



Melatonin Ameliorates Testes against Forced Treadmill Exercise Training on Spermatogenesis in Rats

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Abstract

Introduction: It is well documented that some forced exercises can have bad effects on the genital system. Melatonin is a potent antioxidant that is effective in reducing the physical stress.

Aim: The aim of this study was to evaluate the supportive effect of melatonin on the quality of spermatogenesis, including count, motility, morphology, viability, and apoptosis of sperm following a forced treadmill exercise.

Materials and methods: A total of 40 adult male Sprague-Dawley rats were used in this experimental study. All rats were divided into five groups: control group, sham M group, melatonin (M) group, forced treadmill exercise group (Ft), and melatonin with forced treadmill exercise (MFt) group. The experimental group was trained to force treadmill stress for one hour of forced treadmill exercise daily, five days weekly for eight weeks. Then the sperm quality parameters were measured after dissection and removal of epididymis. Spermatogenesis and germ cell apoptosis were evaluated using Miller and Johnsen's score and TUNEL staining separately.

Results: Results showed the count, motility, morphology, and viability of sperm in forced treadmill-melatonin administrated group, significantly enhanced by melatonin treatment compared to the treadmill exercise group ($p \leq 0.01$). Also the number of apoptotic germ cells significantly decreased in treadmill exercised-melatonin administrated group compared to the treadmill exercised group.

Conclusions: These results suggest that administration of melatonin can protect the testis against the detrimental effect of forced treadmill exercise in adult male rats.

Keywords

forced treadmill exercise, melatonin, rats, sperm quality, spermatogenesis

INTRODUCTION

Infertility is one of the major health and social problems of all human societies in the present age and is one of the major causes of concern in couples who have begun living together. Exposure to external factors before and after pregnancy, and during the early postnatal stages can endanger their reproductive ability and the health of their offspring.^{1,2} According to sport studies, researchers attribute these reproductive disorders to a series of physical activities that affect the male genital system such as the forced swimming exercise.¹⁻³ Some of the factors affecting male infertility depend on professional sport factors. Exercise is likely to decrease testosterone levels.⁴ This change can impair the function of sperm cells which can ultimately decrease sperm quality and sterility. The oxygen consumed by the cells is converted to free radicals or reactive oxygen species (ROS) by the resuscitation of an electron in the mitochondria, as exercise increases the oxygen consumption ten to twenty times, leading to the promotion of ROS production in cells and tissues through the microsomal electron transport system during metabolism.⁵ The drug is also produced in cells and tissues. Consequently, “exercise exerts a severe oxidative stress on cells and tissues”.^{6,7} However, ROS is effectively mediated by the cell’s antioxidant defense system. ROS results in lipid peroxidation, membrane permeability, and apoptosis. This leads to DNA fragmentation of spermatogenesis cells and ultimately apoptosis of spermatogenesis cells. Exogenous antioxidants need to be added outside the cell to compensate for cellular antioxidant defense in order to prevent cellular damage.^{7,8} Antioxidants are molecules that can prevent or slow the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to the oxidizing agent. The oxidation reaction is capable of producing free radicals.^{7,9-11}

Melatonin is a hormone that is made in the body by the pineal gland and a number of other organs such as the retina, lacrimal glands, intestines, and bone marrow or enterochromaffin cells of the gastrointestinal tract.^{4,12} Melatonin is also a potent antioxidant that is readily available. It crosses the cell membrane as an important antioxidant that is active in both water and fat phases. Researchers have noted this.² Melatonin also reduces the effects of testicular injury in rats exposed to nandrolone. On the other hand, the presence of melatonin receptors in the testis has also been demonstrated¹³ given that the amount of testicular antioxidant enzymes are lower than other tissues, such as the liver and kidney, and also given that it is likely that “intense exercise exerts a great deal of oxidative stress on the testis”.^{2,12} Considering the high level of specialized exercise, for example, at the Olympic level or the professional level among male athletes and the wide range of adverse effects, especially sterility^{4,12,14}, it seems important to employ a strategy to reduce these complications. Thus, melatonin is used as a potent antioxidant in this design to reduce the physical stress (exercise), thereby balancing the testicular antioxidant system, improving apoptosis and sperm quality.^{4,15-18}

AIM

Given that no attention has been paid to the molecular mechanism of the effect of melatonin on testicular tissue induced by running exercise, the main purpose of this study was to identify the molecular mechanisms involved in the effect of melatonin on apoptosis in severe exercises such as forced treadmill exercise, so that some strategies may be developed in the future.

