Effects of melatonin on the mitogen-activated protein kinase signaling genes in hypoxic Leydig cells

Mona Ghasemi[1], Adel Saberivand[1]*, Mohammadreza Gholami[2], Vahideh Assadollahi[3], Masoud Alasvand[4], Maryam Saberivand[5], Sahar Rashidi[1]

[1] Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran; [2] Medical Technology Research Center, Institute of Health Technology, Kermanshah University of Medical Sciences, Kermanshah, Iran; [3] Cellular and Molecular Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran; [4] Cancer and Immunology Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran; [5] Connective Tissue Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

Article Info	Abstract
	Leydig cells play a crucial role in male reproductive physiology, and their dysfunction is often associated with male infertility. Hypoxia negatively affects the structure and function of Leydig cells. This study aimed to investigate the impact of melatonin on the c-Jun N-terminal kinase (Jnk), P38, and extra-cellular signal-regulated kinases 1 and 2 (Erk1/2) mitogen-activated protein kinase (MAPK) signaling pathways in TM3 mouse Leydig cells under hypoxia induced by cobalt (II) chloride ($CoCl_2$). The TM3 cell line was utilized as a subject of research, and 100 μM $CoCl_2$ was employed to induce hypoxia. Following the addition of 10.00 ng mL^{-1} melatonin, quantitative reverse transcription-polymerase chain reaction and western blot analyses were conducted to assess the gene expression and protein level of *Jnk*, *p38*, and *Erk1/2*, while enzyme-linked immunosorbent assay was used to measure testosterone secretion. The results showed that melatonin significantly increased testosterone production in the $CoCl_2$ + melatonin group compared to the $CoCl_2$-treated group. Furthermore, melatonin elevated both the protein level and mRNA expression of *Erk1/2*, *Jnk*, and *p38* genes in the $CoCl_2$ + melatonin group compared to the $CoCl_2$ group. In conclusion, melatonin activated the Jnk, p38, and Erk1/2 MAPK signaling pathways and enhanced testosterone production in the presence of $CoCl_2$ in TM3 cells.

Introduction

Male fertility is primarily dependent on male reproductive processes, including testicular steroidogenesis, spermatogenesis, and sexual/erectile functions. However, these processes can be negatively impacted by various factors, including hypoxia.[1]

Hypoxia is a temporary or enduring reduction in the partial pressure of oxygen in the arteries, resulting in a decrease in oxygen delivery to the body's tissues.[2] Hypoxia can generally be categorized as either environmental or pathological. Environmental hypoxia occurs when there is a low partial pressure of inhaled oxygen, often experienced at high altitudes, while pathological hypoxia refers to a compromised delivery of oxygen to cells and tissues due to a pathological condition, such as chronic pulmonary disease, sickle cell disease, and certain

testicular pathologies, like varicocele and testicular torsion.[3,4] The primary factor involved in the body's adaptation to low oxygen levels is hypoxia inducible factor (HIF).[5] When oxygen levels are low, HIF, a protein existing as a heterodimer, attaches to the hypoxia response element[6] to facilitate the activation of various genes being regulated by oxygen.

Conflicting results exist on how hypoxia affects testosterone and gonadotropins. Some studies suggest that hypoxia may suppress testosterone, luteinizing hormone, and follicle stimulating hormone,[7,8] while others indicate that intermittent hypoxia can increase testosterone synthesis in Leydig cells.[9] Hypoxia harms spermatogenesis by reducing adenosine triphosphate production, leading to lower sperm production and quality, causing death and morphological changes in sperm cells, and hindering oxygen delivery to germ cells.[10] It also inhibits

---

*Correspondence:

