies in the results obtained using this method (El Amiri et al., 2015). Most of these mentioned studies focused on the comparison of PAG concentrations between ewes pregnant with one and multiple fetuses (Karen et al., 2006; Barbato et al., 2009; El Amiri et al., 2015). Studies conducted on ewes pregnant with single and twin lambs suggest that the differences in PAG concentrations only become apparent at later stages of pregnancy (Ledezma-Torres et al., 2006; Pickworth et al., 2020; Çebi and Köse, 2024). In a study using a heterologous RIA test, differences in PAG concentration between ewes carrying single and twin lambs were found to be detecteble at 8-9 weeks of pregnancy (Ledezma-Torres et al., 2006). In two other studies, significant differences in PAG concentration were found in ewes with single or twin lambs at 21 weeks of pregnancy (Ranilla et al., 1997) and shortly before parturition (De Carolis et al., 2020). However, studies using the ELISA technique indicate that the difference in PAG concentrations between singleton and twin pregnant ewes becomes more pronounced after the completion of the embryonic period (Alkan et al., 2020; Pickworth et al., 2020; Çebi and Köse, 2024). Moreover, a recent study in ewes reported that PAG levels in animals carrying twin lambs, compared to those carrying a single lamb, decreased from day 30 of pregnancy onward (Akkuş and Yaprakçı, 2022). Similarly, studies in goats have shown that after the completion of the embryonic period, PAG concentrations in goats carrying a single kid, compared to those carrying multiple kids, decreased on days 45 (Singh et al., 2019), 48 (Llanes et al., 2019), and 85 (Lü et al., 2021) of pregnancy. Szelényi et al. (2015) also reported that bovine pregnancy-associated glycoprotein-1 (bPAG-1) concentrations on days 30 and 60 of pregnancy were similar between singleton and twin pregnant cows. They also found that the detection of twin pregnancies using bPAG-1 measurements was only possible from day 85 of pregnancy. When all these results are evaluated, it becomes clear that determining the number of fetuses in multiple pregnancies in the early stages of gestation remains a major challenge. Also in our current study, ELISA-based analysis at the end of the embryonic period showed no significant difference in PAG concentrations between singleton and twin pregnant ewes. Our results are consistent with most studies in the literature and suggest that measurement of PAG concentration in a single blood sample taken on day 35 of pregnancy is not sufficient to discriminate between ewes with single and twin fetuses. The inability to determine litter size by determining PAG concentrations at the end of the embryonic period in Hasak ewes and the resulting limitation in preventing twin pregnancies emphasize the need to find alternative indicators to ultrasonography. It is also recommended that new management strategies be developed, and preventive measures be taken to increase the survival rate of twin lambs that have lower

survival rates at weaning.

Diagnosis of fetal sex during pregnancy in ruminants serves several purposes and has implications for livestock production, including sheep production. The sex of the fetus is determined chromosomally at the beginning of pregnancy, at the same time as fertilization. However, under field conditions, the transition to the fetal stage must be completed to determine the sex of pregnant ewes. Ultrasound-based sexing, which relies on the identification of the genital tubercle, can only be performed after day 60 of gestation and requires an experienced clinician, making it a labor-intensive and time-consuming procedure (Barbagianni et al., 2017). Studies on methods of sex determination based on PAG concentration in maternal blood have not yielded successful results (Ledezma-Torres et al., 2006; Alkan et al., 2020). Also in our current study, plasma PAG concentrations at day 35 of gestation did not show significant differences concerning fetal sex. However, some studies suggest that fetal sex may influence PAG concentrations as pregnancy progresses. Ranilla et al. (1994) reported that maternal PAG concentrations in the blood of ewes with male fetuses increased from 19 weeks gestation. Similarly, De Carolis et al. (2020) suggested that fetal sex may influence PAG concentrations. They suggested that the increased PAG concentrations observed in ewes with male fetuses could be due to the higher weight of male fetuses in utero as gestation progressed. These results suggest that hormonal or placental factors related to fetal development may contribute to the variation in PAG levels in the later stages of pregnancy. Further studies examining the later stages of pregnancy could help clarify the possible relationship between fetal sex and PAG dynamics.

CONCLUSION

In conclusion, it was found that measuring plasma PAG concentration at the end of the embryonic period is not a reliable method for predicting the number and sex of fetuses in Hasak ewes, a meat breed. Similarly designed further studies conducted at advanced stages of pregnancy may be beneficial in assessing the effects of litter size and sex on maternal PAG concentrations.

DECLARATIONS

Ethics Approval

The study was conducted with the approval of the Animal Experiments Local Ethics Committee of Bahri Dagdas International Agricultural Research Institute (25.01.2023/153).

Conflict of Interest

The authors declare that there are no conflicts of interest for this study.

Consent for Publication

Not applicable.

Author contribution

Idea, concept and design: CP, HU, MK[5], NKA, MK[4]

Data collection and analysis: HU, NKA, DS, MK[4]

Drafting of the manuscript: CP, EHU, MOA, DS

Critical review: MK[5], MOA, EHU, DS

Data Availability

The data of this study are available from the corresponding author upon reasonable request.

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