

Ameliorative Effect of Melatonin Against Reproductive Toxicity of Tramadol in Rats via the Regulation of Oxidative Stress, Mitochondrial Dysfunction, and Apoptosis-related Gene Expression Signaling Pathway

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Original Article

Abstract

Background: The aim of the present study was to investigate the protective properties of melatonin (MT) against oxidative stress, mitochondrial dysfunction, and apoptosis induced by tramadol-reproductive toxicity in male rats.

Methods: The rats were divided into the 7 groups of control, melatonin (1.5 mg/kg), tramadol (50 mg/kg), and melatonin (1, 1.5 and 2.5 mg/kg) administered 30 minutes before tramadol and vitamin C group (100 mg/kg). All injections were performed intraperitoneally. After administration for 3 consecutive weeks, the animals were killed and testis tissues were used for assessment of oxidative stress markers including lipid peroxidation (LPO), glutathione (GSH) content and protein carbonyl (PrC), and sperm analysis. Mitochondria were isolated from rat's testis using differential centrifugation technique and were studied in terms of mitochondrial viability, mitochondrial membrane potential (MMP), and mitochondrial swelling. The other part of the tissue sample was placed in RNA protector solution for assessment of Bax and Bcl-2 gene expression through real-time polymerase chain reaction (real-time PCR) assay.

Findings: Tramadol caused a significant decline in epidermal sperm count, motility, and morphology, as well as a significant decrease in GSH level and mitochondrial function, and a significant evaluation of LPO, PrC, MMP, and mitochondrial swelling. In addition, tramadol induced a significant decrease in Bcl-2 gene expression, and increase in Bax gene expression. However, pretreatment of rats with MT improved sperm analysis, and testicular antioxidative status, and mitochondrial function. Furthermore, MT pretreatment regulated testicular Bcl-2 and Bax expressions.

Conclusion: Considering the protective effects of MT against reproductive toxicity induced by tramadol, this compound can be used as a possible agent for the prevention and treatment of tramadol-induced reproductive toxicity.

Keyword: Tramadol; Melatonin; Oxidative stress; Mitochondria; Apoptosis

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Introduction

Today, the prevalence of the use of opioids and sedatives is rising exponentially; however, compared with other substances, opioids have been used more. According to the report by the Research and Training Department of Drug Control Headquarters, Tehran, Iran, about 7 million and 6000 people use drugs in Iran, and the tendency to use opioid-like analgesics, including tramadol (TRA), is increasing rapidly among young people, and especially among teenagers.¹

Tramadol is a synthetic 4-phenylpiperidine codeine analog, which is a selective agonist of μ receptors and has a lower affinity for the δ - and κ -opioid receptors.¹ Its side effects are the same as that of other opioid-like drugs (dizziness, nausea, dry mouth, delayed ejaculation, hypotension, constipation, and itching). However, respiratory depression, which is a fatal complication of this group of drugs, is less likely to occur with tramadol use than morphine use.^{2,3} Today, tramadol is one of the most commonly used drugs worldwide, and its long-term use results in physical and psychological dependence. Tramadol abuse can cause reproductive toxicity by reducing sex hormones, impairing sperm morphology, and lowering normal sperm levels.³ It has been suggested that chronic consumption of opioids, such as tramadol and morphine, for 11 weeks decreased sexual activity and follicle-stimulating hormone (FSH).⁴

The mechanisms of tramadol-induced testicular toxicity are induction of oxidative stress and apoptosis in the spermatocytes.^{5,6} Oxidative stress is an imbalance in the formation of free radicals and the antioxidant defense system, meaning the enzymatic and non-enzymatic defense system of the body is unable to counteract the produced free radicals.⁷ Oxidative stress can cause mitochondrial dysfunction and lead to apoptosis, which in turn damages tissue cells.⁸ Mitochondria, as small intracellular organelles, are the main source of free radical production; therefore, mitochondrial damage can initiate the internal (mitochondrial) pathway of apoptosis.⁹ Apoptosis or programmed cell death is the main mechanism in the development and homeostasis of tissues to eliminate unnecessary, infected, mutated, or damaged cells induced by various physiological and pathological factors.¹⁰ Accordingly, antioxidants are considered to prevent the deleterious effects of free radicals in

enhancing mitochondrial dysfunction and the expression of apoptosis-related genes.

Melatonin (N-acetyl-5-methoxytryptamine; MT) is an endogenous indoleamine widely distributed in nature with multiple functions in single-celled organisms, plants, fungi, and animals. Melatonin is mainly secreted at night and plays a key role in regulating sleep. In addition, it is involved in numerous physiological functions, including antioxidant effects and elimination of free radicals, regulation of increased antioxidant enzymes, anti-apoptotic activities, strengthening of the immune system,¹¹ stopping the growth of cancer cells,¹² and preventing aging.^{13,14} The antioxidant activity of MT has been widely shown, and it has been demonstrated that MT can exert its antioxidant effects by inhibiting superoxide, hydroxyl, and nitric oxide free radicals.¹⁵

Given the favorable analgesic effects of tramadol and its limited side effects compared with other opioids, it is impossible to avoid its use. However, various adverse effects on fertility have limited tramadol use. Moreover, because of an increasing trend in tramadol abuse, especially among young people, considering its side effects and providing a solution to reduce its toxicity and limitations is of great importance. Therefore, this study was conducted to investigate the protective effect of MT on the prevention of tramadol-induced reproductive toxicity through the inhibition of oxidative stress and mitochondrial dysfunction, and expression of apoptosis-related genes in male rats.

Methods

Chemicals and drugs

Tramadol and MT (Cat. No.M5250) were obtained from Sigma Aldrich (St. Louis, MO, USA) and were dissolved in normal saline. Other chemicals were obtained from Merck Ltd. (SRL Pvt. Ltd., Mumbai, India). All reagents and chemicals used were of analytical grade. Tramadol and MT doses were determined based on previous studies and modified during pretests.¹⁶⁻¹⁸ All of the solutions were prepared at the beginning of each test day.

Animals treatment

Male Wistar rats (200-250 g) were housed in an air-conditioned room with controlled temperature of 22 ± 2 °C and maintained on a 12:12 hour light-dark cycle with free access to food and water. All experimental procedures were conducted

according to the ethical standards and protocols approved by the Committee of Animal Experimentation of Mazandaran University of Medical Sciences, Sari, Iran. We tried to minimize the number of animals and their suffering.

Animals were randomly divided into 7 groups (6 rats per group) of control group (normal saline), tramadol group (50 mg/kg intraperitoneal),¹ tramadol plus different doses of MT group (1, 1.5, and 2.5 mg/kg intraperitoneal), and vitamin C group (as positive control) (100 mg/kg intraperitoneal). MT was administered 30 minutes before tramadol. The study was carried out over 21 days (3 weeks) and treatments were administrated as single injection in all groups.

