The effect of chronic stress and obesity on sperm quality and testis histology in male rats; a morphometric and immunohistochemical study

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Summary. Objective. Stress and obesity lead structural and functional abnormalities in many organs. This research investigated the effect of these two risk factors on sperm parameters and histologic structure of testis in rat model.

Materials and methods. Twenty-four male rats were divided into four groups with six rats in each group as control, stress, obesity, and stress-obesity groups. The rats of obesity and stress-obesity groups were fed high-fat diet for 12-week and obesity was created. Control and stress groups were given standard rat chow for the same time. Through last 4-week, stress and stress-obesity groups were applied to chronic mild stress procedure. At the end of the experiment, epididymal sperm was collected from vas deferens and testes were harvested from sacrificed animals. Sperm samples were evaluated in terms of concentration and motility by using Makler Chamber. Sperm smears were stained with Eosin-Y stain for morphological evaluation, and also histochemically for GABA transporter-1 (GAT1) expression assessment. Testis sections were dyed with Hematoxylin-Eosin and Johnsen scores were assessed. GAT1 expression was detected in testis sections by immunohistochemistry, and TUNEL method was used for determining apoptosis in testis.

Results. In comparison with the control samples in stress, obesity, and stress-obesity groups sperm concentration and motility decreased, and also the number of sperm with abnormal morphology increased. Stress, obesity, and stress-obesity groups showed a significantly decreased in sperm concentration and motility in comparison with the control group, and also in these groups had significantly increased number of abnormal sperm compared to control. Additionally, the testicular structure was deteriorated, and Johnsen scores decreased. And also GAT1 expression and apoptosis were prominent. These negative results, especially, testicular weight, sperm concentration, and Johnsen score were more observed in the stress-obesity group.

Conclusions. Stress and obesity may induce male infertility by disrupting both sperm quality and testis histology. When stress and obesity are coexisting, these adverse effects are more severe. And also, increased GAT1 expression may be associated with these effects.

Key words: Chronic stress, Obesity, Sperm quality, Testis histopathology, GAT1 immunohistochemistry

Introduction

Stress is a condition that threatens and strains the physical and mental limits of a person (Baltaş and Baltaş, 1993; Stults-Kolehmainen and Sinha, 2014). Intense stress continuing for a long time can lead to structural and functional abnormalities both in the brain and in other organs (McEwen, 2000). For example, gastric ulcer may be triggered by stress (Hu and Lu,

2015), and stress may influence the onset and progression of coronary heart disease (Tennant, 2000; Hu and Lu, 2015). Stress has harmful effects on various semen parameters associated with semen quality such as sperm concentration, sperm motility and morphology (Clarke et al., 1999; Janevic et al., 2014; Nordkap et al., 2016). In a study conducted by Eskiocak, it was mentioned that acute and chronic stress have hazardous effects on the male reproductive system and semen quality (Eskiocak et al., 2006). In a study carried out by Charpenet, immobilization stress was indicated to reduce the plasma testosterone levels in rats (Charpenet et al., 1982).

Nowadays, obesity is one of the most important health problems (de Oliveira et al., 2014). In one study, it was shown that sperm concentration and the forward-moving sperm count were reduced in proportion with obesity (Hammoud et al., 2008b). Androgen and sex hormone binding globulin (SHBG) levels are low in obese males. Inhibin B levels decrease in obesity, and there is also a negative correlation between the levels of low inhibin B and grade of obesity (Hammoud et al., 2008a). These hormonal changes in obese men can explain the changes in the semen parameters and the increased risk of infertility (Jensen et al., 2004; Hammoud et al., 2008a; Palmer et al., 2012).

Gamma aminobutyric acid (GABA) is the most important presynaptic inhibitory neurotransmitter in the brain and retina. It is synthesized from glutamate in a reaction catalyzed by glutamic acid decarboxylase (GAD) in the GABAergic neurons (Schousboe et al., 2013). GABA accumulates in the vesicles located at the presynaptic terminals. GABA released from the presynaptic neuronal membrane induces a transient hyperpolarization of the postsynaptic membrane, named the inhibitory postsynaptic potential (IPSP). Then, GABA is removed from the synaptic cleft via GABA transporter (GAT) proteins, known as GAT-1, GAT-2, GAT-3, and GAT-4 (BGT-1) found in neurons and glial cells. So, the action of GABA at the synapse is terminated. GABA uptake into glial cells, particularly into astrocytes, is performed by a specific high-affinity GAT. GABA found at the presynaptic end and glial cells is metabolized by GABA transaminase (GABA-T) catalyzing conversion of GABA via succinic semialdehyde to succinate (Bernstein and Quick, 1999; Barakat and Bordey, 2002; Scimemi, 2014).

The GABAergic system plays a role in both stress and obesity. After stress, the balance between glutamate and GABA is disrupted, and GABA level increases. This situation causes neuronal apoptosis in the hippocampus (Gao et al., 2014). Recently studies have shown that forced swimming (Tian et al., 2013) and chronic unpredictable stress (Partridge et al., 2016) promote GABAergic transmission, and restraint stress (Ciccocioppo et al., 2014) and elevate baseline GABAergic responses. In a study, plasma GABA levels of mice fed high-fat diet were found to be significantly higher than those of control mice (Xie et al., 2015).

In recent studies, it was shown that GABA can increase human sperm motility and activation (Hu et al., 2004b) and can induce the acrosome reaction (AR) in human (Shi et al., 1997) and bull sperm (Puente et al., 2011). The GABAergic system can also be found in the peripheral tissues such as β-cells of pancreas, testis and oviduct epithelium except for the central nervous system (CNS) and GAD enzyme is also expressed in these tissues (Hu et al., 2000; Watanabe et al., 2002). It was detected that GAD and glutamic acid are found in the testis (Ma et al., 2000a; Liu et al., 2009; Du et al., 2013a) and GABA has a direct effect on steroidogenesis, sperm viability and motility (Frungieri et al., 1996). The sperm plasma membrane has GABA-specific binding areas (Erdo and Wekerle, 1990). GABA stimulates AR via GABAA and GABAB receptors. GABA reuptake is an important mechanism in the regulation of GABA activity. The inhibition of GABA reuptake increases GABA activity (Soudijn and van Wijngaarden, 2000). This reuptake is regulated by GABA transporter I (GAT1). The excess expression of GAT1 can negatively affect reproduction and cause infertility. GAT1 is found in testis and sperm, but physiologically its function is not known (Hu et al., 2004b).

The studies conducted about the deleterious effects of obesity and stress on testis and sperm are relatively new, and there are many points that need to be investigated. In the literature review, no articles were found about whether chronic stress is a risk factor in terms of male infertility in obese males. In this study, we aimed to investigate the effects of chronic stress and obesity, both separately and coexisting, on the testes and sperms, and to evaluate the presence of GAT1 in just mentioned tissues in the experimental rat models by using morphometric and microscopic analyses.

Materials and methods

Animals and experimental design

Twenty-four 4-5 week old male Sprague Dawley rats weighing about 70-120 g were used in the present study. The animals were obtained from the Medical Experimental Application and Research Center of Atatürk University (ATADEM, Erzurum, Turkey). All experimental procedures used in this study had been approved by the Board of Ethics on Animal Experiments of the Atatürk University (B.30.2.ATA.0.23.85-28). Rats were housed in an air-conditioned room with constant 12-hour light–dark cycle. The animals were fed standard rat chow and had free access to drinking water ad libitum. At the beginning of the 12-week experimental period, rats were randomly divided into 4 groups with 6 animals in each group. Control group (CG) rats were fed standard rat chow throughout the experiment and did not have the Chronic Mild Stress (CMS) procedure applied. Stress group (SG) rats were fed standard rat chow throughout the experiment and had the CMS procedure

applied during the last four weeks of the experiment. Obesity group (OG) rats were fed high-fat diet (HFD) throughout the experiment and did not have the CMS procedure applied. Stress-obesity group (SOG) rats were fed HFD throughout the experiment and had the CMS procedure applied during the last four weeks of the experiment (Table 1).

