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RESEARCH COMMUNICATION

Effects of thymoquinone on testicular structure and sperm production in male obese rats

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Abstract

Thymoquinone (TQ) is a phytochemical compound found in the plant *Nigella sativa*. It has antioxidant and anti-cancer effects. This study investigated the effects of TQ on obesity and testicular structure of high-fat-diet (HFD) fed rats. Obese control (OC) and obese thymoquinone (OT) groups were fed a special diet containing 40% of total calories from fat. Non-obese control (NC) and non-thymoquinone (NT) groups were fed a standard diet for nine weeks. Then, intraperitoneal TQ injections were carried out to the OT and NT groups for six weeks and testes were removed. Catalase and myeloperoxidase activity were determined in rat testis tissue. Stereological, histopathological, and immunohistochemical changes were evaluated in the testes of the rats. In stereological studies, mean volumes of testis and seminiferous tubules, the number of spermatogenic cells and also Leydig cells in the OC group were reduced, but these values significantly increased in the OT group. Apoptotic cells were observed in the OC group in comparison to the OT group. The number of healthy sperms were reduced in the OC group, whereas the majority showed anomalies in the head, neck, and tail. The number of healthy sperm was increased and the anomalies significantly reduced by using TQ in both the NT, and especially the OT group. TQ like antioxidants may improve fertility by means of increasing the healthy sperm number and preventing sperm anomalies.

Abbreviations: BMI: body mass index; CAT: catalase; HE: hematoxylin-eosin; HFD: high-fat-diet; LH: luteinizing hormone; MPO: myeloperoxidase; NC: non-obese control; NT: non-obese thymoquinone; OC: obese control; OT: obese thymoquinone; TQ: thymoquinone

Keywords

Antioxidants, infertility, obesity, oxidative stress, rat, stereology, testis, thymoquinone

History

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Introduction

In recent years, there has been an increase in the number of obesity associated conditions as a result of the increasing uptake of high-energy foods, decreasing physical activity, and a sedentary lifestyle. Oxidative stress caused by obesity is closely related to various diseases such as cardiovascular, hypertension, diabetes, cancers, and infertility [WHO 1997]. Obesity is characterized by increased fat accumulation in the body and it is related to many factors, and is a serious chronic disease. Obesity or excessive lipid accumulation are generally accompanied with increased glucose tolerance and rising serum triacylglycerol concentrations. Also, type 2 diabetes, hypertension, dyslipidemia, cardiovascular disease, infertility, and fast cancer progression are frequently seen in obese individuals [Galinier et al. 2005; Must and Anderson 2003; Wright et al. 1994]. The most important risk factors for

obesity are excessive calorie intake, gender, age, education, marriage, and birth number. In addition, obesity can be caused by hereditary factors. More than 30% of the population in Turkey (men 7.9%, women 23.4%) are obese. Obesity is closely associated with many chronic diseases [Hatemi et al. 2002; Onat et al. 2002].

Known causes of male infertility are testicular torsion, trauma, undescended testis, hypogonadotropic hypogonadism, seminal tract infections, varicocele, and reproductive tract obstruction or gonadal dysgenesis. Lately, in some studies, body mass index (BMI) has been reported to negatively affect male fertility. Several studies revealed a direct correlation between high BMI and decreased free and total testosterone levels in the blood. In addition, obesity may cause oxidative stress as indicated by elevated oxidative stress markers paralleling decreased antioxidant defence enzymes. Oxidative stress causes damages on the major compounds in cells like lipids, proteins, DNA, and carbohydrates. All these changes may affect the semen parameters in obese cases [WHO 1997].

Nigella sativa is a flowering plant that belongs to the Ranunculaceae family. It has been used in many Middle Eastern countries as a natural remedy/treatment [Swamy and

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Table 1. The mean body, testis, and peri-testicular adipose tissue (PTAT) weight (g), testis volume (cm³), and mean volume of the seminiferous tubules (mm³) in all groups.

Groups	Body weight (g)	Testis weight (g)	PTAT Weight (g)	Testis volume (cm ³)	Mean volume of the tubules
NC	337.5 ± 2.59	1.18 ± 0.06	2.76 ± 0.17	2.73 ± 0.21	0.65 ± 0.04
CV	—	—	—	0.14	0.38
OC	394.5 ± 6.71**	0.63 ± 0.05**	5.49 ± 0.46**	1.90 ± 0.14**	0.42 ± 0.02*
CV	—	—	—	0.15	0.11
NT	356 ± 1.67	1.13 ± 0.12	2.4 ± 0.28^	2.70 ± 0.28	0.59 ± 0.03
CV	—	—	—	0.02	0.12
OT	361.8 ± 4.22##	1.3 ± 0.04##	3.8 ± 0.61##	2.13 ± 0.19#	0.55 ± 0.06#
CV	—	—	—	0.18	0.26

NC: non-obese control; CV: coefficient of variation; OC: obese control; NT: non-obese thymoquinone; OT: obese thymoquinone. CV values were estimated in only stereological analyses. Data was expressed as mean ± SEM. * and ** show significant differences between the results of the NC and OC groups at 0.05 and 0.01 levels, respectively. # and ## show significant differences between the results of the OC and OT groups at 0.05 and 0.01 levels, respectively. ^ shows significant difference between the results of the NC and NT groups at 0.05 level.

Tan 2000]. It contains volatile oil, fixed oil, quinines, proteins, alkaloids (nigellimine, nigellidine, nigellicine, and nigellamine), saponins (α -hederin), cumarins, vitamins, and minerals. The seeds or the oil of the seed have analgesic, anti-pyretic, anti-neoplastic, anti-fungal, anti-convulsant, anti-ulcer, anti-bacterial, anti-tumour, anti-diabetic, immune-modulator, anti-inflammatory, anti-viral, anti-hyperlipidemic, hepato-protective, anti-oxidant, anti-histaminic, anti-helminthic, and anti-hypertensive activities [Ali and Blunden 2003; Bruits and Bucar 2000]. Thymoquinone (TQ) is the most important phytochemical compound of the seeds and is responsible for many of these biological effects. But, the influence of TQ on obesity, obesity-induced testicular injury, and sperm production essentially is unknown. To address this issue the possible beneficial effect of TQ on the obesity related changes in adult rats' testis and their functions were examined.