All animals were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) 24 hours after the last injection. Next, the testicular tissue of rats was separated. For each rat, the right testis was immediately stored in RNA protector solution (Cib Zist Fan Co., Iran) and kept at -80 °C; one part was used for gene expression analysis by real-time polymerase chain reaction (real-time PCR), and the other part was used for oxidative stress and sperm parameters assay including glutathione (GSH) concentration, lipid peroxidation (LPO), protein carbonyl (PrC), sperm count, motility, and morphology. The left testis was used for mitochondrial isolation, using a differential centrifugation technique, which was used for mitochondrial function assay including mitochondrial viability, mitochondrial membrane potential (MMP), and mitochondrial swelling.

Measurement of total protein

The protein concentration of the samples was determined using Bradford protein assay. Bovine serum albumin (BSA) was used as standard, homogenate sample mixed with Coomassie blue, and after 10 minutes, absorbance was determined at 595 nm using a spectrophotometer.¹⁹

Oxidative stress markers assay

Measurement of LPO: The level of malondialdehyde (MDA), an index of LPO, was determined using dithiobarbituric acid (TBA) test; 0.25 ml sulfuric acid (0.05 mol/l) was added to 0.2 ml samples (1 mg protein/ml), with the addition of 0.3 ml 0.2% TBA. All the microtubes were incubated in a water bath at 80 °C for 30 minutes. At the end, the tubes were cooled in an ice-bath and 0.4 ml n-butanol was added to each tube. Then, they were centrifuged at 3500×g for 10 minutes.

The amount of MDA formed in each of the samples was assessed through measuring the absorbance of the supernatant at 532 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Tecan, Rainbow Thermo, Austria). Tetrametoxypyrone was used as standard and MDA content was expressed as $\mu\text{mol L}^{-1}$.²⁰

Measurement of reduced GSH: Reduced GSH content was determined using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as the indicator and the developed yellow color was read at 412 nm on a spectrophotometer (UV-1601 PC; Shimadzu, Japan). GSH content was expressed as nmol L^{-1} . Testis homogenates were deprotonated with tricarboxylic acid (TCA) through centrifugation. Then, 0.1 ml of tissue homogenates was added to 0.1 mol L⁻¹ of phosphate buffers and 0.04% DTNB in a total volume of 3.0 ml (pH = 7.4). Then, the absorptions were read at 412 nm on the spectrophotometer. A standard curve was drawn using different specified concentrations of GSH solution. With the help of this standard curve, the results were illustrated in $\mu\text{M}/\text{mg}$ of protein.²¹

Measurement of PrC: Free radicals can attack proteins and alter amino acid (lysine, arginine, proline, and histidine) residues, resulting in the production of carbonylated moieties. Thus, the content of PrC moieties can be used as a marker for protein oxidation. For homogenization, 200 μl of brain tissue is needed. Samples were extracted in 500 μl of 20% (w/v) TCA. Then, samples were maintained at 4 °C for 15 minutes. The precipitates were treated with 500 μl of 0.2% 2,4-dinitrophenylhydrazine (DNPH) and 500 μl of 2 mol L⁻¹ hydrochloride (HCl) for the control group, and samples were incubated at room temperature for 1 hour with vortexing at 5-minute intervals. Then, proteins were precipitated by adding 55 μl of 100% TCA. The microtubes were centrifuged and washed 3 times with 1000 μl of ethanol-ethyl acetate mixture. Then, the microtubes were dissolved in 200 μl of 6 mol L⁻¹ guanidine HCl. The carbonyl content was determined by reading the absorbance at 365 nm wavelength.²⁰

Mitochondrial function assay

Isolation of mitochondria: The tissues were homogenized and mitochondria were isolated using differential centrifugation technique. The homogenates were centrifuged at 1000×g for 8 minutes at 4 °C. Supernatants were collected in fresh Eppendorf, and then, centrifuged at 10000×g

for 10 minutes at 4 °C. The obtained pellets were resuspended in isolation buffer and spun again at 12300×g for 10 minutes at 4 °C. The resulting supernatants were transferred and isolation buffer with Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA) [215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.0 μM EGTA, and pH is adjusted to 7.4 with potassium hydroxide (KOH)] was added and the mixture was again stirred at 12,300×g for 10 minutes at 4 °C. Pellets containing pure mitochondria were resuspended in isolation buffer. All procedures were performed on ice throughout the protocol.²²

Determination of mitochondrial function:

Mitochondrial function was assessed with the use of a dye called tetrazolium salt (MTT). This yellow indicator is reduced to purple formazan by mitochondrial succinate dehydrogenase. The formazan crystals were dissolved in dimethyl sulfoxide and its absorbance was measured using an ELISA reader at the wavelength of 570 nm.²³

Determination of the MMP: To evaluate MMP, the uptake of cationic fluorescence probe rhodamine 123 by mitochondria was determined. Rhodamine 123 (10 μM) was added to isolated mitochondria in MMP buffer [220 mM sucrose, 68 mM d-mannitol, 10 mM potassium chloride (KCl), 5 mM KH₂PO₄, 2 mM MgCl₂, 50 μM EGTA, 5 mM sodium succinate, 10 mM HEPES, 2 μM rotenone], then, a fluorescence spectrophotometer (RF-5000 U, Shimadzu Corporation, Japan) was used at the excitation and emission wavelengths of 490 nm and 535 nm, respectively.²⁴

Determination of mitochondrial swelling:

Isolated testis mitochondria were resuspended in swelling buffer containing (in mM) 120 KCl, 5 KH₂PO₄, 20 3-Morpholinopropane sulfonic acid (MOPS), and 10 Tris ·HCl (pH = 7.4). Mitochondrial swelling was followed by light scattering immunoassay (LIA) in isolated mitochondria. ELISA reader was used to determine absorbance at 540 nm. The decreased absorbance is an indicator of increased mitochondrial swelling.²⁵

Evaluation of apoptosis-associated genes (Bcl-2 and Bax) expressions by real-time PCR

For total RNA extraction, 100 mg of each sample was used. Testis samples were digested in a microtube using Hybrid-R™ total RNA isolation kit (Seoul, South Korea) based on the manufacturer's instructions. The total RNA

obtained was free of proteins and DNA contamination. Next, extracted RNA integrity was determined by certain qualitative and quantitative methods. For quantitative measuring, 3 μl total RNA was mixed with 97 μl diluted water and absorption was measured at 260 and 280 nm using a spectrophotometer (Agilent Technologies, Inc., USA). For qualification testing, agarose gel electrophoresis was utilized. Complementary DNA (cDNA) was synthesized using PrimeScript™ RT reagent Kit (Perfect Real Time), Takara cDNA Synthesis Kit. Real-time PCR was performed for all the samples using the specific primers (Table 1) of Bcl-2 and Bax, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene by a real-time analyzer (Rotor-Gene 6000; Corbett Life Science Pty. Ltd., Sydney, Australia).

