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Melatonin Protects Mouse Type A Spermatogonial Stem Cells against Oxidative Stress via The Mitochondrial Thioredoxin System

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Abstract

Objective: Mitochondrial oxidative stress is an important factor in infertility. The mitochondrial thioredoxin system plays an important role in this condition. N-acetyl-5-methoxy tryptamine (melatonin) plays a role in reducing oxidative stress and apoptosis in spermatogonial stem cells (SSCs). In this study, we explore the probable protective effects of melatonin on the mitochondrial thioredoxin system [thioredoxin 2 (Trx2)/Txnip] in SSCs under oxidative stress.

Materials and Methods: In this experimental study, SSCs were co-cultured two-dimensionally (2D) with Sertoli cells in DMEM culture medium that contained 10% fetal bovine serum (FBS), 1% antibiotics, and 10 ng/ml glial cell-derived neurotrophic factor (GDNF) for 30 days. The cultured cells were subsequently divided into four groups: control; melatonin (250 μM, 24 hours); melatonin (250 μM, 24 hours)+hydrogen peroxide (H_2O_2, 50 μM, 24 hours); and H_2O_2 (50 μM, 24 hours). Intracellular reactive oxygen species (ROS) production was determined by flow cytometry. Malondialdehyde (MDA) levels were measured by Fluorometry. The expressions of apoptotic and antioxidant genes and nuclear factor erythroid 2-related factor 2 (Nrf2), Trx2, and nicotinamide nucleotide transhydrogenase (NNT) proteins were determined by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot. Adenosine triphosphate (ATP) levels were measured by fluorometry.

Results: Melatonin reduced H_2O_2-induced ROS levels and apoptosis in the SSCs. Melatonin also increased mRNA expression of *Nrf2, Trx2, NNT*, Sirtuin 3 (*Sirt3*), and decreased mRNA expression of *Txnip*, and increased protein expressions of Nrf2, Trx2, NNT thereby increasing activity of the mitochondrial thioredoxin system. In addition, melatonin increased ATP levels.

Conclusion: Melatonin increased *Trx2* expression through the *Nrf2* pathway. This study suggests that melatonin may protect SSCs from oxidative stress in diseases related to infertility.

Keywords: Melatonin, Oxidative Stress, Spermatogonia

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Introduction

Mitochondria play a key role in fertility. The primary biological reactions inside mitochondria are oxidative metabolism, energy production, and free radical production (1). This organelle also participates in the regulation of cell death, a function that appears to be directly related to reactive oxygen species (ROS) production (2). Mitochondria are the main source of intracellular superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2); issues in the mitochondrial electron transport chain can lead to ROS production, mitochondrial dysfunction, and cell death (3).

Mitochondrial oxidative stress is one of the important factors in infertility. Nearly half of all infertility cases are caused partly or entirely by male factors. It has been reported that about 30-80% of infertile men have high levels of ROS in their semen (4). Conditions such as smoking, varicocele, chronic stress, genital tract infections, gonadotropins, and hyperthermia induce oxidative stress and increase ROS levels. Also, a damaged mitochondrial respiratory chain and severe lack of adenosine triphosphate (ATP) often cause ROS production in the mitochondria and increase conditions for the development of pathological diseases (5).

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Although normal levels of ROS are necessary to maintain the physiological state of stem cells, high levels of ROS can cause chromosomal abnormalities, mitochondrial DNA damage, and disturbances in stem cell differentiation (6). Spermatogonial stem cells (SSCs) are a group of cells positioned in the basement membrane of seminiferous tubules, which constitute only 0.03% of all testis cells. However, the continuation of spermatogenesis throughout life depends on the proper regulation of SSC self-renewal and differentiation processes. SSCs are sensitive to the overproduction of ROS (7).

Antioxidant molecules that can inhibit oxidative stress by restoring the balance between ROS and antioxidants have long been an interesting subject of research. However, the increase in ROS levels is not always the result of a lack of antioxidant defence, and other cases should be considered. It has recently been shown that an increase in ROS can be caused by "reductive stress" due to over-accumulation of reductant. Therefore, additional amounts of antioxidants do not seem to be beneficial (8, 9). N-acetyl-5-methoxy tryptamine (melatonin) is a small biological molecule secreted from the pineal gland and other organs, including the retina and testis (10). It is commonly found in nature (11). The results of our previous studies show that melatonin, an exogenous antioxidant agent, reduces apoptosis and oxidative stress in SSCs (10, 12, 13). The protective effects of melatonin include reduction of oxidative stress, apoptosis and inflammation, and regulation of mitochondrial function and sex hormones. It also indirectly reduces oxidative stress by regulating antioxidant enzymes, downregulating prooxidant enzymes, and regulating mitochondrial homeostasis as the main source of ROS production. Melatonin treatment (50 μM H_2O_2 and 2500 ng/L melatonin) is associated with regulation of the NF-κB/iNOS and nuclear factor erythroid 2-related factor 2 (Nrf2)/HO-1 signalling pathways (14). Treatment with melatonin reduces the severity of testicular tissue damage in animal models of hyperlipidaemia, testicular torsion, varicocele, and toxicity caused by chemotherapy drugs or environmental toxins (15, 16). The glutathione and thioredoxin-dependent pathways are two thiol-dependent peroxidation pathways that can remove H_2O_2 in the mitochondrial matrix. Both pathways depend on the regeneration of the oxidised disulfide form of an intermediate [e.g., glutathione disulfide (GSSG) or the cys-cys disulfide form] by a reductase. In both of the thiol-dependent pathways, reductase prefers to use NADPH rather than NADH. The main source of NADPH is the integral enzyme nicotinamide nucleotide transhydrogenase (NNT), which is positioned in the inner membrane of mitochondria.

The mitochondrial thioredoxin system consists of thioredoxin 2 (Trx2), thioredoxin reductase (TrxR2) and thioredoxin-dependent peroxidase. Since Trx2 can reduce disulfide bonds, overexpression of Trx can result in increased mitochondrial membrane potential and ATP synthesis. Trx2 deficiency leads to a release of cytochrome C from mitochondria and the activation of caspases 3 and 9. Thus, Trx2 protects cells against mitochondrial oxidative stress and ROS-induced apoptosis (17). NNT is a rich source of NADPH that is required for the glutathione and thioredoxin antioxidant system (18). Evidence shows that melatonin increases the nuclear transcription of Nrf2 (19). The increase in Nrf2 is associated with an increase in the cellular levels of thioredoxin and TrxR2 (11, 20). From a clinical point of view, biomarkers that reflect the level of oxidative stress will be useful for clinicians to evaluate pathological features of various diseases and drug efficacies (21). Melatonin is an antioxidant that can affect the mitochondrial pathway of oxidative stress and maintain cell homeostasis by activating the thioredoxin system, which is a line of defence against oxidative stress. Here, we investigate the effects of melatonin on mitochondrial oxidative stress and its potential to reduce oxidative stress in type A SSCs. The main aim of this study is to determine the effects of melatonin on the mitochondrial thioredoxin system in mouse type A SSCs under oxidative stress conditions, as a future therapeutic solution for infertility.

