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Effect of exogenous Melatonin administration on Spermatogenesis in chronic unpredictable stress rat model

Efecto de la administración exógena de Melatonina sobre la Espermatogénesis en un modelo de rata con estrés crónico impredecible

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ABSTRACT

This study investigated the hormonal, inflammatory, oxidant–antioxidant, and histopathological effects of exogenous Melatonin administration on Spermatogenesis in rats' chronic unpredictable stress model (CUSM). In the study, stress caused a decrease in follicle stimulating–hormone (FSH), luteinizing hormone (LH), Testosterone, Melatonin, Glutathione (GSH), Glutathione peroxidase (GSH–Px), catalase, interleukin 10 (IL–10) levels and motility, and an increase in Corticosterone, nuclear factor kappa beta (NF–kB), tumor necrosis factor–alpha (TNF–α), interleukin 1 beta (IL–1β), interleukin 6 (IL–6), abnormal sperm, dead/live sperm ratio and exogenous Melatonin reduced inflammatory cytokines and oxidative stress and improved spermatological parameters (P<0.05). Melatonin also partially corrected stress–induced changes in testicular morphology. As a result, using Melatonin in rats with CUSM may be effective in improving spermatological parameters through anti–inflammatory and antioxidant mechanisms.

Key words: Chronic stress; spermatogenesis; Melatonin; antioxidant; anti–inflammatory cytokines

RESUMEN

Este estudio investigó los efectos hormonales, inflamatorios, oxidantes–antioxidantes e histopatológicos de la administración exógena de melatonina sobre la espermatogénesis en el modelo de estrés crónico impredecible (CUSM) de ratas. En el presente estudio, el estrés provocó una disminución de los niveles de hormona foliculoestimulante (FSH), hormona luteinizante (LH), testosterona, melatonina, glutatión (GSH), glutatión peroxidasa (GSH–Px), catalasa, interleucina 10 (IL–10) y motilidad, y un aumento de la corticosterona, factor nuclear kappa beta (NF–kB), factor de necrosis tumoral alfa (TNF–α), interleucina 1 beta (IL–1β), interleucina 6 (IL–6), espermatozoides anormales, relación espermatozoides muertos/espermatozoides vivos y la melatonina exógena redujeron las citocinas inflamatorias y el estrés oxidativo y mejoraron los parámetros espermatológicos (P<0,05). La melatonina también corrigió parcialmente los cambios inducidos por el estrés en la morfología testicular. Como resultado, el uso de melatonina en ratas con CUSM puede ser eficaz para mejorar los parámetros espermatológicos a través de mecanismos antiinflamatorios y antioxidantes.

Palabras clave: Estrés crónico; espermatogénesis; melatonina; antioxidante; citoquinas antiinflamatorias

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INTRODUCTION

Stress decreases semen quality male reproductive system [1, 2]. It causes many effects, such as a decrease in testicular antioxidant capacity [3], increases in testicular apoptosis [1], and damage to the blood testicular barrier [4] among others.

Reproductive system pathologies are an actual problem for all species [2, 5, 6, 7]. Stress is known to cause infertility [1, 2, 4]. Infertility is a global health problem affecting approximately 15% of couples [8], and male infertility accounts for 40–50% of all infertility cases [9]. In studies to explain the etiopathogenesis of infertility in chronic stress, it has been argued that chronic stress may cause inhibition of Testosterone production from Leydig cells by glucocorticoid [10], decrease in the expression of some genes involved in Testosterone synthesis [11], inflammation and systemic or local oxidative stress [12].

Melatonin, the main indolamine the pineal gland produces, is small and highly lipophilic, easily crosses biological membranes, and reaches all cell parts [13]. It has been reported that Melatonin, which easily crosses the blood–testicular barrier and has very low toxicity, is protective in male reproductive health [1]. Exogenous Melatonin has antioxidant, anti-inflammatory, and antiapoptotic effects in many tissues, including the testes [13, 14]. Decreased Melatonin levels in stressful situations contribute to stress-induced infertility with different pathways [15].

This study aimed to investigate the possible effects of exogenous Melatonin use on Spermatogenesis and the possible mechanisms of these effects in terms of hormonal, inflammatory, and oxidant–antioxidant in rats with chronic unpredictable stress model (CUSM)

MATERIAL AND METHODS

Ethics, study design, and animals

The study was approved by the Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee (HADYEK) ethical committee decision numbered 2021/03/06. Animal care and experimental protocol complied with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. (12 h light; 12 h darkness; 24 ± 3°C). During the experiment, the rats (*Rattus norvegicus*) were fed with commercial pellet food and tap water *ad libitum*.