Preparation of diet used in obesity model

A diet containing 30% fat was prepared for the purpose of creating an obesity model (Altunkaynak et al., 2007). To eliminate possible changes that may occur due to the lack of carbohydrates in the animals at the end of the experiment, the carbohydrate content of prepared chow was kept at the same rate as standard rat chow. To prepare the HFD, melted bovine abdominal fat was added into the standard rat chow in powder form. The mixture was homogenized and it was shaped by hand, taking small pieces from the dough. This specially prepared chow was dried at room temperature and used for feeding of OG and SOG subjects. So, 30% of the total energy was provided from fat.

Throughout the experiment, the CG and SG rats were given standard rat chow and the OG and SOG subjects were fed specially prepared HFD. To calculate the body mass index (BMI=kg/m^2) of the rats, body weights and lengths of all animals were measured each week and were recorded regularly.

CMS procedure

The CMS procedure was applied to the SG and SOG rats during the last four weeks of the experiment. For the first eight weeks, the rats in the obesity and stress-obesity groups were fed HFD. By the end of the eighth week, the rats had gained weight and become obese, and from the ninth week, the CMS procedure was applied to both the SG and SOG rats concomitantly. During the first eight weeks, the SG rats were kept together with the others under the same conditions. The stressors used in the CMS procedure are described as follows; keeping in a wooden box, exposing to 240 Hz noise, putting in a separate cage, swimming in water at a temperature of 18°C and 31°C at different times, giving a tiny electric shock and attaching a latch 1 cm distant from the root of rat-tail (Willner, 1997; Vollmayr and Henn, 2003; Ozbek

et al., 2009; Gedikli, 2010). The stress procedure was performed during the last 4 weeks of the experiment, and each of the different stressors mentioned above was applied to the rats on different days.

It was shown by scientific research that CMS causes depression-like behavior in rats and mice (Henningsen et al., 2009; Wu et al., 2017). At the end of the experiment, the rats were given 1% sucrose solution and it was detected that the rats had anhedonia to sugar. The sucrose test is used to show whether the depression related to the CMS occurs in rats and results in reluctance related to sucrose (Willner, 1997; Moreau, 2002; Vollmayr and Henn, 2003).

Tissue and organ collection

At the end of week 12, the abdomens were opened by midline incision under ketamine hydrochloride (50 mg/kg, Ketalar®, Pfizer, Turkey) and Xylazine (10 mg/kg, Rompun®, Bayer, Canada) anesthesia. Both testes were removed immediately with epididymis and vasa deferens together. Testes were separated from the epididymis and their weights were recorded by measuring with sensitive scales. Then testes were fixed in Bouin's solution for histopathological evaluation. Sperm samples were obtained from the vas deferens for light microscopic evaluation.

Evaluation of sperm count and motility

Vas deferens was detached from the adipose tissue surrounding it and excised by cutting its upper and lower ends. Immediately, it was placed in pre-warmed Petri dish at 37°C. Vas deferens was slightly squeezed with the help of a fine collet from the bottom to the top (close part of vas deferens to the epididymis). Epididymal sperm from the vas deferens entered the Petri dish, and 10 μl epididymal sperm was taken and placed in another dish, then it was diluted 1:5 by adding 40 μl saline imidazole (SAIM) solution (pH=7.0) containing 0.15 M NaCl and 30 mM imidazole (Celik-Ozenci et al., 2003). The prepared sperm suspension was stirred, 10 μl was placed on a Makler chamber (Makler Counting Chamber, Sefi Medical Instrument, Haifa, Israel), and sperm counts and percentage of motile cells were calculated. The sperm was counted at X200 magnification in ten random squares of the coverslip of

Table 1. Experimental design.

Groups	First 8 weeks	Last 4 weeks
CG	Standard rat chow feeding; No application of the CMS procedure	Standard rat chow feeding; No application of the CMS procedure
SG	Standard rat chow feeding; No application of the CMS procedure	Standard rat chow feeding; Application of the CMS procedure
OG	HFD feeding; No application of the CMS procedure	HFD feeding; No application of the CMS procedure
SOG	HFD feeding; No application of the CMS procedure	HFD feeding; Application of the CMS procedure

CG: Control Group, SG: Stress Group, OG: Obesity Group, SOG: Stress-Obesity Group.

the Makler counting chamber. The obtained number was multiplied by 1 million. Thus, the number of sperm was calculated as million per milliliter. In addition, to determine sperm motility, 100 sperm were counted in ten random squares of the Makler chamber. Motile and immotile sperm counts were recorded separately, and their percentages were estimated.

Evaluation of sperm morphology

The epididymis was minced in a Petri dish by using a lancet and 4 ml SAIM solution was added. Then, this mixture was filtered through double gauze and the epididymal sperm solution was obtained. Epididymal sperm suspension was mixed 1:1 with 1% Eosin Y (Sigma Aldrich, Taufkirchen, Germany) and incubated at room temperature for 30 min (Sailer et al., 1997; Shetty, 2007). The rest was used for immunohistochemical staining of the sperm. A drop was taken from the sperm suspension stained with eosin Y and a smear slide was prepared. Two slides were prepared for each subject; fixed in methanol and dried at room temperature again. The slides were examined under Nikon Eclipse E600 light microscope (x400) and sperm morphology was evaluated. Five hundred spermatozoa were counted for each sample and they were classified as normal or abnormal. Abnormal sperm was assessed mainly in 2 categories; 1. Sperm with head abnormalities; headless sperm, amorphous head sperm, flattened head sperm, banana head sperm, ring shaped head sperm and pinhead sperm. 2. Sperm with tail abnormalities; tailless sperm, double tailed sperm, bent tail sperm, coiled tail sperm and bent neck sperm (Sailer et al., 1997; Narayana et al., 2002; Kuriyama et al., 2005; Shetty, 2007).

Immunocytochemical (ICC) staining of sperm

In order to identify the GAT1 protein, immuno-cytochemical staining was performed using an immunoperoxidase method on sperm smear slides (Ma et al., 2000a). Smear slides were prepared as follows; two circles were drawn on each slide. In each circle, 15 μl sperm solution was placed and phosphate buffered saline (PBS) was dropped on it. Slides were incubated in the humidity chamber at +40°C overnight. Next morning, slides had 3.7% formalin dropped on, were washed with PBS and air-dried at room temperature. After being treated with 3% BSA (Amresco Inc., Solon, Ohio, USA), GAT1 antibody (Gene Tex Inc., Irvine, CA, USA) (1:400) was dropped in the upper circle but only PBS was added to the lower one. After the slides were incubated with primary antibody at 4°C overnight, they were incubated with the secondary antibody (Ultra Vision Plus Detection System, Thermo Scientific Inc.) for 60 min. Slides were incubated in a Streptavidin-HRP (Ultra Vision Plus Detection System, Thermo Scientific Inc.) for 20 minutes and subsequently treated with diaminobenzidine (DAB) (Thermo Scientific Inc.). After they were mounted, they were investigated under a light microscope (x400) (Nikon Eclipse E600, Tokyo, Japan).

Testis histopathology

Fixed testes were embedded in paraffin blocks; sections approximately 4-5 μm thick were taken and stained with hematoxylin and eosin (H-E). Preparations were examined with a light microscope (x400). To evaluate testicular injury and spermatogenesis, the H-E stained histologic sections were graded using the Johnsen score (JS) (Johnsen, 1970). One hundred tubules were assessed for each group, and every tubule was given a score from 1 to 10 (Table 2).