Table 1. Special primers for Bcl-2 and Bax genes and glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene used for quantitative real-time polymerase chain reaction (PCR)

Gene	Forward primers	Reverse primers
GAPDH	5'CCCCCAATGTA TCCGTTGTG3'	5'TAGCCCAGGAT GCCCTTTAGT3'
Bcl-2	5'ACTTCTCTCGTC GCTACCGTCGC3'	5'AGAGCGATGTT GTCCACCAGGG3'
Bax	5'CCAGGACGCAT CCACCAAGAAG3'	5'CCCAGTTGAAG TTGCCGTCTGC3'

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

Table 2 shows the real-time PCR program for Bcl-2, Bax, and GAPDH genes. Gene expression analysis was carried out using the 2^{-ΔΔCT} method.²⁶

Table 2. Real-time polymerase chain reaction (PCR)

Cycle	Cycle Point
Hold	Hold at 95 °C, 15 minutes 0 seconds
Cycling (40 repeats)	Step 1: Hold at 95 °C, 15 seconds Step 2: Hold at 60 °C, 30 seconds Step 3: Hold at 72 °C, 15 seconds, acquiring to Cycling A([Green][1][1])
Melt	Ramp from 72 °C to 95 °C Hold for 90 seconds on the 1 st step Hold for 5 seconds on the next steps, Melt A([Green][1][1])

Sperm characteristics assay

Assessment of sperm count: Semen samples were prepared from the caudal epididymis separated from the testis and placed in a Petri dish. Epididymal spermatozoa were obtained by

mincing the epididymis with scissors. Diluted sperm suspension was placed in physiological saline and incubated at 32 °C for 10 minutes. The epididymal sperm count was determined using a hemocytometer; 5 ml of diluted sperm was placed on the central square of the Neubauer hemocytometer slide. After counting the sperms using a microscope at $\times 40$ magnification in 5 squares, their numbers were expressed as 1 million in 1 ml of sample size.²⁷

Assessment of sperm motility: Sperm motility was measured according to the World Health Organization (WHO) guidelines. The count was evaluated twice for each sample by a microscope at $\times 40$ magnifications and the means were announced. At least 200 sperms per animal were examined. Numbers of motile and non-motile sperm were counted. Sperm motility was expressed as a percentage of motile sperm of total sperm calculated in each replication.²⁸

Assessment of sperm morphology: Sperm was smeared onto glass slides and allowed to air dry overnight for morphological defects detection. The slides were stained with 1% eosin-Y/5% nigrosin. The specimens were examined under a microscope at $\times 100$ magnifications for morphological abnormalities such as amorphous, bicephalic, hookless, coiled, or abnormal tails. At least 200 sperms per animal were examined and the percentage of abnormal sperm was calculated.²⁹

Results are presented as mean \pm standard deviation (SD). All statistical analyses were performed using one-way ANOVA and Kruskal-Wallis tests, followed by Tukey's test in SPSS software (version 14; SPSS Inc., Chicago, IL, USA). Average, standard, minimum, and maximum deviations were calculated for each group of data. Statistical significance was set at $P < 0.05$. For gene expression analysis, Bcl-2 and Bax genes expression in relation to GAPDH as a reference gene were measured using Ct variations and $2^{-\Delta\Delta Ct}$ formula.

Results

Effects of melatonin on malondialdehyde (MDA) formation in testis tissue: The major product of LPO is MDA the concentration of which is generally presented as the total level of LPO products. Tramadol administration led to the enhancement of MDA formation ($P < 0.010$) compared to the control group. The pretreatment with MT for 21 consecutive days at both doses (medium and high)

led to a significant ($P < 0.010$ and $P < 0.001$, respectively) decrease in tramadol-induced LPO compared to the tramadol treated group. There was no significant difference in the MDA levels between lower dose of MT and tramadol group as shown in figure 1-A.

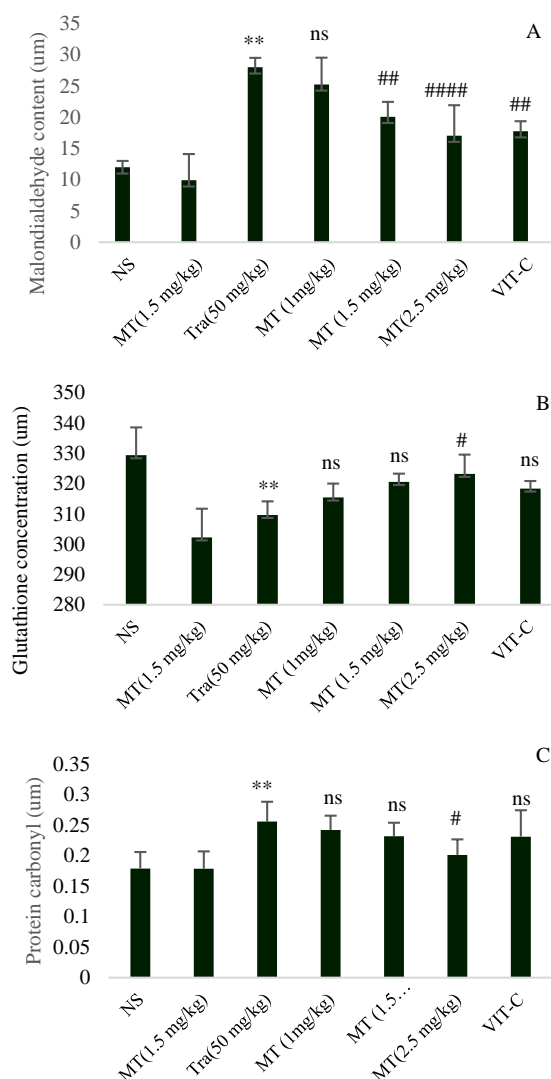


Figure 1. The effects of melatonin on tramadol-induced alterations in the oxidant-antioxidant status of testis tissue of male rats in terms of Malondialdehyde (MDA) content (A), Glutathione (GSH) concentration, and (B) Protein carbonyl (PrC) content (C)

Data are expressed as mean \pm standard deviation (SD) and analyzed by ANOVA followed by Tukey's test ($n = 6$).

