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Protective effects of melatonin against hypoxia-induced TM3 cell damage *via* suppression of the TGF-β pathway

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Abstract

This study aimed to explore whether melatonin protects TM3 Leydig cells from cobalt (II) chloride ($CoCl_2$)-induced hypoxia through the transforming growth factor beta (TGF-β) signaling pathway. Cells were divided into four groups: a control group without treatment (Group 1), a melatonin group (10.00 ng mL^{-1}; Group 2), a group treated with $CoCl_2$ (100 µM) to induce hypoxia (Group 3), and a melatonin + $CoCl_2$ group (Group 4). After 96 hr of incubation, cell viability was assessed using the MTT assay, and *transforming growth factor beta 1*, *activin receptor-like kinase-5*, and *bone morphogenetic protein 4* gene and protein expressions were measured through RT-PCR and western blotting. The $CoCl_2$ and melatonin + $CoCl_2$ groups exhibited significantly diminished cell viability compared to the control. However, melatonin treatment enhanced survival in the $CoCl_2$-exposed cells. Notably, transforming growth factor beta 1 expression was elevated in all groups. *Activin receptor-like kinase-5 (*gene and protein expression increased in $CoCl_2$-treated groups but was lower in the melatonin + $CoCl_2$ group. Melatonin treatment reduced bone morphogenetic protein 4 expression compared to the control, while $CoCl_2$ groups showed increased bone morphogenetic protein 4 levels. These findings suggest melatonin's potential as a therapeutic agent against oxidative stress and hypoxia in TM3 cells through its antioxidant properties.

Introduction

Hypoxia is a major stressor that impacts reproductive health.[1] The main adverse effects of hypoxia on male reproduction include excessive reactive oxygen species (ROS)-mediated oxidative stress, suppression of apoptosis and proliferation, chronic inflammation, and epigenetic alterations.[2] Hypoxia has been demonstrated to diminish male fertility in both animals and humans, resulting in lower sperm count, lower sperm motility, and abnormal sperm morphology.[3]

Leydig cells are a crucial cell type in the testis responsible for androgen synthesis and release. They can stimulate the growth of the male reproductive system and spermatogenesis. Research has indicated that testicular function is closely linked to oxidative stress and Leydig cell death.[4] Therefore, anti-oxidant and anti-apoptotic supplements may be a potential therapy for addressing male infertility.[5]

Melatonin (N-acetyl-5-methoxytryptamine), a hormone produced by the pineal gland, regulates sleep and circadian rhythm, as well as other important physiological functions.[6] It plays a significant role in various biological activities, including immune regulation, anti-inflammatory responses, and oxidative stress responses.[7] Some studies have shown that melatonin enhances cell survival in normal tissues,[8] and that its antioxidant properties may prevent cell death in both healthy and pathological conditions.[6] Research has also demonstrated that melatonin directly influences the release of testosterone,[9] enhances the response of Sertoli cells to follicle-stimulating hormone during testicular development,[10] and modulates testicular cell growth, proliferation, and secretory activity, while also preventing local inflammatory processes and the production of ROS.[11] Furthermore, melatonin administration has been found to reduce the severity of testicular injury in animal models with conditions such as hyperlipidemia, induced gonadal

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torsion, artificial varicocele, or toxicity caused by exogenous substances like anti-cancer medications or environmental toxins.[12] Melatonin also appears to protect human spermatozoa from apoptosis.[13] Additionally, studies have demonstrated that melatonin has a direct effect on mouse Leydig cells by modulating androgen synthesis and regulating the expression of steroidogenic genes.[12]

The transforming growth factor beta (TGF-β) family is responsible for regulating a wide range of biological functions, including cell division, migration, differentiation, and apoptosis.[14] There are five TGF-β isoforms (TGF-β1-5) encoded by various genes.[15] The TGF-β family members control the growth of the gonads and accessory sex glands, spermatogenesis, immunoregulation of pregnancy, embryo implantation, and placental development in reproductive tissues.[14] Specifically, testicular TGF-β1 is a critical regulator that influences male reproductive function. Testicular TGF-β1 regulates Leydig cell steroidogenesis, the structure of peritubular myoid cells, testis development, and spermatogenesis. Several investigations have found that TGF-β1 plays a role in the tight balance of proliferative and apoptotic responses in Leydig cells.[16] The TGF-β1 acts as a signaling molecule by binding to the serine/threonine kinase receptors TGF-β1 type II and TGF-β1 type I, also known as activin receptor-like kinase-5 (ALK5). The TGF-β1 binding to the type II receptor leads to the creation of heteromeric complexes with ALK5, within which the type II receptor phosphorylates ALK5, activating receptor kinase activity. When ALK5 is active, suppressor of mothers against decapentaplegic 2 (Smad2) and/or suppressor of mothers against decapentaplegic 3 (Smad3) are phosphorylated at C-terminal serines, and the activated Smad2 and/or Smad3 form a heterotrimeric complex with Smad4, which translocate to the nucleus to regulate transcription.[17] The TGF-βRII and ALK-5 receptors have been identified in Leydig cells, Sertoli cells, and germ cells from mice, rats, pigs, hamsters, and humans.[13,15] Bone morphogenetic protein 4 (BMP4) is a member of the TGF-β family. The BMP4 is expressed in Leydig cell lineage cells and inhibits stem/progenitor Leydig cell entrance into adult Leydig cells *via* SMAD-dependent and SMAD-independent signaling pathways.[17]

In the present study, the effect of melatonin on the expression of *TGF-β1*, *ALK5*, and *BMP4* genes in TM3 mouse Leydig cells under hypoxic conditions was investigated.