Immunohistochemical (IHC) staining of testis

To examine the expression of GAT1 in the testis at light microscopic level, immunohistochemical staining was performed (Ma et al., 2000a). Approximately 4-5 μm thick testis sections were dewaxed, dehydrated and exposed to heat-induced antigen retrieval in 10% EDTA (pH=8) (Bio Optica, Milan, Italy). Internal peroxidase activity was blocked with 3% hydrogen peroxide incubation at room temperature for 15 min. The sections were incubated with anti-GAT1 antibody (Gene Tex Inc.,

Table 2. Johnsen score system.

Score	Histologic criteria
10	Complete spermatogenesis, many spermatozoa present, germinal epithelium multi-layered and well organized, open tubular lumen
9	Many spermatozoa present, multi-layered but disorganized germinal epithelium, obliterated tubular lumen
8	Germinal epithelium multi-layered but only a few spermatozoa (less than 10) present
7	No spermatozoa but many spermatids, spermatocytes and spermatogonia present
6	No spermatozoa present, less than 10 spermatids but many spermatocytes and spermatogonia present
5	No spermatozoa or spermatids but many spermatocytes and spermatogonia present
4	No spermatozoa or spermatids, less than 5 spermatocytes, spermatogonia present
3	Only spermatogonia present
2	No germ cells, only Sertoli cells present (Sertoli cell-only syndrom)
1	No cells (neither germ cells nor Sertoli cells) present in tubular section

Irvine, CA, USA) at a dilution of 1:400. After incubation, tissue sections had a secondary antibody (Ultra Vision Plus Detection System, Thermo Scientific Inc.) and Streptavidin-HRP (Ultra Vision Plus Detection System, Thermo Scientific Inc.) applied according to the manufacturer's instructions, respectively. The sections were subsequently incubated in DAB solution (Ultra Vision Plus Detection System, Thermo Scientific Inc.). Afterwards, the sections were counterstained with hematoxylin, mounted with entellan and examined with a Nikon Eclipse E600 microscope (Nikon Eclipse E600, Tokyo, Japan).

TUNEL staining of testis

The Terminal Deoxynucleotide Transferase Mediated dUTP Nick End Labeling (TUNEL) method was implemented to detect apoptotic cell death in the testis (Hsiao et al., 2015). After overnight incubation at $37°C$, 4-5 μm thick sections derived from paraffin blocks were deparaffinized with heat. Subsequently, the sections were passed through xylene and alcohol series. To detect the distribution of apoptotic cells, the testis sections were stained with the TUNEL method using the ApopTag® Plus Peroksidase In Situ Apoptosis Detection kit (Chemicon® International Inc., Temecula, CA, USA) according to the procedure of the kit and counterstained with Gill hematoxylin.

Statistical analysis

Data was analyzed by using SPSS 17.0 for Windows. To perform multiple comparisons between all experimental groups, the Kruskal Wallis test was performed. Statistical significance between the various groups was determined by using Mann-Whitney U test and Post Hoc (Tamhane) test. All values are expressed as mean ± standard deviation. A p-value of less than 0.05 was considered statistically significant.

Results

Body mass index (BMI)

In order to assess the presence of obesity in the OG and SOG rats, the BMI of each rat in all groups was calculated and compared with each other statistically. The mean BMI values of subjects in the four experimental groups are shown as 0-week (beginning of the experiment), 8-week and 12-week in Table 3.

At the beginning of the test, there was no statistical difference between the BMI of the CG and the other three groups (p>0.05; Post Hoc Test), but the BMI of the SG was higher than OG and SOG (p<0.05; Post Hoc Test). This difference was considered to be a coincidental difference. At 8 weeks into the test, the BMI's of the OG and SOG rats fed with high fat diet were statistically higher than the BMI's of the CG and SG rats fed with standard rat chow (p<0.05; Post Hoc Test). Thus, it was detected that the obesity model was established in OG and SOG rats (Table 3). Before the stress procedure was applied (at the beginning of 9th week) to the SOG, there was no statistically significant difference between BMI of OG and SOG rats (p>0.05; Post Hoc Test). Four weeks later (at the end of the 12th week) at the end of the stress procedure, it was detected that the BMI of SOG was less than BMI of OG and a statistically significant difference appeared between the OG and SOG rats (p<0.01; Post Hoc Test). At the beginning of the stress procedure, there was a significant difference between the CG and SOG rats (p<0.01; Post Hoc Test), at the end of the procedure this difference was lost (p>0.05; Post Hoc Test). At the beginning of the stress procedure, the BMI's of the OG and SOG were significantly higher than those of CG and SG (p<0.01; Post Hoc Test), but there was no significant difference between the OG and SOG. So, it was ensured that the obesity model was established in these two groups. After stress application, the weight gain of the SOG paused, so the BMI of the SOG was lower than that of OG (p<0.01; Post Hoc Test).

Testis weights

When the groups were compared in terms of testis weight, a statistically significant difference was found between the groups (p<0.01; Kruskal-Wallis test) (Fig. 1). The testis weights of SOG decreased compared to those of CG, and this reduction was significant (p<0.01; Mann-Whitney U test). Testicular weights of the rats in the SOG were significantly lower than both OG and SG (p<0.05, Mann-Whitney U test).

Table 3. The mean BMIs of all groups at the beginning of the test, 8-week and 12-week.

	CG (Mean±SD)	SG (Mean±SD)	OG (Mean±SD)	SOG (Mean±SD)
0-week	5.72±0.13	5.94±0.25[a]	5.55±0.23	5.65±0.25
8-week	5.83±0.15	6.02±0.37	6.50±0.26[b,c]	6.36±0.11[b,c]
12-week	6.13±0.28	6.46±0.21	6.70±0.29	6.04±0.6[d]

CG: Control Group, SG: Stress Group, OG: Obesity Group, SOG: Stress-Obesity Group. SD: Standard deviation. [a] Significantly higher than OG and SOG (p<0,05; Post Hoc Test) (coincidental difference). [b] Significantly increased when compared with CG and SG (p<0,05; Post Hoc Test). [c] No significant difference between the OG and SOG (p>0,05; Post Hoc Test). [d] Significantly decreased when compared with OG (p<0,01; Post Hoc Test).

Sperm count and motility

There were statistically significant differences between the groups (p<0.01; Kruskal-Wallis test) in evaluation of epididymal sperm concentration (Table 4). When compared to the CG, it was observed the epididymal sperm concentration of the SG, OG and SOG were decreased and the difference was statistically significant (p<0.05, Mann-Whitney U test) (Table 4). Between the other three groups except CG, there was no significant difference (p>0.05; Mann-Whitney U Test), but sperm concentration of the SOG was lower than OG and this difference was close to the significance limit (p=0.055; Mann-Whitney U Test).

The mean sperm motility of the SG, OG and SOG were lower than the CG (p<0.01; Mann-Whitney U Test) (Table 4). Also, it was found the sperm motility of OG and SOG was less than the motility of the SG (p<0.01; Mann-Whitney U Test). So, it was determined that obesity was more harmful than stress to sperm motility.

Sperm morphology

For each subject, 500 sperm were counted and these sperm were assessed morphologically. The sperm with head defects (Fig. 2a-e) and tail defects (Fig. 2f-o) were evaluated as abnormal sperm (Table 5).

In the SG, OG and SOG, the sperm count with head defects was higher than the CG (p<0.01; Mann-Whitney U Test). When these three groups are compared with each other, there was no difference (p>0.05; Mann-Whitney U Test). Similarly, the sperm count with tail defect increased in the SG, OG and SOG (p<0.01;

Mann-Whitney U Test). There was no difference between these groups (p>0.05; Mann- Whitney U Test).

