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Melatonin protects methotrexate-induced testicular injury in rats

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Abstract. – OBJECTIVE: Melatonin possesses anti-inflammation and anti-oxidant potentials. However, whether NF-E2 related factor 2 (Nrf2) pathway and nuclear factor-kappaB (NF-κB) pathway are involved in the protective effect of melatonin are unknown. We aim to explore the regulatory effect of melatonin on methotrexate-induced testicular injury.

MATERIALS AND METHODS: Sprague Dawley (SD) rats were randomly assigned in sham group, methotrexate group and melatonin group, with 8 rats in each group. Testis tissues were collected 10 days after animal procedures. Pathological lesions and cell apoptosis in testis tissues were evaluated using HE (hematoxylin and eosin) staining and TUNEL assay, respectively. Oxidative stress in rat testis was accessed using relative commercial kits. Western blot was performed to detect protein expressions of relative genes in Nrf2 pathway and NF-κB pathway in rat testis tissues.

RESULTS: Activities of SOD, GSH, CAT and T-AOC in testis homogenate in melatonin group were remarkably higher than those of methotrexate group ($p < 0.05$). On the contrary, levels of MDA, ROS and inflammatory factors (TNF-α, IL-1β, IL-6 and KC-GRO) were markedly decreased after melatonin treatment. Besides, melatonin group showed alleviated pathological lesions and cell apoptosis in testis. Western blot results demonstrated that melatonin treatment upregulated expressions of Nrf2, GSR, GCLm, HO-1 and NQO-1 in testis. However, protein expressions of NF-κB, TNF-α, VCAM-1, ICAM-1 and MCP-1 were downregulated.

CONCLUSIONS: Melatonin protects methotrexate-induced testicular damage in rats by improving antioxidant capacity and inhibiting inflammatory response via Nrf2 and NF-κB pathways.

Key Words:
Melatonin, Nrf2 pathway, NF-κB pathway, Methotrexate, Testicular injury.

Introduction

Drug-induced testicular injury is resulted from the exposure to toxic or potentially toxic drugs. The clinical manifestations of drug-induced testicular injury are abnormal structure and function of testis[1-4]. Methotrexate is a dihydrofolate reductase inhibitor commonly used in tumor chemotherapy. Long-term, low-dose application of methotrexate would lead to irregular menstruation, amenorrhea and dyszoospermia. Methotrexate exerts drug toxicity leading to chronic testicular damage[5,6]. Hence, prevention on methotrexate-induced testicular toxicity has been well concerned. In-depth researches should be carried out to develop novel strategies for alleviating further secondary damage to other organs after testicular injury. Oxidative stress is an important pathogenic factor of methotrexate-induced testicular injury. Methotrexate itself does not possess redox ability but indirectly contributes to oxidative stress[7]. Oxidation/antioxidant balance is interfered by external stimuli, thereby increasing the level of reactive oxygen species (ROS). Excessive production of ROS can lead to oxidative damage, alteration of membrane structure and function, and lipid peroxidation[8,9]. It is well known that testis are very sensitive to methotrexate toxicity[5,6]. Methotrexate may result in testicular torsion, partial destruction of testicular spermatogenic epithelium, changes in testicular support cell structure and antioxidant enzyme activity. Researches on testicular toxicity models indicated that free radical scavengers, antioxidants, anti-inflammatory cytokines and other drugs could alleviate testicular injury[10-13]. In recent years, the role of the transcription factor NF-E2 related factor 2 (Nrf2) in regulating oxidative stress has received increasing attention. Nrf2

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is an important nuclear transcription factor that exerts the capacity of protecting oxidative stress in cells. More than 200 endogenous protective genes can be encoded by Nrf2[14-21]. In addition, nuclear factor-kappaB (NF-κB) signaling pathway is capable of regulating chronic inflammation and fibrosis-related genes[21-24].

Oxidative stress and inflammatory factor stimulation are considered as important mechanisms of methotrexate-induced testicular toxicity. It is believed that free radical scavengers and antioxidants contribute to prevent methotrexate toxicity[5-10]. Some natural antioxidants, such as vitamin E, olive oil and watermelon oil exert protective effects on methotrexate toxicity as well[3,4]. In recent years, the effects of melatonin on the occurrence and development of organ damage caused by toxins and ischemia have been well recognized[25-29]. In this study, we explored the role of melatonin in alleviating methotrexate-induced testicular injury and its underlying mechanism. Our results provide a solid foundation for preventing and treating toxic testicular injury.