Materials and Methods

Cell culture and treatments. The cell line (TM3) was obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). The cells were cultured in a 1:1 mixture of Ham-12 and Dulbecco's Modified Eagle Medium (Hyclone, Logan, USA) supplemented with 5.00% horse serum (Gibco, Grand Island, USA), 2.50% fetal bovine serum (Hyclone), 100 U mL^{-1} penicillin (Gibco), and 100 U mL^{-1} streptomycin (Gibco). The cells were then incubated at 37.00 ˚C, 5.00% CO_2 and 95.00% humidity, with the culture media being changed every 2 days. A stock solution of cobalt (II) chloride (Merck, Darmstadt, Germany) at a concentration of 25.00 mM was prepared using ultrapure water and added to serum-free medium at a final concentration of 100μM. Melatonin (Sigma-Aldrich, St. Louis, USA) was dissolved in alcohol at a concentration of 1.00 mg mL^{-1} and stored at – 20.00 ˚C. Before treatment, 1.00 mg mL^{-1} of melatonin was diluted in serum-free medium to a final concentration of 10.00 ng mL^{-1}. When the cells reached 80.00% confluence, they were divided into four groups in cell culture plates. The first group received no treatment (Group 1), the second group (Group 2) was treated with a concentration of 10.00 ng mL^{-1} melatonin, the third group (Group 3) had 100μM $CoCl_2$ added to the medium to induce hypoxia,[18] and the fourth group (Group 4) received 100 μM $CoCl_2$ and 10.00 ng mL^{-1} melatonin. After 96hr of incubation at 37.00 ˚C, 5.00% CO_2 and 95.00% humidity, the cells were collected for further analysis.

MTT assay. The MTT assay was used to assess cell viability. A total of 1.00 × 10^4 cells *per* well were plated in 96-well plates and exposed to various predetermined conditions before being placed in an incubator at 37.00 ˚C. After incubation, 20.00 μL of MTT solution (5.00 mg mL^{-1}) was added to each well, and the cells were further incubated at 37.00 ˚C for 4 hr. The supernatants were then collected, and 200 μL of dimethylsulfoxide (Sigma-Aldrich) was added to each well to dissolve the formazan crystals. The cells were treated with dimethylsulfoxide for an additional 10 min. Absorbance was measured at 490 nm With each test conducted in triplicate.

RNA preparation and RT-PCR. For total RNA extraction, the cells were rinsed with ice-cold phosphate-buffered saline and lysed by adding 1.00 mL TRizol (RNX TM reagent; SinaClon, Tehran, Iran) per 10.00 cm^2 area in a culture dish and scraped with pipette tips. After treatment with DNase-I (Cinnagen, Tehran, Iran), cDNA template (2.00 μg) was generated using the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). Real-time PCR analyses were performed using RunMei (RunMei Gene Technology Co. Ltd, Hunan, China) with a final volume of 12.50 μL, containing 3.00 μL cDNA (100 ng), 0.25 μL of each primer (10.00 μM), 6.25 μL SYBR Green I PCR Master Mix (Yekta Tajhiz, Tehran, Iran), and 2.75 μL RNase-free water. The PCR conditions were as follows: 95.00 ˚C for 30sec; 40 cycles of 95.00 ˚C for 5 min, different Tm97 values for 34 sec; 95.00 ˚C for 15 sec, 60.00 ˚C for 60 sec; and 95.00 ˚C for 15 sec. The $2^{-\Delta\Delta CT}$ method was used to evaluate the data, with β-actin employed as the reference gene. Table 1 shows the primer sequences used.

Table 1. Primer sequences of genes transforming growth factor beta (*TGF-β1*), activin receptor-like kinase-5 (*Alk5*), bone morphogenetic protein 4 (*Bmp4*), *β-actin* used in the current study.

Genes	Accession No.	Primer sequence (5'→3')	Product size (bp)
TGF-β1	M57639.1	F: TGGCTTCCTAGCCAACTTCT R: TTGTCGAAGAAGAGCACGGA	192
Alk5	NM_001312868.1	F: ATCTGCCATAACCGCACTGT R: AGCAGTGGTAAACCTGATCCA	149
Bmp4	NM_001310452.1	F: GGAGTTTCCATCACGAAGAACATC R: GAGATCACCTCATTCTCTGGGAT	109
β-actin	NM_007393.5	F: AAGAGCTATGAGCTGCCTGA R: CCACAGGATTCCATACCCAAGA	105

