ARAŞTIRMA MAKALESİ / *RESEARCH ARTICLE*

Investigation of the Protective Effect of Thymoquinone on the Livers of the Obese Rats Induced by a High-Fat Diet

Yüksek Yağlı Diyetle Oluşturulan Obez Sıçanların Karaciğerleri Üzerinde Timokinonun Koruyucu Etkisinin Araştırılması

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ABSTRACT

Aim: *Obesity, which reduces the quality of life and forms the basis of many diseases, is caused by the imbalance between calories consumed and calories expended. The increase in calories consumed and fat accumulation in the body also cause many health problems. Obesity-related fatty liver and liver damage are one of these diseases. Fatty liver causes deterioration of functions and, accordingly, changes in homeostasis. Thymoquinone, an antioxidant, is preferred for liver damage. This study aimed to examine the protective effect of thymoquinone on liver damage caused by obesity.*

Materials and Methods: *In our study, 24 adult male Wistar albino rats were randomly divided into four groups (n: 6). Non-obese control (NOC) and non-obese thymoquinone (NTQ) groups were fed with standard chow; obese control (OC), obese-thymoquinone (OTQ) groups were fed with high-fat diet (40% of calories from fat) for 15 weeks. All treatment groups were given 10 mg/kg thymoquinone i. p. for six weeks. After the experimental study, the subjects were placed under intracardiac perfusion, and liver tissues were removed. Liver samples were subjected to tissue processing and cut into five µm thick sections for stereological and histopathological analyses. The obtained samples were stained with hematoxylin-eosin. The preparations were examined under a camera microscope, liver and sinusoid volumes were analyzed using the Cavalieri method, and hepatocyte counts were analyzed using the physical dissector method.*

Results: *In the volumetric analyses, an increase was observed in both total liver and sinusoid volumes in the OC group compared to the NOC group. Again, in both volume analyses, it was observed that the volumes in the OTQ groups decreased compared to the OC groups. In the examination of the hepatocyte count, an increase was observed in the OC group compared to the NOC group, while it was observed that it decreased in the OTQ groups compared to the OC groups. In addition, it was observed that the number of hepatocytes in the NTQ group increased significantly compared to the NOC group. When histopathological analyses were examined, ballooned hepatocytes were selected in the OC group, while this ballooning was not observed in the OTQ group.*

Conclusion: *Considering the analyses and the data obtained, thymoquinone may have a protective effect on obesity-induced liver damage. In addition, more comprehensive analyses should examine the pathways through which this effect progresses.*

Key words: *thymoquinone; liver; obesity; hepatoprotective effect; stereology*

ÖZET

Amaç: *Yaşam kalitesini düşüren ve birçok farklı hastalığın temelini oluşturan obezite alınan kalori ile harcanan kalori arasındaki dengenin bozulmasından kaynaklanmaktadır. Alınan kalorinin artması ve vücutta yağ birikimin gözlenmesi beraberinde birçok farklısağlık sorununu da ortaya çıkarmaktadır. Obeziteye bağlı karaciğer yağlanması ve karaciğer hasarı bu hastalıklardan biridir. Karaciğer yağlanması fonksiyonların bozulmasına ve buna bağlı olarak homestazın değişmesine sebebiyet vermektedir. Bir antioksidan olan thymoquinone karaciğer hasarlarında tercih edilmektedir. Bu çalışmada obeziteye bağlı olarak oluşan karaciğer hasarında thymoquinone'nun protektif etkisi incelenmesi amaçlanmıştır.*