Materials and Methods

Chemicals and Reagents

Melatonin was purchased from Sinopharm Chemical Reagent (Shanghai, China); Methotrexate was purchased from Qilu Pharmaceutical (Jinan, China); Relative commercial kits of MDA (malondialdehyde), T-AOC (total antioxidant capacity), CAT (catalase), GSH (reduced glutathione) and SOD (superoxide dismutase) were purchased from Jiancheng Bioengineering Institute (Nanjing, China); Coarse balance, electronic thermometer and 721 type spectrophotometer were obtained from Inesa Analytical Instrument (Shanghai, China).

Animal Procedures

24 male Sprague Dawley (SD) rats weighing 200 ± 20 g (Vital River Laboratory Animal Technology, Beijing, China) were maintained in an environment with a 12 h/12 h light/dark cycle. Rats were given to free access to food and water. All rats received intragastrical administration of distilled water (0.01 ml/g) for 28 consecutive days. Rats in methotrexate group received additional intragastrical administration of methotrexate (150 mg/kg·d) on the 7th day 2 h after distilled water administration. Rats in melatonin group were intragastrically administrated with 20 mg/kg melatonin for 28 days and were additionally administrated with 25 mg/kg methotrexate on the 7th day. Blood sample was collected from orbital vein after the animal procedures. Body weight and daily activities of each rat were regularly observed. This study was approved by the Animal Ethics Committee of Nanjing Medical University Animal Center.

Assessment of Testis Growth

Body weight, testicular weight, epididymis weight and testicular index of each rat were daily recorded during the animal procedures. After sacrifice, envelope and fat tissue were peeled off from bilateral testicular tissues for weighing. Testicular index = testicular weight / body weight × 100%.

HE (Hematoxylin And Eosin) Staining

Testicular tissues were cut in coronal section, fixed with 10% paraformaldehyde and stained with hematoxylin and eosin (HE). Histological lesions in testis tissues were assessed by semi quantitative detection of injury and necrosis of seminiferous tubules. Five randomly selected fields of each sample were evaluated for testicular pathological lesions (200×).

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay

Testicular cell apoptosis was detected by TUNEL assay according to the instructions of ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon, Millipore, Billerica, MA, USA). 5-μm paraffin section were counterstained with hematoxylin and TUNEL-positive cells were counted in 5 randomly selected fields (200×).

Biochemical Measurements

Testis tissues were immediately collected after the testis color was turned from red to pale. Testis homogenate was prepared for determining levels of MDA, T-AOC, CAT, GSH and SOD using relative commercial kits.

ROS Detection

For evaluating testicular production of intracellular reactive oxygen species (ROS), intracellular superoxide level assay was detected by a fluorescent microscope (Eclipse Ti-SR, Nikon Co., Tokyo, Japan). The density of the images was detected with a laser scanning confocal microscope (Zeiss Ltd, Göttingen, Germany) in arbitrary units per millimeter square field.

Western Blot

Total protein was extracted using the RIPA (radioimmunoprecipitation assay) protein lysate (Beyotime, Shanghai, China) and quantified by BCA (bicinchoninic acid) method (Pierce, Rockford, IL, USA). Protein sample was separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Millipore, Billerica, MA, USA). The incubation of primary and secondary antibodies was performed based on the standard protocols of Western blot. Chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA) was used to expose the protein bands on the membrane.

Statistical Analysis

SPSS22.0 (Statistical Product and Service Solutions) statistical software package (IBM, Armonk, NY, USA) was used for data analysis. Data were expressed as $\bar{x} \pm s$. The *t*-test was used to analyze the difference between two groups. Categorical data were analyzed using χ^2-test or Fisher's exact test. $p < 0.05$ was considered statistically significant.

Results

Pretreatment with Melatonin Improved Testis Function in Methotrexate-Induced Mice

Body weight, testicular weight, epididymis weight and testicular index of rats in methotrexate group were remarkably decreased compared with those of sham group, indicating the successful construction of methotrexate-induced testicular injury in rats ($p < 0.05$). Compared with those of methotrexate group, the above indexes were remarkably alleviated in melatonin group ($p < 0.05$, Figure 1).