Materials and Methods

The Ethics Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran approved the animal experiments in this research (IR.KUMS.MED.REC.1400.083). Testes were obtained from 80, six-day-old male neonatal BALB/c mice for testicular cell isolation. The animals were kept under standard controlled conditions of a 12-hour light/12-hour dark cycle, room temperature of 20 ± 2ºC, and fed with concentrated food for mice and sufficient water. The male and female mice were placed in a cage and allowed to mate. The pregnant mice were subsequently placed in a separate cage until the birth of the pups. The birth date of the pups was considered to be day zero.

Isolation and purification of spermatogonial stem cells

Testes from the six-day-old mice were used to culture highly proliferative cells according to a two-step enzymatic digestion process for tissue digestion (22). The testicular tissues were placed in 2 ml of DMEM culture medium (DMEM/F-12; Life Technologies, Auckland, New Zealand) that contained 1 mg/ml collagenase IV (Sigma, USA) and 0.05 mg/ml DNase I (DNase I. RNase-free 500 U.1u/μl-MO5401; Sinaclon, Iran), and incubated at 37°C and 5% CO_2 for 15 minutes, followed by centrifugation for 5 minutes at 1200 RPM.

In the second stage of the enzymatic digestion process, 0.25% trypsin (Sigma, USA) and 0.05 mg/ml DNase I were added to the cell plate to purify the spermatogonial cells from fibroblasts, myoglobins, and other impurities. Following isolation of these cells, trypsin activity was stopped by the addition of 10-20% fetal bovine serum (FBS, Gibco, EU-approved, South America) and the cell suspension was filtered through a 70 μm nylon mesh. The solution was then transferred to a flask and placed in an incubator at 37°C for 30 minutes. Subsequently, the

suspended spermatogonial stem cells were separated from the cells that were deposited on the bottom of the flask. The SSCs were co-cultured two-dimensionally (2D) with Sertoli cells in DMEM culture medium that contained 10% FBS (Gibco, EU-approved, South America) and 1% Pen/Strp (100 mg/ml streptomycin, 100 U/ml penicillin, Gibco, USA), and 10 ng/ml glial cell-derived neurotrophic factor (GDNF, Sigma, USA) for 30 days. The SSCs were incubated at 37°C and 5% CO_2 in a humidified atmosphere. The number of viable cells were then determined by the MTT assay. The SSCs were cultured in laminin-coated culture dishes and then sorted and purified by magnetic activated cell sorting (MACS) according to the Invitrogen protocol for MACS (22).

Preparation of a Sertoli cell feeder layer

A layer of Sertoli cells was used as a protective, feeding layer for the SSCs. A mouse Sertoli cell line (NCBI code: C513) was purchased from Pasteur Institute (Tehran, Iran). The Sertoli cells were cultured in a T-25 flask that contained DMEM supplemented with 10% FBS and 1% antibiotics (100 mg/ml streptomycin, 100 U/ml penicillin). Mitotic division of the progenitor cells was stopped by the addition of 10 µg/ml of mitomycin C (Sigma, USA) to the culture medium that contained the serum and the flask was allowed to incubate for two hours at 37°C (22). This medium was subsequently removed and the Sertoli cells were washed 2-3 times with PBS. After isolation and purification, the SSCs were co-cultured with Sertoli cells as a feeder layer (2D culture system). The culture medium consisted of 10% FBS and 10 ng/ml GDNF. The co-culture of SSCs and Sertoli cells was incubated at 37°C and 5% CO_2 in a humidified atmosphere for 30 days to enable the SSCs to form colonies.

Cell viability

The MTT assay was used to assess the cell cytotoxicity of melatonin (Sigma, USA, M5250-1G) and determine optimum concentrations of melatonin and H_2O_2 (Neutron Pharmaceutical Co., Iran) for SSC viability (23). To perform this assay, 4×10^4 cells were placed in each well of a 96-well plate and treated with different concentrations of melatonin (100, 250, 500, 1000, 1500 µM) for 24 hours. The viability of the cultured cells was measured after incubation with 0.5 mg/ml of MTT (Thermo Fisher Scientific, USA). For this measurement, 100 µl of the medium that contained 0.5 mg/ml MTT was added to each well and the plates were incubated in the dark at 37°C for three to four hours. Then, the supernatant was discarded and the resultant formazan crystal was dissolved in 100 µl of dimethyl sulfoxide (DMSO, Sigma, USA). Optical density (OD) of the samples was read at 570 nm with an ELISA reader. In order to evaluate the SSCs viability after oxidative stress, the cells were treated with two different concentrations of H_2O_2 (50 and 100 µM) and the viability of the cultured cells was measured by the MTT assay. We investigated the effects of melatonin under oxidative stress conditions by simultaneously exposing the cells to different concentrations of melatonin (100, 250, 500, 1000, 1500 µM) and 50 µM of H_2O_2 for 24 hours. The cells in the control group did not receive any treatment. Then, four groups of SSCs were examined: control, melatonin (250 µM), melatonin (250 µM)+H_2O_2 (50 µM), and H_2O_2 (50 µM).

Reactive oxygen species

The effect of the protective concentration of melatonin (250 µM) on ROS production in SSCs under oxidative stress conditions (H_2O_2 50 µM) was measured by using 2′-7′-Dichlorodihydrofluorescein (DCFH-DA, Sigma, St. Louis, MO, USA, D6883) and a flow cytometer according to the manufacturer's instructions (24). For this measurement, three groups of SSCs were treated separately with either 250 µM of melatonin, 250 µM of melatonin plus 50 µM of H_2O_2, or 50 µM of H_2O_2. The cells in the control group did not receive any treatment. After 24 hours, the cells were washed with PBS. After resuspension in PBS, the cells were incubated with 1 µM of DCFH-DA dye at 37°C for 30 minutes. The green fluorescence of DCFH-DA in the FL-1 channel was read at 500-530 nm by a flow cytometer. The fluorescence intensity was determined by measuring 10 000 cells per group and examining the average fluorescence intensity in the resultant flow cytometry histograms. The data were statistically analysed with FlowJo software (Flowjo 7.6.1, BD Biosciences, USA).

Malondialdehyde

Lipid peroxidation was calculated by measuring malondialdehyde (MDA) with Fluorometry according to the manufacturer's instructions (Kiazist, KMDA-96, Iran) (25). For this measurement, 50 µl of the homogenate of each sample that contained SSCs treated with a certain concentration of melatonin and H_2O_2 was mixed with 250 µl of the solution that contained 20% trichloroacetic acid and 100 µl of 0.6% thiobarbituric acid. The mixture was heated in a boiling water bath for at least 20 minutes. The samples were allowed to cool before they were centrifuged at 5000 RPM for five minutes to remove the impurities. Then, 200 µl of the supernatant from each sample was transferred to a 96-well plate and the absorbance was read compared to a blank (200 µl of water) at 535 nm by a spectrophotometer (Bio-Tek, Winooski, VT, USA). The amount of MDA produced was determined from a standard curve of 1,1,3,3-tetraethoxypropane (TEP). For this purpose, a 1 mM TEP solution was mixed with 50 ml of 1% sulphuric acid and incubated for one hour. Based on the stock concentration, TEP dilutions of 100, 50, 25, 12.5, and 0.63 nmol/ml were prepared and subjected to the same preparation processes performed on the samples. Absorbance of the standard samples was read at 535 nm and plotted against the standard MDA concentration in order to ascertain a standard MDA curve and determine the line formula. The concentration of the samples was then calculated from the slope of the standard curve.