Immunocytochemical (ICC) staining of sperm

To determine GAT1 protein in the sperm, sperm smears were stained with the immunoperoxidase method using GAT1 antibody as primary antibody and examined under a light microscope (Fig. 3).
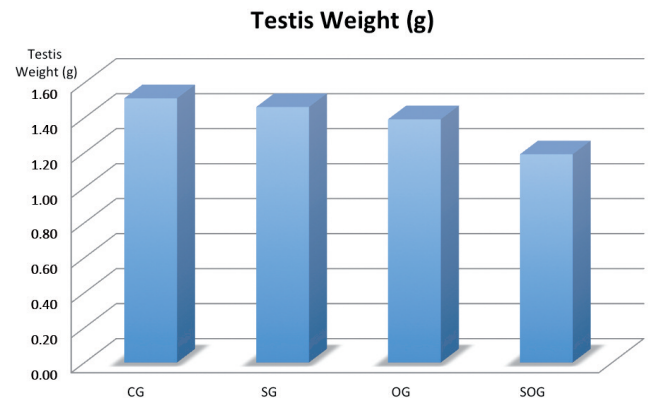
Fig. 1. The graph shows the mean testis weight of the four experimental groups at week 12. Testis weight of SOG (Mean±SD=1.19±0.09) significantly decreased when compared with CG (Mean±SD=1,51±0,07) (p<0.01; Mann-Whitney U test), and both OG (Mean±SD=1.39±0,11) and SG (Mean±SD=1.46±0.14) (p<0.05, Mann-Whitney U test). CG: Control Group, SG: Stress Group, OG: Obesity Group, SOG: Stress-Obesity Group. SD: Standard deviation.

Table 4. Epididymal sperm concentration and sperm motility of all groups.

	CG (Mean±SD)	SG (Mean±SD)	OG (Mean±SD)	SOG (Mean±SD)
Epididymal Sperm Concentration (million / ml)	155,83±5,15	111,50±28,89[a,b]	116,38±13,95[a,b]	90,66±24,31[a,b]
Sperm Motility (%)	90,25±1,21	78,50±1,95[c]	60,08±4,16[c,d]	61,50±5,00[c,d]

CG: Control Group, SG: Stress Group, OG: Obesity Group, SOG: Stress-Obesity Group. SD: Standard deviation. [a] Significantly decreased when compared with CG (p<0.05, Mann-Whitney U test). [b] No significant difference between the groups (p>0.05, Mann-Whitney U test). [c] Significantly decreased when compared with CG (p<0.01, Mann-Whitney U test). [d] Significantly decreased when compared with SG (p<0.01, Mann-Whitney U test).

Table 5. Sperm morphology of all groups.

Groups	Normal sperm count (Mean±SD)	Abnormal sperm count (Mean±SD)		
		Head defect	Tail defect	Total
CG	450.5±15.7 (90.1%)	14.0±5.2 (7.1%)	35.5±12.6 (2.8%)	49.5±15.7 (9.9%)
SG	400.5±12.7 (80.1%)	29.5±6.7[a,b] (14%)	70.0±15.6[a,c] (5.9%)	99.5±12.7[a,d] (19.9%)
OG	392.7±8.4 (78.5%)	25.8±3.9[a,b] (16.3%)	81.5±8.4[a,c] (5.2%)	107.3±8.4[a,d] (21.5%)
SOG	392.9±14.5 (78.6%)	29.8±7.2[a,b] (15.5%)	77.3±9.4[a,c] (5.9%)	107.1±14.5[a,d] (21.4%)

CG: Control Group, SG: Stress Group, OG: Obesity Group, SOG: Stress-Obesity Group. SD: Standard deviation. [a] Significantly increased when compared with CG (p<0.01, Mann-Whitney U test). [b,c and d]: No significant difference between the groups (p>0.05, Mann-Whitney U test).

For each subject, 500 sperm were counted and positive immune reaction was determined (Fig. 4). When compared with the CG, the number of immune positive sperm was increased statistically significantly in the other three groups (SG, OG, and SOG) (p<0.05; Mann-Whitney U Test).

Testis histopathology

In the hematoxylin-eosin stained histological sections of testis obtained from the four experimental groups, the spermatogenesis evaluation was done according to the Johnsen score system (Table 2). For each group, the mean Johnsen score was calculated and is shown in Table 6. When the groups are compared in terms of Johnsen score, there were statistically significant differences between them (p<0.01; Kruskal-Wallis Test). The Johnsen scores of the SG, OG and SOG were significantly lower than the CG (p<0.01; Mann-Whitney U Test). The Johnsen score of the SOG was also less than the OG (p<0.05; Mann-Whitney U Test). However, there was no difference between the SG and obesity groups (OG and SOG) (p>0.05; Mann-Whitney U Test).
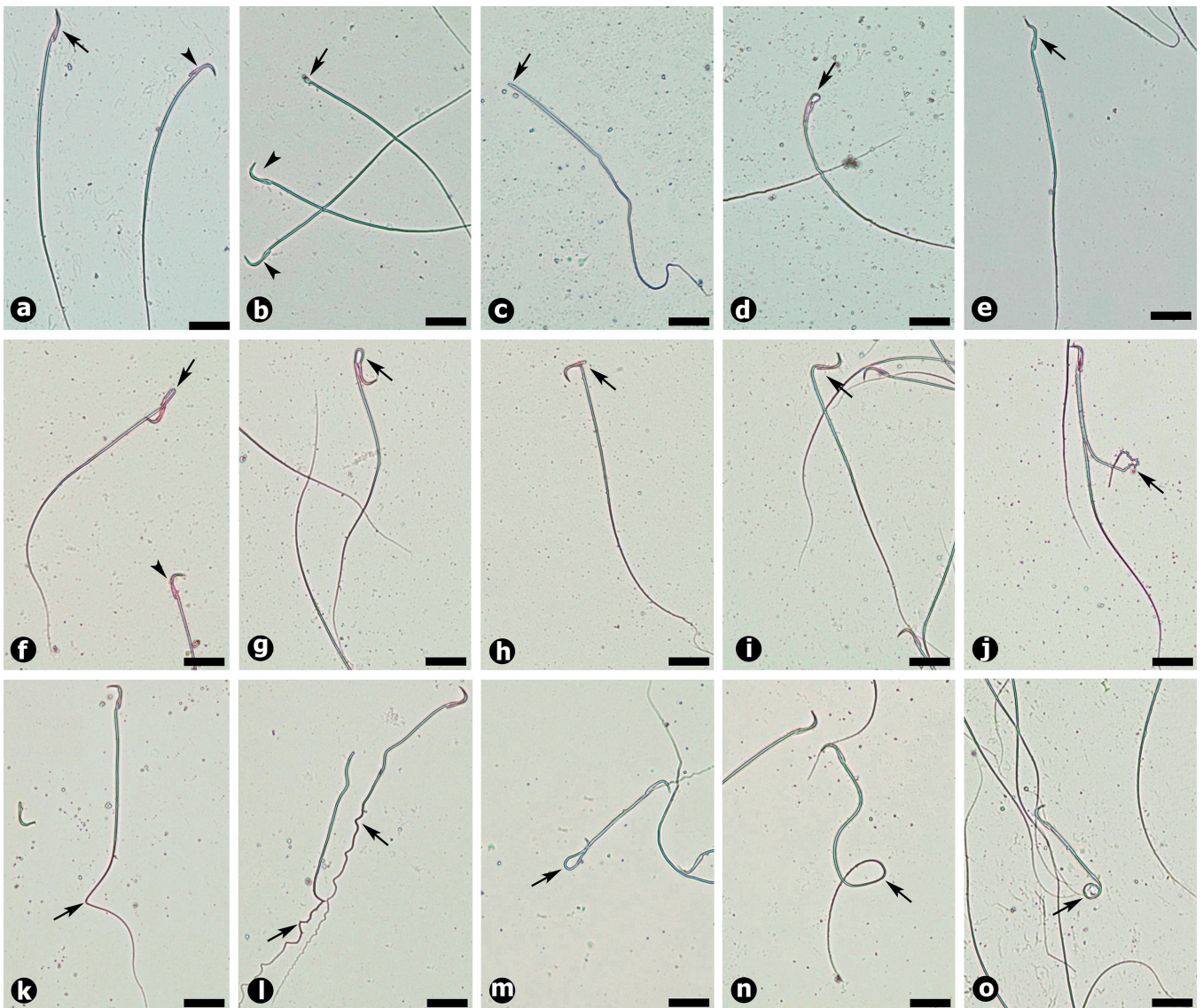
Fig. 2. Light microscopic image of the sperm with head defect **(a-e)** and tail defect **(f-o)** in the smear. **a.** Banana head sperm (arrow). **b.** Pinhead (arrow). **c.** Headless sperm (arrow). **d.** Ring shaped head sperm (arrow). **e.** Amorphous head sperm (arrow). **f-i.** Bent neck sperms (arrows). **j.** Double and coiled tail sperm (arrow). **k.** Bent tail sperm (arrow) and tailless sperm (left). **l.** Headless and bent tail sperm (left) and bent tail sperm (right). **m-o.** Coiled tail sperms (arrows). arrow head, sperm with normal head and tail. Stain: Eosin. Scale bars: 25 µm.

