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Effect of lactation number on milk yield in Holstein dairy cows

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ABSTRACT

Objective: Aim of the present study was to determine the effect of the lactation number on milk production and to detect the most economical lactation period in Holstein dairy cows.

Materials and Methods: The animal materials of the study were 392 Holstein dairy cows with a similar dry period and lactation length. The cows were divided into 6 groups according to the number of lactations. The groups were formed as 1st lactation (Group 1; n=67), 2nd lactation (Group 2; n=124), 3rd lactation (Group 3; n=51), 4th lactation (Group 4; n=62), 5th lactation (Group 5; n=41) and 6th lactation (Group 6; n=47).

Results: Average lactation milk yields for the 1st, 2nd, 3rd, 4th, 5th, and 6th lactations of the cows used in the study were 7860.03 kg, 9010.02 kg, 10207.15 kg, 10165.89 kg, 8430.57 kg and 8069.78 kg, respectively. It was determined that the highest milk production and highest daily income were obtained during the 3rd and 4th lactation periods ($p<0.05$).

Conclusion: In conclusion, milk production increased in the first three lactations, 3 and 4 lactation remained stable and decreased in 5th and 6th lactations. But, regardless of the number of lactations during the first 6 lactation periods, it is considered economical to use healthy cows for milk production in dairy farms.

Keywords: *Cows, Lactation number, Milk production*

INTRODUCTION

Traditionally, breeding programs for livestock animals have focused on the advancement of economically important production traits (Oltenocu and Broom, 2010). For this purpose, milk production has always been one of the most important parameters, and many studies have been carried out to increase milk yield per dairy cow (Zadoks and Fitzpatrick, 2009; Oltenoce and Broom, 2010; O'Hara et al., 2020). In the last three quarter-century, annual milk production per cow has been increased around 4-fold with successful genetic selection and other rehabilitation studies (Abuelo et al., 2019). It has also been reported that it should focus on lifelong production and longevity due to its environmental impact and animal welfare (Oltenoce and Broom, 2010). Due to the increase in milk production, it has become difficult to meet nutritional needs especially during the early lactation period (Walsh et al., 2011). And it is known that the high-yielding dairy cows experience negative energy balance up to the first 6 weeks postpartum (Bisinotto et al., 2012). Consequently, the increased milk yield has been accompanied by an increase in the production of diseases and culling rates (Esposito et al., 2014). On the other hand, pathological and physiological factors affecting milk production must be tackled and kept within ideal limits for sustainable profitability in milk production. However, many pathological and physiological factors affect milk production and are

caused by management inaccuracies in dairy cows (Short and Lawlor, 1992; Leblanc, 2010). Some of the important physiological factors that affect milk production in healthy dairy cows are dry period length (O'Hara et al., 2020) and lactation number (De Vries and Marcondes, 2020). Lactation numbers can affect the milk yield and composition in dairy cows (Vijayakumar et al., 2017). It is also known that aging has an impact on milk production (Holodova et al., 2019). Dairy cows need to be fully matured for optimum milk production (De Vries and Marcondes, 2020). Although dairy cows live longer than 10 lactations, their average productive life is only 3 or 4 lactations (Sott, 1994). Based on this information, it is thought that knowing the most productive lactation periods will have economic benefits. Therefore, we hypothesized that the lactation number might have a role in the annual milk yield of dairy cattle. The present study aimed to determine the effect of the lactation number on milk production and to detect the most economical lactation period in Holstein dairy cows.

MATERIALS and METHODS

Animals and management

For the presented study, the ethics committee approval was obtained from the Local Ethics Committee of Ceyhan Veterinary Faculty, Çukurova University (decision 09.07.2020 and 02/01). The animal material of the present study was 392 Holstein dairy cows with lactation numbers between 1 and 6. The cows were managed in free-stall barns, fed according to their individual needs with free access to water, and milked twice a day. All cows had a dry period length of around 60 days. At the beginning of the dry period, selective dry period treatment was performed on all dairy cows under the control of the farm veterinarian. The dairy cows routinely received early dry period (far-off), late dry period (close-up), fresh cow, early lactation, mid-lactation, and late lactation diets. The chemical content of the ration changed according to the stage of lactation and dry period. The health of the cows was checked periodically by the farm veterinarian. Clinical examinations were performed periodically at every stage of lactation, especially during group changes, and included rectal temperature, respiratory, appetite, physical posture, daily milk production, and routine udder checks. Cows with any health problems were registered in the farm registry system. Cows used in the present study were clinically healthy during the lactation period and did not have a problem that would negatively affect milk yield. The cows were divided into 6 groups according to the number of lactations. The groups were formed as 1st lactation (Group 1; n=67), 2nd lactation (Group 2; n=124), 3rd lactation (Group 3; n=51), 4th lactation (Group 4; n=62), 5th lactation (Group 5; n=41) and 6th lactation (Group 6; n=47) by the randomized grouping method. The colostrum period was defined as the first 5 days postpartum (Tsioulpas et al., 2007), and milk yield after this period was taken into account. Total milk production from initiation of lactation to end of lactation was recorded for each group of the cows. And daily average milk yield was calculated by the ratio of total milk yield to lactation days. Raw milk price was determined according to the national milk council 2019 recommendation price (USK, 2019).

Statistical analysis

Analysis of all datasets was performed statistically with SPSS (Version: 22.0; IBM, USA). The normality test of the data obtained from the research was performed with the Kolmogorov-Smirnov test. As a result of the normality test, one-way analysis of variance (ANOVA) and Duncan multiple comparison test were applied to reveal differences between group averages. The significance level was accepted as $p<0.05$. The results of the study were presented as the mean ± standard deviation of the mean (SD).

Table 1. The effect of lactation number on lactation milk yield in Holstein dairy cows.

Lactation Number	Cow Number	Lactation Milk Yield Mean (kg)	Std. Deviation
1	67	7860.03[a]	1241.02
2	124	9010.01[b]	1235.18
3	51	10207.15[c]	898.58
4	62	10165.89[c]	1100.29
5	41	8430.57[b]	1191.72
6	47	8069.78[a]	898.30
Total	392	8978.69	1427.64

[a, b, c] : The difference between the groups is statistically significant shown with different letters in the same row ($p<0.05$).

RESULTS

In the present study, the mean lactation length between groups was similar with 334.5±10.21 days ($p>0.05$). It was determined that the lactation number of cows affects the annual total milk production. Milk production gradually increased significantly in the first 3 lactations, peaked at the

3rd and 4th lactations, and then began to decline ($p<0.05$). The effect of lactation number on milk yield is given in Table 1. When considered economically, it was determined that the highest daily income was in the 3rd and 4th lactation periods (Table 2).

Table 2. Daily average milk production and milk income

Lactation Number	Cow Number	Daily milk yield Mean (kg)	Std. Deviation	Daily income per cow (1 L Milk=2.30 TL)
1.00	67	25.77[a]	4.06	59.27
2.00	124	29.54[b]	4.04	67.94
3.00	51	33.46[c]	2.94	76.96
4.00	62	33.33[c]	3.60	76.66
5.00	41	27.64[ab]	3.90	63.57
6.00	47	26.45[a]	2.94	60.84
Total	392	29.43	4.68	67.69

[a, b, c]: The difference between the groups is statistically significant shown with different letters in the same row ($p<0.05$).

DISCUSSION

Milk and dairy products have an indispensable place in food and drink consumption. The globalizing modern dairy industry is constantly updated. And the economy and sustainability are always important in the farm industry (Borawski et al., 2020). For sustainable profitability in dairy farms, optimum milk production per dairy cow is required and can be considered as an indicator of financial success (Horvath and Miko, 2016).

The normal life expectancy of dairy cattle is about 20 years (De Vries and Marcondes, 2020). However, it is known that their care on dairy farms for such a long time is not economical for the farm industry. On the other hand, Groenendaal et al. (2004) reported that annual veterinary cost per cow increased by an average of $ 5 each lactation. Therefore, dairy cows are used for milk production during their most economic and productive periods. The milk yield of dairy cows increases with age and decreases inversely proportional to aging after reaching the maximum level (Holodova et al., 2019). With a similar description, Grandl et al. (2016), reported that milk production increases during the first few lactations, reaches a flatness between 5 and 8 lactations, and then begins to decrease. In the present study, data of the first 6 lactation cows were used because there was a problem of finding cows in other lactation. The reason for this is that animals are culled from the herd due to disease and production problems. Similarly, De Vries and Marcondes (2020) reported that the productive life of dairy cows in the most modern dairy industries is average 2.5 to 4 years. They were also declared that their total life span is between 4 and 6 years in most developed dairy industries because they are removed from the herd due to problems caused by high production. But we think that shortening in the average life expectancy of cows may also be related to management conditions. Because cows up to the 6th lactation are commonly used in production.

De Vries and Marcondes (2020) were declared healthy cows mature fully in approximately the 5th lactation and produce the most milk in this period. Similarly, Holodova et al. (2019) found that milk yield reached a peak in the 6th lactation. However, we determined that the lowest milk production was in 1st and 6th lactations and milk yield peaked in the 3rd and 4th lactations ($p<0.05$). When the table is also examined, the milk yield and income of milk obtained per cow has increased by 30% on average during the 3rd and 4th lactation periods compared to the 1st lactation period. These differences are thought to be due to milk yield, genetic differences, and management. However, the common point in our and other researchers' studies is that the milk yield starts to decrease again after reaching the peak level. This indicates that there is a decrease in the function of the udder tissue due to physiological aging. Snijders et al. (2000) were also noticed that dairy cows in 1st lactation had lower milk yield and body weight compared to dairy cows in 3rd lactation. Turiello et al. (2020) were reported that age at first calving associated with milk yield during the first lactation. However, the age and body weight were not taken into consideration in the present study. Dry period length can also affect lactation milk production in dairy cows (O'Hara et al., 2020). Kok et al. (2017) and Boujenane (2019) reported that dry period should be performed for optimum milk production at the next lactation. The ideal dry period length is considered to be 60 days (Boujenane, 2019). In the presented study, an average of 60 days' dry period was applied to all cows.

CONCLUSION

In conclusion, milk yields of healthy Holstein cows were compared during the first 6 lactations in this study. The findings obtained show that milk production increased in the first three lactations, 3 and 4 lactations remained stable and decreased in 5th and 6th lactations. In terms of milk yield and

income, the most economical periods were the 3rd and 4th lactation. Regardless of the number of lactations, it is considered economical to use healthy cows for milk production in dairy farms. However, this situation should be investigated for cows in the 7th lactation and older.

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Author's Contributions: All authors (FE and SK) contributed to the study conception and design. Supervision/Consultancy (SK and FE), Data collecting (FE), Literature research (SK and FE), Writing the article (FE and SK), Critical review (FE and SK). All authors read and approved the final manuscript.

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The evaluation of arterial blood pressure in anesthetized dogs with xylazine and ketamine

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ABSTRACT

Objective: It was aimed to investigate the effects of ketamine combination administered with xylazine used for general anesthesia in dogs on arterial blood pressure, heart rate, and body temperature.

Materials and Methods: In the study, a total of 20 dogs, 14 females and 6 males, from various breeds and ages 1 to 5, which undergone elective ovariohysterectomy or castration according to body weights. 1 mg/kg xylazine (xylazine hydrochloride, 23.3mg/ml, Xylazinbio 2% Bioveta®, Czechia) and 10 mg/kg ketamine (ketamine hydrochloride, 100 mg/ml, Ketasol 10% Richter® Pharma Ag, Austria) combination within a single injection with 21G needle were applied intramuscularly. The food and water access were ceased 12 hours before drug administration. Systolic and diastolic blood pressures, pulse measurements, and body temperatures were measured 3 times before and 5 times during anesthesia (at the 15th, 30th, 45th, 60th and 120th minutes of anesthesia) by using the AM6100 veterinary bedside monitor. Muff was placed to cover 1/3 of the proximal leg and for the artery to be recognizable by the microprocessor. Pulse rates were measured with electrodes connected to the device. Body temperature was measured by a rectal thermometer which was a part of the device. Measurements were taken before anesthesia was considered as control measurements.

Results: In systolic blood pressure, the recordings at 0, 15th, 30th mins have been found as statistically significant in relation to 45th, 60th, and 120th ($p<0.05$). In diastolic blood pressure, there were no significant differences recorded. The change between the preintervention and post-application has been found significant ($p<0.001$). Body temperature has shown a meaningful change in comparison to the starting point after the readings ($p<0.001$).

Conclusion: Eventually, decreases in blood pressure, heartbeat, and body temperature were observed for the dogs that have been anesthetized with the combination of xylazine-ketamine.

Keywords: *Blood Pressure, Body Temperature, Diastolic, Dog, Heart Rate, Systolic*

INTRODUCTION

Nowadays, anesthetics are used in immobilization of wild animals, surgical interventions, and during operations for pain relief, as well as during labor, diagnosis, and treatments of diseases with pain (Atasoy and Karadeniz, 2003). After the administration, differences are seen in body temperature, rate of respiration, heart rate, arterial blood pressure, arterial pH, blood gasses, and hematologic values within the organism. (Allen et al., 1986; Hall et al., 2001; Koç and Sarıtaş, 2004).

Xylazine is the first $\alpha 2$ agonist used in veterinary medicine for premedication (Greene and Thurman, 1988; Bilgili and Doğan, 1991). After parental application, while decrease in respiratory rate, bradycardia, hypothermia form is observed, long

term hypotension forming after intravenous application. (Samy and Othman, 1985; Börkü et al., 2005; Murrel and Hellebrekers, 2005; Lemke, 2007; Cardoso et al., 2014; Kellihan et al., 2015). In some animals, it causes a decrease in intraocular pressure by reducing aqueous humor production (Sinclair, 2003).

Ketamine is a dissociative anesthetic which is a phencyclidine derivative that constitutes dissociation from the surrounding environment like catalepsy (Booth, 1982; Allen et al., 1986; Hellyer, 1996). Depending on the dosage, analgesic, sedative, and anaesthetic effects can be observed (Aantaa and Scheinin, 1993). In recent years, antidepressant-like features were also discovered (Mihara et al., 2012; Franceschelli et al., 2015). When used alongside anaesthetics with depressive effects on the nervous system, hypoxia, hypercarbia and changes in body temperature can occur (Short et al., 1993). It causes increase in heart rate and blood pressure. Temporary respiration depression forms. following anaesthesia, outflow hallucinations may take place. therefore, it is not suggested that it is applied on its own (Colby and Sanford, 1981; Booth, 1982; Haskins et al., 1985; Izci et al., 1993; Gülanber et al., 2001; Topal, 2005; Flecknel, 2016).

The side effects caused by the combination of xylazine-ketamine, which is often used in small animal medicine, are being underestimated. Complications are occurring especially upon usage in animals with an unhealthy cardiovascular system and at absence of essential precautions. In this study, the effect of xylazine-ketamine combination on blood pressure was investigated using the oscillometric technique.

MATERIALS and METHODS

A variety of dog breeds, 14 females and 6 males, aged between 1-5, weighed 21-45 kg, brought to the clinic for spaying and the method ovariohysterectomy, a castrating method, were used as study subjects. 1 mg/kg xylazine (xylazine hydrochloride, 23.3mg/ml, Xylazinbio 2% Bioveta®, Czechia) and 10 mg/kg ketamine (ketamine hydrochloride, 100 mg/ml, Ketasol 10% Richter®, Pharma Ag, Austria) combination within a single injection with 21G needle was applied intramuscularly. The food and water access were ceased for 12 hours prior to the practice. Before practice and during the 15th, 30th, 45th, 60th and 120th minutes of the practice their systolic and diastolic blood pressures, pulse rates and body temperatures were recorded, to produce three repeat measures in total. Data recorded before practice was used as control measurements. Measurements were taken by a special veterinary use bed-side monitor (AM6100 Veterinary Monitor, Shanghai-China). Blood pressures were measured automatically by an oscillometer. According to the literature, muff was placed to cover 1/3 of the proximal leg and for the artery to be recognizable by the microprocessor. Pulse rates were measured with electrodes connected to the device. Body temperature was measured by a rectal thermometer which was a part of the device.

Statistical analysis

Before significance tests, Numeric data obtained was first evaluated by Shapiro-Wilk test to assess the normality of parametric test assumptions, and Levene test to assess homogeneity of the variances. Variance analysis was used for Dependent quantitative data which were repeated more than two times to test variations. In the case of determined differences, Post-hoc Analysis with Bonferroni correction was used to specify which repetition the differences resulted from. All statistical calculations were analyzed with a minimum of 5% error margin. Level of significance was determined as $p<0.05$.

RESULTS

Changes in systolic and diastolic blood pressure, heart rate per minute and body temperature caused by xylazine-ketamine combination before and after application presented in Table 1.

Systolic blood pressure was significant at 0, 15 and 30 minutes after application in comparison with the measurements at 45, 60 and 120 minutes (Figure 1).

No significant change in diastolic blood pressure (Figure 2). Changes in heart rate were statistically significant ($p<0.001$). Heart rate measurements showed a significant decrease after the application (at 45, 60 and 120 minutes) in comparison to before application ($p<0.05$) (Figure 3).

Body temperature showed a significant fall in comparison to values before application ($p<0.001$). Value obtained before application and at 30th minute are statistical meaningful in comparison to values obtained at 45th, 60th and 120th minutes ($p<0.05$) (Figure 4).

Table 1. Data before and after the xylazine-ketamine combination application.

Parameter	Measurements (minutes)						
	0	15	30	45	60	120	P
Systolic Blood Pressure (mmHg)	167.417 ± 4.875[a]	169.117 ± 3.857[a]	176.233 ± 6.225[a]	158.567 ± 4.523[b]	160.167 ± 3.474[b]	157.517 ± 3.711[b]	0.010
Diastolic Blood Pressure (mmHg)	106.283 ± 6.413	118.017 ± 5.189	126.033 ± 7.839	108.667 ± 5.835	112.267 ± 3.971	105.800 ± 5.995	0.051
Heart rate (bpm)	90.950 ± 5.246[a]	79.783 ± 5.346[ab]	90.633 ± 4.557[a]	73.433 ± 4.199[b]	69.100 ± 4.641[b]	72.767 ± 3.776[b]	<0.001
Body temp. (°C)	38.317 ± 0.121[a]	37.710 ± 0.230[ab]	37.925 ± 0.142[a]	37.072 ± 0.198[bc]	37.007 ± 0.203[bc]	36.717 ± 0.187[c]	<0.001

*[a, b, c]: The difference between the groups is statistically significant shown with different letters in the same row ($p<0.05$).