Protein extraction and western blot analysis. Cellular proteins were extracted using a lysis buffer and quantified using the Bradford method. The cultured cells were washed with ice-cold phosphate-buffered saline and then lysed with ice-cold lysis buffer (8.00 M urea, 2.00 M thiourea, Tris 10.00 mM, pH = 8.00). The cells were scraped wiht a pipette tip and agitated in microcentrifuge tubes for 30 min at 4.00 ˚C. The Laemmli method was used to separate proteins on a 12.00% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis gel, and a semi-dry Trans-Blot system (Bio-Rad, Hercules, USA) was used to electroblot the proteins onto nitrocellulose membranes. The blots were briefly blocked with 5.00% skimmed milk, then incubated overnight at 4.00 ˚C with primary antibodies including anti-TGF-β1 mouse monoclonal, beta-actin monoclonal antibody, Alk5 monoclonal antibody, and Bmp4 monoclonal antibody (Sigma-Aldrich, Taufkirchen, Germany). Then, the blots were treated with a horseradish peroxidase-conjugated secondary antibody (1: 5,000 v/v) for 2hr. Photos were captured using the luminal system on a blot scanner from LiCor (Lincoln, USA). The β-actin was used as an internal normalizer for quantitation.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism (version 3.0; GraphPad Software Inc., San Diego, USA). The normality of the data was assayed by the Shapiro Wilk test. The Levene's test was used to evaluate the homogeneity of variances between groups. Comparisons were made using one-way analysis of variance, followed by the Tukey test. Data are presented as mean ± SEM. A p values less than 0.05 was considered statistically significant.

Results

Cell viability. The results of the MTT assay are shown in Figure 1. The viability of cells treated with 100 µM $CoCl_2$ (Group 3) was significantly reduced ($p < 0.05$) compared to both the control (Group 1) and the melatonin-treated (Group 2) groups. The viability of TM3 cells treated with 10.00 ng mL^{-1} melatonin (Group 2) for 96 hr did not significantly differ from the control group (Group 1). However, cell survival in the group treated with both melatonin and $CoCl_2$ (Group 4) for 96 hr was significantly lower ($p < 0.05$) than the control group (Group 1), but significantly increased ($p < 0.05$) compared to the $CoCl_2$ -treated group (Group 3).

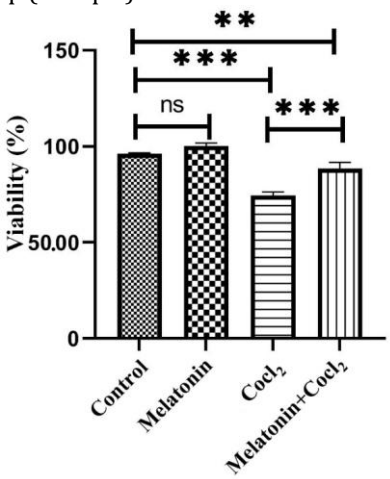

Fig. 1. Effect of melatonin on the cell viability of $CoCl_2$-hypoxia-induced TM3 mouse Leydig cells after 96 hr incubation (n = 3). *** $p < 0.001$, ** $p < 0.01$, and ns=non-significant.

Effect of melatonin on Alk5, Bmp4 and TGF-β1 mRNA expression. The results of RT‑PCR are shown in Figure 2. The mRNA expression of Alk5 and Bmp4 was significantly increased ($p < 0.05$) in the groups treated with $CoCl_2$ (Group 3), and melatonin + $CoCl_2$ (Group 4) compared to the control (Group 1) and melatonin-treated (Group 2) groups (Figs 2A and B). The expression of these genes was also significantly ($p < 0.05$) increased in the $CoCl_2$ -treated group (Group 3) compared to the melatonin + $CoCl_2$ -treated group (Group 4). There was a significant reduction ($p < 0.05$) in the gene expression of Alk5 and Bmp4 in the melatonin-treated group (Group 2) compared to all other groups. As shown in Figure 2C, the mRNA expression of the *TGF-β1* gene was significantly increased ($p < 0.05$) in TM3 cells treated with melatonin (Group 2) compared to the control (Group 1). Moreover, the mRNA expression of the *TGF-β1* gene was significantly increased ($p < 0.05$) in the groups treated with $CoCl_2$ (Group 3), and melatonin + $CoCl_2$ (Group 4) compared to the control (Group 1) and melatonin-treated (Group 2) groups. The expression of the *TGF-β1* gene in the melatonin + $CoCl_2$ -treated group (Group 4) was significantly lower ($p < 0.05$) than in the $CoCl_2$ -treated group (Group 3).