In the H-E stained testis sections of the CG, the morphology of the seminiferous tubules was quite regular and undamaged. Germinal epithelium exhibited a stratified view. Spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids were lined up from the basement membrane towards the lumen. Sertoli cells extended between these cells. There were Leydig cells with eosinophilic cytoplasm and chromatin-poor rounded nuclei, connective tissue fibers and cells and many capillary vessels in the interstitium (Fig. 5a,e).

The seminiferous tubules were damaged in the testis sections of the SG. There were intense hyalinization and vacuolization in the interstitium. The wall of the seminiferous tubules was thinner and their lumens were larger than the CG, and also their Johnsen scores were lower. Irregularities in the tubular epithelium and cytoplasmic degeneration characterized by intercellular edema and cellular swelling especially in the basal part of the epithelium were noticed. In addition, large vacuoles were observed between the spermatogonia and basement membrane in the testes of the SG (Fig. 5b,f).

Intense hyaline accumulation and vacuolization in the interstitium were observed in the H-E stained testis

Table 6. The spermatogenesis results according to Johnsen Score System.

Groups	Johnsen score (Mean±SD)
CG	9,63±0.10
SG	8,17±0.29[a]
OG	8,20±0.17[a,c]
SOG	7,89±0.20[a,b,c]

CG: Control Group, SG: Stress Group, OG: Obesity Group, SOG: Stress-Obesity Group. SD: Standard deviation. [a]: Significantly decreased when compared with CG ($p<0.01$, Mann-Whitney U test). [b]: Significantly decreased when compared with OG ($p<0.05$, Mann-Whitney U test). [c]: No significant difference when compared with SG ($p>0.05$, Mann-Whitney U test).
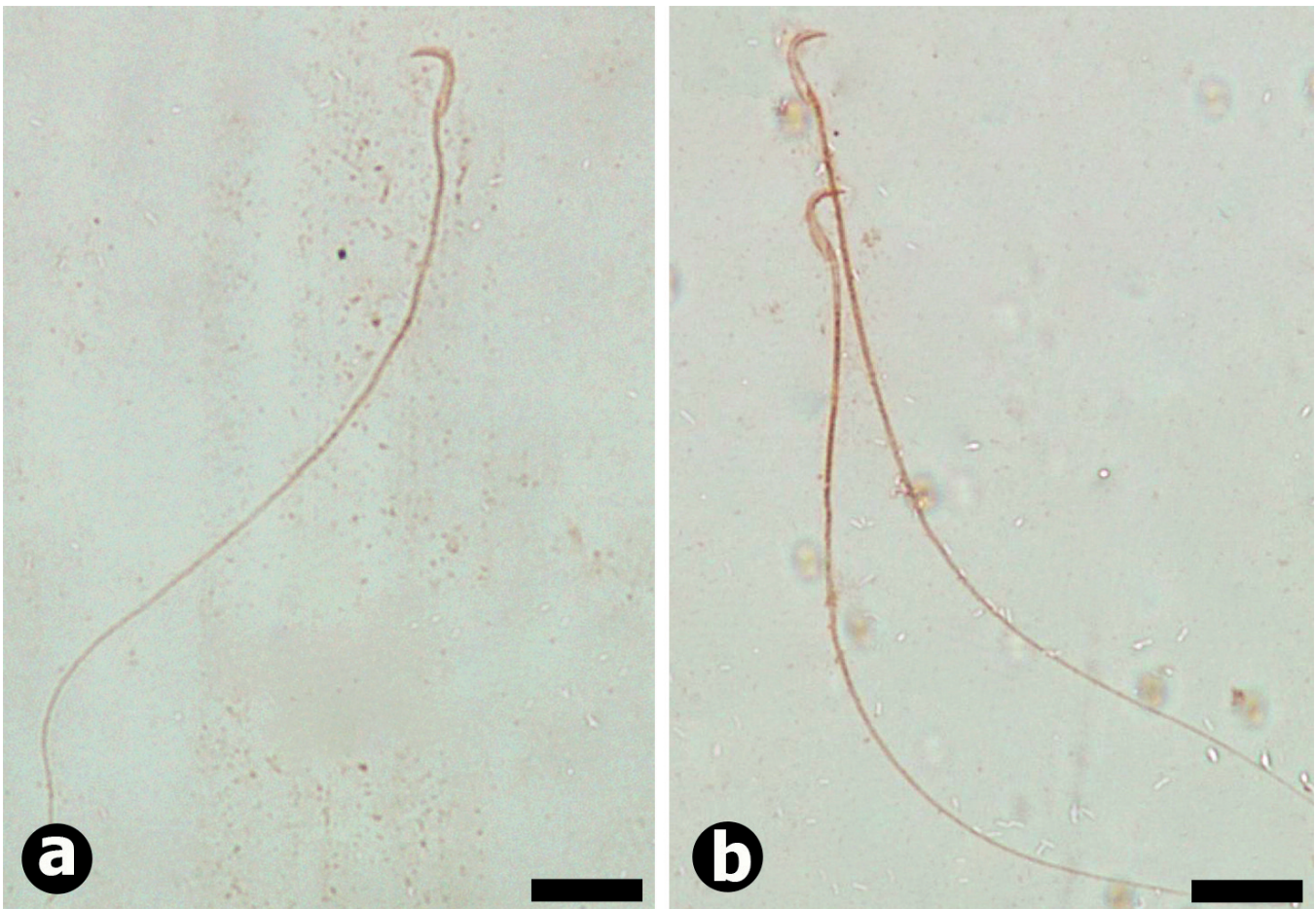
Fig. 3. Light micrographs of sperm smears applied GAT1-specific immunocytochemical stain. **a.** In the negative control not applied GAT1 antibody, the brown staining is not observed in the sperm. **b.** In the smear marked with primary antibody (GAT1 antibody), the positive reaction is seen in the head and neck regions of the sperm. Stain: Immunoperoxidase reaction. Scale bars: 25 μm.

sections of OG. The lumen of some tubules was larger than the control group and they had no sperm. Cytoplasmic swelling in the spermatogenic cells, separations between the cells due to intercellular edema and the presence of apoptotic cells were noticed (Fig. 5c,g). In some tubules, there were large vacuoles between the basement membrane and germinal epithelium. Hence, it was considered that the basement membrane separated from the epithelium wall. However, the thickness of germinal epithelium was lower than the CG. While the primary spermatocytes in diplotene of first meiotic division were found, secondary spermatocytes were not seen (Fig. 5g).