Materyal ve Metot: *Çalışmamızda 24 adet yetişkin erkek Wistar albino sıçan rastgele dört gruba (n: 6) ayrıldı. Non-obez kontrol (NOC) ve Non-obez timokinon (NTQ) grubu standart yem ile beslenirken; obez kontrol (OC), obez-timokinon (OTQ) grupları15 hafta boyunca yüksek yağlı diyet (kalorinin %40'ı yağdan) ile beslendi. Tüm tedavi gruplarına altı hafta boyunca i.p. 10 mg/kg timokinon verildi. Deneysel çalışmadan sonra denekler intrakardiyak perfüzyona alındı ve karaciğer dokuları çıkarıldı. Karaciğer örnekleri doku işlemine tabi tutuldu ve stereolojik ve histopatolojik analizler için 5µm kalınlığında kesildi. Elde edilen örnekler hematoksilen-eozin ile boyandı. Preparatlar kameralı mikroskop altında incelendi ve karaciğer ve sinüzoid hacmi Cavaileri metodu ile hepatosit sayısı ise fiziksel disektör metodu ile analiz edildi.*

Bulgular: *Yapılan hacimsel analizlerde hem total karaciğer hem de sinüzoid hacimlerinde OC grubunda NOC grubuna göre artış izlendi. Yine her iki hacim analizinde de hacimlerin OTQ gruplarında OC gruplarına oranla azaldığı görüldü. Hepatosit sayısının incelenmesinde ise OC grubunda NOC grubuna oranla artış izlenirken; OTQ gruplarında OC gruplarına oranla azaldığı görüldü. Bunların yanı sıra NTQ grubundaki hepatosit sayısınında NOC grubuna göre önemli derecede arttığı izlendi. Histopatolojik analizler incelendiğinde ise OC grubunda balonlaşmış hepatositler seçilirken, OTQ grubunda bu balonlaşmaya rastlanamadı.*

Sonuç: *Yapılan analizler ve elde edilen veriler dikkate alındığında timokinonun obezite kaynaklı karaciğer hasarında protektif etkiye sahip olabileceği düşünülmektedir. Bunun yanı sıra bu etkinin hangi yolaklar üzerinden ilerlediği daha kapsamlı analizler ile incelenmelidir.*

Anahtar kelimeler: *timokinon; karaciğer; obezite; hepatoprotektif etki; stereoloji*

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Introduction

Obesity is an excessive or abnormal accumulation of body fat in adipose tissue that leads to health problems. It is measured by body mass index (BMI) percentile[1]. A BMI above 30 is considered obese[2]. Obesity is an epidemiologic problem with overweight people worldwide. It may cause significant health risks such as cardiovascular problems, diabetes, hyperlipidemia and hypertension, and metabolic syndrome, and it complicates many medical conditions, including critical diseases[3,4]. The most common change observed with increased fat in obesity is liver steatosis. In liver steatosis, changes in the liver, such as minimal liver fat infiltration, hepatocyte ballooning with or without fibrosis, and inflammatory cell infiltration, are observed[5,6]. The inflammatory process occurring in the liver creates changes in oxidative stress homeostasis. Antioxidants tolerate the effects of the free radicals formed. In addition to the body's antioxidant metabolism, antioxidants are taken with different natural compounds. While treating obesity-induced liver damage can be reduced in many ways, antioxidants are one of the most preferred treatments[7–10]. Thymoquinone is an antioxidant that is often preferred in liver damage. Nigella sativa, also known as Black cumin and from the Ranunculaceae family, contains thymoquinone. *Nigella sativa* is a herbaceous plant located in the Middle East[10,11]. *Nigella sativa* is used frequently, especially in the Middle East, India, and Iran. The thymoquinone (TQ) contained in black cumin has anti-inflammatory and antioxidant effects[10,11]. TQ can be effective in reversing the toxicity caused by obesity with both its antioxidant and hepatoprotective effects[12]. In addition, researchers reported TQ's anti-inflammatory, anti-histaminic, and immuno-modulatory effects, which prevent membrane lipid peroxidation in hepatocytes[13]. Studies about the usage of TQ in liver fibrosis have shown that TQ helps reduce the damage caused in the liver by reducing oxidative stress parameters[11]. Likewise, TQ treatment was performed in mice with liver damage by tamoxifen, and it was shown that it significantly inhibits glutathione depletion and lipid peroxidation accumulation in tamoxifen-induced hepatic toxicity. In addition, it has been reported that it normalizes the activity of superoxide dismutase and improves the damage to the liver histopathology[14].