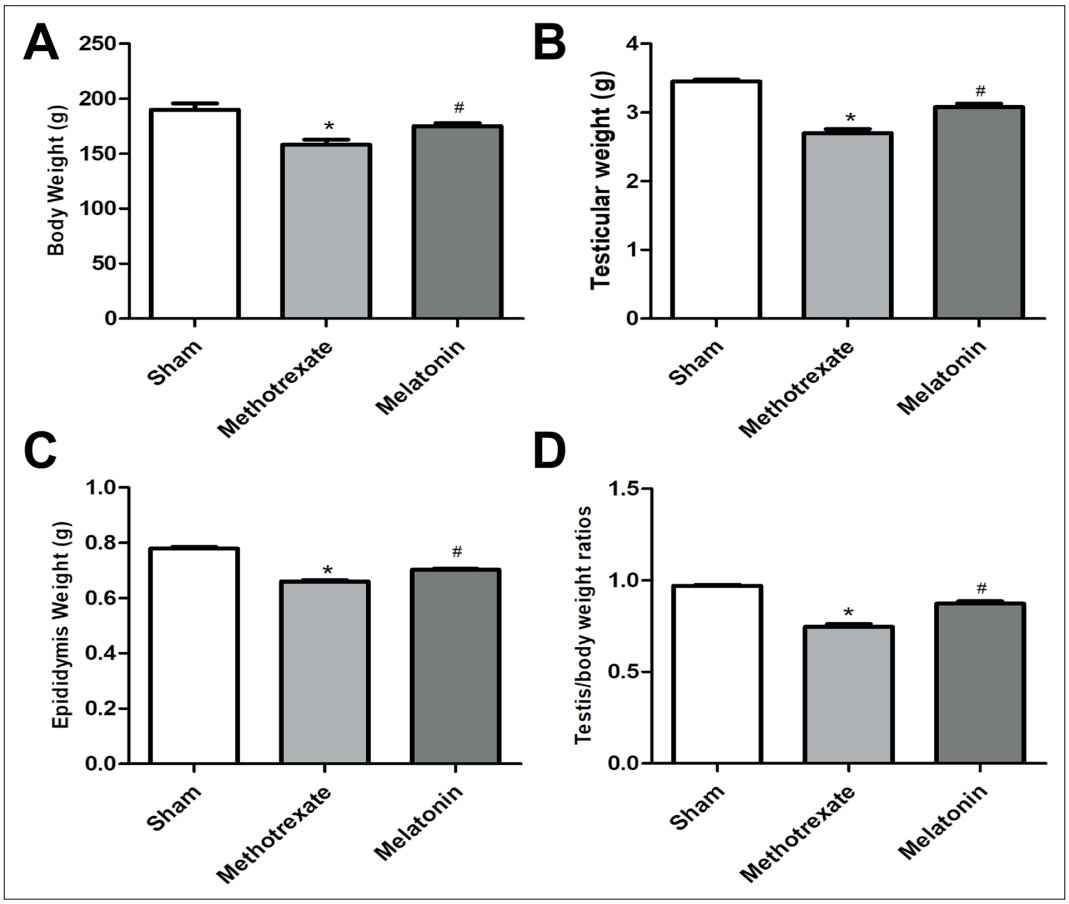

Figure 1. Melatonin conserved testis growth in methotrexate-induced testis injury. ***A,*** Body weight of rats in the different management groups; ***B,*** Testicular weight of rats in the different management groups; ***C,*** Epididymis weight of rats in the different management groups; ***D,*** The ratio of testicular weight/body weight in the different management groups. Data were presented as mean ± SD, *significant difference *vs.* sham group ($p < 0.05$); #significant difference *vs.* Methotrexate group ($p < 0.05$).

Melatonin Preserved Testis Histologic Structure and Mitigated Neutrophil Infiltration

HE staining showed well-ordered testicular cells with regular structure. However, seminiferous tubules were enlarged with disordered testicular cells. Granular degeneration, nuclear condensation, interstitial proliferation and inflammatory cell infiltration were pronounced in methotrexate group. Melatonin group showed alleviated pathological lesions in testis with slight enlargement of seminiferous tubules (Figure 2A and 2B). Pathological grade in methotrexate group and melatonin group was remarkably higher than that of sham group ($p < 0.05$).