Results

Body and testis weight and mean seminiferous tubule and testis volume and cellular characteristics

During the experimental period, weight measurements were done weekly in the animals within the obese and non-obese groups. At the end of the 9th week, measured weight and length parameters were calculated using BMI, and those which were more than 5 kg/m² were considered obese [Altunkaynak et al. 2008]. At the end of the study, the mean body weight and white adipose tissue around the testes in the obese control (OC) group was significantly more than that of the non-obese control (NC), and obese thymoquinone (OT) groups ($p < 0.01$). Following the TQ treatment, the body weight was significantly reduced in the non-obese thymoquinone (NT) ($p < 0.05$) and OT ($p < 0.01$) groups in comparison to those of the NC and OC groups. The testis weight and volume were reduced in the OC group in comparison with the NC group ($p < 0.01$), but the testis weight and volume increased after the TQ treatment of rats in the OT group, in comparison to the OC group ($p < 0.001$ and $p < 0.05$, respectively). The data show that the mean volume of the seminiferous tubule in animals of the OC group decreased in comparison to the NC group ($p < 0.05$). It was also obvious

Table 2. The percentage of sperm with normal morphology or head, neck, and tail anomaly in all groups.

	Normal morphology sperm (%)	Sperm with head anomaly (%)	Sperm with neck anomaly (%)	Sperm with tail anomaly (%)
NC	76 ± 3	16 ± 2	6 ± 1	2 ± 1
OC	29 ± 1**	46 ± 1**	15 ± 1**	10 ± 2**
NT	88 ± 4^	7 ± 2^	3 ± 1^	2 ± 1
OT	65 ± 3##	27 ± 3##	6 ± 1##	2 ± 1##

NC: non-obese control; OC: obese control; NT: non-obese thymoquinone; OT: obese thymoquinone. Data expressed as mean ± SD. ** show significant differences between the results of the NC and OC groups at 0.01 level. ## show significant differences between the results of the OC and OT groups at 0.01 level. ^ show significant differences between the results of the NC and NT groups at 0.05 level.

that the mean volume of the seminiferous tubules showed a significant increase in obese animals following the TQ treatment compared with those of the OC group ($p < 0.05$). There was no significant difference in these parameters between the NC and NT groups (Table 1).

Sperm morphological evaluation results in the samples of sperm smears in all groups are shown in Table 2. In the evaluation of the sperm morphology, the sperms generally had normal morphology in the NC and the NT groups and a few head, neck, and tail anomalies were observed (Figure 1A and C). But, the data revealed that the percent of sperms with normal morphology was significantly decreased ($p < 0.01$) and the percent of sperms with head, neck, or tail anomalies were significantly increased in the OC group compared to those of the NC group ($p < 0.01$). It was notable that the sperm tails were circled around the head in the OC group (Figure 1B). Many Dag defected sperms were formed, and tail-less sperms were observed (Figure 1B). Sperm anomalies were significantly reduced in the OT group in comparison to the OC group (Figure 1B and D). The TQ treatment in both the NT ($p < 0.05$) and OT ($p < 0.01$) groups caused an increase in the number of sperms with normal morphology and caused a decrease in the percentage of sperms with the head, neck, or tail anomalies.

The red stained areas on testis samples of all groups were evaluated as positive in terms of luteinizing hormone (LH)



Figure 1. The sperm smears belonging to non-obese control (A), obese control (B), non-obese thymoquinone (C), and obese thymoquinone (D) groups are shown. Arrowheads: normal sperm morphology; double arrowheads: Dag effect; white filled arrow: tail-less sperm; black arrow: sperms with head and tail anomaly. The general morphology of sperms is seen in the all groups. Tail-less sperms, sperms with head and tail anomalies and also Dag effect are seen in the sperm smears of the OC group. But following TQ application, sperms having normal morphology are seen in the sperm smears of the OT group. Dye: HE; Scale Bars: 25 μ m.

receptor immunoreactivity. The immunoreactivity was only observed in the cytoplasm of positive cells. So, no immunostaining was seen in the nuclei. The intensity of staining in the cell cytoplasm varied from cell to cell in the NC group. Strong positive immunoreactivity was observed in some Sertoli cell cytoplasm (Figure 2A). Slightly positive areas in the cell cytoplasm of spermatogonia, Sertoli, Leydig, and peritubular cells were seen in the OC group (Figure 2B). The strong immunoreactivity appeared in the cell cytoplasm of the endothelial cells of the vessels, connective tissue, and Sertoli and Leydig cells especially in the NT group (Figure 2C). However, the pericytes, smooth muscle cells on vessel walls showed strong immunoreactivity. The cell cytoplasm of Leydig cells, spermatocyte, and spermatids in the OT group exhibited more positivity than those in the OC group (Figure 2D).

Biochemical and histopathological analysis

The total number of Leydig cells significantly decreased in the OC group in comparison to the NC group ($p < 0.01$). After the TQ treatment, the total number of Leydig cells significantly increased in the OT group in comparison to that of the OC group. The total number of spermatogonia ($p < 0.05$), primary spermatocyte ($p < 0.01$), and round spermatids ($p < 0.01$) in animals in the OC group significantly decreased

in comparison to the NC group. The TQ treatment in the OT group significantly increased the total number of spermatogonia ($p < 0.05$), primary spermatocyte ($p < 0.01$), and round spermatids ($p < 0.01$) compared to the OC group. Also, the total number of round spermatids in the NT group significantly increased in comparison to the NC group ($p < 0.05$; Table 3).

Catalase (CAT) and myeloperoxidase (MPO) enzyme activities in all groups are shown in Table 4. The CAT activity significantly increased in the OC group in comparison to the NC group ($p < 0.05$). But, the TQ treatment significantly lowered CAT activity in both the NT and OT groups compared to those of the NC and OC groups ($p < 0.01$). MPO activity significantly increased in the OC group in comparison to the NC group ($p < 0.0001$). Following the TQ treatment, the MPO activity was reduced the OT group in comparison to the OC group ($p < 0.01$).