MATERIALS AND METHODS

Animals and exercise procedure

In this experimental study, the rats were accidentally separated into five different groups with 8 animals each. All animal care was performed according to guidelines of the Mazandaran University of Medical Sciences (Sari, Iran).

Group 1 (control group): without any injection or exercise protocol. Group 2 (sham M): rats received the solvent of melatonin (ethanol %1) as a vehicle (ip); Group 3 (M): rats received 10 mg/kg of melatonin weekly for eight weeks (ip). Group 4 (Ft): the exercise protocol was engaged for one hour of forced treadmill exercise per day, five days a week for eight weeks. Group 5 (MFt): rats received 10 mg/kg/week of melatonin and the exercise protocol was engaged for one hour of forced treadmill exercise per day, five days a week for eight weeks.

Sample collection

Measurement of testes weight, seminal vesicle, prostate and epididymal weights were evidenced along with measuring epididymal sperm count, morphology, viability, and motility. Testes were placed overnight in 10% buffered formaldehyde (37% formaldehyde, Merck, Darmstadt, Germany).

Sperm analysis

The epididymis was minced with scissors in a petri dish containing 5 ml of Ham’s F10 medium and incubated at 37°C for 15 minutes to allow the spermatozoa to exit from the tissue. To analyze sperm motility, 10 µl of sperm suspension was placed on a slide and then covered with a coverslip. The percentage of motile sperms was calculated by selecting 10 microscopic fields at 400× magnifications.^{19,20} To determine sperm viability, 10 µl of sperm suspension was blended with an equal volume of eosin-nigrosine dye. The percentage of live sperms (colourless or light pink) and dead sperms (red or dark pink color) were calculated by counting 200 sperms in each slide with observation by light microscope at 1000× magnification. The sperm count was calculated by mixing 50 µl of sperm suspension with 200 µl of distilled water. 10 µl of this diluted suspension was moved to each of the counting chambers of the Neubau-

er haemocytometer and stand for 5 minutes for cells sedimentation. After this 5 minutes, sperm count was analyzed with a light microscope at 400× magnification.^{3,21} Sperm morphology was evaluated by using eosin Y staining. One drop of sperm suspension was mixed with an equal amount of 1% eosin Y dye. After 30 minutes, smears were prepared and allowed to dry in the air, and were mounted and then covered with a coverslips. Two hundred sperm cells were inspected in each slide to investigate the morphological abnormalities at 1000× magnification.^{22,23} Unusual structure or morphology of head and tail of spermatozoa was considered as abnormal morphology of sperm.

Evaluation of spermatogenesis

In the current study, Johnsen's and Miller's scores were used to evaluate the spermatogenesis. Spermatogenesis was ranked by calculating Johnsen's score (from 1-10) and measuring the number of germinal cell layers in the testes. Ten seminiferous tubules were considered to count germinal epithelial layers according to the Miller's scores. The scores of spermatogenesis quality in seminiferous tubules, were obtained according to the maturity of germ cells.²¹

Histopathological analysis

After fixation, testes were embedded in paraffin wax and then 5- μ m thick sections were obtained by using a rotary microtome. The prepared slides were stained with haematoxylin and eosin and then observed by a light microscope.