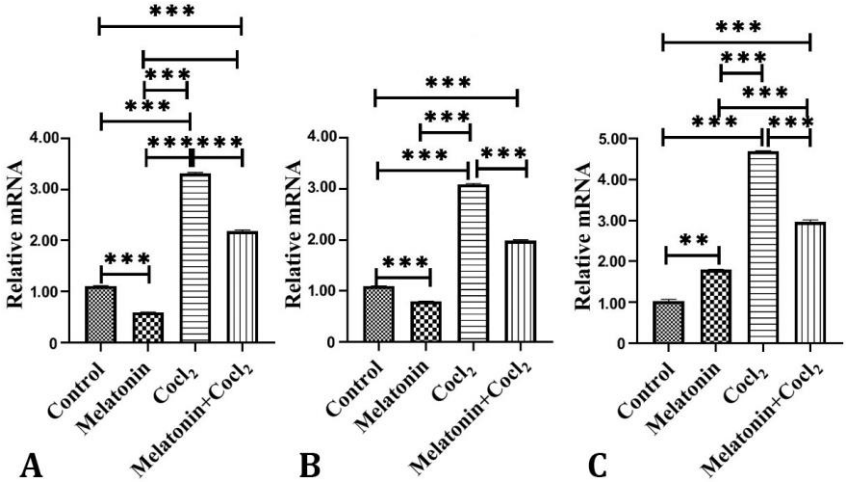

Fig. 2. Effect of melatonin on the expression of transforming growth factor beta (TGF-β) signaling pathway-related genes in hypoxic TM3 cells. **A)** Activin receptor-like kinase-5; **B)** Bone morphogenetic protein 4; and **C)** TGFβ1.

Effect of melatonin on Alk5, Bmp4 and TGF-β1 protein expression. The results of the Western blot are indicated in Figure 3. The protein expression of Alk5, Bmp4 and TGF-β1 was significantly increased ($p < 0.05$) in the group treated with $CoCl_2$ (Group 3) compared to all other groups (Figures 2A-2C). The protein expression of *Alk5* and *Bmp4* genes, but not TGF-β1 was significantly reduced ($p < 0.05$) in the melatonin-treated group (Group 2) compared to the control group (Group 1). Interestingly, the treatment of TM3 cells with melatonin significantly increased ($p < 0.05$) the protein expression of TGF-β1 in comparison to the melatonin + $CoCl_2$ -treated group (Group 4) and control groups (Group 1). The protein expression of *TGF-β1* gene in all groups was significantly higher ($p < 0.05$) than in the control group (Group 1). Also, a significant increase ($p < 0.05$) was observed in the protein expression of the *TGF-β1* gene in $CoCl_2$ group (Group 3) compared to melatonin-treated (Group 2), and the melatonin + $CoCl_2$ -treated (Group 4) groups. The acrylamide gel electrophoresis and blotting produced 53.00, 25.00, 23.00 and 43.00 kDa proteins for *Alk5*, *Bmp4*, *TGF-β1* and *β-actin* genes, respectively (Fig. 4).

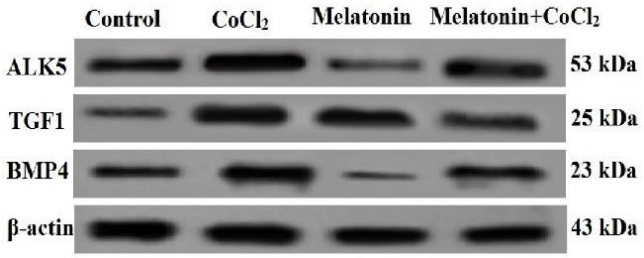

Fig. 4. Western blot results of ALK5, TGFβ1, Bmp4 and β-actin.

Discussion

This study examines the potential effects of melatonin on the expression of several genes involved in TGF-β signaling in chemically induced hypoxia and its protective

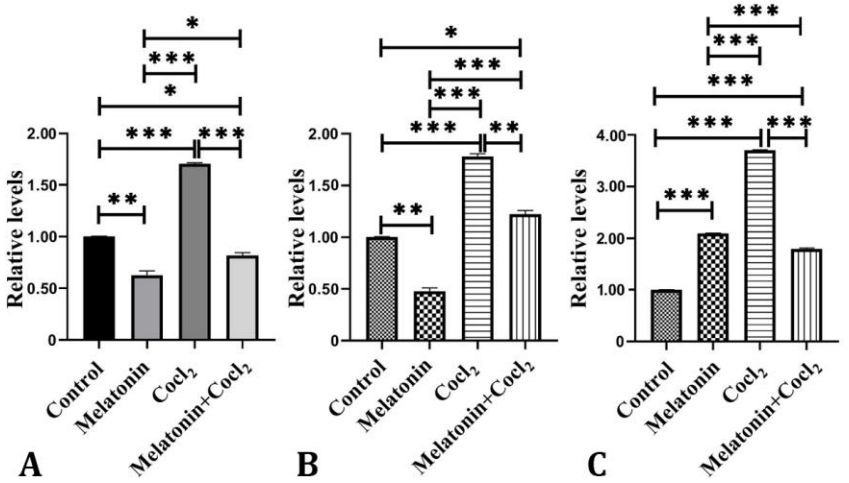

Fig. 3. Relative expression levels of **A)** ALK5, **B)** Bmp4 and **C)** TGFβ1 where β-actin was used as in internal control in hypoxic TM3 cells treated with 10.00 ng mL^{-1} melatonin.