In the testes of the NC group, tunica albuginea, seminiferous tubuli, interstitial connective tissue and Leydig cells were observed in normal histological appearance. The cells belonging to the spermatogenic series were orderly arranged in the tubule wall (Figure 3A and B). Testes in the OC group presented as compromised and many seminiferous tubuli were atrophied. The seminiferous tubule wall in the OC group became thinner than that of the NC group, and the wall structure was degenerated. The separation from the interstitial

area and the increase in the connective tissue were notable (Figure 3C and D). The testis cross sections were in normal histological structure in the NT group. The seminiferous tubules, spermatogenic cell series, and interstitial connective tissue also presented normal morphology (Figure 4A and B). The distances between germ cells in the OT group were extended. In some, the spermatogenic cell lines were damaged and the spermatogenesis was delayed. In these areas, the density of spermatids and spermatozoa in the tubule lumen were reduced compared to the NT group, but they were more than those of the OC group (Figure 4C and D).

The apoptotic index of the all groups is shown in Table 5; apoptotic activity was observed in few spermatogonia of the NC and NT groups (Figure 5A and C). There was no significant difference in terms of the incidence of the apoptotic spermatogenic cells among the NC, NT, and OT groups ($p>0.05$; Figure 5A, C and D). In comparison, OC apoptotic activity was observed in the Leydig, Sertoli, and peritubular myoid cells to a greater extent than those of the other groups ($p<0.01$; Figure 5B). However, apoptotic activity was reduced in the germ cells, especially in the spermatogonia of the OT group compared to the OC group ($p<0.01$; Figure 5D).

Discussion

Obesity, containing social, genetic, metabolic, and physiological behavioral components is a complex, chronic disease [Heysmfield et al. 1989]. It is known that obesity is related to

Table 3. The total number ($\times 10^6$) of the different cell types of the testis seen in the all groups.

Groups	Spermatogonia	Primary spermatocyte	Round spermatids	Leydig cell
NC	1.24 \pm 0.32	2.34 \pm 0.28	1.91 \pm 0.28	0.42 \pm 0.66
CV	0.12	0.16	0.14	0.18
OC	0.91 \pm 0.16*	1.08 \pm 0.11**	1.21 \pm 0.1**	0.25 \pm 0.28**
CV	0.13	0.15	0.14	0.17
NT	1.17 \pm 0.27	2.45 \pm 0.22	2.32 \pm 0.34^	0.38 \pm 0.52
CV	0.11	0.14	0.12	0.13
OT	1.12 \pm 0.45#	1.57 \pm 0.76##	1.87 \pm 0.08##	0.31 \pm 0.30#
CV	0.12	0.11	0.16	0.19

NC: non-obese control; CV: coefficient of variation; OC: obese control; NT: non-obese thymoquinone; OT: obese thymoquinone. CV values were estimated in all analyses. Data was expressed as mean \pm SEM. * and ** show significant differences between the results of the NC and OC groups at 0.05 and 0.01 levels, respectively. # and ## show significant differences between the results of the OC and OT groups at 0.05 and 0.01 levels, respectively. ^ shows significant difference between the results of the NC and NT groups at 0.05 level.

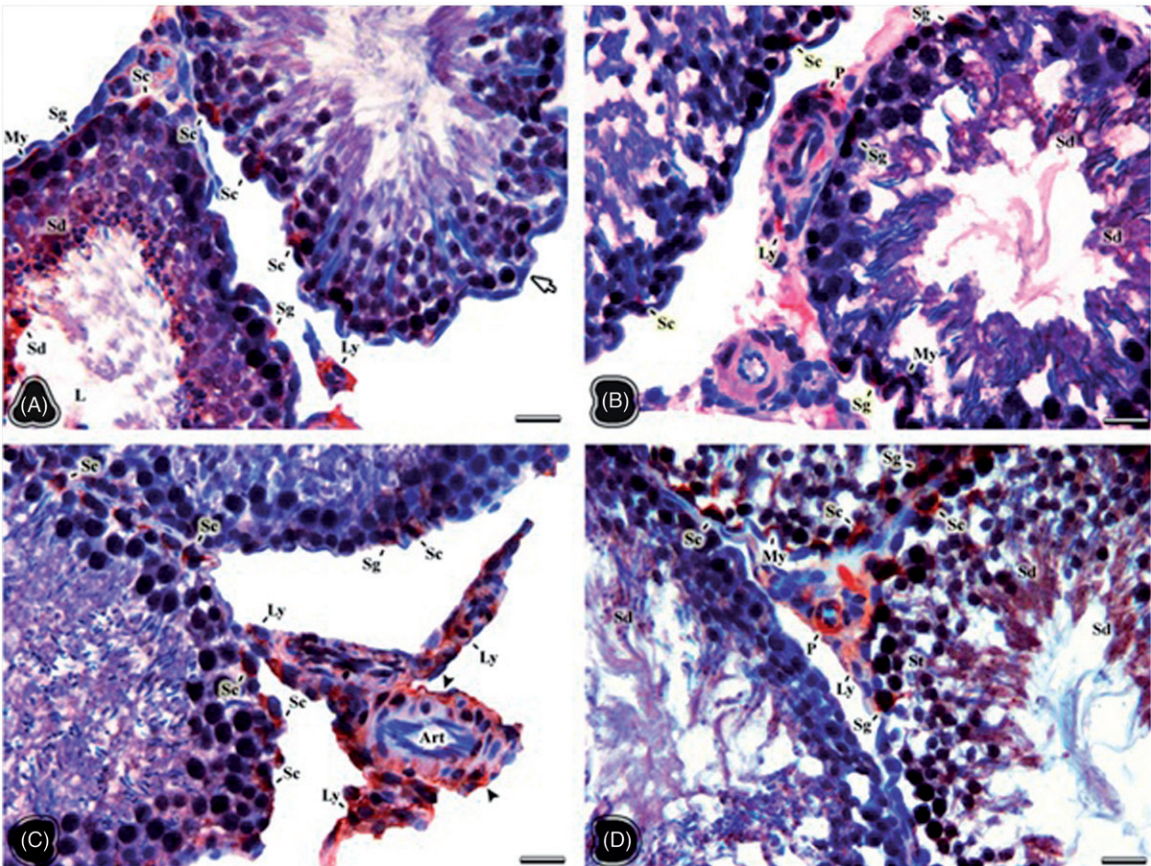


Figure 2. The light microscopical sections of testis belonging to the non-obese control (A), obese control (B), non-obese thymoquinone (C), and obese thymoquinone (D) groups are shown. Sg: spermatogonia, Sc: sertoli cell, Sd: Spermatid, St: spermatocyte, Ly: Leydig cell, My: myoid cell, P: pericytes, L: tubule lumen. (A) Arrow: negative staining of Sertoli cell. (C) Art: arteriole, Arrowhead: vessel wall. Histological structure of testis is seen in the all groups. Luteinizing hormone (LH) receptor positivity is shown by immunohistochemistry in the nuclei of the positive stained cells (signed). LH receptor activity was observed in the cells occupying the connective tissue and vascular walls rather than tubular and Leydig cells of the obese control group. But following thymoquinone application, more positive cells were seen in the light microscopical sections of the obese thymoquinone group. Dye: anti-LH/choriogonadotrophic hormone (CG) receptor immune stain; Scale Bars: 25 μ m.