Adenosine triphosphate

Fluorometry was used to determine the effects of 250 µM of melatonin on ATP levels in SSCs under oxidative stress conditions. The three groups of treated SSCs (250 µM of melatonin; 250 µM of melatonin+50 µM of H_2O_2; and 50 µM of H_2O_2) and the untreated control group were assessed. After 24 hours, the cell deposits were collected and analysed with a Colorimetric/Fluorometric ATP Assay Kit (catalogue number MAK190, Sigma). ATP levels were measured according to a previously published protocol (26).

Quantitative real-time polymerase chain reaction

The expressions of the apoptotic genes [Bcl-2 associated X-protein (*Bax),* Fas cell surface death receptor (*Fas*), B-cell lymphoma 2 (*Bcl2*)] and antioxidant genes [*Nrf2, Trx2, Txnip, NNT,* Sirtuin 3 *(Sirt3)*] were determined by quantitative real-time polymerase chain reaction (qRT-PCR). For this purpose, the three groups of SSCs were treated with either 250 µM of melatonin, 250 µM of melatonin plus 50 µM of H_2O_2, or 50 µM of H_2O_2 for 24 hours at 37°C. The cells in the control group did not receive any treatment. RNA extraction was then performed using a Total RNA Extraction Kit (Pars Tous, Iran) (27) according to the manufacturer's instructions. We determined the concentration and quality of the extracted RNA by measuring light absorbance with a NanoDrop spectrophotometer (Pishro Pajoohesh, Iran). Next, 1 µg of RNA, 1 µl of 10x reaction buffer that contained $MgCl_2$, and 0.5 µl of DNase I that contained 1 unit of the enzyme without RNase were placed in an RNase-free microtube and incubated at 37°C for 30 minutes, followed by the addition of 1 µl of 50 mM EDTA. The product was allowed to incubate at 65°C for 10 minutes. The resultant RNA was used for cDNA synthesis and assessed with an Easy cDNA Synthesis Kit (Pars Tous, Iran, cat: A101162) according to the manufacturer's instructions.

Gene expressions were determined by qRT-PCR with a RunMei Q2000 PCR machine Hunan Runmei Gene Technology Company, Ltd (RunMei, China) and a 2X SYBR Green Real-Time PCR Master Mixmix (+ROX) kit (Pars Tous, Iran, cat: C101022) using SYBR green dye. The reactions were carried out in 50 cycles per gene in a volume of 12.5 µl.

The results were interpreted by comparing ΔΔCt using RunMei QC3 software and the general $2^{-\Delta\Delta Ct}$ formula with *Gapdh* as the calibrator. The *Nrf2*, *Trx2*, *Txnip*, *NNT*, *Sirt3*, *Bax*, *Bcl2*, *Fas*, and *Gapdh* gene sequences were extracted from http://www.ncbi.nlm.nih.gov. Forward and reverse primers were designed using Primer express (version 3.05) software (Table S1, See Supplementary Online Information at www.celljournal.org).

Western blot

Western blot was used to investigate the effect of melatonin on the expressions of the antioxidant proteins Nrf2, Trx2, and NNT in the four assessed groups of SSCs (28). The SSCs were treated in different groups as previously mentioned. After 24 hours, the cells were collected and placed in 200 µl of DMEM and kept in a -80°C freezer until Western blot analysis. A total of 300 µl of each sample of the treated cells was homogenised in lysis buffer (137 mM NaCl, 20 mM Tris HCl, pH=7.4, 5% sodium dodecyl sulfate (SDS), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM Sodium vanadate) using a SpeedMill PLUS homogeniser (Analytik Jena, Germany). The resultant suspension was centrifuged at 2500 RPM for two minutes, and the supernatant was collected and stored in a -80°C freezer until use. The amount of protein in the tissue homogenate was determined by the Bradford assay. For SDS-Polyacrylamide gel electrophoresis, 20 µg of protein from each sample was placed on 8% polyacrylamide gel and subjected to electrophoresis. The proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes and then blocked with 5% nonfat dry milk and 0.1% Tween-20 in TBS for one hour at room temperature. After blocking, the paper was incubated with the primary antibodies for Trx-2 (1:1000; F-10:sc-133201, Santa Cruz Biotechnology, Inc.), Nrf2 (1:1000; D1Z9C XP #12721, Cell Signalling Technology), NNT (1:1000; B-3:sc-390236, Santa Cruz Biotechnology, Inc.), Gapdh (1:1000; D16H11 XP #5174, Cell Signalling), and β-actin (1:1000; C4:sc-47778, Santa Cruz Biotechnology, Inc.) as the internal control gene for 16 to 18 hours. Another incubation was then performed for the secondary antibody (1:2000; mouse anti-rabbit IgG-HRP; sc-2357) in PBS for one hour. An ECL kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) was then used according to the manufacturer's instructions to reveal the proteins. Finally, light-sensitive papers were scanned with a JS 2000 scanner (Wuhan BonninTechnology Ltd, China) and the band density was determined.

Statistical analysis

All data in this paper are written as mean ± SD for at least three independent replicates. The data were analysed with one-way ANOVA and Tukey's post hoc test with GraphPad Prism software (version 7, Dotmatics, USA). For quantitative variables, the normality of distributions was checked by the Kolmogorov-Smirnov test. $P<0.05$ was considered to be statistically significant.

Results

Viability of the isolated spermatogonial stem cells

Mouse SSCs were isolated by a two-step enzymatic

digestion with collagenase IV, DNase I, and trypsin and purified with anti-Thy-I antibody by MACS. Trypan blue staining as measurement of cell viability showed that over 90% of the cells were viable.

Spermatogonial stem cell colony formation

The SSCs cultured on a feeder layer of Sertoli cells were round or oval with large nuclei and small cytoplasms. These cells started to form colonies at the end of the first week of culture. Large numbers of large colonies formed after 20 days (Fig.1).

Effect of N-acetyl-5-methoxy tryptamine (melatonin) on cell viability under oxidative stress conditions

The results of the MTT assay showed no significant difference in viability between the control group and the SSCs treated with different concentrations of melatonin (100, 250, 500, 1000, and 1500 µM) for 24 hours. However, the cells that were treated with lower concentrations of melatonin (100, 250, and 500 µM) had higher viability than those treated at higher concentrations (1000 and 1500 µM). The cells that were treated with H_2O_2 (50 and 100 µM) showed significantly lower viability than the control group ($P<0.001$). The MTT assay results showed that the protective effect of melatonin on the cells under oxidative stress conditions was greater at 250 µM, which was a significant difference compared to the H_2O_2 group. Therefore, 250 µM was chosen as the optimal dose in this study.