In the study, 40 male rats (Wistar albino) (40–50 days old, 180–200 g) were used. Animals were divided into five groups Control (C) (n=8), Vehicle (V) (n=8), Stress (S) (n=8), Melatonin (M) (n=8), and Stress + Melatonin (SM) (n=8).

Stressors (social stress, cage tilt, without sawdust, damp sawdust, foreign object, cage changing, restricted food access, sound of predator noise) were applied to S and SM groups for eight weeks daily [16, 17]. Melatonin (Alpha Aesar>99) (10 mg·kg^{-1}) administration to M and SM groups was performed at 17:00 for 21 d between the sixth and eighth weeks as daily orally [18, 19]. Group V was given 10% Dimethylsulfoxide (DMSO) as Melatonin solvent [20]. At the end of the eighth week, rats were anesthetized with Xylazine Ketamine, and the animals were sacrificed by intracardiac blood collection. Also, spermatological analyzes were performed by collecting epididymal tissue samples.

Serum and testicular tissue analysis

Blood was collected from animals under Xylazine Ketamine anesthesia and centrifuged (Nüve, Nf 800R, Turkey) at 3000 G for 10 min [21]. In the obtained blood serum samples, Follicle stimulating hormone (FSH), Luteinizing hormone (LH), and Testosterone levels were measured by Radioimmunoassay (RIA); Corticosterone, Melatonin, Tumor necrosis factor alpha (TNF-α), Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6) levels were measured with commercial Enzyme-linked Immunosorbent Assay (ELISA) kits (Bioassay Technology Laboratory) and microplate reader (Erba Manheim, Lisascan EM, Czech Republic).

For oxidative stress analysis, testis tissues were weighed (Weightlab WL-303, Turkey) and transferred to glass tubes and then homogenized by adding 1/10 (w/v) Tris Buffer (pH:7.4) to them. After the homogenates were centrifuged (Nüve, Nf 800R, Turkey) at 3000 G for 60 min, the supernate part was separated. Malondialdehyde (MDA), Glutathione (GSH) levels, Glutathione peroxidase (GSH-Px), and catalase activities were measured from the supernatant in tissue with a spectrometer (Shimadzu, UV-1700, Japan) [21].

For testicular cytokines analysis, testes were homogenized by diluting with Phosphate-buffered saline (PBS) (pH 7.4) 1/10 (w/v) and centrifuged (Nüve, Nf 800R, Turkey) at 16000 G +4°C for 15 min, and TNF-α, IL-1β, IL-6, Interleukin 10 (IL-10), Nuclear factor kappa beta (NF-kB) levels were measured on tissue serum samples with commercial ELISA kits (Bioassay Technology Laboratory, Zhejiang, China) and microplate reader (Erba Manheim, Lisascan EM, Czech Republic).

Spermatological analysis

After the testicles were taken out and cleared of fatty tissues, the cauda epididymis was separated. Left and right cauda epididymis, each separately, in a petri dish (Isolab 60 × 15mm) with a 1 mL (PBS containing 0.1 M Glucose and 0.1 M Na-Citrate) diluent pre-warmed to 37°C and under a stereo microscope (Euromex, Nexius Zoom, Netherlands) with a scalpel and it was cut with scissors and incubated for 10 min in an incubator (Nüve, N300, Turkey) at 37 °C in order for the spermatozoa to reach the extender. After incubation, sperm samples were examined to determine spermatological parameters (motility, abnormal, dead-live sperm). To determine motility, 20 µL of sperm content was taken from each sample and placed on a slide, and 300 sperm cells were counted on each slide at 37 °C at 400 magnification. In the count, the ratio of motile spermatozoa making robust, smooth, and linear movement in the forward direction and non-motile spermatozoa was calculated in the area where motile spermatozoa were examined [22].

The Eosin-Nigrosin staining method was used for dead spermatozoa and abnormal sperm analysis [23]. Fifty µL of the semen sample and 50 µL of Eosin-Nigrosin solution were mixed. A smear was prepared from this mixture and allowed to dry on a hot floor at 37 °C. Dried smears were examined under a microscope (Olympus CX31, Japan), at 400 times magnification. Those whose head part of the spermatozoon did not receive dye were considered alive, and those whose heads were dyed red were considered dead. Three hundred spermatozoa were counted for each sample, and the result was determined as % dead spermatozoa. In abnormal sperm analysis, 300 spermatozoa were counted in the counting area for each sample, and those with abnormal morphology were expressed as a percentage.