**Significantly difference compared to the normal saline group (NS) ($P < 0.010$), NS: Not significant compared to the tramadol group ($P > 0.050$), #Significantly different compared to the tramadol group ($P < 0.050$), ##Significantly different compared to the tramadol group ($P < 0.010$); ###Significantly different compared to the tramadol group ($P < 0.001$).

Effects of melatonin on GSH content in testis tissue: Tramadol administration led to the significant depletion of testis GSH level ($P < 0.010$) as compared to the control group. Pretreatment with MT for 21 consecutive days increased these levels significantly ($P < 0.050$) at higher dose as compared to the tramadol treated group. Medium and low doses of MT pretreatment showed no significant changes in GSH level as compared to the tramadol treated group, as shown in figure 1-B.

Effects of melatonin on PrC content of testis tissue: The significant evaluation of PrC was seen in the tramadol treated group compared to the control group ($P < 0.010$) as shown in figure 1-C. Pretreatment with MT for 21 consecutive days was found to be significantly effective in decreasing these oxidations at higher doses ($P < 0.050$) compared to the tramadol treated group. No significant difference was seen in the level of PrC between medium and low doses of MT and the tramadol treated group.

Effects of melatonin on mitochondrial function in testis tissue: As demonstrated in figure 2-A, tramadol administration significantly ($P < 0.001$) decreased mitochondrial function as compare to the control group in testis mitochondria. Pretreatment with MT for 21 consecutive days increased these levels significantly ($P < 0.050$) at medium and high doses as compared to the tramadol treated group. No significant difference was observed in mitochondrial function between low dose of MT and the tramadol treated group.

Effects of melatonin on MMP in testis tissue: MMP collapse as an electrochemical potential is a sign of mitochondrial dysfunction. As shown in figure 2-B, significant ($P < 0.010$) induction MMP collapse was observed after tramadol administration compared to the control group. Pretreatment with MT for 21 consecutive days was found to be significantly effective in the prevention of tramadol-induced mitochondrial MMP collapse at higher doses ($P < 0.050$) as compared to the tramadol treated group. However, medium and low doses of MT did not develop MMP collapse in compare to the tramadol treated group.

Effects of melatonin on mitochondrial swelling in testis tissue: A significant increase in mitochondrial swelling was found after 21 consecutive days in the tramadol treated group as compared to the control group ($P < 0.001$). The pretreatment with MT at higher doses significantly

($P < 0.010$) decreased mitochondrial swelling as compared to the tramadol treated group. Medium and low doses of MT did not cause any significant change in mitochondrial swelling as compared to the tramadol treated group. These results are presented in figure 2-C.

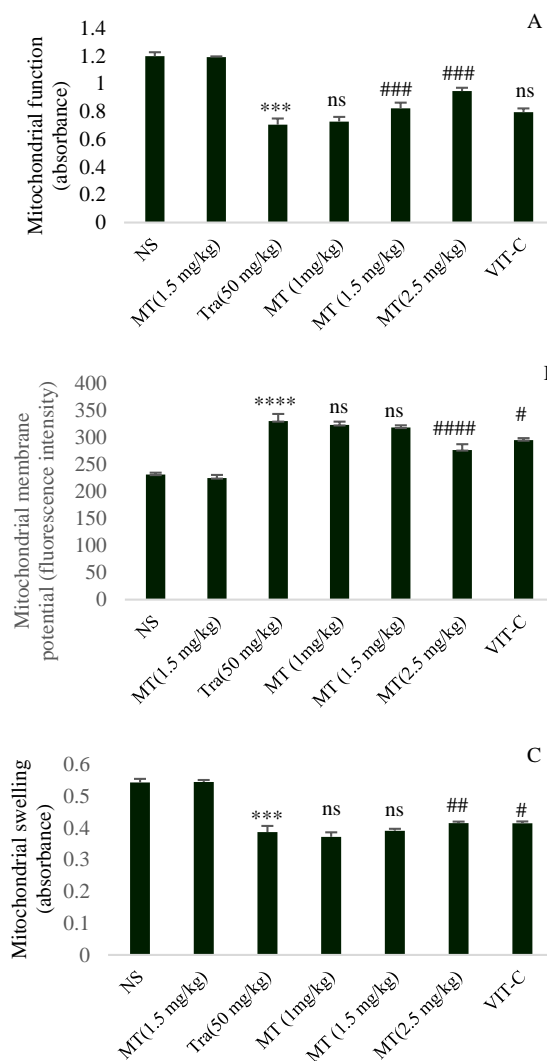


Figure 2. The effects of melatonin on tramadol-induced alteration in the mitochondrial damage in isolated mitochondria of rat's testis in terms of Mitochondrial function (MTT assay) (A), Level of mitochondrial membrane potential (MMP) (B) and mitochondrial swelling (C)

Data are expressed as mean \pm standard deviation (SD) and analyzed by ANOVA followed by Tukey's test ($n = 6$).

*** and **** Significantly different compared to the normal saline group (NS) ($P < 0.001$), NS: Not significant compared to the tramadol group ($P > 0.050$); ## Significantly different compared to the tramadol group ($P < 0.010$), ### and #### Significantly different compared to the tramadol group ($P < 0.001$).

Effects of melatonin on gene expression of Bcl-2 and Bax in testis tissue: According to the real-time PCR analysis, the expression level of Bcl-2 in the tramadol group was significantly decreased compared to the control group. Following pretreatment with higher doses of MT, the level of Bcl-2 expression was increased significantly ($P < 0.010$) as compared to the tramadol treated group. Interestingly, pretreatment with medium and high doses of MT did not cause a significant difference in the level of Bcl-2 expression as compared to the tramadol treated group (Figure 3, A). Moreover, the expression of Bax gene was upregulated in the testis tissue of the tramadol group as compared to the control group. However, medium and high doses of MT inhibited Bax expression compared to the control group ($P < 0.010$ and $P < 0.001$, respectively). Lower doses of MT did not cause any significant changes in Bcl-2 and Bax expression as compared to the tramadol treated group (Figure 3, B).