In the H-E stained sections of SOG, histopathological changes were similar to findings in both stress and obesity groups. Hyalinization and vacuolization in the interstitium, degeneration such as edema and cytoplasmic swelling in the seminiferous tubule epithelium, especially apoptotic changes of the spermatogonia were the main findings. These changes were more intensive than in the other groups. The arrangement of germinal epithelium was disrupted due to edema between the intercellular areas. Cells with dark eosinophil cytoplasm and condensed nuclear chromatin were seen among the spermatogenic cells and it was thought that these cells may be apoptotic cells due to their appearance (Fig. 5d,h).

GAT1 immunohistochemistry of the testis

In the immunostaining with the GAT1 antibody, slightly positive immunoreaction was seen in some Sertoli cells, the spermatozoa found in the apical portion of the Sertoli cells, spermatozoa in the lumen and occasionally in connective tissue in the sections of CG

GAT1-immunopositive sperm count/500 sperm

Fig. 4. The graph shows the mean GAT1 positive sperm counts of the four experimental groups. GAT1 positive sperm counts of SG (Mean±SD=219.17±28.26), OG (Mean±SD= 209.83±26.86), and SOG (Mean±SD= 206.17±18.66) significantly increased when compared with CG (Mean±SD=108.16±12.46) (p<0.05; Mann-Whitney U test). CG: Control Group, SG: Stress Group, OG: Obesity Group, SOG: Stress-Obesity Group. SD: Standard deviation.

(Fig. 6b). In the SG, GAT1 positive areas seen in the seminiferous tubules and interstitial connective tissue were more than the CG. Especially, there was strong positive staining in the spermatozoa in the lumen and the basal parts of the epithelium and connective tissue (Fig. 6c). In the OG and SOG, immunopositivity for GAT1 was similar to those of the SG (Fig. 6d,e). However, when compared with the SG, positive GAT1 reaction was not observed in the spermatogonia in the OG (Fig. 6d).

TUNEL staining of the testis

In the testes of the CG, TUNEL positive apoptotic cells with brown nucleus were observed occasionally in the wall of some seminiferous tubules (Fig. 6g). When the sections of SG were examined, positive stained spermatogonia with the brown nucleus and primary spermatids were seen a lot in the basal part of the germinal epithelium. Also, there were apoptotic Leydig cells with brown nucleus in the interstitium (Fig. 6h). In the testes sections of OG, TUNEL positive apoptotic cells were detected extensively in the epithelium of seminiferous tubule and in the interstitium compared to the CG. Apoptotic spermatids located close to the lumen were remarkably observed (Fig. 6i). In the SOG, both in the seminiferous tubules and in the interstitium, apoptotic cells were present in abundance compared to the other groups. In the early and late spermatids located in both basal parts of the germinal epithelium and close to the tubule lumen, intensive TUNEL positive reaction was detected (Fig. 6j).

Discussion

Biological and psychosocial mechanisms work together to provide balance in organisms. The stress factor disturbs this normal balance and plays an important role in the development of many diseases (Gedikli, 2010; Nargund, 2015). Stress shows its effect on the reproductive system by changing hormone levels by affecting the hypothalamus and hypophysis, or directly affecting the gonads (Tilbrook et al., 2000).
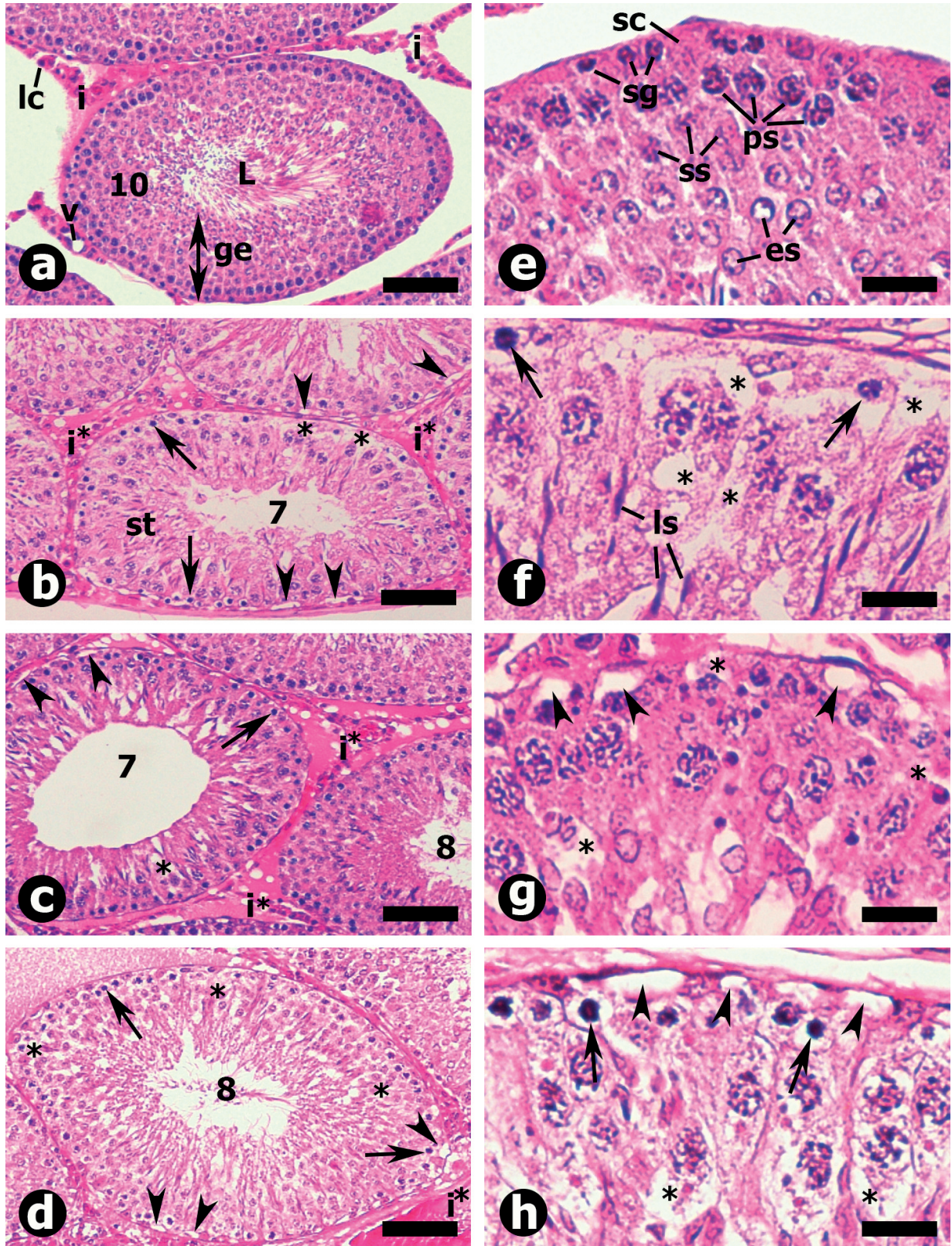
Obesity, characterized by an increase in fat mass in the body, is a chronic disease affecting quality and duration of life negatively. Obesity leads to erectile dysfunction and male infertility by reducing semen quality and causing other physical problems. Also, the increase in abnormal reproduction hormone levels, adipokines associated with obesity, and hormones derived from adipose tissue are other factors affecting the male reproductive system (Cabler et al., 2010). In the present study, we investigated the negative effects of chronic stress and obesity on the male reproductive system.