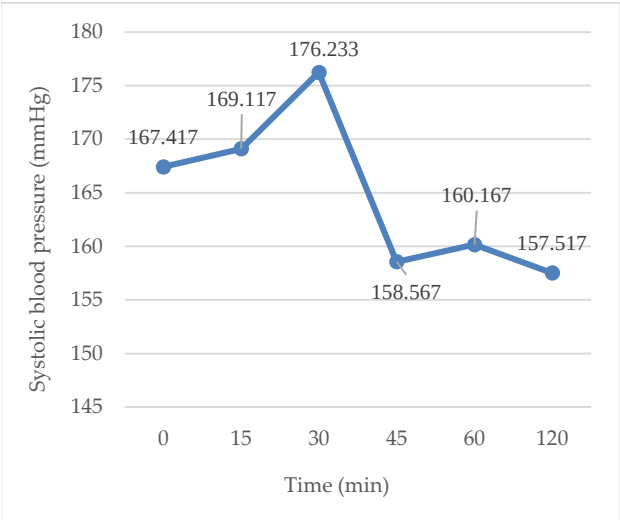

Figure 1. Changes in systolic blood pressure

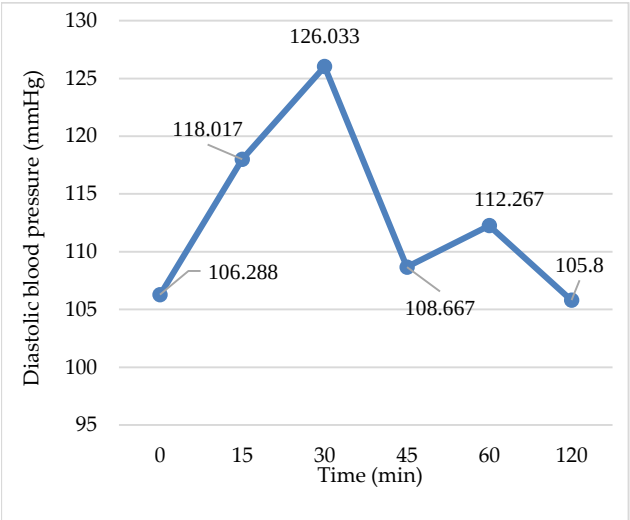

Figure 2. Changes in diastolic blood pressure

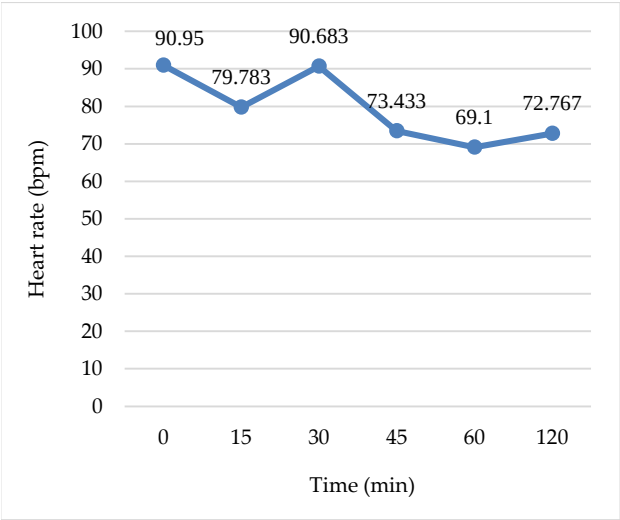

Figure 3. Changes in heart rate

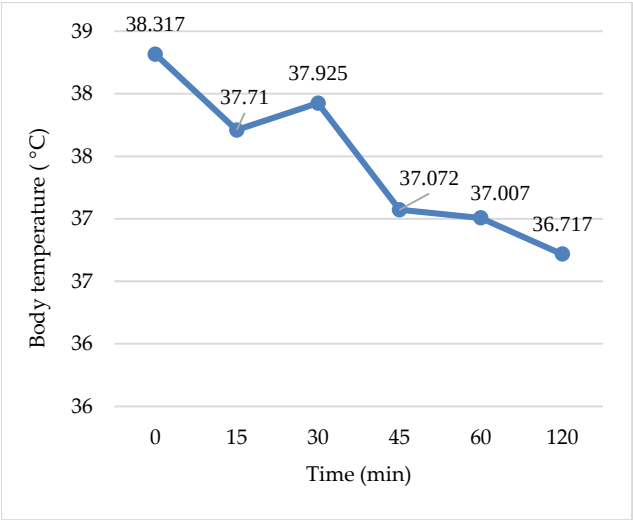

Figure 4. Changes in body temperature due to time

DISCUSSION

Physiological changes occur due to the effects of anesthetic medications in animals' system. In this study aimed at observing the effects of the combination of xylazine- ketamine and determining any potential results of blood pressure on dogs.

Xylazine, causes temporary hypertension in arterial blood pressure (Samy and Othman, 1985; Börkü et al., 2005). Ketamine causes rises in blood pressure and heart rate because of stimulating

cardiovascular system (Colby and Stanford, 1981; Haskins et al., 1985; Izci et al., 1993). Therefore, in our study it is thought that rise in the blood pressure during 15th and 30th minutes cause xylazine to cause temporary hypertension and ketamine stimulating cardiovascular system. In a study conducted by Koç et al., (2002), blood pressure was found to decrease time-dependently, and this decrease indicated that the depressive effect of xylazine in the cardiovascular system was greater than the stimulatory effect of ketamine. Data acquired from our study, drop in arterial blood pressure after 30th minute and reaching out to the minimum value at 120th minute, showed us that our study is compatible with Koç et al., (2002). During our study, significant decrease in heart rate at 60th minute supported literature information about xylazine's depressive effect on cardiovascular system causes a decrease in heart rate (Haskins et al., 1985; Samy and Othman, 1985; Allen et al., 1986; Izci et al., 1993; Koç et al., 2002). Between 15- and 30-minutes rise in heart rate was recorded which showed that the idea of ketamine, rising heart rate temporarily is compatible with literature which specifies the same thing (Colby and Stanford, 1981; Haskins et al., 1985; Samy and Othman 1985; Izci et al., 1993).

As a result of vasodilatation that is caused by xylazine's depressive effect on peripheral sympathetic system, fall in body temperature is indicated (Samy and Othman, 1985; Koç et al., 2002). It is also indicated that, drops in body temperature is because of thermoregulation center is being depressed because of ketamine being used with depressive effective anesthetics (Short et al., 1993). In this case, body temperature reaching its minimum value at 120th minute and showing a significant fall when compared to measurements taken between 0 and 30 minutes are all arise from xylazine depressing peripheral sympathetic system and ketamine depressing thermoregulation center. Facts that obtained from this study are compatible with literature information's (Samy and Othman, 1985; Short et al., 1993; Koç et al., 2002).

CONCLUSION

Consequently, dogs which were put under anesthesia with the combination of xylazine-ketamine have shown decreases in arterial blood pressure, heart rate and body temperature.

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Effect of probiotic on total antioxidant (TAS) and total oxidant (TOS) in treatment of newborn calf diarrhea

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ABSTRACT

Objective: Newborn calf diarrhea causes significant economic losses. Therefore, this study was aimed to determine the effect of adding probiotics on Total Antioxidant (TAS) and Total Oxidant (TOS) levels in routine treatment of calf diarrhea.

Material-Method: The material of the study consisted of 12 calves, routine treatment (Group 1, n=6 calves) and probiotic group. (Group 2; n=6 calves).

Results: TAS values increased statistically after treatment ($P < 0.05$) in group 2 compared to pretreatment. Comparison of the groups after treatment revealed that TAS significantly increased ($P < 0.05$) in group 2. While there was no statistical difference in TOS values between the groups a statistically significant increase ($P < 0.05$) was detected after treatment compared to pretreatment.

Conclusion: It was concluded that adding probiotics may be useful in addition to routine treatment in newborn calf diarrhea.

Keywords: *Antioxidant, Diarrhea, TAS, TOS, Probiotic*

INTRODUCTION

The neonatal period is the most critical period of calf rearing, covering the days between 0 and 28 following birth (Alkan, 1998). This time is the period when the diseases are most common and the mortality rates are the highest, especially in the first 15 days of calf life. Neonatal diseases in calves includes infectious (bacterial, viral, parasitic and mycotic) and noninfectious aetiolody (vitamin, mineral substance and, trace element deficiencies, congenital anomalies, etc.) (Ok et al., 2009; Aslan 1986; Burgu et al., 1986). Diarrhea is the most common problem. Treatment of diarrhea in calves involves; nutrition and prevention of bacteremia / septicemia and, it is important to correct dehydration, acid base and electrolyte balance (Şen et al., 2013). Use of antibiotics may become a public health problem due to development of antibiotic resistance. To reduce the dose and side effects of antibiotic treatment applied in neonatal calf diarrhea and to increase the therapeutic effectiveness probiotic applications are of great importance. Probiotics used in single stomach animals aid in regulation of the normal digestive flora, reducing metabolic disorders thus increase the efficiency in a healthy way (Minssen and Nordberg, 2020).

Probiotics have been started to be used in ruminants (Nageshwar et al., 2016; Valencia et al., 2017). Probiotics protect the host's useful microorganisms (the natural microflora) which can positively effect on the health of the host, help in

food digestion and vitamin production, and inhibit the pathogenic bacteria with substances produced in the intestines (Oelschlaeger, 2011). Application of probiotics in the treatment of diarrhea, shows a progressive effect by strengthening the immune system, and it has also been observed that the gastrointestinal mucosa barrier function, which is impaired in both in vitro and in vivo models, is rearranged by probiotics (Yeoman et al., 2018; Garcia-Lafuente et al., 2001; Madsen et al., 2001). Probiotics, effects on the immune system include, antimicrobial (Lactobacillus acidophilus; Acidophilin, Lactobacillin) secretions, increasing defense release from cryptos in the small intestine, such as preventing the adhesion of pathogens (Salmonella typhimurium) and they also neutralize virulence factors by breaking down toxins (Clostridium difficile toxins). (Solis-Pereira and Lemonnir, 1993). In some studies, the use of probiotics, promotes cytokine production in blood cells and improves the activity of macrophages (Solis-Pereira and Lemonnir, 1993; Shah, 2001). In addition, the use of probiotics in ruminant's benefits from the increase in yield characteristics, it benefits from digestive regulatory effects in condition and rumen (Wallace and Newbold, 1992; Burçak and Yalçın, 2013; Yavuzarslan, 2018; Alıç Ural and Toplu, 2017).

In intestinal cells there are several enzymatic and non-enzymatic antioxidants, including superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) which plays a role in the antioxsidants system (Çadırcı et al., 2007). Reactive Oxygen Species (ROS) that actively contribute to the ongoing pathogenic cascade with oxidative stress increases with the release of different pro-inflammatory mediators and it connects to oxidative stress (Lewis et al., 2008; Moura et al., 2015). In infectious diarrhea intestinal permeability is impaired and Gram (-) bacterial agents cause sepsis which is an important complication and oxidative stress increases in sepsis (Winterbourn et al., 2000; Oldham et al., 2002). Many roles of probiotics in antioxidation have been identified, however, there is no research related to probiotic effects on antioxidant and oxidant system in ruminants and neonatal calves (Shen et al., 2011; Asemi et al., 2013; Kullisaar et al., 2002). Mechanisms of probiotics in antioxidation can be listed as: (1) metal ion chelating ability (Ahire et al., 2013), (2) antioxidant enzymes system capacity (LeBlanc et al., 2008), (3) reduction in antioxidant metabolites (Kullisaar et al., 2002; Endo et al., 2013) and (4) enhanced antioxidant effect by adjusting the microbiota composition (Doron & Gorbach, 2006)

It has been understood that diarrhea and diarrhea complications cause an increase in oxidative stress, and antioxidant capacity in the intestines decreases in conditions and in this regard, no studies have been found on the effect of adding probiotics on TAS and TOS levels in neonatal calf diarrhea. This study was aimed to investigate the effect of probiotic use on the antioxidative mechanism in neonatal calf diarrhea treatments by using a probiotic combination of 2 bacteria (*Lactobacillus spp: L. plantarum, L. casei and Bacillus spp: B. subtilis*) and 1 yeast (*Saccharomyces spp: Saccharomyces cerevisiae*).

MATERIALS and METHODS

This study was carried out with the approval of Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee (YÜHADYEK-code:03, 28.03.2019). The study included 12 calves with diarrhea from Van and its vicinity. The calves were those brought to the Department of Internal Medicine of the Faculty of Veterinary Medicine and / or private veterinary clinics for clinical examinations with the complaint of diarrhea. Diarrhoeic calves with moderate to severe dehydration, possible metabolic acidosis and hyperkalemia, hypothermia, arrhythmia and tachypnea were included in the study. All calves with diarrhea were divided into 2 groups.

I. Group animals (n=6); 1.3% NaHCO3, + 0.9% NaCl + routine diarrhea treatment

II. Group animals (n=6); 1.3% NaHCO3 + 0.9% NaCl + Oral supplement (Probiotic) + routine diarrhea treatment

Blood samples were taken from the groups before treatment (0 hour) and on the third day after treatment,

Serum TAS was measured using the Commercial Kit (Product Code: RL0017, REL Assay Diagnostic, Mega Tıp, Gaziantep, Turkey) developed by Erel (2004) on Biochrom Anthos Zenyth 200 rt Microplate Reader Device. Results were given as millimolar trolox equivalent per liter (mmol trolox equiv / L).

Serum TOS level was measured on Biochrom Anthos Zenyth 200 rt Microplate Reader using the Commercial Kit (Product Code: RL0017, REL Assay Diagnostic, Mega Tıp, Gaziantep, Turkey) developed by Erel (2005).

Statistical evaluation of data obtained in the research were paired t test to determine the statistical difference between the same parameter before and after treatment, variance analysis (ANOVA) to determine the significance of the difference between different groups before and after treatment and Duncan test to determine the difference between groups. SPSS 20.0 Statistical Package Program (SPSS Inc., Chicago, IL, USA) was used for this purpose. Statistical significance was determined as $p < 0.05$. All data are given as arithmetic mean ± standard error of mean.

RESULTS

Clinically, there was dehydration and hypothermia along with diarrhoe and metabolic acidosis and hyperkalemia in sick animals. There was arrhythmia on the cardiac examination. Tachypnea was detected.

Serum TAS values before treatment in 2 groups significantly increased after treatment ($P < 0.05$). When both groups were compared, a statistically significant increase ($P < 0.05$) was observed in group 2 compared to group 1 after treatment (Table 1). While TOS values insignificantly decreased after treatment in group 2, the value significantly increased ($P < 0.05$) after the treatment in group. TOS values significantly differed between the groups after treatment ($P < 0.05$) (Table 1).

Table 1. TAS and TOS levels (x ± SS) of both groups before and after treatment

Groups	TAS (mmol Trolox Eq/L)		TOS (μmol H_2O_2 Eq/L)	
	Before Treatment	After Treatment	Before Treatment	After Treatment
I. Group	1.326±0.84	1.144±0.73[a]	4.480±1.77*	7.784±1.42*
II. Group	1.116±0.59*	1.588±0.71* [a]	5.492±1.68	4.178±1.89

* :The difference between the pre-treatment and post-treatment of the same group is statistically significant ($p<0.05$).

[a] :The difference between groups is statistically significant ($p<0.05$).

DISCUSSION

The neonatal period covers the days 0 to 28 following birth and is the most critical time of calf breeding where, calf diseases especially are most common. Neonatal calf diseases and deaths cause serious economic losses (Alkan, 1998; Ok et al., 2009; Aslan, 1986). Recently, probiotic applications in addition to routine treatment are gaining importance in diarrhea treatments (Nageshwar et al., 2016; He et al., 2017; Le et al 2017). Diarrhea also triggers many local or systemic pathologies and causes an increase in oxidative stress (Lewis et al., 2008; Moura et al., 2015). Probiotics have been shown to reduce oxidative stress by many mechanisms (Ahire et al., 2013; LeBlanc et al., 2008; Endo et al., 2013; Kullisaar et al., 2002; Çatalkaya, 2020). Although there exist a few studies on the antioxidant and oxidant systems of calves. In a study conducted in healthy calves, TOS value was determined as 10.61 ± 3.82 (μmol H_2O_2 Eq/L) and TAS value was 1.72 ± 0.63 (mmol Trolox Eq/L) (Topraktaş, 2019). In another study, in calves with diarrhea, the value of TAS was 0.51 ± 0.02 (mmolTrolox Eq/L) before treatment and 0.55 ± 0.02 (mmolTrolox Eq/L) after treatment and the TOS value was 13.47 ± 0.81 (μmol), (H_2O_2 Eq / L) before treatment and 11.21 ± 0.26 (μmol H_2O_2 Eq / L) after treatment (Kabu et al., 2015). In a study conducted in calves with septicemia; TAS value was 0.50 ± 0.02 (mmolTrolox Eq/L) before treatment and 0.58 ± 0.03 (mmolTrolox Eq/L) after treatment. The TOS value was 5.30 ± 0.74 (μmol H_2O_2 Eq/L) before treatment and 2.13 ± 0.2 (μmol H_2O_2 Eq/L) after treatment in the study by Erkılıç et al., 2016. In this study, TAS value of 1,116 ± 0,59 (mmol Trolox Eq/L) before treatment increased to 1,588 ± 0,71 (mmol Trolox Eq/L) ($P < 0.05$) after treatment in the group 2 while these values did not markedly differ in group did not receive probiotics. When both groups were compared, a marked increase ($P < 0.05$) was observed in group 2 after treatment. This significant increase in TAS value might indicate that probiotics play a role in the rapid response in antioxidant capacity. Reactive Oxygen Species are 30% higher in calves on the first day of birth compared to their mothers and this is thought to cause increased oxidative stress, which is inevitably present in newborns after the first inhalation of atmospheric oxygen (Gaál et al., 2006). In our study, the value of TOS in Group 1 before treatment was 4,480 ± 1,77 (μmol H_2O_2 Eq / L) and 7,784 ± 1,42 (μmol H_2O_2 Eq / L) after treatment. This finding might suggest that probiotics were more effective along with antibiotic use when compared to the antibiotic use only.

In the group 2, the TOS value before the treatment 5,492 ± 1,68 (μmol H_2O_2 Eq / L) decreased to 4,178 ± 1,89 (μmol H_2O_2 Eq / L) after treatment. Although not statistically significant, it was concluded that this reduction may be related to increased capacity of antioxidant due to probiotic use. In many studies, including this study, differences in TAS and TOS values have been observed and this is thought to be due to measurement methods, kit differences or sensitivity of tests, but studies have reported that the TOS value increases and the TAS value decreases in disease condition (Kızıl et al., 2007; Topraktaş, 2019; Kabu et al., 2015; Erkılıç et al., 2016).

CONCLUSION

As a result, the use of additional probiotics in the treatment helps to reduce oxidative stress, if additional probiotics are not used an increase in oxidant capacity could be noted or might not cause a rapid decrease. Besides the local effects of probiotics on microbiota, they play a role in the antioxidant-oxidant mechanism systemically with symbiotic effects. It has been determined that adding the probiotics to neonatal calf diarrhea treatments protocol might cause an increase in antioxidant capacity. But there is a need for further investigation on the role probiotics in mechanism of antioxidant process.

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Author's Contributions: NY, EÇ and MY designed the study. EÇ and MY collected the materials, NY, EÇ, MY and YB performed the analysis. YB evaluated statistically. NY, EÇ, MY and YB wrote the study

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Immunohistochemical investigation of lipid peroxidation in renal coccidiosis of geese

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ABSTRACT

Objective: In this study, we aimed to evaluate the oxidative damage caused by lipid peroxidation due to renal coccidiosis by histopathological and immunohistochemical methods.

