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Effects of Different Stress Applications on Some Reproductive Hormones, Sperm Parameters, Lipid Profile, Immunohistochemical and Immunofluorescent Markers

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ABSTRACT The current study assessed how 5 stress protocols applied affected sperm parameters, lipid profile, and some reproductive hormones. Live materials of the study consisted of 50 rats. The number of rats in the groups was equal and they were randomly assigned. Each group consisted of 10 rats. No stress application was conducted in the control group. The rats in the psychological stress group were subjected to a cycle of 4-hour light and 20-hour dark per day. The rats in the physical stress group were deprived of feed and water for two hours per day. In the psychological stress + physical stress group, the psychological and physical stress protocol was conducted. In the first 4 groups, all the applications were conducted for 14 days. A different stress application was applied to the rats in the depression group every day. It was determined that the abnormal sperm rate was high in the stress and depression groups, but the highest rate was in the depression group. In addition, sperm motility and sperm concentration were the lowest in the depression group. While the stress and depression groups had significantly lower serum triglyceride and HDL levels and LH and FSH levels, cholesterol and LDL values were significantly higher. Bax expression and 8 OHdG expression were severe in psychological stress+physical stress group and depression group. When the findings are evaluated collectively; it was determined that stress negatively affected sperm parameters, lipid profile, reproductive hormones, immunofluorescence and immunohistochemical parameters.

Keywords: *Bax, LDL, LH, Sperm, Stress, Triglyceride.*

ÖZ

Farklı Stres Uygulamalarının Bazı Üreme Hormonları, Sperm Parametreleri, Lipid Profili, İmmünohistokimyasal ve İmmünofloresan Belirteçler Üzerine Etkileri

Mevcut çalışma, uygulanan 5 stres protokolünün sperm parametrelerini, lipid profilini ve bazı üreme hormonlarını nasıl etkilediğini değerlendirdi. Çalışmanın canlı materyalini 50 sıçan oluşturdu. Gruplardaki sıçan sayısı eşitti ve rastgele dağıtıldılar. Her grup 10 sıçandan oluşuyordu. Kontrol grubuna herhangi bir stres uygulaması yapılmadı. Psikolojik stres grubundaki sıçanlar günde 4 saat aydınlık, 20 saat karanlık döngüsüne tabi tutuldu. Fiziksel stres grubundaki sıçanlar günde 2 saat yem ve sudan mahrum bırakıldı. Psikolojik stres + fiziksel stres grubunda psikolojik ve fiziksel stres protokolü uygulandı. İlk 4 grupta tüm uygulamalar 14 gün süreyle yapılmıştır. Depresyon grubundaki sıçanlara her gün farklı bir stres uygulama uygulandı. Anormal sperm oranının stres ve depresyon gruplarında yüksek olduğu ancak en yüksek oranın depresyon grubunda olduğu belirlendi. Ayrıca sperm motilitesi ve yoğunluğu depresyon grubunda en düşüktü. Stres ve depresyon gruplarında serum trigliserit ve HDL düzeyleri ile LH ve FSH düzeyleri anlamlı olarak düşük bulunurken, kolesterol ve LDL değerleri anlamlı olarak yüksekti. Bax ekspresyonu ve 8 OHdG ekspresyonu psikolojik stress + fiziksel stres grubu ve depresyon grubunda şiddetli düzeydeydi. Bulgular toplu olarak değerlendirildiğinde; stresin sperm parametrelerini, lipid profilini, üreme hormonlarını, immünofloresan ve immünohistokimyasal parametreleri olumsuz etkilediği belirlendi.

Anahtar Kelimeler: *Bax, LDL, LH, Sperm, Stres, Trigliserit.*

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INTRODUCTION

While industrial and technological developments make life easier, they also bring problems. The reproductive system is very sensitive to such environmental factors and is heavily affected by them (Fahim et al. 2019). People are placing more importance on reproductive health than they used to because their lifestyles are changing and they're exposed to more stress, both of which in turn tend to have a negative impact on them and their reproductive health (Awadalla et al. 2011). While short-term (acute) stress affects the process of adaptation to the environment, long-term and excessive (chronic) stress adversely influences health (Lee et al. 2015). It is known that an appropriate micro-environment (appropriate temperature, blood supply and hormonal stimulation) is required for testicular morphology and function (Damber and Janson 1978; Shalet 2009).

