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Determination of local expressions of IGF-1, LC3B and NF-kB in white muscle disease in lambs by immunohistochemical method

ABSTRACT

White muscle disease (WMD) is also known as Stiff Lamb Disease or Nutritional Muscular Dystrophy. Selenium and/or Vitamin E deficiency constitutes the etiology of the disease. This study aimed to immunohistochemically evaluate local protein expressions of Nuclear factor kappa B (NF-kB), Insulin-like growth factor-1 (IGF-1) and Microtubule-related protein 1A/1B-light chain 3 beta (LC3B) in WMD. The material of the study consisted of 15 WMD, and 6 healthy lamb heart samples. The heart tissues of the autopsied lambs were subjected to routine tissue processing and paraffin blocks were obtained. Then, it was stained with Hematoxylin-Eosin and immunohistochemical methods. Control group lambs had normal macroscopic appearance. Macroscopically, hyaline degeneration and zenker's necrosis, calcification areas were observed in WMD tissues. Microscopically, degenerative and necrotic muscle fibers, calcification areas, fibrosis, mononuclear cell infiltrates and macrophage infiltrates were detected in WMD heart tissues. Immunohistochemically, significant increases were detected in IGF-1 ($p<0.001$), LC3B ($p<0.001$) and NF-kB ($p<0.05$) in the WMD group compared to the control group. Immunoreactivity in the relevant primers was detected commonly in degenerative and necrotic muscle fibers. In addition, occasional immunoreactivity was observed in the relevant primers in inflammatory cell infiltrates. In conclusion, NF-kB, IGF-1 and LC3B protein expressions were evaluated immunohistochemically for the first time in lambs with WMD. Our findings show that IGF-1 and LC3B proteins are highly expressed in heart tissue in WMD. Additionally, it is possible to say that IGF-1 and LC3B can be used in the diagnosis of WMD.

Keywords: Histopathology, IGF-1, LC3B, NF-kB, white muscle disease

INTRODUCTION

White muscle disease (WMD) is also known as Stiff Lamb Disease or Nutritional Muscular Dystrophy. Selenium (Se) and/or Vitamin E (Vit E) deficiency constitutes the etiology of the disease. WMD is a major muscle degeneration disease of domestic animals (lambs, kids and calves) characterized by tissue destruction in striated muscles (Abutarbush and Radostits, 2003; Sobiech and Żarczyńska, 2020). WMD usually causes death due to heart failure in young animals. In WMD, degeneration, necrosis, fibrosis and calcification develop in the heart muscle. The incidence of WMD in our country is reported to be between 20-30%. The disease is generally seen in Central Anatolia, Eastern Anatolia and Southeastern Anatolia regions (Karakurt et al., 2021; Karatas and Akcakavak, 2024; Yavuz, 2017).

Nuclear factor *kappa* B (NF-kB) is involved in various cellular processes such as cell proliferation and apoptosis, neurodevelopment, response to infection, inflammation (Zinatizadeh et al., 2021). In the

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Research Article

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resting state, NF-kB is inactivated in the cytoplasm by inhibitory proteins such as p105 and IkB in the cells. Reactive oxygen species (ROS) and some other free radicals affect these inhibitory proteins, causing the release of NF-kB and its migration to the nucleus (Biswas and Bagchi, 2016; Zinatizadeh et al., 2021).

Insulin-like growth factor-1 (IGF-1) is known as the essential mediator of growth hormone (GH). IGF-1 is part of a network of many growth factors, receptors and binding proteins involved in important processes such as cellular proliferation, differentiation and apoptosis (Bailes and Soloviev, 2021; Gusscott et al., 2016; Yoshida and Delafontaine, 2020). GH induces the production and release of IGF-1, which then binds to IGF-1R on the surface of cells. In addition, after the IGF-1-IGF-1R interaction, intracellular tyrosine kinase domains become activated. Thus, PI3K/Akt and Raf/MEK/ERK result in the activation of multiple signaling pathways (Gusscott et al., 2016; Yoshida and Delafontaine, 2020).

Autophagy is an intracellular catabolic process involving the degradation of intracellular components by lysosomes through the formation of autophagosomes. Autophagy plays important roles in maintaining cell homeostasis (Ichimiya et al., 2020). Microtubule-associated protein 1A/1B-light chain 3 (LC3), the mammalian homolog of yeast Atg8p, is considered an important component of autophagosomes (Meng et al., 2020). Although LC3 has several homologues in mammals, LC3B is the most commonly used for autophagy measurements. LC3B is expressed primarily in the heart, brain, skeletal muscle, and testis (Mizushima and Yoshimori, 2007; Wang et al., 2022).

In recent years, many studies have been carried out to elucidate the pathophysiology of WMD in domestic animals. For this purpose, different processes such as oxidative stress, apoptosis, etc. are evaluated (Karakurt et al., 2021; Tunca et al., 2009; Yildirim et al., 2019; Yumusak et al., 2018). Thus, new perspectives on the pathophysiology and diagnosis of WMD is revealed. This study aimed to immunohistochemically evaluate NF-kB, IGF-1 and LC3B protein expressions in WMD, a metabolic disease in lambs.

MATERIALS AND METHODS

Animal materials

The material of the study was heart tissue samples of 15 lambs (1-6 months, Merino) with WMD detected at necropsy and 6 healthy lambs (1-6 months, Merino) in Yozgat, Sivas and Konya regions.

Histopathological examination

Heart samples taken after necropsy were fixed in neutral formaldehyde for 24-48 hours. Afterwards, it was obtained in paraffin blocks by going through routine tissue tracking procedures. Sections were taken from paraffin blocks, stained with Hematoxylin-Eosin and examined under light microscopy (Luna, 1968).

Immunohistochemical examination

Sections were taken from paraffin blocks onto adhesive slides. Immunohistochemical examination was performed with the UltraVision Detection System Anti–Polyvalent, HRP (Ready–To–Use, TP–060–HL, Lab Vision, USA) kit in accordance with the manufacturer recommendations. Anti-LC3B (Santacruz Biotechnology, sc-271625, 1/200 dilution), Anti-IGF-1 (Santacruz Biotechnology, sc-518040, 1/200 dilution), and Anti-NF-kB (Bioss, Bs-0465R, 1/200 dilution) antibodies were used as primers. 3.3 diaminobenzidine (DAB) was used as chromogen and counterstaining was performed with Mayers-Hematoxylin. In the negative control, PBS was inoculated instead of antibody. Immunohistochemical scoring was performed semi-quantitatively (0; none, 1; mild, 2; moderate, 3; severe) (Akcakavak et al., 2023).

Statistical analysis

Evaluation of data between groups was done with SPSS (version 25.0, Inc., Chicago, USA) statistical program. Prior to analysis, immunohistochemical

data were assessed for normal distribution and then subjected to independent sample t-test. The accepted significance limit was $p<0.05$.

RESULTS

Macroscopic results

Heart tissues of control group lambs had normal macroscopic appearance. Hyaline degeneration and zenker's necrosis, dystrophic calcification areas were observed in WMD heart tissues (Figure 1A). The relevant lesions were located in the endocardium and epicardium. Areas of hyaline degeneration and zenker's necrosis were generally pale in color and resembled fish and/or chicken flesh (Figure 1A-B). Dystrophic calcification areas were detected in the heart in 9 lambs.

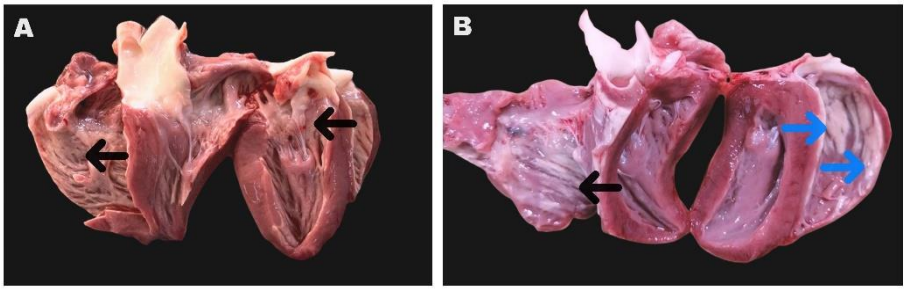

Figure 1. Macroscopic view of White muscle disease (WMD) heart tissues. **A.** Dystrophic calcification foci (arrows) in the endocardial section. **B.** Hyaline degeneration and zenker's necrosis (blue arrows), dystrophic calcification (black arrow) in the endocardium.

Microscopic results

Histopathological results

The heart of control animals showed normal structure (Figure 2A). Degenerative and necrotic muscle fibers were detected in WMD heart tissues. Striation was lost in these muscles, and they appeared swollen and pink (Figure 2B-C). Additionally, dystrophic calcification foci and areas of fibrosis were found occasionally. Mononuclear cell infiltrates were detected in the interstitial area and macrophage infiltrates were detected around necrotic muscle fibers (Figure 2D).

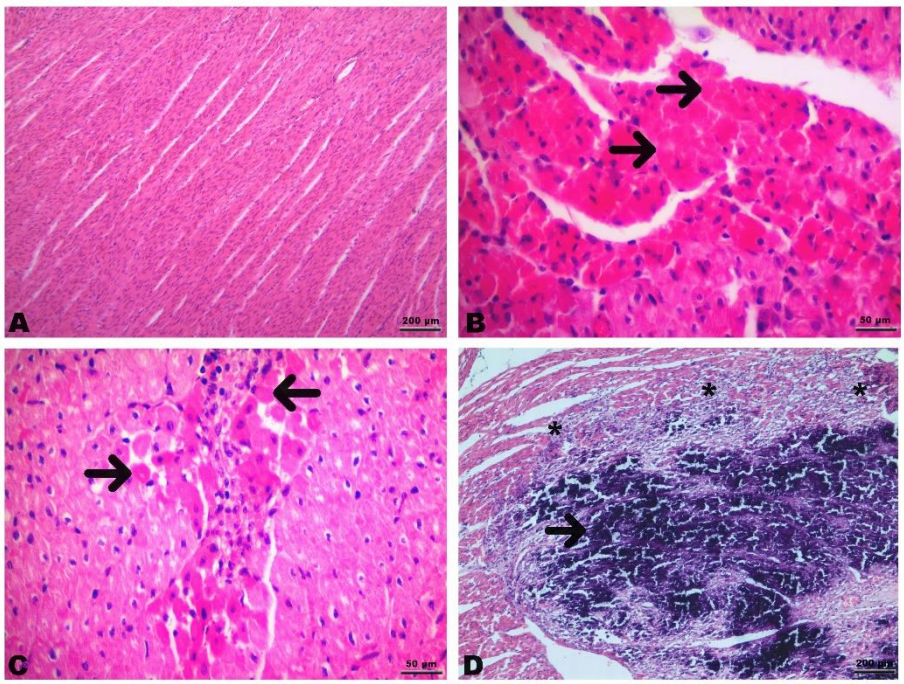

Figure 2. Histopathological examination of control and White muscle disease (WMD) lambs, Hematoxylin-Eosin, **A**. Normal histological appearance in control animals. **B-C.** Hyaline degeneration and zenker's necrosis (arrows) in lambs with WMD. **D.** Dystrophic calcification (arrow), fibrosis and inflammatory cell infiltration (stars) in WMD lambs.

Immunohistochemical results

Immunohistochemical scores for NF-kB, IGF-1 and LC3B are given in Table 1. In the control group, the immunoreactivity of the relevant primers was very mild or absent (Figure 3). Significant increases were detected in IGF-1 ($p<0.001$), LC3B ($p<0.001$) and NF-kB ($p<0.05$) in the WMD group compared to the control group. Immunoreactivity in the relevant primers was detected commonly in degenerative and necrotic muscle fibers. In addition, occasional immunoreactivity was observed in the relevant primers in inflammatory cell infiltrates (Figure 3).

Table 1. Statistical scores of IGF-1, LC3B and NF-kB in control and WMD lambs.

Primers	Control (n=6)	WMD (n=15)
IGF-1	0.83 ± 0.17^{b}	2.50 ± 0.22^{a}
LC3B	0.50 ± 0.22^{b}	2.17 ± 0.17^{a}
NF-kB	0.33 ± 0.21^{b}	1.5 ± 0.22^{a}

$^{a-b}$: Different letters on the line indicate statistical significance ($p<0.05$). WMD: White muscle disease, IGF-1: Insulin-like growth factor-1, LC3B: Microtubule-associated protein 1A/1B-light chain 3 beta, NF-kB: *Nuclear* factor *kappa* B.

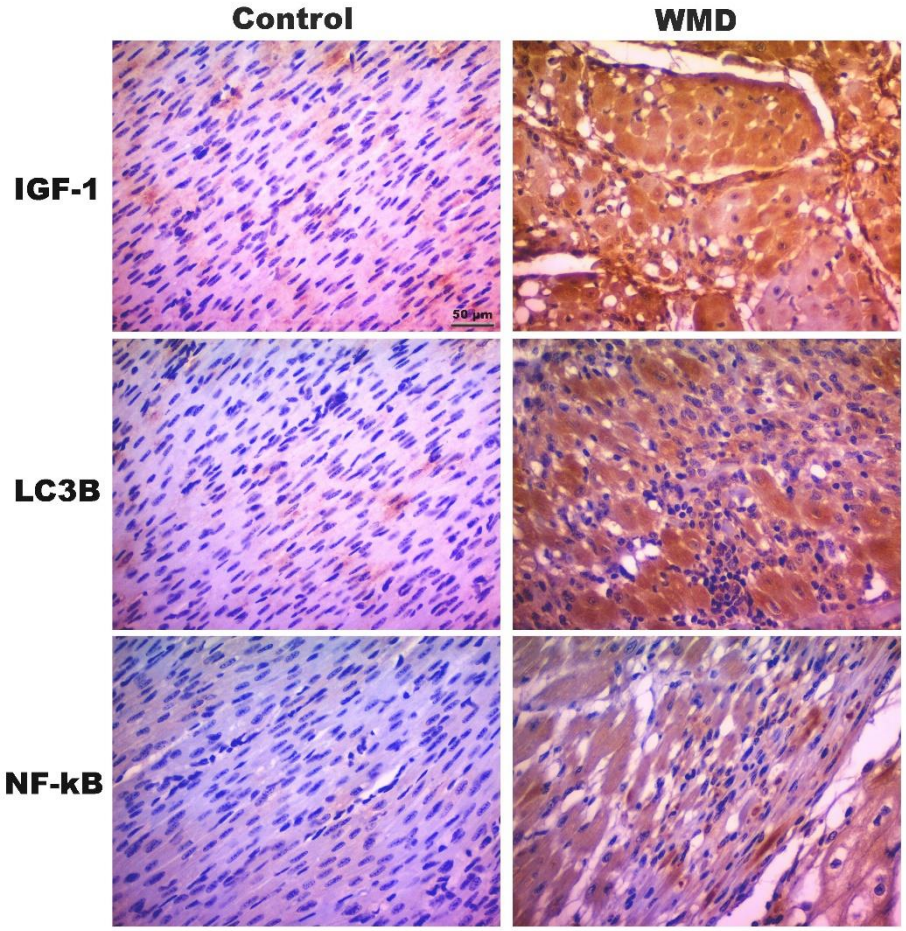

Figure 3. Immunohistochemical staining (DAB) of IGF-1, LC3B and NF-kB in WMD and control, bar; 50 µm. WMD: White muscle disease, IGF-1: Insulin-like growth factor-1, LC3B: Microtubule-associated protein 1A/1B-light chain 3 beta, NF-kB: *Nuclear* factor *kappa* B.

DISCUSSION

White muscle disease, which is an important metabolic disease of lambs, is frequently encountered in our country, especially in the Central Anatolia, Eastern Anatolia and Southeastern Anatolia regions, and causes important economic loses in lamb breeding. Recently, many studies have been conducted on the pathophysiology, diagnosis and prognosis of WMD (Karakurt et al., 2021; Karatas and Akcakavak, 2024; Kozat et al., 2011; Kozat et al., 2007; Yumusak et al., 2018, Yıldırım et al., 2021). In this study, NF-kB, IGF-1 and LC3B protein expressions were revealed immunohistochemically in WMD in lambs and

their effects on the pathophysiology of the disease were evaluated.

White muscle disease occurs in two different clinical forms: acute and subacute. The acute form is known as the cardiac form and is manifested by degeneration of the heart muscle and sudden death in young animals. The subacute form presents with skeletal muscle degeneration (Dabak et al., 2002). In the study conducted by Yavuz (2017) on 39 lambs, macroscopically, areas of pallor and calcification were detected in the heart, and microscopically, hyaline degeneration, zenker's necrosis, inflammation and calcification areas were detected in the heart. Karakurt et al., (2021) reported in their study that they detected macroscopic necrotic areas on the epicardial and endocardial surfaces and ventricular walls in lambs. They also reported that they detected microscopic degenerative and necrotic muscle fibers, inflammatory cell infiltrates and fibrosis. They also identified areas of calcification in necrotic muscle fibers. The macroscopic and microscopic findings of the current study are consistent with previous studies.

Some free radicals formed as a result of the decrease in antioxidant defense as a result of deficiency of Se and vit E cause oxidative stress. In Se and vit E deficiencies, lipid peroxidation and hydrogen peroxide cannot be cleared from the muscles due to the decrease in glutathione peroxidase (GSH-Px) activity. Moreover, ROS levels serve as markers of oxidative stress, and lipid peroxidation and imbalance of the redox system are associated (Ataollahi et al., 2013; Karakurt et al., 2021; Kozat et al., 2011; Kozat et al., 2007, Yıldırım et al., 2019). It has been reported in many studies that oxidative stress plays a very important role in the pathogenesis of WMD (Karakurt et al., 2021; Kozat et al., 2007; Yumusak et al., 2018; Yıldırım et al., 2019). In this context, oxidative stress is considered an important cause of degenerative and necrotic changes in relevant tissues.

Insulin-like growth factor-1 is a growth factor known as an anabolic and pro-myogenic factor important for the development and regeneration of skeletal muscle (Al-Shanti and Stewart, 2012). IGF-1 plays important roles in maintaining homeostasis in skeletal muscle by activating molecular steps critical for muscle homeostasis (O'Neill et al., 2015). It has been reported that increases in IGF-1 expression protect dystrophic muscle from necrosis (Grounds et al., 2008). Monocytes/macrophages recruited to the lesioned area after muscle injuries represent the first source of IGF-1 (Tidball and Welc, 2015). Previous studies have highlighted the importance of IGF-1 in promoting muscle regeneration through stimulation of myoblast proliferation and differentiation (Pelosi et al., 2007; Tonkin et al., 2015). Ye et al., (2013) attributed IGF-1 overexpression to alleviating muscle damage and accelerating muscle regeneration in their study. Sukhanov et al., (2007) reported in their study that increasing circulating IGF-1 reduced systemic and vascular oxidative stress. In the current study, IGF-1 expressions in WMD heart tissues were significantly higher than in control animals ($p<0.001$). IGF-1 expression was especially intense in degenerative and necrotic muscle fibers and macrophage cells. This situation has been interpreted as resulting from the body's response to the degenerative and necrotic damage occurring in the heart tissue due to WMD. Additionally, its intense expression in degenerative and necrotic muscle fibers may be caused by the response to oxidative stress.

Autophagy plays a role in important biological processes such as stress responses, programmed cell death and the elimination of damaged organelles. It is also known as an important sensor of the redox signal (Nichenko et al., 2016). It has been reported that the autophagy process tends to reduce oxidative stress (Yun et al., 2020). LC3 is known as a marker that plays an important role in the autophagy process (a component of the

autophagosome). Many studies conducted in recent years state that the autophagy process increases after muscle damage and is critical for functional recovery and muscle renewal (Nichenko et al., 2016; Paolini et al., 2018). In the current study, it was determined that LC3B protein expressions were significantly increased in lambs with WMD compared to the control group ($p<0.001$). Current findings show that there may be upregulation of the autophagy process due to the oxidative stress process that occurs in WMD in lambs.

Nuclear factor kappa B is the transcription factor that regulates important processes such as inflammation and immune response (Zinatizadeh et al., 2021). Many studies have reported an increase in NF-kB expression after muscle damage and it is stated that it causes a worse pathology (Koshimizu et al., 2013; Nascimento et al., 2019; Wang et al., 2023). Oxidative stress and NF-kB are closely related and oxidative stress is known as an important inducer of NF-kB (Cuevas et al., 2005; Schreck et al., 1992). In the current study, increases in NF-kB levels were detected in lambs with WMD compared to the control ($p<0.05$), and it was thought that this situation may be due to oxidative stress. Because ROS and some other free radicals affect IkB inhibitor proteins, causing the release of NF-kB and its migration to the nucleus (Zinatizadeh et al., 2021).

The current study has some limitations. The most important limitation is the lack of blood concentrations values of the relevant proteins. Determination of blood concentrations of relevant proteins in future studies may provide a more comprehensive perspective on the diagnosis of WMD.

CONCLUSION

As a result, NF-kB, IGF-1 and LC3B protein expressions were evaluated immunohistochemically for the first time in lambs with WMD. Our findings show that IGF-1 and LC3B proteins are highly expressed in heart tissue in WMD. Additionally, it is possible to say that IGF-1 and LC3B can be used in the diagnosis of WMD.

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

Ethical statement or informed consent: The study was approved by the Sivas Cumhuriyet University Animal experiments local ethics committee (02.02.2024, Decision no: 09).

Author Contributions: The study was designed by GA and MT. GA, OK, OD collected the relevant samples. ATC and OD laboratory performed tissue tracking procedures. GA and OK performed the immunohistochemical analyses. All authors read and approved the final version.

Availability of data and materials: All data and materials of the study are available in contact with the corresponsible author.

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The effect of ginger (*Zingiber officinale*) essential oil on catalase in rat kidney tissue

ABSTRACT

We were aimed to investigate the effect of ginger (Zingiber officinale) essential oil on catalase release in rat kidney tissue by histopathological and immunohistochemically method. This study, 21 male Wistar albino rats were used. Rats were divided into three groups: control, 100 mg/kg ginger essential oil (G100), and 500 mg/kg ginger essential oil (G500). Hematoxylin-eosin staining method was used for histopathological evaluations. Immunohistochemically localization of catalase in kidney tissue was determined by streptavidin-biotin peroxidase method. As a result of histopathological evaluations, an increase in glomerulus diameter was observed in kidney tissues of G100 and G500 groups. In addition, vacuolar degeneration was observed in the proximal and distal tubule epithelial cells in the renal cortex of the G100 group. The immunoreactivity of catalase in the renal cortex region; In the control group, it is strong in the proximal tubules and very weak in the collecting ducts. In the G100 group, catalase immunoreactivity was weak in the proximal tubules and distal tubules and strong in the collecting ducts. In G500 group, weak catalase immunoreactivity was observed in only proximal tubules. Strong catalase immunoreactivity was detected in the proximal tubules of the kidney medulla regions of the rats in all groups. Furthermore, there was strong catalase immunoreactivity in collecting ducts in the medullary region of the G100 group. We think that ginger essential oil can be used in appropriate doses and durations to reduce kidney damage.

Keywords: Catalase, ginger essential oil, kidney

INTRODUCTION

Ginger (*Zingiber officinale*) belonging to the *Zingiberaceae* family is a medicinal plant whose root or rhizome has been used as a spice or herbal medicine for many years (Karna et al., 2012). Ginger root is used to relieve or treat some common ailments such as headaches, colds, nausea, and vomiting. Ginger contains 1-3.3% essential oil (Karna et al., 2012). Ginger essential oil and its components have been studied mostly for their flavor and fragrance. However, in recent years, it has attracted a lot of attention around the world due to its multi-purpose functional uses.

Having numerous therapeutic effects, ginger is widely used in public medicine for its many health benefits in various diseases including diabetes (Al Hroob et al., 2018), cancer (Chen et al., 2018), ulcers (Liu et al., 2015), obesity (Suk et al., 2017), chronic diseases such as Alzheimer's (Cuya et al., 2018), cardiovascular (Liu et al., 2013) diseases and depression (Kukula-Koch et al., 2018).

Oxidative stress occurs due to the imbalance between production and elimination of reactive oxygen species (ROS), one of the free radicals, such as superoxide anions (O_2-) and hydroxyl radicals (OH-). Overproduction of ROS causes oxidation of cellular compounds and cell

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Research Article

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death (Alonso-Alconada et al., 2012; Nita and Grzybowski, 2016). Excessive oxidative stress is responsible for the initiation and progression of cell damage/death in organs (Cayir et al., 2011; Galle, 2001; Zhu et al., 2011). There are endogenous antioxidant defense mechanisms, including antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase, in protecting or reducing oxidative stress (Akbulut et al., 2014; Cayir et al., 2011; Forbes et al., 2008).

Catalase is a tetrameric enzyme consisting of 60 kDa subunits containing a heme group and a NADPH molecule (Scibior and Czeczot, 2006). It plays a role in preventing or delaying oxidative damage by catalyzing hydrogen peroxide (H_2O_2) and converting it to H_2O and O_2 (Yu et al., 2007).

Glomerular diameter is an indicator of glomerular hypertrophy. Focal segmental glomerulosclerosis (FSGS) is linked to a variety of illnesses, including unilateral renal agenesis, diabetes, eclampsia, and a high protein diet (Fogo, 2000).

In this study, it was aimed to investigate the protective effect of ginger essential oil on kidney tissue.

MATERIALS AND METHODS

Animals and experimental design

We used 200-250 g male *Wistar albino* rats housed in a sterile environment at 22 ± 3°C and 60–65% humidity in a room under a 12-hour light-dark cycle. Animals were permitted access to pellet feed and tap water *ad libitum*. Groups was formed from randomly selected rats as 7 rats in each group as follows.

Control group (n = 7): No application was made to the rats in this group. 100 mg/kg/day ginger essential oil (G100 Group) (n = 7): The rats in this group were administered 100 mg/kg/day ginger essential oil (Hekimhan Herbal-Antalya, Türkiye) by oral gavage for 10 days (Jeena et al., 2011). 500 mg/kg/day ginger essential oil (G500 Group) (n = 7): The rats in this group were administered 500 mg/kg/day ginger essential oil by oral gavage for 10 days.

The experiment was finished by following the ethics committee rules throughout the study. At the end of the experiment, rats in all groups were anesthetized with 15 mg/kg xylazine (Rompun; Bayer, İstanbul, Türkiye) and 75 mg/kg ketamine (Ketalar; Pfizer, İstanbul, Türkiye) before sacrifice by cervical dislocation. The kidney tissue samples were removed and placed in 10% formalin.

Histopathological procedure

After the kidney tissue samples were fixed in 10% formaldehyde solution, they were blocked in paraffin after routine histological procedures. Hematoxylin-Eosin (H&E) staining technique was applied to the 5 µm sections taken from the blocks to examine the general structure of the tissue.

Glomerulus diameters were measured using Image J (v1.50i) software from kidney tissue samples. A total of 975 glomerulus diameters were measured, including 325 glomerulus diameters from each group.

Immunohistochemical procedure

The streptavidin-biotin-peroxidase technique, one of the indirect methods, was used to the sections (5 µm) taken to the lams coated by chrome aluminum gelatin (Hsu et al., 1981). The sections were then incubated in 3% H_2O_2 prepared in methanol for 15 min to prevent the endogenous peroxidase activity. After the deparaffinization and rehydration processes. They were then applied heat at the maximum temperature in a microwave oven for 10 min (800 watt) in the citrate buffer solution (Ph 6.0) to bring antigens into the open after washing with the PBS. The blocking solution A was dripped to prevent the nonspecific binding (Histostain-Plus IHC Kit, HRP, broad-spectrum Ref.) after washed by PBS. The anti-catalase (Santa Cruz sc271358, it was diluted at the rate of 1/500) were applied on the sections in a humid

environment at the ambient temperature for 1 h. The Broad-Spectrum Antibody was dripped on the sections since it was against the type produced by the primary antibody. The HRP streptavidin was incubated at the ambient temperature for 15 min after washing with PBS. The 3,3'-Diaminobenzidine tetrahydrochloride (DAB) substrate solution (0.5 mg DAB/ml; Dako Corporation, Carpinteria, USA) was added for the chromogen practice and then, Gil III hematoxylin was used for the background staining. The slides were examined in a research microscope and their photos were taken (Leica DM4000B, Germany). The immunohistochemically evaluation was made by considering staining characteristic and staining density of the target cells. The evaluation was made by two independent observers by giving values from 0 to 4 in accordance with the characteristics including no staining (-), very weak staining (+), weak staining (++), moderate staining (+++), and strong staining (++++) (Aras et al., 2023).