effects on the TM3 cell line. Chemical hypoxia induced by $CoCl_2$ is a commonly used hypoxia mimic. To ensure the induction of hypoxia in TM3 cells by cobalt chloride, the expression of the hypoxia-inducible factor *(HIF)1α* gene was identified. Since this article is part of a broader research project, information regarding the expression of this gene has been reported in the article published by Karimi *et al*.[19] Under normoxic conditions, $CoCl_2$ significantly stabilizes HIF1 α and HIF2 α, providing a sustained stabilization for several hours. This model allows for a broader time window to manipulate and analyze samples under normoxic conditions.[20]

Hypoxia is characterized by an increase in ROS, leading to oxidative stress,[21] and has been shown to be a potent inducer of apoptosis.[22] The high altitude-induced hypoxia caused various types of degeneration and atrophy in the seminiferous tubules and interstitial tissue during postnatal development in rats.[1] In our study, we observed that hypoxia induced by $CoCl_2$ resulted in a reduction in the viability of TM3 cells compared to the control group. In a similar study, different concentrations of cobalt chloride were found to decrease the viability of TM3 cells.[19]

In this study, treatment of TM3 cells with a concentration of 10.00 ng mL^{-1} melatonin did not significantly alter cell survival rate. Similarly, other studies have reported that concentrations ranging from 1.00×10^{-6} to 1.00 mM of melatonin did not significantly affect the survival of TM3 cells.[19] Previous studies have shown that a concentration of 10.00 ng of melatonin exhibits the most effective activity.[8] In this regard, supplementation of 10.00 ng of melatonin into pig granulosa cells culture media stimulates the expression of genes related to the regulation of cell cycle proliferation and anti-apoptosis,[23] which aligns with our findings. However, concentrations of 10.00 and 100ng mL^{-1} of melatonin have been shown to increase the survival and proliferation of TM3 cells.[5] Furthermore, it has been shown that a concentration of 10.00 ng of melatonin can improve the survival of granulosa cells of mice treated with palmitic acid.[24] Additionally, melatonin has been reported to protect brain endothelial cells from damage caused by oxygen-glucose deprivation.[25]

Melatonin, a primary secretion of the pineal gland, serves as a potent antioxidant and free radical neutralizer.[26] Due to its small size and high lipophilic properties, melatonin easily passes through the cell membrane and spreads throughout the cell. Its concentration in the nucleus is very high, and it protects DNA against destructive factors.[27] The present study showed that administering 10.00 ng of melatonin for a period of 96 hr, reduces the negative effects of $CoCl_2$ on cell viability. These changes are likely caused by the strong antioxidant properties of melatonin, which can stimulate the activity or gene expression of antioxidant enzymes such as superoxide dismutase, glutathione reductase, and glutathione peroxidase.[28] Additionally, the anti-apoptotic properties of melatonin prevent the death of TM3 cells by inhibiting the process of apoptosis. Previous findings have also demonstrated the anti-apoptotic effects of melatonin on mouse Leydig cells and various other tissues.[5]

In this study, it was shown that hypoxia increases the expression of TGF-β1 at mRNA and protein levels in TM3 cells treated with $CoCl_2$ compared to the control group. Increased TGF-β1 has been linked to Leydig cell hyperplasia/hypertrophy, according to the literature.[29] The TGF-β1 has been shown to increase ROS production and suppress the antioxidant system, thus causing oxidative stress or redox imbalance.[30] It also leads oxidative modifications and increases apoptosis in hamster pancreatic β-cell cells by accumulating reactive oxygen species and suppressing catalase and glutathione peroxidase.[31]

The TGF-β1 has been shown to regulate testicular Leydig cell function *in vivo* and *in vitro*.[32] It is a post-transcriptional regulatory molecule that is essential for the expression of various genes in physiological and pathological processes.[16] Overexpression of TGF-β1 in the testis can interfere with the body's natural spermatogenic activity.[33] There is increasing evidence that differential expression profiles of TGF-β1 are linked to infertility and disorders of sexual development and function in men.[34] The TGF-β1 receptor blockers have been shown to significantly reduce the density and motility of rat spermatozoa.[35] Mice mutated for TGF-β1 survive until reproductive age, but the level of testosterone in the testis and plasma of these mice shows a significant decrease.[14] Hypoxia has been shown to increase TGF-β1 protein expression in various cell types, including fibroblasts.[36] hepatic stellate and smooth muscle cells.[37]

Antioxidants such as selenium, quercetin, and carvacrol have been demonstrated to reduce the expression of TGF-β1.[38] In the present study, it was observed that the expression of TGF-β1 significantly decreased when cells were treated with melatonin in addition to hypoxia, compared to the group induced with $CoCl_2$ and hypoxia. Kim *et al*. have shown that melatonin can prevent the transformation and differentiation of renal interstitial fibroblasts into myofibroblasts by suppressing the expression of TGF-β1.[39] It has also been proven that melatonin is able to reduce the adverse effects of hypoxia during the differentiation of mouse embryonic stem cells into myocardial cells.[40] Therefore, it is likely that the reduction in TGF-β1 expression in the melatonin + cobalt group in this study is due to the antioxidant properties of melatonin.