many chronic diseases [Abdel-Halim 2005; Buschemeyer and Freedland 2007; Gupta et al. 2010; Kutlutürk et al. 2011; Logue et al. 2011; Zimmerman et al. 2011]. The relationship between obesity and infertility has begun to be studied intensely [Abdel-Halim 2005; Hammoud et al. 2012]. In the studies of the last 50 years, it was detected that the sperm number was significantly reduced in obese individuals [Swan et al. 2000]. Increased oxidative stress was considered

to be one of the major causes in the emergence of obesity complications [Bakos et al. 2011; Tunc et al. 2011]. Many studies reported that insufficiency occurred in antioxidant defence mechanisms of obese individuals, and this situation may cause different complications related to obesity [Colette et al. 2003; Puchau et al. 2010; Stefanović et al. 2008; Tunc et al. 2011]. Therefore, antioxidant treatment is a potential method for many diseases and it has become increasingly important in recent years [Puchau et al. 2010].

This study was carried out to examine the effects of the oxidative stress caused by obesity, and the antioxidant feature of thymoquinone on male fertility was examined in high-fat-diet (HFD) fed obese rat testes. Gu et al. [2012] observed that the body weight, visceral fat/body weight proportion, visceral fat, and insulin resistance index of a group of rats feeding on HFD during 22 weeks increased compared to another group feeding on a standard diet. Cattaneo et al. [1997] observed that although obesity developed in a group of rats at the end of the nutrition period, on the contrary, no development occurred in another group of female rats feeding on HFD for 7 months. In our study, we observed that the mean weight of testes and the mean weight of the adipose tissue around the testis were rather higher in the OC group than in the NC group. It was observed that the weight increase followed increased fat

Table 4. The values of catalase (CAT) and myeloperoxidase (MPO) enzyme activity in all groups.

Groups	CAT Activity (U/min/mg tissue)	MPO Activity (U/min/mg tissue)
NC	0.6 ± 0.26	8.34 ± 2.41
OC	0.9 ± 0.09*	190.74 ± 7.35***
NT	0.2 ± 0.07^^	12.31 ± 4.93
OT	0.3 ± 0.11##	111.67 ± 1.19##

NC: non-obese control; OC: obese control; NT: non-obese thymoquinone; OT: obese thymoquinone. Data was expressed as mean ± SD. * and *** show significant differences between the results of the NC and OC groups at 0.05 and 0.001 levels, respectively. ## show significant differences between the results of the OC and OT groups at 0.01 level. ^^ shows significant difference between the results of the NC and NT groups at 0.01 level.