In response to daily stress, people tend to eat excessively and this results in weight gain (de Oliveira et al., 2014; Miller and Lumeng, 2018). However, if the exposed stress is severe, food consumption and fat
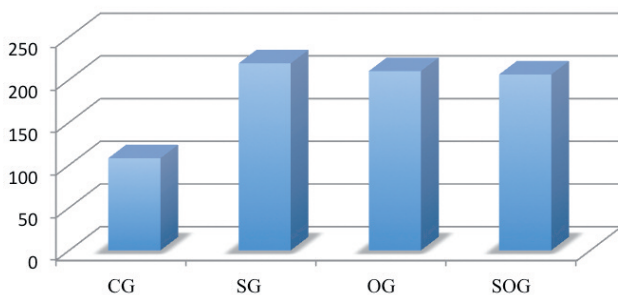
Fig. 5. Light micrographs of the sections obtained from the testis of the CG **(a, e),** SG **(b, f),** OG **(c, g) a**nd SOG **(d, h).** i, interstitium; L, sperm filled lumen of the seminiferous tubule; ge, germinal epithelium; sc, Sertoli cell; sg, spermatogonia; ps, primary spermatocyte; ss, secondary spermatocyte; es, early spermatid; ls, late spermatid; st, seminiferous tubule; i*, hyalinization and vacuolization in the interstitium; *, cellular swelling and intercellular edema seen in the germinal epithelium; arrow head, large vacuoles found between the basement membrane and spermatogonia; 7, 8 and 10, the scores of the seminiferous tubules according to the Johnsen criteria; arrow, apoptotic cells. Stain: H-E. Scale bars: a-d, 100 μm; e-h, 25 μm.

Stress, obesity and male infertility
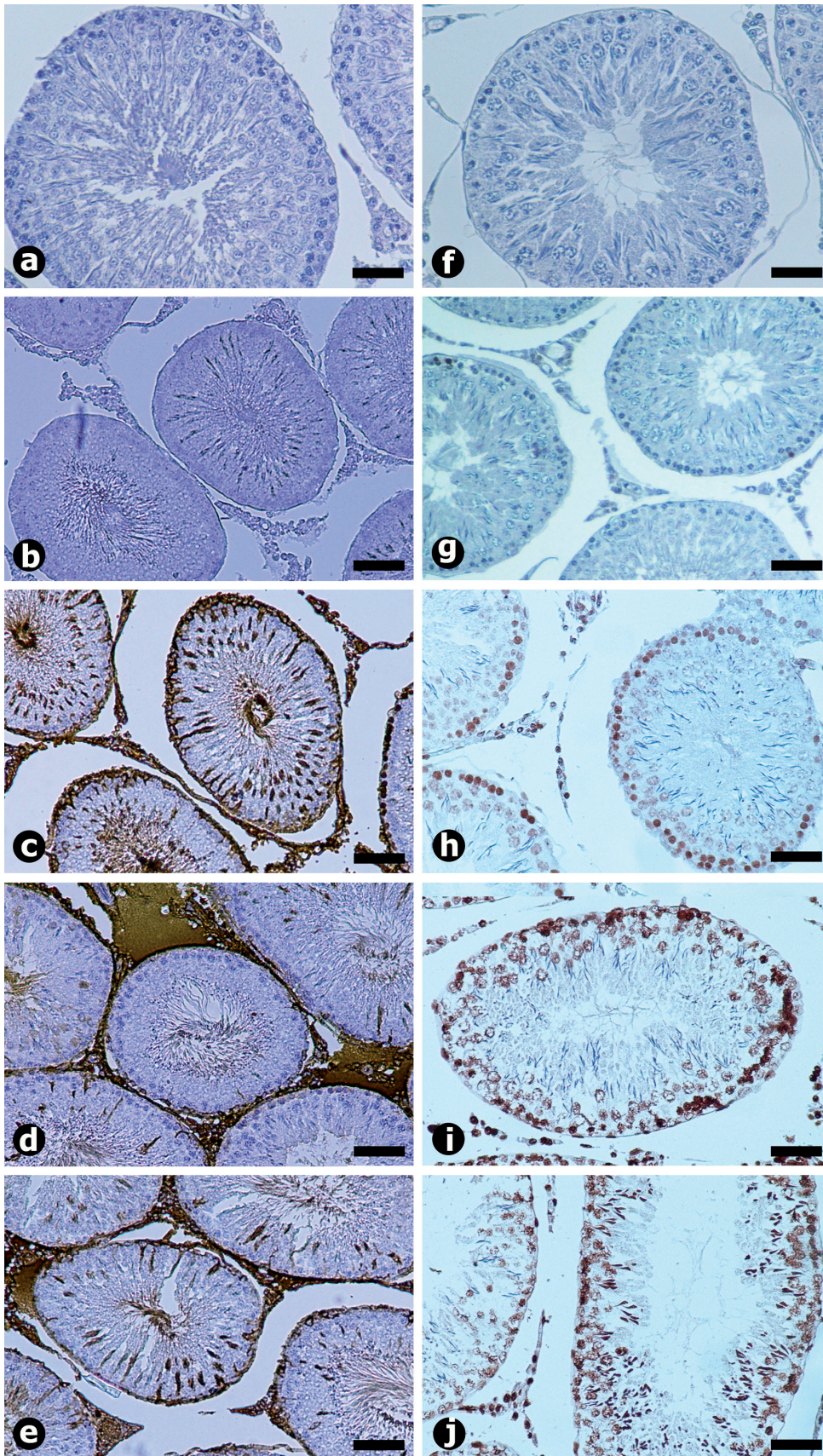
Fig. 6. Light micrographs of the immuno- and TUNEL-stained testis sections obtained from CG (**b, g**), SG (**c, h**), OG (**d, i**) and SOG (**e, j**) and the sections prepared as negative controls for GAT1-specific testis immunohistochemistry (**a**) and TUNEL staining method (**f**) obtained from the rats of the stress group. GAT1 immunopositive areas (**b-e**) and TUNEL positive cells (**g-j**) were seen as brown. Stain: immunoperoxidase reaction and TUNEL (Terminal Deoxynucleotide Transferase mediated dUTP Nick End Labeling) staining method. Scale bars: 50 μm.

accumulation are reduced in the body. It was reported that laboratory animals respond to stressors such as swimming, inactivity, social stress etc. by reducing food intake and fat accumulation (Foster et al., 2006). In our study, although it was not observed in the SG, the significant decrease in the BMI of the SOG may be interpreted as indicating the response of obese rats to the stressors is more excessive.

It was also reported that immobilization (Suarez et al., 1996), electroshock (Mukherjee et al., 1990), chronic restraint stress (Arun et al., 2016) and chronic social defeat stress (Wang et al., 2017) cause decreased testicular weight in laboratory animals. According to some authors, obesity increases testicular weight (El-Sweedy et al., 2007), while others have indicated it does not alter it (Bakos et al., 2011). In a study conducted by Miao, it was reported that high-fat diet-induced obesity led to reduced testicular weight in rats (Miao et al., 2018). In the current study, we determined that there was a statistically insignificant decrease in testicular weight in the rats of the SG and OG. But, the reduction in testicular weight in the SOG was statistically significant. It is considered that this reduction may develop due to thinning of the tubule epithelium, cell death and decrease in the number of spermatozoa. It is also possible that excess fat tissue found around the testes prevents testicular development in obesity.

Recently, studies have focused on the idea that stress may cause infertility in men by reducing the quality of semen. Psychological stress disturbs the quality of semen in males (Guo et al., 2017). Physiological stress decreases sperm concentration and progressive motility, and increases abnormal sperm counts (Li et al., 2011). Recently studies have shown that obesity significantly reduces sperm concentration and motility, especially progressive sperm motility (Kort et al., 2006; Hammoud et al., 2008b; Ghanayem et al., 2010; Miao et al., 2018). In the current study, we determined that sperm concentration and sperm motility values were significantly lower in the SG, OG, and SOG compared to the CG. In terms of sperm concentration, there was no statistical difference among these three groups. However, sperm motility in the OG and SOG were significantly lower than in the stress group. In other words, obesity was more harmful to sperm motility than stress.