Melatonin Decreased Methotrexate-Induced Testis Tubular Cells Apoptosis

TUNEL staining indicated that TUNEL-positive cells in methotrexate group were much more than sham group. However, fewer TUNEL-positive cells were observed in testis tissues of melatonin group than those of methotrexate group ($p < 0.05$, Figure 2C-2E).

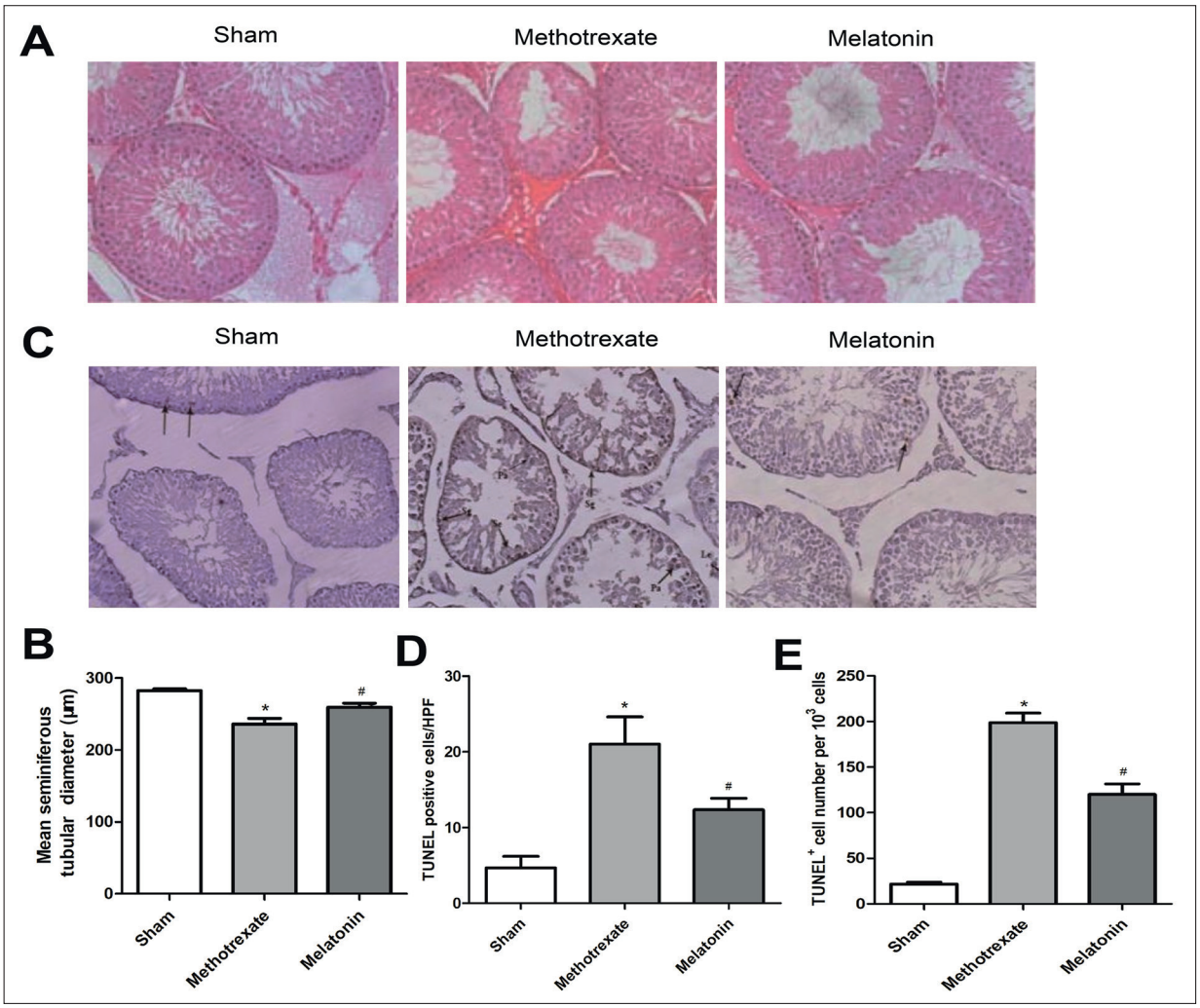

Figure 2. Melatonin prevents methotrexate-induced testis injury in testis morphology. Testis sections were stained with hematoxylin and eosin and examined using light microscopy (200×). ***A,*** H&E staining of testis tissues in sham, methotrexate group, and melatonin group; ***B,*** Number of seminiferous tubule/unit area of testes in the different management groups; ***C,*** Representative images (200×, scale bar = 50 μm) of TUNEL immunostaining in the methotrexate-induced testis injury; ***D,*** Apoptotic index of testis in the different management groups; ***E,*** TUNEL-positive cells per 10^3 germ cells of testis in the different management groups. Data were expressed as mean ± SD. *significant difference *vs.* sham group ($p < 0.05$); #significant difference *vs.* methotrexate group ($p < 0.05$).

Melatonin Reduced Inflammatory Cytokine Levels in Methotrexate-Induced Testis Injury

To further investigate the protective role of melatonin on methotrexate-induced inflammation in testis, inflammation-related indicators were detected. Our data found that the levels of TNF-α, IL-1β, IL-6 and KC-GRO in rat testis homogenate were markedly elevated, which were partially reversed by melatonin treatment (Figure 3A-3D).