Germ cell apoptosis by TUNEL assay

Germ cell apoptosis was evaluated by TUNEL staining according to the TUNEL [terminal deoxynucleotidyl transferase (TdT) enzyme-mediated dUTP nick end labeling] assay kit protocol. 5- μ m thick paraffin-embedded sections were deparaffinised and then rehydrated in graded alcohol. Sections were incubated in blocking solution (3% H₂O₂) to neutralize endogenous peroxidases for 10 minutes. Then, sections were washed with PBS and were incubated with TdT for 60 minutes at 37°C. After washing the slides with PBS, they were incubated with anti-digoxigenin peroxidase antibodies. DAB substrate was applied for 10 minutes to stain positive apoptotic cell brown. At least, 10 seminiferous tubules were selected in each section for counting apoptotic cells by light microscope observation.

RESULTS

Body weight changes and accessory sex organs weight

Body weight changes significantly decreased in the Ft (5.86±0.65) and MFt (8.10±0.72) groups ($p<0.001$) via

forced treadmill exercise as compared to the control (11.36±0.45), sham M (11.03±0.56) and M (11.60±0.30) groups. Melatonin treatment increased the body weight changes in the MFt group (8.10±0.72) in comparison to the Ft group (5.86±0.65) ($p<0.01$).

Seminal vesicle weight in the Ft group (0.13±0.05) was significantly lower than in the control (0.36±0.05), sham M (0.36±0.05) and M groups (0.36±0.05) ($p<0.01$). Also, seminal vesicle weight in the MFt group (0.16±0.05) was lower than in the control, sham M, and M groups ($p<0.05$). Melatonin treatment increased the seminal vesicle weight in the MFt group as compared to the Ft group, but this change was not significant. No significant differences were observed in prostate weight of different groups (0.56±0.05, 0.50±0.10, 0.50±0.10, 0.41±0.01, and 0.46±0.01, respectively in the control, sham M, M, Ft and MFt groups, $p>0.05$) (Fig. 1).

Sperm parameter analysis

Mean sperm normal morphology percentage in the Ft (65.33±2.08) and MFt (71.33±2.51) groups were significantly lower than in the control group (85.33±4.16) ($p<0.001$). Melatonin treatment in the MFt group (71.33±2.51) increased sperm normal morphology percentage as compared to the Ft group (65.33±2.08); however, this difference failed to reach significance. The mean sperm concentration in Ft group ($138.30 \times 10^6 \pm 2.08$) significantly decreased as compared to control ($166.00 \times 10^6 \pm 8.88$), sham M ($157.30 \times 10^6 \pm 4.04$), and M ($165.01 \times 10^6 \pm 6.55$) groups ($p<0.001$). Also, sperm concentrations in the MFt group ($143.70 \times 10^6 \pm 1.52$) was significantly lower than in the control, sham M, and M groups ($p<0.01$). No significant difference was observed in the mean sperm concentration of Ft and MFt groups. The mean percentage of progressive forward motility significantly decreased in the Ft group (26.67±4.50) as compared to the control (61.60±4.93), sham M (59.01±6.24), and M groups (60.01±1.01) ($p<0.001$). Also, the mean percentage of progressive motility in MFt group (43.33±0.57) was significantly lower than in the control, sham M, and M groups ($p<0.01$). Melatonin treatment in MFt group (43.33±0.57) increased the progressive motility percentage as compared to the Ft group (26.67±4.50) ($p<0.01$). The mean percentages of sperm viability in the Ft (55.33±5.50) and MFt (64.33±4.04) groups were significantly lower than in the control (88.34±1.15), sham M (86.67±1.52), and M (83.01±2.00) groups ($p<0.001$). The mean percentage of sperm viability significantly increased in the MFt group (64.33±4.04) as compared to that in the Ft group (55.33±5.50, $p<0.05$) (Fig. 2).