Statistical analyses

SPSS 18 (IBM Corp., New York, USA) package program was used to evaluate the data obtained in the study. The Kolmogorov-Smirnov test was utilized to assess the normality of the group data. Data determined to be normally distributed were tested with one way analysis of variance (ANOVA) followed by post-hoc Duncan test. P values below 0.05 ($P<0.05$) were considered statistically significant.

RESULTS

Histopathological results

In the kidney tissue samples taken from all groups, 325 glomerulus diameters were measured in randomly selected areas from each group. When the control and experimental groups were compared, there was no significant difference between the control and G500 groups, and between the G100 and G500 groups. But the difference was significant between the control and G100 groups ($p<0.05$, Table 1, Figure 1).

Table 1. Statistical evaluation of glomerulus diameter levels of rats according to groups (um)

Groups	n	Average ± S.D.
Control	325	90,29±13,57
G100	325	95,12±14,09*
G500	325	92,67±15,69

*: $p<0.05$ relative to control was accepted.

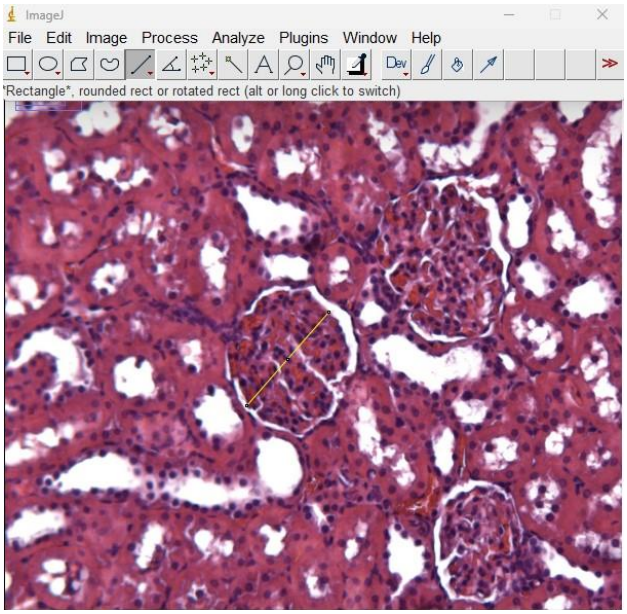

Figure 1. Sample glomerulus measurement with Image J (v1.50i) application. Yellow line is glomerulus diameter.

Renal corpuscles containing glomerulus in the cortex region of the control group kidney tissue and both distal and proximal tubules around it were in normal structure. When compared to the kidney tissues of the control group, an increase in Bowman's space was observed in the kidney tissue of the G100 group. And vacuolar degeneration was observed in the proximal and distal tubule epithelial cells in the cortical region. In the G500 group kidney tissue, an enlargement of the Bowman space of some glomerulus was observed, while a narrowing of the Bowman space was observed in some regions. In addition, casts were showed in some cortical proximal tubule lumens in the kidney tissue of the G500 group (Table 2, Figure 2).

Table 2. Comparison of catalase immunoreactivity between groups.

Cortex and Medulla Cortex	Control	G100	G500
Proximal tubules	++++	++	++
Distal tubules	-	++	-
Mesangial cells	-	-	-
Collecting ducts	+	++++	-
Medulla			
Proximal tubules	++++	++++	++++
Distal tubules	-	-	-
Collecting ducts	-	++++	-
Vascular endothelium	-	-	-

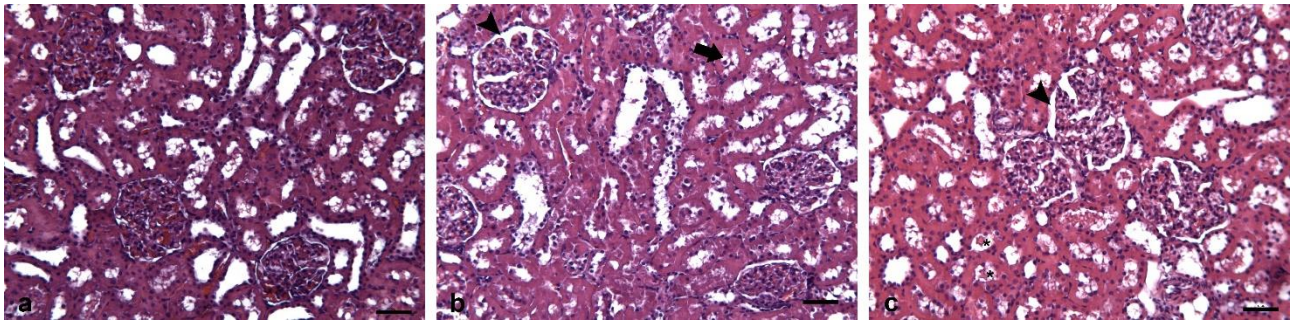

Figure 2. Rat kidney tissues. a) Control group, b) G100 group, c) G500 group. Arrowhead: Bowman spaces, Black arrow: Vacuole degeneration, p: Proximal tubules, d: Distal tubules, *: Casts. Hematoxylin & Eosin Staining (H&E). Bar 50 µm.

Immunohistochemical results

Catalase immunoreactivity was examined separately in cortex and medulla regions of rat kidney tissues in all groups. In the cortex region; In the control group, there was strong (++++) catalase immunoreactivity in proximal tubules and very weak (+) catalase immunoreactivity in collecting ducts, but no catalase immunoreactivity in distal tubules and mesangial cells. In the G100 group, there was no catalase immunoreactivity in mesangial cells, but there was weak (++) catalase immunoreactivity in proximal and distal tubules, as well as strong (++++) catalase immunoreactivity in collecting ducts. In the G500 group, weak (++) catalase immunoreactivity was observed in the proximal tubules. No catalase immunoreactivity was observed in distal tubules, mesangial cells and collecting ducts (Figure 3).

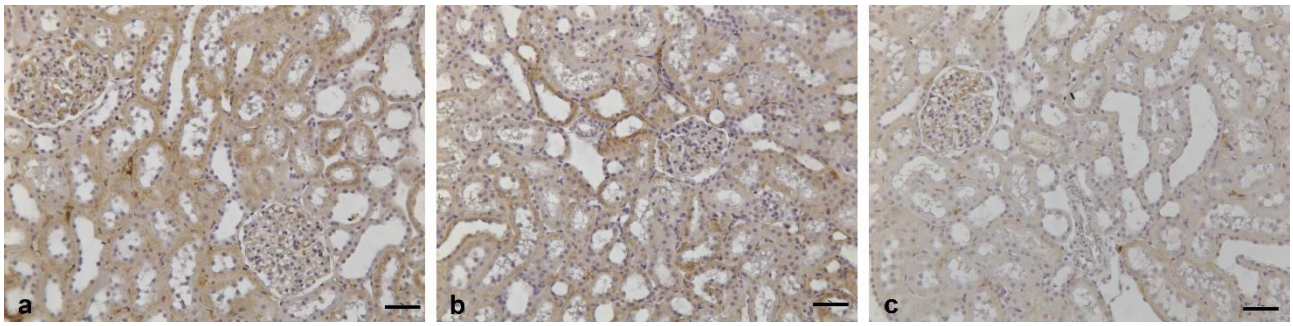

Figure 3. Rat kidney (cortex) tissue. a) Control group, b) G100 group, c) G500 group. Arrow (m): Mesangial cell, c: Collecting ducts, p: Proximal tubules, d: Distal tubules. Catalase immunoreactivity. Bar: 50 µm.

When the medulla region of the kidney tissue of all groups was examined. In the control and G500 groups, there was strong (++++) catalase immunoreactivity in the proximal tubules, but no catalase immunoreactivity was observed in the distal tubules and collecting ducts. In the G100 group, there was strong (++++) catalase immunoreactivity in the proximal tubules and collecting ducts. There was no catalase immunoreactivity in distal tubules. No catalase immunoreactivity was observed in the vascular endothelium in the kidney tissues of all examined groups (Figure 4).

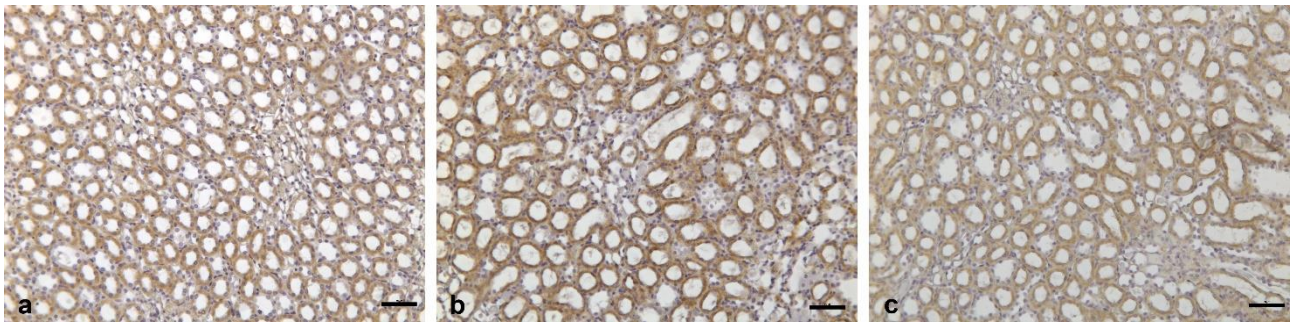

Figure 4. Rat kidney (medulla) tissue. a) Control group, b) G100 group, c) G500 group. c: Collecting ducts, p: Proximal tubules, d: Distal tubules Catalase immunoreactivity. Bar: 50 µm.

DISCUSSION

The use of plants or components obtained from plants is increasing day by day due to the natural antioxidants they contain. Along with this increase, the incidence of some side effects increases due to errors caused by use (for example, misdiagnosing the plant, high doses, long-term use). As a result of such adverse reactions, serious side effects have been reported in important organs such as the liver, kidney, heart and brain (Shaw, 2010; Shaw et al., 2012).

Products from plants may protect against kidney damage by increasing endogenous antioxidants (Palipoch, 2013). In the study conducted with *Oenanthe javanica* extract, almost no kidney damage was found in glomeruli and kidney tubules in all groups (Tae et al., 2014). In addition, it has been reported that administration of 600, 750 and 900 mg/kg *Moringa stenopetala* extract did not show significant histopathological changes in mouse kidney compared to the control group (Ghebreselassie et al., 2011). Many herbs can cause damage to kidney tissue depending on the increase in the applied dose (Parveen et al., 2010; Paul and Didia, 2012). For example, it has been shown that significant cytoplasmic vacuolation occurs in the renal tubular cells of rats administered 50 mg/kg *Teucrium polium* (Khleifat et al., 2021). It has been reported that *Moringa oleifera* methanolic extract, 3.5mg/kg extract in Guinea pig kidney tissue has a normal histological structure in the kidney tissue, and 4.6 mg/kg extract application causes deterioration in the distal convoluted tubules and glomerulus in the kidney sections, and occlusion of the interlobular vein. In addition, it has been reported that the application of 7.0 mg/kg extract caused interstitium infiltration with inflammatory cells in the kidney sections, disruption in the proximal convoluted tubule and glomerulus, and formation of tubular lumina containing amorphous eosinophilic material (Paul and Didia, 2012).

It has been reported that the ginger plant is protective against kidney damage caused by lead, iron doxorubicin, gentamycin, metalaxyl. In addition, ginger rhizome extract has been shown to play a protective role against kidney damage caused by diabetes by improving oxidative stress, inflammation, and apoptosis (Ademiluyi et al., 2012; Ajith et al., 2008; Al

Hroob et al., 2018; Gholampour et al., 2017; Reddy et al., 2014; Sakr et al., 2011).

Measurement of glomerulus diameter in kidneys is important in the histopathological evaluation of various diseases (Kotyk et al., 2016). Therefore, kidney glomerulus diameters of the study groups were evaluated. It has been reported that ethephon, which is used to develop nephrotoxicity experimentally, causes a decrease in the glomerulus diameter. In addition, it has been shown that the application of fresh garlic extract together with the ethephon substance increases the decreasing glomerulus diameter (Albrakati, 2021). In our study, there was an increase in glomerulus diameters in the experimental groups. And this increase is thought to be due to the antioxidant properties of ginger.

In our study, histopathological changes in kidney tissue of two different doses of ginger essential oil (100 and 500 mg/kg/day) were investigated on healthy rats without any chemical or disease formation. In the control group kidney tissue, renal corpuscles containing glomerulus in the cortex region and tubules with distal and proximal folds around them were found to be in normal structure. An increase in Bowman's space was observed in the kidney tissue of the G100 group, and vacuolar degeneration was observed in the proximal and distal tubule epithelial cells in the cortical region. In the kidney tissue of the G500 group, an increase in Bowman's space was observed in some regions and narrowing in some regions. In addition, adhesions were observed between the parietal and visceral leaves of Bowman's capsule in some glomeruli. In addition, casts were seen in some cortical proximal tubule lumens in the G500 group.

In previous studies, it has been reported that catalase immunoreactivity is seen in tubular cells, especially in the distal tubules in control group (Lee et al., 2019). Tae et al., (2014) reported that catalase immunoreactivity is weak in the distal tubules of the kidney in control group. Bakir et al., (2017), while catalase immunoreactivity was not observed in the distal tubules, a strong catalase immunoreactivity was observed in the proximal tubules in control group. In our study, catalase immunoreactivity in the kidney tissues of the control group was examined in the cortex and medulla regions. Consistent with the study of Bakir et al., (2017) catalase immunoreactivity was strong in the proximal tubules in the cortex region of the control group. When the medulla regions were examined, strong catalase immunoreactivity was observed in the proximal tubules in all groups.

It has been reported that administration of *Populus tomentiglandulosa* extract significantly increases the immunoreactivity intensity of catalase in the kidney (Lee et al., 2019). It has been suggested that application of *Ocimum basilicum* leaf extract increases the immunoreactivity concentration of catalase in the kidney in acetaminophen-induced kidney damage in mice (Karaali et al., 2018). It has been shown that the intensity of catalase immunoreactivity in kidney tissue of *Oenanthe javanica* extract increased approximately 2-fold compared to the control group (Tae et al., 2014). In another study, it was reported that the immunoreactivity of catalase in the kidney medulla of mice administered *Onosma nigricaule* was quite weak. It has also been reported that catalase immunoreactivity was not seen in the distal tubules. It has been shown that catalase immunoreactivity is quite intense in the renal cortex and proximal tubules (Bakir et al., 2017).

In our study, weak catalase immunoreactivity was observed in the cortex region, proximal tubules and distal tubules, and strong catalase immunoreactivity was observed in the collecting ducts in the G100 group, while weak catalase immunoreactivity was observed only in the proximal tubules in the G500 group. When the medulla regions of the kidney tissues of the rats in all groups were examined, strong catalase immunoreactivity was observed in the proximal

tubules in all groups, while strong catalase immunoreactivity was observed in the collecting ducts only in the G100 group. No catalase immunoreactivity was observed in the vascular endothelium and distal tubules in the kidney tissues of all studied groups.

CONCLUSION

As a result, in this study, it was observed that the essential oil obtained from the ginger plant, which has been used as a spice for many years, causes some damage to the kidney tissue, and at the same time, it reduces the release of catalase, which is one of the endogenous enzymatic antioxidants, with the increase in the dose.

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Ethical statement or informed consent: This study was carried out at Kafkas University Research Animals Application Center. This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine, Kafkas University (KAU-HADYEK, Ref No:2021/035 Date: 25.03.2021).

Author Contributions: YYA, MM and MÖ contributed to the project idea, design and execution of the study. ŞYA, HA and AG contributed to the acquisition of data. MM and HA analyzed the data. YYA and TŞ drafted and wrote the manuscript. HA and MÖ reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

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

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Investigation of cytogenetic, electrophoretic, histopathological and biochemical effects of walnut (*Juglans regia* L.) leaf extract in rats experimentally induced diabetes by streptozotocin

ABSTRACT

In this study, the effects of *Juglans regia* L. (JR) leaf extract on histological damage and cytogenetic, electrophoretic, and biochemical parameters in the liver and kidney tissues of diabetic rats were investigated. In the study, 60 male rats (*Sprague-Dawley*) were separated into six groups. According to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) data, it was determined that there were increases and decreases in different serum protein expressions because of the treatment of JR leaf extract in the diabetes group. In the kidney tissue of the diabetes group, an increase in volume in the glomeruli and narrowing of the Bowman's space as well as thickening of the walls of tubules were detected. Vacuolization and shedding were observed in the epithelial cells of tubules in the cortical regions in kidney. In diabetic + JR extract groups administered JR leaf extract at doses of 250 mg/kg and 500 mg/kg, serum AST and ALT levels were reduced compared to the diabetic group. Diabetic rats' livers showed spotted necrosis and fibrosis in the portal area, biliary tract proliferation, mild inflammation, increased vascularization, unicellular necrosis in hepatocytes, and sinusoidal dilatation. The JR leaf extract group did not exhibit these problems. JR leaf extract influences reducing hepatotoxic and oxidative damage due to diabetes and increasing the level of antioxidant enzymes. As a result, it was concluded that the application of JR leaf extract may have a protective effect against damage caused by diabetes.

Keywords: Diabetes, *Juglans regia* L., kidney, liver, walnut

INTRODUCTION

Diabetes is a fatal disease that affects 285 million people worldwide and is a chronic metabolic condition that disrupts carbohydrate, protein, and lipid metabolism (Giacco and Brownlee, 2010). Therefore, early diagnosis is important and patients need to be treated appropriately and effectively. There are two types of diabetes: Type 1 (insulin dependent) and Type 2 (non-insulin dependent). Type 1 diabetes is an autonomic disease that appears suddenly in childhood. It is characterized by the autoimmune destruction of B cells. Type 2 diabetes occurs with insulin resistance and B cell dysfunction. It occurs in children and adults and is thought to be associated with obesity (Hoogwerf, 2020).

Walnut, which is widely available in the world, is traditionally a plant of great importance. Green walnuts, shells, kernels, bark, seeds, and leaves are widely used in the cosmetic and pharmaceutical industries (Stampar et al., 2006). While walnut leaf is used in traditional medicine in the treatment of venous insufficiency and hemorrhoid symptoms, the plant's antidiarrheal, anthelmintic, and antidiabetic properties are also used.

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Research Article

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A high amount of ellagic acid was found in the analyses made on the extract obtained from the leaf of the walnut (Ozer et al., 2007). The dried parts of walnut leaves are widely consumed in some European and Asian countries as tea. Green bark and leaf parts are rich in phenolic substances and flavonoids. These substances provide a protective effect against degenerative diseases by preventing oxidative stress and macromolecular oxidation. It also shows anti-carcinogenic properties with its free radical scavenging effects (Pereira et al., 2007). The most well-known active ingredient in walnuts is juglone (5-hydroxy-1,4-naphthoquinone), which is found in excess in young green leaves. This substance has very strong antioxidant and antimicrobial properties (Yiğit et al., 2009). Studies have shown that the antioxidant effects of the extracts obtained from plants are directly related to the phenolic content of the plant, and this feature does not change even after the plant has been stored for years (Halvorsen et al., 2002). Diabetes occurs with the insufficiency of insulin secretion of the pancreatic gland, which is characterized by hyperglycemia and the impairment of the response of the relevant tissues to insulin (Lipinski, 2001).

Mohammadi et al., (2011) found that due to the phenolic compounds in walnut leaf extract, such alkaloids, flavonoids, and saponins, it has potential in the control of type I diabetes.

In this study, it was aimed to define the cytogenetic, electrophoretic, histopathological, and biochemical effects of walnut leaf extract in rats with diabetes experimentally induced by streptozotocin.

MATERIALS AND METHODS

Walnut leaf extract

In June 2020, walnut leaf samples were collected near Kars-Kağızman and identified at Kafkas University's Department of Botany. The samples were dried in the dark, sunlight-free laboratory. The samples were dried in a sunlight-free laboratory, ground with an IKA A11 (Staufen, Germany) grinder, and 50 g of the ground material was placed in a 500 mL Soxhlet extractor. Ethanol was used as a solvent, and extraction was performed for about 10 hours (10-15 siphons) until it became clear. The liquid extracts obtained were filtered through a <2 µm pore size blue band filter paper (Grade 589/3, Whatman, UK), and the solvents were evaporated at 50ºC with a rotary evaporator. The resulting walnut leaf extract was weighed with 0.1 mg sensitivity, stored at +4ºC, and prepared for further study (Gundogdu et al., 2016; Uluman and Aksu-Kılıçle, 2020).

Animals and experimental design

In this study, we used 60 male rats (*Sprague Dawley*), aged 2-3 months, which had never been mated or used in previous research. The rats were kept in standard cages at 22 ± 2ºC, under a 12-hour light-dark cycle, and were fed ad libitum with pellet feed and drinking water for a one-week acclimatization.

The rats were divided into six groups: (G-I) Control group (n = 10), (G-II) Diabetic group (n = 10), (G-III) 250 mg/kg JR leaf extract group (n = 10), (G-IV) 500 mg/kg JR leaf extract group (n = 10), (G-V) D+250 mg/kg JR leaf extract group (n = 10), and (G-VI) D+500 mg/kg JR leaf extract group (n = 10). Diabetes was induced in the Diabetic group using 50 mg/kg streptozotocin (STZ) administered intraperitoneally, and fasting blood glucose levels were measured 3 days after STZ application from tail veins using glucometers. Rats with a fasting blood glucose level of 250 mg/dL were accepted to have diabetes (Yapislar et al., 2022). The JR leaf extract was administered orally for 21 days in the treatment groups, with dosages based on previous research (Çelik and Koç, 2019).

Determination of mitotic index

The femoral bones of rats were removed, with one used for the mitotic index and the other for the micronucleus test Bone marrow from the femur was collected in a centrifuge tube with fetal calf serum. Mitotic activity was assessed following Preston's method, with metaphase preparations stained using 10% Giemsa for 10 minutes. Under an Olympus CX21 microscope, 1000 cells were randomly counted from each animal sample at 1000x magnification to determine mitotic activity. Cell numbers in the metaphase stage and their percentages were recorded (Preston et al., 1987).

Detection of micronucleus frequency

The femur bone was cut at both ends. Bone marrow was transferred to a centrifuge tube containing 3 mL of calf serum with the help of an injector. Then, it was prepared and stained using a method developed by Schmid and adapted to our laboratory's working conditions. Under an Olympus CX21 brand light microscope at 1000 magnification, 2000 pieces of PCE were randomly selected from each preparation, and the number of MNPCEs within them was determined, and their percentages were calculated (Schmid, 1975).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Sds-Page) method

Blood samples were centrifuged for 10 minutes at + 4ºC and 3000 rpm and their sera were removed and kept at - 20ºC until the study period. Protein concentrations of the samples were measured by the biuret method (Eisenthal and Danson, 1993). The SDS-PAGE procedure was performed according to Laemmli, (1970) and O'Farrell, (1975) methods. Bovine albumin (66 kD), egg albumin (45 kD), trypsinogen (24 kD) and lysozyme (14 kD) were used as protein standards in electrophoresis applications. The molecular weights of the proteins were determined according to the method of Weber et al., (1972).

Histopathological procedures

At the end of the experiment, liver and kidney tissue samples were taken from the rats by cervical dislocation under anesthesia. Tissue samples were fixed in 10% formalin and blocked-in paraffin after routine histological procedures. Crossman's triple staining technique and Hematoxylin Eosin staining were applied to the 5-7 µm sections taken from the blocks to show the general structure of the tissue (Luna, 1968). To observe the plasma cells and pyroninophilic cells, tissue samples were also fixed in alcohol-formol solution for 48 hours and methyl green pyronin staining was performed on the sections.

Biochemical analysis method

Serum total antioxidant status (TAS), total oxidant status (TOS) (Rel Assay Diagnostics, Clinical Chemistry Solutions, Türkiye) (Erel, 2004). aspartate aminotransferase (AST) (EASTR-100; EnzyChrom™ Aspartate Transaminase Assay Kit, USA), alanine aminotransferase (ALT) (EALT-100; EnzyChrom™ Alanine Transaminase Assay Kit, USA), quantities were determined spectrophotometrically using the commercial kit. AST and ALT values were calculated by measuring absorbances at 340 nm at 5^{th} and 10^{th} minutes as specified in the kit procedure.

Statistical analyses

The Kolmogorov-Smirnov test was utilized to assess the normality of the group data. Data determined to be normally distributed were tested with ANOVA followed by post-hoc Tukey HSD test. P values below 0.05 ($P < 0.05$) were considered statistically significant. Results are presented as mean ± standard deviation (SD). SPSS 22 (IBM Corp., New York, USA) was used for all calculations.

RESULTS

Cytogenetic results

Statistical analysis revealed a significant increase in MN numbers for the diabetic control

group and other diabetic groups (P < 0.001). However, MN numbers did not differ significantly in groups treated with walnut extract alone (P > 0.05). However, it was determined that the MN numbers of the groups in which diabetes was created and walnut extract was applied decreased compared to the diabetes group (P < 0.001) and this decrease was proportional to the dose of walnut extract applied. Mitotic index values decreased in diabetic groups (P < 0.001) but started to increase when different doses of walnut extract were applied (P < 0.001) (Table 1 and 2; Figure 1 and 2).

Table 1. Control and experimental groups mitotic index data.

Group	Number of subjects	Total cell count	Number of interphase cells	Number of metaphase cells	Group Mean ±SD	Metaphase cell ratio average (%)
Negative control	10	10000	9685	315	31.5 ±1.65[a]	3.15
D	10	10000	9833	167	16.7 ±2.06[d]	1.67
250 mg/kg JR	10	10000	9684	316	31.6 ±1.26[a]	3.16
500 mg/kg JR	10	10000	9678	322	32.2 ±1.99[a]	3.22
D+250 mg/kg JR	10	10000	9726	274	27.4 ±1.07[b]	2.74
D+500 mg/kg JR	10	10000	9766	234	23.4 ±0.97[c]	2.34

[a-d]: The difference between groups in columns with different character is significant (P<0.001). D: Diabetic, JR: *Juglans regia* L.

Table 2. Control and experimental groups micronucleus data.

Groups	Total PCE	MNPCE	MNPCE (%)	Mean ± SD
NK	20000	58	0.29	5.80 ± 1.14[d]
Diabetes	20000	257	1.28	25.70 ± 1.49[a]
250 mg/kg JR	20000	56	0.28	5.60 ± 0.84[d]
500 mg/kg JR	20000	59	0.29	5.90 ± 1.45[d]
D+250 mg/kg JR	20000	227	1.13	22.70 ± 1.49[b]
D+500 mg/kg JR	20000	159	0.79	15.90 ± 1.20[c]

The MN numbers of the diabetic control group and other diabetic groups increased statistically significantly (P < 0.001), but there was no difference in the MN numbers of the groups that only applied walnut extract (P > 0.05). [a-d]: The difference between groups in columns with different character is significant. D: Diabetic, JR: *Juglans regia* L.

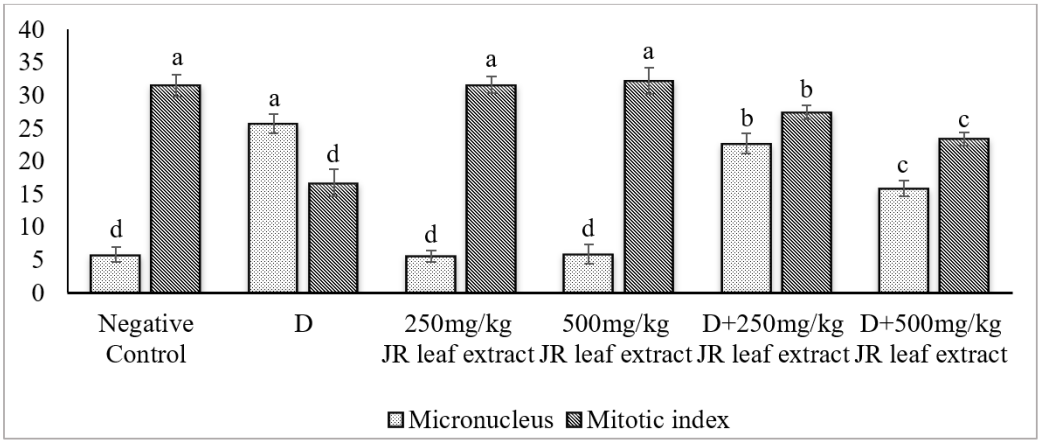

Figure 1. Micronucleus and mitotic activity values of control and treatment groups. D: Diabetic, JR: *Juglans regia* L.