Material-Method: The material of this study was made up of tissue samples taken from 139 geese whose average age was 10 weeks, who were brought to our department dead between 2013-2020. Tissue samples taken were fixed in 10% buffered formaldehyde solution. 5 µm-thick sections were taken from the paraffin blocks prepared after routine tissue follow-up procedures. Hematoxylin & Eosin staining was applied to the sections in order to detect histopathological changes. Sections were examined and photographed under a light microscope.

Results: Various clinical signs such as fever, respiratory distress, weakness, anorexia, tremors, inability to get up from the ground, balance disorders, rotational movement, diarrhea, wheezing were detected in geese. In systemic necropsies of geese, large and small white nodular structures were detected in the kidney. In histopathological examinations, coccidiosis agents (*E. truncata*) were found in the tubular epithelium of the kidney. Necrosis and mononuclear cell infiltration were observed in the tubules due to the presence of *E. truncata*. In addition, edema and hyperemia in the lungs, multifocal necrosis in the liver, cell infiltration in the portal spaces and enteritis were other important histopathological findings. In one case, aspergillosis was detected together with renal coccidiosis. We observed that MDA expression was more severe in oocyst stages, which is the mature form of the parasite, compared to other parasitic life stages.

Conclusion: Based on the results obtained from this study, it was revealed that renal coccidiosis in geese caused lipid peroxidation / oxidative damage through the increase in MDA expression.

Keywords: *Histopathology, Goose, Lipid peroxidation, MDA, Renal coccidiosis*

INTRODUCTION

Coccidiosis is a serious protozoan disease that causes hemorrhagic diarrhea, depression, weakening, wing drooping, sensitivity to other diseases and loss of body weight, as well as deaths, especially in young animals, caused by various *Eimeria* species (Sarı and Çakmak, 2008; Liu et al., 2018; Fortuoso et al., 2019). Coccidiosis, which is common in many parts of the world, causes serious problems in many animal species such as cattle, sheep, goats, dogs, cats, pigs and rabbits, especially in poultry (Dumanlı and Aktaş, 2015; Song et al., 2017). Avian coccidiosis has a high morbidity and mortality rate and the economic loss it causes is more than 3 billion dolllars annually (Galli et al., 2019; Griss et al., 2019). Parasites continue their development in the interstitial canal or kidneys according to the species differences and do not need

any intermediate hosts during their development (Dai et al., 2005; Dalloul and Lillehoj, 2006). The disease is more severe in young animals than adults, and the risk of infection is higher, especially in 3-12 week old goslings. The acute form of renal coccidiosis has a high mortality rate of 80% (Hilbert, 1951; Dumanlı and Aktaş 2015). This parasite causes deaths in young animals, and the elderly animals that survive the disease become susceptible, causing them to play a role in carrying the disease as a carrier (Arslan et al., 2002; Dumanlı and Aktaş 2015; McDougald, 2020). A total of 17 *Eimeria* species have been isolated in domestic and wild geese. 7 species have been seen in domestic geese, it has been reported that only one species is *Tyzzeria* and the others belong to the *Eimeria* lineage (Karaer and Çiçek, 2013; Song et al., 2017). *E. anseris*, *E.cotlani*, *E. noces*, *E. parvula*, *E.stigmosa*, *E. truncata* and *Tyzzeria anseris* are coccidiosis species isolated in geese (Hanson et al., 1957; Arslan et al., 2002). It has been reported that intestinal coccidiosis is caused by E. anseris and renal coccidiosis is generally caused by *E.truncata* (Montgomery, 1978; Arslan et al., 2002). Endogenous development of *E. truncata* occurs in the tubular epithelial cells of geese kidneys (Entzeroth et al., 1981). Cases of renal coccidiosis are diagnosed by the presence of oocysts in the kidneys and cloaca near the urethra (McDougald 2020).

Free radicals are highly active chemical products that occur during metabolism in the body. The most important free radicals in biological systems are radicals formed from oxygen and these are called Reactive Oxygen Species (ROS) (Atmaca and Aksoy, 2009). Reactive oxygen species initiate lipid peroxidation by causing oxidation in polyunsaturated fatty acids (PUFA) found in biological membranes (Özcan et al., 2015). By cleavage of polyunsaturated fatty acids containing three or more double bonds, one of the most important indicators of lipid peroxidation is Malondialdehyde (MDA), a three-carbon dialdehyde (Tabakoğlu and Durgut, 2013).

In this study, we aimed to evaluate the oxidative damage caused by lipid peroxidation due to renal coccidiosis in geese by histopathological and immunohistochemical methods.

MATERIALS and METHODS

Animals

The material of this study was made up of tissue samples taken from 139 geese whose average age was 10 weeks, who were brought to our department dead between 2013-2020. Information on age, clinical symptoms, parasitic forms and the severity of MDA immune positive expressions for all animals are given in Table 1.

Ethical Approval

The ethics committee report of this study was obtained from Kafkas University Animal Experimentals Local Ethics Committee (Authorization number: KAU-HADYEK-2020/166).

Histopathological Investigations

After systemic necropsy of geese, tissue samples were fixed in 10% buffered formalin solution. After routine procedures paraffin blocks were cut to 5 µm thickness and Hematoxylin & Eosin (H&E) staining was applied to the sections in order to detect histopathological changes. In order to reveal the presence of Aspergillus fungi, Periodic acid–Schiff (PAS) staining was applied to the sections as suggested by Facepath company. Sections were examined and photographed under a light microscope.

Immunohistochemical Investigations

Avidin-Biotin Peroxidase method was used as immunohistochemical method. For immunohistochemical staining, the sections of 4 µm in thickness taken to poly-L-lysine coated slides were deparaffinized and rehydrated in graded alcohols. In order to prevent endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide solution in Phosphate Buffered Saline (PBS) for 15 minutes. For antigen retrieval, the sections were boiled in Citrat Buffer Solution (pH 6) for 25 min in the microwave oven (at 800 watt). In order to prevent nonspecific staining, the sections were incubated for 10 min with non-immune serum (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL) at room temperature. Diluted antibodies (MDA: Abcam, ab6463, Dilution Rate: 1/250) were incubated for overnight (+ 4 °C in refrigerator). The sections were washed 3 times in PBS solution for 5 minutes, and the biotinylated secondary antibody (Thermo Scientific, Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL) was applied to them at room temperature for 10 minutes. After washing in PBS (3-5 min), all sections were incubated with peroxidase-bound Streptavidin (Thermo Scientific, Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL) for 10 minutes at room temperature. A solution of 3.3-diaminobenzidine tetra hydrochloride (DAB)

(Thermo Scientific, REF: TA-125-HD) was used as a chromogen for 15 minutes. The sections were treated with Mayer's Hematoxylin for 30 second and washed in running water for 5 min, dehydrated in graded alcohols, cleared in xylene and coated with entellan. Primary antibodies were omitted from the negative control sections and were treated with diluted normal serum. The slides prepared after the covering were examined under a light microscope (Olympus Bx53) and photographed via the Cell^P program (Olympus Soft Imaging Solutions GmbH, 3,4). Analyzes of the images were done with Image J Program. Results were evaluated as negative (-), mild (+), moderate (++) and severe (+++).

Table 1. Age, clinical symptoms, microscopic results and severity of MDA expressions of all animals

Case No	Age (week)	Clinical Symptoms	Parasitic form	MDA expressions
1	9	Fever, anorexia	Oocyst	+++
2	9	Anorexia, depression	Oocyst	++
3	6	Weakness, anorexia	Macro/micro gamet	+
4	12	Balance disorders, diarrhea	Oocyst	+++
5	12	Rotational movement around its axis, diarrhea	Oocyst	+++
6	10	Diarrhea, emaciation	Oocyst	++
7	9	Diarrhea	Oocyst	+++
8	8	Tremor	Micro/macro gamet	+
9	10	Inability to get up from the ground	Micro/macro gamet	++
10	12	Diarrhea, balance disorders	Oocyst	+++
11	12	Diarrhea, balance disorders	Oocyst	+++
12	10	Rotational movement around its axis	Oocyst	++
13	10	Rotational movement around its axis	Oocyst	+++
14	10	Rotational movement around its axis	Oocyst	+++
15	10	Inability to get up from the ground	Oocyst	+++
16	8	Respiratory distress, wheezing	Micro/macro gamet	++
17	9	Diarrhea, balance disorders	Micro/macro gamet	+

RESULTS

Clinical Symptoms

Various clinical symptoms such as fever, respiratory distress, weakness, anorexia, emaciation, tremor, inability to get up from the ground, balance disorders, rotational movement around its axis, diarrhea, wheezing.

Macroscopical Results

We observed large and small yellowish-white nodular structures in the kidneys of 17 (12.23%) of 139 geese examined macroscopically (Figure 1).

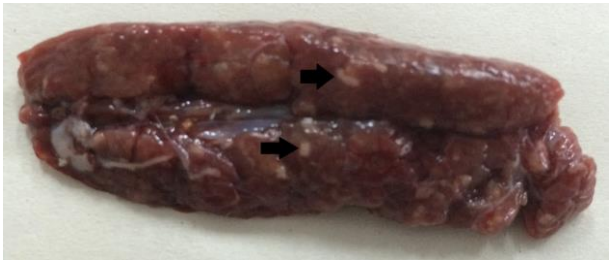

Figure 1 Yellowish-white nodular structures (arrows) in the kidney.

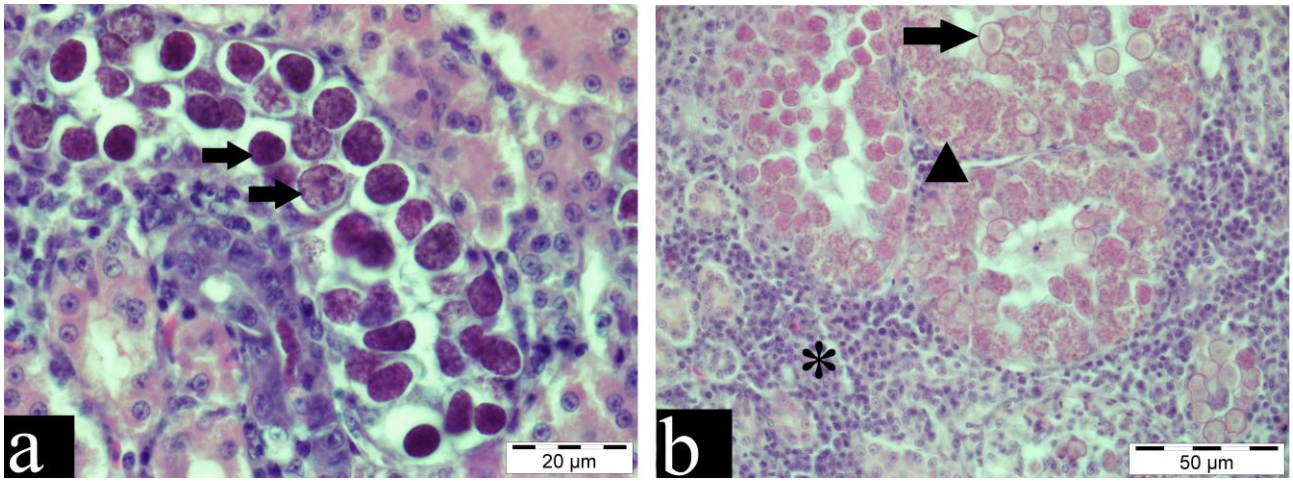

Figure 2 a. *E. truncata* oocysts (arrows) in renal tubule epithelium, H&E, Bar= 20µm, **b.** Macrogamet (arrow) and microgamet (arrowhead) stages of *E. truncata* in renal tubule epithelium and nephritis (star), H&E, Bar = 50µm

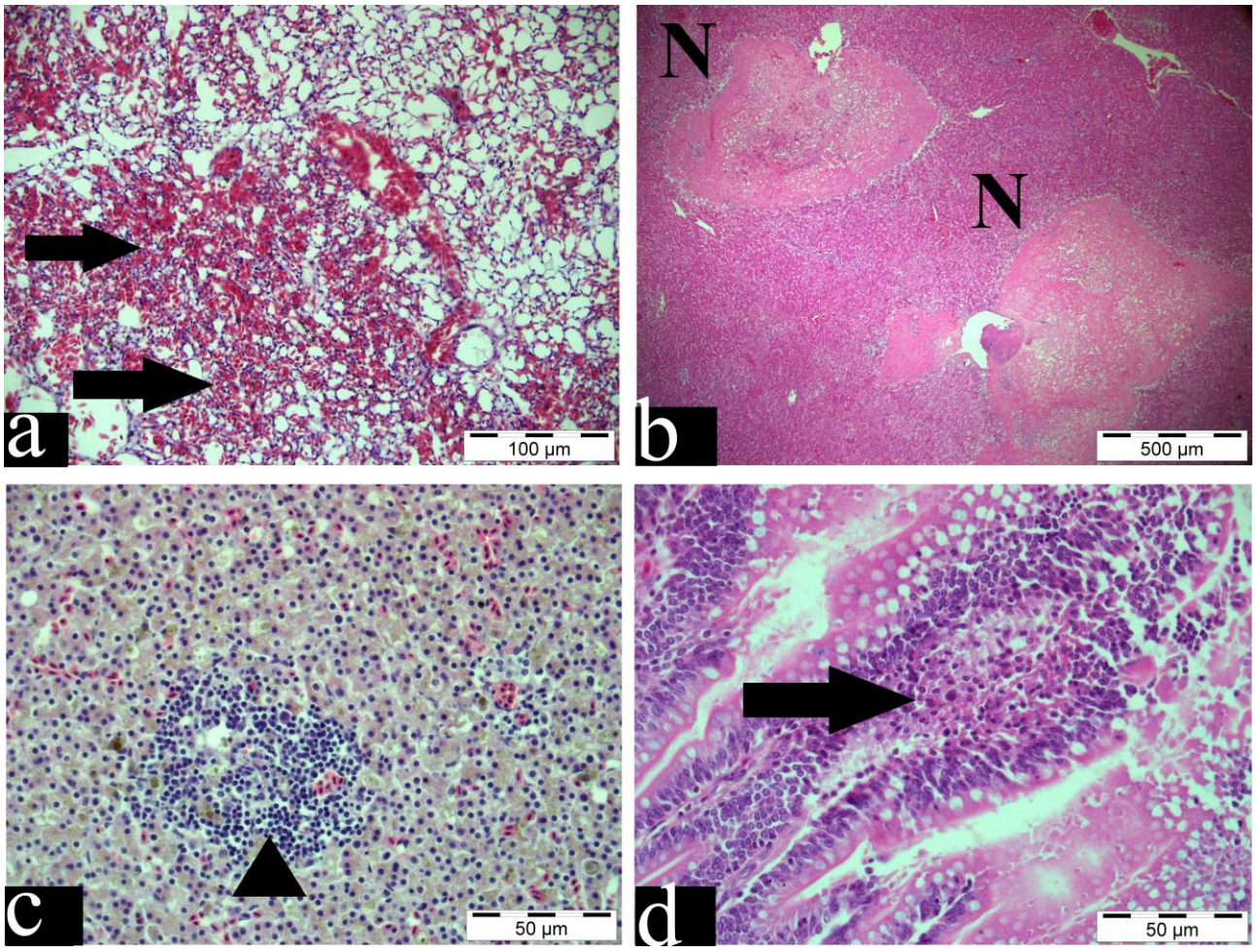

Figure 3 a. Lung, hyperemic areas (arrows) in lungs, H&E, Bar = 100 µm**, b.** Liver, necrotic areas (N), H&E, Bar = 100 µm**, c.** Liver, mononuclear cell infiltration (arrowhead), H&E, Bar = 50 µm**, d.** Intestine, diphteroid necrotic enteritis, H&E, Bar = 50 µm

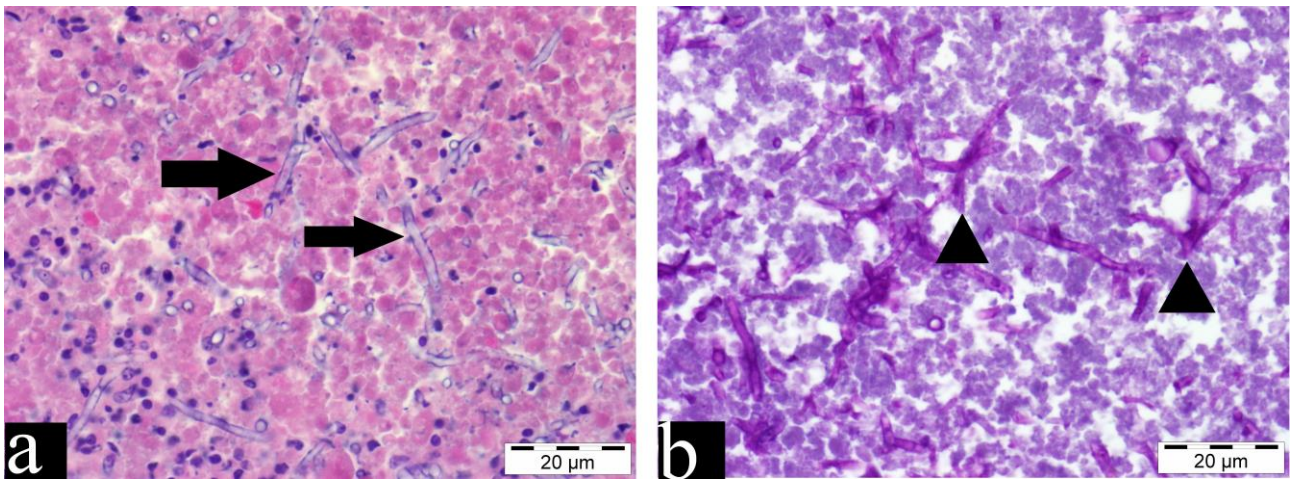

Figure 4 a. Lung, hyphae (arrows), H&E, Bar = 20 µm, **b.** Lung, hyphae (arrowheads), PAS staining, Bar = 20 µm

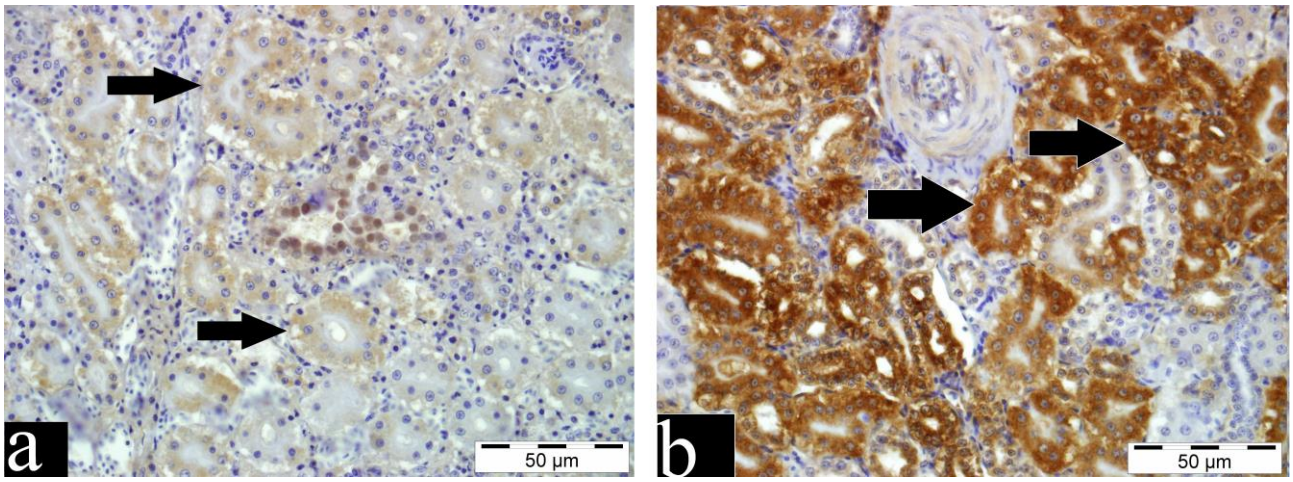

Figure 5 a. Kidney, oocyst form, MDA expressions in degenerated and necrotic tubular epithelium (arrows), IHC, Bar=50 µm, **b.** Kidney, oocyst form, severe intracytoplasmic MDA expressions in degenerated and necrotic tubular epithelium around the inflammatory area (arrows), IHC, Bar=50 µm

Microscopical Results

We identified different life forms of coccidiosis factors in renal tubular epithelium (Figure 2 a-b). In addition to these, nonpurulent nephritis, necrosis in the renal tubular epithelium, edema and hyperemia in the lungs, necrosis in liver and hepatitis, and diphteroid necrotic enteritis in the intestines were among the other important histopathological findings (Figure 3 a-d). We detected pulmonary aspergillosis (PAS positive) in only one goose (Figure 4 a-b).