During stress, cortisol levels normally elevate, and when the stressful situations end, cortisol levels lower. However, cortisol accumulation increases when the organism is exposed to chronic stress (Stephens and Wand 2012). In this case, cortisol affects muscle, liver and adipose tissues to provide the necessary fuel. Thus, the fat accumulation in the tissues is mobilized and the lipid levels in the blood increase (Bower and Sergerstrom 2001). Hyperlipidemia has a major role in testicular damage (Mehta et al. 2003).

In case of chronic stress, various body activities are inhibited and cellular proliferation and differentiation are negatively affected (Rosmond and Bjorntorp 2000). Chronic stress causes very serious problems in reproduction (decrease in reproductive hormones, impaired sperm quality, erectile dysfunction, decreased sexual desire) (Kennedy et al. 1999; Baldwin 2001). In addition, chronic stress causes atrophy in seminiferous tubules, decreases tubule diameters, increases thickness of basement membrane, and causes necrosis in Leydig cells and interstitial edema (Arun et al. 2016; Fahim et al. 2019).

Numerous studies have emphasized that chronic stress causes very serious problems in male reproduction. However, they generally used a single stress protocol. In the present study, 5 stress protocols were applied. The aim of this study is to determine to what extent different stress protocols affect sperm parameters, reproductive hormones, lipid profile, immunohistochemical and immunofluorescent markers.

MATERIAL AND METHODS

Animals and Experimental Groups

All experimental applications were carried out based on the guidelines of the National Institutes of Health on the care and use of laboratory animals. Before the study, approval was obtained from the Animal Experiments Local Ethics Committee of Van Yuzuncu Yil University with the "decision dated 30.0.2022 and numbered 2022/06-02". 50 male Wistar albino rats, aged 3 months (200–250 g) provided by the Experimental Research Unit of Van Yuzuncu Yil University, were used in the study. They were randomly selected and divided into 5 groups including 10 rats in each. In order to make a statistically sound comparison, the number of rats in each group was adjusted equally. Rats were grouped considering their similar physical characteristics (especially their weight). In addition, attention was paid to the same age, species and gender. The number of subjects was kept high in order to increase the reliability, validity and sensitivity of the study and to guarantee the results. Caging was done on a group basis and 10 rats in each group were kept in a single cage. The rats were housed in a ventilated, 50±5% humidity environment under standard conditions during the study period, in laboratory conditions with a 12-hour light-dark cycle. Sterile corn cobs (MBD Feed Mill, Kocaeli, Turkey) were used as litter and changed daily. The rats were fed with standard rat pellet feed (Bayramoglu Feed Factory, Erzurum, Turkey).

In order to minimize the effects of these five protocols applied, a 1-week run-in period was applied. During this 1-week period, the same researcher checked the rats at the same time every day to adapt to the environment, the researcher, and routine procedures.

Control group, (n=10): No application was made on the rats in this group.

Psychological stress group (PS), (n= 10): In this group, rats were deprived of light. For this purpose, it was kept in laboratory conditions with a 4-hour light and 20-hour dark cycle for 14 days (Bulmus 2018).

Physical stress group (FS), (n=10): Rats in this group were placed in a semi-cylindrical acrylic tube with only ventilation holes (4.5 cm wide and 12 cm long). They were not allowed to take feed and water for 2 hours a day (between 10:00 and 12:00 in the morning) for 14 days.

Psychological stress + physical stress group (PS+FS), (n=10): Applications in the psychological and physical stress protocol were performed.

Depression group (DEP), (n=10): Rats in this group were deprived of food and water for 24 hours on the first day. On the second day, they were placed in 50 ml falcon tubes (between 8:00 and 11:00). On the third day, they were left in wet and dirty cages for 24 hours (between 08:00 and 08:00). On the fourth day, they were forced to swim for 5 minutes in a glass aquarium filled with ice water. On the fifth day, they were again placed in wet and dirty cages and deprived of food. On the sixth day, they were exposed to restrictive stress and crowded cages. On the seventh day, they were placed in empty cages and forced to swim in icy water. The same applications were applied to the rats in the depression group in the same order for 14 days (Basar and Ertugrul 2005).