Effect of N-acetyl-5-methoxy tryptamine (melatonin) on reactive oxygen species levels under oxidative stress conditions

Flow cytometry data showed that H_2O_2 resulted in higher DCF fluorescence compared to the control group with a mean fluorescence intensity of (228 MFI compared to 105 MFI). Treatment with melatonin significantly ($P<0.001$) reduced the impact of H_2O_2 (151 MFI versus 228 MFI) (Fig.2).

Effect of N-acetyl-5-methoxy tryptamine (melatonin) on malondialdehyde levels under oxidative stress conditions

Fluorometry results showed that exposure to 50 µM of H_2O_2 for 24 hours significantly ($P<0.001$) increased lipid peroxidation levels in the SSCs (MDA level) compared to the control group. Co-treatment with 250 µM of melatonin significantly reduced MDA levels, and returned them to normal in the control group (Fig.3).

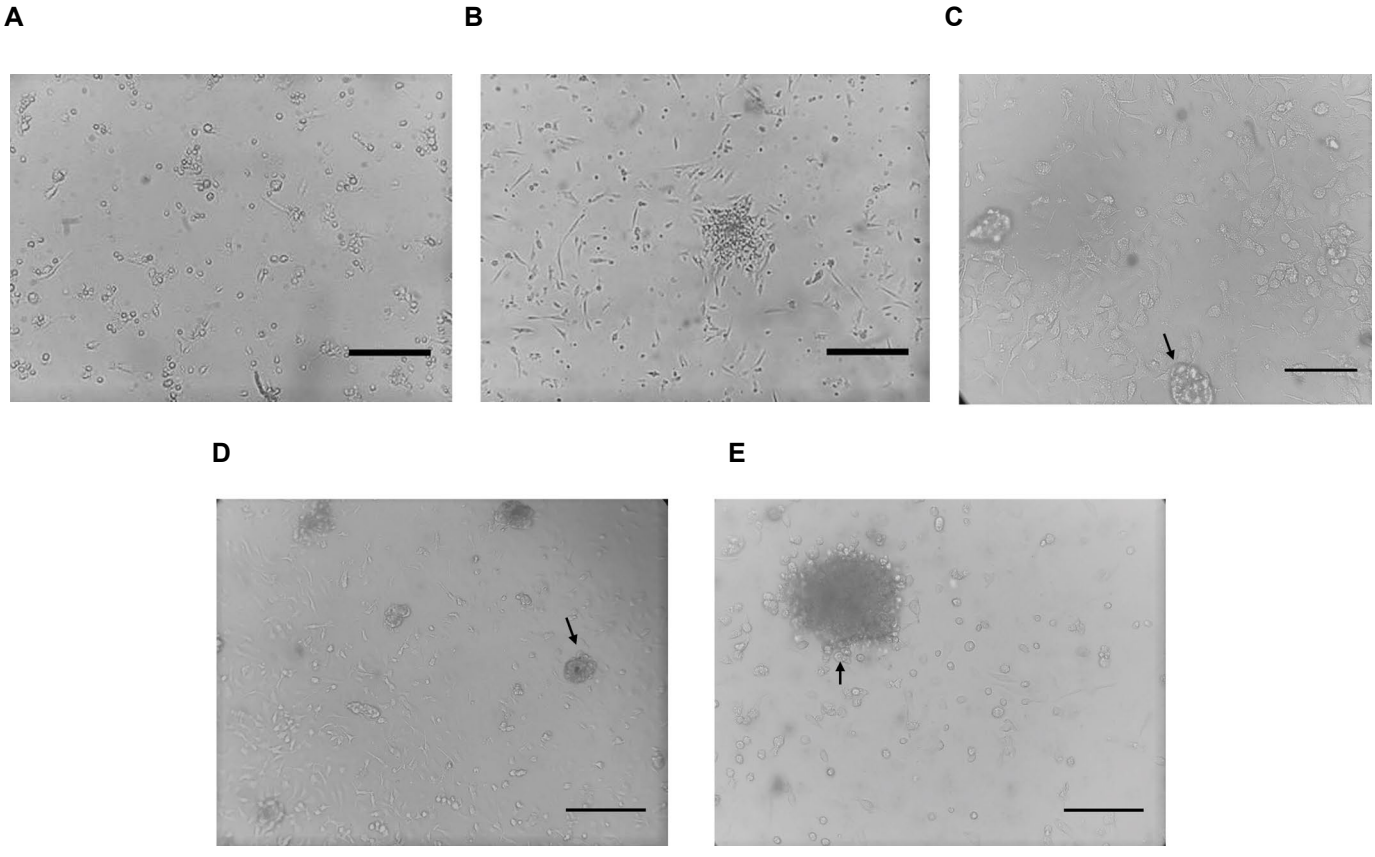

Fig.1: Spermatogonial stem cells (SSCs) culture and morphology. **A.** Mouse type A SSCs after enzymatic digestion with trypsin-EDTA (10x magnification). **B.** Sertoli cell morphology (10x magnification). **C-E.** Arrows indicate SSCs colony after 20 days of co-culture with a feeder layer of Sertoli cells in a 2D culture system (40x magnification, scale bar: 100 µm).