Immunohistochemical Results

We detected MDA immune positive expression especially in degenerated and necrotic tubular epithelium. We observed that MDA expression was more severe in oocyst stages, which is the mature form of the parasite, compared to other life stages (macrogamet and microgamet) (Figure 5 a-b).

DISCUSSION

While goose breeding is an important sector after chicken and turkey breeding in many parts of the world, it is not yet at the desired size in our country. In our country, goose breeding is mostly carried out in the Eastern Anatolia Region (Kars, Ardahan, Muş, Ağrı and Van) at the family level and the interest in goose breeding is increasing every year. The presence of geese grown in Turkey, North East Region, especially Ardahan and Kars provinces constitute approximately half of the total assets of the country geese (Demir et al., 2013; Otlu, 2016). One of the most important problems encountered in goose breeding is the fight against infectious

diseases and protection (Sarı and Saatcı, 2020). One of the most important of these infectious diseases is coccidiosis infection caused by parasites related to *Eimeria* lineage, which causes significant losses in goose breeding (Liu et al., 2018; Wang et al., 2020).

In this study, in accordance with the literature data (Entzeroth et al., 1981; Tuggle and Crites, 1984; Dumanlı and Aktaş, 2015) the mean age range of the geese with renal coccidiosis was 10 weeks. Various clinical symptoms such as diarrhea, depression, anorexia, and emaciation have been reported in previous studies (Arslan et al., 2002; Dai et al., 2005; Song et al., 2017; Liu et al., 2018; Wang et al., 2020) in geese with renal coccidiosis. In our study, in addition to these clinical symptoms, we identified different clinical symptoms such as respiratory distress, tremor, inability to get up, balance disorders, rotational movement around their axis, and wheezing. We thought that this diversity observed in clinical symptoms may be related to the fact that parasitic factors weaken the immune system and predispose to the formation of other diseases (Liu et al., 2018; Fortuoso et al., 2019).

Similar to the literature (Hilbert, 1951; Montgomery, 1978; Gajadhar et al., 1982; Tuggle and Crites, 1984) we also observed large and small white nodular structures in the kidneys in systemic necropsies of geese. Parallel to previous studies, in the histopathological examination of the kidneys, we detected different parasitic forms of *E.truncata* in the renal tubular epithelium (Klimeš, 1963; Entzeroth et al., 1981; Gajadhar et al., 1982; Gajadhar et al., 1986), severe degeneration and necrosis (Oksanen, 1994; Arslan et al., 2002) and inflammatory infiltration in which the majority of mononuclear cells (Montgomery, 1978; Tuggle and Crites, 1984) were formed. Findings such as hepatitis, enteritis, and pulmonary aspergillosis, which we thought to be caused by avian coccidiosis increasing predisposition to other disease factors, were among the other important histopathological changes we found (Liu et al., 2018; Wang et al., 2020).

Oxidative stress is an imbalance between antioxidant and oxidant status (Tabakoğlu and Durgut, 2013). This imbalance is related to the overproduction, or slowing down of the removal of these free radicals, such as ROS (Özcan et al., 2015). ROS overproduction causes to damage of nucleic acids, lipids and proteins. Oxidative stress plays a serious role in the initiation and progression of many infectious diseases such as coccidiosis (Griss et al., 2019). It has been reported that coccidiosis in animals causes an increase in ROS and reactive nitrogen species (RNS), causes changes in antioxidant enzyme activities and a decrease in the concentrations of antioxidants (Khatlab et al., 2019). Fortuoso et al., (2019) found that serum ROS levels and lipid peroxidation increased in broiler chickens experimentally infected with *Eimeria*. In another study, an increase in intestinal ROS production with lipid peroxidation has been reported in chicks experimentally infected with *Eimeria* species (Galli et al., 2019). In addition, an increase in MDA levels, which is an important marker of lipid peroxidation, was detected in the experimentally induced *Eimeria* infection in broiler chickens (Muraina et al. 2020). According to literature (Fortuoso et al., 2019; Galli et al., 2019; Griss et al., 2019; Khatlab et al., 2019; Muraina et al. 2020) we found that the expression of MDA in renal coccidiosis of geese increased significantly in different life forms of the parasite. We interpreted this increase in MDA expression as lipid peroxidation-based oxidative stress may play an important role in the pathogenesis of the disease.

CONCLUSION

According to clinical, macroscopic and microscopic findings, the presence of renal coccidiosis was 12.23% (17/139) in 139 dead geese samples brought to our department in the last 8 years in Kars, which is an important goose breeding region. We concluded that coccidiosis plays an important role in goose deaths. In the literature searches, we did not find any studies in which lipid peroxidation was evaluated immunohistochemically by means of MDA expression in renal coccidiosis of geese. In this respect, we thought that the results obtained from our study would contribute to the literature on the pathogenesis of renal coccidiosis of geese.

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Author's Contributions: EK and AY designed the study. AY collected the goose samples. He did immunohistochemical and H&E staining, source scanning, and photography. EK performed microscopic evaluations of the staining.

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Effects on the wound healing process using ozonated oils (*Sesame*, *Nigella sativa*, *Hypericum perforatum*) in rats

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ABSTRACT

Objective: In this study, the effects of three different ozonated oils (*Sesame*, *Nigella sativa* and *Hypericum perforatum*) on wound closure rate, healing process and possible complications were examined macroscopically and microscopically.

Materials and Methods: Twenty-one adult Wistar albino female rats were used in the study. Subjects were divided into three groups, early wound healing, (7 days), medium wound healing, (14 days) and late wound healing (21 days). Four full-thickness skin wounds of equal size (10 mm in diameter) were formed on the back regions of all rats. This region was chosen for preventing self-inflicted injuries and reducing external irritation. The wound was left open during the healing process. While the first wound (control) received no treatment in the second wound, ozonated *Sesame* oil, in the third wound ozonated *Nigella sativa* oil and in the fourth wound ozonated *Hypericum perforatum* oil were used. No group obtained parenteral drug administration. First, second and third main groups were euthanized on days 7, 14 and 21, respectively. The wound healing was assessed macroscopically daily. Wound sizes of individual rats were measured with a caliper and digitally photographed every day from the day of injury. After euthanasia, all wound sites of the subjects were evaluated histopathologically.

Results: There were no significant differences in wound healing between treatments in the first day 7. On they 14, it was found that the healing was better in the group applied *Nigella sativa* and *Sesame* oils ($p \leq 0.05$). On day 21 wound healing was completed in all subjects with a resultant of better outcome in *Nigella sativa* oil treatment compared with others ($p \leq 0.01$).

Conclusion: In this study the best wound healing outcome was achieved with *Nigella sativa* oil and *Sesame oil* where three different minced oils were used.

Keywords: *Wound healing, Ozonated oil, Sesame, Nigella sativa, Hypericum perforatum*

INTRODUCTION

The definition of a wound is interruption of ordinary congruity of structures typically limited to those caused by physical means and cause damage and injury (Lazarus et al., 1994). It is wound types that are performed in a manner compatible with anatomical and functional repair within normal healing period. Under appropriate conditions, healing occurs within 20 to 30 days. These types of injuries are usually short-lived tissue injuries that are not very invasive surgical procedures, and healing is short-term and occurs in four distinct stages (Strodtbeck, 2001; Li et al., 2007). Wound healing is one of the most complex biological events after birth. It is a complex process of the replacement of dead tissue by a vital tissue. The

response of the body to local injury begins during in the process of inflammation, and results in repair and regeneration. Wound healing consists of four distinct phases, namely hemostasis phase, inflammatory phase, proliferative phase and maturational or remodeling phase, intermingled with each other. These phases can not be completed within the time of the failure should occur, or may occur in any one phase may result in the delay or closure of the wound healing (Cotran et al., 1999). Strong antibacterial, antiviral and antifungal effect, immunomodulatory effect, due to its positive effect on the transport and release of oxygen in the tissues, as well as its quick and efficient healing properties, medical ozone can be used in a wide range of indications (Viebahn-Haensher, 2002). The few studies on the therapeutic effects of ozonated oils on acute skin scarring in animal models does not analyze the dose / behavior response, expressed as the amount of peroxide present in the ozonated derivative used (Bocci, 2007). Recently, a quantitative evaluation of the therapeutic effect of locally applied ozonized sesame oil on acute skin scarring in mice as an animal model has been developed (Kim et al., 2009). The results indicate that low (<1000) and high (>3000) doses, expressed in terms of peroxide value (see the corresponding section in this article), delay skin healing. Such evidence is reinforced by a number of results between groups where the "average" concentration (about 1500) has the most beneficial effect in accelerating the rate of wound closure (Bocci, 2007). *Nigella sativa* has been found that broad spectrum medicines applied to diseases seen in animals affect people who consume them by accumulating in the animals' structures. This effect has been shown to impair people's immune system. It has antihistaminic, anti-inflammatory, anti-infective properties and has bronchodilator and vasodilator characteristics (Burits and Bucor, 2000; Ali and Blunden, 2003). The healing effect of the wounds of *H. perforatum* is well known. Human health and well characterized from the wound in Anatolia are used in many more people because of the positive effect. *H. perforatum* wound-healing effect, as well as sedative, antiseptic, antioxidant, anti-depression, spasm, antiviral and antimicrobial, hepatoprotective, diuretic effect and is referred to by the presence of antibiotic (Greeson et al., 2001; Hunt et al., 2001; Öztürk et al., 2007).

H. perforatum L. oily preparations, topical minor burns, wounds, skin infections and are used for various pains. Plant preparations are used in anxiety and depressive problems (Di Carlo et al., 2001). Wound-healing effect of *H. perforatum* prepared from St. John's Wort oil has been known for a long time. This drug leads to photosensitization when used extensively and exposed to sunlight, inflammation and dermatitis occur in the mucosa and skin (Cingi, 1991; Çırak, 2006). The most important characteristic of sesame oil is resistance to oxidative degradation. Sesame oil high stability; in the composition of sesamol, sesaminol located just tocopherols from compounds acting on other edible oil from the oil-specific and potent antioxidant than these, hydrocarbons and some is due to the antioxidant effect of certain sterol (Anonymous 1). Sesame oil has many physiological functions such as lowering estrogenic activity, blood lipids and arachidonic acid level. The key step in determining the color, composition and quality of sesame oil is roasting. Antioxidant factors that provide stability are affected by roasting parameters (Agosa, 2011). Considering these advantages of ozonated oils, in this study, it was aimed at investigating which of the following results would be better using *Nigella sativa*, *Sesame* seeds and *Hypericum perforatum* in treatment of an experimental wound induced in rats.

MATERIALS and METHODS

Twenty-one 2-month-old female Wistar albino rats weighing 220-250 g were used and the study was conducted in the Fırat University Experimental Research Center. Animals were maintained and fed under standard conditions; they were left free in the cages. This study approved by Local Ethics Experimental Animal Committee of Fırat University, Turkey (19.12.2016-219). General anesthesia of rats was performed i.m. injection of 10 mg/kg Xylazine HCI (Rompun, Bayer®) and 80 mg/kg Ketamine Hydrochloride (Ketalar, Parke-Davis®). Following adequate anesthesia depth, i.e. loss of the pedal and eyelid reflex, the rats were placed on the operation table in the abdominal position and their back regions were shaved, scrubbed and painted using povidone iodine, covered with sterile surgical drapes and prepared for aseptic surgery. All operation procedures were performed following strict aseptic and atraumatic surgical guidelines. In the back region of the rats four full-thickness skin wounds of 1 cm in diameter were created with biopsy punch (Figure 1) on the back of each rat. The wound sites were divided into four groups according to agents used. While the

right cranial wound site was served as a control group, the left cranial which marked with "S" was allocated to group OS (ozonated sesame oil), right caudal marked with "Kt" (Control Group) to group ON and left caudal marked with "Ç" to group OH (ozonated *Hypericum perforatum* oil) (Figure 2). While control group wound site received no agent throughout the study, group OS, ON (ozonated *Nigella sativa* oil) and OH wound sites were applied ozonated sesame oil, ozonated *Hypericum perforatum* oil and ozonated *Nigella sativa* oil twice a day (morning and evening), respectively. All wounds were left uncovered throughout the study. The status of wound healing was evaluated macroscopically, the size of individual wounds in each rat were measured by a caliper (Figure 3) and photographed every day, beginning on the day of wounding. The rats were also divided randomly into three groups, days 7 (group 1), 14 (group 2) and 21 (group 3) of animals with equal number (no: 7) according to the day of euthanasia in order to evaluate the early, middle and late findings of wound healing. Group 1 (early wound healing) subjects were euthanized on postop day 7, group 2 subjects (middle-term wound healing) on post-op day 14, group 3 subjects (late wound healing) on postop day 21 by carbon dioxide inhalation. The wound sites were harvested with a scalpel and placed into %10 formalin liquid and presented to pathology department for histopathological evaluation. Histopathological evaluation was performed according to the wound healing assessment criteria shown in Table 1. Inflamation, ulseration and vascular proliferation was scored non (-), mild (+), moderate (++), and severe (+++). Closure of the wound surface, epithelization and fibroblast activity increase were graded as present (+) or absent (-). The mean scores of the histopathological findings were calculated scoring system, i.e. non (-), mild (+), moderate (++) and severe (+++) with 1, 2, 3 and 4 points, respectively.

Figure 1. Creation of full-thickness skin wound with biopsy punch.

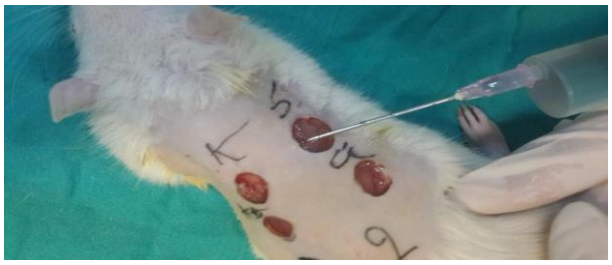

Figure 2. Each letter depicts which treatment had been applied for each wound. Ozonated sesame oil administration to the wound site ''S''.

Table 1. Evaluation criteria for the variables used in wound healing score.

Inflammation	Ulceration	Vascular Proliferation	Surface Closure	Epithelisation	Increased Fibroblast Activity
No	No	No	No	No	No
Mild	Mild	Mild (less than 5 vessels)	Yes/Have	Yes/Have	Yes/Have
Moderate	Moderate	Moderate (6-10 vessels)	-	-	-
Severe	Severe	Severe (More than 10 vessels)	-	-	-

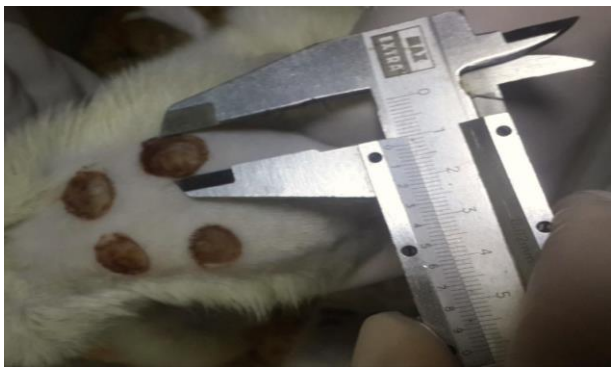

Figure 3. Measurement wound size with vernier caliper**.**

Statistical analysis

All data was presented as mean ± SD. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Dunnet's post hoc test where multiple comparisons were made (SPSS22.0, USA). Differences set to $p<0.01$ and $p<0.05$ were considered statistically significant.