Germ cell apoptosis

Forced treadmill exercise in the Ft group (5.2±0.54) dramatically led to an increase in the number of apoptotic germ cells as compared to the control (0.89±0.99), sham M (0.9±0.44), and M (0.69±0.18) groups ($p<0.001$). Interest-

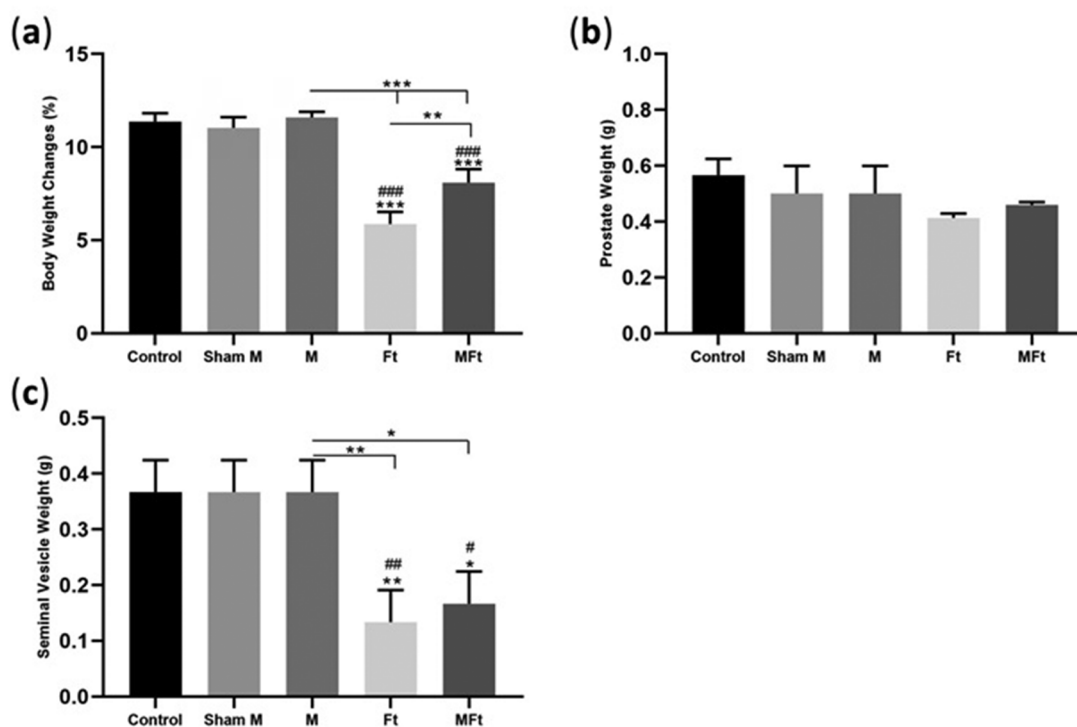


Figure 1. Effects of melatonin on the body weight changes and accessory sex glands weight in forced treadmill exercised rats. (a) the body weight changes, (b) prostate weight, (c) seminal vesicle weight. * $p < 0.05$: in comparison with the control group, ** $p < 0.01$: in comparison with the control group. *** $p < 0.001$: in comparison with the control group, # $p < 0.05$: in comparison with the sham M group, ## $p < 0.01$: in comparison with the sham M group, ### $p < 0.001$: in comparison with the sham M group.