In order to observe whether stress behavior occurred in rats, observations were made using components such as tail hanging and forced swimming (rising on the hind legs, standing still, etc.).

Sample Collection

Cardiac blood samples were taken under general anesthesia by inserting a needle from the lower end of the thorax into the area of the beats. They were centrifuged (5 minutes at 3.000 x g) and their serum was separated. These serums were utilized to assess reproductive hormones and lipid profiles. One testis was resected before the body cooled down following the blood collection process and its spermatological parameters were analyzed. The other testis was left in a 10% solution of formalin and then analyzed for histopathological and immunohistochemical examinations. For euthanasia, the ketamine/xylazine combination was used at four times the anesthetic dose (Belhan et al. 2017).

Spermatological Examination

The cauda epididymis of the testis, which was resected from the body after blood collection procedure, was first used to evaluate motility. This evaluation was made under a light microscope with a heating plate. The semen was

diluted in saline at 37 °C. While assessing the motility, the researchers did their best to avoid cooling down the cauda epididymis part and wasting time. The mixture obtained by slicing this part in 2 ml of saline was used to evaluate the sperm concentration and abnormal sperm ratio (Aksu et al. 2015; Turk et al. 2008).

Evaluation of Reproductive Hormones and Lipid Profile

Follicle stimulating hormone (FSH), Luteinizing hormone (LH), and lipid parameters were measured in the Abbott Architect I6200 SR device, using the chemiluminescence microparticle immunology method (Belhan et al. 2020).

Histopathological Examination

Tissue samples left in formaldehyde (10%) solution were fixed for 48 hours. Afterwars, routine tissue follow-up procedures were performed and embedded in paraffin blocks. Preparations were prepared by taking 4-µm thick sections from each block. These preparations were stained with hematoxylin-eosin (HE) and analyzed using light microscopy (Olympus BX 51, JAPAN). Examples were rated as none (-), mild (+), moderate (++), and severe (+++) in histopathological examination (Belhan et al. 2020).

Immunohistochemical Examination

The tissue sections were first taken on adhesive (poly-L-Lysin) slides to carry out immunoperoxidase examination. They were then deparaffinized and dehydrated. This process was followed by inactivation of endogenous peroxidase by keeping it in 3% H_2O_2 for 10 minutes. In the next step, the tissues boiled in 1% antigen retrieval solution were left to cool down at room temperature. Nonspecific background staining of the tissues was prevented by incubating the sections in protein block for 5 minutes. Tissues were incubated after instillation of primary antibody = Bcl-2-associated X protein (Bax) (Bax Cat No: sc-7480, Dilution Ratio: 1/100, US). 3-3' Diaminobenzidine chromogen was used as chromogen. The stained sections were analyzed by using a light microscope (Zeiss AXIO GERMANY) and based on their immune positivity. They were evaluated as none (-), mild (+), moderate (++), and severe (+++) (Temel et al. 2020).

Immunofluorescence Examination

Immunofluorescence examination was performed by taking the sections on adhesive (poly-L-Lysin) slides and then deparaffinizing and dehydrating them. This process was followed by inactivation of endogenous peroxidase by keeping it in 3% H_2O_2 for 10 minutes. In the next step, the tissues boiled in 1% antigen retrieval solution were left to cool down at room temperature. Their nonspecific background staining was prevented by incubating the prepared sections in protein block for 5 minutes. Then, primary antibody = 8-hydroxy-2-deoxyguanosine (8 OHdG) (8 OHdG Cat No: sc-66036, Dilution Ratio: 1/100, US) was dripped onto the tissues and incubated in accordance with the use instructions. Immunofluorescence secondary antibody (FITC Cat No: ab6717, Diluent Ratio: 1/1000, UK) was used as a secondary marker and incubated in the dark for 45 minutes. Finally, DAPI with mounting medium (Cat no: D1306, Dilution Ratio: 1/200 UK) was dripped onto the sections and incubated in the dark for 5 minutes, and the sections were covered with a coverslip. The stained tissues were analyzed under a fluorescent microscope (Zeiss AXIO GERMANY). The immune positivity of the sections was rated as none (-), mild (+), moderate (++), and severe (+++) (Belhan et al. 2017).