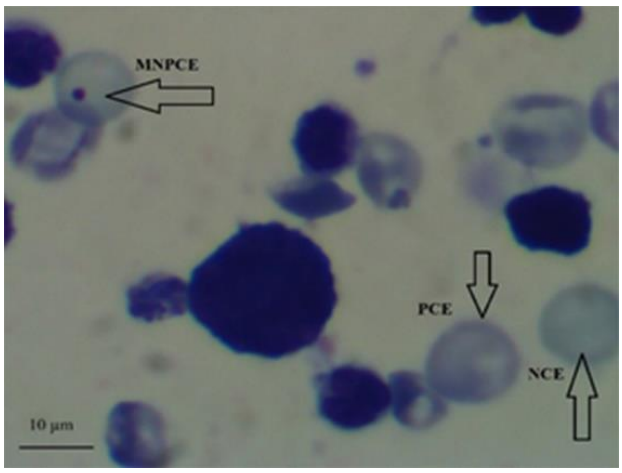

Figure 2. Polychromatic erythrocyte with micronucleus (MNPCE), Polychromatic erythrocyte (PCE) and Normochromatic erythrocyte (NCE) image.

Electrophoretic results

In the electropherogram obtained from sodium dodecyl sulphate polyacrylamide gel electrophoresis of serum samples, it was observed that the protein expressions of 13 kD, 40 kD and 45 kD increased, while the protein expression of 24 kD decreased in the samples belonging to the animals in the diabetic group. 13 kD, 16 kD, 40 kD, 45 kD, 56 kD, 82 kD and 103 kD protein expressions were increased in diabetic animals treated with JR leaf extract, and 24 kD protein expression decreased in diabetic animals treated with 500 mg/kg JR leaf extract (Figure 3).

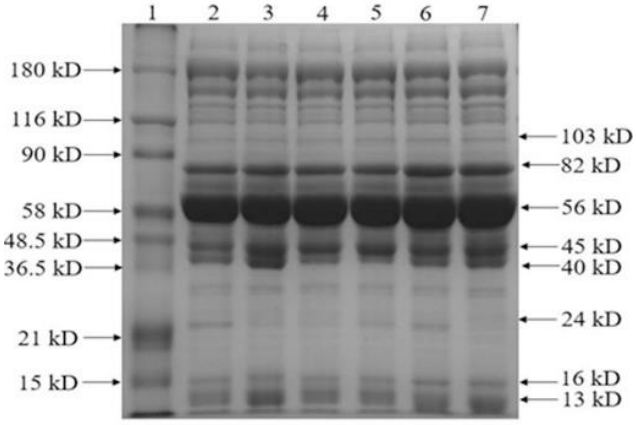

Figure 3. Electropherogram obtained from SDS-PAGE of animals in control and administration groups. 1: Standard proteins, 2: Negative control, 3: D, 4: 250 mg/kg JR, 5:500 mg/kg JR, 6: D+250 mg 7kg JR, 7: D+500 mg/kg JR. D: Diabetic, JR: *Juglans regia* L.

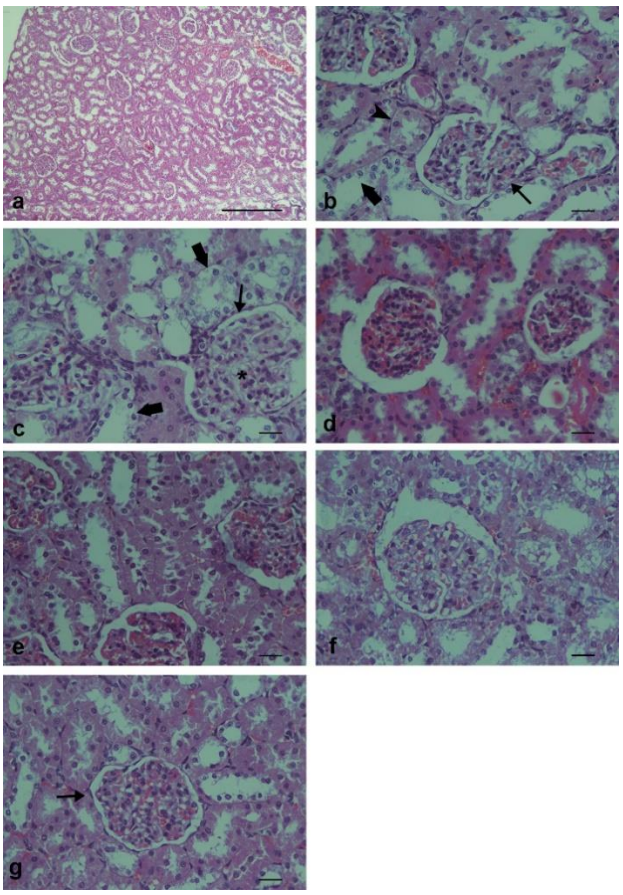

Figure 4. Rat kidney tissue. a: Control group. Crossman's triple staining, 100 µm, b: Diabetes group, increase in Glomerus volume (asterisk), narrowing in Bowman's space (arrow), thickening of the walls of the distal (thick arrow) and proximal tubules (arrowhead), c: Diabetic group, Increase in glomerus volume (asterisks) and Bowman area narrowing (arrow), Vacuolization in epithelial cells of proximal and distal tubules (thick arrow), d: D+250 mg/kg JR leaf extract group, e: D+500 mg/kg JR leaf extract group, f: 250 mg/kg JR leaf extract group, g: 500 mg/kg JR leaf extract group, constriction in Bowman capsules in glomeruli (arrow). H&E staining, 10µm. D: Diabetic, JR: *Juglans regia* L.

Histopathological results

Normal histological structure was detected in kidney tissues from the G-1, G-III, G-V, and G-VI groups. In the kidney tissue of diabetic rats, an increase in glomerulus volume, narrowing in Bowman's space, and thickening of the walls of the distal and proximal tubules were detected. Vacuolization and epithelial cell shedding were observed in the epithelial cells of the proximal and distal tubules. The narrowing of the Bowman capsules of the glomeruli was detected in the G-IV (Figure 4).

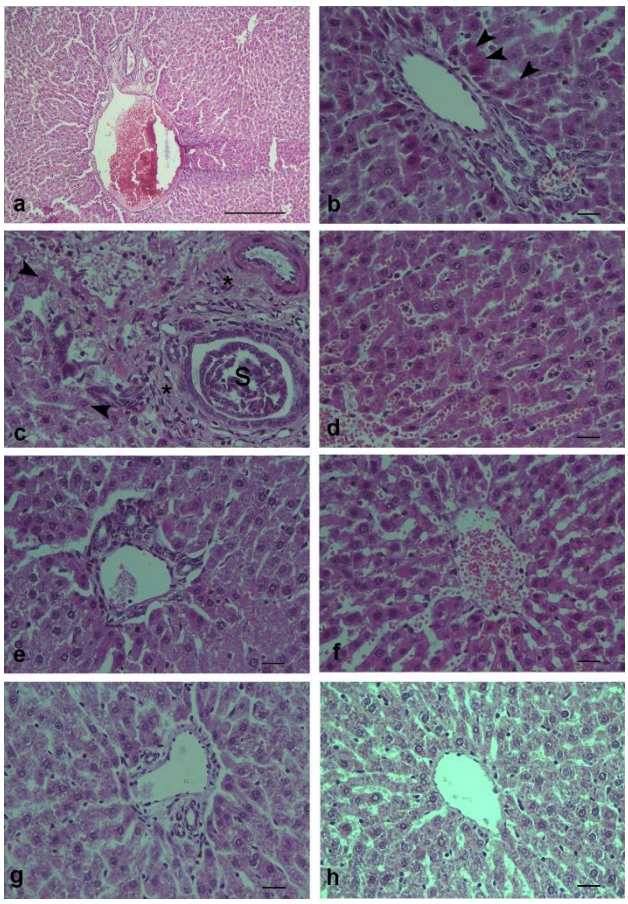

Figure 5. Rat liver tissue. a: Control group. Crossman's triple staining, 100µm, b: Diabetes group, Spotty necrosis areas (arrowheads), c: Diabetes group, Proliferation in bile ducts (S), portal area fibrosis (stars), mild inflammation, increased vascularity and single cell necrosis in hepatocytes (arrowheads), d: Diabetic group, Dilatation in sinusoids, e: D+250 mg/kg JR leaf extract group, f: D+500 mg/kg JR leaf extract group, Dilatation in sinusoids, g: 250 mg/kg JR leaf extract group, h: 500 mg/kg JR leaf extract group. H&E staining, 10µm. D: Diabetic, JR: Juglans regia L.

The liver tissue of rats in the G-I, G-III, G-IV, and G-V groups was shown to have normal histological structure. In the liver of rats with diabetes, spotty necrosis and fibrosis in the portal area, proliferation in the bile ducts, mild inflammation, increased vascularization, single cell necrosis in hepatocytes, and dilatation in sinusoids were observed. In the G-VI group, dilatation was detected in the sinusoids (Figure 5).

Biochemical results

Due to the application of JR leaf extract at doses of 250 mg/kg and 500 mg/kg, the liver AST ($P < 0.001$) and ALT ($P < 0.05$) enzyme levels were increased in rats with diabetes with streptozotocin compared to the G-I group. It has been determined that it has a decreasing effect on diabetes-related hepatotoxic damage, thus reducing the levels of this enzyme. However, the decrease in enzyme levels did not show a statistical difference compared to the G-I group ($P > 0.05$) (Table 3).

Antioxidant enzyme level increased against oxidative damage caused by diabetes ($P < 0.001$), but no statistical difference was found in oxidant enzyme level ($P > 0.05$). JR leaf extract application was found to increase the antioxidant enzyme level ($P < 0.001$), and the oxidant enzyme level was similar to the G-I group ($P > 0.05$) (Table 3).

Table 3. Liver and antioxidant / oxidant enzyme levels of animals in control and treatment groups.

Groups	AST (U/L)	ALT (U/L)	TAS (mmolTrolox Equiv./L)	TOS (µmol H_2O_2 Equv./L)
Control	136.57 ± 17.06 ^{c}	31.00 ± 5.35 ^{b}	0.46 ± 0.14 ^{c}	17.95 ± 3.63 ^{ab}
Diabetes	188.14 ± 33.14 ^{a}	38.14 ± 6.07 ^{a}	1.27 ± 0.37 ^{a}	19.58 ± 3.94 ^{a}
250 mg/kg JR	138.71 ± 19.21 ^{bc}	31.86 ± 3.98 ^{ab}	1.36 ± 0.28 ^{a}	17.99 ± 3.40 ^{ab}
500 mg/kg JR	135.71 ± 13.82 ^{c}	32.29 ± 2.63 ^{ab}	0.84 ± 0.20 ^{b}	13.18 ± 2.94 ^{b}
D + 250 mg/kg JR	168.14 ± 7.24 ^{ab}	37.29 ± 2.29 ^{ab}	1.07 ± 0.18 ^{ab}	17.46 ± 3.49 ^{ab}
D + 500 mg/kg JR	161.71 ± 12.75 ^{abc}	35.71 ± 3.15 ^{ab}	1.08 ± 0.20 ^{ab}	17.27 ± 5.66 ^{ab}

AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; TAS: Total antioxidant status; TOS: Total oxidant status. $^{a-d}$: The difference between groups in columns with different character is significant. D: Diabetic, JR: *Juglans regia* L.

DISCUSSION

Diabetes mellitus is one of the diseases with high rates of mortality and morbidity in the World (Memisogullari, 2005). In addition, diabetes hyperglycemia can both increase the formation of free radicals and cause the endogenous antioxidant defense system to deteriorate (Saxena et al., 1993). Superoxide radical, hydroxyl radical, hydrogen peroxide, nitric oxide, and transition metals are blamed for oxidative stress in diabetes (Memisogullari, 2005). Although some other living things, especially humans, have antioxidant defense systems that protect and restore oxidative damage caused by free oxygen radicals, these systems may fail to prevent oxidative damage (Yoshikawa et al., 2000). Oxidative stress is an important tool in the development of chronic complications (neuropathy, nephropathy, and retinopathy) in diabetes (Lipinski, 2001; Memisogullari, 2005). Today, the therapeutic potential of many natural and nanotechnology-based compounds against diabetes is being investigated (Caylak and Nur, 2024a; Caylak and Nur, 2024b; Deprem et al., 2015; Yıldız et al., 2015).

Walnuts are cancer-preventing and also contain polyphenols that destroy free radicals and have metal chelate (binding) activity qualities. For example, ellagic acid, a polyphenol, is known for its anticancer properties as well as enhancing the immune system (Cerda et al., 2005). Walnut leaves have high anti-diabetic properties due to the highly hydrophilic components in the *Juglans regia* leaf extract (Forino et al., 2016).

In a study, the cholesterol profile of type II diabetes patients who ate 30 g of walnuts per day improved. It is stated that the reason for this is probably due to the high content of omega-3 acids in walnuts (Tapsell et al., 2004). Omega-3 fatty acids also play a key role in the fluidity of cell membranes, and membrane stiffness, which develops in the absence of these fatty acids, negatively affects transport functions, receptor interactions and numbers. For example, while the increase in membrane fluidity increases the number of insulin receptors, the solidification of the membranes causes a decrease in the number of these receptors and leads to diabetes and insulin resistance (Konukoglu, 2008).

Genotoxicity was investigated by micronucleus tests in rat bone marrow cells and peripheral blood in which experimental diabetes was induced by STZ. Arora et al., (2005) reported that extracts obtained from three medicinal plants (*Acacia nilotica*, *Juglans regia* and *Terminalia chebula*), using vitotox and comet assay tests, showed antimutagenic properties at certain concentrations. An anti-apoptotic effect of walnut mallow extract against UVB-induced apoptotic responses has been reported. This protective effect is due to the high amount of flavonoid and antioxidant content in the extract (Muzaffer et al., 2018). We think that the walnut leaf extract used in our study may have genotoxicity properties similar to the above studies and the decrease in micronucleus number may be due to the antimutagenic properties of the extract.

While it was determined that the total protein levels in the brain and kidney tissues of rats treated with carbon tetrachloride increased and decreased in the liver tissue, it has been reported that due to the application of *Juglans regia* L. extract with CCl_4, due to the phytochemical compounds it contains, the total protein levels in the brain and kidney tissues decreased and increased in the liver tissue (Aydin et al., 2015). In another study, it was reported that serum total protein levels decreased in rats treated with CCl_4, and total protein levels increased due to the application of *Juglans regia* L. extract (Hosseini et al., 2018). In this study, an increase and a decrease in different protein expressions were found in the diabetic groups in the serum protein expressions and in the diabetic groups treated with JR extract.

Diabetes have effects such as hypertrophy in the glomeruli of rat kidneys, enlargement of the mesangium, glomerular sclerosis, thickening of the glomerular basement membrane, enlargement of the kidneys, and increased permeability through pores (Li et al., 2019). In addition, with STZ injection, it was found that the rate of kidney structure deterioration and apoptosis was more intense, but renal damage was significantly reduced by treatment with paricalcitol (Liu et al., 2019). In another study, walnut leaf application removes the damage caused by diabetes in kidney and liver tissues (Mollica et al., 2017). In our study, an increase in glomerular volume, narrowing of Bowman's space and thickening of the walls of distal and proximal tubules were detected in the kidney tissue of diabetic rats. In addition, vacuolization and epithelial cell shedding were observed in epithelial cells of proximal and distal tubules. The liver tissue of diabetic rats has degeneration and necrosis in hepatocytes, especially the cytoplasm of hepatocytes in the periacinal region has single or multiple small vacuoles, enlargement in the portal spaces, fibrosis, bile duct proliferation and inflammatory cell infiltration, but it has been reported that these damages are significantly reduced with the application of acorn extract (Yaman and Doğan, 2016). Diabetic groups showed apoptotic nuclei, hydropic degeneration and central vein occlusion in liver tissue (Asokan et al., 2019). In our study, the liver of diabetic rats showed punctate necrosis and fibrosis in the portal region, proliferation of bile ducts, mild inflammation, increased vascularization, single cell necrosis in hepatocytes and dilatation of sinusoids.

There are many studies on the use of natural compounds as preservatives against hepato and nephrotoxic agents (Deveci et al., 2021; Nur et al., 2023). The application of *Juglans regia* extract in rats with liver damage with carbon tetrachloride reduces liver enzyme levels by showing hepatoprotective effect, but also increases antioxidant enzyme activities such as superoxide dismutase and catalase (Eidi et al., 2013). In a study in which *Juglans regia* L. leaf extract was applied to diabetic patients, the ALT level, which was high before the extract application, decreased significantly (Rabiei et al., 2018). AST and ALT enzyme levels decreased significantly due to the application of the ethanolic extract of the walnut fruit core to diabetic rats (Ghiravani et al., 2016). The application of *Juglans regia* extract against the oxidative damage caused by isoprenaline showed quite important antioxidant properties (Sharma et al., 2021). Similarly, Rusu et al., (2020) stated that the *Juglans regia* extract significantly reduced the level of reactive oxygen species. In the present study, applying different doses of JR leaf extract to diabetic rats decreased serum AST and ALT levels, as well as increased TAS levels and decreased TOS levels in rats by showing protective properties against liver damage due to diabetes.

CONCLUSION

As a result of this study, walnut leaf extract has an effect on reducing kidney damage due to diabetes, hepatotoxic and oxidative damage, increasing the antioxidant enzyme level, and also decreasing the increase in micronucleus frequency and increasing the mitotic index.

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

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Incidence of some reproductive problems following a voluntary waiting period in cows on family-type cattle breeding farms in Van Province

Research Article

ABSTRACT

This study aimed to identify some reproductive problems in cows on family-type cattle breeding farms in rural areas of Van province using transrectal ultrasonographic examination. The study included 196 cows (at least 50 days postpartum and non-pregnant) of various breeds and ages. Postpartum days and current body condition scores (BCS) were recorded. Genital organs of each animal were examined twice using rectal palpation and ultrasonography, at intervals of 7-11 days. Cows with a detected corpus luteum in their ovaries were classified as cyclic, while those without were classified as non-cyclic, also categorized according to anovulatory anestrus types. The average number of days postpartum for cows was determined to be 94.42 days. Based on ovarian examination results, 51.03% (n = 99) of the cows were classified as cyclic, while 48.97% (n = 95) were classified as non-cyclic. According to anovulatory anestrus types, 15% (n = 15) of the cows were classified as Type I anovulatory anestrus, 26% (n = 26) as Type II anovulatory anestrus, 54% (n = 54) as Type III anovulatory anestrus, and 5% (n = 5) as Type IV anovulatory anestrus. In addition, pyometra was diagnosed in two of the cows. In the study, it was concluded that the cows in family-type cattle breeding farms in rural areas of Van Province were delayed in starting postpartum cycle activities and high anestrus rates may be primarily due to nutrition. It was concluded that the widespread use of ultrasonography in family-type cattle breeding farms would have significant and positive effects on the reproductive success of cows and sustainable animal breeding.

Keywords: Anovulatory anestrus, dairy cattle, infertility, reproduction, theriogenology

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INTRODUCTION

Livestock plays a crucial role in the social and economic fabric of a country. It has a wide range of functions, not only limited to animal food production but also creating employment, contributing to national income, supplying raw materials to the industry, reducing unemployment in rural areas, and preventing migration to city centers. Especially with the global population increasing, sustaining these roles requires maintaining high-performance animal husbandry practices (Luperto Telli, 2017).

Fertility is a critical factor that affects the success of cattle breeding, which should be kept at an optimal level and is important for the sustainability of the sector (Alcay et al., 2022; Ata, 2013; Koca et al., 2023). In cattle breeding, focusing on calf and heifer productivity is the key to ensuring economical production in cattle farms. In order to ensure economical production, the primary aim is to obtain calves from cows within the optimum period of time. Otherwise, it is not possible to make

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a profit from this sector (Ata, 2013; Cevik, 2023; Koca et al., 2024; Yalcın, 2008). For cattle farms to carry out profitable livestock farming, the life cycle of cows, encompassing parturition, lactation, pregnancy, dry period, and calving to conception, must unfold within a defined timeframe (Noakes, 2009; Ocal, 2007). For pregnancy to occur again after parturition, several crucial processes must occur physiologically, including the completion of uterine involution in the postpartum period, the elimination of bacterial contamination, the regeneration of the endometrium, and the resumption of cyclic activity in the ovaries (Ocal, 2007; Sheldon, 2004).

Negativity and disruptions related to reproduction, by extending the time between calvings, causes a decrease in calf and milk yield throughout a cow's life (Peters and Lamming, 1990). To achieve the goal of having a calf within the optimum period, a cow must become pregnant at the optimal time after the voluntary waiting period. It is understood that a cow should conceive within 80-100 days postpartum, and the postpartum period should not exceed the physiological limit of days (Mwaanga and Janowski, 2000; Rhodes et al., 2003; Roche et al., 2000; Yavas and Walton, 2000). Normally, the interval from calving to first ovulation in dairy cows ranges from 2 to 4 weeks (Crowe 2008; Darwash et al., 1997; McCoy et al., 2006). Dairy cows experiencing postpartum problems are reported to have lower pregnancy rates at the first insemination and require a higher number of inseminations per pregnancy compared to cows with early ovulation (Lamming and Darwash, 1998). Disruptions arising from follicular dynamics and ovulation mechanism in the postpartum period result in follicles on the ovary not entering the follicular wave (inactive ovary), the follicle entering the follicular dynamics atresia without becoming the dominant follicle, the dominant follicle forming but not ovulating and turning into a cystic structure, or the corpus luteum forming after the dominant follicle ovulates showing activity for a long time (Peter et al., 2009; Song et al., 2021).

This study aimed to evaluate reproductive findings detected through ultrasonographic examinations of non-pregnant animals that had completed the voluntary waiting period on family-type cattle breeding farms in Van province, Türkiye. In this way, the objective is to raise awareness about reproductive health in family-type cattle breeding farms in our country. These farms, although small-scale, have a positive impact on local economies by efficiently utilizing natural resources and offer sociological advantages such as reducing rural-to-urban migration. The goal is to support productivity in the livestock sector and contribute to the sustainability of rural livestock farming.

MATERIALS AND METHODS

Animals

The study utilized 196 cows of various breeds and ages, all of which had calved at least once, in rural areas of Van province. The study received ethics committee approval from the Van Yuzuncu Yil University Animal Experiments Local Ethics Committee (Approval no: 2023/11-02).

Examinations of the genital organs

Family-type cattle breeding farms in rural areas of Van province were visited and cows that had completed at least a 50-day voluntary waiting period after birth and were not pregnant were included in the study. The last calving dates of the cows were recorded and the number of days after birth was determined. After each animal underwent a clinical examination and scoring of body condition score (BCS) between 1-5 (Atalay et al., 2019; Yolcu, 2024), their genital organs were examined in detail through rectal palpation and ultrasonography (Honda HS-1500), conducted twice at intervals of 7-11 days.

Cows (n=194) with a detected corpus luteum in their ovaries were categorized as cyclic, whereas those without were classified as non-

cyclic. Also, anovulatory anestrus cows (n=100) were classified according to ovarian findings as specified by Senünver and Nak (2015). Accordingly, cows in which no corpus luteum or dominant follicle was detected in both examinations and no follicular activity (having follicles <5 mm in both examinations) were classified as Type I anovulatory anestrus. Cows in which a dominant follicle is detected on examination (having a follicle up to 8 mm in at least one of the two examinations) but no corpus luteum is detected are classified as Type II anovulatory anestrus. Cows in which ovulatory-sized and larger follicle/follicles are detected during examination, but no corpus luteum is detected in both examinations, are classified as Type III anovulatory anestrus. Cows with persistent corpus luteum detected in both examinations, prolonged luteal phase, and no estrus detected in the anamnesis were classified as Type IV anovulatory anestrus. Cows (n=2) with uterus filled with purulent contents and corpus luteum were accepted as pyometra.

Statistical analysis

The descriptive statistics for continuous variables in the study were expressed as number (n), percentage (%), mean, standard deviation, minimum, and maximum. The SPSS statistical package program (IBM SPSS for Windows, ver.26) was used for calculations.

RESULTS

The study determined that the average number of days after birth for the cows included was 94.42 day (Table 1).

Table 1. The number of days after parturition for the animals included in the study.

Days after parturition	Mean ± Standard deviation	Minimum	Maximum
	94.42±39.01	51.00	280.00

Based on the results of ovarian examination, 51.03% (n=99) of the cows were found to be cyclic, while 48.97% (n=95) were found to be non-cyclic (Fig. 1).

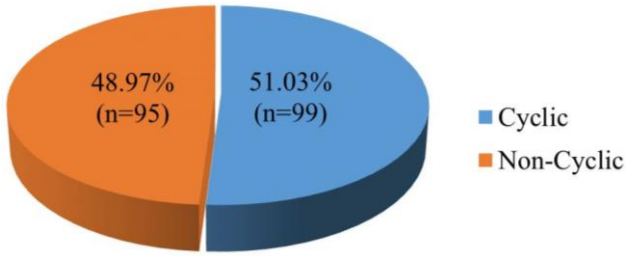

Figure 1. Results of the ovarian examination.

According to the anovulatory anestrus types, 15% (n = 15) of the cows were classified as Type I anovulatory anestrus, 26% (n = 26) as Type II anovulatory anestrus, 54% (n = 54) as Type III anovulatory anestrus, and 5% (n = 5) were identified as Type IV anovulatory anestrus (Table 2). Also, pyometra was diagnosed in two of the cows.

Table 2. Anovulatory anestrus classifications.

Types	Rate
Type I anovulatory anestrus, % (n)	15 (n=15)
Type II anovulatory anestrus, % (n)	26 (n=26)
Type III anovulatory anestrus, % (n)	54 (n=54)
Type IV anovulatory anestrus, % (n)	5 (n=5)

BCS was 2.25-2.75 in Type I and Type II anovulatory anestrus cows, 2.75-3.25 in Type III anovulatory anestrus cows and 2.75-3.50 in Type IV anovulatory anestrus cows.

DISCUSSION

In the study, the average number of days after calving determined in our material, which consisted of cows without a current pregnancy,

was 94.42 day. This rate is notably high for cows that are not currently pregnant, indicating that approximately 50% of the cows exceed the limit reported in fertility parameters (Alacam, 2007). Ensuring that animals become pregnant again within ideal and acceptable periods is essential for the sustainability of family-type cattle breeding farms.

While it is normal or physiological for animals to not have a sexual cycle for a few weeks after parturition, a prolonged absence of a sexual cycle can have a negative effect on their ability to become pregnant again (Gautam et al., 2010; Klm et al. 2012). Reproductive efficiency in dairy cattle is put at risk due to the long anestrus period after parturition (Lucy, 2007). In our study, among animals that completed at least a 50-day voluntary waiting period after birth, the cyclic cow rate was determined to be 51.03% and the non-cyclic cow rate was 48.97%. Large variations in the incidence rates of postpartum anestrus in dairy cattle have been reported, with prevalence at the end of the voluntary waiting period varying between 10- 38% (Darwash et al., 1997; Francos and Mayer, 1988; Rhodes et al., 2003; Walsh et al., 2007). This wide range in prevalence is attributed to the genetics of the cows, their management, climatic conditions, and some other factors (age, stress, uterine infections, etc.)(Mwaanga and Janowski, 2000; Rhodes et al., 2003). Gautam et al., (2010) state that the first postpartum ovulation occurring after the 35th day negatively affects the calving-reconception interval and pregnancy rate. Therefore, they emphasize that the threshold period for the first ovulation after birth is 35 days. Gautam et al., (2010) reported an anestrus rate of 18.6% after 49 days postpartum. In another study (Gautam, 2023) conducted on a dairy cow farm, where 60 days after birth was used as a threshold, the anestrus rate was found to be 61%. In the same study, when 90 days after birth was considered as the threshold, the rate was determined as 31.4%, indicating that approximately 30% of the cows experienced their first estrus between 60 and 90 days after parturition. In our study, we found the rate of non-cyclic cows to be significantly higher than the wide range of 10-38%. However, it is a lower rate than reported in the study by Gautam, (2023). Data from a farm with poor management practices may have contributed to this situation.