Adel Saberivand. DVM, PhD

Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

E-mail: a.saberivand@tabrizu.ac.ir

germ cell proliferation and spermatogenic function. High-altitude-induced hypoxia is linked to lipid peroxidation, oxidative DNA damage, and reduced sperm quality and concentration.[11] Low testosterone levels from hypoxia can reduce libido and sexual activity by affecting dopamine regulation[12] and genital reflexes.[13] Hypoxia-induced issues include reduced DNA methylation, and sperm count and motility, and increased DNA fragmentation.[14] It can cause mitochondrial dysfunction and activate enzymes, like xanthine oxidase and inducible nitric oxide synthase, generating reactive oxygen species (ROS), leading to oxidative stress.[2] Excess ROS-mediated oxidative stress, HIF-1α-mediated germ cell death and proliferation suppression, systemic inflammation, and epigenetic alterations are among the potential pathways implicated in hypoxia-induced male reproductive damage.[6]

Testosterone is essential in the male reproductive system as it promotes sperm maturation and maintains male secondary sex characteristics. Additionally, testosterone stimulates the function of reproductive organs, muscle protein synthesis, bone growth, calcium and phosphorus deposition, and red blood cells production. Research has linked androgen deficiency not only to male sexual dysfunction, erectile dysfunction, and reduced reproductive capacity but also to cardiovascular disease,[15] diabetes,[16] osteoporosis, and other diseases.[17]

The principal androgen-producing cells in mammals are Leydig cells. Located adjacent to the seminiferous tubules of the testis, Leydig cells are responsible for producing 95.00% of a man's testosterone.[18] Steroido-genesis regulation is a multi-compartmental process involving numerous signaling pathways.[19] A significant body of evidence suggests that distinct mitogen-activated protein kinase (MAPK) cascades play a role in steroidogenesis regulation through genetic and non-genomic influences.[20,21] One of the most significant signaling pathways, the MAPK signaling pathway, is involved in cell proliferation and differentiation, apoptosis, stress, inflammatory responses, and response to environmental stimuli.[22] The MAPK regulates cell proliferation, differentiation, and death in the mammalian testis and is considered an essential regulator of sperm development. The MAPK can indirectly influence animal germ cell development by affecting the function of Leydig cells.[23] The signals mediated by extra-cellular signal-regulated kinases 1 and 2 (Erk1/2), c-Jun N-terminal kinase (Jnk), and p38 kinase, which are all capable of affecting steroidogenesis, are three downstream signal relays that the MAPKs can use to transmit growth factor signals to steroidogenic cells.[21]

N-acetyl-5-methoxytryptamine (melatonin), a neuro-hormone generated and secreted predominantly by the pineal gland, is essential for animal reproduction.[24] Melatonin has been shown to protect the testis,[25] sperm,[26]

and Leydig cells in males.[27] Melatonin's role in male infertility has recently received a lot of interest. It has been demonstrated to play a role in male reproduction by altering steroid hormone secretion[28] as well as spermatogenic cell proliferation.[29] Melatonin has been shown to play a crucial protective and regulatory role in Leydig cells. Studies have shown that melatonin can decrease Leydig cells apoptosis, increase testosterone production, and improve the quality of sperms in melatonin-enriched transgenic mice.[30]

To the best of our knowledge, no study has been conducted to investigate the effect of melatonin on the expression of the primary genes *p38*, *Jnk*, and *Erk1/2* in the MAPK signaling pathway in TM3 mouse Leydig cells under hypoxic conditions. In line with that, the present study was conducted to evaluate the effect of melatonin on mRNA expression and protein level of three MAPK genes, including *Jnk*, *p38*, and *Erk1/2* subfamily, in TM3 mice Leydig cells under hypoxic conditions induced by cobalt (II) chloride ($CoCl_2$).