This study aimed to investigate the histopathological changes in the livers of rats fed high-fat diets to cause obesity and the possible effects of thymoquinone, which has antioxidant activity, on these changes.

Materials and Methods

Animals and Experimental Procedure

The Ethical permissions for this study were obtained from the Experimental Animal Studies Ethics Committee of Ondokuz Mayis University (HADYEK/35, 27.04.2011). Twenty-four adult male Wistar albino rats were randomly divided into four groups: non-obese control (NC group), obese control (OC group), non-obese thymoquinone (NTQ group) and obese-thymoquinone (OTQ group) groups. All groups had six rats. The non-obese control and non-obese thymoquinone groups received a conventional diet for 15 weeks, but the obese control and obese-thymoquinone groups were fed a high-fat diet (40% calories from fat). The rats weighing 300–350 grams were considered obese. After the ninth week, the TQ group received an intraperitoneal injection of 10 mg/kg thymoquinone (Sigma-Aldrich, catalog no: 274666) dissolved in 0.04 ml saline in addition to their diet for six weeks per day. The rats were kept in a conventional cage with a 12-hour light/12-hour dark cycle, 50% humidity, and temperatures between 20 and 24°C with ad libitum feeding.

Tissue Procedures and Analysis

After the experimental processes, the rats were administered intramuscular ketamine and prilocaine hydrochloride (0.5 ml/300 g ketamine and 0.25 mg/100 g prilocaine hydrochloride; Sigma Chemical Comp., St. Louis, MO, USA). Then, the livers were removed and placed in a formaldehyde solution for fixation. After that, liver tissues processed with graded alcohol and xylene were embedded in a paraffin block and cut with 5 μm thickness by rotary microtome (Leica RM 2135, Germany). Sections were stained with hematoxylin-eosin for histopathological and stereological analyses.

Stereological Methods

This study used stereological methods to determine the total liver volume, sinusoidal volume, and total number of hepatocytes. The Cavalieri methods with point counting grids were used to estimate the volumes of the liver and sinusoids. The physical disector was used to determine the total number of hepatocytes.

Volume Estimation with the Cavalieri Principle

5 μm sections of liver tissue stained with hematoxylin-eosin were used for the volume analysis. Volume analyses were estimated using the Cavalier method in the