Histopathological evaluation

Testicular samples were fixed in 10% buffered formalin (pH 7.2–7.4). After fixation, it was reduced to 4 mm thickness and washed. It was blocked in paraffin after being passed through graded alcohol (50, 80, 96 and 100%), xylol, and paraffin series according to routine methods. Sections taken from the prepared four micron-thick tissue blocks cut with a microtome blade (Pfm medical, Feather A35, United Kingdom)

were first thawn in a 37°C water bath, then transferred to slides and dried in an oven at 54°C for 30 min. These sections were deparaffinized in xylol, passed through 100, 96, 80, and 70 alcohol series, and stained with Hematoxylin Eosin (H–E). They were passed through graded alcohol (80, 96 and 100%) and xylol again and closed with a coverslip with the help of an adhesive (Entellan). They were examined under a light microscope and their microphotographs (Olympus DP12, Japan) were taken.

Statistical analysis

Data were evaluated statistically. For all variables obtained, parametric test assumptions were applied before the significance tests. Variables were analyzed with the Shapiro–Wilk test for normality and the Levene test for homogeneity. One-way analysis of variance (ANOVA) was then used to examine the difference between the variables statistically. Tukey test was used as the Posthoc test for the variables in which the difference between the groups was significant. All statistical analyses were conducted with a minimum margin of error of 5%. IBM SPSS 23.0 software package was used in all statistical analyses.

RESULT AND DISCUSSION

Spermatological parameters

There was no statistical difference between the spermatological parameters (motility, abnormal sperm, dead–live ratio) of the C, V, and M groups. The S group showed decreased motility and increased abnormal and dead–live ratios (compared to C, V, and M groups). In the SM group, motility, abnormal sperm, and dead–live ratio were measured as 63.12 ± 0.58, 12.38 ± 0.5, and 22.88 ± 0.44 (P<0.05 compared to the S group). Spermatological parameters are shown in Fig. 1.

Serum hormone and cytokine levels

Serum FSH, LH, and Testosterone levels were similar in groups C and V. It was similar in the M and S groups and significantly lower than in control. The lowest hormone levels were in the SM group (P<0.05).

Serum corticosterone levels of groups V and M were similar to the control group. While there was a significant increase in the S group compared to the control, Melatonin treatment in the SM group significantly decreased it compared to the stress group (P<0.05). The highest serum Melatonin level was measured in group M (P<0.05). While the serum Melatonin levels of the V and SM groups were similar to those of the C group, a statistically significant decrease was observed in the serum Melatonin levels in the S group.

Serum TNF–α and IL–6 levels were highest in the S group compared to the other groups (P<0.05), and there was no statistically significant difference in the C, V, M, and SM groups. Moreover, serum IL–10 levels were significantly lower in the S group than in the other groups (P<0.05). No statistically significant difference existed in the C, V, M, and SM groups. Serum hormone and cytokine levels are shown in Fig. 2.

Testicular tissue oxidative stress, and cytokine levels

Testicular tissue MDA levels were similar in the C, V, M, and SM groups but were higher in the S group than in the other groups (P<0.05). GSH levels were also similar in the C, V, M, and SM groups but significantly decreased in the S group compared to the other groups (P<0.05). GSH–Px and catalase levels were not statistically different in the C, V, and M groups but significantly lower in the S group and higher in the SM group than the S group (P<0.05).