In the present study, the expression of the *ALK5* gene at both the mRNA and protein levels in TM3 cells under hypoxic conditions was found to be increased. Furthermore, treatment with melatonin in hypoxic conditions resulted in a reduction of the *Alk5* expression

gene at both the mRNA and protein levels. Previous reports have indicated that hypoxia can induce the expression of Alk5.[41] It has been identified that increased oxidative stress is associated with elevated ALK5 expression. Hypoxia promotes an increase in ROS, leading to oxidative stress, and reducing ROS production can be an effective strategy for managing oxidative stress damage.

One of the key receptors involved in TGF-β1 functions is Alk5, also known as the TGF-β1 receptor.[14] The presence of Alk5 receptors has been widely documented in mammalian reproductive cells.[29,42] It has been shown that the reduction in fertility of men experiencing high altitude was probably due to an uncontrolled ROS production.[3] Melatonin has been found to be a potent antioxidant that effectively reduces oxidative stress.[3,8,27,39] Research has indicated that Alk5 could be a potential target for antioxidant treatment.[2] Considering the role of Alk5 in regulating the functions of Leydig cells, reducing the expression of this gene in hypoxic conditions may be an effective treatment to mitigate the harmful effects of oxidative stress on these cells.

In our study, we observed an increase in Bmp4 expression at both the mRNA and protein levels in hypoxic TM3 cells. Additionally, treatment of TM3 cells with melatonin in hypoxic conditions resulted in a significant decrease in Bmp4 expression compared to the $CoCl_2$-treated group. In contrast to our findings, a different study has shown that osteoblastic cells affected by oxidative stress caused by H2O2 exhibit decreased Bmp4 expression, and simultaneous treatment with the antioxidant Apigenin leads to an increase in Bmp4 expression.[43] Another study has demonstrated that the antioxidant Dioscin can mitigate myocardial infarction damage caused by oxidative stress in hypoxic conditions by modulating Bmp4.[44] Similarly, boldin, another antioxidant, exerts its effects by inhibiting excessive ROS production through Bmp4-dependent mechanisms. Endothelial dysfunction in diabetic mice has been associated with increased oxidative/nitrosative stress and up-regulation of Bmp4, and boldin treatment has been shown to restore impaired endothelial function and prevent ROS overproduction and Bmp4 upregulation.[45]

The BMP4 is a crucial member of the TGF -β family and is expressed in the Leydig cell lineage.[46] The transcription factor HIF-1 plays a pivotal role in controlling the expression of numerous genes during the adaptive response to hypoxia.[47] Gao *et al*., demonstrated that HIF-1α binds to the promoter region of Bmp4, leading to increased Bmp4 expression in H9C2 rat cardiomyocytes under hypoxic conditions.[48] Previous research has also shown that HIF-1α regulates the transcriptional activation of Bmp4 expression in various cell types.[49]

In conclusion, melatonin at a concentration of 10.00 ng had protective effects on TM3 cells under hypoxic conditions induced by $CoCl_2$. Additionally, melatonin was found to decrease the expression of TGF-β1 and its receptor Alk5, and Bmp4 which are associated with oxidative stress and cell damage in hypoxic conditions. Overall, these findings support the potential beneficial effects of melatonin in protecting TM3 cells against hypoxia-induced oxidative damage through its antioxidant properties, thus suggesting its potential as a therapeutic agent in conditions characterized by oxidative stress and hypoxia.

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Conflicts of interest

The authors declare no conflicts of interest.

References

1. Mohammed HM, Tingari M. Effect of high altitude hypoxia on the postnatal development of rat testis: histological and histometric study. Int J Morphol 2016; 34(2): 610-615.
2. Li Z, Wang S, Gong C, et al. Effects of environmental and pathological hypoxia on male fertility. Front Cell Dev Biol 2021; 9: 725933. doi: 10.3389/fcell.2021.725933.
3. Verratti V, Mrakic-Sposta S, Fusi J, et al. Fertility impairment after trekking at high altitude: a proof of mechanisms on redox and metabolic seminal changes. Int J Mol Sci 2022; 23(16): 9066. doi: 10.3390/ijms 23169066.
4. Wang S, Ren X, Hu X, et al. Cadmium-induced apoptosis through reactive oxygen species-mediated mitochondrial oxidative stress and the JNK signaling pathway in TM3 cells, a model of mouse Leydig cells. Toxicol Appl Pharmacol 2019; 368: 37-48.
5. Xu G, Zhao J, Liu H, et al. Melatonin inhibits apoptosis and oxidative stress of mouse Leydig cells *via* a SIRT1-dependent mechanism. Molecules 2019; 24(17): 3084. doi: 10.3390/molecules24173084.
6. Slominski RM, Reiter RJ, Schlabritz-Loutsevitch N, et al. Melatonin membrane receptors in peripheral tissues: distribution and functions. Mol Cell Endocrinol 2012; 351(2): 152-166.
7. Fischer TW, Kleszczyński K, Hardkop LH, et al. Melatonin enhances antioxidative enzyme gene expression (CAT, GPx, SOD), prevents their UVR-induced depletion, and