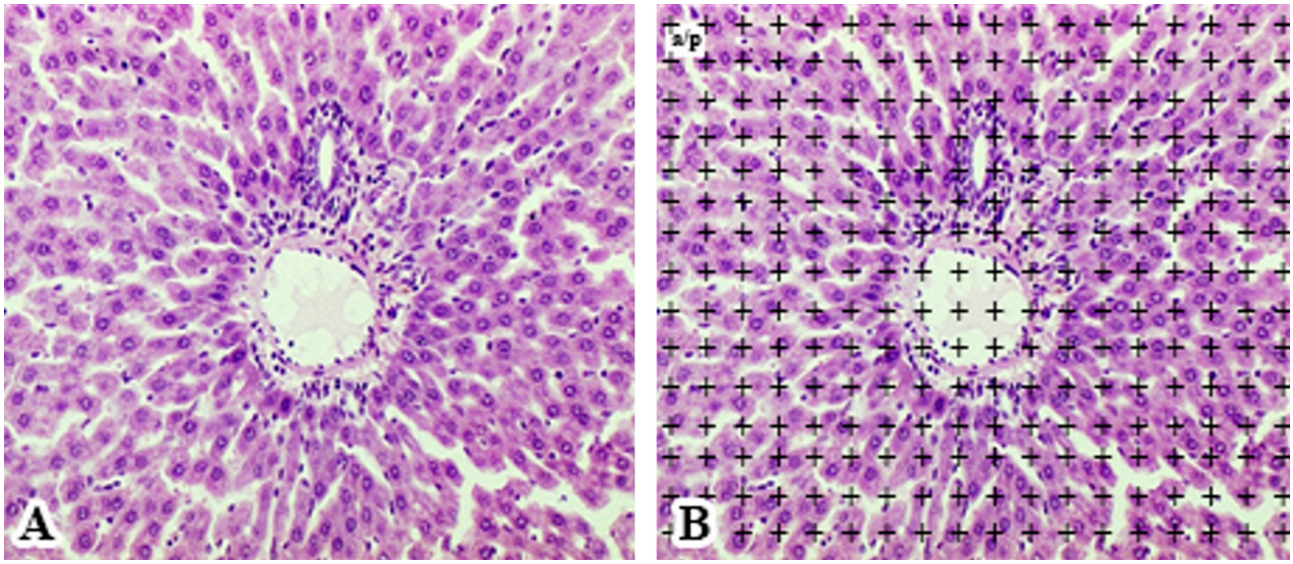

Figure 1. *A liver section and a superimposed dot-counting grid*

ImageJ program25. The liver tissue images were taken with a microscope with a camera attachment (Olympus BH-2, Tokyo, Japan). A 9000 μm 2 point counting grid for the sinusoid and the total volume were used for estimation (Figure 1).

The point-counting grid was randomly fixed on the image, and the points that hit each area were counted (Figure 1). The volume was estimated by the formula that was given below:

Volume: $t \times a/p \times \sum P$

In this equation, "t" stands for the section's thickness, "a/p" for each point's area on the point-counting grid, and "∑P" for the total number of points that hit the area of interest[15].

Estimation of Hepatocytes Number

Disector pairs were taken from the liver by systematic random sampling. And 15–20 section pairs were obtained from each animals. All liver images were taken with a microscope (Olympus BH-2, Tokyo, Japan). Hepatocyte nucleoli were accepted as dissector particles. Particles observed in the reference section but not in the look-up section were counted, and then the process was repeated by changing the sections to increase the particle count. In the unbiased counting frame placed on the sections, the particles in the countable areas were included in the count for the disector method[26]. The mean numerical density of hepatocytes (N_V (HC)) per mm^3 was estimated using the following formula:

$Nv\,(HC)=\sum Q^{-}(HC) / t\,(af)$

In this equation, "ΣQ-(HC)" stands for the total number of disector particles, "t" for section thickness, and "af" for the area of the unbiased counting frame.

Statistical Analyses

Data were statistically analyzed using the IBM Statistical Package for Social Sciences (SPSS) program version 21.0 (IBM; Chicago, USA). One-way ANOVA and Tukey tests were used to determine significant differences between groups. In contrast, the Shapiro-Wilk test was performed to determine if the data had a normal distribution. In the analysis, values with $p<0.05$ were deemed statistically significant, while those with $p<0.01$ were considered statistically highly significant.

Result

The Total Volume of Liver

Total liver volumes were estimated using the Cavalieri principle, and the results are summarized in Table 1 and Figure 2.

The liver volume was 7.61 mm^3 in the OC group and 6.507 mm^3 in the NOC group. There were significant differences between the NOC and OC groups. The liver volume in the OC group was higher than in the NOC group ($p\leq0.05$). The volume of the liver was 5.37 mm^3 in the OTQ group. The volume of the OTQ group was lower than the OC group, and the volume of the liver decreased in the NQT and OTQ groups compared to the NOC group.

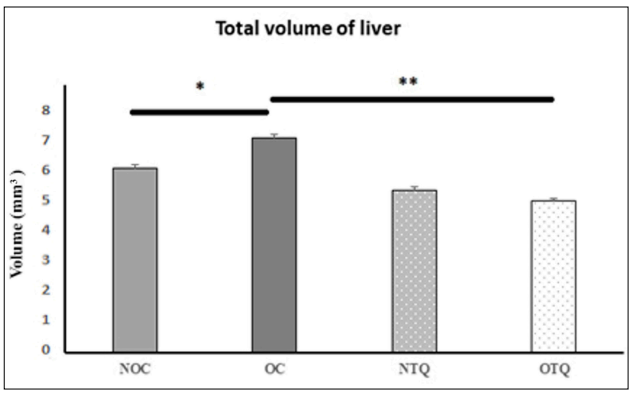

Figure 2. *The total volumes of the liver are shown. (**NOC:** non-obese control group; **OC:** obese control group; **NTQ:** non-obese thymoquinone group; **OTQ:** obese thymoquinone group; ***:** Significant differences; ****:** High significant differences)*