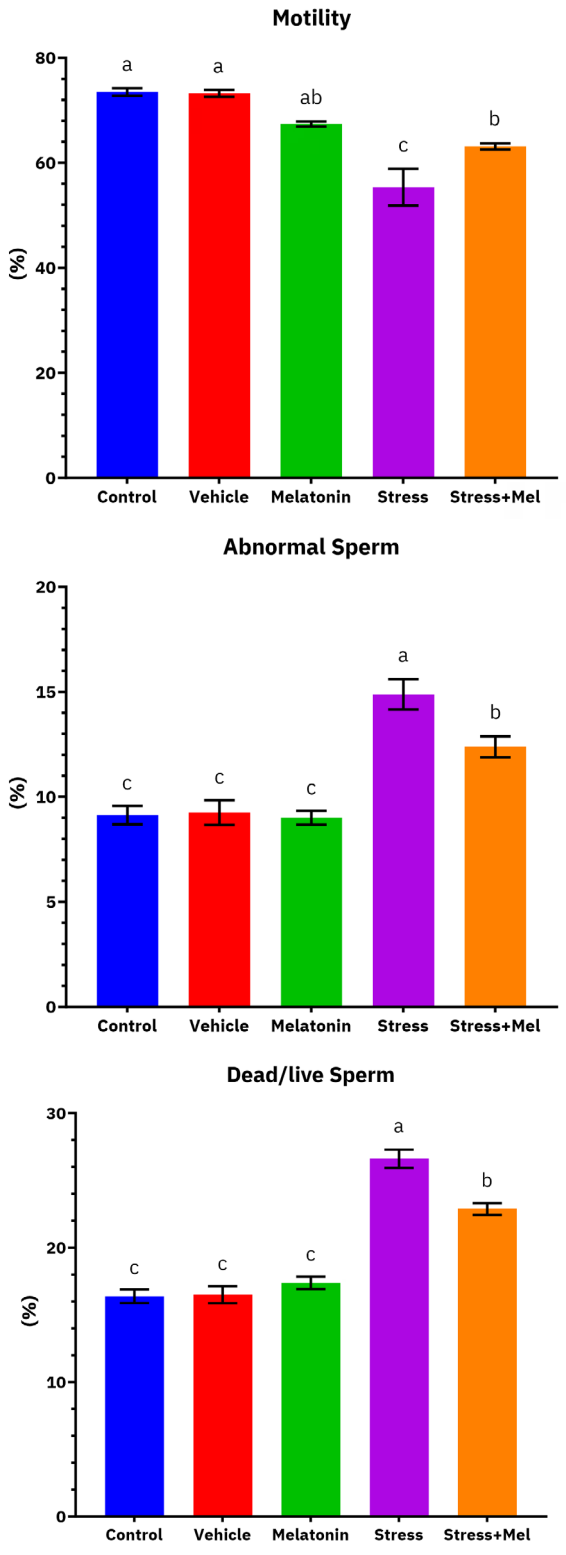

FIGURE 1. Spermatological parameters. Values are Mean±SEM and different letters (a, b, c) indicated represent statistical significance between groups (P<0.05)