Materials and Methods

Cell culture. The TM3 cells were cultured in 35.00 mm dishes using a 1:1 mixture of Ham-12 and Dulbecco's MEM (Cytiva - HyClone, Logan, USA) included 5.00% horse serum (Gibco, Grand Island, USA) and 2.50% fetal bovine serum (Cytiva - HyClone). The culture media were replaced every two days. Cells were cultured at 37.00 ℃ in a humidified atmosphere containing 95.00% air and 5.00% CO_2. After 12 hr of adherence, the dishes were divided into four groups, including no treatment, as well as treatments with 100 μM $CoCl_2$,[31] 10.00 ng mL^{-1} melatonin,[32] and 100μM $CoCl_2$ plus 10.00 ng mL^{-1} melatonin, and incubated for 96 hr. The required concentrations of melatonin and $CoCl_2$ were prepared by dissolving the powders of these substances in the culture medium. Cell supernatants were collected for testosterone concentration assay. Total cell protein and RNA were extracted for western blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses, respectively. Each experiment was performed in triplicate.

Testosterone concentration assay. The enzyme-linked immunosorbent assay kit (Monobind Inc., Lake Forest, USA) was used to measure testosterone concentrations in the medium samples using a chemi-luminescence immunoassay according to the manufacturer's instructions. The sensitivity of the testosterone assay was 0.576 pg. The results were expressed as ng mL^{-1}.

Real-time PCR analysis. For the real-time PCR analysis, total RNA was extracted using RNX TM reagent (CinnaGen, Tehran, Iran). After DNase-I treatment (CinnaGen), cDNA was generated from purified total RNA

(2.00 μg) following the manufacturer's protocol (Takara, Kusatsu, Japan). Real-time PCR analyses were conducted using RunMei (Hunan Runmei Gene Technology Co., Hunan, China) in a final volume of 12.50 μL, containing 3.00 μL cDNA (100.00 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 ℃ for 30 sec, 40 cycles of 95.00 ℃ for 5 min, with different melting temperature values for 34 sec, 95.00 ℃ for 15 sec, 60.00 ℃ for 60 sec, and 95.00 ℃ for 15 sec. The $2^{-\Delta\Delta CT}$ method was used to analyze the data, with β-actin serving as the reference gene. The primer sequences used are detailed in Table 1.

Protein extraction and western blot analysis. Cellular proteins were extracted using a lysis buffer (Sigma-Aldrich, Taufkirchen, Germany) including 8.00 M urea, 2.00 M thiourea, and 10.00 mM Tris; pH: 8.00, and the Bradford method was employed to quantify the proteins colorimetrically. The proteins were separated by 12.00% sodium dodecyl-sulfate (Sigma-Aldrich) poly-acrylamide gel electrophoresis using the Laemmli method and then, electroblotted onto a nitrocellulose membrane using a semi-dry Trans-Blot device (Bio-Rad, Hercules, USA). The blots were briefly blocked with 5.00% skimmed milk, and incubated with primary antibodies against p38 (mouse monoclonal), β-actin (monoclonal), Erk1/2 (monoclonal), and Jnk (monoclonal) overnight at 4.00 ℃, followed by treatment with a horseradish peroxidase-conjugated secondary antibody (1:5,000 v/v; Sigma-Aldrich) for 2 hr. Images were captured using the luminal system on a blot scanner (LiCor, Lincoln, USA). For quantification, β-actin was used as an internal normalizer.[33]

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 3.0; GraphPad Software Inc., San Diego, USA) *via* one-way analysis of variance. The data were expressed as mean ± standard error of the mean. The $p < 0.05$ was considered statistically significant.

Results

Effect of melatonin on testosterone production. Figure 1 shows the levels of testosterone concentration. Induced hypoxia with $CoCl_2$ resulted in a significant decrease in testosterone production by TM3 cells compared to the control ($p < 0.0001$). The level of testosterone in cells treated with melatonin was significantly lower than that in the control ($p = 0.0012$). Simultaneous treatment of TM3 cells with 10.00 ng mL^{-1} of melatonin and 100 μM of $CoCl_2$ caused a significant decrease in testosterone levels compared to the control group ($p < 0.0001$). It appears that the addition of melatonin resulted in a further increase in testosterone production in the induction of hypoxia with $CoCl_2$ (simultaneous treatment group with melatonin and $CoCl_2$ *vs*. $CoCl_2$ alone group; $p = 0.0005$). Additionally, the amount of testosterone production in cells treated with melatonin was significantly higher than that in cells treated with $CoCl_2$ ($p < 0.0001$).