Statistical Analysis

SPSS package software (Version 21) was used for statistical analysis of lipid profile and reproductive hormones. The Shapiro-Wilk test was applied to analyze the compatibility of the data for normal distribution. The groups were normally distributed; therefore, Kruskal Wallis test was run to identify the presence of significant differences between the groups in terms of the same parameter. Post hoc analysis was run to determine which group caused the differences. The *P* value of ≤0.05 was accepted as significant. Descriptive statistics were presented as mean and standard deviation.

Five random areas were selected from each of immunohistochemical and immunofluorescent staining images and analyzed using the ZEISS Zen Imaging Software software to determine the intensity of positive staining. Data were statistically presented as mean and standard deviation (mean±SD) for % value of the area. Mann-Whitney U test was run in comparison of positive immunoreactive cells and immunopositive stained areas with healthy controls. As a result of the test, a p value of <0.05 was accepted as significant.

RESULTS

Sperm Parameters

It was determined that sperm parameters showed significant differences between the groups. Sperm concentration and motility were found to be lowest in the depression group. It was found that the rate of abnormal sperm was higher in the stress groups compared to the control group. However, the highest rate of abnormal sperm was found in the depression group. Findings regarding sperm parameters are presented in Table 1.

Table 1: Sperm parameter findings of the rats in the control group and the groups applied different stress protocols.

Groups	Motility (%)	Sperm Concentration (x106)	Abnormal sperm rate (%)
Control group	76.20±1.22^{a}	111.10±1.28^{a}	10.80±0.88^{e}
Psychological stress group	65.00±0.66^{b}	95.80±1.13^{b}	17.50±0.97^{d}
Physical stress group	60.10±1.37^{c}	84.90±0.99^{c}	21.60±0.96^{c}
Psychological stress + physical stress group	51.60±2.01^{d}	64.00±1.24^{d}	28.70±0.82^{b}
Depression group	38.50±1.58^{e}	57.60±1.50^{e}	32.70±1.33^{a}

$^{a, b, c, d}$ p*: Different letters in the same column indicate the difference between the groups (p<0.001).

Reproductive Hormones and Lipid Profile Findings

Triglyceride values of the stress and depression groups were significantly lower than the control group (p<0.001). However, the most significant decrease in triglyceride values was detected in the depression group (p<0.001). The highest cholesterol level was found in the depression group, and the lowest cholesterol levels were found in the control group (p<0.001).

When high-density lipoprotein (HDL) levels were examined in serum samples, the most significant decrease was detected in the psychological stress group ($p<0.001$). Additionally, HDL values of the depression group were interestingly higher than both the psychological and psychological+physical stress groups ($p<0.001$). Low-density lipoprotein (LDL) levels in the psychological+physical stress and depression groups were significantly higher than the control group ($p<0.001$). However, LDL values in the physical stress group were lower than the other groups ($p<0.001$).

LH and FSH levels were lower in the stress and depression groups than in the control group ($p<0.001$). The decrease in LH levels was even more pronounced, especially in the psychological stress, psychological + physical stress and depression groups. FSH levels in the physical stress, psychological+physical stress and depression groups were lower than the control group ($p<0.001$). No significant difference was detected between FSH levels of the control and psychological stress groups ($p\geq0.05$). Both physical stress and depression groups caused a significant decrease in FSH levels ($p<0.001$). Findings regarding the lipid profile and reproductive hormones of all groups are presented in Table 2 and Figure 1.

Histopathological Findings

Histopathological examination revealed that the testicular tissues in the control group had a normal histological structure (Figure 2). In the testes of the psychological stress group, moderate edema in the intertubular spaces and hyperemia in the vessels, mild degeneration in some spermatocytes and a moderate decrease in the number of sperm in the tubular lumens were detected (Figure 2). In the physical stress group, in addition to the symptoms in the psychological stress group, hyperemia in the vessels was found to be severe and there was moderate degeneration in spermatocytes (Figure 2). In the psychological stress + physical stress group; It was determined that the edema and hypermia observed in the psychological stress and physical stress groups were severe, and there was a significant decrease in the number of sperm in the tubules (Figure 2). There was a statistically significant difference between this group and the control group. In the depression group; In addition to the symptoms seen in the psychological stress and physical stress groups, advanced degeneration of spermatocytes in the tubular wall was detected (Figure 2). It was determined that this group had a statistically significant difference with the control group. Table 3 shows the histopathological findings.