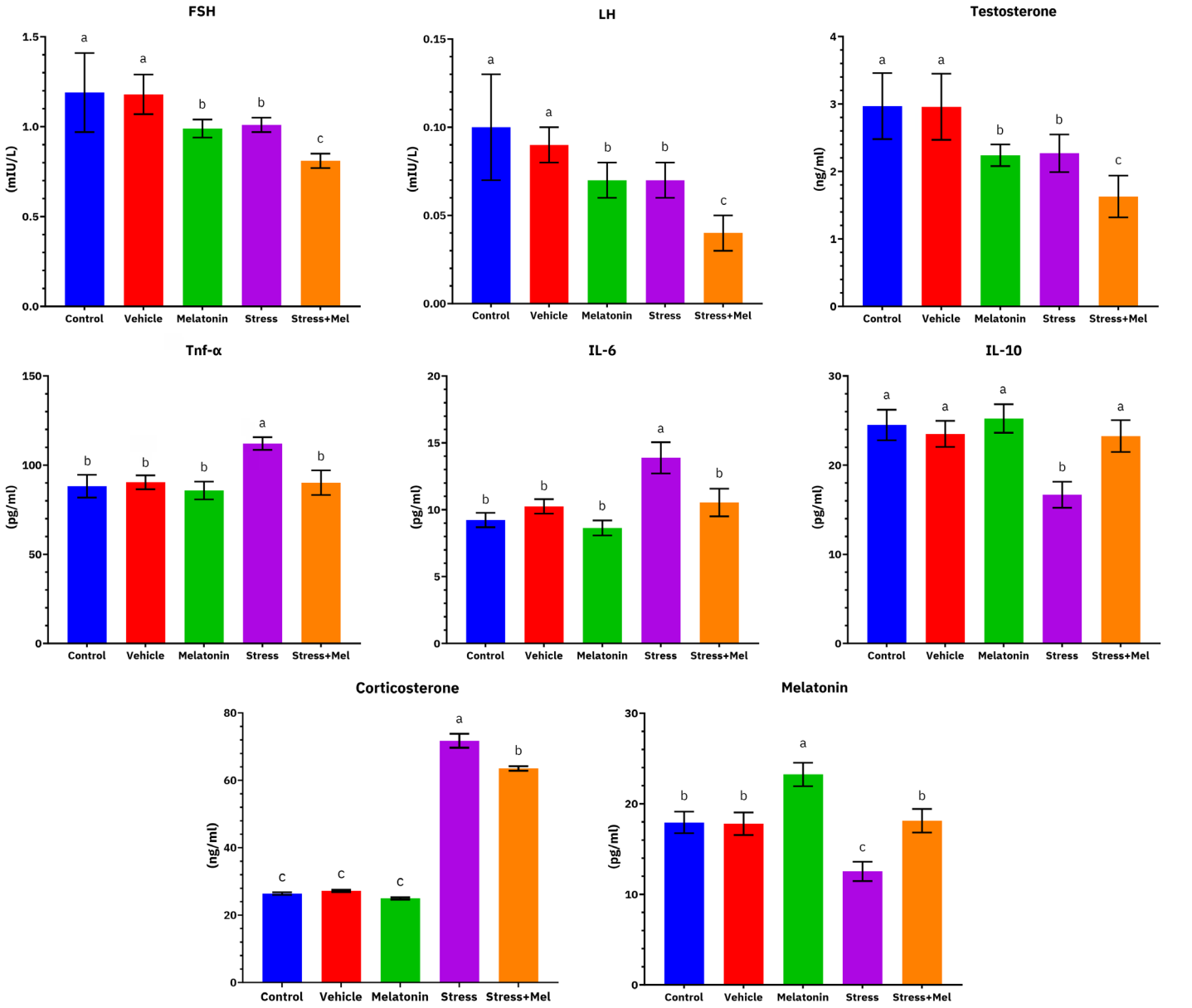

FIGURE 2. Serum hormone and cytokine levels. Values are Mean ± SEM and different letters (a, b, c) indicated represent statistical significance between groups (*P*<0.05)

Testicular pro-inflammatory cytokine (NF-κB, TNF-α, IL-1β, and IL-6) and anti-inflammatory cytokine (IL-10) levels of the V, M, and SM groups were similar to that of the control group. In group S, there was an increase in proinflammatory cytokine levels and a decrease in anti-inflammatory cytokine levels compared to other groups (*P*<0,05). Testicular tissue oxidative stress and cytokine levels are shown in Fig. 3.

Histopathological findings

In the current study, when the histological sections of the seminiferous tubules (ST) of the C, V, and M groups were examined under 20 and 100 µm magnification, it was seen that the ST were regular in terms of shape, structure and mature sperm density in the tubule lumen. There was no significant difference between them. Morphologically, in the S group, deformations in seminiferous tubule shape and structures and decreases in sperm density in the seminiferous tubule lumen were observed compared to the control group. In the SM group, the shape and structure of the ST were similar to the control group, while the sperm density in the seminiferous tubule lumen was similar to the stress group. The histopathological images are shown in Fig. 4.

In this study, which was conducted to examine the changes that stress can cause in the reproductive system in male rats and the possible effects of Melatonin on this situation; To confirm whether a chronic unpredictable stress pattern has occurred, serum corticosterone levels were measured, and serum corticosterone levels were found to be high, it is the primary glucocorticoid corticosteroid