Table 1. *The average, CV and SEM values (mm³) of the total liver volume*

	NOC	OC	NTQ	OTQ
Average	6.507	7.61[a]	5.72	5.37[b]
CV	0.04	0.03	0.02	0.02
SEM	0.11	0.11	0.07	0.05

NOC: non-obese control group; OC: obese control group; NTQ: non-obese thymoquoinone group; OTQ: obese thymoquoinone group; CV, coefficient of variation; SEM, standard error mean; a: Significant differences between NOC and OC groups; b: Significant differences between OC and OTQ groups.

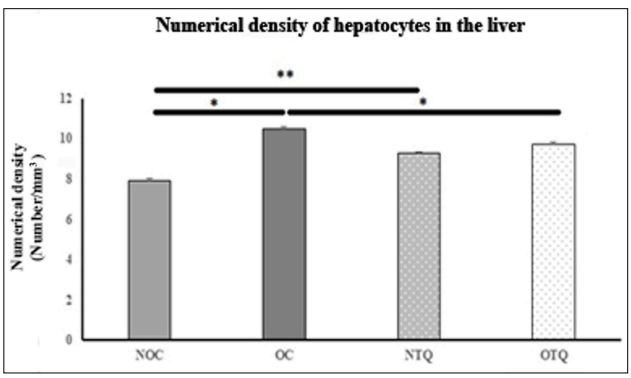

Figure 3. *The numerical density of hepatocytes is shown. (**NOC:** non-obese control group; **OC:** obese control group; **NTQ:** non-obese thymoquinone group; **OTQ:** obese thymoquinone group; ***:** Significant differences; ****:** High significant differences)*

Table 2. *Average numerical density of the hepatocyte (number/mm³) in the liver*

	NOC	OC	NTQ	OTQ
Average	7.97	10.58[a]	9.27[b]	9.62[c]
CV	0.02	0.03	0.02	0.02
SEM	0.13	0.23	0.08	0.09

NOC: non-obese control group; OC: obese control group; NTQ: non-obese thymoquoinone group; OTQ: obese thymoquoinone group; CV, coefficient of variation; SEM, standard error mean; a: Significant differences between NOC and OC groups; b: Significant differences between OC and OTQ groups.

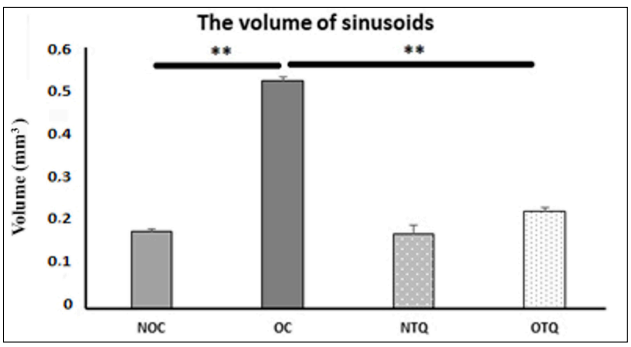

Figure 4. *The volumes of sinusoids are shown. (**NOC:** non-obese control group; **OC:** obese control group; **NTQ:** non-obese thymoquinone group; **OTQ:** obese thymoquinone group; ***:** Significant differences; ****:** High significant differences)*

Table 3. *The average, CV and SEM values (mm³) of the sinusoid volume*

	NOC	OC	NTQ	OTQ
Avarage	0.17	0.53[a]	0.19	0.22[b]
CV	0.05	0.02	0.02	0.07
SEM	0.004	0.005	0.013	0.007

NOC: non-obese control group; OC: obese control group; NTQ: non-obese thymoquoinone group; OTQ: obese thymoquoinone group; CV, coefficient of variation; SEM, standard error mean; a: Significant differences between NOC and OC groups; b: Significant differences between OC and OTQ groups.

The Number of Hepatocytes

The physical disector estimated the number of hepatocytes, and the results are given in Table 2 and Figure 3.