The classification of cows with anestrus problems according to ovarian ultrasonographic findings is important for the rational diagnosis and treatment of the underlying physiological condition. According to this classification, in type I anovulatory anestrus, follicles, grow until they emerge from the follicular pool, but there is no deviation. The pathophysiology of this condition is not fully understood, but it is attributed to extreme malnutrition (Wiltbank et al., 2002). We found the incidence of Type I anovulatory anestrus to be 15% (n = 15) in our study. Less than 10% of the dairy population in a normal herd may experience this situation during the postpartum period (Peter et al., 2009). Type I anovulatory anestrus may occur in postpartum animals due to malnutrition and a severe energy deficit, resulting in a lack of LH support necessary to maintain follicular growth and dominance (Jolly et al., 1995). The Type II anovulatory anestrus rate in the study was determined as 26% (n = 26). In this type of anestrus, follicular deviation and growth occur, followed by atresia or regression. In some cases, regression or atresia occurs only after the follicle reaches a dominant state (McDougal et al., 1995). Under normal conditions, when the frequency of LH oscillation waves reaches 1 per hour, the dominant follicle usually completes the final maturation process leading to ovulation (Crowe, 2008). These animals are reported to have low LH pulse frequencies, typically once every 3 to 4 hours (Peter et al., 2009). In type III anovulatory anestrus, there are waiting follicles of ovulatory size or follicles that have exceeded the ovulatory size, the animals detected in this type are 54% (n=54) with ovulatory follicle size exceeded. In this type of anestrus, it may be due

to the insensitivity of the hypothalamus to the positive feedback effect of estradiol produced by the follicles or to the changing follicular response to the gonadotropic support regulated through metabolic hormones (e.g. insulin-like growth factor and insulin) (Peter et al., 2009). The rate of Type IV anovulatory anestrus, which occurs due to the onset of cyclic activity after birth and the prolongation of the luteal phase, was determined as 5% (n = 5). The occurrence of this anestrus may be caused by the absence of an estrogenic dominant follicle at the expected time of luteal regression (Wiltbank et al., 2002), and many factors increase the risk of a prolonged luteal phase, including parity, dystocia, health problems in the first month of lactation, heat stress, and perhaps ovulation immediately after calving (Opsomer et al., 2000). Additionally, uterine infection (Mateus et al., 2002) or pyometra can prolong the lifespan of the corpus luteum (Sheldon et al., 2006).

BCS monitoring in enterprises is important in terms of minimizing reproductive problems, controlling defects in care and feeding, and determining negative energy balance. BCS during calving in cows is one of the most important factors affecting postpartum re-conception. BCS losses occur in animals that are malnourished after calving (Yolcu, 2024). In our study, BCS was 2.25-2.75 in Type I and Type II anovulatory anestrus cows, 2.75-3.25 in Type III anovulatory anestrus cows, and 2.75-3.50 in Type IV anovulatory anestrus cows. Anestrus problems were encountered due to inappropriate care, feeding, and management facilities in the visited farms and that care, feeding, and management should be carried out in accordance with the physiological conditions of the cows for optimum reproductive efficiency.

Implementation of appropriate nutritional strategies is crucial to optimize reproductive performance in dairy cattle, particularly in addressing the anestrus problems mentioned above (Aksoy and Deniz 2024). One of the main reasons for anestrus in animals is the negative energy balance (NED) caused by malnutrition (Okur and Polat 2019). As a result of NED, it causes the formation of endogenous opioids due to the use of the body reserves of the animals, and as a result, the concentration of non-esterified fatty acids (NEFA) increases in the circulation (Erdogan and Ural, 2020; Okur and Polat 2019). High circulating NEFA concentration reduces the frequency of LH release, leading to disruptions in the ovulation mechanism (Okur and Polat 2019). Lack of energy, especially after birth, is considered the most important reason for the late-onset or disruption of ovarian functions (Butler and Smith 1989). For this reason, it is of great importance that the animals are fed with appropriate rations after birth and that estrus monitoring is carried out properly in order to ensure optimum fertility and achieve the specified goals.

It is normal for cows to have a contaminated uterus after parturition (Sheldon et al., 2002). Although the majority of cows can eliminate this bacterial contamination within 5 weeks postpartum, bacterial infection can lead to uterine disease in 10-17% of animals (LeBlanc et al., 2002). In our study conducted on animals that completed at least 50 days postpartum, pyometra was detected in two of cows. The occurrence of uterine infections is related to the individual immune status of the animals, the type and density of microorganisms they are exposed to, other problems experienced during the involution process, and the defense mechanism of the uterus. In order to maintain optimal fertility in farms, it is important to manage the postpartum period effectively (Avcilar et al., 2023; Sheldon et al., 2006). Uterine infections cause significant economic losses on farms. Therefore, preventive measures against uterine infections should be taken and effective

protection strategies for farms are economically important (Ganaie et al., 2018).

CONCLUSION

As a result, the resumption of postpartum cyclic activity was delayed in cows on family-type cattle breeding farms in rural areas of Van province, and the rates of anestrus, which were mainly due to nutrition and management, were high. The most important factor for animals to resume their cyclic activities after birth and become pregnant at the ideal time is the postnatal energy balance. Thus, farms must take care to feed animals with an appropriate ration. Also, it was concluded that the widespread use of ultrasonography in family-type cattle breeding farms would have significant and positive effects on the reproductive success of cows and sustainable animal husbandry.

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Investigation of *Brucella* spp. presence of amphibians collected from some regions in Türkiye

ABSTRACT

Brucellosis is a zoonotic disease that causes economic losses in many countries worldwide, especially in livestock. Firstly, the African bullfrog and then *B. inopinata* and *B. microti*-like strains have been identified in various frog species worldwide. This study aimed to determine the presence of *Brucella* in amphibian frogs for the first time in Türkiye by bacteriological and molecular methods. Internal organ samples (spleen, liver, lung, kidney, etc.) of 150 frogs from different regions of Türkiye (Edirne/Ipsala (53 pieces), Adıyaman/Kâhta (97 pieces)) were used for the isolation of *Brucella* spp. As a result of *Brucella* genus-specific PCR (Polymerase Chain Reaction) and Multiplex PCR methods performed with these extracts, no positivity was detected in the frog samples taken from Edirne province, while in 4 of the frog samples taken from Adıyaman province, bands of approximately 250, 600, 700, 1000, 1500 and 3000 bp were observed in Multiplex PCR. *Sphingomonas paucimobilis* (*S. paucimobilis*) was identified by analysing the culture obtained from these samples with VITEK®2:Healtcare. As a result, the presence of *Brucella* spp. could not be detected both bacteriologically and molecularly in the study samples. However, observing similar multiple bands in multiplex PCR suggested that this bacterium and *Brucella* species are phylogenetically close. It was determined that *S. paucimobilis*, which belongs to the same class as *Brucella* species under the Alphaproteobacteria, may be dangerous for people who work on farms where frogs are raised for human consumption. This is because the bacteria can cause opportunistic infections, particularly in immunocompromised humans. Therefore, it may be imperative to take the appropriate precautions.

Keywords: Bruce-ladder, *Brucella* spp., Frog, Isolation, PCR, *S. paucimobilis*

INTRODUCTION

Brucellosis, also known as Malta fever, is a zoonotic infection caused by *Brucella* species, which are facultative, intracellular, immobile, aerobic, and Gram-negative coccobacilli (Buttigieg et al., 2018). Brucellosis is a disease characterized by reproductive disorders with a wide range of hosts and causes significant economic losses, especially in livestock. It is recognized as one of the most important bacterial zoonoses worldwide, with 500.000 human cases per year (Godfroid et al., 2005). Since there is currently no approved human vaccine, treatment is difficult and prolonged and carries the risk of recurrence, it is essential to update and increase the knowledge about the pathogenesis, diagnosis and treatment of brucellosis to control and manage this infection, especially in endemic areas (Amjadi et al., 2019). In addition to the six classical species affecting terrestrial mammals, the diversity of animal hosts has been expanded to include marine mammals and primates, bringing the number to 12 (Jaÿ et al., 2020; Whatmore et al., 2014). This classification is defined according to host preference, reproduction and biochemical properties (Scholz et al., 2008; WOAH, 2018).

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In recent years, more atypical *Brucella* spp. have been isolated from cold-blooded animals. Frogs are the cold-blooded animals from which the most isolations are made (Eisenberg et al., 2012; Leclercq et al., 2020; Mühldorfer et al., 2017). *S. paucimobilis* is a Gram-negative bacillus with a single polar flagellum, aerobic, yellow-pigmented, non-fermenting glucose, oligotrophic, non-spore-forming, non-spore-forming Gram-negative bacillus, which is very closely related to the genus *Brucella* and is reported to be widely distributed in natural and mainly aquatic environments. *Sphingomonas* is considered to be an opportunistic pathogen, and although it is rarely reported in clinical settings, it has been reported to be closely associated with meningitis cases (Tai and Velayuthan, 2014; Walayat et al., 2018). It has been reported to grow in distilled water, haemodialysis fluids and sterile drug solutions. Since it has been reported that it can be transmitted, especially to people with chronic diseases or immunocompromised diseases, it is essential for public health (Göker et al., 2017). *S. paucimobilis* was isolated from frogs for the first time in Türkiye (Dökenel and Özer 2019).

The natural host range of Brucellosis disease has expanded to amphibians in recent years due to reports of some atypical *Brucella* spp isolated from frogs. In France in 2017, a *Brucella* strain was isolated for the first time from animals in a frog (*Pelophylax ridibundus*) farm produced for human consumption and identified as *B. microti*-like. Following this first isolation, *B. microti*-like strains were isolated in the samples taken from the frog farms and their environment, indicating that the agent can survive in the host environment. This situation reveals the zoonotic and pathogenic potential of atypical *Brucella* species and shows that they may pose a possible risk to consumers and workers (Jaý et al., 2018). This strain from the frog showed genetic and phenotypic characteristics similar to the isolates obtained from the field mouse (*Microtus arvalis*) in the Czech Republic (Hubálek et al., 2007). In addition, atypical *Brucella* isolations from various frog species such as African bullfrog (*Pyxicephalus edulis*) (Eisenberg et al., 2012), big-eyed tree frog (*Leptopelis vermiculatus*) (Fischer et al., 2012), white tree frog (*Litoria caerulea*) (Whatmore et al., 2015) were realised (Jaý et al., 2018). Significant research is being conducted to expand the current knowledge on atypical *Brucella* isolated from amphibians worldwide and to address the challenges faced (Mühldorfer et al., 2017). It has been reported that Türkiye exported an average of 642 tons of edible frogs. The number of farms in Türkiye has increased over the years due to commercial frog breeding and its great potential as a food source. The demand for frogs is rising and global frog consumption is rising as a result of the decline in frog populations. Most nations that import wild and farmed frogs are Türkiye as well as Indonesia, China, Thailand, India, and Vietnam (Şimşek et al., 2022).

In this study, organ materials (liver, spleen, spleen, lung, heart, kidney, ovaries and skin) of 150 frog specimens of different species and sizes collected from Edirne/İpsala and Adıyaman/Kâhta provinces/districts were investigated for *Brucella* spp, and it was aimed to obtain data on the presence of *Brucella* in amphibia for the first time in Türkiye.

MATERIALS AND METHODS

Animal materials

In this study, organ materials (liver, spleen, lung, heart, kidney, ovaries, and skin) of a total of 150 frog specimens (53 examples from farms in the Edirne/İpsala region and 97 samples from nature from Adıyaman/Kâhta region) of different species and sizes were used as examination samples for the diagnosis of *Brucella* spp. in the spring months of 2022-2023.

Culture and biotyping

Tryptic Soy Agar (TSA) (CM0131, Oxoid) was prepared according to the manufacturer's protocol and sterilized by autoclave at 121 °C for

15 min. After the medium was cooled to 50-55 °C, Farrell's medium was prepared by adding *Brucella* Selective Supplement (SR0083A, Oxoid) and inactivated bovine serum (5%) (Biochrom, S0115, Germany) under sterile conditions to the prepared TSA medium. Tissue homogenates prepared from organ materials of frog samples were simultaneously inoculated into Farrell's agar and enrichment liquid medium containing vancomycin (20 µg/ml), amphotericin B (1 µg/ml), 1% dextrose and 5-10% serum. The cultured sample materials were incubated in an oven containing 5-10% CO2 under microaerophilic conditions for six weeks. During this period, passages were made on TSA and Farrell's medium. Classical bacterial methods were applied for the diagnosis of *Brucella* spp. (Alton et al., 1988; WOAH, 2022). DNA extraction was performed from the isolates studied for *Brucella* spp.

DNA extraction

A commercial isolation kit (High Pure FFPET DNA Isolation Kit, 06650767001, Roche) was used for DNA extraction from suspected cultures and tissue homogenates simultaneously.

Reference materials

B. melitensis Rev 1 strain, Tbilisi, Izatnagar, and R/C phages and monospecific sera (A, M) were obtained from Harran University, Faculty of Veterinary Medicine, Department of Microbiology Laboratory.

Species specific and multiplex PCR (Bruce-Ladder)

For the genus-specific DNA amplification, a 223 bp sequence present on a gene encoding the BCSP31 protein, which is present in all *Brucella* species and weighs 31 kDa, was considered by the previously described protocol (Baily et al., 1992). To amplify this target sequence, primers B4 F (5′ TGG CTC GGT TGC CAA TAT CAA 3′) and B5 R (5′ CGC GCT TGC CTT TCA GGT CTG 3′) (Sigma Aldrich) were used. The multiplex PCR method was performed according to the method reported by Mayer-Scholl et al., 2010. The obtained DNA extracts were prepared with 2x Taq PCR Mastermix (206143, Qiagen) protocol, with a total volume of 25 µl in each tube, containing 12.5 µl Qiagen master mix, 0.2µM of each of 9 primer pairs, nine µl water and 1 µl template DNA. The contents of the mixture were amplified in a Thermal Cycler (Thermo Fisher). The cycles determined for amplification were performed by the commercial mastermix content. For this purpose, after initial denaturation at 95 ºC for 15 min, amplification was completed at 94 ºC for 30 s, initial binding at 58 ºC for 90 s, 72 ºC for 3 min (first synthesis), and final synthesis at 72 ºC for 10 min, for a total of 30 cycles. At the end of the process, amplicons were subjected to electrophoresis (Thermo Scientific EC300 XL). Agarose gel (Sigma, A9539) was prepared at a concentration of 1.5%. At the end of the procedures, specific bands in the gel were investigated with a gel imaging device (Major Science UVCI-1100).

RESULTS

Bacteriological analyses were performed according to the standards set by WOAH (WOAH 2022). All cultures were incubated in Farrell's broth at 37 °C, 5-10% CO2 for six weeks. During this period, Farrell's agar was inoculated weekly, and as a result of the inoculations, specific honey-colored, smooth-looking suspicious colonies similar to *Brucella* spp. were found. The suspicious colonies were subjected to an agglutination test with A and M monospecific sera, but no agglutination was observed. These isolates were also not lysed by Tblisi, Izatnagar and R/C phage. As a result of the *Brucella* spp. and Bruce-Ladder PCR method performed on suspected colonies isolated from the cultured and incubated media and amplicons obtained by DNA extraction from tissue homogenates simultaneously, no positivity was

received in the samples collected from the Edirne/Ipsala region. As a result of the Bruce-Ladder PCR method performed on samples collected from the Adıyaman/Kâhta region, approximately 250, 600, 700, 1000, 1500 and 3000 bp bands were detected in 4 of 97 samples, but no positivity was obtained in any of the samples as a result of the genus-specific PCR for *Brucella* spp. (Figure 1). As a result of the identification (VITEK®2:Healtcare automatic identification device) of the isolates exhibiting different band profiles as a result of multiplex PCR analysis, samples 61, 62, 63 and 129 were identified as *S. paucimobilis*.

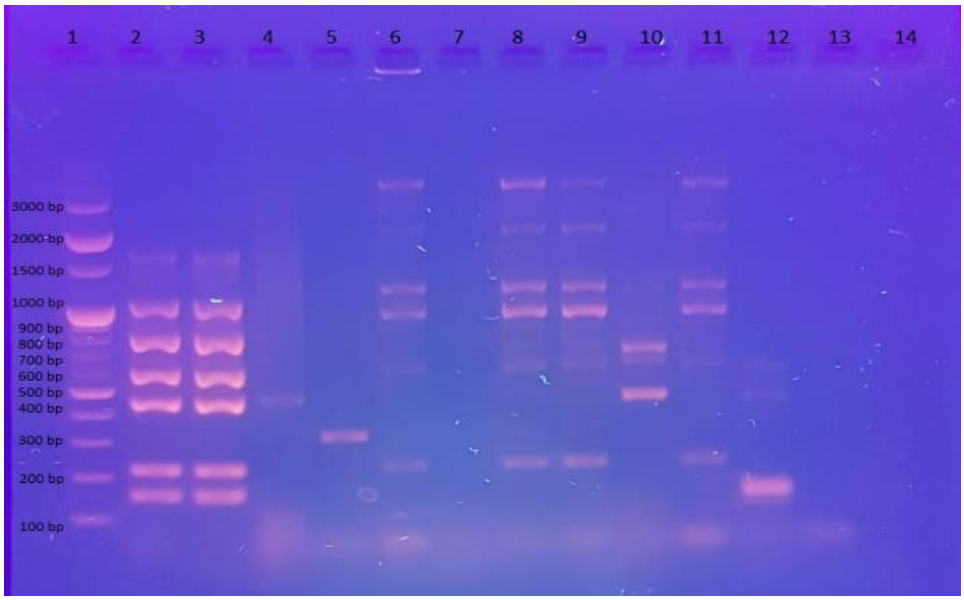

Figure 1. Leadder (Qiagen GelPilot Mid Range katalog no: 239135) 2,3: Positive control (B. melitensis Rev 1), 6,8,9,11: Sphingomonas paucimobilis, 4,5,7,10,12,13; Negative samples, 14;Negative control

DISCUSSION

Until recently, the genus *Brucella* was thought to be a clonal group of genetically identical bacteria isolated only from mammals, but the recent isolation of *Brucella* group bacteria from cold-blooded animals such as frogs, lizards and fish has radically changed this idea. In contrast to the known classical *Brucella* species, the recently reported species isolated from exotic frogs have a different ecology, are genetically diverse and may represent several new *Brucella* species (Scholz et al., 2016; Whatmore et al., 2007). This study is essential as the first study investigating *Brucella* spp. in frogs in Türkiye. *Sphingomonas* is very closely related to *Brucella*. It is reported that *S. paucimobilis* shares genes related to intracellular vitality with *Brucella* species, and genes related to adhesion and movement are co-expressed with *Legionella* species (El Beaino et al., 2018). Although considered to have low virulence, *S. paucimobilis* can cause infections in chronically ill and immunocompromised hosts (Göker et al., 2017).

Atypical *Brucella* isolates have been isolated from many frog species and identified as the cause of localized infection, including skin and soft tissue abscesses, panophthalmitis, spinal arthropathy as well as systemic disease (Mühldorfer et al., 2017). Systemic lesions were found in the skin and internal organs of some healthy frogs used in this study. Some researchers have isolated *Brucella* spp. from various frog species for cultural and molecular studies (Jaÿ et al., 2020; Latheef et al., 2020). The present study determined that the suspicious colonies obtained from various tissue samples due to the culture method were not identified as *Brucella* spp. no positivity was obtained in the PCR study performed at the genus level (Bcsp31). However, the detection of multiple bands in the species level (Bruce-Ladder) study indicates that *Brucella* spp. can be colonized in amphibians by other organisms in the same

genus and can continue its presence in their environment. The cultures analyzed on the VITEK®2:Healtcare automatic identification device were identified as *S. paucimobilis*, included with *Brucella* spp. in the *Alphaproteobacteria* class. It has been reported that *S. paucimobilis* has been isolated from many patients as case reports in Türkiye (Aşkın et al., 2022; Göker et al., 2017; Gün et al., 2014; Özekinci et al., 2022). Although many studies are isolated from humans in hospitals, *S. paucimobilis* was identified for the first time in frogs in Türkiye. In a study conducted in Germany to investigate the presence of atypical *Brucella* in amphibians, *S. paucimobilis* was found in 11 of 27 isolates and *Ochrobactrum* in 16 of 27 isolates (Mühldorfer et al., 2017). This result, which is compatible with our study, suggests that *S. paucimobilis* is in close association with *Brucella* species.

Occupational exposure through direct contact with infected animals and foodborne transmission through consumption of raw animal products are recognised as the main routes of brucellosis transmission to humans (Corbel et al., 2006). For this reason, *Brucella* spp. is important in terms of a possible risk for workers and consumers due to the pathogenic and zoonotic potential of similar atypical species. There are frog breeding farms in our country, which are seen as luxury consumption food and are exported abroad in large quantities or for domestic consumption (Gülçiçek, 2021). It is essential to determine the prevalence of *B. microti* and *B. inopinata*-like organisms among the frog population raised here and to investigate their presence in the farm environment (Jaý et al., 2018).

CONCLUSION

In this study, *Brucella* spp. could not be isolated from amphibians. However, both the relatively small sample size and the fact that the clinical samples were taken from healthy frogs may have caused this result. Future studies aim to investigate the presence of *Brucella* spp. in amphibians, especially by taking more samples with lesions from different locations.

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The effect of birth type and gender on growth performance, live weight gain, growth pattern modeling, and survival rate in Anatolian Merino lambs

Research Article

ABSTRACT

In this study, the aim was to determine the effect of birth type and gender on growth performance, live weight gain, growth modeling, and survival rate in Anatolian Merino lambs. Growth performance was determined in 28 singletons (14 females/14 males), 80 twins (38 females / 42 males), and 54 triplets (22 females / 32 males) lambs in the study. The body weight of the lambs was measured on the day of birth (day 0), and at 10, 20, 30, 45, and 60 days of age. Birth type was effective on growth performance in Anatolian Merino lambs ($P < 0.001$). The effect of gender on growth performance was significant at 30 days of age and beyond ($P < 0.05$). Except for the period between 0-20 days, the effect of birth type was statistically significant for all other measurement days on daily live weight gain until the day of measurement ($P < 0.05$). However, gender influenced daily live weight gain between 0-45 days and 0-60 days ($P < 0.05$). Survival rates up to 60 days of age in Anatolian Merino lambs was not statistically different according to birth type and gender ($P > 0.05$). The total survival rate was 95.3%. In growth modeling of Anatolian Merino lambs, the highest regression value was in singleton males ($r^2 = 0.910$), and the lowest r^2 value was in triplet males ($r^2 = 0.746$). In conclusion, birth type may affect the growth performance and daily live weight gain of Anatolian Merino lambs. Moreover, the similar survival rate according to birth types indicates that this productive breed is both productive and has high survivability.

Keywords: Anatolian Merino, growth performance, lambs, live weight gain, survival

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INTRODUCTION

Given Türkiye's geographical conditions, sheep farming stands out as a significant livestock production activity, both culturally and economically. Sheep effectively utilize poor pastures and convert them into economically valuable animal products. Consequently, sheep farming generates economic benefits for family-type farms engaged in this activity (Boğa Kuru & Kuru, 2022; Kaymakçı, 2016; Kırbaş et al., 2022; Koyuncu, 2019; Turgut et al., 2023; Yılmaz et al., 2022). According to the Turkish Statistical Institute (TÜİK) data for 2023, there are 42,060,470 sheep in Türkiye (TÜİK, 2023) .

Anatolian Merino carries 80% German Mutton Merino and 20% White Karaman genotype. Generally, it is raised in Central Anatolia. Anatolian Merino, which is a meat-wool dual-purpose breed, is one of Türkiye's strong local sheep breeds that adapts well to pasture conditions and flock management. Females are polled, while males may have horns. Female mature body weight ranges from 30 to 78 kg (average 55 kg), while males mature to 80-90 kg. Birth weight in Anatolian Merino lambs

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ranges from 2.1 to 6 kg in males (with an average of 4.5 kg) and from 2 to 6.0 kg in females (with an average of 4 kg). These sheep do not face many infertility problems, and their lambing rate is 1.3. Survival rates in lambs from birth to weaning and to puberty are 85% and 83.7%, respectively (Alçayır & Karabacak, 2019; Boğa Kuru et al., 2024; Resmi Gazete, 2004; TAGEM, 2009).

Birth weight in lambs is an important criterion for the profitability of enterprises (Habtegiorgis et al., 2022; Odabaşıoğlu, 1990). Furthermore, birth weight in lambs can significantly influence survival rate by directly affecting growth and growth traits (Akçapınar, 1994; Kaymakçı, 2016; Koyuncu, 2019; Öztürk et al., 2018). A decrease in birth weight can lead to an increase in lamb mortality. The average birth weight of lambs generally ranges from 3 to 5 kg (Akçapınar, 1994; Alçayır & Karabacak, 2019; Ceyhan et al., 2015; Koyuncu, 2019; Odabaşıoğlu, 1990; Schreurs et al., 2010). There are many factors that affect birth weight, such as feeding, birth type, maternal age, breed, and season (Akçapınar, 1994; Alçayır & Karabacak, 2019; Dellal, 2002; Karakuş, 2023; Sveinbjörnsson et al., 2021).

Growth in animal husbandry is defined as an increase in animals' body measurements, particularly their live weight (Aytekin et al., 2009; Lamy et al., 2012). Mature live weight is recognized as a crucial criterion for evaluating animals' growth and developmental characteristics (Castillo et al., 2023; Koyuncu, 2019; Topal et al., 2004). While a higher mature live weight is desirable, it can also bring about drawbacks like birthing difficulties and increased expenses. Therefore, it is essential to regulate growth rates in accordance with the intended purpose, considering factors such as animals' genotypic makeup and nutrition (Aytekin et al., 2009; Lamy et al., 2012; Owens et al., 1993; Topal et al., 2004).

The aim of this study was to determine the effects of birth type and gender on growth performance, live weight gain, growth pattern modeling, and survival rate in Anatolian Merino lambs.

MATERIALS AND METHODS

Ethical approve

This study commenced following approval from the Animal Experiments Local Ethics Committee of Kafkas University (KAÜ-HADYEK-2024/11), Kars, Türkiye.

Location

The research was conducted at a commercial sheep farm located in the Sandıklı district of Afyonkarahisar province, Türkiye. Sandıklı is situated at 38°27'53.7"N latitude and 30°16'22.7"E longitude, with an elevation of 1095 meters above sea level.

Animals

A total of 170 Anatolian Merino lambs were utilized in the study. Body weights were measured for 162 of these lambs over a 60-day period. Data from 8 lambs that died at different times were not included in the study. Growth performance was determined for 28 single lambs (14 females/14 males), 80 twin lambs (38 females/42 males), and 54 triplet lambs (22 females/32 males).

Housing and feeding

No additional practices were implemented for the housing and feeding criteria of the lambs, and routine care and feeding conditions on the farm were maintained. In addition, the sheep were supplemented with a balanced concentrate formulated to meet their milk yield requirements (NRC, 2007).

Lambs stayed with their mothers in pens for the first 7 days. From the 7th to the 21st day, lambs stayed with their mothers in collective pens for socialization. After the 21st day, lambs

were moved to separate pens. Between the 21st and 45th days, lambs had free access to their mothers through a system called the "kaşak" system, allowing them to consume both mother's milk and feed and water. After the 45th day, lambs stayed with their mothers for 30 min in the morning and evening to encourage them to focus more on feed intake.

Starting from the 15th day, lambs were provided with as much dry hay and starter lamb feed (Şampiyon Kuzu Yemi®, Emek Yem, Balıkesir) as they could consume. From the 30th to the 60th day, lambs were fed with dry hay, barley, and starter lamb feed. Access to fresh and clean water was provided at all times since the start of feeding lambs.

Measurement of body weight

The body weight of the lambs was measured using a digital scale on the day of birth (day 0), as well as at 10, 20, 30, 45, and 60 days of age.

Statistical analysis

The Shapiro-Wilk test was employed to assess the normality of the data. Subsequently, General Linear Model (GLM) was utilized to investigate the effects of gender and birth type (single, twin, and triplet) on growth performance and live weight gain in lambs. Duncan's multiple comparison test was employed for pairwise comparisons of differences between birth types at the same time point. To evaluate potential disparities in mortality and survival rates, the chi-square test was implemented, considering the influences of gender and birth type. Linear regression models based on gender and birth type were constructed to elucidate their influence on live weights. Data are presented as mean ± standard deviation (SD). Statistical analyses were conducted using GraphPad Prism® (Version 9.5.1, GraphPad Software Inc., San Diego, CA, USA) and SPSS® (Version 26.0, SPSS Inc./IBM Group, Chicago, IL, USA) software. A significance level of $P < 0.05$ was established for group comparisons.