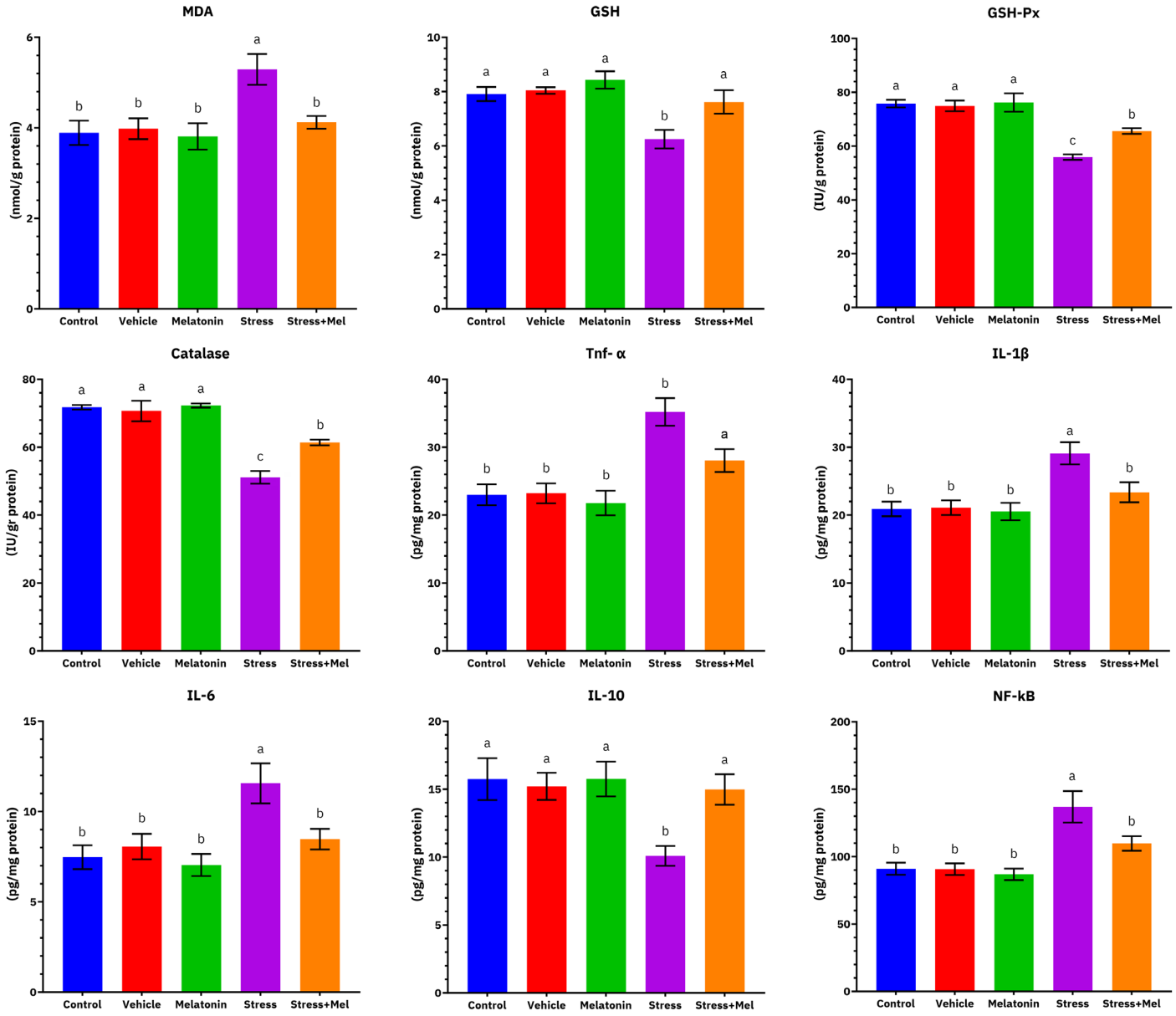

FIGURE 3. Testicular tissue oxidative stress and cytokine levels. Values are Mean ± SEM and different letters (a, b, c) indicated represent statistical significance between groups (*P*<0.05)

secreted from the adrenal gland in rats (Wistar albino) [24]. This finding shows that the chronic stress model applied is effective.

Studies have shown that exogenous Melatonin causes an increase in Melatonin levels in both serum and testes [1, 15]. In the study, it was measured the serum Melatonin levels to reveal the presence of orally administered Melatonin in the circulatory system, and it was found to be significantly higher. Due to the toxicity of DMSO, which is used as a solvent for some substances, it is recommended not to use more concentrated than 10%. However, in cases where it is used, it is recommended to add a new group to the study as an additional solvent group [20]. Because it was dissolved Melatonin in 10% DMSO in the present study, it was added the solvent group to the study. Furthermore, it was found that the related parameters were not different from the control group.

Guvenc *et al.* [21] and Guo *et al.* [1] showed that sperm parameters were negatively correlated with MDA levels and positively correlated with antioxidant enzyme levels. This study shows a similar correlation between oxidative stress parameters and spermatological parameters among the Stress and Stress+Melatonin groups. In addition, in the present study, it was determined that the use of Melatonin for three weeks in the M group did not cause a change in the testicular oxidant–antioxidant balance. Guo *et al.* [1] showed that Melatonin treatment did not affect testicular oxidant–antioxidant capacity in healthy male mice (*BALB/c*) (*Mus musculus*).