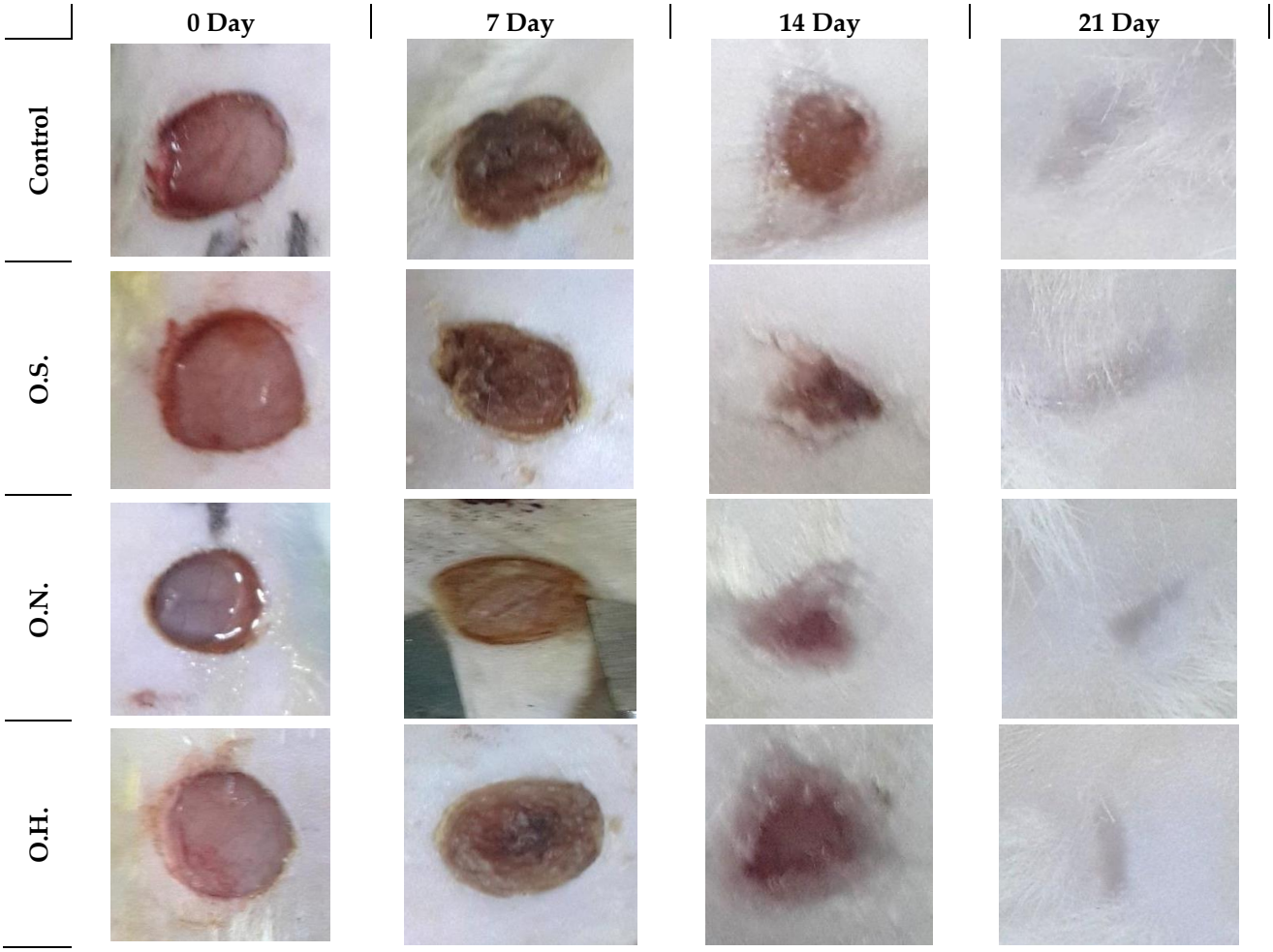

Figure 4. Wound healing processes throughout the study in four groups (control, groups OS, ON, OH) based on wound sites.

RESULTS

During the experimental study, no postoperative complications including wound infection and abnormal health status were recorded. Based on the statistical results, no significant difference was found between control as well as groups OS, ON and OH in post-op day 7 (group 1). However, when the data of group 2 (post-op 14) were statistically evaluated the healing rate of wound sites treated with ozonated *Nigella sativa* oil (group ON) was significantly higher ($p<0.01$), which is followed successively by the ozonated sesame oil (group OS, $p<0.01$) and ozonated *Hypericum perforatum* oil (group OH, $p<0.05$) compared to the control group. On post-op day 18, while all wound sites treated with ozonated oils (groups OS, ON, OH) reached

full recovery, healing in control wound site were still continuing, which were also observed to reach fully recovered on post-op day 19. As a result, healing processes in all wound sites (control, group OS, ON, and OH) of all subjects completed before post-op day 21 (Figure 4).

Table 2. Distribution of the variables according to histopathological evaluation in rats euthanized on day 7 (group 1).

Variables/Groups	Control	Sesame oil	*Nigella sativa* oil	*Hypericum perforatum* oil
Inflammation	3	2	1	2
Ulceration	2	1	1	1
Vessel Proliferation	3	2	1	1
Surface Closure	−	−	_	−
Epithelisation (p≤0.01)	−	−	+	_
Fibroblast Activity (p≤0.01)	−	+	+	+

Table 3. Distribution of data according to histopathological evaluation in rats euthanized on day 14 (group 2).

Variables/Groups	Control	Sesame oil	*Nigella sativa* oil	*Hypericum perforatum* oil
Inflammation	2	0	0	1
Ulceration	1	0	0	0
Vessel Proliferation	2	1	0	1
Surface Closure (p≤0.01)	-	+	+	+
Epithelization (p≤0.01)	-	+	+	+
Fibroblast Activity Increase (p≤0.01)	+	+	+	+

Histological Results

Histopathological findings in group I (Day 7):

According to the histopathological scoring data, in control group wound sites contained a quite high inflammation and vascular permeability rates, newly formed fibroblast proliferation but no epithelization development. In group ON, there was a relatively less distinctive inflammation but high vascular permeability and high fibroblast proliferation compared to control group. In this group unlike control group epithelization process started. In group OS and OH moderate inflammation, increased vascular permeability and the edema formation were present. In both groups fibroblast proliferation also indicated (Figure 5) (Table 2).

On the seventh day, in all the groups, the lesions were characterized by skin ulcer including inflammatory infiltration, complete epithelial loss and dermatitis. Inflammatory reaction consisted of the neutrophils, macrophages and lymphocytes. There was no difference between the control and experimental groups in terms of inflammatory response, epithelization and connective tissue formation in all groups.

Histopathological Findings in Group 2 (Day 14):

According to the histopathological scoring data, control and OH groups showed moderate levels of inflammation and increased vascular permeability with newly formed fibroblast proliferation. In these groups' epithelization appeared to occur in most parts of the wound area. Unlike the control, in groups OS and *ON*, no inflammation was observed. In the control group it was determined that there was little edema compared to groups OS and ON. There was less increased vascular permeability in group ON than in group OS (Table 3).

In all groups of rats examined on day 14 of the study, fibrous connective tissue regeneration with no sebaceous and sweat glands was evident. Control group wound sites contained no epithelial regeneration but include irregularly laid down fibrous ligament. In the experimental groups, i.e. groups OS, OH and ON, had well developed epithelial regeneration. In the experimental groups, epithelization rate can be ordered form the most down to the lowest as group ON, OH and OS (Figure 6).

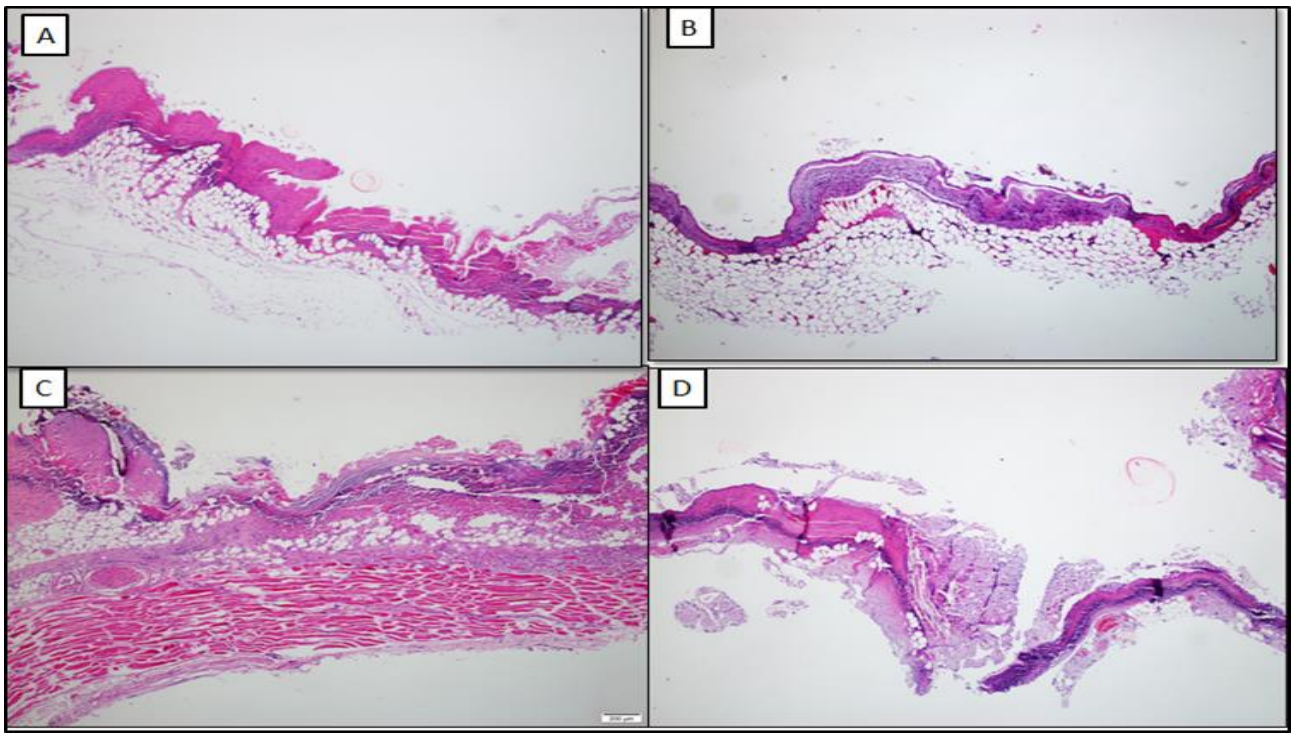

Figure 5. Skin ulcers characterized by necrotic debris and inflammatory infiltration with total absence of epidermis in all groups, including the control group, i.e. **A:** Control group, **B:** ON group, **C:** OH group, **D:** OS group, H-E, x4.

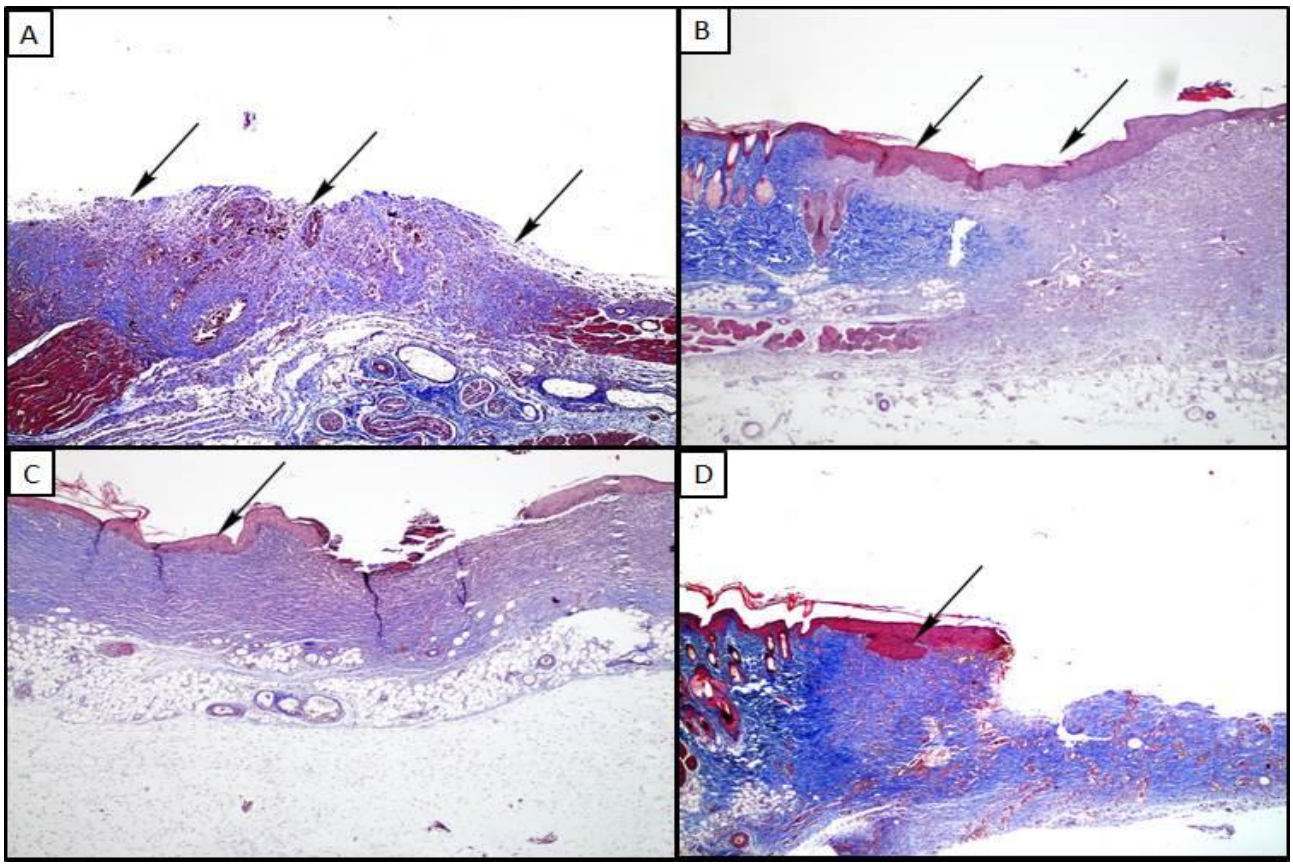

Figure 6. A: Complete removal of epidermis and irregular granulation tissue (arrows) in control group, MT, x10, B. **B:** Complete recovery of epidermis and development of granulation tissue (arrows), MT, x10. **C:** Partially epidermal (arrow) and dermal regeneration, (arrows) MT, x10. **D:** Partial epidermal (arrow) and dermal regeneration (arrows), MT, x10 in group OS.

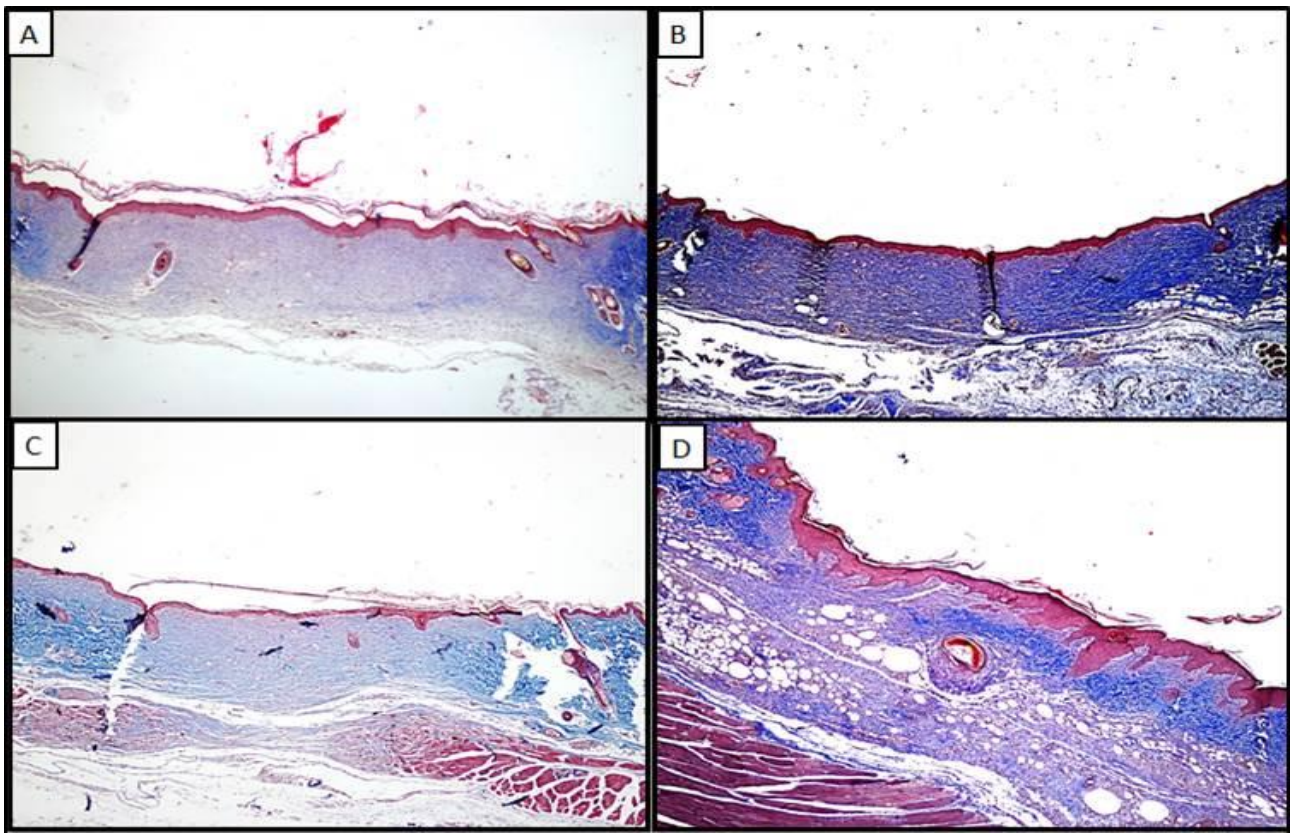

Figure 7. A: Epidermatal regeneration and granulation tissue formation in the control group, MT, x10, **B:** Full recovery of the epidermis and granulation tissue development, MT, x10. **C:** Partial epidermal and dermal regeneration, MT, x10. **D:** Partial epidermal (arrow) and dermis regeneration, MT, x10 in group OS.

Histopathological Findings in Group 3 (Day 21):

In the third group, lesions in all experimental and epidermal regeneration and granulation tissue formation in the control group (Figure 7).

DISCUSSION

Wound healing in a defect tissue consists of a number of repair and reorganization processes, during which coagulation systems become active, acute and chronic inflammatory responses arise, new vascular events occur through angiogenesis and vasculogenesis, cells become proliferative, divide, apoptotic, extracellular matrix accumulates (Witte, 1997).

There are many products used under the name of dressing materials for therapeutic purposes in wound healing. Many herbal and fat-containing products have antioxidant activity and these products have been tested for topical wound healing promotion. Recently, *H. perforatum*, known as kantaron oil, has come to the agenda as a topical treatment agent especially in burns wound care (Blumenthal, 1998). Hammer et al. (2007) in their study reported that considering its antimicrobial, antioxidant, anti-inflammatory and immunomodulatory effects its anti-inflammatory activities kantaron oil can be uses as active ingredient to induce wound healing. Dunic (2009) showed that kantaron oil contains high amounts of quercetin and biapigenin (129 µg / ml and 52 µg / ml, respectively). Lavagne and Secci (2001) showed that topical use of *H. perforatum* containing oils has positive effects on epithelial regeneration of surgical wounds.

Sesame oil has recently begun to be used in wound healing (Valacchi et al., 2011). Suja (1998) reported that antioxidant property of sesame oil is due to ingredients such as sesamin, sesamolin and sesamol present in its content. Sesame oil also contains 55% lipid and 20% protein. Kang (2000) revealed in vivo and in vitro studies that the sesamol is the most important free radical scavenger agent. In the same studies, these agents, i.e. sesamin, sesamolin and sesamol, were shown to have inhibitory effects in membrane lipid peroxidation.

In a study conducted by Valacchi et al. (2011), was investigated the topical effects of ozonated sesame oil on wound healing, where it revealed that ozonated sesame oil interacts with polyunsaturated fatty acids and therefore has antioxidant effect. This

product promotes angiogenesis in wound healing and increases vascular endothelial growth factor as well as cyclin D1 expression.