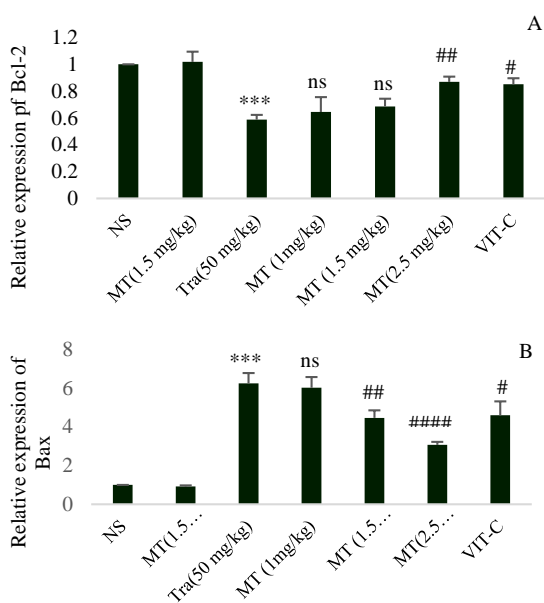


Figure 3. The effects of melatonin on tramadol-induced alteration in the apoptotic-related gene expression in testis tissue of male rats in terms of Anti-apoptotic relative gene expression (Bcl-2) (A) and Pro-apoptotic relative gene expression (Bax) (B)

GAPDH served as an internal standard. Experiments were repeated 3 times, and similar results were obtained. Data are expressed as mean \pm standard deviation (SD) and analyzed by ANOVA followed by Tukey's test ($n = 3$).

*** and **** Significantly different compared to the normal saline group (NS) ($P < 0.001$); NS: Not significant compared to the tramadol group ($P > 0.050$); ## Significantly different compared to the tramadol group ($P < 0.010$); #### Significantly different compared to the tramadol group ($P < 0.001$).

Effects of melatonin on the sperm characteristics: As shown in figures 4-A and B, epididymal sperm analysis in the tramadol treated group revealed a significant reduction in sperm count and motility ($P < 0.001$) after 21 consecutive days compared to the control group. There was poor or slight improvement in sperm count after pretreatment with high doses of MT compared to the tramadol treated group ($P < 0.050$). Interestingly, medium and high doses of MT significantly improved ($P < 0.010$ and $P < 0.001$, respectively) sperm motility compared to the tramadol treated group.

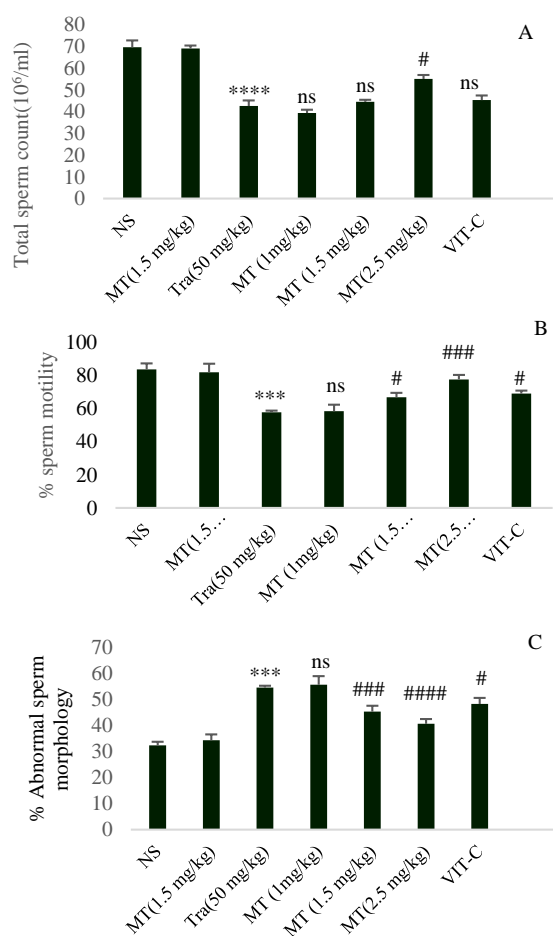


Figure 4. The effects of melatonin on tramadol-induced toxic alteration in sperm parameters in rats in terms of Sperm count (A), Sperm motility (B) and Sperm morphology (C)

Data are expressed as mean \pm standard deviation (SD) and analyzed by ANOVA followed by Tukey's test ($n = 3$).

*** and **** Significantly different compared to the normal saline group (NS) ($P < 0.001$); NS: Not significant compared to the tramadol group ($P > 0.050$); # Significantly different compared to the tramadol group ($P < 0.050$); ### and #### Significantly different compared to the tramadol group ($P < 0.001$)

The impact of MT on sperm morphology in rats treated with tramadol is shown in figure 4-C. Tramadol significantly increased the percentage of sperm abnormality as compared to the control group ($P < 0.001$). Pretreatment with MT for 21 consecutive days decreased these levels significantly ($P < 0.001$) at medium and high doses as compared to the tramadol treated group. There was no significant difference in sperm motility and morphology between low dose of MT and tramadol treated group.

Discussion

The results of this study demonstrated that tramadol exposure in adult male rats for 21 consecutive days caused oxidative damage in testis via oxidation of lipids and proteins and decreased GSH levels, disturbed mitochondrial function through loss of MMP and mitochondrial swelling, increased apoptosis-related gene expression, and decreased sperm count, motility, and normal morphology. Increased levels of oxidative stress and apoptosis seem to be crucial in the pathogenesis of testicular injury in response to tramadol administration. MT pretreatment ameliorated the oxidative stress state, mitochondria damage, and apoptosis in the testes induced by tramadol exposure.

Oxidative stress has a key role in the pathogenesis of many diseases and toxicities such as hepatotoxicity, cardiotoxicity, nephrotoxicity, and reproductive toxicity.³⁰ Furthermore, the role of oxidative stress in tramadol-induced reproductive toxicity has been illustrated in several studies.^{4,6} Tramadol interacts with molecular oxygen and initiates a cascade of reactions producing free radicals, such as superoxide, hydrogen peroxide, hydroxyl, and peroxynitrite by which it can induce LPO and reduce antioxidant enzymes in different tissues.³¹⁻³³ These radicals lead to various oxidative damages of critical mitochondrial membrane integrity, and consequently, induce spermatogenic cell death.²⁸ Spermatogenic cells are sensitive to redox reactions due to their high content of unsaturated fatty acids and a limited amount of cytoplasmic antioxidants, which cause toxicity in testicular tissue.³⁴ Furthermore, our data revealed that tramadol exposure increases testicular susceptibility to reactive oxygen species (ROS) by decreasing the level of GSH (as a non-enzymatic

antioxidant) in testis. In this context, because of the reduced antioxidant defenses in spermatogenic cells, testis tissue is more vulnerable to LPO.³⁵ MT pretreatment reversed the oxidative parameters of testis tissue. In addition, our data was in agreement with that of the study by Adikwu and Bokolo who found that MT inhibited oxidative stress and tramadol-induced nephrotoxicity in rats by increasing GSH levels, decreasing free radicals, decreasing lipid oxidation, and increasing superoxide dismutase activity.³⁶

Oxidative stress can also induce mitochondrial dysfunction via oxidation of thiol groups in mitochondrial membrane proteins that is one of the most important reasons for alterations in mitochondrial permeability.³⁷ In addition, damage to mitochondrial membrane integrity and the opening of the mitochondria permeability transition pores is a key determinant in apoptosis mechanisms.³⁸ MTT results showed that the function of testicular mitochondria decreased significantly after 3 consecutive weeks of tramadol injection, which is consistent with the results of previous studies.^{39,40} In addition, studying MMP and mitochondrial swelling revealed damage to testicular mitochondria due to tramadol administration. It should be noted that pretreatment with MT improved mitochondrial function, the integrity of MMP, and reduced swelling in the testicular mitochondria.