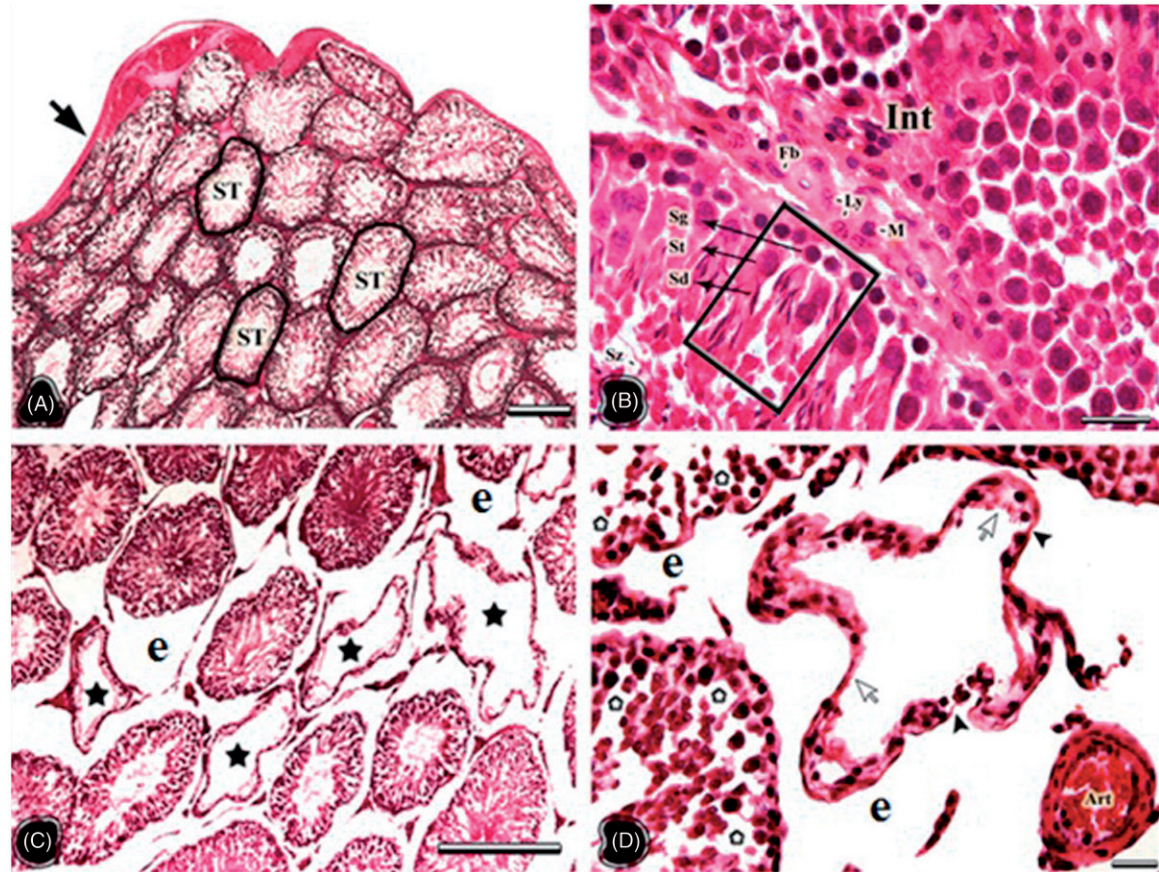


Figure 3. The light microscopical sections of testis belonging to non-obese control (NC) and obese control (OC) groups are shown. (A and B) The light microscopical sections of testis belonging to the NC group. ST: seminiferous tubules, Black arrow: Tunica albuginea, Int: interstitial area, Fb: fibroblast, Ly: Leydig cell, M: macrophage, Framed area: spermatogenic cell series, Sg: spermatogonia, St: spermatocytes, Sd: spermatid, Sz: spermatozoon. (C and D) The light microscopical sections of testis belonging to the OC group. The stars show atrophic seminiferous tubules, asterisks show degenerated spermatogenic cell series, Arrowheads: the thin seminiferous tubule wall, e: enlarged peritubular tissue, Arrows: no spermatogenic cells within tubule epithelium, Art: arteriole. Histological structure of testis is seen in the NC and OC groups. In the OC group degenerations in the seminiferous tubules, enlarged peritubular tissue, and tubular atrophy are important findings. Dye: HE; Scale Bars: (A–C) 250 µm; (B–D) 25 µm.

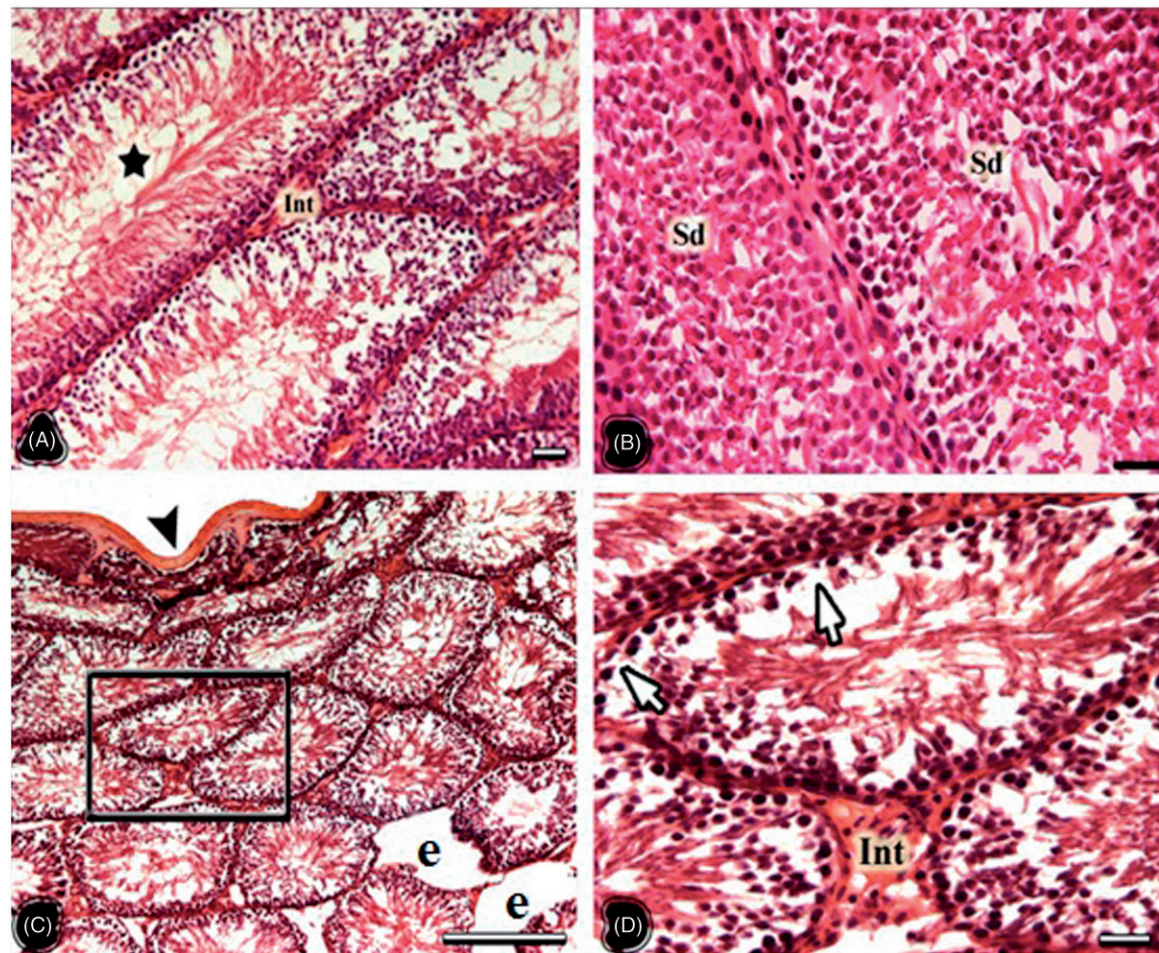


Figure 4. The light microscopical sections of testis belonging to the non-obese thymoquinone (NT) and obese thymoquinone (OT) groups are shown. (A and B) The light microscopical sections of testis belonging to the NT group. Star: intensive sperm in the tubule lumen, Sd: intensive spermatid in the tubule lumen, Int: interstitial area. (C and D) The light microscopical sections of testis belonging to the OT group. Arrowhead: tunica albuginea, e: enlarged peritubular tissue, arrows: germ cell loss. Framed area of C was indicated in D at higher magnification. Histological structure of testis is seen in the NT and OT groups. In the NT group more spermatogenic cells were seen on the seminiferous tubule walls compared to those of the NC group. Degenerated cells were decreased and no atrophic tubules were seen in OT group. Dye: HE; Scale Bars: (B–D) 25 μ m; (A–C) 150 μ m.

Table 5. Comparison of examined incidence of apoptotic cells with TUNEL staining.

Groups	Incidence of apoptotic cells (%)
NC	8
NT	6
OC	35**
OT	12##

NC: non-obese control; NT: non-obese thymoquinone; OC: obese control; OT: obese thymoquinone. ** shows significant difference among the OC and the other groups at 0.01 level. ## shows significant difference between the results of the OC and OT groups at 0.01 level.

around the testis in the OC group feeding on a HFD. The mean testis wet weight in the OC group was reduced more than the other experimental groups. We considered that the testes wet weight could have decreased due to obesity caused by HFD. The decrease in weight in the OT group as compared to that of the OC group showed that the TQ application may affect weight loss in obese individuals.