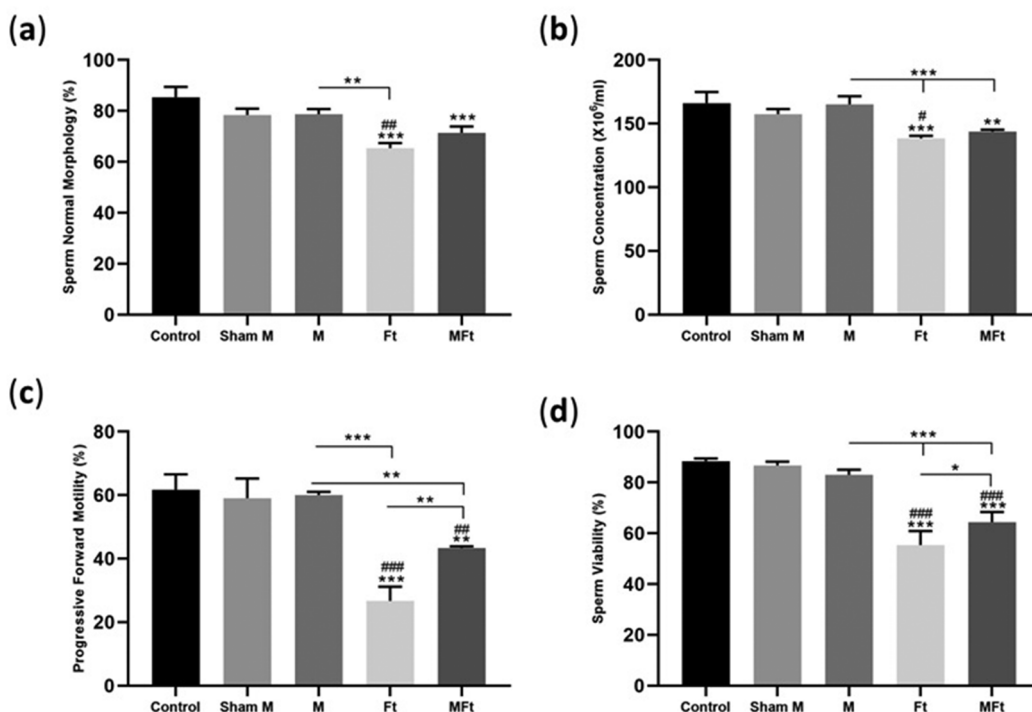


Figure 2. Effects of melatonin on the sperm quality parameters in forced treadmill exercised rats. (a) Sperm normal morphology, (b) Sperm count, (c) Sperm forward motility, (d) Sperm viability. * $p < 0.05$: in comparison with the control group, ** $p < 0.01$: in comparison with the control group. *** $p < 0.001$: in comparison with the control group, # $p < 0.05$: in comparison with the sham M group, ## $p < 0.01$: in comparison with the sham M group, ### $p < 0.001$: in comparison with the sham M group.

ingly, melatonin treatment in the M group (0.69 ± 0.18) significantly decreased the number of apoptotic germ cells as compared to that in the control group (0.89 ± 0.99) ($p < 0.01$). Also, melatonin treatment and forced treadmill exercise in the MFt group (1.8 ± 0.27) significantly decreased apoptotic germ cell in comparison to the non-treated forced exercise group (group Ft) (5.2 ± 0.54 , $p < 0.001$) (Table 1, Fig. 3).

Johnsen's scores

Evaluation of Johnsen's scores in histopathological samples indicated that forced treadmill running exercise led to a significant decrease of spermatogenesis quality in the Ft group (8.1 ± 0.83) as compared to that in the control (9.9 ± 0.52) and sham (9.8 ± 0.31) groups ($p < 0.01$). Melatonin treatment in forced exercise rats (group MFt) increased the Johnsen's scores (9.0 ± 0.67) as compared to the Ft group (8.1 ± 0.83), but this change was not significant (Table 1, Fig. 3).

DISCUSSION

In the current study, we assessed the protective effect of melatonin treatment on harmful effects of forced treadmill running exercise in adult rats. An eight-week forced exercise led to an increase in the apoptotic germ cells in the testes tissue, and to a decrease in the sperm quality parameters, Johnsen's scores, body weight changes, and seminal vesicle weight. We found that melatonin treatment can protect against forced treadmill running exercise harmful effects via decreasing the apoptotic germ cells, increasing the sperm quality parameters (sperm viability and sperm progressive motility), and preventing body weight reduction.