RESULTS

Birth type in Anatolian Merino lambs significantly impacted growth performance throughout all measurement days ($P < 0.001$). Single lambs had significantly higher live weights compared to both twin and triplet lambs ($P < 0.001$). Interestingly, twin and triplet lambs exhibited statistically similar growth performance ($P > 0.05$). The effect of gender on growth became apparent from day 30 onwards ($P = 0.027$), with significant differences persisting on days 45 ($P = 0.001$) and 60 ($P < 0.001$). Table 1 summarizes the changes in live weight observed in Anatolian Merino lambs at birth, and at 10, 20, 30, 45, and 60 days of age, categorized by birth type and gender.

Table 1: Changes in growth performance (kg) of Anatolian Merino lambs according to measurement days, birth type, and gender (mean ± standard deviation).

Items		n	Day 0*	Day 10	Day 20	Day 30	Day 45	Day 60
Birth type	Single	28	6.27 ± 0.84^{a}	8.10 ± 1.19^{a}	9.95 ± 0.94^{a}	12.73 ± 1.09^{a}	16.01 ± 2.15^{a}	18.95 ± 3.48^{a}
	Twins	80	4.37 ± 0.93^{b}	6.21 ± 1.04^{b}	8.04 ± 1.15^{b}	10.62 ± 1.21^{b}	13.55 ± 2.33^{b}	15.88 ± 3.01^{b}
	Triplet	54	4.36 ± 1.89^{b}	6.55 ± 2.10^{b}	8.16 ± 2.21^{b}	10.17 ± 2.04^{b}	12.54 ± 2.11^{b}	14.74 ± 2.25^{b}
	P value	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Gender	Female	74	4.53 ± 1.50	6.57 ± 1.64	8.33 ± 1.67	10.62 ± 1.77	13.22 ± 2.56	15.31 ± 3.02
	Male	88	4.85 ± 1.48	6.73 ± 1.65	8.48 ± 1.75	11.04 ± 1.73	13.99 ± 2.42	16.63 ± 3.22
	P value	-	0.10	0.47	0.365	0.027	0.001	<0.001
Total		162	4.70 ± 1.49	6.65 ± 1.64	8.41 ± 1.71	10.84 ± 1.75	13.64 ± 2.51	16.03 ± 3.19

*: Birth weight; $^{a-b}$: Within each column, groups with different letters show statistically significant differences from each other.

Birth type significantly influenced daily live weight gain across measurement days, while gender's effect was evident from 20-30 days of age onwards (Table 2). Except for the 0-20-day interval, birth type significantly impacted daily live weight gain until the final measurement day ($P < 0.05$). Conversely, gender affected daily live weight gain between 0-45 and 0-60 days ($P < 0.05$). Table 2 presents the inter-measurement and age-specific daily live weight gains in Anatolian Merino lambs.

Table 2: Effect of birth type and gender on daily live weight gain (mean ± standard deviation) in Anatolian Merino lambs.

Daily live weight gain between measurements (g)							
Items		n	Day 0-10	Day 11-20	Day 21-30	Day 31-45	Day 46-60
Birth type	Single	28	183.2 ± 53.5^{b}	185.0 ± 37.6^{a}	277.1 ± 113.3^{a}	219.3 ± 131.2^{a}	195.5 ± 100.3^{a}
	Twins	80	183.6 ± 47.1^{b}	182.9 ± 41.2^{a}	258.1 ± 109.9^{a}	195.0 ± 120.6^{a}	155.2 ± 62.8^{b}
	Triplet	54	218.5 ± 79.1^{a}	160.6 ± 41.2^{b}	201.1 ± 60.6^{b}	158.0 ± 93.2^{b}	146.8 ± 58.4^{b}
	P value	-	0.001	0.006	0.001	0.047	0.003
Gender	Female	74	203.8 ± 75.2	176.6 ± 41.1	229.3 ± 95.7	173.2 ± 107.7	139.2 ± 56.7
	Male	88	188.0 ± 48.5	175.1 ± 42.6	253.4 ± 104.4	198.3 ± 121.3	176.3 ± 77.4
	P value	-	0.098	0.404	0.048	0.029	<0.001
Total		162	195.2 ± 62.5	175.8 ± 41.8	242.4 ± 101.0	186.9 ± 115.7	159.3 ± 71.0
Daily live weight gain (g)							
Items		n	Day 0-10	Day 0-20	Day 0-30	Day 0-45	Day 0-60
Birth type	Single	28	183.2 ± 53.5^{b}	184.1 ± 25.0	215.1 ± 41.3^{a}	216.5 ± 58.6^{a}	211.2 ± 65.7^{a}
	Twins	80	183.6 ± 47.1^{b}	183.3 ± 32.6	208.2 ± 38.6^{ab}	203.8 ± 58.4^{ab}	191.6 ± 55.5^{ab}
	Triplet	54	218.5 ± 79.1^{a}	189.5 ± 45.1	193.4 ± 32.5^{b}	181.6 ± 40.6^{b}	172.9 ± 35.3^{b}
	P value	-	0.001	0.434	0.034	0.012	0.004
Gender	Female	74	203.8 ± 75.2	190.2 ± 41.3	203.2 ± 40.1	193.3 ± 54.4	179.7 ± 49.9
	Male	88	188.0 ± 48.5	181.5 ± 30.8	205.5 ± 36.1	203.1 ± 54.3	196.4 ± 54.9
	P value	-	0.098	0.353	0.258	0.036	0.002
Total		162	195.2 ± 62.5	185.5 ± 36.1	204.5 ± 37.8	198.6 ± 54.4	188.8 ± 53.1

$^{a-b}$: Within each column, groups with different letters show statistically significant differences from each other.

Birth type significantly influenced both weekly and monthly live weight gain in Anatolian Merino lambs ($P < 0.05$). Table 3 presents the weekly and monthly live weight changes and their significance levels for Anatolian Merino lambs categorized by birth type and gender. The survival rates up to 60 days of age in Anatolian Merino lambs was not significantly affected by birth type or gender ($P < 0.05$). A total of 8 lambs died throughout the study, resulting in an overall survival rate of 95.3% (Table 4). Table 4 also presents the number of lambs that died, the total mortality rate, and the survival rate by birth type and gender on the measurement days during the study. Figure 1A-E presents the results of simple linear regression analysis on growth performance data for Anatolian Merino lambs up to 60 days of age, categorized by both gender and birth type. The highest regression value for Anatolian Merino lambs was observed in single males ($r^2 = 0.910$, $P < 0.001$), with the regression equation $Y = 0.2526*X + 5.507$, where Y represents weekly growth performance and X represents days (Figure 1A). In terms of birth type, singles ($r^2 = 0.851$) again had higher regression values compared to twins ($r^2 = 0.833$) and triplets ($r^2 = 0.736$) (Figure 1E).

Table 3: Effect of birth type and gender on weekly and monthly live weight gain (mean ± standard deviation) in Anatolian Merino lambs.

Weekly live weight gain (kg)					
Items		n	Week 0-4	Week 4-8	Week 0-8
Birth type	Single	28	1.62 ± 0.32[a]	1.56 ± 0.85[a]	1.59 ± 0.49[a]
	Twins	80	1.58 ± 0.30[ab]	1.33 ± 0.63[ab]	1.44 ± 0.42[ab]
	Triplet	54	1.47 ± 0.25[b]	1.15 ± 0.46[b]	1.31 ± 0.27[b]
	P value	-	0.05	0.011	0.006
Gender	Female	74	1.54 ± 0.31	1.19 ± 0.55	1.36 ±0.38
	Male	88	1.55 ± 0.27	1.41 ± 0.68	1.48 ± 0.41
	P value	-	0.305	0.001	0.002
Total		162	1.55 ± 0.29	1.31 ± 0.63	1.42 ± 0.40
Monthly live weight gain (kg)					
Items		n	Month 0-1	Month 1-2	Month 0-2
Birth type	Single	28	6.45 ± 1.24[a]	6.22 ± 3.37[a]	6.36 ± 1.97[a]
	Twins	80	6.25 ± 1.16[ab]	5.25 ± 2.49[ab]	5.78 ± 1.66[ab]
	Triplet	54	5.80 ± 0.97[b]	4.57 ± 1.83[b]	5.21 ± 1.06[b]
	P value	-	0.034	0.010	0.004
Gender	Female	74	6.10 ± 1.20	4.69 ± 2.19	5.42 ± 1.49
	Male	88	6.16 ± 1.08	5.62 ± 2.71	5.92 ± 1.64
	P value	-	0.257	0.001	0.001
Total		162	6.13 ± 1.14	5.19 ± 2.52	5.69 ± 1.59

[a-b]: Within each column, groups with different letters show statistically significant differences from each other.

Table 4: Effect of birth type and gender on mortality count and survival rate of Anatolian Merino lambs by day.

Birth type	Gender	The daily mortality count (n)					Mortality (n / Total n)	Survival rate, % (n / Total n)
		0-10	11-20	21-30	31-45	46-60		
Single	Female	-	-	-	-	-	0/14	100 (14/14)
	Male	1	-	-	-	-	1/15	93.3 (14/15)
	Total	1	-	-	-	-	1/29	96.6 (28/29)
Twins	Female	1	-	1	-	-	2/40	95 (38/40)
	Male	1	-	-	-	1	2/44	97.7 (42/44)
	Total	2	-	1	-	1	4/84	95.2 (80/84)
Triple	Female	1	1	-	-	-	2/24	91.7 (22/24)
	Male	-	-	1	-	-	1/33	96.7 (32/33)
	Total	1	1	1	-	-	3/57	94.7 (54/57)
Total	Female	2	1	1	-	1	5/79	93.7 (74/79)
	Male	2		1	-		3/91	96.7 (88/91)
	Total	4	1	2	-	1	8/170	95.3 (162/170)

Note: There is no statistically significant difference in the intergroup survival rates based on birth type and gender (P > 0.05).

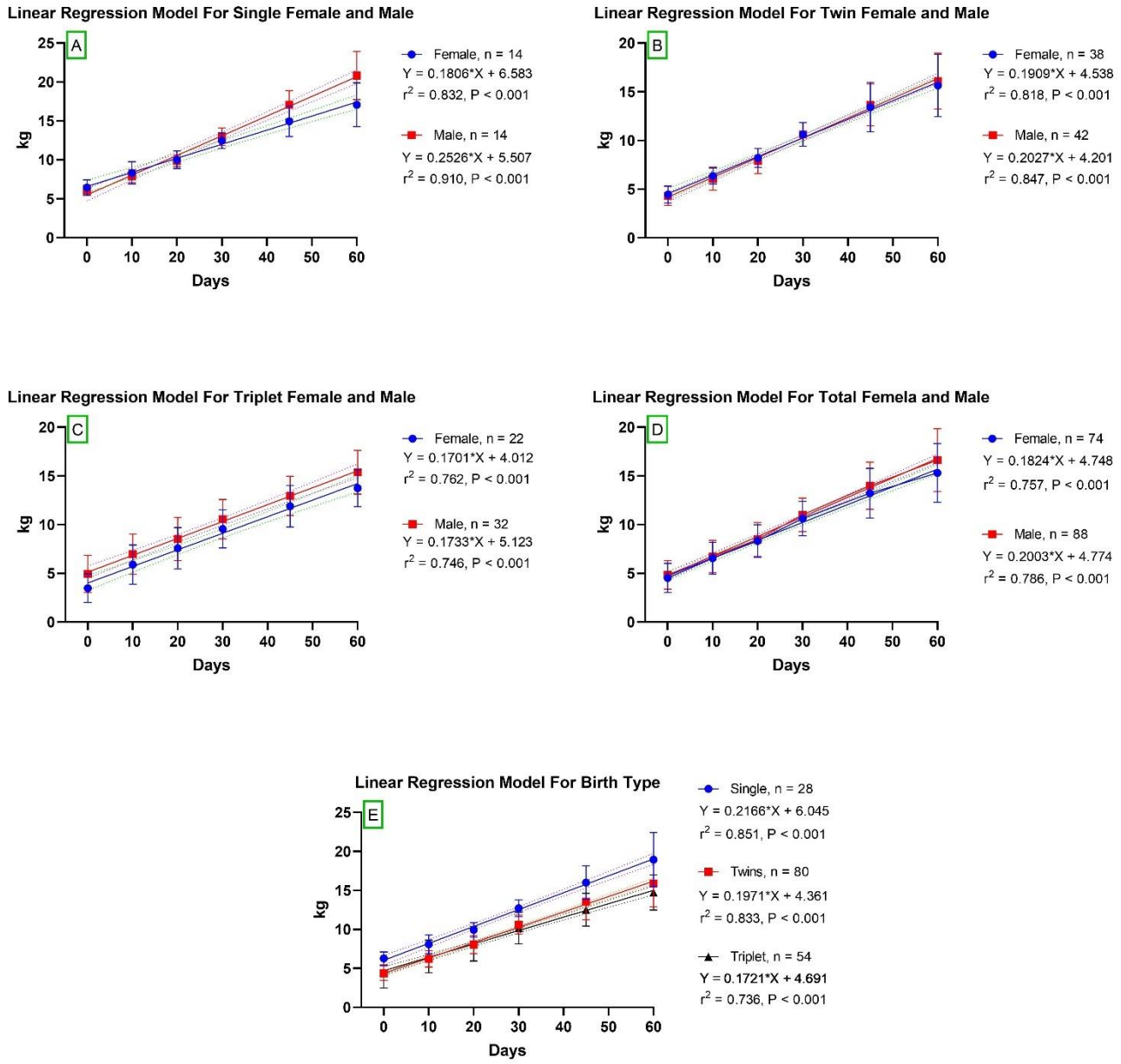

Figure 1: Linear regression models for gender (A-D) and birth type (E) in Anatolian Merino lambs. Y: Live weight, X: Days.

DISCUSSION

In herd management, acquiring body measurement-based data is crucial for monitoring or evaluating the growth and development of farm animals. Particularly in herd management, the methods used to estimate live weight in ruminants must be reliable (Aytekin et al., 2009; Coşkun et al., 2023; Guvenoglu, 2023; Keskin & Dağ, 2006; Tırınk, 2022; Wangchuk et al., 2018). This study aimed to investigate the effects of birth type and gender on growth performance, live weight gain, growth modeling, and survival rate in Anatolian Merino lambs from birth to 60 days of age, along with the variations in these characteristics across sampling days.

The effect of gender and birth type on growth performance and live weight gain from birth to weaning was determined in Central Anatolian Merino lambs (Aktaş et al., 2016). In Central Anatolian Merino lambs, birth weight and 45-day live weight were 4.71 kg and 14.12 kg for males, and 4.41 kg and 13.33 kg for females. Additionally, mean birth weight and 45-day live

weight in singletons were 5 kg and 4.24 kg, and in twins were 14.52 kg and 13.03 kg. Both gender and birth type influence the growth performance of Central Anatolian Merino lambs (Ünal & Akçapinar, 2001). In another study conducted with Central Anatolian Merino lambs, the birth weight and 45-day live weight of singletons and twins were 4.69 kg - 4.04 kg and 15.5 kg – 12.2 kg for males, and 4.47 kg – 3.81 kg and 14.7 kg and 11.7 kg for female singletons and twins, respectively (Yalçın et al., 1980). In Central Anatolian Merino lambs, the average birth weight was 5.50 kg for singles, 4.47 kg for twins, 5.15 kg for males, 4.64 kg for females, and 4.86 kg overall. The effects of birth type and gender on the same traits were found to be significant (Odabaşıoğlu, 1990). In Konya Merino lambs, birth, 30-day, and 60-day live weights were 4.8 kg, 12.5 kg, and 20.2 kg for singles, 4 kg, 9.9 kg, and 16.9 kg for twins, 4.2 kg, 10.6 kg, and 17.7 kg for females, and 4.6 kg, 11.8 kg, and 19.3 kg for males. Overall, singles had significantly higher live weights than twins. Additionally, males were significantly heavier than females at different stages of the study (Aktaş et al., 2013). In Karacabey Merino lambs, the average birth weight for single males, single females, twin males, and twin females was 4.48 kg, 4.32 kg, 4.21 kg, and 4.06 kg, respectively, with 60-day weights of 22 kg, 20.7 kg, 18.5 kg, and 17.9 kg. Moreover, male lambs developed faster than females, and singles grew faster than twins (Oğan, 1994). In this study, Anatolian Merino lamb birth weights and live weights at 30, 45, and 60 days were as follows: for singles, 6.3 kg, 12.7 kg, 16 kg, and 19 kg; for twins, 4.4 kg, 10.6 kg, 13.6 kg, and 15.9 kg; for females, 4.5 kg, 10.6 kg, 13.2 kg, and 15.3 kg; and for males, 4.9 kg, 11 kg, 14 kg, and 16.6 kg. Consistently across studies (Aktaş et al., 2013, 2016; Odabaşıoğlu, 1990; Oğan, 1994; Ünal & Akçapinar, 2001; Yalçın et al., 1980), singles had higher live weights than twins on all measurement days. Moreover, male lambs exhibited superior live weights compared to females from 30 days onwards. Additionally, it was found that environmental factors such as dam age, lamb birth year, gender, birth type, and season could significantly affect lamb weights at various growth stages in Merino lambs (Alçayır & Karabacak, 2019; Yalçın et al., 1980). The live weight results obtained in this study from different Merino sheep breeds (Aktaş et al., 2013; Odabaşıoğlu, 1990; Oğan, 1994; Ünal & Akçapinar, 2001; Yalçın et al., 1980) appear to be within similar ranges. However, the presence of triplets in this study has had an effect on the average live weight of both female and male lambs. Nevertheless, significant differences in live weight measurements did not occur at different times for both female and male lambs. Therefore, the presence of triple births in Anatolian Merino lambs does not create a significant disadvantage in terms of live weights and could potentially provide more economic benefits for the farm.

In Central Anatolian Merino lambs, daily live weight gain from birth to weaning, weaning to 120 days, and birth to 120 days was determined to be 191.0 g, 215.3 g, and 200.1 g, respectively. Similarly, during the birth to weaning period, females had a daily weight gain of 184.3 g, males 197.7 g, singles 207.4 g, and twins 174.5 g. In this context, both gender and birth type have an impact on daily live weight gain in Central Anatolian Merino lambs (Aktaş et al., 2016). In a study conducted with Merino lambs, daily weight gain varied between 198-216 g during the 0-30-day period and 224-250 g during the 0-60-day period. Additionally, birth type and gender have been effective on daily weight gain at birth, 15, 30, 45, and 60 days of age (Akmaz et al., 1992). In this study, birth type influenced live weight gain from 0-30, 0-45, and 0-60 days, while gender affected daily live weight gain between 0-45 and 0-60 days. Daily live weight gain from 0-60 days was 211.1 g for singles, 191.6 g for twins, 172.9 g for triplets, 179.7 g for females, and 196.4 g for males. These findings are similar to a study on Orta Anatolian Merino lambs (Aktaş et al., 2016) but lower than those

reported for Merino lambs (Akmaz et al., 1992). Triplet births may have particularly influenced the differences in daily live weight gain. Additionally, nutrition, breed, and birth type could have contributed to varying results in daily live weight gain.

In Central Anatolian Merino lambs, the overall survival rate was approximately 96.5% (Yalçın et al., 1980). In another study, the survival rate of Central Anatolian Merino lambs ranged from 88.7% to 92.6% (average 90.8%) (Aktaş et al., 2016). Additionally, the effect of gender on the 30-day survival rate in Central Anatolian Merino lambs was found to be negligible, while birth type could be influential. However, neither gender nor birth type had an effect on the survival rate at older ages in lambs (Ünal & Akçapinar, 2001). In Karacabey Merino lambs, the 60-day survival rate was determined to be 97.5% (Oğan, 1994). Similarly, in Karacabey Merino lambs, the 90-day survival rate was 92.5% for singles, 93% for twins, and an overall average of 92.9% (Başpınar et al., 1997). In a different study, the survival rate in Karacabey Merino lambs was found to be 97.6% for singles, 94.9% for twins, and 90% for triplets, with the birth type significantly affecting survival (Boztepe, 1994). In Konya Merino lambs, the survival rates ranged from 94.1% to 100% up to 30, 60, and 90 days of age (Aktaş et al., 2013). The survival rates obtained from Anatolian Merino lambs in this study are within the reported ranges for Central Anatolian Merino lambs (Aktaş et al., 2016; Yalçın et al., 1980), Karacabey Merino lambs (Başpınar et al., 1997; Oğan, 1994), and Konya Merino lambs (Aktaş et al., 2013). Additionally, the survival rate of triplets in this study was higher than that reported for Karacabey Merino lambs (Boztepe, 1994), although the difference in the number of days monitored may have influenced these rates. Particularly in multiple births, the mortality rate can be higher due to low birth weight (Koyuncu & Duymaz, 2017). However, birth type did not affect survival rates in this study, indicating that Anatolian Merino lambs may have robust characteristics and high survival performance. These findings demonstrate that the growth and survival performance of Anatolian Merino lambs are satisfactory. Supporting and increasing the population of this breed as an alternative to cattle farming for red meat production could contribute to both red meat production and lamb meat production. Furthermore, promoting and supporting Anatolian Merino lamb farming could increase employment in rural areas and contribute to the local economy.

In Anatolian Merino lambs, simple linear regression curves can be used to calculate growth curves in two different rearing methods (Aytekin et al., 2009; Keskin & Dağ, 2006). The determination coefficient of the simple linear regression model in Anatolian Merino lambs was found to be high (0.990) (Keskin & Dağ, 2006). In the linear model, the r^2 values for growth curve patterns up to 420 days of age in Kıvırcık and Dağlıç lambs were determined as 0.993 and 0.997, respectively (Akbaş et al., 1999). In another study, a linear model with an r^2 value of 0.990 provided a good fit for live weight changes in Akkaraman and Awassi x Akkaraman lambs between 1-29 weeks of age (Kocabaş et al., 1997). In this study, a simple linear regression model was used for Anatolian Merino lambs. The highest r^2 value in the study was for single males (0.910), and the lowest was for triplet males (0.746). The r^2 value we obtained is lower than in previous studies (Akbaş et al., 1999; Keskin & Dağ, 2006; Kocabaş et al., 1997). The observed lower r^2 value in our study compared to previous findings (Akbaş et al., 1999; Keskin & Dağ, 2006; Kocabaş et al., 1997) could potentially be attributed to two main factors: a shorter follow-up period for lamb growth compared to prior studies, and potential variations in the number of lambs included

within each treatment group. Future research endeavors investigating the growth patterns of Anatolian Merino lambs with extended monitoring durations and standardized group sizes could provide a more comprehensive understanding of their growth trajectory and further refine growth curve models for this breed.

CONCLUSION

In conclusion, the birth type of Anatolian Merino lambs may have an effect on growth performance and daily live weight gain, while the gender effect on growth performance and daily live weight gain may become clearer in later ages. Additionally, due to the robust structure of Anatolian Merino lambs, despite being higher in single births, the similarity in survival rates according to birth types demonstrates that this productive breed also has high survival capabilities. Furthermore, linear regression models can be constructed for live weight, allowing for age prediction. Projects and support aimed at increasing the number of Anatolian Merino sheep can contribute to both livestock activities and economic benefits. Especially, raising awareness among rural communities in this regard, providing on-site employment, will promote both red meat and wool production as well as rural development.

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

Ethical statement or informed consent: The study was approved by the Local Ethics Committee on Animal Experiments of Kafkas University (KAU-HADYEK-2024/11), Kars, Türkiye.

Author contributions: BBK: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. VÖ: Project administration, Methodology, Investigation, Data curation. All authors edited, read, and approved the final manuscript.

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

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The effect of lycopene and ascorbic acid on the post thaw Angora buck semen parameters

ABSTRACT

The main goal of this research was to examine the impact of antioxidants, specifically lycopene and ascorbic acid, on the viability of goat sperm following cryopreservation and thawing. Goat rearing plays a vital role worldwide by supplying animal protein, creating employment opportunities, and providing raw materials for various industries. Improving the reproductive efficiency of goats is crucial for genetic conservation, as well as for enhancing profitability and sustainability for breeders. This study illustrates that both lycopene and ascorbic acid, either alone or in combination, have a positive influence on motility, plasma and acrosome integrity, and mitochondrial activity after thawing. Semen samples were obtained from four mature goats and segregated into four groups: control (C), lycopene at 2 mM/mL (L2), ascorbic acid at 5 mM/mL (A5), and a combination of lycopene at 2 mM/mL + ascorbic acid at 5 mM/mL (L2A5). Following dilution in Tris/egg yolk diluent, the semen samples were cryopreserved in liquid nitrogen and then thawed for assessment. The L2A5 group displayed the highest values across all evaluated parameters (motility, plasma and acrosome integrity, and mitochondrial activity) when compared to the control group ($p<0.05$). These results indicate that the concurrent use of lycopene and ascorbic acid can significantly enhance the quality of cryopreserved goat semen, thereby contributing to improved reproductive outcomes and genetic preservation in goats.

Keywords: Ascorbic acid, buck, cryopreservation, lycopene, semen

INTRODUCTION

Due to its excessive hardiness and performance to harsh conditions outdoors, the goat is distributed in many geographical areas. Goat farming is a commercial enterprise that seeks to maximize sales, create capital, and provide employment. It also focuses on the availability of animal protein in the meat and dairy sector for human consumption and the provision of raw materials and garments to the leather and garment industries. The total number of goats raised worldwide is approximately 1.2 billion. Goat consumption has continuously increased, implying the influence of demands and the economic and environmental value of goats in different geographical locations. Goat meat is an essential food source throughout the world, especially in developing countries, which are mostly in the tropics. Despite its significance and consumption in developing countries, where over 90% of goat populations exist, with 56%, but 1.8% of goats are available in Europe. Goat meat is widely consumed in Asia, Africa, and the Caribbean. On the other hand, goat milk is ranked third globally in dairy production but consumed by more people than all other dairy products (Raheem, 2024; Hussein et al., 2023).

The main function of a semen extender is to provide the spermatozoa with nutrients, protect them from the harmful effects of cold shock, and

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Research Article

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keep the optimal conditions for sperm in the rice straw of the cryocenter. Cryopreservation remains one of the most challenging aspects of applications involving the preservation of goat origin sperm (Purdy, 2006). The buffer used to preserve the spermatozoa in goats includes Tris and citric acid, which is the same as that which is effective in preserving spermatozoa of a buck (Mishra et al., 2010, Narlicay and Uslu, 2022). In most cases, diluted semen of goats is done using the two most commonly utilized sex dampen, which are sex extender based on skim milk or egg yolk. Egg yolk contains lipids that are hydrolyzed by a specific lipase enzyme, which is an element of the bulbourethral gland of the goat. A lipase enzyme interacts with skim milk loin by hydrolyzing triglycerides and develops a compound that is detrimental to sperm vigor in males (Sias et al., 2005). The resultant interaction that happens in goat semen is not with bull sperm, boar sperm, or ram sperm, but it is exclusively observable in goat semen. The protein is the causal factor for the decrease in sperm lively and it is the protein fraction called SBU III originated from the parotid of the goat (Pellicer-Rubio et al., 1997). Cryopreservation may cause DNA fragmentation, acrosomal defects, membrane lipoid peroxidation (Atiken, 2020), mitochondrial potentialization (Shah et al., 2016) and apoptotic changes (Agarwal and Majzoub, 2017) of spermatozoa. Spermatozoan births are mainly induced by an increased manufacture of reactive oxygen (Klalique et al., 2023). The impact of ROS on spermatozoa is associated with a reduction in sperm vigorous and an increment in dead and slow spermatozoa, decreases in fertilization, implantation, and pregnancy rates, decreased cleavage, decreased quality of the embryo, and anoreduction in blastocyst initiation by Simon et al., (2017). The authority of ROS transactions within the physiological system must be understood in governance, which results in isotopic loss. Many studies have been conducted concerning providing the reactive balance of ROS and search sperm as out as a point of sperm during preservation by administering exogenous antioxidants namely lycopene and Vitamin C (ascorbic Acid) (Al-Mutary, 2021).

Lycopene, a naturally occurring carotenoid with strong antioxidant properties, can be commonly found in foods such as tomatoes, watermelon, papayas, and pink grapefruit. It plays a crucial role in protecting cells and tissues from lipid peroxidation by engaging in processes that involve quenching singlet molecular oxygen and scavenging peroxy radicals. The antioxidant activity of lycopene is primarily catalytic in nature (Velmurugan et al., 2004). Research indicates that lycopene supplementation can enhance sperm motility, cell membrane integrity, and DNA integrity by up to 70-73% (Bucak et al. 2015; Uysal and Bucak, 2007; Zini et al. 2010). In contrast, vitamin C (ascorbic acid) is an enzyme-free, water-soluble antioxidant known for its high efficacy in combating reactive oxygen species. Studies suggest that there might be saturation levels of vitamin C in certain tissues. Acting as a scavenger for reactive oxygen species, vitamin C has been shown to protect sperm by minimizing damage to sperm membranes. This protective effect can ultimately improve sperm motility, vitality, and potentially aid in preserving sperm health (Amini et al., 2015).