The numerical density of hepatocytes was 10.58 (number/mm³) in the OC group and 7.97(number/mm³) in the NOC group. Compared to the NOC group, the OC group had a higher numerical density of hepatocytes ($p \leq 0.05$). The numerical density of the OTQ group was 9.62(number/mm³), and the differences between OC and OTQ were quite noticeable. The density of the hepatocytes in the NTQ group was 9.27 (number/mm³). Compared to the NOC group, the NTQ group had a higher numerical density of hepatocytes ($p \leq 0.05$). Additionally, the OTQ group's hepatocyte density was lower than that of the OC group ($p \leq 0.05$).

The Volume of Sinusoids

The volume of sinusoids was estimated using the Cavalieri principal, and the results are summarized in Table 3 and Figure 4.

There were significant differences between the NOC and OC groups. The sinusoid volume in the OC group was 0.53 mm3, and the sinusoid volume in the NOC

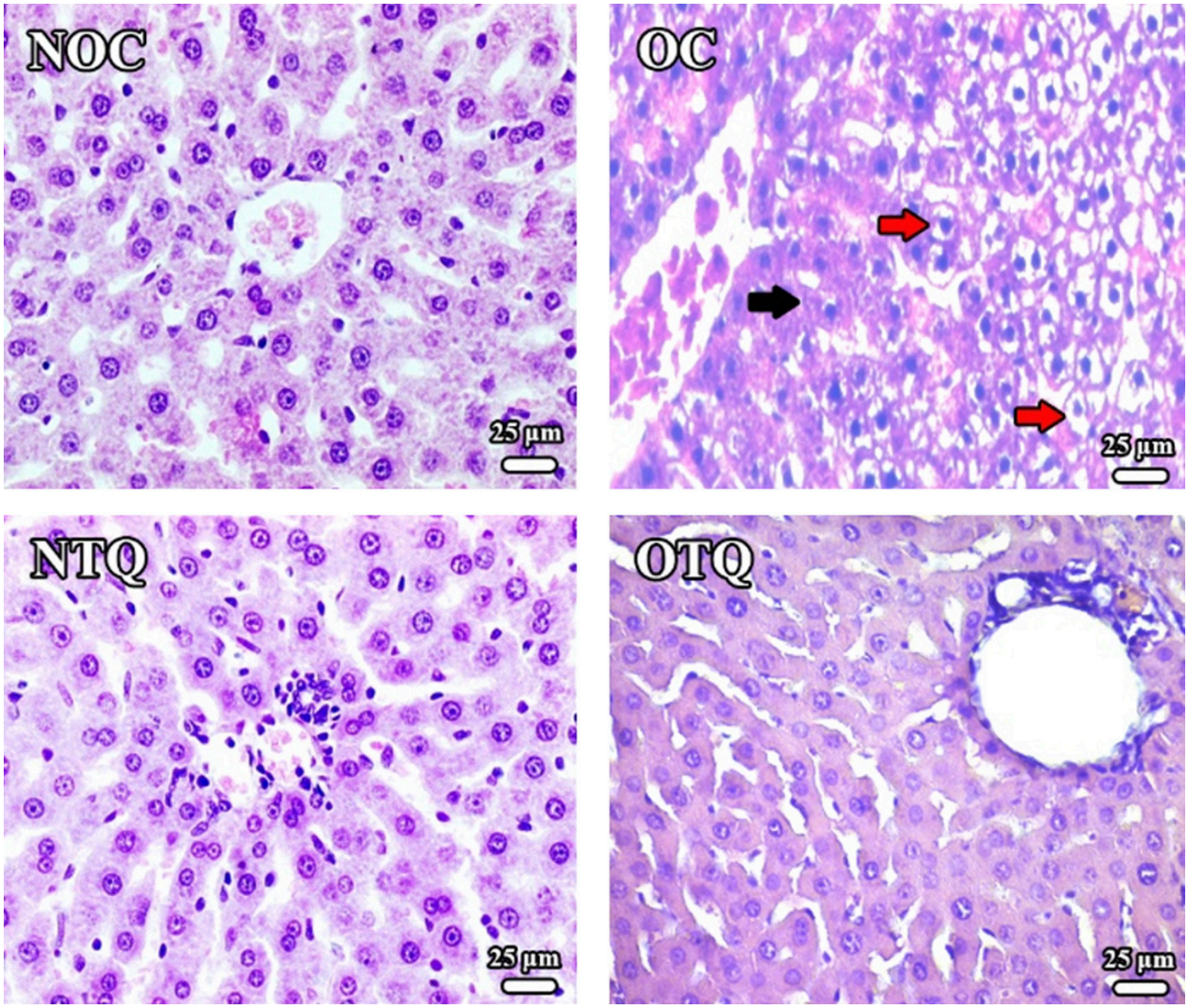

Figure 5. *Liver tissues of all groups are seen.* ***Red color arrow:*** *Hypertrophic hepatocytes with impaired nuclei,* ***black arrow:*** *Hepatocyte with unclear cell borders. Stained with hematoxylin and eosin (****NOC:*** *non-obese control group;* ***OC:*** *obese control group;* ***NTQ:*** *non-obese thymoquinone group;* ***OTQ:*** *obese thymoquinone group)*

groups was 0.17 mm^3. The volume in the OC group was higher than in the NOC group ($p \leq 0.01$). The volume of the OTQ group was lower than the OC group. The volume of the OTQ group was 0.22 mm^3.

Histological Results

In the light microscopic examination of the liver tissues, it was observed that the control group had a healthy morphology. The nuclei of hepatocytes were round and centrally located, hepatocytes arranged in cords. In the OC group, hepatocyte cords were observed as scattered, while hypertrophic hepatocytes with impaired nuclear borders and pathological appearance were remarkable (Figure 5). In the treatment groups treated with thymoquinone, hepatocytes had a healthy appearance, clear nuclear borders, and prominent nucleoli (Figure 5). Among the cords of regularly monitored hepatocytes, sinusoids, central vein, and portal areas had clear borders and healthy vascular structures (Figure 5).