Studies on the antimicrobial properties of Nigella sativa are available in the literature (Islam et al., 1989; Burits and Bucor, 2000; Arici et al., 2005). A study (Arici et al., 2005) determined that antimicrobial properties of *Nigella sativa* have a potential to accelerate wound healing. In a study by Arici et al. (2005), the antibacterial activity of *Nigella sativa* oil was investigated in vitro and a total of 24 antibacterial effects, which were mediated by the combination of thymoquinone, p-cymene and carvacrol components, were observed. Islam et al. (1989) tested the antifungal activity of *Nigella sativa* oil against 24 fungal organism of pathogenic and industrial strains and at the end of the study, they found that *Nigella sativa* oil had significant activity and stronger and wider range of actions against fungi. Bruits and Bucor (2000) found that *Nigella sativa* oil had especially antioxidants and free radical sweeping features but not pro-oxidant.

Flavanoids and triterpenoids are well-known to enhance wound healing. Because flavonoids and triterpenes are also components of *Nigella sativa* we hypothesized that the wound healing in this group would take place in a shorter period. Ozone, a powerful oxidant, is known as one of the strongest disinfectants because of this property. It is also used in the treatment of certain disorders such as dermatitis, alone or in combination with other agents, e.g. ozonated oil. Ozone gas reacts with oil via carbon-carbon double bonds (oleic acid, linoleic, linolenic acid, etc.) found in unsaturated fatty acids of vegetable oils (Suja, 1998). As a result of this reaction different products such as hydrogen peroxide, hydroxyhydroperoxide, aldehyde and ozonit are released (Lazarus et al., 1994). In the light of all these studies, we planned a study showing the effects of ozonated *H. perforatum* oil (St. John's Wort oil), sesame oil and *Nigella sativa* oil on topical wound healing. In this study, mean epithelialization days of *Nigella sativa*, sesame oil, *H. perforatum* oil and control (K) groups were measured as 5.08±0.36, 16.17±0.46, 16.83±0.46 and 16.93±0.46, respectively. These data shows that *Nigella sativa* application has positive effects on wound epithelization rate. Additionally, in our study, wound closure rates were assessed daily throughout the study and it was found that this rate was significantly higher ($p<0.001$) in the treatment groups compared to the control groups. After 7th day and 14th day in wound treatment, macroscopical findings demonstrated that the granulation tissue was smoother and more alive and histological parameters showed that wound healing developed healthier and vascularization reduced more markedly in experimental groups compared to control.

The wound area is the most obvious symptom of wound healing and can be evaluated measured with the closure rate of the wound surface. Contraction, known as the centripetal motion of the wound edges, which speeds up the closing process of open wounds, is governed by the myofibroblast and the extracellular matrix around it. Wound contraction is 80% effective in closing open wounds in where with loose skin texture (Robbins, 1992; Gençcelep et al., 2001; Engin, 2009). In our study, it was observed that contraction rate was significantly higher in *H. perforatum* group which were proceeded by *Nigella sativa* and sesame oil groups compared to control.

CONCLUSION

We investigated the effects of sesame oil, N*igella sativa* oil and *H. perforatum* oil on wound healing considering their antiseptic and antibacterial properties. The wound healing rates were statistically more significant in the experimental groups than in the control groups except the 7th day. Macroscopic evaluation showed that recovery rate was faster in *Nigella sativa* oil and sesame oil group however when the experimental groups (sesame oil, *H. perforatum* oil) were compared with respect to histopathologic result no difference between experimental groups in terms of regeneration formation was found. The quantitative data of this study showed that ozonated *Nigella sativa* oil promoted wound healing significantly ($p<0.001$) as compared to other groups. These results demonstrate that ozonated oils, especially the ozonated *Nigella sativa* oil may improve acute cutaneous wound repair, mainly via shortening the duration of epitalization. Topical application of specific ozonated oil may be considered as an alternative therapeutic modality to enhance cutaneous wound healing. Therefore, we believe that *Nigella sativa* oil can be used as an adjunct or an alternative agent to existing treatments in the future wound healing due to its antimicrobial, antioxidant, anti-inflammatory and immunomodulatory.

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Author's Contributions: Creating an experimental 1 cm wound on rats and applying ozonated oils on them until the wound healed were performed by İC and TR. Histopathological examinations were performed by YE. Collecting, evaluating and writing the experimental data was done by İC and TR.

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Evaluation of the effect of Carvacrol on retinal neovascularization in rats

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ABSTRACT

Objectives: To compare the effect of intraperitoneal administered Carvacrol with bevacizumab in an oxygen-induced retinopathy (OIR) model in rats.

Materials and Methods: Twenty-eight newborn rats were included in the study and the OIR model was created with the 50/10% oxygen style. The study consisted of 4 groups and each rat in the groups received an intraperitoneal injection of 0.01 mL once on the postnatal 14th day. While the OIR model was not created in Group I (control group), it was created for Groups II, III, and IV. Groups I and II were injected with 0.9% NaCl solution, Group III with bevacizumab, and Group IV with carvacrol. The rats were sacrificed on the postnatal 18th day.

Results: Histopathological and immunohistochemical studies showed that the number of retinal vascular endothelial cells (RVECs) and nuclear factor (NF)-κB levels decreased similarly in Group III and Group IV compared to Group II. RVECs values for Group I, Group II, Group III and Group IV were measured as 1.26±0.80, 27.10±3.63, 7.54±1.38, and 6.22±1.22, respectively and it differed significantly between groups ($p<0.001$). Likewise, NF-κB levels were recorded as 0.61 ± 0.30, 4.36±0.65, 2.68±0.44, and 2.85±0.58, respectively and it differed significantly between groups ($p<0.001$). On the other hand, RVECs and NF-κB levels were similar between Group III, and Group IV (p values were 0.58 and 0.91, respectively).

Conclusions: The study demonstrated that carvacrol significantly reduced retinal pathological neovascularizations, RVECs, and NF-κB levels. Moreover, the observed effects were comparable to those of bevacizumab.

Keywords: *Bevacizumab, Carvacrol, Neovascularization, Nuclear factor-κB, Oxygen-induced retinopathy, Retinal vascular endothelial cell*

INTRODUCTION

Although neovascularization (NV) is physiologically vital, it is also the main cause of many diseases. Excessive NV's may result in vision loss and blindness in the eye (Ferris et al., 1984). Diabetic retinopathy (DRP), retinopathy of prematurity (ROP), age-related macular degeneration (AMD), and pathological NVs due to retinal vascular occlusions (RVOs) are the major causes of vision loss (Engerman, 1989; Ferris et al., 1984; Green, 1999). In these diseases, as a result of the triggering of inflammatory cytokines, retinal NVs and edema develop, and a decrease in visual acuity occurs (Engerman, 1989; Green, 1999; Klaassenet al., 2013). Although many mediators are blamed for this process, intertwined pro-inflammatory and anti-inflammatory stages are mentioned (Klaassen et al., 2013). Among the prominent mediators include vascular endothelial growth factor (VEGF) and nuclear factor-κB (NF-κB) (Ascaso et al., 2014).

The oxygen-induced retinopathy (OIR) model imitates the vaso-occlusive and NV phases of ROP and enables the trial of new drugs to be used for treatment (Smith et al., 1994). In this model, the normal vascular development of the retina and pathological NV formation can also be examined (Smith et al., 1994). Hypoxia occurring in the retina for any reason causes the release of pro-angiogenic factors and the formation of NV (Adamis et al., 1994). NF-κB, released and controlled by retinal vascular endothelial cells (RVECs), has an important role in the NV formation process (Moraiset al., 2009; Zhanget al., 2011). NF-κB indirectly contributes to VEGF production. When inhibited, it inhibits VEGF, thus angiogenesis (Halleet al., 2011; Zhang et al., 2011). In addition, when VEGF is inhibited, NF-κB and NV formation have been shown to be inhibited (Lopezet al., 2019).

Bevacizumab (Avastin®), is a monoclonal antibody that inhibits VEGF activity and is increasingly used for the treatment of various proliferative retinopathies (Maguire et al., 2016; Rich et al., 2006). Treatment with anti-VEGF agents (such as aflibercept, ranibizumab, and bevacizumab) prevented the pathological NVs seen in DRP, AMD, and ROP, reduced macular edema, decreased vision loss, and increased visual acuity (Maguire et al., 2016; Talks et al., 2016). In addition, it is a known fact that bevacizumab indirectly reduces NF-κB and NV since it inhibits VEGF (Lopez et al., 2019).

Carvacrol is the most important ingredient in thyme's essential oils (Baser, 1994). Carvacrol, which is widely used in medicine, pharmacy, and agriculture, has been used since ancient times with its medicinal benefits, and new benefits are discovered day by day (Altundağ and Aslım, 2005). Carvacrol has a wide range of therapeutic effects, such as anti-angiogenic, anti-inflammatory, and anti-oxidant actions (Liet al., 2019; Mahmoodi et al., 2019). Studies have shown that carvacrol regulates the balance between pro-inflammatory and anti-inflammatory mediators and suppresses the inflammatory response (Liet al., 2019; Mahmoodi et al., 2019). Besides these effects, carvacrol additionally reduces VEGF, NF-κB, and suppresses NV (Bayramoglu et al., 2014; Li et al., 2019; Mahmoodi et al., 2019). It has been reported that due to the ability of Carvacrol to inhibit NF-κB, it can also prevent NV development and metastasis in cancers (Cui et al., 2015).

Although the anti-angiogenic effect of carvacrol has been studied in many tissues and organs, including cancers, the number of studies on the retinal NV is quite limited (Bayramoglu et al., 2014; Husseinet al., 2017). Therefore, we planned to investigate the effect of carvacrol administered intraperitoneally in the OIR model on retinal NV and to compare it with the routinely used bevacizumab.

MATERIALS and METHODS

The present study was carried out in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research guidelines. The study was approved by the Bolu Abant İzzet Baysal University Experimental Animal Studies Ethics Committee as the local ethics committee (No: 2021/02).

Twenty-eight newborn Sprague Dawley rats were included in the study. To establish the OIR model, the newborn pups were placed with their mothers in an oxygen-regulated environment Oxycycler (Biospherix Ltd., Lacona, NY) within 4 hours after birth, where they were exposed to 50% oxygen for 24 hours followed by 10% oxygen for 24 hours (Pennet al., 1994). A 12-hour light: 12-hour dark environment was created in the care room with a temperature of 22-24°C and relative humidity of 45-60%. Food and beverage restrictions were not applied. This 50/10% oxygen cycle was repeated seven times until the 14th postnatal day (PND). Oxygen and carbon dioxide levels were monitored daily and calibrated as needed. In PND 14, pups were placed in a room with ambient air for 4 days.

A total of four groups were formed, including seven rats in each group. Ip injections were administered to the rats only once on PND 14 when they were taken to the care room.

Group I: 0.01 ml ip 0.9% NaCl solution was administered without creating the OIR model (control group).

Group II: OIR model was created and 0.01 ml ip 0.9% NaCl solution was administered (Untreated OIR group).

Group III: OIR model was created and 0.01 ml (2.5 mg/kg) ip bevacizumab (Altuzan, Roche, Istanbul, Turkey) treatment was applied (Acun et al., 2018).

Group IV: OIR model was created and 0.01 ml (73 mg/kg) ip carvacrol (Sigma-Aldrich, St. Louis, USA) treatment was applied (Şen et al., 2014).

In this OIR model, the best NV formation is assumed to be 18-20th PNDs. Therefore, after anesthesia was provided with intramuscular ketamine/xylazine, approximately 5 ml of blood was taken from all pups by intracardiac puncture,

and sacrification was performed in PND 18 and their right eyes were enucleated. Tissues were taken for histopathological and immunohistochemical analyses.

Quantification of retinal vascular endothelial cells and immunohistochemistry

The paraffin-embedded eyes were fixed in 10% neutral formalin and paraffin-embedded serial sections (4 μm) of eye tissue were obtained sagittally then stained with Hematoksilen and Eosin (H&E). The nuclei of retinal vascular endothelial cells (RVEC) on the vitreal side of the retinal inner limiting membrane (ILM) were counted in ten sections of each eye at x40 objective as previously described (Şen et al., 2014). The mean number of the endothelial cell nuclei of each eye was counted and calculated by two independent reviewers blind to the experiment.

Immunohistochemistry was performed using a biotin-streptavidin HRP detection kit (ab93697; Abcam, Cambridge, UK). Formalin-fixed and paraffin-embedded eye tissue sections (4 μm) were deparaffinized in xylene, and rehydrated in graded ethanol series. Citrate buffer was used as antigen retrival. Endogenous peroxidase was blocked with H2O2 (3%) in methanol. The sections were treated by a blocking serum (Histostatin plus kit broad-spectrum; Invitrogen, California, USA). The sections were then incubated with anti-NF-κB P65 antibody (ab 16502), and the biotinylated secondary antibodies (Mouse and Rabbit Specific HRP Plus (ABC) Detection IHC kit (ab93697; Abcam) at room temperature. 3,3-diaminobenzidine (DAB kit 88-2014, Invitrogen, California, USA) was used to visualize the peroxide complex. Finally, the sections were counterstained with Mayer's hematoxylin (Invitrogen, California, USA), dehydrated, and mounted with Entellan. The images were observed with a light microscope and photographed. Each section was graded as: no expression, 0; weak, 1; moderate, 2; strong, 3; and very strong expression, 4. The percentage of positive cells was defined as 0, <5%; 1, 6% to 15%; 2, 16% to 50%; 3, 51% to 80%; and 4, >80% positive cells (Figure 2) (Gocmezet al., 2015). The mean value was determined by analysing 3 retinal sections of each rat.

Statistical Analysis

Data analyzes were performed using the SPSS statistical software package, version 25.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard deviations (SDs) for each data set. Statistical significance was accepted as $p < 0.05$. Statistical analysis of the data was performed using one-way analysis of variance test and post hoc analysis after the homogeneity and normality of sample distribution was confirmed.

RESULTS

Quantitative analysis of RVEC with H&E staining

The nuclei of retinal vascular endotelial cells were counted, and quantified to confirm the effects of carvacrol on NV (Figure 1). Group I have a histology of normal retina (Figure 1A). In Group II, many new blood vessels growing in the vitreous humor (Figure 1B, black arrow) and enlarged intraretinal vessel profiles were seen in the ILM, resulting as a characteristic of OIR. In Group IV, there was a reduced number of RVEC and vascular tufts on the vitreal side of the retinal ILM (Figure 1D) similar to Group III (Figure 1C). RVECs values for Group I, Group II, Group III, and Group IV were measured as 1.26±0.80, 27.10±3.63, 7.54±1.38, and 6.22±1.22, respectively and it differed significantly between groups ($p<0.001$).

Expressions of NF-κB

Immunochemistry was also performed to investigate expression levels of NF-κB (Figure 2). In Group I, NF-κB expression levels were weakly seen only in the GCL layer (Figure 2A). However, in Group II, brown stained nuclei were strongly detected in the GCL, INL, IPL and OPL (Figure 2B). The expression levels of NF-κB in Group III and Group IV were lower in the retinal layers with reduced neovascularization on the vitreal side of the retinal inner limiting membrane (ILM) compered to Group II (Figure 2 C and Figure 2D). NF-κB levels for Group I, Group II, Group III, and Group IV were recorded as 0.61±0.30, 4.36±0.65, 2.68±0.44, and 2.85±0.58, respectively and it differed significantly between groups ($p<0.001$).

When the post hoc test results were evaluated, the RVECs and NF-κB levels of Group II were significantly higher than Group I, Group III, and Group IV ($p<0.001$ for each). On the other hand, RVECs and NF-κB levels were similar between Group III, and Group IV ($p=0.58$, $p=0.91$, respectively). It was observed that the RVECs values of Group III were higher than Group I and quite lower than Group II ($p<0.001$ for each). Similarly, NF-κB levels of Group IV were found to be higher than Group I and significantly lower than Group II ($p<0.001$ for each).

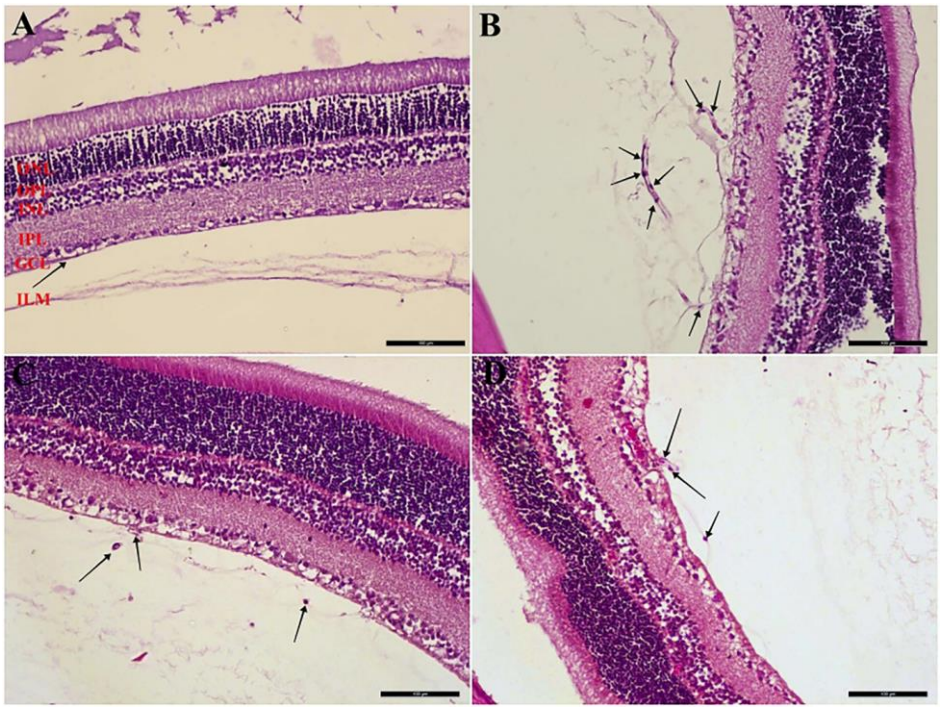

Figure 1. The representative retinal sections of retinal vascular endothelial cell nuclei (arrow) breaking through the ILM, stained with H&E. A: Control group (Group I), B: Untreated OIR group (Group II), C: OIR group treated with bevacizumab and D: OIR group treated with carvacrol. ILM: Inner limiting membrane, GCL: Ganglion cell layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OIR: Oxygen-induced retinopathy, ONL: Outer nuclear layer, OPL: Outer plexiform layer.