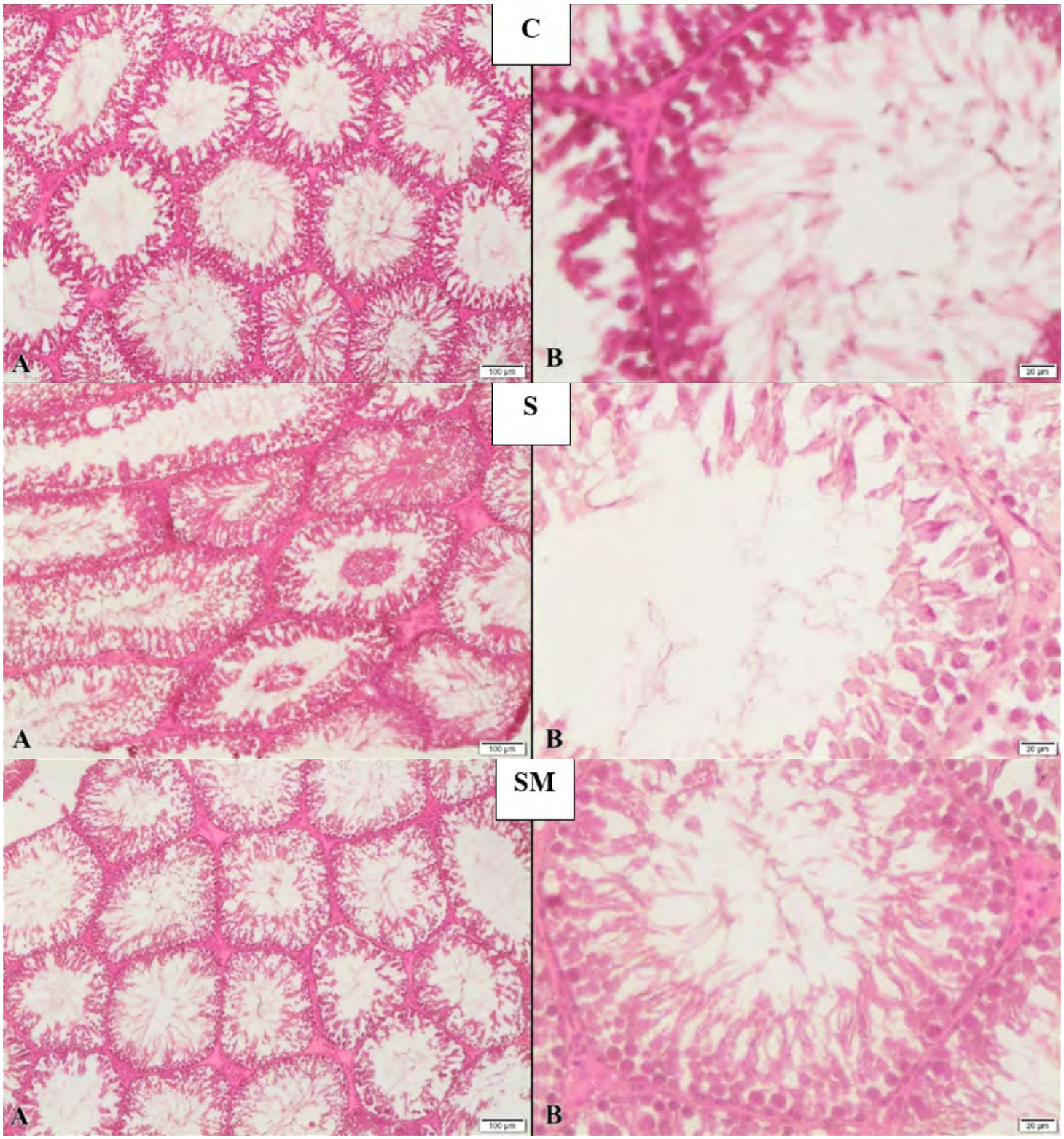

FIGURE 4. Seminiferous tubule histological images of Control (C), Stress (S) and Stress+Melatonin (SM) groups A: Histological structure of the seminiferous tubules (ST) of the magnification, H&E × 100 µm B: Histological structure of the ST lumen of the magnification, H&E × 20 µm

In the present study was found that stress increased proinflammatory cytokine levels in serum and testicular tissue and decreased anti-inflammatory cytokine (IL-10) levels. Also, it was found that the use of Melatonin caused a decrease in increased proinflammatory cytokine levels and an increase in decreased anti-inflammatory cytokine levels in the SM group. Similar findings are seen in previous studies [1, 16]. A study in mice showed that chronic unpredictable stress causes deterioration in spermatological parameters (sperm count, motility, abnormal acrosome) [4]. Nirupama *et al.* [3] stated in their study that most of the semen damage caused by stress is irreversibly affected. However, Guo *et al.* [1] reported that exogenous Melatonin increased sperm density, was ineffective on testicular weight and was curative against testicular damage in restraint stress mice. In addition, the use of Melatonin in testicular damage caused by different stimuli causes an increase in sperm count, motility, and sperm viability and a decrease in abnormal sperm [25]. In the current study, while stress caused deterioration in sperm, Melatonin statistically improved the sperm damage caused by stress in the SM group.