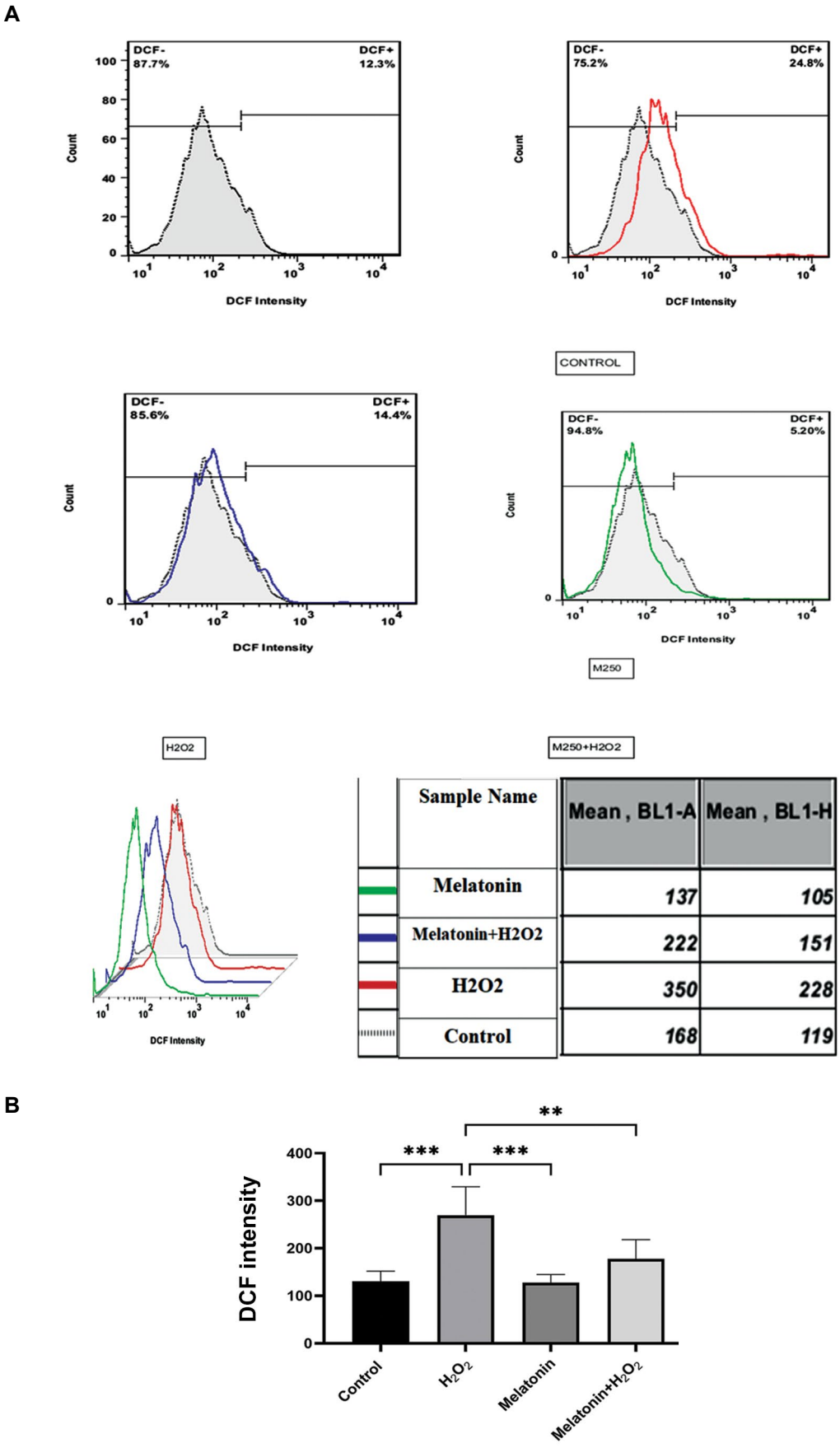

Fig.2: Protective effects of melatonin on ROS production induced by H_2O_2 in SSCs. **A.** Representative flow cytometry histograms show intracellular ROS levels measured by DCF fluorescence. **B.** The quantitative data are shown as mean ± SD. The SSCs were treated separately with 250 µM of melatonin, 250 µM of melatonin plus 50 µM of H_2O_2, or 50 µM of H_2O_2 for 24 hours. The cells in the control group did not receive any treatment. ROS; Reactive oxygen species, SSCs; Spermatogonial stem cells, DCF; Dichlorofluorescein, **; P<0.01, and ***; P<0.001.

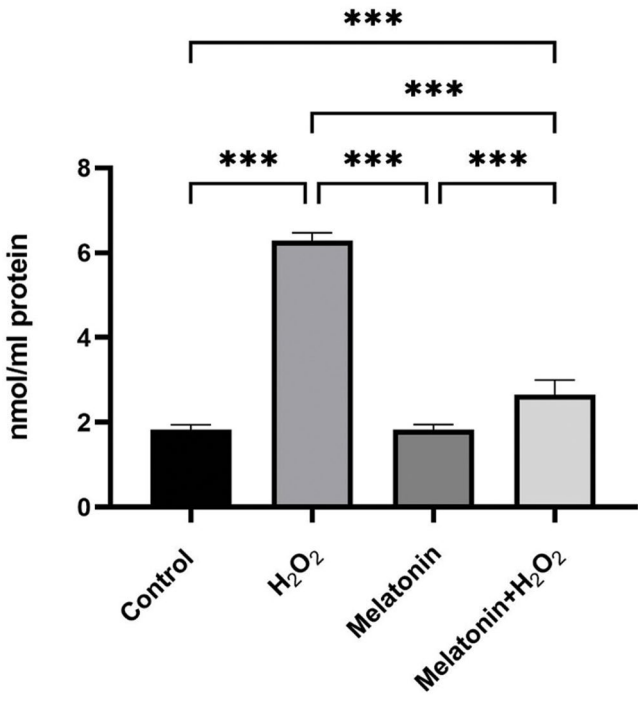

Fig.3: Protective effects of melatonin on MDA levels under oxidative stress conditions. SSCs were treated with either 250 µM of melatonin, 250 µM of melatonin plus 50 µM of H_2O_2, or 50 µM of H_2O_2 for 24 hours. The cells in the control group did not receive any treatment. Data are shown as mean ± SD. MDA; Malondialdehyde, SSCs; Spermatogonial stem cells, and ***; P<0.001.

Effects of N-acetyl-5-methoxy tryptamine (melatonin) on adenosine triphosphate levels under oxidative stress conditions

Fluorometry results showed that 24 hours of exposure to melatonin significantly increased (P<0.001) ATP levels in the SSCs (1.2 nmol/µg protein) compared to the control group (1 nmol/µg protein). Exposure to H_2O_2 decreased the ATP levels, whereas melatonin caused an increase in ATP under oxidative stress (Fig.4).

Effect of N-acetyl-5-methoxy tryptamine (melatonin) on apoptotic gene expression under oxidative stress conditions

Exposure to H_2O_2 significantly increased the expressions of the apoptotic genes *Bax* and *Fas* in the SSCs. Melatonin significantly reduced the expressions of these genes (P<0.001) and restored them to the level of the untreated control group. Treatment with melatonin also increased *Bcl2* expression, which was significantly lower in the H_2O_2-treated group compared to the control group (P<0.001, Fig.5).

Effects of N-acetyl-5-methoxy tryptamine (melatonin) on the expression of antioxidant genes under oxidative stress conditions

Exposure to H_2O_2 significantly increased expressions of *Nrf2* and its downstream genes in the mitochondrial thioredoxin system (*Trx2, NNT*, and *Sirt3*; P<0.001). Treatment with melatonin caused a greater increase in expressions of these genes. The cells also showed significantly lower expression of *Txnip*, an inhibitor of *Trx2*, in the presence of melatonin (Fig.5).

Effect of N-acetyl-5-methoxy tryptamine (melatonin) on the expression of Nrf2, Trx2, and NNT proteins under oxidative stress conditions

The results showed that oxidative stress conditions (H_2O_2, 50 µM) increased expression of the antioxidant pathway proteins Trx2, Nrf2, and NNT (Fig.6). Melatonin, an exogenous antioxidant, significantly increased the expressions of these proteins under oxidative stress conditions (P<0.001). Western blot results also showed that 24 hours incubation of SSCs with 250 µM of melatonin increased the expressions of Nrf2, TRX2, and NNT compared to the control group; this effect was greater under oxidative stress conditions. Western blot results showed that melatonin increased NNT protein expression in both oxidative stress (1.63 ± 0.09) and normal (1.44 ± 0.07) conditions compared to the control group (1.00 ± 0.05). However, there was no significant difference in NNT protein expression observed between the H_2O_2 and melatonin plus H_2O_2 groups during oxidative stress.