Immunohistochemical Findings

It was determined that cytoplasmic Bax expression in spermatocytes was negative in the control group (Figure 2), moderate in the psychological stress group and physical stress group (Figure 2), and high in the psychological stress + physical stress group and depression group (Figure 2). A statistically significant difference was found in the depression group compared to the control, psychological stress and physical stress groups (Table 4). Table 4 shows the immunohistochemical findings.

Immunofluorescent Findings

In the evaluation of testicular tissues using the immunofluorescence method; It was observed that 8-OHdG expression was negative in the control group (Figure 2), mild-moderate in the psychological stress group (Figure 2), moderate in the physical stress group (Figure 2), and severe in the psychological stress + physical stress group (Figure 2). Psychological stress + physical stress group; showed a statistically significant difference compared to the control, psychological stress and physical stress groups (Table 4). Severe 8-OHdG expression was detected in the testicular tissues of the depression group (Figure 2). Depression group; showed a statistically significant difference according to the control, psychological stress and physical stress groups (Table 4). Immunofluorescence findings are presented in Table 4.

Table 2: Mean and standard deviation values of lipid profile and reproductive hormone levels measured in serum samples.

	Control group	Psychological stress group	Physical stress group	Psychological stress + physical stress group	Depression group	p value
Triglyceride (mg/dL)	77.20±7.51^{a}	66.00±6.93^{b}	69.25±8.14^{b}	69.13±4.89^{b}	51.44±5.99^{c}	0.001
Cholesterol (mg/dL)	37.07±2.83^{c}	52.13±4.64^{b}	53.78±3.15^{b}	54.60±4.45^{b}	62.22±6.22^{a}	0.001
HDL (mg/dL)	50.88±4.12^{a}	32.79±3.26^{d}	46.54±3.19^{b}	34.73±2.91^{d}	39.19±3.15^{c}	0.001
LDL (mg/dL)	4.93±0.42^{c}	5.27±0.52^{bc}	2.86±0.23^{d}	5.31±0.37^{b}	5.68±0.26^{a}	0.001
LH (mlU/mL)	0.57±0.05^{a}	0.39±0.03^{c}	0.44±0.03^{b}	0.35±0.04^{d}	0.4±0.03^{bc}	0.001
FSH (mlU/mL)	0.27±0.01^{a}	0.26±0.02^{ab}	0.24±0.01^{bc}	0.22±0.02^{cd}	0.21±0.03^{d}	0.001

LDL: Low density lipoprotein, HDL: High density lipoprotein, LH: Luteinizing hormone, FSH: Follicle stimulating hormone,

^{a,b,c,d}p: Values with different letters in the same row are significant when compared to each other ($p<0.001$).