The primary goal of this study was to investigate the effect of antioxidants like lycopene and ascorbic acid on the properties of goat sperm after thawing their cryopreserved samples. With goats currently numbering into growing populations and circles worldwide, enhancing reproductive efficiency to save genetic capacity is of great importance. Improvements in sperm quality can result in significant gains for breeders regarding profitability and sustainability. Then, if cryopreservation techniques for goat semen improve development begins to occur, it undoubtedly will have a positive effect on population genetics. This study shows that when

used alone or in combination, lycopene and ascorbic acid will positively affect parameters such as motility, plasma and acrosome integrity, and mitochondrial activity after freezing and thawing of goat semen.

MATERIALS AND METHODS

Reagents

All chemicals and reagents used in study (Citric Acid C0706, Glycerol G2025, Fructose F2543, Lycopene L9879, L-Ascorbic Acid A4544, FITC-PNA L7381, Trisma Base T6066) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Biological Industries (1 % Penicillin-streptomycin-amphotericin B mixture, 03- 033-1B) and Thermo Fisher Scientific, Waltham, MA, USA (Live/Dead™ Viability Kit L7011, JC-1 T3168). Lycopene dosage was prepared by modifying from Ren et al., (2018). Ascorbic acid dosage was prepared by modifying from Paudel et al., (2010).

Collection of semen

In the study, 4 adult goats (aged 2-5 years) were used. The care and feeding of the animals were carried out at Prof. Dr. Hümeyra Özgen Research and Application Farm. The research was conducted during the breeding season. Ejaculates were collected three times a week with the help of an electroejaculator. Mass and motility examinations were performed on the collected ejaculates. Ejaculates with a mass score of 3 or higher and a motility rate of over 80% were mixed and transferred to a water bath at 37°C. Tris extender (Fructose 82.66 mM, citric acid 96.32 mM, tris 297.8 mM, 1 egg yolk, 5% glycerol; pH: 7, 300 mosm) was used as the basic diluent for sperm. The mixed ejaculates were divided into four equal volumes as follows:

1. Control (C)
2. Lycopene 2 mM/mL (L2)
3. Ascorbic acid 5 mM/mL (A5)
4. Lycopene 2 mM/mL + Ascorbic acid 5 mM/mL (L2A5)

The samples were diluted in Tris/egg yolk diluent at 37°C to approximately 400 x 10^6 spermatozoa per milliliter. Following dilution, the semen samples were drawn into 0.25 mL French straws and equilibrated at +4°C for 3 hours. After equilibration, the semen samples were frozen in liquid nitrogen vapor (~-100°C) for 15 minutes and then stored in liquid nitrogen at -196°C. At the end of equilibration and after being stored in liquid nitrogen for at least 24 hours, the samples were thawed at 37°C for 25 seconds and evaluated for sperm quality parameters (motility, plasma and acrosome integrity, and mitochondrial activity).

Mass activity

According to Evans and Maxwell (1987), a drop of freshly obtained semen was placed on a slide and examined under a heated stage microscope with a 4x objective to assess the general movement of the sperm. The assessment was scored on a scale from 1 to 5.

Motility evaluation

Motility evaluation was conducted at 37°C using a heated stage phase-contrast microscope at 400x magnification. Semen sample (10 µL) was placed between a slide and coverslip, and motility was assessed by examining at least 7 different microscope fields. The average motility values from these fields were recorded as the motility rate (Evans and Maxwell, 1987).

Assessment of plasma membrane integrity

SYBR-14/PI was employed to evaluate spermatozoa viability (Garner and Johnson, 1995). Thawed sperm samples were diluted 1:3 with PBS (2 x 10^6 spermatozoa/mL). Subsequently, 30 µL of the sample was combined with 2.5 µL propidium iodide (PI) (2 mg PI in 1 ml distilled water) and 2.5 µL SYBR stock solution (diluted 1:5 with DMSO), followed by an incubation in darkness at 37°C for 15 minutes. The samples were then fixed with

10 µL Hancock solution. Evaluation was done using a fluorescent microscope (Leica DM 3000). Spermatozoa bearing green staining were deemed to possess intact plasma membranes, whereas those displaying red staining were interpreted as having compromised plasma membranes (Figure 1).

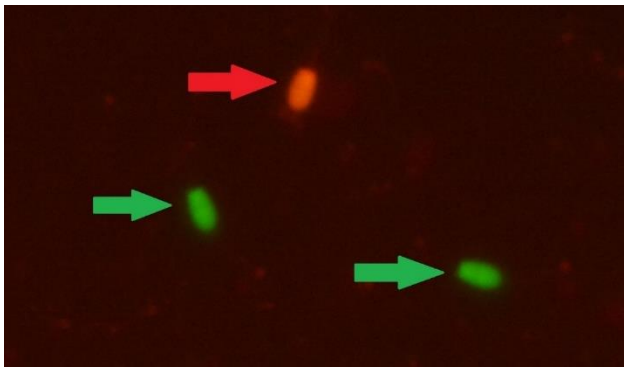

Figure 1. SYBR-14/PI staining for plasma membrane staining. Green spermatozoa heads represent intact plasma membrane (green arrows), red spermatozoa head represents damaged plasma membrane (red arrow).

Assessment of acrosome membrane integrity

A modified staining technique using Fluorescein isothiocyanate conjugated to Arachis hypogaea/PI (FITC-PNA/PI) was employed, as described by Nagy et al., (2003), to evaluate spermatozoa acrosome integrity. In this method, 5 µl of FITC-PNA stock solution (100 µg FITC-PNA/1 mL PBS) and 2.5 µl of PI solution were combined with 60 µl of diluted sperm sample at 37°C. The mixture was then dark-incubated at 37°C for 15 minutes followed by fixation with 10 µL Hancock solution. Acrosome integrity was assessed using a fluorescent phase-contrast microscope where spermatozoa exhibiting green, fluorescent acrosomes were classified as having damaged acrosomes, while those lacking green fluorescence were considered to have intact acrosomes (Figure 2).

Assessment of mitochondrial activity

Spermatozoon mitochondrial function was assessed using a modified JC-1/PI staining technique modified by Garner et al., (1997). A stock solution of JC-1 (1.53 mM) was prepared in DMSO. The cryopreserved spermatozoa were thawed in a 37°C water bath, diluted 1:3 with phosphate-buffered saline (PBS), and combined with 2.5 µL of JC-1 and 2.5 µL of PI in a 300 µL diluted sperm sample. Following a 15-minute incubation at 37°C in darkness, 10 µL of Hancock solution was added to stop reaction. Evaluation involved placing the sperm sample on a microscope slide, covering it with a coverslip, and examining 200 spermatozoa under a fluorescence microscope at 400× magnification. Mitochondrial activity was indicated by yellow-orange or bright green fluorescence in the midpiece, while pale dull green fluorescence signified the absence of mitochondrial activity (Figure 3).

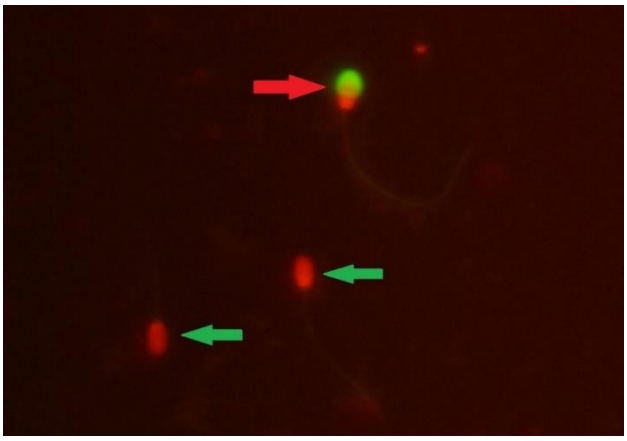

Figure 2. FITC-PNA/PI staining for acrosome membrane integrity. Spermatozoon with green hat represent damaged acrosome membrane (red arrow), spermatozoon without green hat represent intact acrosome membrane (green arrow).

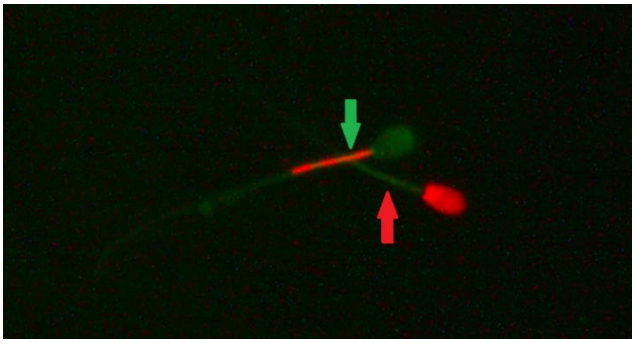

Figure 3. JC-1/PI staining for mitochondrial activity. Spermatozoa with orange midpiece represents the presence of mitochondrial activity (green arrow), spermatozoa with the pale green midpiece represents the absence of mitochondrial activity.

Statistical analysis

The research was replicated five times. Data was presented as the mean ± SEM. The average

values were assessed through ANOVA and Duncan's post-hoc test to identify notable variations in all parameters. These computations were carried out using SPSS/PC software version 25.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was denoted by $p<0.05$.

RESULTS

In the presented study, the values of motility, plasma and acrosome integrity, and mitochondrial activity in the K, L2, A5, and L2A5 groups are shown in Figure 4.

After freezing and thawing, the highest values for motility, plasma and acrosome integrity, and mitochondrial activity were obtained in the L2A5 group (63%, 72.96%, 69.91%, and 63.13%, respectively) compared to the control group ($p<0.05$).

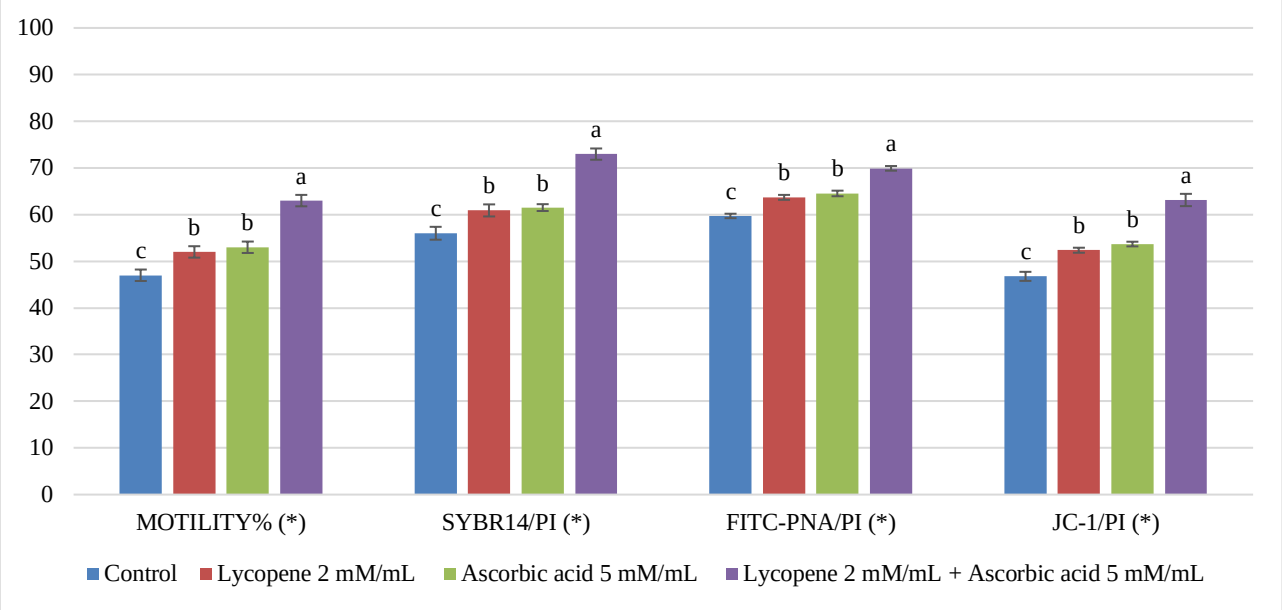

Figure 4. The effects of lycopene, ascorbic acid, and the combination of these two antioxidants on motility, plasma membrane integrity (SYBR14/PI), acrosomal membrane integrity (FITC-PNA/PI), and mitochondrial activity (JC-1/PI) in frozen-thawed Ankara buck semen. (*): Means with different letters (a,b,c) within each column indicate significant differences ($p < 0.05$).

DISCUSSION

The number of goats is increasing worldwide, necessitating the preservation of genetic capacity and the enhancement of reproductive efficiency. Improvements in sperm quality can result in significant gains for breeders regarding profitability and sustainability. In this context, advancements in cryopreservation techniques for buck semen will undoubtedly positively impact population genetics. Supplementing cryopreservation media with antioxidants enhanced semen quality by mitigating damage caused by free radicals (Bucak et al., 2019; Bucak et al., 2020; Bucak et al., 2024; Karaşör et al., 2022). The problem of antioxidants having different effects on sperm cryopreservation is still not solved, which is a big problem (Bucak et al., 2009). This study demonstrates that when used alone or in combination, lycopene and ascorbic acid positively affect parameters such as sperm motility, plasma and acrosome integrity, and mitochondrial activity after freezing and thawing processes. These findings significantly contribute to goat breeding and reproductive biotechnology, aiding in developing sustainable livestock practices.

Lycopene, present in tomatoes and red fruits, is recognized as the most powerful antioxidant

among various carotenoids. This polyene hydrocarbon features an unsaturated acyclic chain with 13 double bonds, 11 of which are conjugated and arranged in a linear fashion, providing its efficient antioxidant properties (Sen, 2019). Its antioxidant attributes include the ability to neutralize singlet oxygen and capture peroxyl radicals (Stahl et al., 1996). Ascorbic acid, also known as vitamin C, is a secure and water-soluble antioxidant. It has the capability to disrupt chain reactions and counteract the free radicals that initiate them, thus diminishing peroxidation. Ascorbic acid functions as a co-antioxidant, impeding the inclination toward lipid peroxidation. Acting as a robust free radical scavenger, ascorbic acid generates monodehydroascorbate radicals as end products—these are inert radicals that do not undergo reactions with other molecules or oxygen to produce highly reactive radicals (Bechara et al., 2022). This study illustrates the impact of lycopene, ascorbic acid, and their combined form on Ankara buck semen post freeze-thawing, as illustrated in Figure 1. The findings indicate that lycopene and ascorbic acid, both solo and in synergy, substantially safeguarded sperm motility, plasma and acrosome integrity, and mitochondrial activity compared to the control group post freeze-thawing ($p<0.05$). Notably, the combined dosage of 2 mM/mL lycopene and 5 mM/mL ascorbic acid exhibited superior protective effects on semen in comparison to all other groups ($p<0.05$).

In their study on Cashmere bucks, Ren et al. (2018) used lycopene doses of 0, 0.5, 1.0, 2.0, and 4.0 mg/mL. They obtained the highest motility, acrosomal and plasma membrane integrity, and mitochondrial activity rates with the 1 mg/mL lycopene dose. In our study, we used Ankara Bucks and added 2 mM lycopene. When calculated for molarity, 2 mM/mL is approximately equivalent to ~1 mg/mL. In our study, similar to the control group, the lycopene group showed higher motility and plasma and acrosomal membrane integrity, and mitochondrial activity integrity results. This suggests similar results can be obtained from sperm of the same species but different breeds. In their research on Sapudi rams, Bintara et al. (2023) used ascorbic acid and lycopene at concentrations of 1%, 2%, 3%, and 4%. They reported that the 3% lycopene and 3% ascorbic acid combination preserved semen quality after freeze-thawing. In another study on rams (Bucak et al., 2014), the 1×10^{-3} g/mL lycopene dose was reported to maintain mitochondrial activity and reduce DNA damage after freeze-thawing. Bucak and Uysal (2007) used an 800 µg/mL lycopene dose in Akkaraman rams and reported that it preserved sperm characteristics after freeze-thawing. In another study on bulls (Tuncer et al., 2014), the 500 µg dose of lycopene was reported to preserve semen parameters after freeze-thawing. Lycopene has also been reported to be effective under short-term storage conditions (Akalın et al., 2016). In their study on dogs, Sheikholeslami et al. (2020) used 500 and 750 µg of lycopene and incubated the semen at +4°C for 72 hours, reporting that both doses preserved progressive motility values. Besides bulls, rams, and dogs, studies have also reported the effectiveness of lycopene in mice (Babaei et al., 2021) and rabbits (Rosato et al., 2012). In their study on Boer buck semen, Memon et al. (2012) investigated ascorbic acid doses of 2.5, 4.5, 6.5, and 8.5 mg/mL. They observed that the 8.5 mg/mL dose preserved motility, viability, and acrosomal membrane integrity better than the other groups. In their research on Holstein Friesian x Haryana crossbred bulls, Paudel et al. (2010) tested the effects of ascorbic acid, chlorpromazine, and catalase at doses of 10 mM, 0.1 mM, and 200 IU, respectively, as well as their combination, and found that all antioxidant groups preserved sperm better than the control group. Ascorbic acid has also been effective in short-term studies. Akhter et al. (2023) tested the 1 mg/mL doses of Vit C and Vit E individually and in combination over a short term (72 hours) in Kail rams. They

reported that both individual and combined doses preserved sperm quality.

Research on the combined doses of lycopene and ascorbic acid in bucks is quite limited. The studies show that both lycopene and ascorbic acid preserve sperm parameters after freeze-thawing. In our study, the combined dose of lycopene and ascorbic acid provided the highest motility, plasma and acrosome integrity, and mitochondrial activity rates compared to all other groups ($p<0.05$). The lycopene used in our study belongs to the carotenoid class. The combination of two powerful antioxidants in our study worked synergistically to provide the highest sperm parameter values. Lycopene itself is a potent antioxidant (Stahl and Sies, 2003). As an antioxidant, ascorbic acid (Vitamin C) neutralizes free radicals and is oxidized to dehydroascorbic acid (DHA). Dehydroascorbic acid can be reduced back to ascorbic acid in the body through various enzymatic and non-enzymatic pathways. This redox cycle allows ascorbic acid to remain in an active form and maintain its antioxidant capacity (Buettner, 1993). Carotenoids, especially beta-carotene, effectively prevent lipid peroxidation (oxidation of lipids by free radicals). In this process, carotenoids trap free radicals and oxidize themselves, and these oxidized forms are reduced back by ascorbic acid, thus preserving the antioxidant efficacy of carotenoids (Sies et al., 1992).

This study reveals that combining lycopene and ascorbic acid significantly enhances the quality of cryopreserved goat semen, improving sperm motility, plasma and acrosome integrity, and mitochondrial activity. These findings suggest incorporating these antioxidants into semen extenders could benefit reproductive outcomes and genetic preservation, leading to increased profitability and sustainability in goat farming. Future research should optimize dosages for different breeds and explore long-term effects on fertility and offspring viability. Investigating other antioxidants and their potential synergistic effects could further enhance semen cryopreservation techniques.

CONCLUSION

In conclusion, it can be said that the quality of cryopreserved goat semen could efficiently be improved by using antioxidants such as lycopene and ascorbic acid. These antioxidants so help to enhance sperm motility, plasma membrane, acrosome membrane along with mitochondrial activity and reproductive outcomes after thawing. The most effective were extracts obtained from the combination of lycopene and ascorbic acid, since they gave that control group the highest values for all parameters tested. These results emphasize the ability of these antioxidants to reduce or overcome such damages due to cryopreservation that contribute towards maintaining the functional integrity of goat sperm. On the other hand, this can not only contribute to genetic conservation but also improve its profitability and sustainability.

On the basis of these promising results, we believe that there is a scope for research to optimize the cryopreservation protocols for buck sperm. Subsequent studies should also work in determining the optimal doses of lycopene and ascorbic acid which help to achieve maximum protective effect across various breeds under ambient stress. Furthermore, assessing the effects of these antioxidants on fertility and offspring viability in subsequent generations on a larger scale may provide greater evidence about their effectiveness. There is also room to explore other antioxidants combined with them which can be more protective against environmental stresses imparted by cryopreservation. These breakthroughs are vital in providing the livestock sector with sustainable breeding processes, meaning for thousands of

years to come farm animals are genetically safeguarded.

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Comparison of some diabetic and oxidative status parameters in three different experimental type 2 diabetic rat models

ABSTRACT

The aim of the study was to compare the levels of postprandial glucose, oral glucose tolerance test, and malondialdehyde parameters in 3 different experimental type 2 diabetic models induced rats. In the study, 18 Wistar albino rats were divided into 3 groups. The high-fat diet and streptozotocin (35 mg/kg, SC) were administered to the rats in the first group, water containing 20% fructose was administered to the second group, and nicotinamide (110 mg/kg, IP) and streptozotocin (60 mg/kg, SC) were administered to the third group. Oral glucose tolerance test, postprandial glucose, and malondialdehyde analyzes in 3 different experimental type 2 diabetic rat models were performed and they were euthanized at the 70th days. The postprandial glucose level was higher in the 1st and 3rd model groups than in the 2nd model group, while malondialdehyde level was no difference between the groups. Moreover, the second model group was significantly lower than the other two groups at all times according to oral glucose test results. In conclusion, the results of this research will contribute to researchers choosing the right model and parameters in experimental type 2 diabetic models in rats in the future.

Keywords: Malondialdehyde, oral glucose tolerance test, postprandial glucose

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) characterize hyperglycemia, insulin secretion and/or insulin activity defects (Punthakee et al., 2018). In the early stages, T2DM progresses with insulin resistance, hyperinsulinemia, and hyperglycemia, whereas there is observed loss of beta-cell function leading to inadequate synthesis of insulin hormone and elevation of blood glucose levels in the later stages of the disease (Chatterjee et al., 2017; Khan et al., 2020).

Diabetes mellitus, one of the most important metabolic diseases, leads to the loss of millions of lives worldwide (Khan et al., 2020). The global annual prevalence of T2DM is 6.1%, and it is estimated that the number of diabetic individuals will reach 1.3 billion by the year 2050 (Watkins & Ali, 2023) The diabetic complications such as diabetic nephropathy, neuropathy, retinopathy, adversely affects the quality of life in individuals with diabetes mellitus with glycated hemoglobin (HbA1c) levels between 6-9% (Chatterjee et al., 2017; Quinn, 2002). Diabetes mellitus has also been reported in certain animal species such as cats and dogs, similarly to humans (Kerem et al., 2023; Nelson & Reusch, 2014).

Insulin plays a crucial role in the utilization and storage of energy-providing molecules in tissues such as the adipose tissue, skeletal muscles, and liver as well as in maintaining the balance of energy metabolism. Moreover, insulin provides glucose uptake into cells, inhibits hepatic glucose production by suppressing gluconeogenesis and

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Research Article

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glycogenolysis, while promoting glycogen synthesis (Ruan & Lodish, 2003). Insulin resistance is a significant condition for T2DM, and it is characterized decreased insulin receptor sensitivity in muscles and the liver. Additionally, increased gluconeogenesis in the liver leads to elevated glucose release, while impaired glucose oxidation in muscles contributes to an increase in plasma glucose levels. The increased glucose levels are stored as fat in various tissues, primarily in the liver, and it leads to hepatic steatosis. The increased glucose and lipid levels induces oxidative stress, glucotoxicity, lipotoxicity, insulin resistance, and activation of inflammatory pathways in pancreatic β-cells, ultimately disrupting insulin secretion (DeFronzo et al., 2015; Scheen, 2003).

Insulin resistance and impaired glucose tolerance are present in prediabetic patients. The insulin resistance is one of the main treatment targets, especially in the management of T2DM (Sah et al., 2016; Saini, 2010; Young et al., 2019).

In a chronic disease like diabetes mellitus, it is important to control treatment costs and prevent the development of complications. Animal models have historically critical role in investigating the pathophysiology of the disease and evaluating new therapeutic agents in vivo. It is very important to choose the right experimental diabetic rat model for the research and development of new drugs in the treatment of T2DM (Al-Awar et al., 2016).

Until today, many in vivo studies have been conducted on the pathophysiology and treatment of T2DM. However, it is important to choose the right animal model for the research results to be reflected in the clinic. In order to choose the right animal model, the reliability, repeatability and costs of the model should be carefully analyzed (Frode & Medeiros, 2008).

The majority of T2DM models performed in experimental animals are models induced by streptozotocin (STZ). STZ tends to accumulate in pancreatic beta cells via glucose transporter 2 (GLUT2) and it causes cytotoxicity and reduces insulin secretion. The DNA alkylating activity of the methyl-nitrosourea part of STZ creates toxic effects and causes DNA fragmentation. Therefore, STZ-only models are more similar to type 1 diabetes mellitus models (Al-Awar et al., 2016). In addition, the method may cause undesirable effects such as kidney damage, oxidative stress in various organs, inflammation and endothelial dysfunction. It has been emphasized that STZ should be administered together with a high-fat diet in order to achieve symptoms similar to clinical T2DM (hyperglycemia, insulin resistance, altered lipid profile and hyperglycemia) (Ergel & Ertuğrul, 2022; Magalhães et al., 2019).

A more specific (proportional glucose increase and insulin decrease) T2DM model can be created in the diabetes model induced by the application of both STZ and nicotinamide. While STZ causes DNA fragmentation via GLUT2, poly (ADP-ribose) polymerase (PARP-1) activity increases to repair DNA. PARP-1 activity is limited and depletion of NAD+ and ATP in cells is prevented with the protective effect of nicotinamide. In this model, administration of nicotinamide before STZ prevents complete damage to β cells. Thus, hyperglycemia occurs while the insulin level decreases slightly (Szkudelski, 2012). Fructose causes chronic hyperinsulinemia and obesity. T2DM develops as a result of developing obesity and oxidative stress. However, this pattern can take weeks to induce (Basciano et al., 2005).

In the current study, it was aimed to reveal oral glucose test analysis, malondialdehyde (MDA) and post prandial glucose levels at the 10th week in 3 different experimental T2DM models in rats.

MATERIALS AND METHODS

Experimental design

In this study, 18 Wistar Albino male rats (8-12 weeks) were divided 3 groups. Experimental T2DM diabetes was induced in these rats in 3 different ways.

1. Model Group (n=6): In this model, animals were fed with a high-fat diet (58% of metabolic energy from animal fat) for two weeks and then low-dose STZ (STZ, 35 mg/kg, sc) was administered. Animals with a fasting blood glucose level ≥ 250 mg/dL were considered to have T2DM (Soetikno et al., 2020; Srinivasan et al., 2005).
2. Model Group (n=6): In this model, animals were given drinking water containing 20% fructose throughout the experiment (Incir et al., 2016).
3. Model Group (n=6): In this model, animals were injected with nicotinamide (i.p.) at a dose of 110 mg/kg and 15 minutes after this injection, STZ injection (s.c.) was administered at a dose of 60 mg/kg (Sayeli & Shenoy, 2021).

The blood samples were taken from the heart under thiopental Na anesthesia (40 mg/kg, ip) at the end of the 10th week, and the serum were frozen at -80°C until analysis.

Oral glucose tolerance test (OGTT)

The animals were fasted for 12 hours and fasting glucose (0th hour) was detected with glucose test strips (VivaCheck Eco, China) for OGTT analysis on the last day of the experiment. Subsequently, glucose at a dose of 2 g/kg was administered to all rats by oral gavage. Then, blood samples were taken from the tail vein at the 30th, 60 th, 90th and 120th minutes and blood glucose values were determined with glucose test strips.

Biochemical analyzes

Post prandial glucose was measured from frozen serum samples using by autoanalyzer (Abbott c8000, Chicago, USA). MDA (Rat Malondialdehyde Cat No: E0156Ra, Bioassay Technology Laboratory, Shangai, China) was analyzed on an ELISA reader (Bio-Tek Instruments Inc., MWGt Lambda Scan 200) via a rat-specific commercial ELISA kit.

Statistical analysis

The data were statistically analyzed using the SPSS 25.0 program (SPSS, Inc, Chicago, IL, USA). They were analyzed using one-way analysis of variance (ANOVA) and post hoc Duncan test. P<0.05 value was considered statistically significant.