Furthermore, the secretion of testosterone in adjacent cells treated with melatonin was significantly higher than production of testosterone in cells treated with melatonin and $CoCl_2$ ($p = 0.0007$).
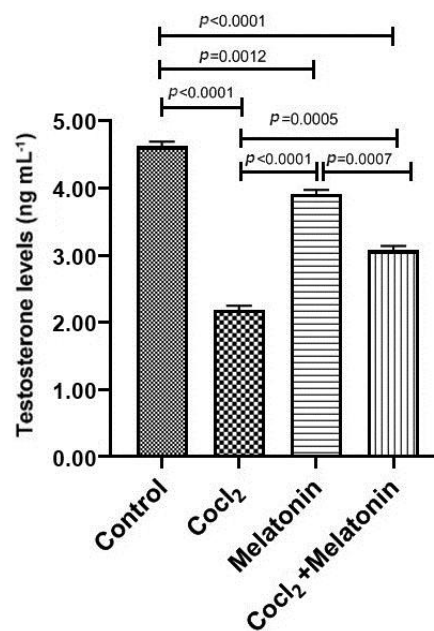

Fig. 1. The testosterone concentrations in the hypoxic TM3 cells treated with melatonin (10.00 ng mL^{-1}), Cobalt (II) chloride ($CoCl_2$; 100 μM), and melatonin (10.00 ng mL^{-1}) plus $CoCl_2$ (100 μM) compared to the control group.

Table 1. Primer sequences of the genes used in the real-time polymerase chain reaction analysis of TM3 cells.

Genes	Accession number	Primer sequence (5'→3')	Product size (bp)
P38	NM_001168508.1	F: AGCAACCTAGCTGTGAACGA R: CACGGACCAAATATCCACTGTC	168
Erk1/2	NM_011952.2	F: AGCCCCAGAGATCATGCTTA R: AGGATAACACCACAGCTCCA	159
Jnk	NM_001310452.1	F: AAGATCCCGGACAAGCAGTT R: CATTGCTGCACCTGTGCTAA	149
β-actin	NM_007393.5	F: AAGAGCTATGAGCTGCCTGA R: CCACAGGATTCCATACCCAAGA	105

Effect of melatonin on *p38*, *Jnk*, and *Erk1/2* mRNA expressions. As shown in Figure 2A, compared to the control, expression of *p38* in the CoCl$_2$, melatonin, melatonin + CoCl$_2$ treated cells was increased about 3.60, 2.30, and 4.60-fold, respectively ($p < 0.0001$). However, the expression of p38 in the melatonin + CoCl$_2$ group was increased compared to the CoCl$_2$ alone group ($p = 0.0002$). Expression level of p38 was significantly higher in CoCl$_2$-treated cells than melatonin-treated ones ($p < 0.0001$). While, the expression level of this gene in the group treated with melatonin + CoCl$_2$ was significantly higher than the group treated with melatonin ($p < 0.0001$). The CoCl$_2$ treatment induced about 1.80-fold up-regulation of Jnk ($p = 0.0003$), and treatments with melatonin and melatonin + CoCl$_2$ caused respectively about 2.30- and 2.80-fold increases in the expression of *Jnk* gene compared to the control group ($p < 0.0001$). Also, the expression level of *Jnk* gene in the cells treated simultaneously with CoCl$_2$ and melatonin was significantly higher than cells treated with CoCl$_2$ alone ($p = 0.0002$). The expression of this gene in the group treated with melatonin was also higher than the CoCl$_2$-treated group ($p = 0.002$). While, the expression of Jnk in the melatonin- treated group was lower than the melatonin + CoCl$_2$-treated group ($p = 0.0025$; Fig. 2B). As shown in Figure 2C, the expression of *Erk1/2* gene in the groups treated with CoCl$_2$, melatonin, and melatonin + CoCl$_2$ was increased respectively by 2.10 ($p = 0.0005$), 2.50 ($p = 0.0002$), and 3.40 ($p < 0.0001$) times compared to the control group. Also, compared to the CoCl$_2$-treated group, the expression of this gene was increased significantly in the melatonin treated groups ($p = 0.0201$) and melatonin + CoCl$_2$ ($p = 0.0002$). The expression of this gene in the group treated with melatonin alone was significantly decreased compared to the group treated with melatonin + CoCl$_2$ ($p = 0.0009$).