In physiological stress conditions, there is a decrease in the number of sperm with normal morphology (Clarke et al., 1999; Li et al., 2011). In a study, the examination of semen samples taken from men during the post-war period revealed an increase in the percentage of sperm with abnormal morphology (Abu-Musa et al., 2007). Wang reported that chronic social defeat stress significantly decreased sperm concentration; however, it increased abnormal sperm in male mice (Wang et al., 2017). Hofny reported that obesity reduces sperm concentration and motility, and increases the number of abnormal sperm (Hofny et al., 2010). In a study performed by Shayeb, it was stated that semen volume

was lower and morphologically abnormal spermatozoa were higher in obese men compared to non-obese ones (Shayeb et al., 2011). In another study, it was pointed out that obesity decreased sperm motility while it did not affect sperm concentration and morphology (Fariello et al., 2012). In the current study, we observed that the number of tail and head defective spermatozoa increased statistically significantly in the SG, OG and SOG compared to the CG. There was no significant difference among the three groups except compared with the CG. Stress and obesity had a similar effect and in terms of effect on sperm morphology.

Chronic intermittent stress, social defeat stress, heat stress and obesity cause suppression of spermatogenesis (Nirupama et al., 2013; Durairajanayagam et al., 2015; Wang et al., 2017; Jia et al., 2018). In our study, testicular sections stained with H-E were examined with a light microscope and Johnsen's testis biopsy scoring (Johnsen, 1970) was performed. As a result of our experiment, both stress application and obesity significantly reduced the Johnsen score in the rat testis. This reduction in the Johnsen score was even more pronounced in the SOG. That is, when stress and obesity coexist, spermatogenesis is more negatively affected.

Recent studies have shown that stress leads to testicular damage by degenerating the spermatogonial cells lining the seminiferous tubules and by causing interstitial edema and vacuoles in the tubules (Sakr et al., 2015; Wang et al., 2017). High-fat diet-induced obesity leads to atrophy of seminiferous tubules by reducing both spermatogenic series cells and Sertoli cells in rats (Miao et al., 2018). Light microscopic examination revealed similar histopathologic changes in the SG, OG and SOG when compared with the CG, such as disruption of seminiferous tubule structure, decrease in epithelial thickness, and enlarged and empty tubule lumen due to decreased sperm production. In the germinal epithelial spermatogenic series cells, degenerations such as edema, cytoplasmic swelling and nuclear picnosis were observed. This structural damage was more prominent in the SOG. In other words, obesity and stress alone affected the histological structure of the testis in a similar way, but when they coexist, the severity of these adverse effects increased.

Apart from the central nervous system, GABA is also present in the testis, epididymis, vas deferens, prostate, and seminal vesicle. It was shown that GAD enzyme is present in Leydig cells and spermatocytes in the rat testis and in the post acrosomal region in human sperm (Aanesen et al., 1996; Geigerseder et al., 2003; Redecker et al., 2003). At low doses, GABA stimulates the sperm AR (Aanesen et al., 1996; Li et al., 2008) and capacitation (Hu et al., 2004a,b) by mimicking the effect of progesterone. Following the binding of progesterone to the mammalian sperm membrane, calcium entry increases into the cell and AR is initiated, and also progesterone increases GABA uptake into sperm (Aanesen et al., 1996). GABA controls the spermatogonial stem cell proliferation as a negative

regulator and maintains the spermatogenesis (Du et al., 2013b). GABA uptake is mediated by the transport proteins [GAT1, GAT2, GAT3, GAT4 (BGT-1)] located on the sperm surface (Aanesen et al., 1995, 1996; Ma et al., 2000a). Also, it was shown that GAT1 can initiate AR by causing depolarization in the sperm membrane (Ma et al., 2000a).

In a recent study, it was observed that excessive fat accumulation is found in interscapular, subcutaneous, epididymal, mesenteric and other adipose tissue regions, especially in the retroperitoneal region in transgenic mice where GAT1 is overexpressed. It was stated that excessive production of GAT1 can affect GABA-mediated functions and thus cause obesity (Ma et al., 2000b). Previous studies have shown that GAT1 is present in the testis and sperm surface and this situation is associated with the testicular function (Aanesen et al., 1996; Redecker et al., 2003; Li et al., 2008). It was reported that GAT1 was found in the tail and in the entire head except for the equatorial part of the sperm. Immunocytochemically, it was reported that GAT1 was present only in spermatozoa and spermatids in the testis, and no immunoreaction was observed in Sertoli cells and spermatocytes (Ma et al., 2000a; Li et al., 2008). According to our findings, the number of spermatozoa showing GAT1 positive immunoreactivity in the SG, OG and SOG was significantly higher than the CG; however, there was no statistical difference in their binary comparison. Brown staining was observed in both the head and tail of sperm showing immunoperoxidase positive reaction. In contrast to the literature, in the sections of the testis of the experimental groups, we observed GAT1-positive immunostaining both in Sertoli cells and in the connective tissue including the basal membrane, and also in the spermatogonia of the stress-exposed groups. As a result of the immunostaining seen both in the testis sections and sperm smears, we think that chronic stress and obesity might increase the level of GAT1 when they were present both separately and coexisting. In studies performed on transgenic mice, it was shown that overexpression of GAT1 results in decreased thickness of the germinal epithelium in the seminiferous tubules, vacuolar formation between epithelial cells, degeneration of spermatogenic cells and decreased testicular weight (Frungieri et al., 1996; Hu et al., 2004a,b; Zhang et al., 2009). The morphological changes we observed in the stress and obesity models that we created support the GAT1 increase in these regions. In our study, we found a decrease in testicular weight in the other three groups compared to the control group. Therefore, this may be related to the GAT1 increase. We detected that the GAT1 protein increased in both sperm smears and testis sections in the stress, obesity and stress-obesity groups in our study. Thus, the structural changes observed in seminiferous tubules of these groups may be explained by suppression of spermatogenesis via GAT1. Based on the knowledge that GAT1 removes GABA from the synaptic interval in the CNS (Frungieri et al., 1996; Hu et al., 2004b; Liu et al.,

2007), we think that the increase in GAT1 might reduce the GABA level in the testis also.

In the testis, programmed cell death usually occurs in spermatocytes and spermatogonia (Beumer et al., 2000) and this apoptosis in the reproductive cells is essential for the normal development of spermatozoa (Jefferson et al., 2000). High temperature, radiation, and cold stress are factors that increase apoptosis in germ cells in testis (Shiraishi et al., 2010). Sasagawa showed that apoptosis increases especially in the spermatogonia, in testis of chronic immobilization stress-exposed rats (Sasagawa et al., 2001). Also, it can be considered that apoptosis, necrosis, and impairment of the chromatin structures in the spermatozoa negatively affect sperm morphology in stressful conditions (Collodel et al., 2008; Zini et al., 2009). Similar to stress, obesity influences spermatogenesis and sperm morphology, either by destroying the sperm chromatin structure or by impressing the HPA and the hypothalamic-pituitary-gonadal (HPG) axes (Du Plessis et al., 2010; Palmer et al., 2012; Davidson et al., 2015). Obesity leads to male infertility through direct and indirect mechanisms. Low inhibin B and androgen levels accompanying high estrogen levels, and increased scrotal temperature lead to impairment of sperm parameters and increased sperm DNA fragmentation index (Kort et al., 2006; Du Plessis et al., 2010). In the current study, we used the TUNEL method in order to mark the DNA fragmentation in the apoptotic cells in testis. We observed more apoptotic cell death in both stress-exposed and obesity groups compared to control in TUNEL-stained testis sections.

In summary, chronic stress and obesity cause testicular dysfunction and male infertility by negatively affecting testicular histopathology, sperm parameters and morphology in rats. When chronic stress and obesity coexist, especially the negative effects on testicular weight, sperm concentration and Johnsen score were more prominent. Also, the presence of GAT1 protein in testis and sperm may be associated with these effects of chronic stress and obesity. The use of selective GAT1 inhibitor drugs may be another research subject in chronic stress- and obesity-induced testicular dysfunction.

It is believed that this study will guide new studies associated with male infertility and new approaches to its treatment.

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