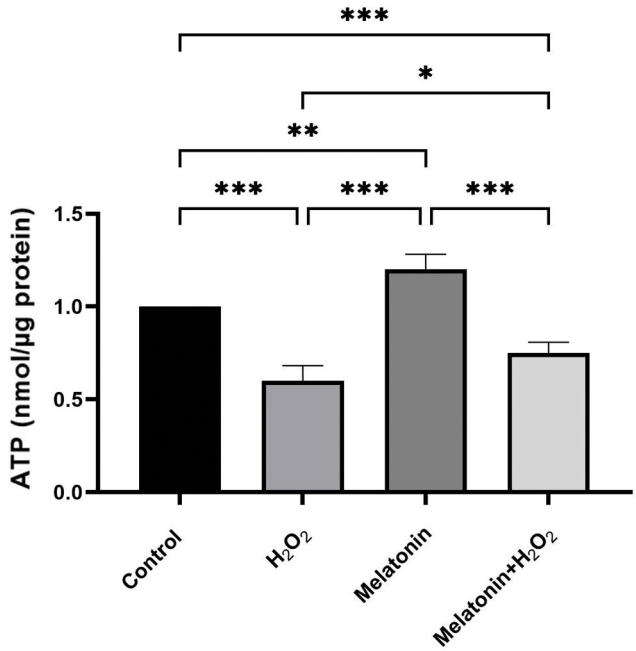

Fig.4: Protective effects of melatonin on ATP levels in oxidative stress conditions. SSCs were treated with either 250 µM of melatonin, 250 µM of melatonin plus 50 µM of H_2O_2, or 50 µM of H_2O_2 for 24 hours. The cells in the control group did not receive any treatment. Data are shown as mean ± SD. ATP; Adenosine triphosphate, SSCs; Spermatogonial stem cells, *; P<0.05, **; P<0.01, and ***; P<0.001.

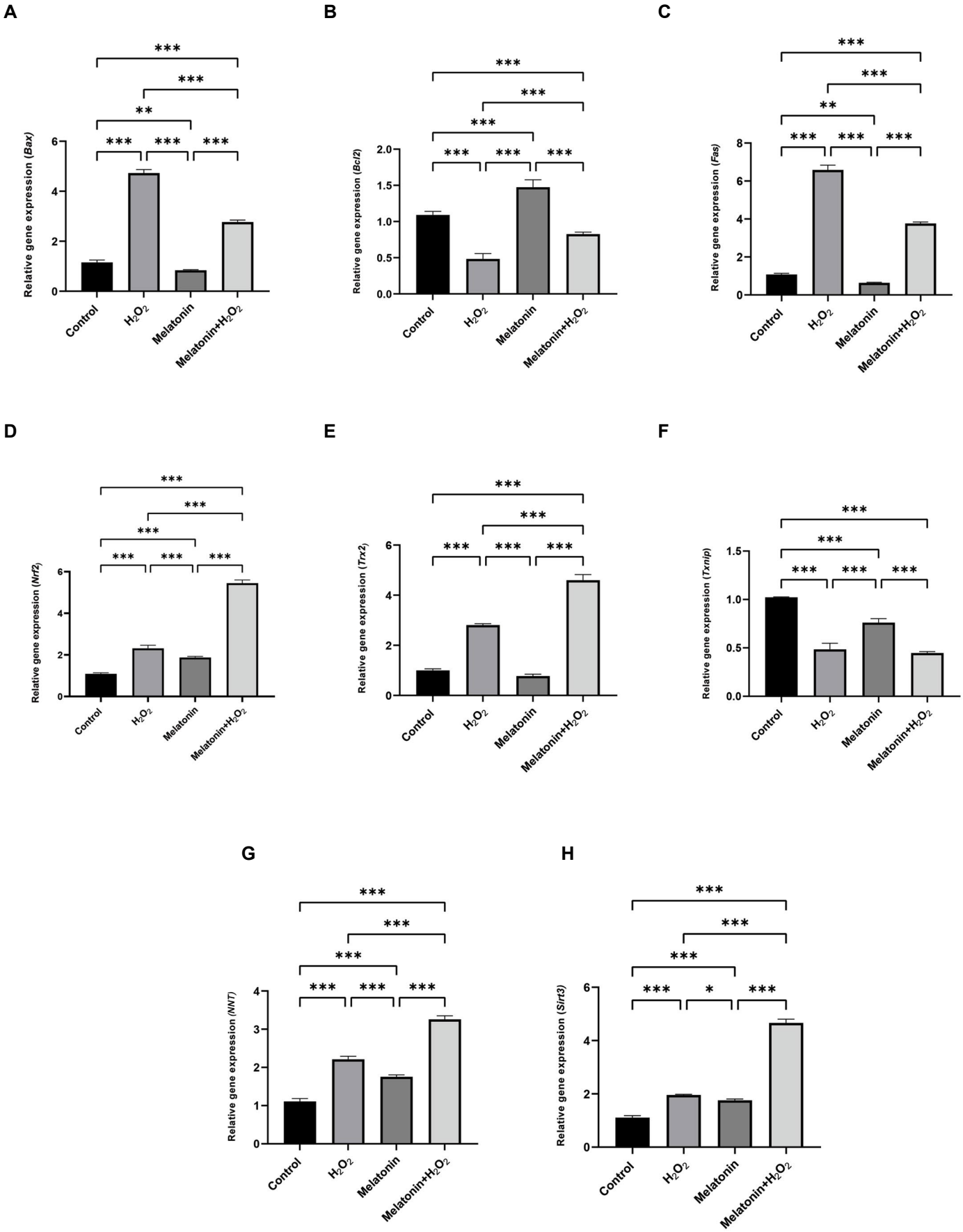

Fig.5: Effects of melatonin and H_2O_2 on apoptotic and antioxidant gene expressions. qRT-PCR analysis for **A.** *Bax*, **B.** *Bcl-2*, **C.** *Fas,* **D.** *Nrf2,* ***E.*** *Trx2,* **F.** *Txnip,* **G.** *NNT,* and **H.** *Sirt3* after exposure of cells with either 250 µM of melatonin, 250 µM of melatonin plus 50 µM of H_2O_2, or 50 µM of H_2O_2 for 24 hours. The cells in the control group did not receive any treatment. mRNA expression was normalised using *Gapdh* mRNA as an internal standard. Data are shown as mean ± SD. qRT-PCR; Quantitative real-time polymerase chain reaction, *; P<0.05, **; P<0.01, and ***; P<0.001.