Chronic stress causes decreases FSH, LH, and Testosterone levels through Gonadotropin-releasing hormone (GnRH [26], LH reduction also contributes to the reduction of Testosterone levels [27, 28]. On the other hand, glucocorticoid levels that increase with stress cause apoptosis in Leydig cells and decrease Testosterone production [27]. In this study, 8-week stress decreased rats' serum FSH, LH, and Testosterone levels.

The effects of Melatonin on reproductive system hormones have been studied for a long time, but its effects are still unclear. Wu *et al.* [29] showed that rats that were using Melatonin inhibited Testosterone release via cyclic adenosine monophosphate (cAMP) in a dose-dependent manner by affecting Leydig cells, and the Melatonin receptor antagonist luzindole eliminated this effect. The effect of Melatonin is also variable in studies conducted on women. For example, it has been observed that chronic Melatonin administration reduces LH levels [30], and acute Melatonin use causes an increase in LH levels in premenopausal women with regular cycles [31]. It has been argued that LH release was higher in patients who received low-dose Melatonin therapy for one year for sleep disorders than in healthy adults. However, it is early to argue for a definitive role of Melatonin as a central or peripheral modulator of the reproductive system [32]. It has been reported that the levels of FSH, LH, and Testosterone in male rats administered Melatonin decrease depending on the dose and time [33]. On the other hand, no correlation was found between low Testosterone levels in people taking low doses of Melatonin [34]. In the present study, it was observed that the use of Melatonin decreased serum FSH, LH, and Testosterone levels in both healthy and stressed rats. It will likely decrease reproductive hormones by showing a cumulative effect when Melatonin and stress are applied together.

Paracrine/autocrine factors play important roles in Spermatogenesis. Testicular cytokines and growth factors are produced in many places in the testis, including Sertoli cells, Leydig cells, Peritubular cells, spermatogonia, differentiated spermatogonia, and even spermatozoa. It has been shown to affect the function and secretion of Leydig and Sertoli cells [35]. Guo *et al.* [1] stated that Melatonin has an antiapoptotic effect using the NFKB/i-NOS pathway and an antioxidant effect using the NRF2-HO-1 pathway in spermatological parameters that decrease in restraint-stressed mice. According to Bahrami *et al.* [36], exogenous Melatonin has an antioxidant effect by increasing total antioxidant capacity, antioxidant enzyme levels such as GSH, decreasing MDA levels, which is an indicator of lipid peroxidation levels, and an anti-inflammatory effect by reducing proinflammatory cytokine levels such as IL-1β and TNF-α, which has a healing effect on spermatological parameters in reproductive damage. In this study, spermatological parameters were not adversely affected despite the decreased hormone levels. This situation can be explained by the fact that Spermatogenesis is a complex process, and spermatological parameters are also affected by non-hormonal mechanisms.

Stress also causes histological changes in testicular tissue. For example, it is known that chronic stress causes deterioration in testicular functions by affecting the tight junction proteins in the blood testicular barrier structure [4]. Koksal *et al.* [37] showed that Melatonin brought the histopathological changes closer to the control in the ipsilateral and wholly normalized in the contralateral in rats with testicular ischemia-reperfusion. A study examining Melatonin's protective effect in acute testicular torsion found that Melatonin partially corrected histological deterioration [38]. In the present study was found that the morphological changes caused by chronic unpredictable stress in rats were partially corrected by exogenous Melatonin.

CONCLUSION

In the current study, stress application causes decrease in spermatological parameters by increasing serum corticosterone levels, oxidative stress, inflammatory cytokines, and decreasing reproductive hormone levels. It was observed that the administration of Melatonin in stress groups caused improvements in spermatological parameters by improving the oxidant-antioxidant balance and reducing inflammatory cytokines. In conclusion, 10 $mg \cdot kg^{-1} \cdot day^{-1}$ of Melatonin for three weeks reduces serum glucocorticoid levels. It stimulates antioxidant and anti-inflammatory mechanisms, resulting in improvements in sperm parameters. However, in pathophysiological conditions such as stress, hormonal status should be considered when used for longer than the specified dose and time.

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Conflict of Interests

The authors of this study declare that there is no conflict of interest with the publication of this manuscript.

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