In the study of Jensen et al. [2004], 1,558 cases were examined and in the individuals with BMI >25 kg/m², the number and concentration of sperm was determined to be 22% lower than healthy controls. Fernandez et al. [2011] showed increased estradiol level, reduced sperm activity, and lower sperm quality in rats feeding on HFD. They observed that HFD caused reproductive disorders and reduced the fertility potential of rats. In our work, germ cell number was reduced significantly in the OC group as compared to the NC group. The increase of the spermatid number in the OT group was supported by histological findings. TQ application increased the sperm number, and caused an increase in the seminiferous tubule volume, but this increase was little, therefore there was no important difference in terms of the testis volume.

Amirkhizi et al. [2007] suggested that a long continuing period of obesity supports the increase of oxidative stress. In our study, there was a significant increase in the CAT activity of the OC group and after thymoquinone this level was decreased in both the NT and OT groups in comparison to the NC and OC groups. Ari et al. [2008] observed that the MPO activity was significantly increased in the antioxidant condition criterion compared to the control group of rats fed a HFD. Accordingly, in our study, the MPO activity increased

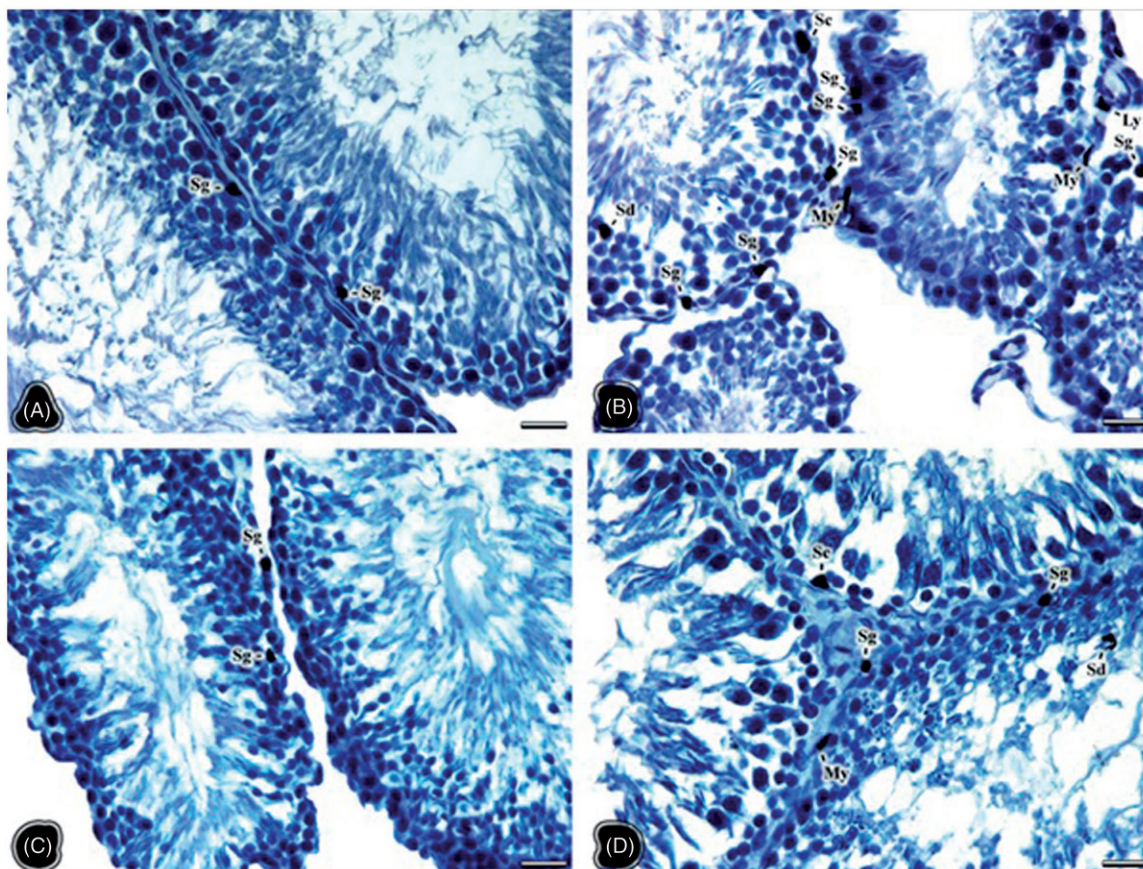


Figure 5. The light microscopical sections of testis belonging to the non-obese control (A), obese control (B), non-obese thymoquinone (C), and obese thymoquinone (D) groups are shown. Sg: spermatogonia, Sc: sertoli cell, Sd: spermatid, St: spermatocyte, Ly: Leydig cell, My: Myoid cell. Histological structure of testis is seen in all groups. Apoptotic activity is shown by TUNEL method in the nuclei of the positive stained cells (signed). The most apoptotic cells are seen in the OC group. But following TQ application, less apoptotic cells are seen in the light microscopical sections of the OT group. Dye: TUNEL stain; Scale Bars: 25 µm.

in the OC group compared to the NC group. These results are suggestive of an obesity caused inflammatory response in the testes, therefore the MPO activity increased significantly, and the oxidative damage was intensely based on obesity in this region. But, TQ application caused a significant decrease in the MPO activity in the OT group as compared to that of the OC group. Relating to our work, Badary et al. [2000] determined that the TQ caused an important elevation of the antioxidant enzymes like SOD, CAT, and GPx.

Upon histopathologic evaluation, the histological images of the cells belonging to the seminiferous tubule, interstitial tissue, and spermatogenic series were normal in testes of the rats in the NC group. The spermatid and spermatozoon intensity was notable in the tubule lumen of the testes in the NT group. However, great damage was observed in the OC group. The degenerated tubules were seen which had no spermatogenic cells and thinner wall including only one layer of myoid cells. The tubules were in places which had thicker peritubular connective tissue than normal. However, these structures were not determined in the OT group, except for the loss of germ cells and separated tissues in some regions. This is similar to the work of Wang et al. [2007] who showed spermatogenic cell decay and decreased mature sperm in an obese group of rats feeding on a HFD for 6 weeks. As a result, feeding on a HFD for a long time caused obesity on rats, and negatively affected the testis functions.