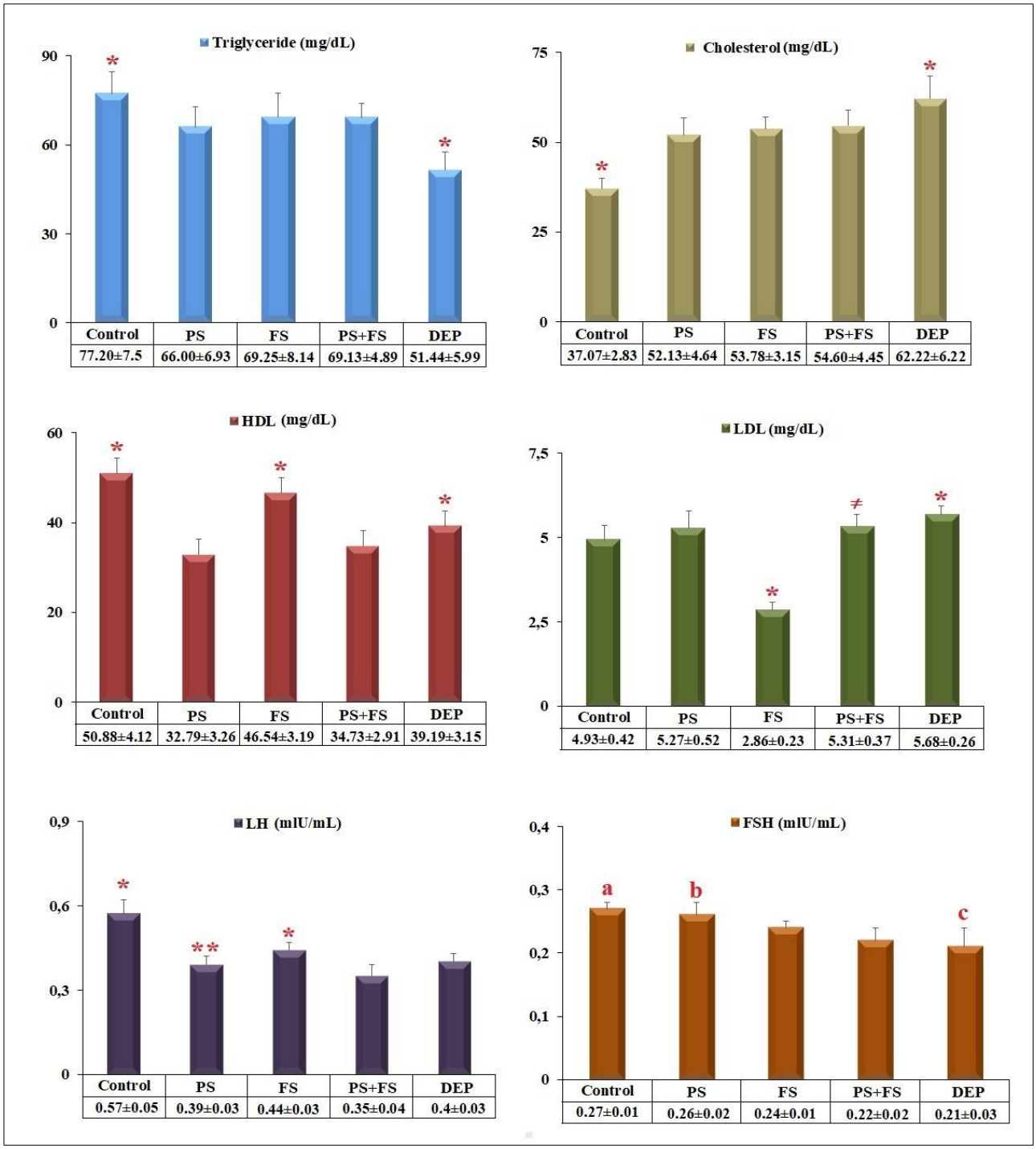

Figure 1: Comparison of the lipid profile and reproductive hormone levels measured in serum samples.

Triglyceride (mg/dL)

LDL; Low density lipoprotein (mg/L)

HDL; High density lipoprotein (mg/dL)

LH; Luteinizing hormone (mlU/dL)

FSH; Follicle stimulating hormone (mlU/dL)

*p: Significant when compared to other groups (p<0.001),

≠p: Significant when compared to psychological stress and psychological+physical stress groups (p<0.001),

[a]p: Significant when compared to psychological stress, psychological+physical stress, and depression groups (p<0.001),

[a]p: Significant when compared to psychological+physical stress and depression groups (p<0.001) Significant when compared to the control group (p<0.001),

[c]p: Significant when compared to the control, psychological stress, and physical stress groups.

Table 3: Scoring the histopathological, immunohistochemical, and immunofluorescence findings observed in testicular tissues.

	Control group	Psychological stress group	Physical stress group	Psychological stress + physical stress group	Depression group
Edema in intertubular spaces	-	++	++	+++	+++
Hyperemia in veins	-	++	+++	+++	+++
Degeneration of spermatocytes	-	+	++	++	+++
Decrease in the sperm count	-	++	++	+++	+++
Bax expression	-	++	++	+++	+++
8 OHdG expression	-	++	++	+++	+++

Table 4: Statistical assessment of immunohistochemical and immunofluorescence findings observed in testicular tissues.