Discussion

Obesity is characterized by fat accumulation in the body if the calories consumed and the calories expended are unbalanced. It is a multifactorial disease with a very high prevalence[4]. While fat accumulation is concentrated in certain parts of the body, the accumulation of adipose tissue on specific organs leads to

functional losses. Increasing fat may cause many metabolic diseases such as insulin resistance, fatty liver, and cardiovascular disease[4,16]. Obesity causes balloon-like patterns in hepatocytes called balloon degeneration, fatty degeneration in liver tissue, and decreased metabolic functions[15,17].Fatty liver has been caused by fat accumulation in hepatocytes, and over time, the inflammatory process in which immune cells play a role begins. If the treatment is not carried out correctly, the disease becomes complicated by hepatic fibrosis[18]. Obesity-induced fattening occurs physiologically and biochemically with changes in oxidative stress parameters and the liver's morphological changes. For this reason, regulation of oxidative stress parameters will minimize morphological and physiological changes resulting from fat accumulation. Antioxidant systems regulate oxidative stress parameters. Antioxidants affect free oxygen radicals and cause stress levels to decrease. Considering the changes in the liver, thymoquinone is thought to be the most suitable antioxidant for the liver. Thymoquinone has both hepatoprotective and antioxidant effects. For this reason, it is used as a therapeutic agent in many cultures. This study aimed to examine the possible impact of the mentioned properties of thymoquinone on the liver damaged by obesity.