Mitochondria produce excessive amounts of free radicals under pathological conditions that cannot be neutralized by the antioxidant system. The mechanism of ROS-mediated apoptosis appears to involve damage to macromolecules, including mitochondrial membrane proteins, and cell membrane rupture, and release of cytochrome C from mitochondria.⁴¹ Cellular apoptosis is regulated by some mitochondrial proteins, including B-cell lymphoma 2 (Bcl-2) family proteins, which are divided into pro-apoptotic and anti-apoptotic groups and play a key role in accelerating the initiation or inhibition of apoptosis. Bcl-2 is one of the most famous proteins to inhibit apoptosis that, in addition to preventing cytochrome C release from the mitochondria, prevents apoptosis by maintaining mitochondrial membrane integrity.^{37,42}

The level of apoptosis in the testis was evaluated via real-time PCR assay. Bax gene expression decreased due to a significant increase in Bcl-2 gene

expression in tramadol-treated rats compared to the control group. Therefore, this finding is consistent with previous studies that have also shown that tramadol increases the expression of Bax and Caspase-2, and decreases anti-apoptotic Bcl-2 protein by producing free radicals, decreasing intracellular GSH levels, and consequently, damaging the mitochondrial membrane leading to apoptosis in the cerebral cortex.^{43,44} However, in our study, the pretreatment of MT significantly reduced apoptosis indices compared to the tramadol group. In fact, in groups treated with a combination of tramadol and MT, a decrease in anti-apoptotic expression and an increase in pro-apoptotic expression were prevented compared with the group treated with tramadol alone. Regarding MT-protected apoptosis, our results were in line with the findings of Reiter et al., who suggested that MT could decrease apoptosis induction in animal models through free radical scavenging and lipid oxidation and an increase in the body's antioxidant capacity.⁴⁵

The results of the analysis of semen parameters were also in agreement with that of previous studies.⁴⁶⁻⁴⁸ In the present study, statistically significant decreases in sperm count and motility were observed in the tramadol-treated group, and pre-treatment with MT resulted in increased sperm count and motility. Furthermore, another study demonstrated that ROS may induce sperm oxidative damage, thus inducing decreased sperm count and motility, and increased percentage of abnormal sperm morphology.⁴⁹ Moreover, Sawy and Abdel Malak reported that tramadol infusion caused impairment in the function of seminiferous tubules and also reduced fertility due to sperm loss in rats.⁵⁰ Accordingly, it has been reported that MT can protect sperm from the damage induced by tramadol through its effective anti-oxidant and anti-apoptotic potential.

References

1. Candeletti S, Lopetuso G, Cannarsa R, Cavina C, Romualdi P. Effects of prolonged treatment with the opiate tramadol on prodynorphin gene expression in rat CNS. *J Mol Neurosci* 2006; 30(3): 341-7.
2. Baghishani F, Mohammadi-pour A, Hosseinzadeh H, Hosseini M, Ebrahimzadeh-Bideskan A. The effects of tramadol administration on hippocampal cell apoptosis, learning and memory in adult rats and neuroprotective effects of crocin. *Metab Brain Dis* 2018; 33(3): 907-16.
3. Takzare N, Samizadeh E, Shoar S, Majidi ZM, Naderan M, Lashkari A, et al. Impacts of morphine addiction on spermatogenesis in rats. *Int J Reprod Biomed (Yazd)* 2016; 14(5): 303-8.
4. Ghoneim FM, Khalaf HA, Elsamanoudy AZ, Helaly AN. Effect of chronic usage of tramadol on motor cerebral cortex and testicular tissues of adult male albino rats and the effect of its withdrawal: Histological, immunohistochemical and biochemical study. *Int J Clin Exp Pathol* 2014;

Conclusion

In this study, MT showed significant antioxidant effects in preventing oxidative damage, mitochondrial injury, and tramadol-induced apoptosis. It also decreased apoptosis in testicular tissue by decreasing and increasing pro-apoptotic and anti-apoptotic gene expressions, respectively. This may be due to the antioxidant effects of MT on mitochondrial function and the maintenance of mitochondrial membrane integrity in testicular tissue. Therefore, given the beneficial effects of MT on the reduction of oxidative stress, mitochondrial damage, and initiation of tramadol-induced apoptosis in testicular Sertoli cells, it can be used to reduce infertility and the sexual side effects of the long-term use of tramadol. It may also be used in clinical trials; however, further studies are required in this regard.

Conflict of Interests

The authors have no conflict of interest.

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Authors' Contribution

FS, gave the idea, NA, were advisors; MK did the study and participated in the literature search and drafted the article; FS participated in drafting and editing the article. EM and MK helped in performing the experimental part of the study. All authors were involved in data analysis and interpretation. FS supervised whole study. All authors read and approved the final version.