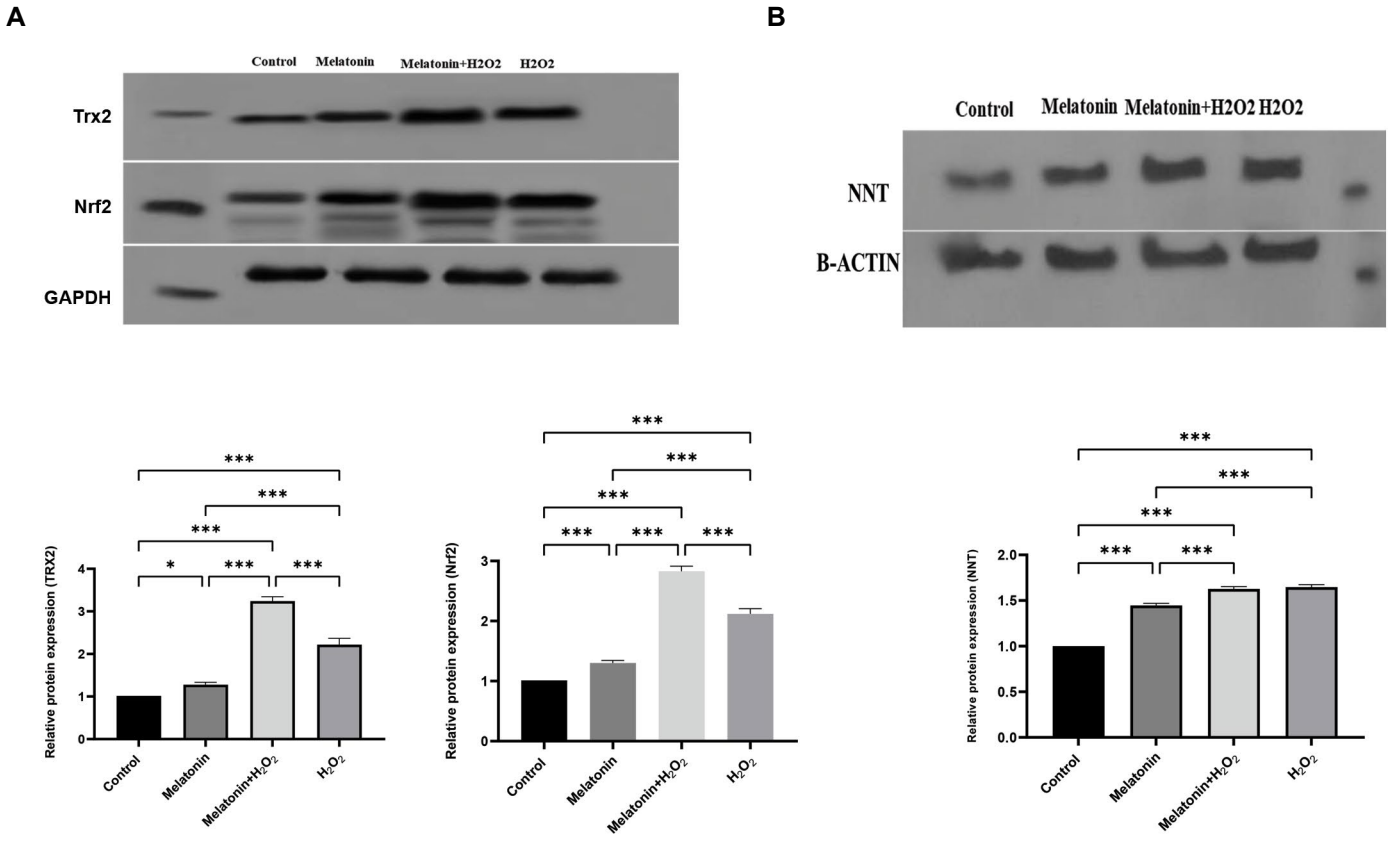

Fig.6: The effects of melatonin and H_2O_2 on the expressions of antioxidant proteins of the thioredoxin system. Western blot analysis for: **A.** Nrf2 and Trx2, and **B.** NNT after exposure of cells to either 250 µM of melatonin, 250 µM of melatonin plus 50 µM of H_2O_2, or 50 µM of H_2O_2 for 24 hours. The cells in the control group did not receive any treatment. Data are shown as mean ± SD. *; P<0.05 and ***; P<0.001.

Discussion

This study investigated the effects of melatonin and its associated mitochondrial pathways on mouse type A SSCs under oxidative stress conditions. The results suggest that melatonin can reduce H_2O_2-induced ROS production and apoptosis in SSCs by activating the mitochondrial thioredoxin system. It can also increase protein and mRNA expression of *Nrf2* under oxidative stress conditions, which is expected to increase antioxidant activity of the mitochondrial thioredoxin system. According to the results, melatonin can mitigate the damaging effects of H_2O_2-induced ROS by increasing both gene and protein expressions of *Trx2* and decreasing gene expression of *Txnip* (Trx2 inhibitor), in addition to increasing gene and protein expressions of *NNT* and gene expression of *Sirt3*. Melatonin increases the amount of energy available to the cells by elevating ATP levels.

The effects of melatonin on SSCs have been examined in multiple studies. Li et al. (29) reported that melatonin increased the expression of superoxide dismutase (SOD) and regulated busulfan-induced ROS production. They observed that melatonin also increased *Sirt1* expression, which is involved in the deacetylation of *p53*; lower concentrations of deacetylated *p53* enhance the resistance of SSCs to apoptosis. There is evidence that melatonin can reduce bisphenol A-induced lipid peroxidation in mouse testes, restore mitochondrial marker enzymes, and improve the activity of enzymatic and non-enzymatic mitochondrial antioxidants (30). Treatment with melatonin reduces the severity of testicular tissue damage in animal models of hyperlipidaemia, testicular torsion, varicocele, and toxicity caused by chemotherapy drugs or environmental toxins (15, 16). Jou et al. (31) showed that melatonin significantly inhibited ROS production under normal conditions and during the initial stages of H_2O_2-induced oxidative stress. They concluded that melatonin prevents ROS from depolarizing the mitochondrial membrane and opening mitochondrial membrane pores, which prevents the release of cytochrome C. Melatonin also suppresses palmitic acid-induced apoptosis by regulating the expressions of apoptosis-related proteins, including *Bcl2, Bax, C-caspase 3*, and *C-caspase 12* in type-B SSCs (32). In our study, we observed an association of melatonin with favourable changes in the expressions of apoptotic genes (decreased *Bax* and *Fas,* and increased *Bcl2*), a decrease in ROS and MDA levels, and an increase in activity of the thioredoxin antioxidant system under oxidative stress conditions. According to He et al. (33), Nrf2 suppresses the basal expression of *Txnip* in heart cells, which is important because *Txnip* induces ROS production and apoptosis by inhibiting *Trx*. Nrf2 also inhibits *Txnip* by binding to its promoter to prevent transcription, which increases thioredoxin activity.

Similarly, we found that melatonin treatment increased both gene and protein expressions of *Nrf2* under oxidative stress, and led to increased gene and protein expression of *Trx2* and reduced expression of *Txnip*. This suggests that melatonin, as an antioxidant, contributes to more effective ROS removal by increasing Nrf2 and its downstream pathway, namely the thioredoxin system.

Zhou et al. (34) reported that the decrease in cellular ROS in melatonin-pretreated microglia was due to the inhibition of ROS production rather than the direct removal of ROS. According to this study, melatonin inhibits NADPH oxidase activation in amyloid-beta-activated microglia but has no apparent inhibitory effect on activated NADPH oxidase. This study provided evidence that melatonin can disrupt the NADPH oxidase accumulation in amyloid-beta-activated microglia and inhibit ROS production in a dose-dependent manner. However, melatonin's ability to directly inhibit superoxide was not significant at 100 μM and only notable at 250 μM. In our study, the best antioxidant effects of melatonin were observed at the 250 μM dose. However, no significant difference was observed between the 100 μM and 250 μM doses of melatonin under normal cellular conditions. Hence, it is suggested that the desired doses should be measured at 2-, 6-, and 12-hour intervals. Pretreatment with melatonin should be investigated in future studies. Florido et al. (26) reported that 0.5 and 1 mM doses of melatonin caused apoptosis by driving mitochondrial reverse electron transport to induce ROS production in head and neck squamous cell carcinoma cell lines. This finding indicated that high doses of melatonin could induce ROS.