There are many different studies examining the effects of obesity on liver tissue[19,20]. Studies have shown that obese individuals have impaired liver function and that the changes in the liver examined by ultrasound are 4.6 times higher in obese individuals compared to healthy individuals[19]. In this study, the effects of obesity on the liver were examined both stereologically and histopathologically. In the examinations, it was seen that in the non-obese control group, hepatocytes were arranged in the form of chords, they had healthy morphology, and the borders and nuclei of the hepatocytes were distinct. In the obese control group, it was noticed that the cord arrangement of the hepatocytes was disrupted, lipid accumulation was observed inside them, and their borders were irregular. This result is similar to the results in the literature. In a study conducted on 30 Wistar Albino rats in 2014, it was reported that fibrosis and ballooning in hepatocytes were observed in the livers of rats fed a high-fat diet[21]. In another study, fat accumulation was observed in the hepatocytes of rats fed a high-fat diet[22]. The fat accumulation observed histopathologically was also examined stereologically. In the stereological analyses, increases were observed in total liver and sinusoid volumes. In a study conducted in 2019, liver and sinusoid volumes of rats fed a high-fat diet were examined using the Cavalieri method, and an increase was observed in the group fed a fatty diet[15]. This is an expected result considering the fatty liver, ballooning, and inflammation processes that occur with obesity. Likewise, an increase in the number of hepatocytes was observed. The irregularity of the cords can explain this result.

Fatty liver due to obesity has many different pathophysiologies and occurs due to different factors. Oxidative stress, one of these factors, increases considerably in the case of obesity. Antioxidant mechanisms are mechanisms that reduce oxidative stress and protect organ physiology. Liver damage caused by increased fat in obesity can be treated with antioxidants. Thymoquinone is one of the most frequently preferred antioxidants and is the essential oil of Nigella sativa. It has anti-diabetic, antioxidant, hepatoprotective, neuro and nephroprotective, anti-tumor, and anti-mutagenic effects[23]. Many studies in the literature show positive effects of TQ on the liver[11,24,25]. The effects of TQ on energy metabolism, glucose and lipid mechanisms, and oxidative stress in mitochondria provide regulation of organ functions[13]. In a study using fifty male Wistar rats, liver damage was performed by creating a lipopolysaccharide-induced inflammation model and treated with TQ. It was shown that fibrosis in the liver was decreased with TQ and returned to a healthy state[11]. TQ was used for the treatment in the study on the damage of fatty liver disease and steatohepatitis in liver tissue with hyperthyroidism. It was shown that an intralobular inflammatory reaction is observed associated with significant increases in the density of resident hepatic macrophages, activated hepatic stellate cells, and alpha-smooth muscle actin observed with hyperthyroidism in disease groups; decreased damage in the livers was observed after treatment with TQ, and the inflammatory effect was reduced[26]. All these effects of TQ are thought to occur by reducing oxidative stress parameters. Reducing free radicals that cause tissue damage will also change the formation and appearance of the inflammatory response. A study showed that liver damage due to diabetes mellitus is eliminated with TQ on the morphological change and oxidative stress enzymes[25]. Another study showed that TQ treated AST, ALP levels, and oxidative stress parameters in rats with diazinon exposure[27]. In this study, stereological and histopathological analyses were performed to investigate the possible effects of TQ on the liver tissues of rats, and an obesity model was created. Stereological analyses showed changes in total liver and sinusoid

volumes. When the total liver and sinusoid volumes were examined, the thymoquinone-treated obese group had significantly lower volumes than the obese control group. These results support thymoquinone's antioxidant and hepatoprotective effects by positively affecting the liver. In the analysis of hepatocyte density, it was observed that the density decreased in the obese group that received thymoquinone injection compared to the obese group. These results suggest that TQ causes a decrease in total liver volume by regulating the sinusoidal volume. Considering the anti-lipidemic property of thymoquinone, it can be suggested that obesity in the sinusoid capillaries reduces the sinusoid volume by decreasing the increased lipid accumulation[28]. The histopathological analyses in this study show that TQ groups showed decreased ballooning appearance, degeneration, and fibrosis and that the livers had a healthy pattern compared to the obese control group. The regularity of hepatic cords and the absence of ballooning patterns, which we observed in the histopathological results, also support our stereological findings and show that TQ may improve liver damage through many mechanisms.

When all these results are considered, thymoquinone may have a protective effect. This effect of TQ is obtained by reducing oxidative stress. However, the study has limitations because oxidative stress parameters were not analyzed within the scope of the study, and the mechanisms were not investigated with different analysis methods. More analysis is needed to explain the mechanism of action.

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