Immunohistochemical analysis evaluated the LH receptor using antibody localization and degree of staining in all groups. Immunoreactivity was reduced due to the heavy damage in the OC group. Biochemical studies indicated that the level of LH decreases in obese individuals [Alonso et al. 2012]. It appears that TQ caused an increase in the LH positivity and this may increase testosterone production. Hajshafiha et al. [2013] have observed associations between BMI values and sexual hormone levels when comparing fertile and subfertile/infertile men.

There is only one study suggesting that obesity can increase apoptosis of spermatogenic cells in the rat testis [Chen et al. 2011]. In the current study, testis TUNEL staining indicated apoptotic activity was generally localized in the spermatogonia of the NC and NT groups. In the OT group, the spermatogonia and Sertoli cells in the damaged regions showed apoptotic activity, but the germ cell apoptosis was reduced significantly compared to the OC group. After the TQ treatment testicular damage was significantly reduced, and the apoptotic germ cell activity was also reduced.

The relationship between BMI and male infertility relating to the sperm number, activity, and morphology is being actively studied [Cabler et al. 2010; Mitchell et al. 2011; Mydlo 2004]. Hammoud et al. [2008] suggested that the sperm number and motility were reduced, and sperm

morphology was negatively affected, with the number of morphologically abnormal sperm increasing with obesity. In the study presented above the reduction in the number of sperm following coil-shaped, head, neck, and tail anomaly sperms were the most pronounced in the OC group. In comparison, these structures were reduced significantly in the OT group suggesting that sperm anomalies in obese rats were reduced with the application of thymoquinone. This supports the view that thymoquinone was effective over oxidative stress caused by obesity, increasing the sperm number. Perhaps the increase in the consumption of plants which contain antioxidants may be effective to avoid many diseases, and in addition, prevent and treat obesity and infertility problems.

Materials and Methods

Animals, diets, and dosage

This study was approved by the Experimental Research Ethical Committee of Ondokuz Mayıs University. In this experiment, 24 adult male *Wistar albino* rats aged 8–10 w with 200 g body weight were used. Rats were provided from the Experimental Animal Research and Application Center of Ondokuz Mayıs University (Samsun, Turkey). During the experiment, all rats were kept in 12:12-h day/night cycle at $22 \pm 2^\circ\text{C}$ with a humidity of 50 ± 5 percent. Rats were allowed to eat food and drink water *ad libitum*. The rats were randomly separated into 4 groups ($n = 6$). While the OC and OT groups, which would generate an obesity model, were fed on a special diet containing 40% calories from/of fat, the NC and NT groups were fed a standard commercial diet for 9 w. At the end of 9 weeks (63 d), obesity conditions were evaluated by calculating BMI.

Intraperitoneal injections were carried out simultaneously to the OT and NT groups. For the injections, TQ (Catalogue No: 274666, Sigma Aldrich, Interlab A.S. Istanbul, Turkey) was dissolved in phosphate buffered saline at a dose of 10 mg/kg/d. Injections were carried out daily during 42 d (6 w) by starting on the 64th day [Alenzi et al. 2010; Gokce et al. 2010].

After this treatment, all animals were anesthetized by intramuscular ketamine (Ketalar[®]-100 mg/kg; Pfizer, Istanbul, Turkey) and xylazine injection (Alfazyne[®]-5 mg/kg; Alfasan, Turkey). Then, the removed testes were prepared for histological, stereological, and biochemical studies, and morphological sperm analyses.

Histological studies

Sperm analysis

To evaluate the sperm morphology, the left testis was put under anaesthesia and $\frac{1}{4}$ section of it was dissected and semen samples were obtained. Then the semen samples were spread on the slides and stained with hematoxylin-eosin (HE). Afterwards, photographs were taken from the slides under the light microscope (Leica LMD 7000, Leica Inc; Wetzlar, Germany), and morphologically evaluated by the Kruger-Strict criteria. On each semen sample, 200 sperms were evaluated in terms of head, neck, and tail anomalies, and normal sperm parameters [Kruger et al. 1986].

Light microscopy

The right testis tissues were kept in 10% formalin for fixation. Remainders of the left testes ($\frac{3}{4}$) were kept in the freezer at -80°C for biochemistry studies. Following the fixation, the testis samples were exposed to routine histological procedures and embedded in paraffin and serially cut by a rotary microtome (Leica RM2255; Nussloch, Germany) [Kumtepe et al. 2010]. The light microscopic sections at the thicknesses of 20 and 7 μm were used for stereological analysis and histopathological examination, respectively. All slides were stained with HE and examined under the light microscope (Leica LMD 7000, Leica Inc; Wetzlar, Germany).

TUNEL assay

To detect DNA breaks, *in situ* cell death detection kits for the TUNEL method were purchased from Roche Applied Science (Penzberg, Germany). The sections were mounted on positive charged slides, deparaffinized, and treated with proteinase K solution (20 $\mu\text{g}/\text{mL}$ in PBS) for 15 min at room temperature. Subsequently, the sections were washed in distilled water and immersed in 3% hydrogen peroxide for 15 min. After several washings in PBS (50 mM sodium phosphate and 200 mM NaCl at pH 7.4), the sections were immersed in equilibration buffer at room temperature for 20 min. The sections were then incubated in terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C for 1 h in a humidified chamber, and the reaction was stopped by immersion in a stop/wash buffer. After several washings, the sections were incubated in antidigoxigenin-peroxidase for 30 min at room temperature. The reaction was revealed with 0.06% 3,3-diaminobenzidine tetra hydrochloride (Sigma Chemical, Interlab A.S.; Istanbul, Turkey) in PBS for 3–6 min and the sections were counterstained with Mayer's haematoxylin [Altunkaynak et al. 2009; Liu et al. 2002]. These sections were examined and photographed under a light microscope (Leica LMD 7000, Leica Inc; Wetzlar, Germany). Also, the number of apoptotic spermatogenic cells were calculated in five independent areas at high magnification ($\times 400$) followed by calculation, it is defined as the ratio of per 100 cells. Apoptotic index is calculated using the following formula [Selzner and Clavien 2000]:

$$\frac{(\text{number of the apoptotic cell})}{(\text{total cell number})} \times 100$$

Immunohistochemical staining of (LH/CG) receptor antibody

Immunohistochemical staining was applied by the streptavidin-peroxidase method. The LH/choriogonadotrophic hormone (CG) receptor antibody was used for the immunohistochemical study of the paraffin-embedded testis samples. Summarily, sections were incubated with 3% H_2O_2 for 10 min and treated and blocked in 5% BSA for 10 min at room temperature. Sections were then probed with the LH/CG receptor antibody (Catalogue Number: AB9549, Millipore; Istanbul, Turkey) at a concentration of 5 $\mu\text{g}/\text{mL}$ for 1 h at room temperature. Then, sections were washed three times with PBS and incubated with biotinylated anti-rabbit IgG for a

period of 30 min at 37°C. Immunological activity was detected with a streptavidin-biotin complex kit (ScyTek Laboratories, SitoGen Biomedikal Ltd; İstanbul, Turkey) and developed with diaminobenzidine tetrahydrochloride. The sections were counterstained with Mayer hematoxylin and images are obtained under a light microscope (Leica LMD 7000, Leica Inc) [Bekar et al. 2014].