There is a general consensus that intensive exercise stress can lead to testis tissue dysfunction and decrease spermatogenesis quality.⁴ Previous studies have reported that intensive exercise increases germ cell apoptosis in testes.^{13,22,24,25} Oxidative stress in testicular tissue is the main cause of infertility following intensive exercise. Due to enhancement of oxygen consumption during exercise, reactive oxygen species (ROS) are excessively generated and affect testicular normal structure and function.^{7,11,24}

Testis is a susceptible tissue to the oxidative stress because of the high level of cell division and existence of high level of unsaturated fatty acids.²⁶ Also, during extensive exercise, the blood supply of testis decreases and

subsequently testosterone secretion declines and hypoxia in testis leads to apoptosis of germ cells.²⁷ There are several research studies both in animals and humans which have demonstrated that intensive exercise leads to the reduction of sperm quality parameters and production of reproductive hormone, and to the increase in the oxidative stress and lipid peroxidation.^{7,8,11,18,28,29} Intensive exercise induces oxidative stress in testis tissue via decreasing the anti-oxidative enzymes (superoxide dismutase and glutathione peroxidase) and increasing oxidative enzyme (malondialdehyde).³⁰ Swimming exercise is one of the best studied animal models of intensive forced exercise. Previously, Moayeri et al. have reported that melatonin treatment could hamper the detrimental effects of forced swimming exercise against oxidative stress in testis tissue and spermatogenesis.⁴ Similar to our study, their study shows that forced swimming exercise leads to the reduction of sperm quality parameters and anti-oxidative enzymes and to the increase of germ cell apoptosis.⁴ They reported that melatonin treatment significantly reduced apoptosis of germ cells and increased progressive motility and antioxidative enzymes as compared to non-treated animals.

Beneficial effects of melatonin treatment as an antioxidant agents has been reported in different oxidative stress related disorders. In accordance with previous studies, we found that forced treadmill exercise decreased prostate and seminal vesicle weights.^{4,22} Intensive exercise can change energy metabolisms and reduce the secretion of some reproductive hormones such as testosterone, which decreases the testes weight and the accessory sex organs.^{31,32} Similar to our findings, a previous study has reported that melatonin could increase accessory sex organ.⁴ Significant reduction in the spermatogenesis and sperm parameters quality after intensive exercises may be due to a reduction of testosterone production and increase of the oxidative stress.

Based on the literature data, overproduction of ROS causes cell damages in the testicular tissue.²⁴ Melatonin has been shown to have protective effects against the detrimental effect of ROS overproduction during intensive exercise.⁴ Melatonin treatment has indicated the beneficial effects after ischemic/reperfusion injuries of animal model of testicular torsion/detorsion.³³ Previously, it has been stated that melatonin ameliorates testicular torsion/detorsion injuries and increases spermatogenesis quality via increasing the Johnsen's score and serum inhibin B.

Table 1. Effects of melatonin on the number of apoptotic germ cells and Johnsen's scores in forced treadmill exercised rats

	Control	Sham M	M	Ft	MFt
Johnsen's scores	9.9±0.52	9.8±0.31	9.1±0.11	8.1±0.83 ^{**,#}	9.0±0.67
Apoptotic germ cells (n)	0.89±0.99	0.9±0.44	0.69±0.18 ^{**,#}	5.2±0.54 ^{***,###}	1.8±0.27 ^{\$\$\$}

M: melatonin; Ft: forced treadmill exercise; MFt: melatonin + forced treadmill exercise. Values are mean ± SEM; ** $p < 0.01$, in comparison with the control group; *** $p < 0.001$, in comparison with the control group; # $p < 0.01$, in comparison with the sham M group; ### $p < 0.001$, in comparison with the sham M group; \$\$\$ $p < 0.001$, in comparison with the Ft group.