RESULTS

The postprandial glucose levels and MDA levels in rats in which T2DM was induced with three different experimental models are presented in Figure 1 and Figure 2, respectively. Although the MDA level decreased in group 3, there was no statistical change between the groups. While the post prandial serum glucose level was highest in the third model group, the first model group was statistically similar to the third model group. The glucose level in the second group did not exceed the limit for diabetes in rats (250 mg/dL).

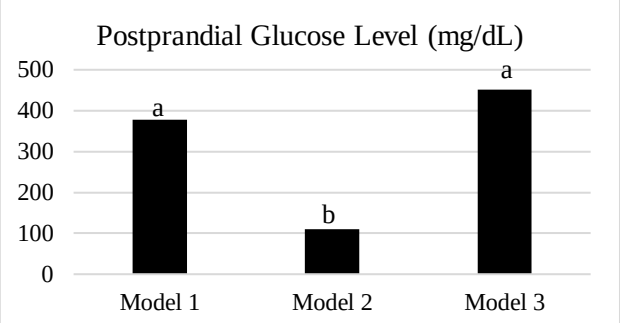

Figure 1. The postprandial glucose level in three different experimental T2DM models in rats.

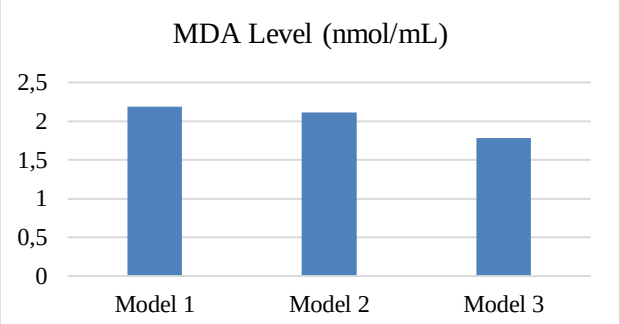

Figure 2. The MDA levels in three different experimental T2DM models in rats.

The OGTT results at the end of the experiment are presented in Table 1. The glucose level in OGTT was statistically lower in the second model group than in the other groups at all sampling times.

Table 1. Effects of three different experimental T2DM models on oral glucose tolerance test in rats (mean ± SD).

Sampling Time/Groups	1.Model Group	2.Model Group	3.Model Group
OGTT 0. min	275.0±114.9[a]	113.5±24.8[a]	305.8±195.1[a]
OGTT 30. min	447.8±127.9[a]	141.0±8.2[b]	398.8±136.3[a]
OGTT 60. min	418.8±127.0[a]	147.0±16.4[b]	452.8±131.4[a]
OGTT 90. min	430.0±116.1[a]	153.3±56.6[b]	436.3±128.9[a]
OGTT 120. min	409.5±168.5[a]	119.8±3.71[b]	380.7±144.4[a]

[a,b] : Values within line with no common superscripts are significantly different(P <0.05).

DISCUSSION

T2DM is a chronic metabolic disease characterized by high glucose levels. Early diagnosis of the pathophysiological changes in diabetes is important for the treatment of the disease and costs (Al-Awar et al., 2016; Chatterjee et al., 2017; Khan et al., 2020). Diabetes models induced in various ways are needed to evaluate the disease diagnostically and to determine the treatment effectiveness of various substances (Al-Awar et al., 2016; Incir et al., 2016; Sayeli & Shenoy, 2021; Soetikno et al., 2020; Srinivasan et al., 2005).

The inflammation and insulin resistance developed in rats fed a high-fat diet for 10 weeks (Lee et al., 2011). The glucose levels increased after 30 days in rats in which an experimental diabetes model was induced with high-fat diet and STZ. In addition, glucose levels after OGTT analysis and high HOMA-IR index showed that insulin resistance developed (Magalhães et al., 2019). In the 20% fructose-induced diabetes model, glucose level increased after 8 weeks (Mamikutty et al., 2014). In the T2DM model induced by STZ and nicotinamide, insulin resistance developed and glucose level increased on the 30th day (Sayeli & Shenoy, 2021). The hyperglycemia and oxidative stress caused renal and liver degeneration after 45 days in rats induced by STZ (65 mg/kg) and nicotinamide (110 mg/kg) application (Murugan & Pari, 2006). High glucose is reabsorbed into the bloodstream by the kidneys and contribute to hyperglycemia, which continues in a vicious cycle. As a result, this condition further contributes to abnormal glucose homeostasis, causing insulin resistance (DeFronzo et al., 2012). When blood glucose concentration does not return to baseline levels within 60 minutes after glucose challenge, insulin secretion has reduced, increasing the risk of insulin resistance and T2DM (Abdul-Ghani et al., 2010).

In the current study, post prandial glucose and glucose levels after OGTT analysis were higher in the STZ-high fat diet and STZ-nicotinamide groups (Models 1 and 3 group) during the experiment, compared to other models. In these groups, hyperglycemia may have occurred due to β cell damage caused by STZ leading to decreased insulin secretion. However, the experimental period may be short since fructose may increase glucose by causing insulin resistance. It can be speculated that model 1 and model 3 are more realistic for future short-term T2DM models and that the possibility of insulin resistance may be higher in these models according to the post prandial glucose and OGTT results.

Previous studies have shown that MDA increases due to the development of T2DM (Ibuki et al., 2020). Additionally, MDA levels have been shown to increase significantly as a complication of T2DM (Lu et al., 2010). Lipid peroxidation (MDA), which occurs due to hyperglycemia, can be inhibited by antioxidant enzymes in living organisms. However, the

levels of both free radicals and antioxidant enzymes increase with the development of diabetic complications in the later stages of the disease (Ahmed et al., 2006). In the current study, MDA level may have increased in all three T2DM model groups due to diabetic complications and no difference was observed between the models.

CONCLUSION

Researcher are carried out on different models on experimental animals for various complications and treatments of T2DM disease. However, it is very important for researchers that these experimental models should cheap, easy to create, and reflect the real symptoms of the disease. In the current study, the model in which STZ and nicotinamide combined model and the high-fat diet and STZ combined model were generally similar. However, the model created with 20% fructose did not reflect T2DM very well. As a result, current research results will be guiding for the experimental T2DM rat model that researchers will choose based on postprandial glucose and oral glucose tolerance testing. However, new experimental designs are required in which more parameters are analyzed, and longer-term studies are conducted.

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Ethical statement or informed consent: The study procedure was approved by Selcuk University Veterinary Faculty Ethics Committee (Ethical approval number 2024/083).

Author contributions: BD: Design of the project, creation of the model and preparation of the text by making statistical evaluations. OT: Carrying out the treatment protocol, performing analyzes and contributing to the writing of the text by evaluating the analysis results. TMP: Carrying out the treatment protocol, performing analyzes and contributing to the writing of the text by evaluating the analysis results.

Availability of data and materials: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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Diameter and sarcomere length of skeletal muscle fibers in the tissue of the tongue during the developmental process of the sheep

Research Article

ABSTRACT

The tongue plays an important role in the holding, chewing, and swallowing of food. This study was designed to determine the diameter and sarcomere length of skeletal muscle fibers in different parts of the tongue during the developmental process of sheep. For this purpose, small pieces of tissue were taken from the apex, the body, and the root parts of the tongue of sheep aged 6-12 months (G1, n:6), 1-2 years (G2, n:6), and 3-5 years (G3, n:6). Tissue samples were fixed in %10 formaldehyde solution and Crossmon's triple staining was applied to the tissue sections after routine histological processing. The diameter and sarcomere length of the skeletal muscle fibers in the tongue's apex, body, and root parts were measured. The diameter and sarcomere length of skeletal muscle fibers did not vary statistically between tongue regions in sheep in any age group ($p>0.05$). The sarcomere length of the skeletal muscle fibers in the apex, body, and root parts of the tongue did not change statistically with the development of the sheep ($p>0.05$). However, the diameter of skeletal muscle fibers at the apex and body parts of the tongue was statistically higher in G3 compared to G1 and G2, and the diameter of skeletal muscle fibers at the root part of the tongue was statistically higher in G3 compared to G1 ($p<0.05$). As a result, sarcomere length did not change throughout the development of the sheep, but skeletal muscle fiber diameter increased. Furthermore, skeletal muscle fiber diameter and sarcomere length did not vary between regions of the tongue.

Keywords: Fiber diameter, sarcomere length, sheep, skeletal muscle fiber, tongue

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INTRODUCTION

The digestive system of ruminants involves a special process known as rumination, and the digestive process begins in the mouth. The tongue is located in the oral cavity and is responsible for collecting, chewing, and swallowing of food. Nutrients taken into the mouth are swallowed and sent to the rumen after a simple digestive process. With the help of muscle movement, water, and saliva in the rumen, the food is thoroughly mixed and broken down. Bacteria and other micro-organisms also digest and ferment food in the rumen. The tongue helps to mix the food in the rumen and makes fermentation more efficient. The food is then returned to the mouth, where it is chewed again, thoroughly soaked, and broken down into smaller pieces. Therefore, in ruminants, the tongue is very important for a healthy digestive process as it assists in food collection, preparation, and rumen fermentation and facilitates the swallowing process (Hofmann, 1989; Reddy & Hyder, 2023).

The tongue is a flexible organ. Its main structure consists of skeletal muscle fibers. The skeletal muscle fibers extend longitudinally, vertically,

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and transversely, giving the tongue the ability to move in multiple directions. The tongue is covered with stratified squamous keratinized epithelium. The lamina propria under the epithelium covers a narrow area. The submucosa contains serous, mucous, and sero-mucous lingual glands. The histological structure of the tongue is designed to support the functions of the tongue. For example, the mucosal layer provides protection against harmful substances from the external environment, while the tongue papillae and taste buds enable the ability to taste. The tongue muscles control the movement of the tongue and support functions such as speaking and swallowing (Gülmez, 2011; Tanyolaç, 1999).

The skeletal muscle fibers show transverse striations. Myofibrils are formed by the regular assembly of actin and myosin myofilaments, the two basic proteins of the skeletal muscle fibers. The light and dark areas formed by actin and myosin myofilaments are located along the myofibrils, and a transversely striped appearance is created by lining up areas of the same tone in the same line. The light-colored parts are called Isotropic (I) bands and the dark-colored parts are called Anisotropic (A) bands. A very thin and dark "Z" line is in the middle of the I band. The distance from one "Z" line to another "Z" line is known as the sarcomere (Ertbjerg & Puolanne, 2017; Kahraman et al., 2018). Sarcomeres are the functional unit of force generation in the skeletal muscle fibers, and sarcomere length is known to be an indicator of active muscle force (Son et al., 2018).

Understanding skeletal muscle growth and development is one of the most important goals in animal science. Therefore, muscle mass is mainly determined by the number and size of muscle fibers. However, the number of muscle fibers does not change after birth. During postnatal growth, the increase in skeletal muscle mass is mainly due to an increase in muscle fiber size (Rehfeldt et al., 2000). So, the measurement of muscle fiber diameter is often used as a parameter for skeletal muscle growth (Hegarty & Hooper, 1971).

Studies on the tongue in ruminants have focused especially on the tongue papillae (Can et al., 2016; Delibaş et al., 2023; Jabbar, 2014; Kurtul & Atalgın, 2008; Madkour & Mohammed, 2021; Mahdy et al., 2021; Tadjalli & Pazhoomand, 2004; Unsal et al., 2003). However, there are no studies on the development of the skeletal muscle fibers, which form the main structure of the tongue. This study aimed to reveal the diameter and sarcomere length of skeletal muscle fibers in the tongue depending on the development of sheep.

MATERIALS AND METHODS

Animal material and tissue processing

The tissue material used in this study was obtained from sheep brought to abattoirs in Siirt province for slaughter. Three different study groups were formed by collecting tongue tissues from sheep aged 6-12 months (G1, n:6), 1-2 years (G2, n:6), and 3-5 years (G3, n:6). Small pieces were taken from the apex, body, and root parts of each sheep's tongue. Tissue samples were fixed in % 10 formaldehyde (pH = 6.9-7.1) for 24 hours at room temperature. After routine histological processing, the tissues were blocked in paraffin. They were cut to a thickness of 5 microns. Crossmon’s triple staining was applied to tissue sections for histological examination and histometric measurements. Prepared sections were examined under a light microscope (DM750, Leica) equipped with a digital camera (MC170, Leica).

Histomorphometric measurements

Ten randomly selected areas from the apex, body, and root parts of each animal's tongue were photographed at 40x magnification. The diameter of 20 longitudinal skeletal muscle fibers was then measured separately from the apex, body, and root parts of each sheep’s tongue. In addition, the sarcomere length of the skeletal muscle fibers was measured, as

described below. Sections containing at least ten sarcomeres from longitudinal skeletal muscle fibers were measured. Measurements were made from the beginning of the A-band to the end of the A-band of the other sarcomere. By counting the I-bands in the intermediate region, the number of sarcomeres was determined. The average sarcomere length was calculated by substituting the length of the measured region and the I band numbers into the formula below (Kahraman et al., 2018) (Figure 1).

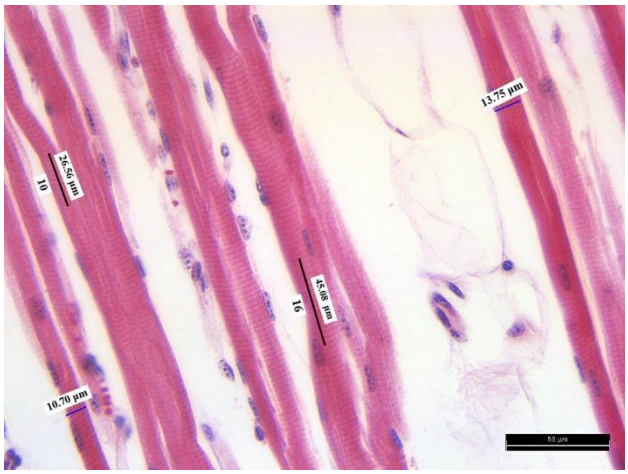

Figure 1: Representative histometric measurements. blue line: Measurement of the diameter of the skeletal muscle fibers (13.75 µm, 10.70 µm), black line: The distance measured from the beginning of the A-band of one sarcomere to the end of the A-band of the other sarcomere (26.56 µm, 45.08 µm), the number of I-bands: (10 and 16). Crossmon's triple staining. Bar: 50 µm.

Average sarcomere length (µm) = Length of measured area (µm) / Number of I-bands.

In this study, a total of 1080 muscle fiber diameters and 1080 sarcomere length measurements were taken using Image J software (Image J, US National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analysis of data was performed using Minitab® (v21.4.1). The Anderson-Darling normality test was used to assess the normal distribution of the data. Parametric tests were preferred because the data were normally distributed. One-way analysis of variance (ANOVA) was used to compare muscle fiber diameter and sarcomere length between age groups and parts of the tongue. The statistical significance level was evaluated as $p<0.05$.

RESULTS

Histological findings

The tongue of the sheep was covered with stratified squamous keratinized epithelium (Figure 2A). Skeletal muscle fibers, nerve plexuses, blood vessels, and lymph vessels were seen in the lamina propria and submucosa under the layer of epithelium (Figure 2A, B). Lingual glands and excretory ducts were observed in the body and root parts of the tongue, excluding the apex (Figure 2E-F). At the apex, body, and root parts of the tongue, the skeletal muscle fibers extended in the longitudinal, transverse, and vertical directions and constituted the majority of the submucosa (Figure 2B, C). The skeletal muscle fibers had numerous nuclei located just beneath the sarcolemma (Figure 2D). Transverse striations were noted in the skeletal muscle fibers, which extend in longitudinal and vertical directions (Figure 2D).

Histomorphometric findings

There was no statistically significant difference in the diameter of the skeletal muscle fibers between the apex, body, and root parts of the tongue in the G1, G2, and G3 groups ($p>0.05$) (Table 1). However, significant changes were observed in the diameter of the skeletal muscle fibers in the apex, body, and root parts of the tongue, depending on the development of the sheep. The diameter of the skeletal muscle fibers in the apex and body parts of the tongue was statistically higher in G3 compared to G1 and G2 ($p<0.05$) (Table 1). The diameter of the skeletal muscle fibers in the root part of the tongue was statistically higher in G3 compared to G1 ($p<0.05$) (Table 1).

Table 1: Diameter of skeletal muscle fibers in the apex, body, and root of the tongue of sheep aged 6-12 months (G1), 1-2 years (G2), and 3-5 years (G3).

Diameter of skeletal muscle fibers				
Groups	**Apex**	**Body**	**Root**	**p-value**
G1	14.86±0.61^{c}	16.53±0.61^{c}	16.60±0.53^{c}	>0.05
G2	15.28±0.32^{c}	17.36±0.42^{c}	16.94±0.54^{b,c}	>0.05
G3	17.21±0.56^{b}	19.04±0.76^{b}	18.43±0.20^{a,b}	>0.05
p-value	0.013	0.035	0.017	-

^{a,b,c}: different letters on the same column show significant differences between the mean values of the groups.

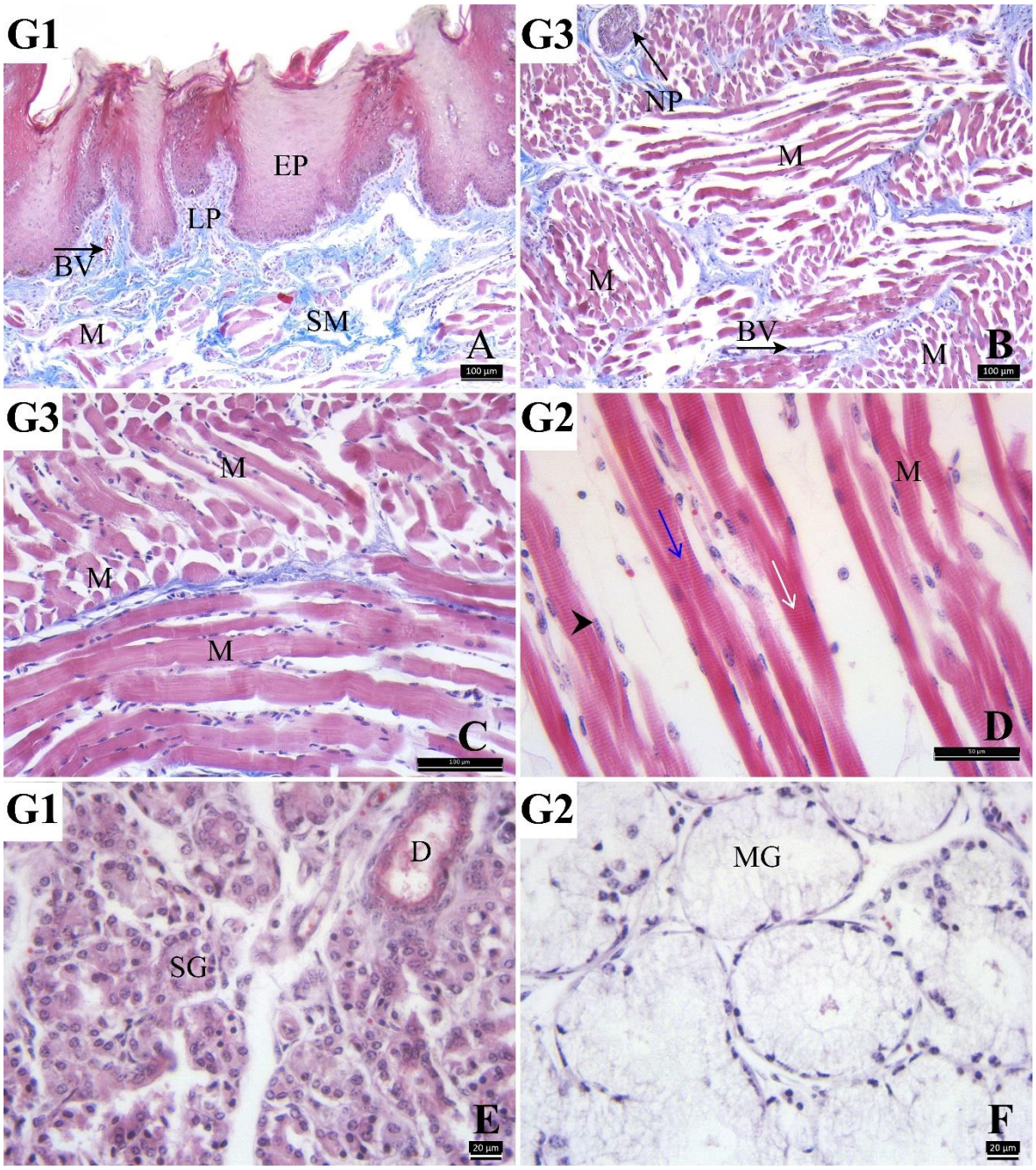

Figure 2: Histological appearance of the tongue of sheep aged 6-12 months (G1), 1-2 years (G2), and 3-5 years (G3). BV: blood vessel; D: excretory duct; EP: epithelial layer; LP: lamina propria; M: muscle fiber; MG: mucous gland; NP: nerve plexus; SM: submucosa; SG: serous gland; blue arrow: A band; white arrow: I band; arrowhead: nucleus. Crossmon's triple staining. Bar: A, B, C: 100 µm; D: 50 µm; E, F: 20 µm.

There was no statistically significant difference in the sarcomere length of skeletal muscle fibers between the apex, body, and root parts of the tongue in the G1, G2, and G3 groups (p>0.05) (Table 2). The sarcomere length of the skeletal muscle fibers in the apex, body, and root parts of the tongue did not change statistically with the development of the sheep (p>0.05) (Table 2).

Table 2: Sarcomere length of skeletal muscle fibers in the apex, body, and root of the tongue of sheep aged 6-12 months (G1), 1-2 years (G2), and 3-5 years (G3).

Sarcomere length of skeletal muscle fibers				
Groups	**Apex**	**Body**	**Root**	**p-value**
G1	2.66±0.02	2.58±0.06	2.78±0.06	>0.05
G2	2.69±0.05	2.60±0.08	2.76±0.03	>0.05
G3	2.57±0.03	2.68±0.05	2.70±0.05	>0.05
p-value	>0.05	>0.05	>0.05	-

DISCUSSION

The tongue, together with other organs in the oral cavity, plays an important role in the nutritional process (Steele & Van Lieshout, 2009). The tongue varies in size and shape in all mammalian species. Structural differences in the tongue between species reflect differences in the habitat and food resources of each species (Erdoğan & Sağsöz, 2018; Kadhim, 2016).

Erdoğan & Sağsöz, (2008) reported that the sheep tongue is surrounded by a multilayered keratinized epithelium and that blood vessels, nerve plexuses, and longitudinal, vertical, and transverse striated muscle fibers are present in the lamina propria and submucosa beneath the epithelial layer. Researchers detected lingual glands and excretory ducts in the body and root parts of the tongue except for the apex. In the present study, similar histological findings were observed in sheep tongues in all study groups.

Differentiated muscle cells lose their ability for division and proliferation (Sağlam et al., 2008). Previous studies have reported that there is no increase in the number of muscle fibers during postnatal development in mice (Rowe & Goldspink, 1969), rats (Brown, 1987; Rosenblatt & Woods, 1992; Schadereit et al., 1995), cattle (Wegner et al., 2000), chickens (Smith, 1963), and quail (Fowler et al., 1980). After birth, the total number of muscle fibers in mammals and birds does not change, and the increase in muscle mass occurs with the transverse and longitudinal growth of the myofibrils in the muscle fibers (Ertbjerg & Puolanne, 2017, Rehfeldt et al., 2000). Most muscle fiber growth takes place from birth to maturity (Ertbjerg & Puolanne, 2017). The ribosome organelle in muscle fibers synthesizes muscle proteins. These proteins are added to existing myofibrils and, when the myofibrils reach a certain thickness, they are split in the longitudinal direction. Thus, the number of fibrils in the muscle fibers increases and, accordingly, the diameter of the muscle fibers increases (Sağlam et al., 2008).

The sheep's tongue consists of the apex, body, and root parts (Kadhim, 2016). Al-Bazii et al., (2020) measured the height, width, and thickness of the apex, body, and root parts of the tongue of lambs and adult sheep. The researchers found that the height, width, and thickness of the tongue were statistically greater in adult sheep than in lambs. They stated that the sheep's tongue grows as its body grows.

There is no current study that shows the change in diameter of the skeletal muscle fibers, which form the main structure of the tongue, as the tongue grows during ruminant development. However, Fattah & El-Din, (2021) found that the

diameter of the skeletal muscle fibers in the tongue tissue of old mice was significantly reduced compared to adult mice. In this study, the researchers examined the changes in the diameter of the skeletal muscle fibers in the tongue due to aging. With aging, degeneration of muscle fibers occurs due to changes in muscle fiber structure, decreased contraction ability of muscle fibers, and increased muscle fatigue (Kletzien et al., 2018). However, the present study revealed changes in the diameter of the skeletal muscle fibers in different parts of the tongue depending on the development of the sheep. In this study, it was determined that the diameter of the skeletal muscle fibers in the apex and body parts of the tongue was statistically increased in G3 compared to G1 and G2, and the diameter of the skeletal muscle fibers in the root part of the tongue was statistically increased in G3 compared to G1. These results indicate that the diameter of the skeletal muscle fibers in the tongue tissue increases as the sheep grow. However, while changes in the diameter of the skeletal muscle fibers due to the development of sheep showed similar characteristics in the apex and body parts of the tongue, the root part of the tongue showed different characteristics compared to the apex and body parts. It is thought that this situation is related to the development of sheep depending on their nutritional characteristics.

In a study conducted on the tongue of rats, it was found that the diameter of the skeletal muscle fibers increased as they moved away from the tip of the tongue toward the back (Cullins & Connor, 2017). The researchers detected that the skeletal muscle fibers in the anterior part of the tongue contract faster, while the posterior part of the tongue has a higher proportion of slower fibers. They have stated that smaller diameter muscle fibers in the anterior region of the tongue allow for motor control, while larger diameter muscle fibers in the middle and posterior regions of the tongue may contribute to bolus driving forces for propulsion into the hypopharynx (Cullins & Connor, 2017). However, in this study, it was found that the diameter of skeletal muscle fibers did not show a significant difference between the regions of the tongue throughout the development of the sheep. It is thought that this may be due to differences in dietary characteristics between species.

Sarcomeres are the smallest contractile units and serve as the primary force-generating machines of striated muscles. In skeletal muscle fibers, contraction is mediated by the interaction of actin and myosin myofilaments of sarcomeres arranged in series along contractile myofibrils. With the contraction, the skeletal muscle fibers are shortened and muscle strength is produced. Together with this basic mechanism, the skeletal muscle fibers perform various functions in the body (Gokhin et al., 2014).

The sarcomere length is a fundamental measurement that determines the ability of a muscle fiber to shorten and lengthen when it is contracted (Gokhin et al., 2014). The sarcomere length varies in a skeletal muscle-specific manner. Therefore, there are differences in sarcomere length between muscle types and animal species. In addition, there is no passive tension along a muscle fiber, so sarcomere length varies even within the same myofibril (Ertbjerg & Puolanne, 2017). In this study, the sarcomere length of the skeletal muscle fibers in sheep at different stages of development did not show statistically significant differences between regions of the tongue. According to this finding, it can be said that the skeletal muscle fibers in the sheep tongue have the same contractile capacity in different parts of the tongue to perform their functions.

Sarcomeres are constantly renewed as muscle fibers grow (Ertbjerg & Puolanne, 2017). However, it is known that the sarcomere lengths along a muscle fiber reach an optimum degree of filament overlap immediately after birth and remain unchanged thereafter (Williams & Goldspink, 1971). In a study conducted on the

skin of mice, it was determined that the sarcomere length in different muscle types did not change with aging (Hooper, 1981). Similarly, in this study, it was determined that the sarcomere length of the skeletal muscle fibers in the apex, body, and root parts of the tongue did not change statistically during the developmental process of sheep.

CONCLUSION

It was found that the diameter and sarcomere length of the skeletal muscle fibers, which form the main structure of the tongue, did not differ between regions of the tongue in sheep of different ages. However, it was observed that as the sheep grew, the diameter of the skeletal muscle fibers in the tongue increased, but the sarcomere length did not change.

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Author contributions: BK designed the study, collected tissue samples, performed laboratory procedures, analyzed study data, and prepared the original draft of the manuscript.