Melatonin Decreased ROS Production and Tissue Impairment by Enhancing Antioxidant Capacity

To evaluate the oxidative stress in rat testis induced by methotrexate administration, the activities of relative oxidation indicators were detected. MDA activity was decreased, whereas SOD, CAT and T-AOC activities were increased in melatonin group than those of methotrexate group (Figure 4A, 4C-4F). Besides, lower ROS level was found in rat testis of melatonin group compared with that of methotrexate group (Figure 4B).

Melatonin Upregulated Nrf2 and its Downstream Gene Expressions by Increasing Nrf2 Nuclear Translocation

We extracted cytoplasm and nucleus of rat testicular cells in each group, respectively. Western blot results indicated that cytoplasmic levels of Nrf2 and its downstream factors (GSR, GCLm, HO-1 and NQO-1) were all upregulated than those of nuclear levels in melatonin group (Figure 5A). Subsequently, protein expressions of relative genes in NF-κB pathway were detected by Western blot. The data showed that protein levels of NF-κB, TNF-α, VCAM-1, ICAM-1 and MCP-1 were higher in melatonin group than those of methotrexate group (Figure 5B).

Discussion

Testicular injury is a common pathological phenomenon showing abnormalities in testicular structure and function. Testicular toxic damage is frequently observed after cardiac vascular