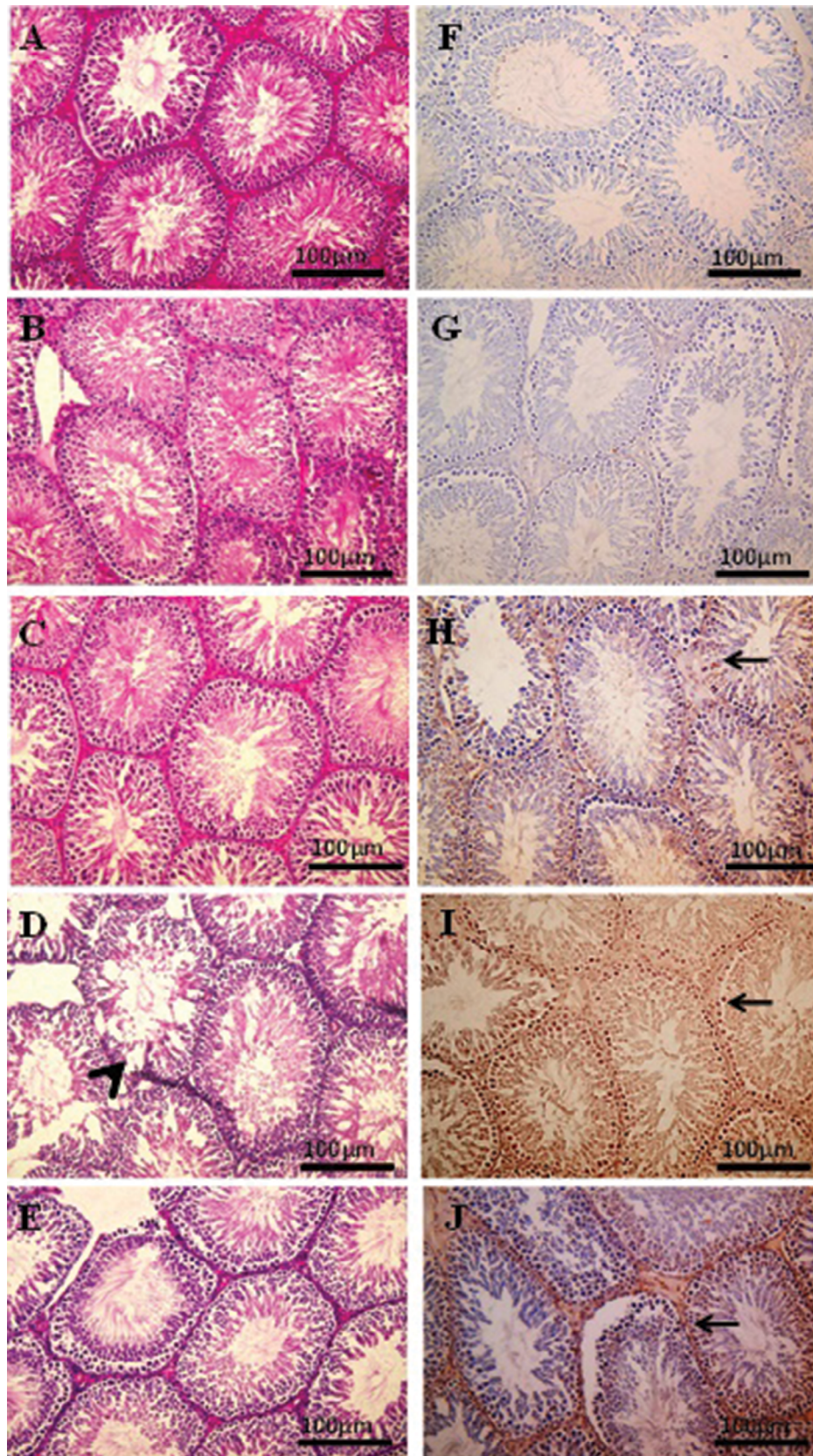


Figure 3. Light micrographs of hematoxylin and eosin (H&E) staining. (A) control group, (B) sham group, (C) melatonin group, (D) forced treadmill exercised group, and (E) exercise + melatonin group demonstrated spermatogenic cell density, and Miller's and Johnsen's scores of the seminiferous tubules. F, G, H, I and J showed the apoptotic index of testis seminiferous tubules with tunnel staining in (F) control group, (G) sham, (H) melatonin group, (I) forced treadmill exercised group, and (J) exercise + melatonin group. Arrow shows the apoptotic cell and arrowhead indicates the spermatogenic cells.