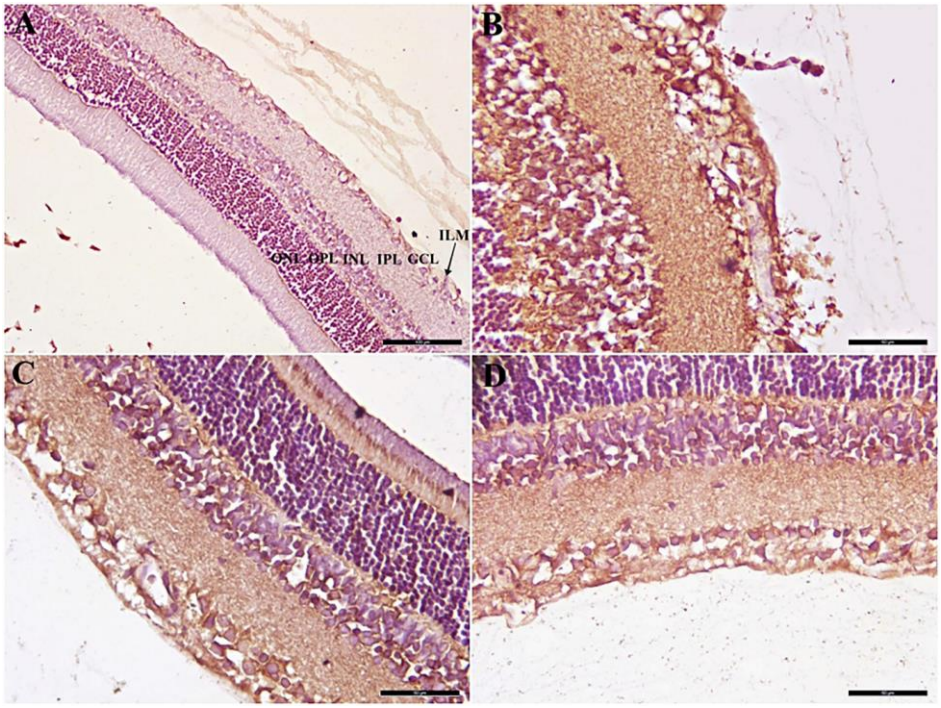

Figure 2. NF-κB immunohistochemical staining of the retina in the OIR model. A: Control group (Grade 0), B: Untreated OIR group (Grade 4), C: OIR group treated with bevacizumab (Grade 2) and D: OIR group treated with carvacrol (Grade 2). ILM: Inner limiting membrane, GCL: Ganglion cell layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OIR: Oxygen-induced retinopathy, ONL: Outer nuclear layer, OPL: Outer plexiform layer.

DISCUSSION

The results in the present study showed that intraperitoneal carvacrol and bevacizumab treatment reversed the oxygen-dependent RVEC and NF-κB elevation and suppressed angiogenesis in the OIR model. It is also important that the effect of carvacrol is similar to bevacizumab.

Bevacizumab is a powerful anti-angiogenic agent used in the treatment of colon cancer. It has found wide use in eye clinics after it has been found to suppress retinal NV. Carvacrol is a monoterpene with a phenolic structure (Baser, 1994). In addition to its anti-inflammatory and anti-oxidant properties, it has anti-angiogenic properties in cancer cases (Bayramoglu et al., 2014; Cui et al., 2015; Li et al., 2019; Mahmoodi et al., 2019). In many studies, it has been shown to suppress NV formation in different tissues and organs, but there is no study demonstrating its effect on retinal NV (Cui et al., 2015; Khan et al., 2019). In the present study, it was observed that carvacrol suppresses angiogenesis both directly and indirectly by affecting the formation of retinal NV.

The RVECs have an important role in angiogenesis. Ischemia and hypoxia in the retina lead to increased RVECs and production of angiogenic cytokines, thus leading to retinal NV formation (Cornel et al., 2015). Bevacizumab has been used in eye clinics for the treatment of pathological NV and macular edema for a long time (Maguire et al., 2016; Rich et al., 2006; Talks et al., 2016). Its anti-VEGF property is already known, but its effect on RVECs has not been studied. Likewise, the effect of carvacrol on RVECs is not known. In this study, it was found that both agents reduce RVECs in H&E staining. Therefore, it can be said that both carvacrol and bevacizumab directly suppress NV formation at this step.

NF-κB suppresses VEGF by being released from RVECs and thus inhibits the formation of NV (Morais et al., 2009; Zhang et al., 2011). In studies other than eye diseases, carvacrol has been reported to suppress NF-κB (Aristatile et al., 2013; Kara et al., 2015). Likewise, Lopez et al. (2019) stated that bevacizumab suppresses NF-κB in colorectal carcinoma (Lopez et al., 2019). However, the effect of carvacrol and bevacizumab on NF-κB in retinal tissues has not been reported in the literature. In the present study, as a result of retinal immunohistochemical staining, it was observed that both bevacizumab and carvacrol significantly decreased NF-κB and it was concluded that these two agents could also suppress angiogenesis indirectly.

There were some limitations in our study. Since the effective dose of intravitreal injection of carvacrol is not known and it is thought that intravitreal application in puppies may increase intraocular inflammation, the injections were administered as a single dose ip. Only RVECs and NF-κB were evaluated by H&E staining and semi-quantitative immunohistochemistry staining. Therefore, the parameters examined were limited and they were not supported by other advanced methods, such as Western blotting and real-time PCR. If anti-inflammatory cytokines were also studied, the scope and power of the study could increase. Despite all these limitations, it is important to determine in our study that carvacrol and bevacizumab can inhibit angiogenesis directly and indirectly by suppressing RVECs and NF-κB. In addition, it is important because it is the first study to reveal the effect of carvacrol on retinal NV and may guide more comprehensive studies on this subject.

CONCLUSION

In conclusion, carvacrol prevented pathological NV formation by suppressing RVECs and NF-κB in the experimental OIR model. In this respect, carvacrol can play an active role in the treatment of many eye diseases with pathological retinal NV formation. Therefore, further studies are needed in which carvacrol is studied in repeated doses intravitreally and more comprehensive parameters are evaluated.

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The distribution of activating transcription factor 6 (ATF6) and nerve growth factor (NGF) in the duodenum tissue of diabetic and non-diabetic rats

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ABSTRACT

Objective: This study was conducted with the purpose of investigating the distribution of the Activating Transcription Factor 6 (ATF6) and the Nerve Growth Factor (NGF) in the duodenum tissue of diabetic and non-diabetic rats.

Material and Method: Eighteen female *Sprague dawley* rats were randomly divided into three groups as the control, sham and diabetes groups. Routine histological and immunohistochemical methods were applied on the duodenum tissues collected at the end of the study.

Results: It was determined that the villus length measurements showed a statistically significant difference between the control and diabetes groups. There was NGF immunoreactivity which was moderate and diffuse cytoplasmic in the villus intestinalis and muscularis layer in all groups, weak in the crypts and glands in the control and sham groups and moderate and diffuse cytoplasmic in the diabetes group. ATF6 immunoreactivity was determined moderate in the villus intestinalis, crypts, glands and muscularis layer in the control and sham groups and strong diffuse cytoplasmic in the diabetes group. **Conclusion:** It was determined that both NGF and ATF6 immunoreactivity increased in the duodenum tissue of the rats on which diabetes was induced experimentally.

Keywords: *ATF6, Diabetes, Duodenum, NGF*

INTRODUCTION

In diabetes, which is a chronic metabolic disease, lack of insulin or problems in insulin utilization occur, and the organism cannot sufficiently utilize carbohydrates, fats and proteins (Irak et al., 2018). As a result of long-term continuation of diabetes, complications such as diabetic diarrhea or constipation, urinary incontinence and sexual dysfunctions in men and women occur (Oztekin Kazancıbaşı, 2014). Patients experiencing diabetes-related intestinal problems encounter issues such as constipation (affecting about 60% of patients) as a result of reduced intestinal motility, diarrhea as a result of an increase in the bacteria in the small intestines or fecal incontinence. The development mechanism of these intestinal diseases usually resembles the upper GIS (gastrointestinal system) involvement of diabetes. Other causes of diarrhea include insufficient pancreatic enzymes, excess fat in the excrement, bile salt absorption problems and drugs (Ohlsson et al., 2006; Gangula et al., 2007; Lee and Lee, 2013). Small intestines; It is one of the parts of the digestive system that has an important role in meeting the energy and building block needs of living things. For this reason, changes that may occur in the morphological structure of the small intestine also affect its functions negatively (Koca,

1993; Koca, 1996). The mucus in the content of the intestine provides a natural defense line by preventing pathogenic bacteria from clinging to the intestinal epithelium (Allen et al., 1993; Gu et al., 2002). The Activating Transcription Factor 6 **(**ATF6) is a transcription factor related to the membrane of the endoplasmic reticulum (ER) (Yoshida et al., 1998). As a result of induction of ER stress, ATF6 is transferred from ER to the Golgi apparatus, and here, it is separated by site-1 and site-2 proteases (Haze et al., 1999; Ye et al., 2000; Chen et al., 2002). The structure of ER is highly developed in pancreatic β cells. It was reported that this is associated with their excessive participation in insulin secretion. For this reason, proper functioning of ER is an important factor for the survival of β cells, and it was reported that insulin secretion is directly influenced in the case of any disruption in ER functions (Harding and Ron, 2002; Oyadomari et al., 2002). The Nerve Growth Factor (NGF) is one of the first discovered members of the neurotrophin family (Bayar et al., 2010). NGF has functions such as neuroblast multiplication, dorsal root ganglion maturation and axon growth. Additionally, it is a trophic protein which has a message recipient role between tissue showing a reaction to peripheral stimulation and the nerves stimulating this tissue (Friess et al., 1999; Faydacı et al., 2004; Berker, 2015). In islet cell cultures performed in diabetes, the insulin secretion in β cells decreased by 80%, while NGF and glucose secretion increased 10-fold. The increase in the synthesis of NGF may be an endogenous reaction for the survival of cells and prevention of diabetes formation (Larrıeta et al., 2006). Exogenous neurotrophic factors may induce intestinal myoelectric activities in rats, and NGF expression increases in invasive and acute watery diarrhea (Chai et al., 2003; Sarker et al., 2010).

This study was conducted with the purpose of investigating the distribution of the Activating Transcription Factor 6 (ATF6) and the Nerve Growth Factor (NGF) in the duodenum tissue of diabetic and non-diabetic rats.

MATERIALS and METHODS

Material

A total of 18 female *Sprague-Dawley* rats were used in the study. The rats were kept at 22±2°C, in standard cages under 12-h light-12-h dark conditions and fed *ad libitum* using standard rodent chow and tap water. The rats were divided into 3 groups including 6 animals in each group.

Method

The rats were randomly divided into three groups:

1. Control Group (n=6): No intervention was made on the rats in this group.
2. Sham Group (n=6): Sodium citrate solution was applied to the rats in this group by 50 mg/kg intraperitoneally (i.p.).
3. Diabetes Group (n=6): Streptozotocin (STZ) (50 ml citric acid + 40 ml disodium hydrogen was dissolved in a phosphate buffer solution, and the pH was adjusted as 4.5) was applied by 50 mg/kg i.p. as a single dose to the rats in this group.

Experimental Induction of Diabetes

Blood was collected from the tail vein of the rats after 8 hours of fasting, measured by a glucometer (On Call Plus), and the blood glucose levels were determined (day 1). On the same day, STZ application was made. Three days later, fasting blood sugar values were measured by collecting blood from the rats that were kept fasting again for 8 hours. The rats with a fasting blood glucose value of 250 mg/dL were accepted as having type I diabetes. Likewise, also at the end of the study (day 17), blood was collected from the tail vein of the rats after 8 hours of fasting, and the blood glucose levels were determined. At the end of the study, the rats were sacrificed under deep anesthesia, and their duodenum tissues were collected.

Histological Examinations

The collected duodenum tissue samples were fixed in a 10% formaldehyde solution for histological and immunohistochemical examinations. They were embedded in paraffin blocks by passing through graded alcohols, methyl benzoate and benzol. The 5-μ cross-sections obtained from the paraffin blocks were subjected to Crossman's triple staining, Hematoxylin-Eosin staining (Luna, 1968), and to determine the goblet cells that secrete neutral mucin in the intestines, PAS (Periodic acid - Schiff) staining (Bancroft et al., 1994).

Statistical Analyses

The SPSS (20.0) package software was used to analyze the data obtained in the study. In the analysis of villus lengths and goblet cell counts, one-way ANOVA was used to determine the differences between the groups. To compare the

significant differences between the groups, Duncan's test was used.

Immunohistochemical Examinations

The cross-sections taken on slides coated with chrome alum gelatin were subjected to the indirect method of Streptavidin-biotin peroxidase (Hsu et al., 1981). After deparaffination and rehydration processes, the cross-sections were shaken in PBS (0.1 M, pH 7.2), and to prevent endogenous peroxidase activity, they were incubated for 15 min. in 3% H_2O_2 prepared in 0.1 M PBS. After washing with PBS, to reveal antigens, heat was applied in a microwave oven at the highest power for 10 min. in a citrate buffer solution. They were then washed again with PBS. To prevent non-specific bonding, they were incubated for 10 min. with a Large Volume Ultra V Block solution. Afterwards, at room temperature, for 1 hour and in a humid environment, the anti-ATF6 (Bioss-Bs1634R) (1/200 dilution) and anti-NGF (Abcam-AB6198) (1/600 dilution) primary antibodies were applied on the cross-sections. The cross-sections were washed with PBS, Biotinylated Goat Anti B Polyvalent solution which corresponded to the species where the primary antibody was produced from was dripped onto the cross-sections and incubated at room temperature for 30 min. After this, the cross-sections were washed with PBS, Streptavidin Peroxidase solution was dripped, and they were incubated at room temperature for 30 min. After washing with PBS again, for chromogen application, DAB-H_2O_2 (Diaminobenzidine hydrogen peroxide) (Shu et al., 1988) solution was added, and modified Gill III hematoxylin solution was used for counterstaining. For the purpose of determining whether the ATF6 and NGF primary antibody immunoreactivity was specific or not, all procedures were exactly applied without adding primary antibodies to the cross-sections (negative control). Immunohistochemical assessment was made based on the staining properties of the target cells and the staining intensity in these cells. The assessment was made by two independent observers by assigning values from 0 to 3 for no staining (0), weak staining (1), moderate staining (2) and strong staining (3) (Zhu, 1989; Seidal et al., 2001).

The cross-sections prepared for histological and immunohistochemical analyses were assessed and photographed under a light microscope (Olympus BX51; Olympus Optical Co. Osaka, Japan). In the duodenum tissue of all groups, villus length measurements and goblet cell counts were made by using the image-j (vI. 50i) software. Villus length measurements on the duodenum tissue were made from a total of 33 areas on 6 different cross-sections in each group. Goblet cell counts were made in each group on 6 areas on 6 different cross-sections (Akgül et al., 2015).

RESULTS

Blood Glucose Results

The blood glucose values of the rats were statistically analyzed, and the results are presented in Table 1. It was determined that the blood glucose results of the diabetes group were significantly increased in comparison to the control group on the days 3 and 17 of the study ($p<0.05$).

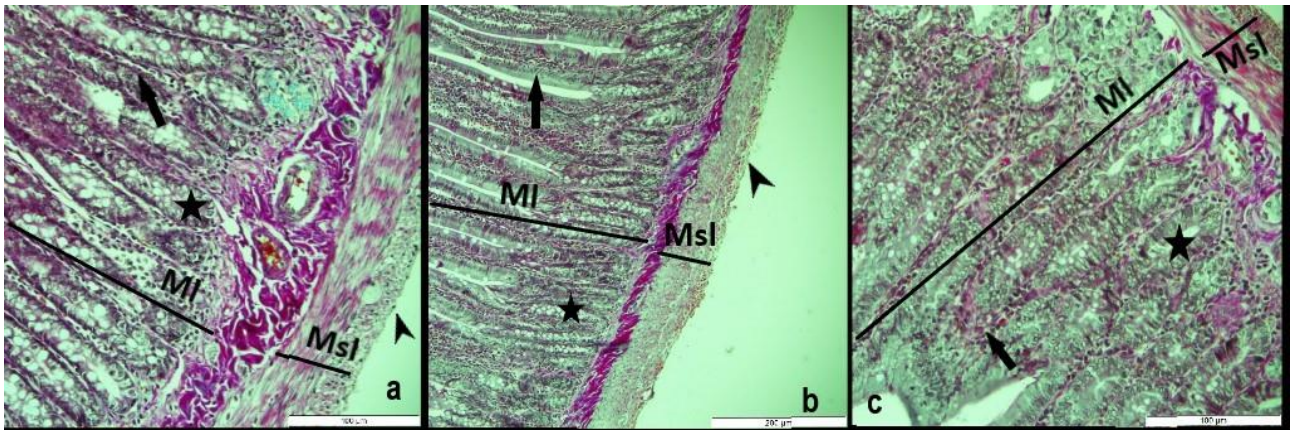

Figure 1. Rat duodenum tissue. a: Control group, b: Sham group, c: Diabetes group. Ml: Mucosa layer, Msl: Muscularis layer, Arrowhead: Serosa layer. Arrow: Villi intestinalis, Star: Crypts, Triple staining.