	Control group	**Psychological stress group**	**Physical stress group**	**Psychological stress + physical stress group**	**Depression group**
Bax	86.55±2.78[a]	256.73±14.22[b]	248.22±9.85[b]	330.56±7.65[c]	335.42±6.38[c]
8 OHdG	123.48±2.56[a]	358.22±11.45[b]	362.28±8.79[b]	483.32±6.53[c]	488.73±9.46[c]

The results in the same row were compared with each other. Different letters include significant results.

For the statistical differences among groups ($p<0.05$), the results were expressed as mean±SD.

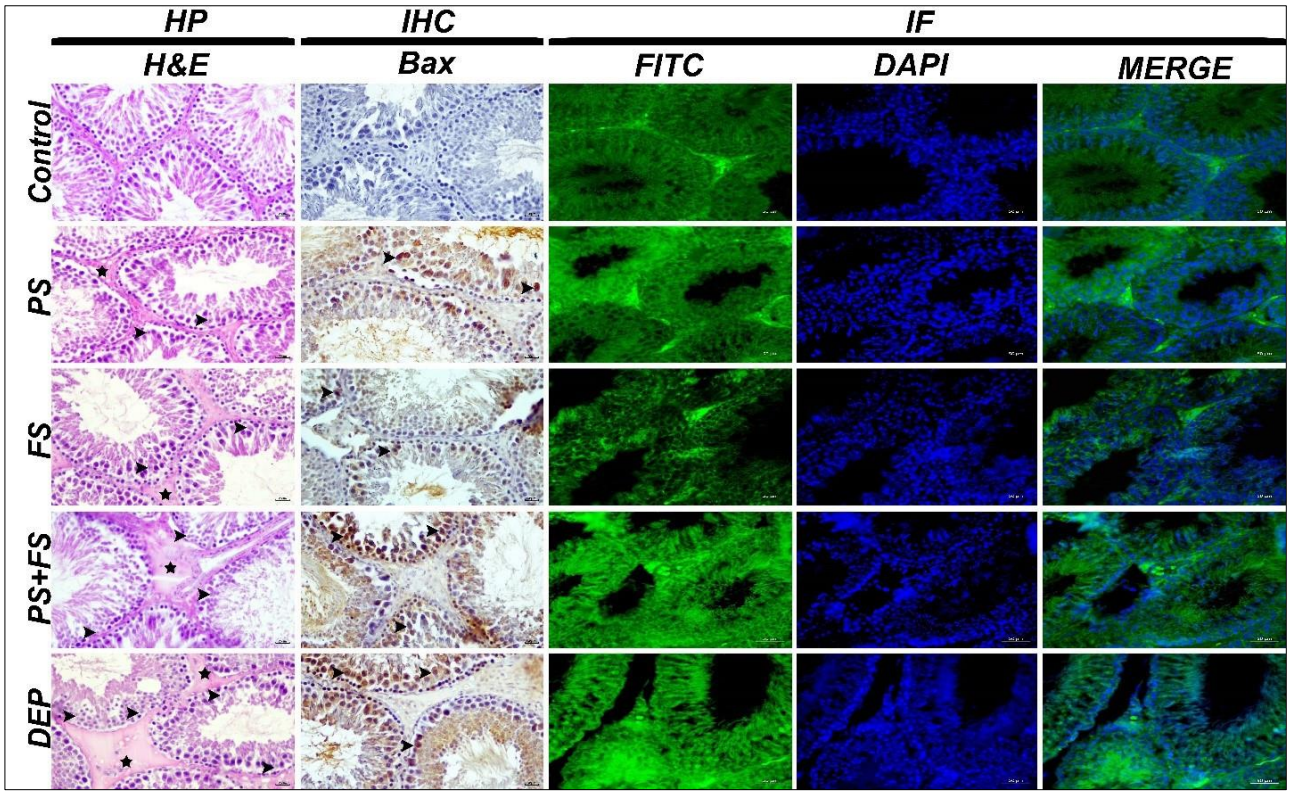

Figure 2: Testicular tissue, H&E-IHC, Bar: 20 µm. IF, Bar: 50 µm.