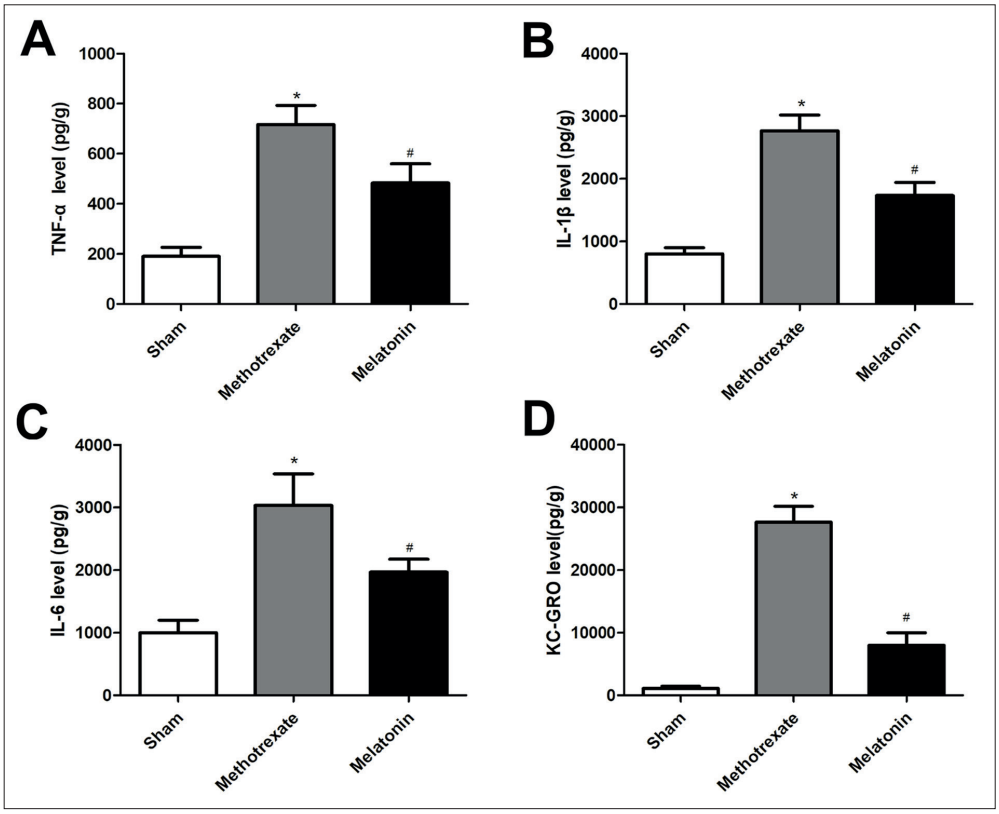

Figure 3. Melatonin reduced testis inflammatory cytokines in methotrexate-induced testis injury. ***A,*** Content of TNF-α in testis tissues. ***B,*** Content of IL-1β in testis tissues. ***C,*** Content of IL-6 in testis tissues. ***D,*** Content of KC-GRO in testis tissues. Data were expressed as mean ± SD. *significant difference *vs.* sham group ($p < 0.05$); #significant difference *vs.* methotrexate group ($p < 0.05$).