- 7(11): 7323-41.
5. Ibrahim MA, Salah-Eldin AE. Chronic addiction to tramadol and withdrawal effect on the spermatogenesis and testicular tissues in adult male albino rats. *Pharmacology* 2019; 103(3-4): 202-11.
 6. Ahmed MA, Kurkar A. Effects of opioid (tramadol) treatment on testicular functions in adult male rats: The role of nitric oxide and oxidative stress. *Clin Exp Pharmacol Physiol* 2014; 41(4): 317-23.
 7. Vahidirad M, Arab-Nozari M, Mohammadi H, Shaki F. Protective effect of edaravone against nephrotoxicity and neurotoxicity of acute exposure to Diazinon. *J Mazandaran Univ Med Sci* 2018; 28(165): 175-82. [In Persian].
 8. Schulz E, Wenzel P, Munzel T, Daiber A. Mitochondrial redox signaling: Interaction of mitochondrial reactive oxygen species with other sources of oxidative stress. *Antioxid Redox Signal* 2014; 20(2): 308-24.
 9. Zhao Y, Li S, Childs EE, Kuharsky DK, Yin XM. Activation of pro-death Bcl-2 family proteins and mitochondria apoptosis pathway in tumor necrosis factor-alpha-induced liver injury. *J Biol Chem* 2001; 276(29): 27432-40.
 10. Marzetti E, Privitera G, Simili V, Wohlgemuth SE, Aulisa L, Pahor M, et al. Multiple pathways to the same end: Mechanisms of myonuclear apoptosis in sarcopenia of aging. *ScientificWorldJournal* 2010; 10: 340-9.
 11. Carrillo-Vico A, Guerrero JM, Lardone PJ, Reiter RJ. A review of the multiple actions of melatonin on the immune system. *Endocrine* 2005; 27(2): 189-200.
 12. Goncalves NN, Colombo J, Lopes JR, Gelaleti GB, Moschetta MG, Sonehara NM, et al. Effect of melatonin in epithelial mesenchymal transition markers and invasive properties of breast cancer stem cells of canine and human cell lines. *PLoS One* 2016; 11(3): e0150407.
 13. Pandi-Perumal SR, Srinivasan V, Maestroni GJ, Cardinali DP, Poeggeler B, Hardeland R. Melatonin: Nature's most versatile biological signal? *FEBS J* 2006; 273(13): 2813-38.
 14. Rodriguez C, Mayo JC, Sainz RM, Antolin I, Herrera F, Martin V, et al. Regulation of antioxidant enzymes: A significant role for melatonin. *J Pineal Res* 2004; 36(1): 1-9.
 15. Meki AR, Hussein AA. Melatonin reduces oxidative stress induced by ochratoxin A in rat liver and kidney. *Comp Biochem Physiol C Toxicol Pharmacol* 2001; 130(3): 305-13.
 16. Matthiesen T, Wohrmann T, Coogan TP, Uragg H. The experimental toxicology of tramadol: An overview. *Toxicol Lett* 1998; 95(1): 63-71.
 17. Huang F, Ning H, Xin QQ, Huang Y, Wang H, Zhang ZH, et al. Melatonin pretreatment attenuates 2-bromopropane-induced testicular toxicity in rats. *Toxicology* 2009; 256(1-2): 75-82.
 18. El-Shenawy SM, Abdel-Salam OM, Baiuomy AR, El-Batran S, Arbid MS. Studies on the anti-inflammatory and anti-nociceptive effects of melatonin in the rat. *Pharmacol Res* 2002; 46(3): 235-43.
 19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.
 20. Koohsari M, Shaki F, Jahani D. Protective effects of edaravone against methamphetamine-induced cardiotoxicity. *Braz Arch Biol Technol* 2016; 59: e16160093.
 21. Shaki F, Koohsari M. Protective effects of edaravone against methamphetamine-induced neurotoxicity. *Proceedings of 22th Iranian Congress of Physiology and Pharmacology*; 2015 Sep 7-11; Kashan, Iran.
 22. Brown MR, Sullivan PG, Dorenbos KA, Modafferi EA, Geddes JW, Steward O. Nitrogen disruption of synaptoneuroosomes: An alternative method to isolate brain mitochondria. *J Neurosci Methods* 2004; 137(2): 299-303.
 23. Shokrzadeh M, Shaki F, Mohammadi E, Rezagholizadeh N, Ebrahimi F. Edaravone decreases paraquat toxicity in a549 cells and lung isolated mitochondria. *Iran J Pharm Res* 2014; 13(2): 675-81.
 24. Baracca A, Sgarbi G, Solaini G, Lenaz G. Rhodamine 123 as a probe of mitochondrial membrane potential: Evaluation of proton flux through F(0) during ATP synthesis. *Biochim Biophys Acta* 2003; 1606(1-3): 137-46.
 25. Mehdizadeh H, Pourahmad J, Taghizadeh G, Vousooghi N, Yoonessi A, Naserzadeh P, et al. Mitochondrial impairments contribute to spatial learning and memory dysfunction induced by chronic tramadol administration in rat: Protective effect of physical exercise. *Prog Neuropsychopharmacol Biol Psychiatry* 2017; 79(Pt B): 426-33.
 26. Mohammadi SS, Ahmadi HM, Mohammadi E, Sheikhesmaeili F, Nikkhou B. Sirt1 gene expression and gastric epithelial cells tumor stage in patients with *Helicobacter pylori* infection. *Asian Pac J Cancer Prev* 2018; 19(4): 913-6.
 27. Hassan E, Kahilo K, Kamal T, El-Neweshy M, Hassan M. Protective effect of diallyl sulfide against lead-mediated oxidative damage, apoptosis and down-regulation of CYP19 gene expression in rat testes. *Life Sci* 2019; 226: 193-201.
 28. Uygur R, Aktas C, Caglar V, Uygur E, Erdogan H, Ozen OA. Protective effects of melatonin against