It is of note that melatonin can stimulate antioxidative enzymes which boosts its antioxidative properties and protects against DNA damage.¹² Also, melatonin can protect testis tissue via stimulating testosterone production and angiogenesis properties.³³ There are different studies consistent to our study which have proved the protective role of melatonin against germ cell apoptosis.³⁴⁻³⁶ For instance, a study reported that melatonin treatment decreased the apoptotic cells in testes of Busulfan-treated mice which confirmed the protective role of melatonin against chemotherapeutic agents and enhancement of fertility after cytotoxic therapy.³⁴ In another study, melatonin showed protective effects against cisplatin testicular damages as chemotropic agents.³⁷ Cisplatin administration led to a significant reduction of the testes weight and accessory sex glands; however, melatonin treatment ameliorated these adverse changes.^{8,17,37,38} Also, melatonin could counteract the adverse effects of cisplatin on decreasing epididymal sperm count, motility, and morphology. Take et al. reported that melatonin could protect testis tissue against degenerative changes and cell death of ionizing irradiation exposure.^{25,39} This result may be due to free radical scavenging, antioxidant and anti-apoptotic (caspase-3 inhibition) properties of melatonin.

CONCLUSIONS

These results suggest that administration of melatonin can protect the testis against the detrimental effect of forced treadmill exercise in adult male rats. Forced treadmill running exercise results in testicular oxidative stress and induces testicular injuries. Taken together, melatonin can protect testis tissue and sperm quality parameters.

Acknowledgments

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Conflict of Interest

The authors have no conflicts of interest to declare.

Author contributions

S.A.A.M and M.N.B. performed all experiments and wrote the manuscript; H.G.H. contributed to concept and design; A.M. analyzed TUNEL data; M.Z. did the manuscript editing; Z.M and M.V. analyzed sperm parameter; Z.M did the statistical analysis. All authors read and approved the final manuscript.

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Мелатонин улучшает работу яичек после принудительной тренировки на беговой дорожке в отношении сперматогенеза у крыс

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Резюме

Введение: В литературе имеется достаточно много данных о пагубном влиянии принудительных упражнений на репродуктивную систему. Мелатонин является мощным антиоксидантом, который эффективен в снижении физического стресса.

Цель: Целью данного исследования было оценить поддерживающий эффект мелатонина на качество сперматогенеза, включая количество сперматозоидов, подвижность, морфологию, жизнеспособность и апоптоз после форсированных упражнений на беговой дорожке.

Материалы и методы: В этом экспериментальном исследовании использовали в общей сложности 40 взрослых самцов крыс породы Sprague-Dawley. Все крысы были разделены на 5 групп: контрольная группа, группа ложного мелатонина, группа мелатонина (M), группа принудительной нагрузки на беговой дорожке (Ft) и группа мелатонина принудительной нагрузки на беговой дорожке (MFt). Экспериментальную группу подвергали стрессу, заставляя работать на беговой дорожке в течение 1 часа или ежедневно, пять дней в неделю в течение восьми недель. После рассечения и удаления придатка яичка измеряли параметры качества спермы. Сперматогенез и апоптоз зародышевых клеток оценивали отдельно с использованием шкалы Miller and Johnsen и окрашивания TUNEL.

Результаты: Результаты показали, что подвижность, морфология и жизнеспособность сперматозоидов в группе мелатонина с принудительной нагрузкой на беговой дорожке были значительно повышены по сравнению с группой, подвергшейся принудительной нагрузке на беговой дорожке ($p \leq 0.01$). Кроме того, количество апоптотических зародышевых клеток значительно уменьшилось в группе мелатонина с принудительной нагрузкой на беговой дорожке по сравнению с группой, подвергшейся принудительной нагрузке на беговой дорожке.

Заключение: Эти результаты показывают, что введение мелатонина может защитить семенники от вредного воздействия принудительной нагрузки на беговой дорожке у взрослых самцов крыс.

Ключевые слова

принудительная нагрузка на беговой дорожке, мелатонин, крысы, качество спермы, сперматогенез