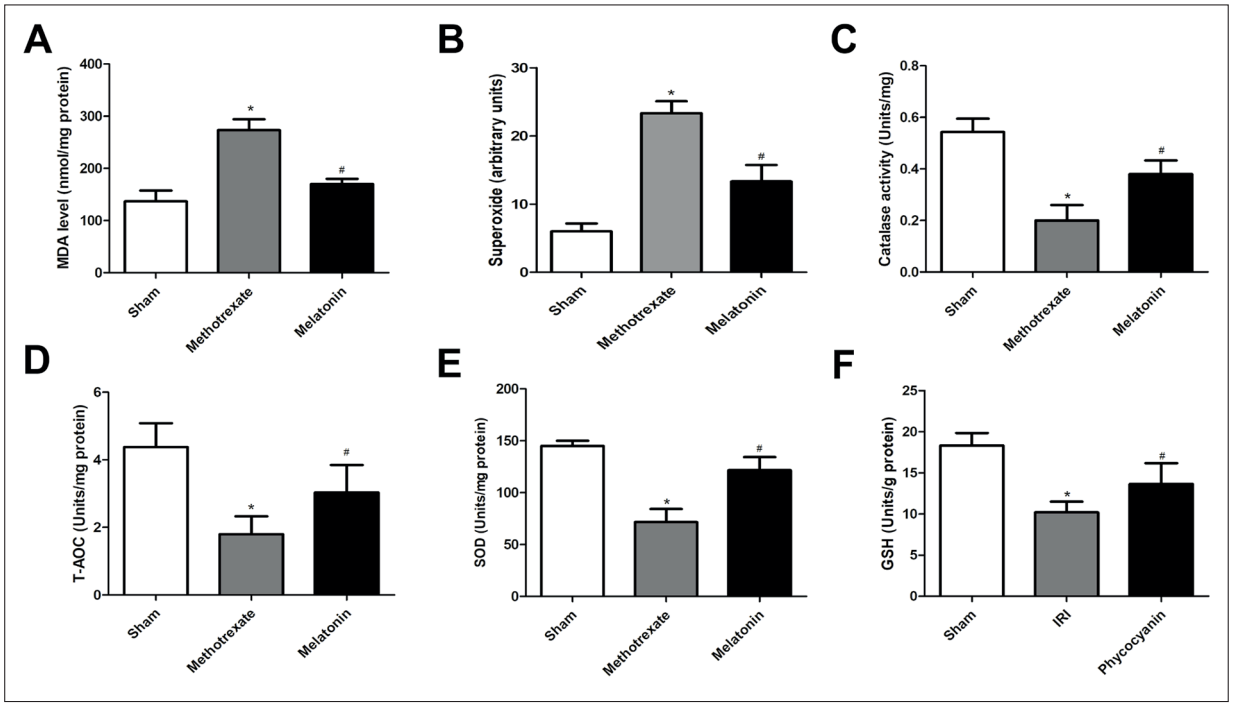

Figure 4. Melatonin attenuated oxidative stress injury by the assessment of biochemical parameters. ***A,*** Content of malondialdehyde (MDA) in testis tissues; ***B,*** Density of ROS was reported as arbitrary units per millimetre squarefield; ***C,*** Content of catalase (CAT) activity in testis tissues; ***D,*** Content of total antioxidant capacity (T-AOC) in testis tissues; ***E,*** Content of superoxide dismutase (SOD) in testis tissues; ***F,*** Content of reduced glutathione (GSH) in testis tissues. Data were expressed as mean ± SD. *significant difference *vs.* sham group ($p < 0.05$); #significant difference *vs.* methotrexate group ($p < 0.05$).

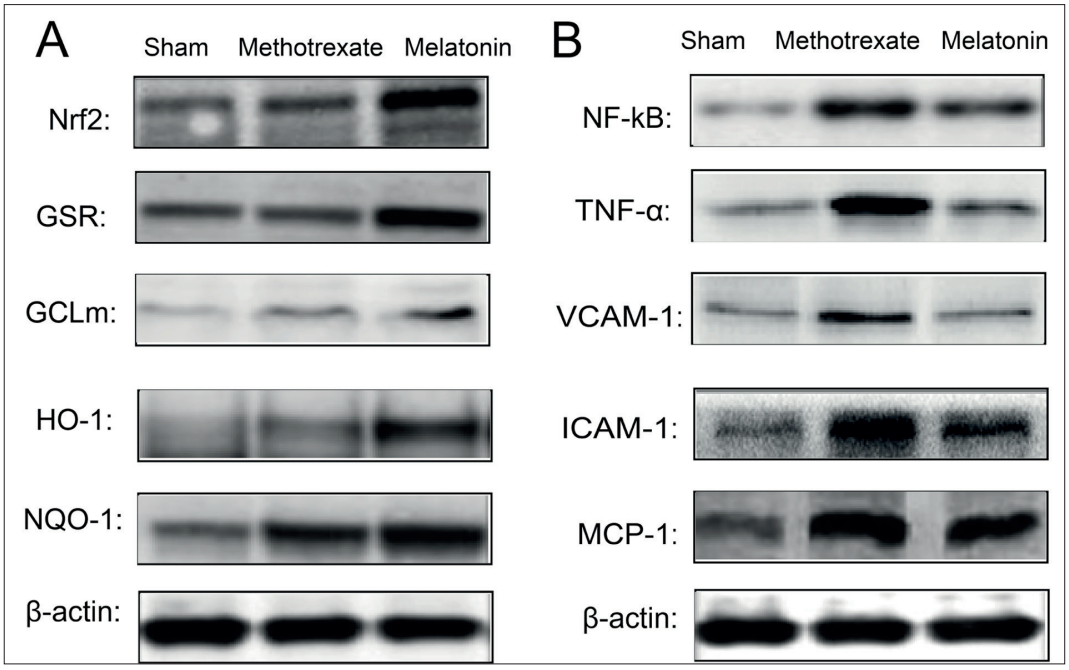

Figure 5. Melatonin supplementation enhanced nuclear translocation of Nrf2, and decreased protein expression of NF-κB. ***A,*** Protein expressions of Nrf2, GSR, GCLm, HO-1 and NQO-1 protein expression in different groups; ***B,*** Protein expressions of NF-κB, TNF-α, VCAM-1, ICAM-1 and MCP-1 in different groups. β-actin was used as a protein control to normalize volume of protein expression. Protein levels were determined by densitometric analysis and normalized to the β-actin signal. Data were expressed as mean ± SD. *significant difference *vs.* sham group ($p < 0.05$); #significant difference *vs.* methotrexate group ($p < 0.05$).