protects against the formation of DNA damage (8-hydroxy-2'-deoxyguanosine) in *ex vivo* human skin. J Pineal Res 2013; 54(3): 303-312.
8. Espino J, Bejarano I, Ortiz A, et al. Melatonin as a potential tool against oxidative damage and apoptosis in ejaculated human spermatozoa. Fertil Steril 2010; 94(5): 1915-1917.
9. Frungieri MB, Mayerhofer A, Zitta K, et al. Direct effect of melatonin on Syrian hamster testes: melatonin subtype 1a receptors, inhibition of androgen production, and interaction with the local cortico-tropin-releasing hormone system. Endocrinology 2005; 146(3): 1541-1552.
10. Heindel JJ, Jackson FL, Berkowitz AS. Role of the pineal in the alteration of hamster Sertoli cell responsiveness to FSH during testicular regression. J Androl 1984; 5(3): 211-215.
11. Rossi S, Windschuettl S, Matzkin ME, et al. Melatonin in testes of infertile men: evidence for anti-proliferative and anti-oxidant effects on local macrophage and mast cell populations. Andrology 2014; 2(3): 436-449.
12. Frungieri MB, Calandra RS, Rossi SP. Local actions of melatonin in somatic cells of the testis. Int J Mol Sci 2017; 18(6): 1170. doi: 10.3390/ijms18061170.
13. Espino J, Ortiz Á, Bejarano I, et al. Melatonin protects human spermatozoa from apoptosis *via* melatonin receptor- and extracellular signal-regulated kinase-mediated pathways. Fertil Steril 2011; 95(7): 2290-2296.
14. Rocio G, Saul C. Silvia Ines G. Testicular expression of the TGF-β1 system and the control of Leydig cell proliferation. Adv Biosci Biotechnol 2013; 4: 1-7. doi: 10.4236/abb.2013.410A4001.
15. Itman C, Mendis S, Barakat B, et al. All in the family: TGF-β family action in testis development. Reproduction 2006; 132(2): 233-246.
16. Wang T, Zhang D, Song T, et al. Advances in research of TGF-B1 in human testis. Food Sci Technol 2022; 42: 22521. doi: 10.1590/fst.22521.
17. Ye L, Li X, Li L, et al. Insights into the development of the adult Leydig cell lineage from stem Leydig cells. Front Physiol 2017; 8: 430. doi: 10.3389/fphys. 2017.00430.
18. Kumar A, Rani L, Dhole B. et al. Oxygen as a regulator of MA-10 cell functions: effect of cobalt chloride on vascular endothelial growth factor production. Andrologia 2012; 44(Suppl 1): 615-620.
19. Karimi S, Jalili C, Mansouri K, et al. Effect of melatonin on steroidogenesis-related enzyme expression and testosterone synthesis following $CoCl_2$-induced hypoxia in TM3 Leydig cells. Iran J Basic Med Sci 2023; 26(9): 1041-1046.
20. Muñoz-Sánchez J, Chánez-Cárdenas ME. The use of cobalt chloride as a chemical hypoxia model. J Appl Toxicol 2019; 39(4): 556-570.
21. Smith KA, Waypa GB, Schumacker PT. Redox signaling during hypoxia in mammalian cells. Redox Biol 2017; 13: 228-234.
22. Isaja L, Mucci S, Vera J, et al. Chemical hypoxia induces apoptosis of human pluripotent stem cells by a NOXA-mediated HIF-1α and HIF-2α independent mechanism. Sci Rep 2020; 10(1): 20653. doi: 10.1038/s41598-020-77792-7.
23. Liu Y, Yang Y, Li W, et al. Effects of melatonin on the synthesis of estradiol and gene expression in pig granulosa cells. J Pineal Res 2019; 66(2): e12546. doi: 10.1111/jpi.12546.
24. Chen Z, Lei L, Wen D, et al. Melatonin attenuates palmitic acid-induced mouse granulosa cells apoptosis *via* endoplasmic reticulum stress. J Ovarian Res 2019; 12(1): 43. doi: 10.1186/s13048-019-0519-z.
25. Song J, Kang SM, Lee WT, et al. The beneficial effect of melatonin in brain endothelial cells against oxygen-glucose deprivation followed by reperfusion-induced injury. Oxid Med Cell Longev 2014; 2014: 639531. doi: 10.1155/2014/639531.
26. Sahoo DK, Chainy GBN. Hormone-linked redox status and its modulation by antioxidants. Vitam Horm 2023; 121: 197-246.
27. Mirhoseini M, Talebpour Amiri F, Karimpour Malekshah AA, et al. Protective effects of melatonin on testis histology following acute torsion-detorsion in rats. Int J Reprod Biomed 2017; 15(3): 141-146.
28. Hacışevki A, Baba B. An overview of melatonin as an antioxidant molecule: a biochemical approach. In: Drăgoi CM, Nicolae AC (Eds). Melatonin molecular biology, clinical and pharmaceutical approaches. Rijeka, Croatia: IntechOpen 2018. doi: 10.5772/ intechopen.74993.
29. Gonzalez CR, Matzkin ME, Frungieri MB, et al. Expression of the TGF-beta1 system in human testicular pathologies. Reprod Biol Endocrinol 2010; 8: 148. doi: 10.1186/1477-7827-8-148.
30. Liu RM, Desai LP. Reciprocal regulation of TGF-β and reactive oxygen species: as perverse cycle for fibrosis. Redox Biol 2015; 6: 565-577.
31. Islam KN, Kayanoki Y, Kaneto H, et al. TGF-beta1 triggers oxidative modifications and enhances apoptosis in HIT cells through accumulation of reactive oxygen species by suppression of catalase and glutathione peroxidase. Free Radic Biol Med 1997; 22(6): 1007-1017.
32. Park E, Song CH, Park JI, et al. Transforming growth factor-β1 signaling represses testicular steroidogenesis through cross-talk with orphan nuclear receptor Nur77. PLoS One 2014; 9(8): e104812. doi: 10.1371/journal.pone.0104812.
33. Yao HHC, Ungewitter E, Franco H, et al. Establishment of fetal Sertoli cells and their role in testis morphogenesis. In: Griswold MD (Ed). Sertoli cell