Effect of melatonin on *p38*, *Jnk*, and *Erk1/2* protein levels. As shown in Figures 3 and 4, western blot analysis revealed significantly higher level of p38 protein in TM3 cells treated with CoCl$_2$ ($p < 0.0001$), melatonin ($p = 0.0002$), and CoCl$_2$ + melatonin ($p < 0.0001$) compared to the control group. While, the p38 level in the melatonin-treated group was significantly lower than that in the CoCl$_2$-treated group ($p = 0.0008$); its level in the group treated with melatonin + CoCl$_2$ was significantly higher than that in the group treated with CoCl$_2$ alone ($p = 0.0006$).

Additionally, the level of p38 protein in the melatonin-treated group was significantly lower than that in the melatonin + CoCl$_2$-treated group ($p < 0.0001$).

Further western blot analysis revealed a significant elevation in Jnk level in CoCl$_2$-treated ($p = 0.0050$), melatonin-treated ($p = 0.0003$), and CoCl$_2$ + melatonin-treated ($p < 0.0001$) groups compared to the control. The level of Jnk in the melatonin-treated ($p = 0.0038$) and CoCl$_2$ + melatonin-treated ($p = 0.0002$) groups significantly increased compared to the CoCl$_2$-treated group. Additionally, western blot analysis showed that Jnk level in the melatonin-treated group alone was significantly decreased compared to the CoCl$_2$ + melatonin group ($p = 0.0032$).

The level of *Erk1/2* in the CoCl$_2$-treated ($p = 0.0269$), melatonin-treated ($p = 0.0019$), and CoCl$_2$ + melatonin-treated groups significantly ($p = 0.0008$) increased compared to the control group. The level of *Erk1/2* in the melatonin-treated and CoCl$_2$ + melatonin-treated groups significantly ($p < 0.02$) increased compared to the CoCl$_2$-treated group. Furthermore, the level of *Erk1/2* showed a significant increase in TM3 cells treated with CoCl2 + melatonin compared to the melatonin-treated group.
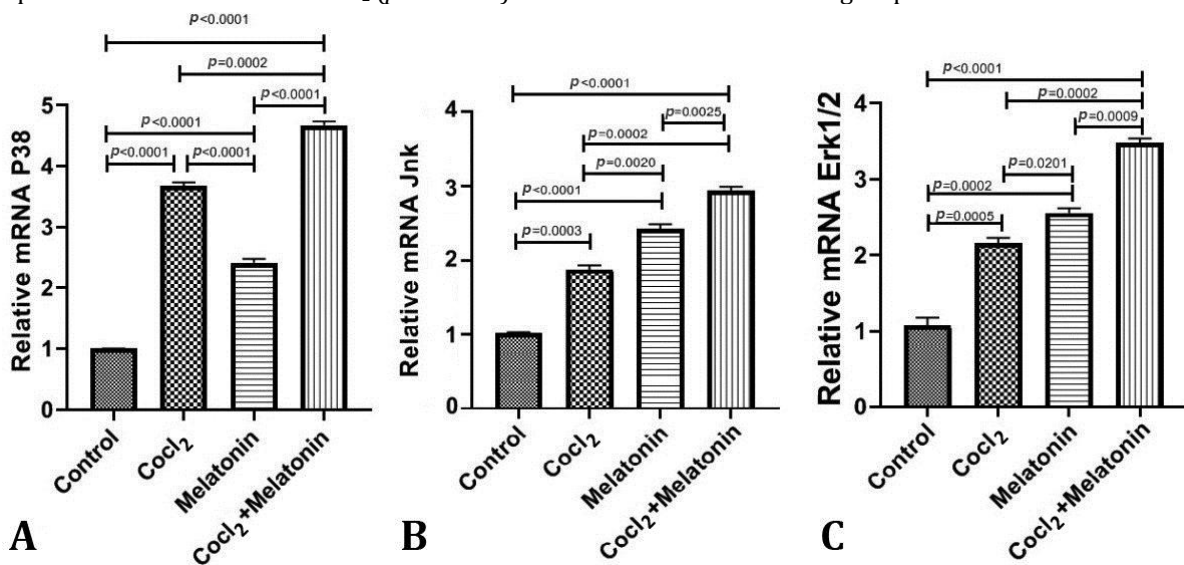