Availability of data and materials: Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Effectiveness of May Grunwald Giemsa staining on tissues fixed with sugar cane molasses

Research Article

ABSTRACT

Formaldehyde commonly used in laboratories for fixation of tissues is found in many areas, including industry, household materials, dentistry coatings manufacturin. Sugar cane molasses, a very viscous product with a long shelf life, is normally produced by boiling the water obtained from sugar cane without adding any other additives. In this study, we aimed to compare the potential impact of the both fixation solusions of buffered formol-saline and low-cost sugar cane molasses on rat biosystem tissues stained with May Grunwald Giemsa. Liver, spleen, kidney, skin, testicle, small intestine, large intestine, brain, cerebellum and lung tissue samples of 4 healthy adult rats of both genders were used as materials. Tissue samples were divided into two parts and fixed in 30% sugarcane molasses (Group A) and 10% buffered formal-saline (Group B) for 24 hours at room temperature. The collected tissues evaluated in terms of chromatin distribution, nucleus separation and cytoplasm staining. As a result, intense cell loss was observed in the skin and small intestines. Considering the chromatin distribution, nucleus separation and cytoplasm staining in other tissues (liver, spleen, kidney, testis, large intestine, brain, cerebellum and lung), tissues fixed with sugarcane molasses showed similar properties to tissues fixed with buffered formal-saline.

Keywords: Fixation, formaldehyde, histology, May Grunwald Giemsa, sugar cane molasses

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INTRODUCTION

Formaldehyde (CH_2O), an important member of the aldehyde family, is obtained as a liquid from the oxidation of methanol. Formaldehyde is a colorless, pungent, irritating, low molecular weight poisonous gas with a highly reactive property due to its strong electrophilic property, which can rapidly turn into gas at room temperature, burn, dissolves very well in water (Shaham et al., 1996; Smith, 1992). Formaldehyde taken into the organism from outside is not stored in the body. Formaldehyde is metabolized to formic acid in the liver and erythrocytes via the dehydrogenase (FDH) enzyme, and this chemical agent is excreted through urine and feces or by respiration as oxidized to carbon dioxide (Smith, 1992; Usanmaz et al., 2002). Formaldehyde is highly irritant to mucous membranes (Smith, 1992) and tends to combine strongly with proteins, nucleic acids and unsaturated fatty acids in a non-enzymatic way. This combination creates denaturation in proteins, causing cytotoxicity, inflammatory reaction, necrosis, allergy and mutagenic effects. It shows fixation function and antimicrobial activity in tissues that have lost their vitality (Bolt, 1987; Heck and Casanova, 1999; Usanmaz et al., 2002).

Molasses (Grape molasses), a traditional Turkish food, is produced from fresh or dried grapes, as well as mulberry, fig, apple, plum, carob,

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watermelon, sugar cane and sugar millet. Although the composition elements of molasses vary in comparsion with the fruit composition, the fundamental component is carbohydrate (Simsek and Artık, 2002). Molasses is a great source of vitamins such as thiamine (B1), pyridoxine (B6) and niacin (B3), which are necessary for healthy life (Batu, 1993).

In addition to its global utilization in the sugar industry, sugar cane molasses is also manufactured within our country, particularly in the Adapazarı province. Due to its high sugar concentration, it is easier to extract molasses from sugar cane because of (Batu, 2006). Sugar cane molasses has also been found to possess a high amounts of sugar, minerals and organic acids (Batu, 1993; Batu, 2001).

Histology and pathology laboratories are where tissue samples are stained and examined under a microscope for sample evaluation. Exposure to formaldehyde used in these processes threatens the health of technicians, histologists, pathologists, anatomists and scientists working in the laboratory. Therefore, there is a significant need to find healthy and natural alternatives to the use of formaldehyde. Numerous studies have proven that honey can replace formaldehyde in routine histochemical and immunohistochemical staining procedures (Lalwani et al., 2015; Nerune et al., 2018; Pandiar et al., 2017; Priyadarshi et al., 2022; Rahma and Bryant, 2006; Sah et al., 2022; Singh et al., 2015). However, honey is not widely available all over the world, so its high cost makes it unsuitable for practical use. Therefore, due to the high cost of formaldehyde and honey, it is important to search for substances that can overcome these shortcomings. Various studies conducted to date on honey, sugar and sugarcane reveal that these natural fixatives fulfill almost all the requirements of an ideal fixative (Patil et al., 2013; Patil et al., 2015).

In this study, the staining properties of tissues fixed with low-cost sugar cane molasses, which is a natural sugar and produced organically in Turkey, shown by May Grunwald Giemsa (MGG) staining method were compared with the staining properties of tissues fixed with buffered formol-saline.

MATERIALS AND METHODS

Experimental Procedure

In this study, experimental animals were obtained from Selçuk University Experimental Medicine Application and Research Center (dated 21.03.2017; decision no. 2017-14). Laboratory studies were carried out at Selçuk University Central Research Laboratory. Liver, spleen, kidney, skin, testicle, small intestine, large intestine, brain, cerebellum and lung tissue samples of 4 healthy adult rats of both genders were used as materials. Tissue samples were divided into two parts and fixed in 30% sugarcane molasses (Group A) and 10% buffered formal-saline (Group B) for 24 hours at room temperature. The collected tissues will be evaluated in terms of chromatin distribution, nucleus separation and cytoplasm staining.

Preparation of Fixative Solutions

Group A - 30% Sugar Cane Molasses - Organically produced sugar cane molasses from the market was diluted with distilled water to a concentration of 30%. The sugar content of the product used was examined in Konya Food Control Laboratory and it was reported that it only naturally contains fructose and glucose.

Group B - 10% buffered formal saline - 10% buffered formal-saline solution with a pH of 7 was prepared with 0.1 M phosphate buffer.

Histological Procedure

After 24 hours of fixation, tissue samples were washed, dehydrated and polished with known histologic techniques and blocked in paraffin. May Grunwald Giemsa (MGG) staining method was applied to 5 µm thick sections taken from the blocks. After the staining process, the preparations were covered with entellan and examined under a light microscope (Olympus, CX23).

RESULTS

Sugarcane Molasses Solution (30%)

Liver: Pale staining was observed. However, hepatocytes were clearly distinguished (Figure 1a).

Spleen: Pale staining was observed. Red and white pulp areas were distinguished from each other. Cell nuclei were prominent at high magnifications (Figure 2a).

Kidney: Renal corpuscles and nephrons were well differentiated. Cell nucleus was well stained, but chromatin detail was not visible. The vessel walls were well stained (Figure 3a).

Skin: Pale staining was observed. Cellular details in the hair follicle and epithelial tissue were not well defined (Figure 4a).

Testicle: Tubules were well stained. However, Leydig cells could not be distinguished. The spermatozoa in the lumen were quite prominent. Cytoplasm, nucleus and chromatin distribution in the cells were well distinguished (Figure 5a).

Small intestine: The lamina epithelialis layer of the vilus intestinalis was not clear. Tissue integrity was disrupted. However, cell nuclei were well differentiated in non- macerated tissues (Figure 6a).

Large intestine: Tissue integrity was preserved and staining quality was quite good. However, cellular loss was observed in the connective tissue. The cytoplasm and nuclei were well stained and chromatin distribution was evident (Figure 7a).

Heart: Cardiac muscle and transverse bandings were well distinguished. The tissue was generally well stained. Cell nuclei and muscular fibers were quite prominent. Cell nuclei were well differentiated in capillaries and endothelial cells (Figure 8a).

Brain: Pale staining was observed. However, cell nuclei were well differentiated. The layers were clearly distinguished from each other (Figure 9a).

Cerebellum: Nerve wires were clearly observed. Purkinje cells were easily distinguished. The appearance of the layers was distinct. Str. Granulosum layer was well stained (Figure 10a).

Lung: A very pale staining was observed. However,bronchi and bronchioles were easily distinguished from each other. Cell nuclei were evident at high magnifications (Figure 11a).

10% Buffered Formal-Saline Solution

Liver: Pale staining was observed. Chromatin distribution was evident in hepatocytes at high magnifications (Figure 1b).

Spleen: The tissue was well stained. Red and white pulp areas were well differentiated from each other (Figure 2b).

Kidney: Pale staining of the cytoplasm was observed, but cell nuclei were quite prominent. Chromatin distribution was well differentiated (Figure 3b).

Skin: Epithelial tissue hair follicles and other layers of the skin were prominent and well stained (Figure 4b).

Testicle: The cells and chromatin distribution in the tubules were quite evident. Tissue integrity was preserved and cells were well stained (Figure 5b).

Small intestine: Although disintegration was observed in some villi, the tissue was generally well stained (Figure 6b).

Large intestine: Tissue integrity and cellular lines were quite evident (Figure 7b).

Heart: Pale staining was observed. Transverse bandings were not well differentiated (Figure 8b).

Brain: The layers were quite distinct and nerve cells and nuclei were well differentiated (Figure 9b).

Cerebellum: Purkinje cells and nerve fibers were well differentiated. Cell nucleus and chromatin distribution was evident (Figure 10b).

Lung: Good staining was observed. Bronchial and bronchiolar epithelia were well stained. Cell nucleus and chromatin distribution were evident (Figure 11b).

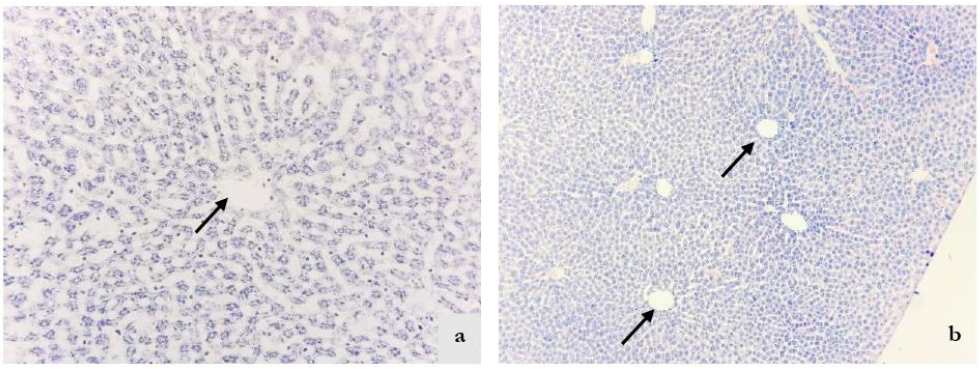

Figure 1. Liver, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X40, b) The tissue fixed with Formol Solution (10%), vena centralis (arrows), X10.

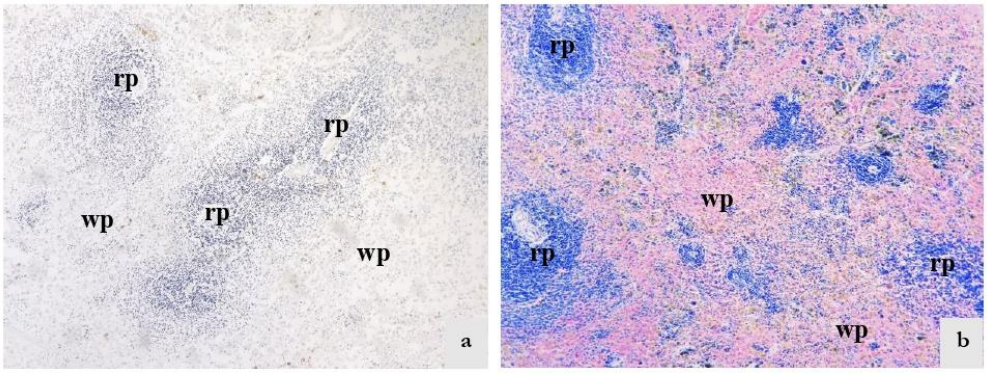

Figure 2. Spleen, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X40, b) The tissue fixed with Formol Solution (10%), Red pulp (rp), white pulp (wp) areas, X40.

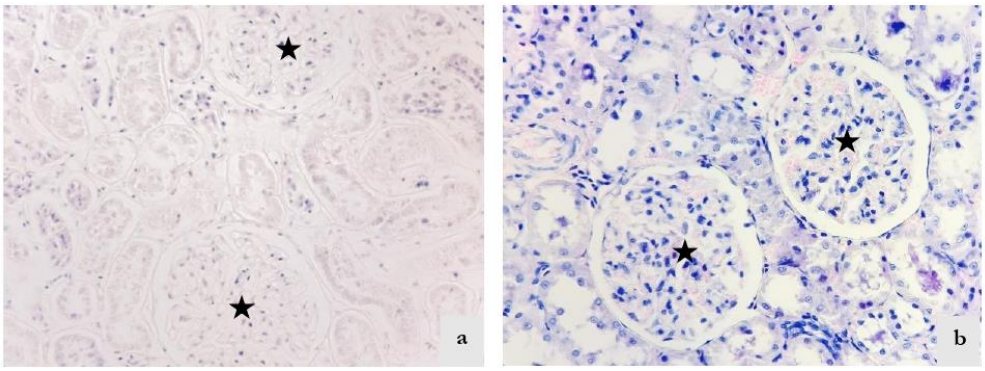

Figure 3. Kidney, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X40, b) The tissue fixed with Formol Solution (10%), Renal corpuscle (stars), X40.

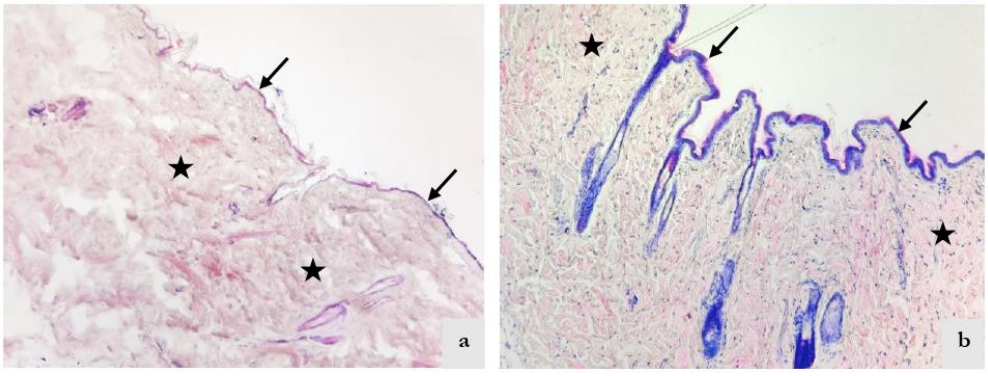

Figure 4. Skin, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X4, b) The tissue fixed with Formol Solution (10%), Epidermis (arrows), hypodermis (stars), X4.

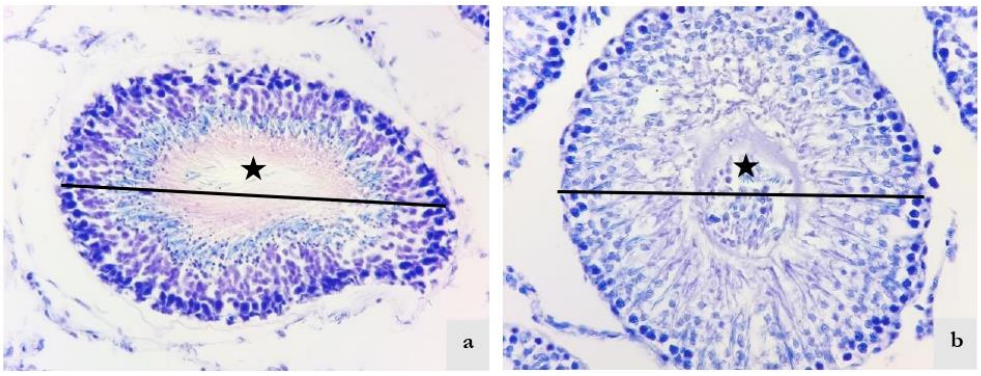

Figure 5. Testicle, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X10, b) The tissue fixed with Formol Solution (10%), Tubulus seminiferus contortus (stars), X10.

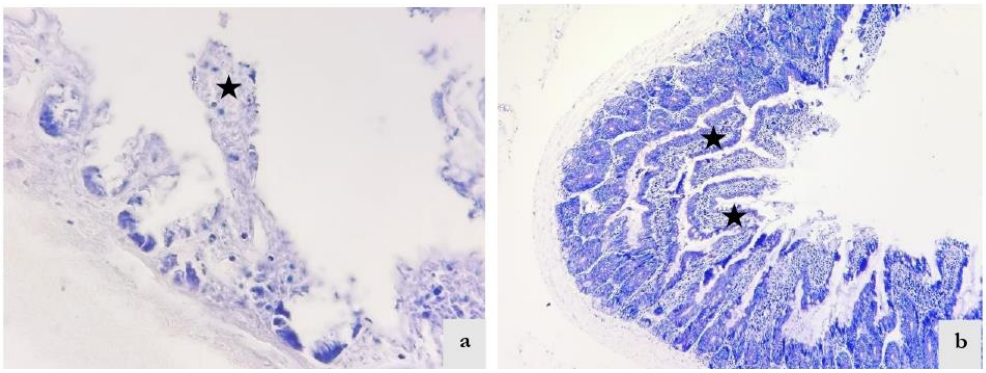

Figure 6. Small intestine, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X10, b) The tissue fixed with Formol Solution (10%), villus intestinalis (stars), X10.

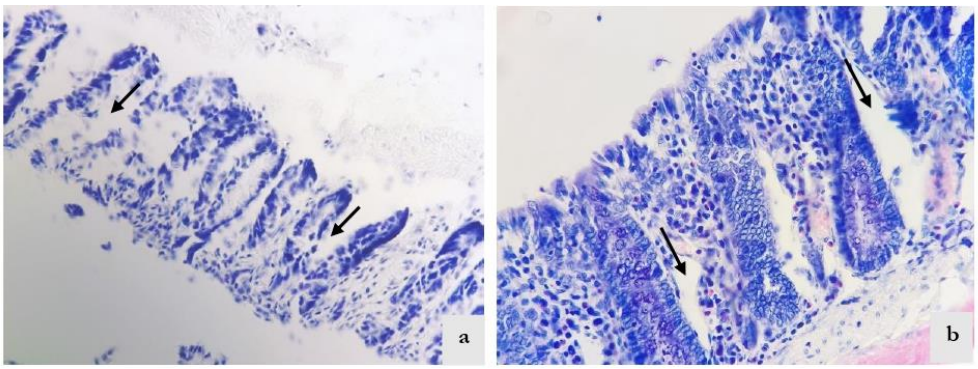

Figure 7. Large intestine, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X10, b) The tissue fixed with Formol Solution (10%), Intestinal crypts (arrows), X10.

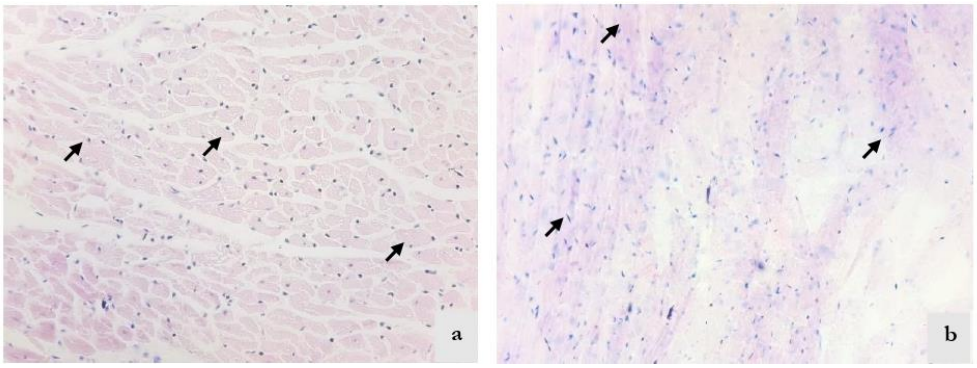

Figure 8. Heart, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X10, b) The tissue fixed with Formol Solution (10%), transverse and longitudinal muscle fibers (arrows), X10.

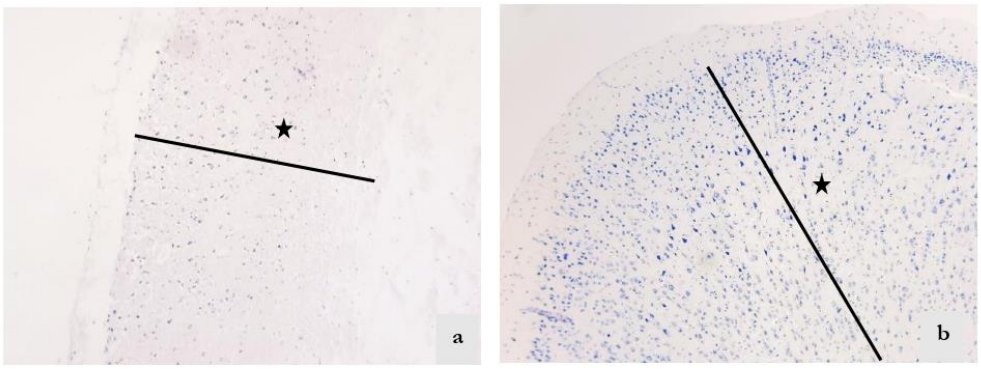

Figure 9. Brain, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X10, b) The tissue fixed with Formol Solution (10%), Neurons arranged in layers (stars), X10.

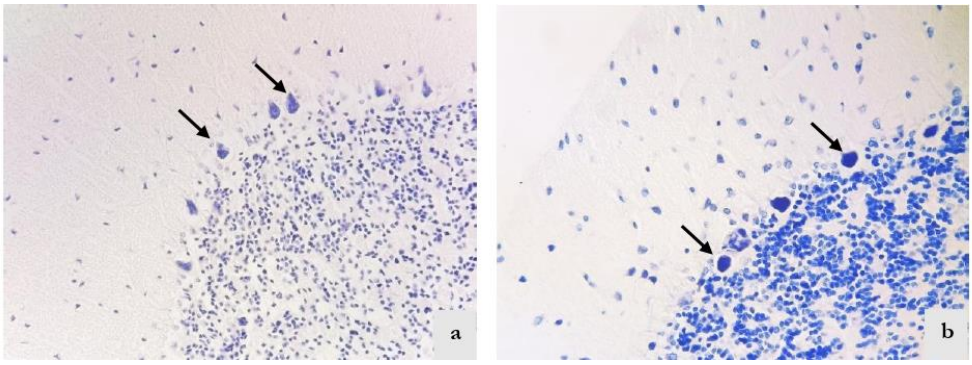

Figure 10. Cerebellum, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X40, b) The tissue fixed with Formol Solution (10%), Purkinje cells (arrows), X40.

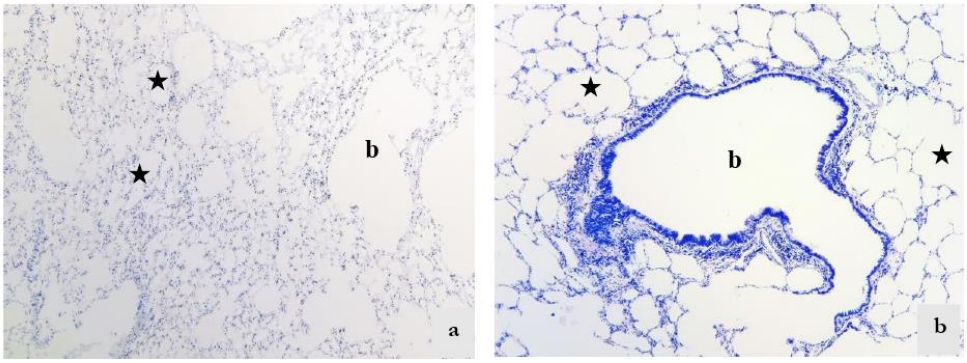

Figure 11. Lung, My-Grünwalt Giemsa (MG) staining method. a) The tissue fixed with Sugarcane Molasses Solution (30%), X10, b) The tissue fixed with Formol Solution (10%), Bronchiole (b), alveolus (stars), X40

DISCUSSION

Fixation is an initial and important step in preparing the tissue for microscopic examination. The main purpose of fixation is to keep tissues in the form closest to vitality, to prevent bacterial destruction, to prevent autolysis and to increase the index of better examination of the tissue.

In recent years, scientists have conducted a number of studies on the fixative properties of honey and sugar cane. Generally, fixatives with low pH do not support the preservation of cytoplasmic organelles; however, they act as a good nuclear fixative. Honey has been known to have anti-bacterial, acidic and dehydrating properties for several centuries. The anti-autolysis and tissue hardening, wound healing and anti-bacterial properties of honey have been emphasized in studies (Sabarinath et al., 2014). These show that honey is a very good fixative as well as a good preservative. Lalwani et al., compared the fixative properties of formaldehyde with processed and unprocessed honey in oral tissues (Lalwani et al., 2015). The fixation and staining quality of processed and unprocessed honey were evaluated in terms of staining efficiency in parallel with neutral

buffered formaldehyde. The staining quality of the nucleus, cytoplasm and evaluation of tissue morphology were determined as 100%, 92% and 75%, respectively. The results of the study indicate that processed honey and unprocessed honey are safe to use as an alternative for formaldehyde (Lalwani et al., 2015). Singh et al., (2015) analyzed the fixation efficiency on cytological smear samples using ethanol and 20% unprocessed honey. They also compared the efficiency between the two fixatives. The results showed that the honey-fixed smear was adequately fixed compared to the ethanol-fixed smear. They concluded that both ethanol- and honey-fixed smears were equal to each other and that honey could be used safely to replace ethanol (Singh et al., 2015). Sabarinath et al., (2014) conducted a study to determine the effectiveness of honey as a fixative by comparing honey and formaldehyde. The results of the study showed that nuclear details in both honey- and formaldehyde-fixed samples were similar with no difference in staining and microscopic morphology. However, cytoplasmic staining was sufficient to ensure the integrity of the tissue. No changes were seen in the cytoplasm of epithelial cells and connective tissue cytoplasm showed good staining by H&E with complete homogenization effect on collagen fibers (Sabarinath et al., 2014).

Patil et al., (2013) compared the tissue fixation properties of 20% honey, 20% sugar syrup and 30% sugar cane syrup (dark brown unrefined sugar obtained from sugarcane) with 10% buffered formaldehyde by staining with Hematoxylin Eosin (H&E). They found that the fixation of sugarcane molasses was excellent and tissue sections showed good overall morphology, nuclear, cytoplasmic details and staining in clearly distinguishable cellular outlines (Patil et al., 2013). In another study, Patil et al., (2015) examined the fixative properties of 30% cane sugar and 20% honey for 6 months (10% buffered formaldehyde was used as control) and stained the results with Hematoxylin Eosin (H&E), Periodic Acid Schiff (PAS) and Masson-Trichrome (MT). They evaluated the suitability of the fixatives. At the end of 6 months, they reported that all three stained sections (H&E, PAS, MT) had the same staining quality as formaldehyde-fixed tissues (Patil et al., 2015).

Nerune et al., (2018) compared the fixative properties of 95% ethyl alcohol and 20% honey on buccal mucosa and concluded that 20% processed honey could be used efficiently in cytological smear fixation to preserve cellular details (Nerune et al., 2018).

Priyadarshi et al., (2022) They compared smears fixed in 20% honey as a cytological fixative with 95% ethyl alcohol and found a strong agreement between both fixatives (kappa value varying between 0.896 and 0.942) and a p value of <0.05 (Priyadarshi et al., 2022).

Sah et al., (2022) in their study to evaluate the effectiveness of 20% honey and 20% jaggery as a fixative for oral exfoliative cytology; reported that low concentration of honey is an excellent alternative to ethanol (95%) and jaggery as a fixative for oral exfoliative cytological samples (Sah et al., 2022).

In this study, May Grunwald Giemsa staining quality of tissues fixed with 30% sugar cane molasses was compared with tissues fixed with buffered formal-saline. When all tissues were evaluated anatomically, the general morphology of the tissues showed that they preserved tissue integrity and that there was no color change in the tissues. In addition, tissue stiffness was at the required level, supporting previous studies.

CONCLUSION

In line with the histological findings, considering chromatin distribution, nucleus separation and cytoplasm staining, tissues fixed with sugarcane

molasses showed similar properties to tissues fixed with buffered formal-saline (except for the skin and small intestine, where intense cellular loss was observed). We believe that this study will be a resource for researchers who will study this subject, as it is the first study conducted with this staining (May Grunwald Giemsa) in the literature.

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Conflict of interest: The authors have no conflicts of interest to report.

Ethical statement or informed consent: This study was carried out at Selçuk University Reserch Animals Application Center. This research was approved by Selçuk University Experimental Medicine Application and Research Center Animal Experiments Ethics Committee (SÜDAM, Ref No: 2017-14, Date: 03/2017).

Author contributions: The research idea, obtaining materials, various processes in the laboratory and evaluation of the results were carried out by HYK and AY.

Availability of data and materials: Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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