- arsenic-induced apoptosis and oxidative stress in rat testes. *Toxicol Ind Health* 2016; 32(5): 848-59.
29. Kabel AM. Zinc/alogliptin combination attenuates testicular toxicity induced by doxorubicin in rats: Role of oxidative stress, apoptosis and TGF-beta1/NF-kappaB signaling. *Biomed Pharmacother* 2018; 97: 439-49.
30. Edrees NE, Galal AAA, Abdel Monaem AR, Beheiry RR, Metwally MMM. Curcumin alleviates colistin-induced nephrotoxicity and neurotoxicity in rats via attenuation of oxidative stress, inflammation and apoptosis. *Chem Biol Interact* 2018; 294: 56-64.
31. Mohamed TM, Ghaffar HM, El Husseiny RM. Effects of tramadol, clonazepam, and their combination on brain mitochondrial complexes. *Toxicol Ind Health* 2015; 31(12): 1325-33.
32. Lemarie A, Grimm S. Mutations in the heme b-binding residue of SDHC inhibit assembly of respiratory chain complex II in mammalian cells. *Mitochondrion* 2009; 9(4): 254-60.
33. Zhang YT, Zheng QS, Pan J, Zheng RL. Oxidative damage of biomolecules in mouse liver induced by morphine and protected by antioxidants. *Basic Clin Pharmacol Toxicol* 2004; 95(2): 53-8.
34. Jalili C, Salahshoor MR, Naseri A. Protective effect of *Urtica dioica* L against nicotine-induced damage on sperm parameters, testosterone and testis tissue in mice. *Iran J Reprod Med* 2014; 12(6): 401-8.
35. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. *Oxid Med Cell Longev* 2008; 1: 616821.
36. Adikwu E, Bokolo B. Prospects of N-acetylcysteine and melatonin as treatments for tramadol-induced renal toxicity in albino rats. *Pharm Sci* 2017; 23(3): 172-81.
37. Attardi LD. The role of p53-mediated apoptosis as a crucial anti-tumor response to genomic instability: lessons from mouse models. *Mutat Res* 2005; 569(1-2): 145-57.
38. Mohammadi-Bardbori A, Ghazi-Khansari M. Alternative electron acceptors: Proposed mechanism of paraquat mitochondrial toxicity. *Environ Toxicol Pharmacol* 2008; 26(1): 1-5.
39. Petrosillo G, Moro N, Ruggiero FM, Paradies G. Melatonin inhibits cardiolipin peroxidation in mitochondria and prevents the mitochondrial permeability transition and cytochrome c release. *Free Radic Biol Med* 2009; 47(7): 969-74.
40. Lopez A, Garcia JA, Escames G, Venegas C, Ortiz F, Lopez LC, et al. Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. *J Pineal Res* 2009; 46(2): 188-98.
41. Hosseini MJ, Shaki F, Ghazi-Khansari M, Pourahmad J. Toxicity of Arsenic (III) on isolated liver mitochondria: A new mechanistic approach. *Iran J Pharm Res* 2013; 12(Suppl): 121-38.
42. Youle RJ, Strasser A. The BCL-2 protein family: Opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008; 9(1): 47-59.
43. Awadalla EA, Salah-Eldin AE. Molecular and histological changes in cerebral cortex and lung tissues under the effect of tramadol treatment. *Biomed Pharmacother* 2016; 82: 269-80.
44. Atici S, Cinel L, Cinel I, Doruk N, Aktekin M, Akca A, et al. Opioid neurotoxicity: Comparison of morphine and tramadol in an experimental rat model. *Int J Neurosci* 2004; 114(8): 1001-11.
45. Reiter RJ, Guerrero JM, Garcia JJ, Acuna-Castroviejo D. Reactive oxygen intermediates, molecular damage, and aging. Relation to melatonin. *Ann N Y Acad Sci* 1998; 854: 410-24.
46. Esua IS, Uno UU, Ekaluo U. Effect of tramadol on sperm profile of male albino rats. *Asian Journal of Research in Biochemistry* 2019; 3(4): 1-6.
47. Farag AGA, Basha MA, Amin SA, Elnaidany NF, Elhelbawy NG, Mostafa MMT, et al. Tramadol (opioid) abuse is associated with a dose- and time-dependent poor sperm quality and hyperprolactinaemia in young men. *Andrologia* 2018; 50(6): e13026.
48. Esua I, Uno UU, Okey F, Ekaluo U. Histopathological effects of tramadol treatments on the testes of male albino rats. *Asian J Biotechnol Bioresour Technol* 2018; 4(4): 1-7.
49. Shi TY, Chen G, Huang X, Yuan Y, Wu X, Wu B, et al. Effects of reactive oxygen species from activated leucocytes on human sperm motility, viability and morphology. *Andrologia* 2012; 44(Suppl 1): 696-703.
50. Sawy M, Abdel Malak H. Effect of tramadol abuse on testicular tissue of adult albino rats: A light and electron microscopic study. *Egyptian Journal of Histology* 2015; 38(2): 356-66.

اثر حفاظتی ملاتونین در کاهش سمیت تولید مثلی ناشی از ترامادول در موش صحرایی با مکانیسم مهار استرس اکسیداتیو، مهار اختلال عملکرد میتوکندری و جلوگیری از بیان ژن‌های دخیل در مسیر آپوپتوز

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مقاله پژوهشی

چکیده

مقدمه: هدف از انجام پژوهش حاضر، ارزیابی میزان اثر حفاظتی ملاتونین بر روی استرس اکسیداتیو، عملکرد میتوکندری و مهار القای آپوپتوز در سمیت تولید مثلی ناشی از ترامادول در موش صحرایی نر بود.

روش‌ها: در این مطالعه، موش‌های صحرایی به هفت گروه (شش موش صحرایی در هر گروه) شاهد، ملاتونین (۱/۵ میلی‌گرم بر کیلوگرم و داخل صفاقی)، ترامادول (۵۰ میلی‌گرم بر کیلوگرم و داخل صفاقی) و سه گروه از ابتدا به ترتیب دزهای ۱ و ۱/۵ و ۲/۵ میلی‌گرم بر کیلوگرم ملاتونین تقسیم شدند و بعد از ۳۰ دقیقه به آن‌ها ۵۰ میلی‌گرم بر کیلوگرم ترامادول به صورت داخل صفاقی تزریق گردید. پس از سه هفته متوالی تزریقات، حیوانات کشته شدند و بافت بیضه جهت ارزیابی شاخص‌های استرس اکسیداتیو شامل اندازه‌گیری پراکسیداسیون لیپیدی، غلظت گلوتاتیون و پروتئین کربونیل و تحلیل پارامترهای مرتبط با اسپرم جدا شد. میتوکندری‌ها با استفاده از روش سانتریفوژ متعدد، از بافت بیضه تازه جداسازی گردید و جهت ارزیابی عملکرد میتوکندری، اندازه‌گیری میزان پتانسیل غشای میتوکندری و تورم میتوکندری مورد استفاده قرار گرفت. قسمت دیگری از نمونه‌های بافت بیضه به منظور ارزیابی میزان بیان ژن‌های پروآپوپتوتیک (Bax) و آنتی‌آپوپتوتیک (Bcl-2) به روش Real-time polymerase chain reaction (Real-time PCR) در محلول RNAlater قرار داده شد.

یافته‌ها: دریافت ترامادول به صورت قابل ملاحظه‌ای موجب کاهش تعداد، تحرک و مورفولوژی اسپرم و همچنین، کاهش گلوتاتیون بافتی و فعالیت میتوکندری شد. از سوی دیگر، افزایش معنی‌داری در پراکسیداسیون لیپیدی، سطح پروتئین کربونیل، پتانسیل غشای میتوکندری و تورم میتوکندری نیز بدنبال دریافت ترامادول مشاهده شد. علاوه بر این، دریافت ترامادول بصورت معناداری منجر به کاهش معنادار بیان ژن آنتی‌آپوپتوتیک Bcl-2 و افزایش بیان ژن پروآپوپتوتیک Bax در بافت بیضه مشاهده گردید؛ در حالی که پیش‌درمان موش‌های صحرایی با ملاتونین، منجر به بهبود شاخص‌های آنالیز اسپرم، وضعیت آنتی‌اکسیدانی بیضه و عملکرد میتوکندری شد. علاوه بر این، پیش‌درمان ملاتونین سبب تنظیم بیان ژن‌های Bcl-2 و Bax گردید.

نتیجه‌گیری: با توجه به اثر محافظتی ملاتونین علیه سمیت تولید مثلی ناشی از ترامادول، می‌توان از ملاتونین در شرایط پاتولوژیک ناشی از القای آپوپتوز و آسیب میتوکندری استفاده نمود.

واژگان کلیدی: ترامادول؛ ملاتونین؛ استرس اکسیداتیو؛ میتوکندری؛ آپوپتوز

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