Fig. 2. Effect of melatonin on the expression of mitogen-activated protein kinase signaling pathway-related genes in hypoxic TM3 cells. **A)** p38; **B)** C-Jun N-terminal kinase (Jnk); and **C)** Extra-cellular signal-regulated kinases 1 and 2 (Erk1/2).
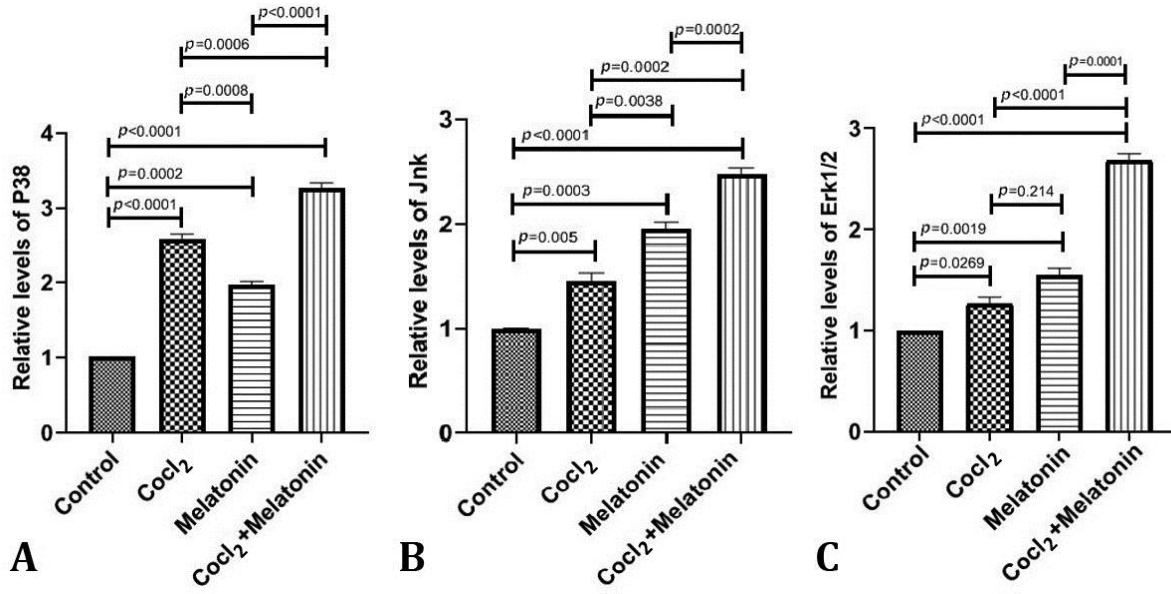
Fig. 3. The relative protein levels of mitogen-activated protein kinase pathway-related proteins in hypoxic TM3 cells treated with 10.00 ng mL^{-1} melatonin. **A)** p38; **B)** C-Jun N-terminal kinase (Jnk); and **C)** Extra-cellular signal-regulated kinases 1 and 2 (Erk1/2).
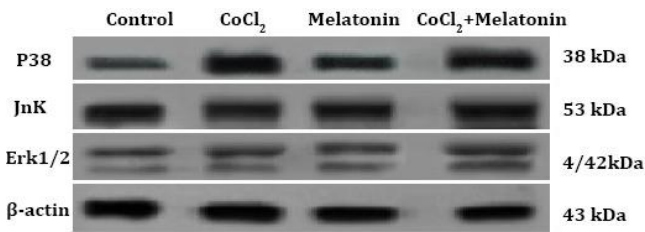