Therefore, it is possible that 250 μM of melatonin could act as an important transcription factor for expression of phase II antioxidant enzymes. These enzymes comprise a group of vital proteins that carry out the detoxification process by increasing hydrophilicity and increasing excretion of xenobiotics through increasing Nrf2 expression. The Keap1 protein is an important factor for Nrf2 function and its transfer into the nucleus. Nrf2 activating agents such as melatonin can affect this factor, and should be investigated in future studies on the Keap1 protein. Yu et al. (35) assessed the effects of melatonin on the thioredoxin system during myocardial ischemia-reperfusion (MI/R) injuries. They found that melatonin treatment reduced myocardial apoptosis; significantly increased Notch1/Hes1/Akt signalling pathway activity; improved the activity of the intracellular thioredoxin system by increasing Notch1, N1ICD, Hes1, and p-Akt activities; and decreased *Txnip* expression. Therefore, they concluded that melatonin reduces the activity of the thioredoxin system by decreasing the expression of *Txnip* through the Notch1/Hes1/Akt signalling pathway in a membrane receptor-dependent manner. We investigated the protective effects of melatonin on Trx/Txnip after induction of oxidative stress. Our results showed that melatonin changed the expression levels of Trx and Txnip. Trx expression significantly increased at the mRNA and protein levels in the groups treated with melatonin alone and under oxidative stress conditions; on the other hand, it caused a decrease in Txnip expression at the mRNA level. Inhibition of Nrf2, an inhibitor of Txnip, is proposed to increase Txnip expression to some extent. Hence, the effect of melatonin on Txnip expression at the protein level and its function, and the effect of inhibition of Nrf2 by trigonelline should be studied. It is also possible that melatonin directly increases Trx activity, which can be related to the regeneration of the oxidised form of Trx by melatonin, or that melatonin may directly inhibit Txnip or Trx activity. More studies that assess the effect of melatonin on Txnip and Trx2 at the protein and activity levels are suggested.

One of the limitations of this study was the unavailability of a Trx2 activity assay kit and 3D culture system for SSCs. Along with combining SSCs with Sertoli cells in a 2D environment, embedding SSCs in a 3D culture system can also provide a structure that imitates the complex structure found in living testes. Multiple studies have shown that NNT is a physiological source of NADPH in mitochondria (36, 37). Silencing of small interfering RNA of NNT in PC12 cells decreases cellular NADPH levels, thereby changing the cell's redox state by decreasing the GSH/GSSG ratio and increasing H_2O_2 levels, which leads to increased redox potential and disruption of electron flow to redox components, and ultimately results in mitochondrial dysfunction (37). We observed that, in the H_2O_2 group, expression of *NNT* increased to provide the amount of NADPH required by the mitochondrial redox system to overcome the oxidative stress conditions. In addition, melatonin contributed to more effective ROS removal by increasing *NNT* expression.

Sirt3 is a member of the Sirtuin family that is primarily positioned in the mitochondria, and protects the tissue against oxidative stress-related conditions such as MI/R. It has been shown that melatonin has positive effects on repairing the damage caused by MI/R. Zhai et al. (38) reported that MI/R injury significantly decreased myocardial *Sirt3* expression and activity. Treatment with melatonin regulated *Sirt3* expression and activity, thereby leading to decreased SOD-2 acetylation. They noted that melatonin increased *Bcl2* expression and decreased the expressions of *Bax* and *Caspase 3* after MI/R. However, the cardioprotective effects of melatonin were largely restricted by the selective *Sirt3* inhibitor (3-TYP), which indicates that Sirt3 plays an essential role in mediating the protective effects of melatonin. This suggests that melatonin alleviates MI/R injury by reducing oxidative stress and apoptosis through activation of the Sirt3 signalling pathway. We also observed that melatonin led to increased Sirt3 expression and ATP levels in SSCs under oxidative stress conditions. Jiang et al. (39) reported that while Nrf2 does not affect the quantity of mitochondrial antioxidant enzymes, it regulates NADPH by controlling gene expression in PPP, which is the main source of cytoplasmic NADPH. In this way, it indirectly increases mitochondrial NADPH to activate the Trx2 system, and thus mobilises the mitochondrial thioredoxin system for

better H_2O_2 removal. In a study by Rao et al. (40), it was noted that NNT is a key enzyme in the mitochondrial inner membrane that regulates the NADPH required by thiol-dependent peroxidase systems of mitochondria. These researchers observed significant impairment in mitochondrial function in response to angiotensin II (Ang II), which was associated with increased superoxide and H_2O_2 levels. They reported an increase in NNT expression and activity in response to the mitochondrial dysfunction and oxidative stress associated with Ang II treatment. In their study, decreased NNT activity led to significantly increased mitochondrial ROS production and reduced glutathione peroxidase and glutathione reductase activity, which they linked to a reduced NADPH/$NADP^+$ ratio, as well as impaired ATP production. We observed elevated *NNT* gene and protein expressions in response to the increased H_2O_2 level. Our results showed that melatonin, as an exogenous antioxidant, increased both gene and protein expressions of *NNT* under oxidative stress conditions, and helped supply the necessary amount of NADPH needed by the thioredoxin system for effective ROS removal. Therefore, our results showed that melatonin may be considered a natural antioxidant to reduce oxidative stress and its action pathways in SSCs. However, additional studies are needed to assess the effects of melatonin on SSCs and oxidative stress pathways, especially the glutathione peroxidase pathway. More research on the effects of melatonin on redoxisome and its role in diseases such as male infertility is needed.

Conclusion

The results of this study show that melatonin is a strong antioxidant that can affect the mitochondrial antioxidant system by increasing *Nrf2* expression at the gene and protein levels. Melatonin can induce the Trx2 system and the Trx/Txnip pathway, elevate expression of *NNT* at the gene and protein levels, and increase the amount of energy available to the cell by increasing ATP levels. Melatonin increases mRNA expression of *Sirt3* and decreases mRNA expression of *Txnip*, thereby enhancing the activity of the mitochondrial thioredoxin system. It seems that melatonin can play an effective role in reducing oxidative stress in SSCs under pathological conditions by activating the cytoplasmic and mitochondrial antioxidant pathways (Nrf2, Trx2); therefore, it can be used in the future as an effective antioxidant in the clinic and for infertility treatments.

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Authors' Contributions

M.Gh., S.H., Z.R.; Project administration, Conceptualization, and Methodology. M.Gh., S.H., Z.R., K.M., B.M.; Collected spermatogonial stem cells, Conducted the experiments, and Data analysis. Z.R., S.H., M.Gh., K.M., I.R., C.J.; Consulted in qRT-PCR and Western blot analyses. All authors read and approved the final manuscript.

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