Stereological studies

The number of Leydig cells, spermatogonia, spermatocytes, and spermatids were calculated by an optic dissector and unbiased counting frame. The mean volumes of seminiferous tubules and testis were calculated by the Cavalieri method.

Optical fractionator method

The stereological analyses were made by a special computerized analysis system (Sterioinvestigator® 9.0, Microbright-field; Gantenbein LTD; Ankara, Turkey). Before beginning to work, the necessary strategy was determined by optical fractionator on the parts of the testis tissue [Altunkaynak et al. 2012]. According to that, the section thickness and section sampling fraction were determined as 20 µm and 1/250, respectively. After this sampling procedure, 16 sections were obtained from each testis. The frame area had 40 µm long edges and the area was 1600 µm² (40 × 40), so as to count 6 interesting cells in each unbiased counting frame. Counting was made under 40× objective. The number of spermatogonia, spermatocytes, spermatids, and Leydig cells were counted on each testis sample by using 4 different markers (Supplementary Figure 1).

The total number of spermatogonia, spermatocytes, spermatids, and Leydig cells in the testis was estimated by the following formula:

$$N = \Sigma Q \frac{1}{ASF} \frac{1}{TSF} \frac{1}{SSF}$$

Where, N is the total number of the interesting cell (spermatogonia, spermatocytes, spermatids, or Leydig cells), ΣQ is the counted number of the interesting cell, ASF is the area sampling fraction, and TSF is the thickness sampling fraction. The efficiency of the sampling procedure and convenience of sampled cells for the total number analysis of the interesting cell was checked by the estimation of the coefficient of error (CE) and coefficient of variation (CV), as previously described [Altunkaynak et al. 2011]. The CE of the sampling schedule of the testis was validated by a pilot study (CE must be ≤10%). It is also possible to estimate the CV within the testis in each group. These are valuable data to see whether the number of subjects in each group is adequate.

Cavalieri method

The volume of testes and seminiferous tubules was counted by using the Cavalieri principle [Altunkaynak and Altunkaynak 2007]. The area and volume were calculated on 7 µm cross sections with 1/250 sampling rate as to the applied systematic random sampling procedure. To estimate the testis volume, first, borders of the testicular area were determined

under 5x objective, and the point interval was found as 150 µm by using a counting frame scale. Then, the counting frame scale was randomly put on the determined testis area and the hitting points over the testis were counted. Finally, the volume of each section was calculated by multiplying the area was calculated by multiplying the total number of these points and section thickness.

To calculate the mean volume of the seminiferous tubule, 10 tubules with drawn borders were randomly selected in each cross section, and the point interval was determined as 50 µm using a counting frame scale. This point counting scale was randomly put on the images of cross sections, and the points which hit over the selected tubules were counted. The mean volume of the seminiferous tubules was calculated by multiplying the total points over the selected tubules and section thicknesses. The value obtained from the calculation was divided to the number of selected tubules (10) to detect the mean volume of the seminiferous tubules (Supplementary Figure 1):

$$\text{Volume}(V) = t \times a(p) \times (\Sigma P)$$

Where V is the volume of interest (seminiferous tubules, testis) in one section plane, t is the section thickness, $a(p)$ is the interpoint area, and Σ is the number of points hitting the object of interest in that section. After this formula was applied to other sections, the total volume to be estimated was obtained from:

$$\text{Total volume} = V_1 + V_2 + \dots + V_n.$$

The volume fractions for each region were estimated by dividing the total point number that was superimposed on the related region by the sum of the points superimposed on the whole testis:

$$\text{Volume fraction}_{(\text{region/testis})} = \frac{P_{(\text{region})}}{P_{(\text{testis})}}$$

where P is the number of points hitting the region of interest or whole testis in that section.

Biochemistry

MPO activity

MPO activity was evaluated according to the modified method of Bradley et al. [1982]. The homogenized samples were frozen and thawed 3 times, and then centrifuged at 1500 rpm for 10 min at 4°C. MPO activity was determined by adding 100 µL of the supernatant to 1.9 mL of 10 mmol/L phosphate buffers (pH 6.0) and 1 mL of 1.5 mmol/L o-dianisidine hydrochloride containing 0.0005% (wt/vol) hydrogen peroxide. The changes in the absorbance of each sample were recorded at 410 nm on a UV-vis spectrophotometer (Shimadzu UV-MINI 1240; Shimadzu; İstanbul, Turkey). MPO activity in all tissues was expressed as micromoles per min per milligram of tissue.

Catalase activity

Decomposition of H₂O₂ in the presence of CAT was performed at 240 nm. CAT activity was defined as the

amount of enzyme required to decompose 1 nm of H₂O₂ min at 25°C and pH 7.8. Results were expressed as millimole per min per milligram of tissue (mmol/min/mg tissue) [Aebi 1984].

Statistical analyses

Statistical comparisons were made using SPSS 15.0 for Windows®. Significance among the incidence of TUNEL positive cells was determined by the aid of chi-square test and Fisher exact test. Stereological data were compared using a One-Way Anova in conjunction with the Bonferroni t test for all pair wise multiple comparisons. Differences were considered significant when $p < 0.05$. Data were presented as the Mean \pm SEM.

Declaration of interest

The authors report no conflicts of interest.

Author contributions

Conceived and designed the experiments: BZA, NHT; performed the experiments: NHT, MEA; Analyzed the data: BZA, MEA; Contributed reagents/materials/analysis tools: SK; Wrote the manuscript: BZA, NHT, SK. All authors approved revisions and the final paper.

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Supplementary material available online

Supplementary Figure.