biology. 2nd ed. Massachusetts, USA: Academic Press 2015; 57-79.
34. Endo T, Freinkman E, de Rooij DG, et al. Periodic production of retinoic acid by meiotic and somatic cells coordinates four transitions in mouse spermatogenesis. Proc Natl Acad Sci 2017; 114(47): E10132-E10141.
35. Oral O, Uchida I, Eto K, et al. Promotion of spermatogonial proliferation by neuregulin 1 in newt (*Cynops pyrrhogaster*) testis. Mech Dev 2008; 125(9-10): 906-917.
36. Mingyuan X, Qianqian P, Shengquan X, et al. Hypoxia-inducible factor-1α activates transforming growth factor-β1/Smad signaling and increases collagen deposition in dermal fibroblasts. Oncotarget 2017; 9(3): 3188-3197.
37. Wiafe B, Adesida A, Churchill T, et al. Hypoxia-increased expression of genes involved in inflammation, dedifferentiation, pro-fibrosis, and extracellular matrix remodeling of human bladder smooth muscle cells. In Vitro Cell Dev Biol Anim 2017; 53(1): 58-66.
38. Ram C, Gairola S, Syed AM, et al. Carvacrol preserves antioxidant status and attenuates kidney fibrosis *via* modulation of TGF-β1/Smad signaling and inflammation. Food Funct 2022; 13(20): 10587-10600.
39. Kim JY, Park JH, Jeon EJ, et al. Melatonin prevents transforming growth factor-β1-stimulated trans-differentiation of renal interstitial fibroblasts to myofibroblasts by suppressing reactive oxygen species-dependent mechanisms. Antioxidants (Basel) 2020; 9(1): 39. doi: 10.3390/antiox9010039.
40. Lee JH, Yoo YM, Lee B, et al. Melatonin mitigates the adverse effect of hypoxia during myocardial differentiation in mouse embryonic stem cells. J Vet Sci 2021; 22(4): e54. doi: 10.4142/jvs.2021.22.e54.
41. Kim BH, Guardia Clausi M, Frondelli M, et al. Age-dependent effects of ALK5 inhibition and mechanism of neuroprotection in neonatal hypoxic-ischemic brain injury. Dev Neurosci 2017; 39(1-4): 338-351.
42. Goddard I, Bouras M, Keramidas M, et al. Transforming growth factor-beta receptor types I and II in cultured porcine Leydig cells: expression and hormonal regulation. Endocrinology 2000; 141(6): 2068-2074.
43. Jung WW. Protective effect of apigenin against oxidative stress-induced damage in osteoblastic cells. Int J Mol Med 2014; 33(5): 1327-1334.
44. Zhang Z, Zhao X, Gao M, et al. Dioscin alleviates myocardial infarction injury *via* regulating BMP4/NOX1-mediated oxidative stress and inflammation. Phytomedicine. 2022; 103: 154222. doi: 10.1016/j.phymed.2022.154222.
45. Lau YS, Tian XY, Mustafa MR, et al. Boldine improves endothelial function in diabetic db/db mice through inhibition of angiotensin II-mediated BMP4-oxidative stress cascade. Br J Pharmacol 2013; 170(6): 1190-1198.
46. Li X, Fang Y, Chen L, et al. Bone morphogenetic protein 4 inhibits rat stem/progenitor Leydig cell development and regeneration *via* SMAD-dependent and SMAD-independent signaling. Cell Death Dis 2022; 13: 1039. doi: 10.1038/s41419-022-05471-8.
47. Semenza GL. HIF-1 and human disease: one highly involved factor. Genes Dev 2000; 14(16): 1983-1991.
48. Gao LT, Yuan JQ, Zhang ZY, et al. Hypermethylation of the Bmp4 promoter dampens binding of HIF-1α and impairs its cardiac protective effects from oxidative stress in prenatally GC-exposed offspring. Cell Mol Life Sci 2023; 80(3): 58. doi: 10.1007/s00018-023-04703-0.
49. Pramono A, Zahabi A, Morishima T, et al. Thrombopoietin induces hematopoiesis from mouse ES cells *via* HIF-1α-dependent activation of a BMP4 autoregulatory loop. Ann N Y Acad Sci 2016; 1375(1):38-51.