Fig. 4. The acrylamide gel electrophoresis results for p38, Jnk, Erk1/2, *and* β-actin proteins.

Discussion

In this study, we evaluated the impact of melatonin on testosterone secretion from mouse Leydig cells (TM3 cell line), as well as the expressions of *p38*, *Jnk*, and *Erk1/2* genes at both the mRNA and protein levels in the MAPK signaling pathway under hypoxic conditions induced by CoCl$_2$ *in vitro*.

The use of hypoxia models in cell culture has enabled the characterization of the hypoxia response and assessment of the effects of various chemicals and medications on hypoxia at cellular, biochemical, and molecular levels. Cobalt chloride-induced chemical hypoxia is one of the most commonly utilized models.[34] Studies have shown that hypoxia induced by CoCl$_2$ and its consequent up-regulation of HIF1 can lead to a decrease in testosterone production in mouse Leydig cells (TM3).[8] Our current investigation revealed that testosterone synthesis in TM3 cells treated with CoCl$_2$ was significantly lower compared to the control group, contrasting with the results reported by Hwang *et al.*, in which hypoxia stimulated cell proliferation and testosterone release in Leydig cells.[35]

Melatonin administration has been shown to reduce the severity of testicular injury in animal models with hyperlipidemia, experimental gonadal torsion and varicocele, or toxicity caused by exogenous substances, such as anti-cancer medications or environmental toxins.[36] Xu *et al.* found that 10.00 ng mL^{-1} melatonin stimulated proliferation while inhibiting apoptosis in Leydig cells.[32] In the current study, testosterone release was significantly higher in cells being simultaneously treated with CoCl$_2$ and 10.00 ng mL^{-1} melatonin than that in cells being only treated with CoCl$_2$. Melatonin has been shown to directly influence testosterone release by binding to specific receptors.[36] It has also been demonstrated to stimulate testosterone production in mammalian Leydig cells.[19] In a recent review paper, Nikolaev *et al.*, summarized the current knowledge regarding the membrane melatonin receptors (MT1 and MT2) activated cell signaling pathways in physiology and pathology and their relevance to certain disease conditions, including cancer in humans.[37] Today, it is believed that the site of melatonin's effect in the reproductive system includes several organs, such as the hypothalamus, pituitary gland, gonads, male and female reproductive organs, mammary glands, accessory sexual glands, or a combination of these organs. Melatonin plays an important role in several aspects of mammalian reproduction. This hormone can directly affect the function of ovaries and reproductive system, and may have a role in the pathophysiology of many reproductive problems. In this regard, the expressions of MT1 and MT2 receptors in many tissues or organs involved in reproduction support this point.