DISCUSSION AND CONCLUSION

The reaction occurs when the body is exposed to stress and varies depending on the intensity, unpredictability and uncontrollability of the stimulus (Koolhaas et al. 2011). In this case, increased glucocorticoid levels promote gluconeogenesis, mobilizeamino acids, and stimulate fat breakdown to maintain circulating glucose levels (Whirledge and Cidlowski 2010).

It is known that immobilization stress causes both psychological and physiological stress as it creates aggression and subsequent burnout (Yazawa et al. 1999). Animal models used to mimic the development and progression of clinical depression include chronic stress models like chronic unpredictable mild stress, chronic restraint stress, separation from the mother, and social isolation (Stepanichev et al. 2014; Wang et al. 2016). It is known that physical and psychological stress can affect reproductive ability in men. Various stressors activate the hypothalamic-pituitary-adrenal axis, suppress the hypothalamic-pituitary-testicular axis and cause dysfunction in the male reproductive system (Kirby et al. 2009).

The results obtained in the current study regarding sperm parameters are compatible with the depression study performed by applying the forced swimming test and unpredictable mild stress study (Roboon et al. 2016; Salami et al. 2020; Bagheri et al. 2021; Moustafa 2021). It is known that stress increases the formation of reactive oxygen species (ROS) in the male reproductive system. Therefore, ROS resulting from stress may have caused problems in sperm parameters (García-Díaz et al. 2015).

In histopathological evaluation, it can be said that the decrease in spermatogenic cell lines is compatible with the previous study (Fahim et al. 2019). Bax expression, which was detected at a severe level in the psychological stress + physical stress group and depression group, and at a moderate level in the physical stress and psychological stress groups, was compatible with previous stress studies (Fahim et al. 2019; Zou et al. 2019).

Findings obtained after immunofluorescence staining of testicular tissues in the current study; It supports the results of studies in which depression was induced by applying corticosterone and chronic restraint stress (Uchihara et al. 2016; Liu et al. 2019).

The hormonal evaluation revealed that the stress and depression groups had significantly lower FSH and LH values. This result supports the results of previous studies (Bagheri et al. 2021; Moustafa 2021). However, it differs from the result of another study (Mohamadpour et al. 2017). Because Mohamadpour et al (2017) stated in their study that FSH and LH levels elevated in stress groups. This may be due to the difference in the stress protocol and the duration of the application applied by the researchers

The results obtained regarding cholesterol levels in serum samples support the results of previous studies (Neves et al. 2009; Devaki et al. 2013; Zeeni et al. 2013; Kopalli et al. 2019). However, other studies reported that cholesterol and LDL levels decreased in stress groups (Lee et al. 2019; Pan et al. 2019). This may be due to the time difference in the applied stress.

The results of this study regarding triglycerides support previous studies (Lee et al. 2019; Pan et al. 2019). However, other studies reported that triglyceride values increased (Neves et al. 2009; Devaki et al. 2013; Kopalli et al. 2019). The reason for this increase may be the time difference in the applied protocols. In this study, the result showing that HDL values were low in the stress and depression groups is compatible with previous studies (Devaki et al. 2013; Zeeni et al. 2013; Kopalli et al. 2019; Lee et al. 2019; Pan et al. 2019).

In the current study, while the rate of abnormal sperm was found to be high in the stress and depression groups, the highest rate was found in the depression group. In the stress and depression groups, serum triglyceride and HDL levels, as well as LH and FSH levels, were found to be significantly lower, while cholesterol and LDL levels were significantly higher. In addition, cytoplasmic Bax and 8-OHdG expression was observed to be moderate in the psychological stress and physical stress groups, and severe in the psychological stress + physical stress and depression groups. It is thought that the results of this study will contribute to the literature on the relationship between stress and male fertility and may guide clinical studies in this field.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: SB, SA
Supervision / Consultancy: SB
Data Collection and / or Processing: SB
Analysis and / or Interpretation: SB, ZH, SY, SA
Writing the Article: SB
Critical Review: SB, ZH, SY, SA

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