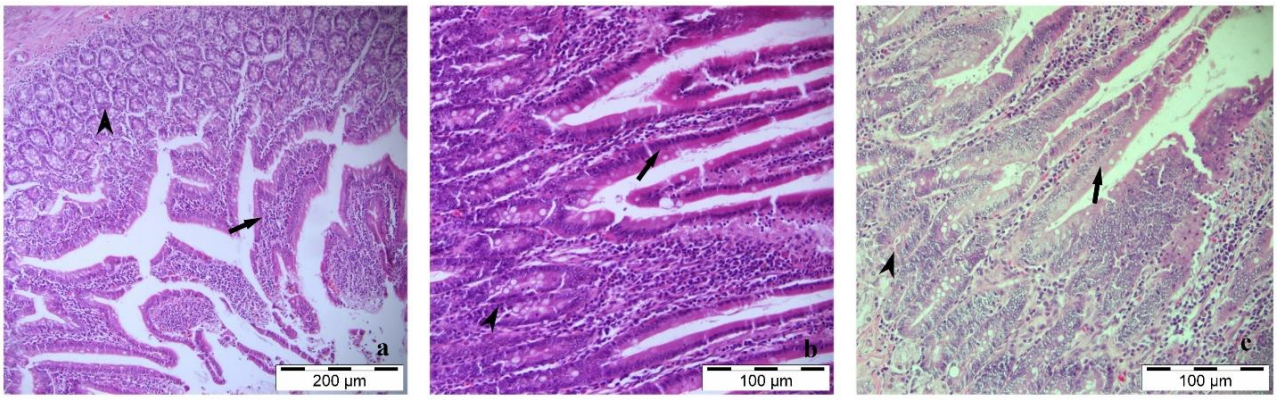

Figure 2. Rat duodenum tissue. a: Control group, b: Sham group, c: Diabetes group. Arrow: Villi intestinalis, Arrowhead: Crypts. H-E staining

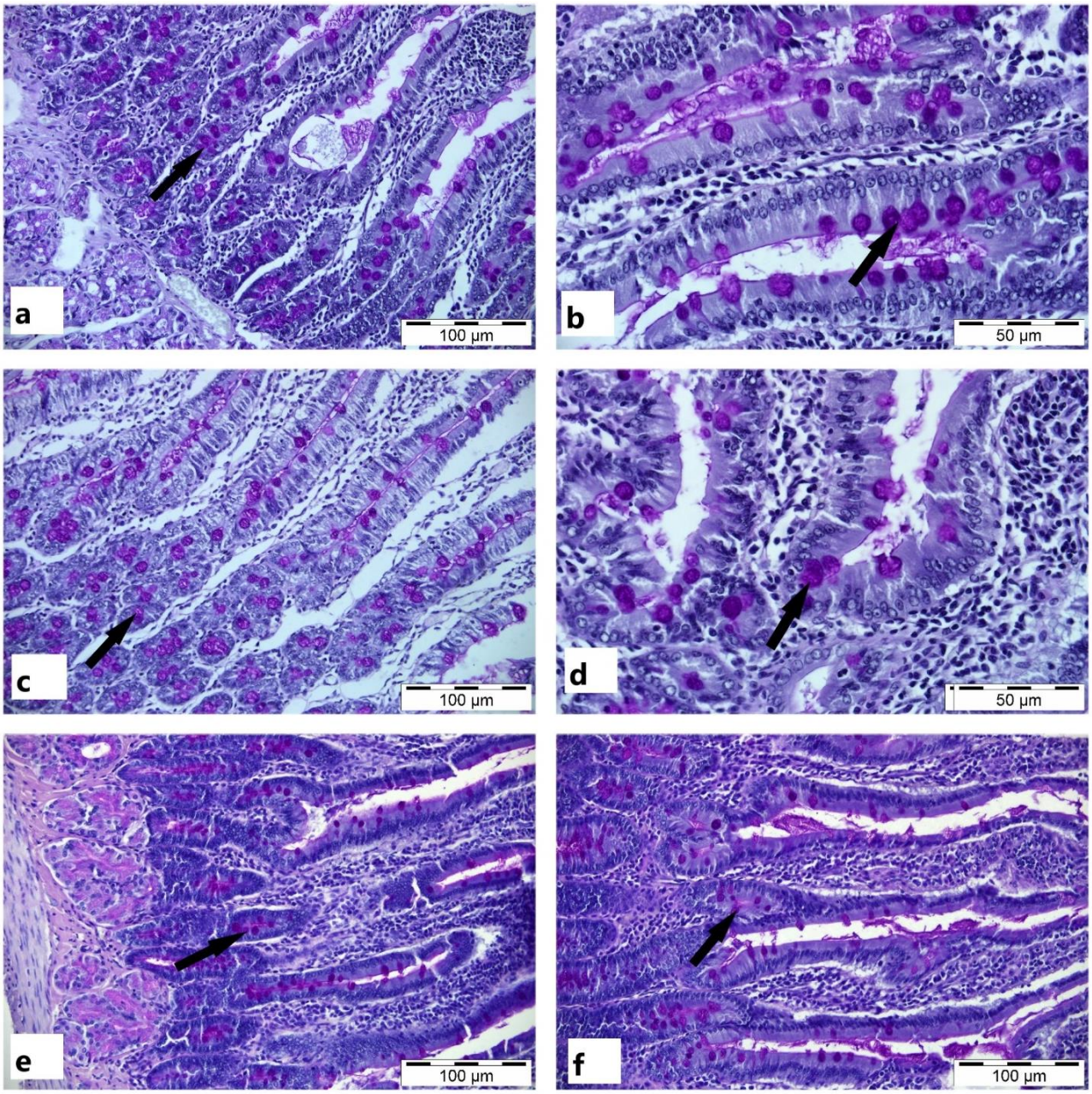

Figure 3. Rat duodenum tissue. a, b: Control group, c, d: Sham group, e, f: Diabetes group. Arrow: Goblet cells. PAS staining.

Table 1. Statistical analysis of blood glucose results.

Days	Control (mg/dl)	Sham (mg/dl)	Diabetes (mg/dl)	p
1st day	88± 3.68 ^{a}	101.67± 0.67^{ab}	95.50± 3.26^{b}	0.014
3rd day	83.17± 1.38^{a}	78± 1.93^{a}	414.67±40.12^{b}	0.000
17th day	95± 1.37^{a}	95± 4.60^{a}	270.67±19.38^{b}	0.000

$^{a, b}$: The difference between the mean values is statistically significant shown with different letters in the same row ($p<0.05$).

Table 2: Histomorphometric results obtained from the duodenum tissues of all groups.

Group	Villus length (µm)	Goblet cells/100 (µm)
Control (n=6)	197.57^{a} ± 4.90	161.50 ± 15.36
Sham (n=6)	204.59^{ab} ± 5.42	161.17 ± 18.45
Diabetes (n=6)	218.58^{b} ± 6.13	165.67 ± 16.83
p	0.02	0.978

$^{a, b, c}$: The difference between the groups is statistically significant shown with different letters in the same row ($p<0.05$).

Histomorphometric Results

There was a significant difference between the control and diabetes groups in terms of the villus lengths ($p<0.05$). There was no significant difference among the groups in terms of the goblet cell counts ($p>0.05$) (Table 2).

Histological Results

The duodenum tissue in all groups consisted of the mucosa, submucosa, muscularis and serosa layers. The villi intestinalis in the lamina epithelialis were almost the same size, and the epithelium was not interrupted. The crypts in the lamina propria, glands in the submucosa and tunica muscularis layer had a normal histological structure (Figure 1-2). The goblet cells that were localized on the epithelium layer covering the surfaces of the villi intestinalis and crypts showed a PAS-positive reaction (Figure 3).

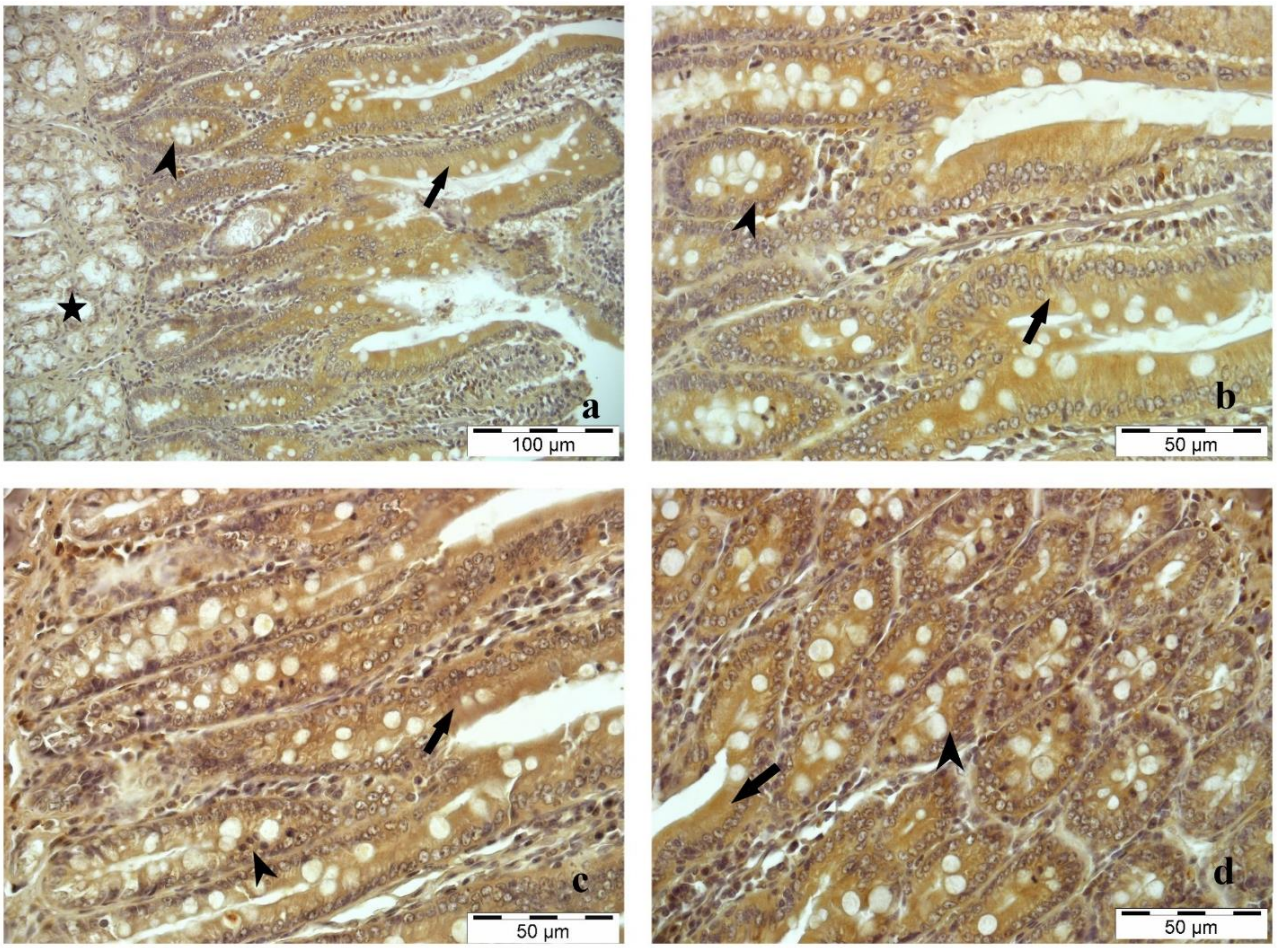

Figure 4. ATF-6 immunoreactivity in the rat duodenum tissue. a, b: Control group, c, d: Diabetes group. Arrow: Villi intestinalis, Arrowhead: Crypts, Star: Glands.

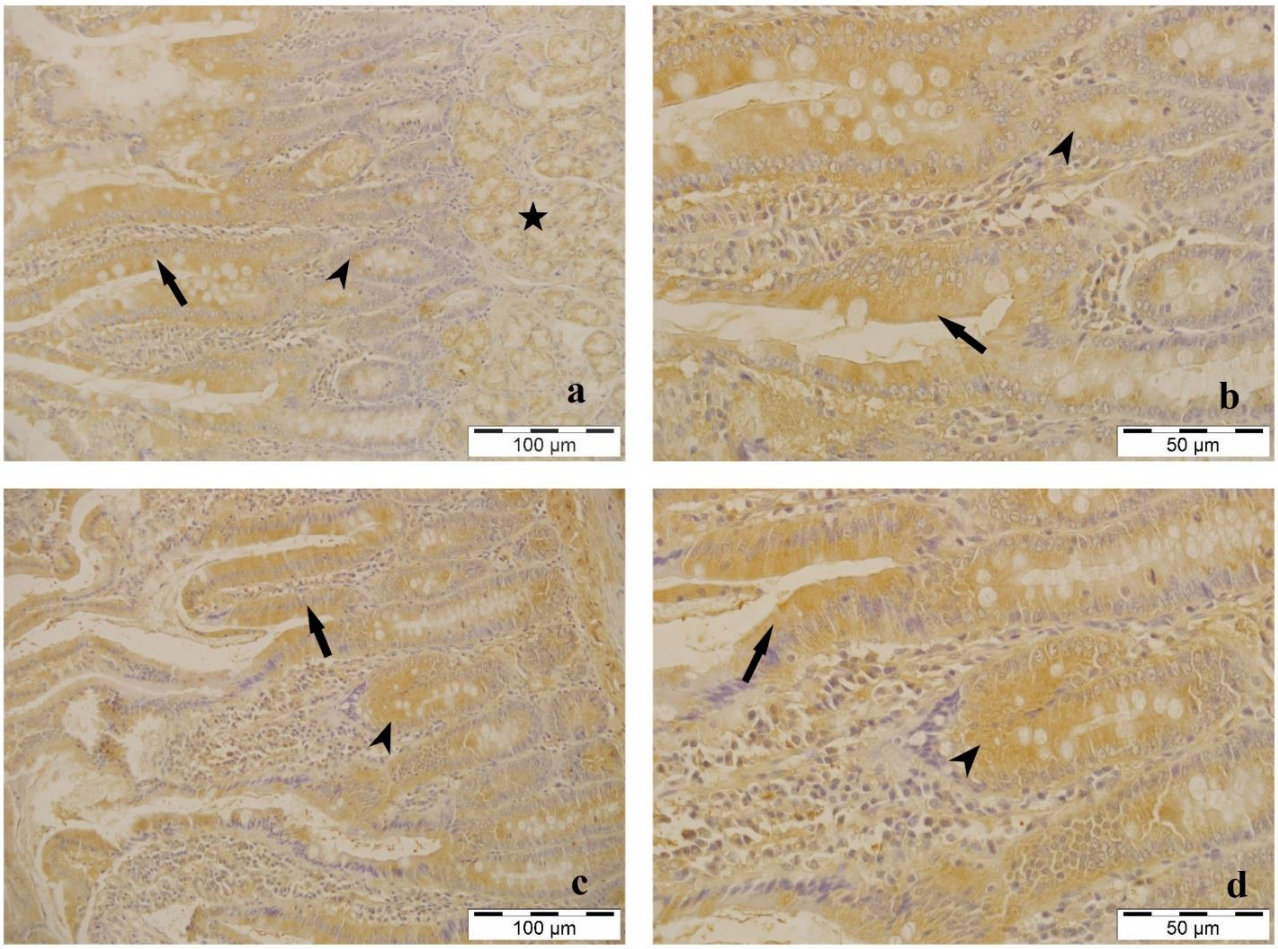

Figure 5. NGF immunoreactivity in the rat duodenum tissue. a, b: Control group, c, d: Diabetes group. Arrow: Villi intestinalis, Arrowhead: Crypts, Star: Glands.

Immunohistochemical Results

There was ATF6 immunoreactivity in the duodenum tissues of the rats in all groups. The ATF6 immunoreactivity was moderate in the villi intestinalis, crypts, glands and muscularis layer in the control and sham groups, while it was strong diffuse cytoplasmic in the diabetes group (Figure 4). There was NGF immunoreactivity in the duodenum tissues of the rats in all groups. The NGF immunoreactivity was moderate and diffuse cytoplasmic in the villi intestinalis and muscularis layer in the duodenum tissue of the rats in all groups, weak in the crypts and glands in the control and sham groups, and moderate and diffuse cytoplasmic in the diabetes group (Figure 5).

DISCUSSION

While the symptoms in the problems of the gastrointestinal system observed in diabetes are not specific, they may sometimes be strong enough to reduce the quality of life of patients. The pathophysiological mechanisms of symptoms are highly complicated, whereas it is believed that multiple factors could be effective. Similarly, it was stated that sensorimotor dysfunctions that are frequently seen in diabetes patients may be closely related to diabetic autonomic neuropathy (DAN) (Frokjaer et al., 2007; Brock et al., 2013; Yarandi and Srinivasan, 2014). Moreover, it was reported that enteric nervous system (ENS) disorders have started to be included in diabetic autonomic neuropathy (Yarandi and Srinivasan, 2014). Intestinal mucins are negatively charged and large-structured glycoproteins. They are synthesized and stored by goblet cells (Kemper and Specian, 1991). Goblet cells firstly form in the crypts, and then, they mature and migrate to the villi. It was reported that goblet cell count and volume are associated with villus size, diet, microbial flora and environmental factors (Miller et al., 1981; Yunus et al., 2005; Brown et al., 2006; Gersemann et al., 2009). Goblet cells in intestinal villi decreased in rats on which experimental ischemia was induced (Dağ et al., 2010). It was also determined that goblet cell accumulation took place on the tops of villi after superficial ischemia-reperfusion (IR) damage in the

small intestines, and this could be effective in the repair of intestinal damage by increasing mucin production and secretion in connection to the increase in goblet cell counts (Chang et al., 2005). A noticeable reduction was seen in goblet cell counts in the case of fasting, and it was stated that this could be related to disruption in the surface epithelium and crypts (Gül et al., 2004). It was determined that there was a statistically significant difference in the blood glucose levels of the diabetes group on the 3rd and 17th days of the study ($p<0.05$). Our results suggested that the experimental diabetes continued until the end of the study. Moreover, in our study, no pathological finding was observed in the duodenum tissue of the rats on which diabetes was induced experimentally, and it was determined that the villi intestinalis were almost the same size, and the epithelium was not interrupted. The finding that there was also no significant difference among the groups in terms of the goblet cell counts suggested that experimentally induced diabetes does not lead to a negative effect on the duodenum tissue.

Endoplasmic reticulum (ER) stress is a process that involves the signaling system known as the unfolded protein response (UPR). In this process, expression of mutant proteins that disrupt normal protein folding occurs in ER (Hetz, 2012). ER stress plays a role in the pathogenesis of diabetes by causing not only the loss of functional beta cells but also development of insulin resistance (Eizirik et al., 2008). In addition to this, UPR affects beta cells in two ways: while it is useful for cells under physiological conditions or mild stress, it damages cells in pathological conditions or under chronic stress by triggering beta-cell dysfunction and apoptosis (Rabhi et al., 2014). Considering the effects of ATF6 in diseases of the digestive system, it was reported that there is ATF6 expression in lesions experiencing pre-cancer atypical changes in cancers related to ulcerative colitis and those that are not related to it (Hanaoka et al., 2018). Looking at the effects of ATF6 on chronic pancreatitis, it was stated that ATF6 signaling regulates the progression of chronic pancreatitis (CP) by modulating pancreatic acinar cell apoptosis, and this may have positive effects in the diagnosis and treatment of CP based on ER stress (Zhou et al., 2019). It was also documented that ATF6 creates a natural immune response in regulation of intestinal dysbiosis and colorectal tumorigenesis (Coleman et al., 2018). In our study, it was determined that the ATF6 immunoreactivity increased in the duodenum tissues of the rats in the diabetes group in comparison to the control group. Considering the association between ATF6 and diseases of the digestive system (Coleman et al., 2018; Hanaoka et al., 2018), it was thought that ATF6 levels could be affected in connection to changes that may occur in the duodenum in diabetes.

It has been reported that diabetes is effective in the histomorphological and biomechanical reshaping of the small intestine and the colon in type I diabetes patients and diabetic animals, and this shaping is closely associated with sensorimotor dysfunctions (Zhao et al., 2003; Zhao et al., 2006; Frokjaer et al., 2007; Zhao et al., 2009). NGF is a nerve-specific growth factor, but later studies showed that NGF also has roles outside the nervous system (Okada et al., 2004). NGF distributions were checked in various segments of the intestinal tissues of rats on which colitis was experimentally induced, and it was determined that the NGF levels in the rats with colitis increased in comparison to the control group. Based on these results, it was stated that NGF may have roles in limiting or solving inflammation that occurs in the intestinal tissue (Barada et al., 2007). It was reported that NGF treatment has positive effects in early intestinal stress and prevention of diseases that may occur in the gastrointestinal system in relation to early intestinal stress (Wong et al., 2019). In our study, it was determined that the NGF immunoreactivity increased in the crypts and glands of the duodenum tissues of the rats on which diabetes was experimentally induced in comparison to the rats in the control group, and these results suggested that experimentally induced diabetes may affect NGF levels in the duodenum tissue.

CONCLUSION

Consequently, several organs and systems may be affected in the long term in diabetes, which is a chronic disease. Several complications also occur in relation to diabetes in the digestive system. In our study, it was determined that the villus length measurements in the duodenum tissues of the rats in the diabetes group and those in the control group had a statistically significant difference, while there were also increased NGF and ATF6 immunoreactivities in the diabetes group. These results that we obtained would provide positive contributions to the literature in terms of the treatment of diabetes and prevention of complications that may occur in relation to diabetes.

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