surgery, shock, and toxic stimulation, which is caused by accumulation of oxygen free radicals in testis[1-4]. Studies[14-21] have shown that nuclear Nrf2 protects against oxidative stress *via* regulating expressions of various downstream antioxidant genes after binding to antioxidant elements. In addition, NF-κB pathway could regulate chronic inflammation and fibrotic gene expressions[22-24]. Melatonin, a potent anti-oxidant and anti-inflammatory drug, has been shown to possess anti-oxidative, anti-inflammatory and anti-apoptotic effects. It is presumed to exert protective effects against toxin-induced testicular injury[25-29]. Oxidative stress is the leading cause for methotrexate-induced testicular injury[7]. T-AOC, ACT, GSH and SOD are crucial in scavenging free radicals[8-10]. GSH is a self-protective antioxidant in liver tissue for detoxification. GSH reduces peroxidation through catalyzing the reduction of hydrogen peroxide, reflecting the antioxidant capacity in cells[9]. CAT and SOD are considered as protective enzymes for the removal of ROS. SOD is widely present with the capacities of removing superoxide anion radicals and maintaining the homeostasis of free radicals. SOD activity can indirectly reflect the ability to scavenge oxygen free radicals[10]. Under normal conditions, endogenous anti-oxidant enzymes could protect the body from oxidative stress damage[11-13]. However, external stimuli result in abundant ROS accumulation, thereafter destroying the redox balance. Subsequently, multiple pathological changes are observed, including inflammatory response, immune disorders and tumor development[12]. Melatonin can eliminate free radicals, enhance the antioxidant capacity of cells, and delay the rate of cell senescence and death, thereby protecting biological functions of cells[25, 26]. Nrf2 is an important transcription factor involved in oxidative stress response. Inactivate Nrf2 is distributed in the cytoplasm binding to Keap1, which is easily degraded[14-18]. Oxidative stress stimulates the dissociation and translocation of Nrf2. Nuclear Nrf2 immediately binds to the anti-oxidation element (ARE), inducing the downstream genes to alleviate oxidative stress damage[19-21].

Inflammatory cytokines are multifunctional protein peptides, which are synthesized and secreted by immune cells. They are mainly involved in the regulation of immune response[30-32]. For example, IL-1β upregulates the proliferation and differentiation of synovial cells and lymphocytes[33]. IL-6 is a pro-inflammatory cytokine that stimulates B-lymphocytes to produce various inflammatory mediators involved in immune regulation[34]. TNF-α can stimulate the production of IL-1β and IL-6. It induces angiogenesis *via* activating dinoprostone and collagenase. TNF-α also regulates tumor development *via* activating macrophages and neutrophils, as well as stimulating the proliferation and differentiation of T and B lymphocytes[35]. Studies have shown that methotrexate induction upregulates mRNA expressions of cyclooxygenase and inflammatory factors (IL-1β, IL-6 and TNF-α). The production of inducible NO synthase and interferon is stimulated, whereas anti-inflammatory cytokines are decreased after methotrexate induction[36,37]. In the present research, methotrexate treatment stimulated expressions of TNF-α, IL-1β, IL-6 and KC-GRO in testis tissue, indicating that methotrexate leads to inflammatory reaction. NF-κB is a transcription factor that inhibits the expressions of pro-inflammatory genes. Inactivated NF-κB is normally present in the cytoplasm in a dimeric form binding to the inhibitory protein IκB[22]. IκB kinase (IKK) activation phosphorylates IκB to detach from the NF-κB complex[23]. Activated NF-κB is rapidly translocated to the nucleus and binds to the specific κB sequence of the target gene, inducing transcription of inflammatory cytokines[19, 22-24]. Melatonin (N-acetyl-5-methoxytryptamine), is an endogenous amide in plants[25,26]. Existing studies have shown that melatonin is involved in plant development, including germination, seedling growth and senescence[27, 28]. In addition to acting as a dark signal and a regulator of plant growth, melatonin also protects antioxidants from internal and environmental oxidative stress[29]. Animal experiments have confirmed the protective effect of melatonin on organ damage. However, the effect of melatonin on testicular toxic damage has not been reported. In this report, methotrexate group showed significant pathological lesions in testis, which were markedly alleviated in melatonin group. Oxidative stress was pronounced in methotrexate group, whereas melatonin treatment remarkably decreased levels of oxidative stress indicators. Western blot results indicated that melatonin treatment upregulated Nrf2 expression and downregulated NF-κB expression after methotrexate induction in testis, indicating the involvement of Nrf2 and NF-κB pathways in melatonin-induced protection of testicular injury.

Conclusions

We showed that melatonin protects methotrexate-induced testicular damage in rats by improving antioxidant capacity and inhibiting inflammatory response *via* Nrf2 and NF-κB pathways.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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