Valenti *et al.* reported a dose-dependent suppression of testosterone release in the presence of melatonin in rat

Leydig cells.[38] However, rats given melatonin showed no discernible alterations in their serum testosterone levels. Despite the fact that in our investigation melatonin administration to Leydig cells considerably decreased testosterone secretion relative to the control group, testosterone secretion in the $CoCl_2$-treated cells was significantly lower. A study found that treating Leydig cells with 10.00 ng/ml of melatonin lowers testosterone release by these cells, being consistent with the current findings.[24] According to Frungieri *et al.*, physiological levels of melatonin have a direct inhibitory effect on testosterone production in Leydig cells from reproductively active Syrian (golden) hamsters (*Mesocricetus auratus*).[39]

Numerous studies have revealed a link between the MAPK pathway and male reproductive health.[40-43] The MAPKs are highly expressed and evolutionarily conserved proteins being essential for controlling signaling pathways and playing key roles in male reproductive processes in mammals. These proteins help transmit signals through phosphorylation cascades, regulating various intra-cellular functions, such as germ cell development in the male gonads, physiological maturation of spermatozoa in the epididymis, and regulation of motility during ejaculation in the female reproductive tract.[44] The MAPK pathways consist of three pathways, including the p38, Jnk, and Erk pathways, playing important roles in spermatogenesis, as well as germ cell development, maturation, and apoptosis.[8] It is know well known that the MAPK signaling plays an important role in regulating steroid synthesis. The Jnk and p38 signaling pathways are mostly associated with cell stress and inflammation, while the Erk signaling route is associated with cell proliferation and differentiation.[45] Hypoxia has been shown to activate the Erk/Jnk/p38 MAPK signaling pathway.[34,46]

In this research, we initially measured MAPK pathway activation levels in the testis after induction of hypoxia by $CoCl_2$ treatment. The results showed that hypoxia increased the expressions of p38, Jnk, and Erk, as well as the protein levels of p38, Jnk, and Erk, indicating that the MAPK pathway was activated in TM3 cells after exposure to $CoCl_2$. The results also showed that melatonin significantly increased the levels of MAPKs-related proteins, as well as mRNA expressions of Erk and Jnk compared to the control group. However, the expression of p38 showed a significant decrease in both the protein and mRNA levels after treatment with melatonin compared to the $CoCl_2$ group. In the group where TM3 cells were simultaneously treated with $CoCl_2$ and melatonin, melatonin activated the MAPK pathway in Leydig cells *in vitro* by up-regulating the expression of key MAPK genes (*p38*, *Jnk*, and *Erk*) and proteins (p38, Jnk, and Erk).

The HIF-1 activation requires p38 MAPK signaling, according to the literature.[47] It has also been suggested that hypoxic stress increases the phosphorylation of both Erk1/2 and p38.[48]

Overall, these findings indicated that melatonin can activate the MAPK pathway in Leydig cells. Melatonin significantly increased the expressions of *p38*, *Jnk*, and *Erk* genes and proteins in accordance with the results published earlier.[49-50] Additionally, a study has shown that melatonin can mitigate the effects of oxidative stress and inflammation by inhibiting the p38 pathway.[47] Further-more, melatonin has been observed to reduce the phosphorylation levels of the MAPKs Erk and Jnk in hamster Leydig cells.[31]

It was shown that a decrease in the level of phosphorylated Erk1/2 down-regulates the steroid-related genes, inhibiting steroidogenesis in Leydig cells.[40] Li *et al.*, also revealed that nano-Tio2 may down-regulate ERK1/2/PKA/PKC signaling pathway, decreasing testosterone secretion in Leydig cells.[42] These differences can be attributed to variations in melatonin dose and duration studied by the researchers, as well as differences in animal species and cell lines used in both *in vivo* and *in vitro* studies.

In conclusion, supplementation of melatonin activated the Jnk, p38, and Erk1/2 MAPK signaling pathways in mRNA and protein stages and enhanced testosterone production in hypoxic Leydig cells line induced by $CoCl_2$. Since the MAPK signaling pathway is a key player in connecting environmental stimuli, such as oxidative stress, hypoxia, and inflammation, understanding how this pathway interacts with these factors could offer valuable knowledge for improving treatments for male infertility. However, TM3 cells, being a specific cell line, may not fully represent the physiological responses of Leydig cells *in vivo* and results obtained from the present *in vitro* study may not translate accurately to *in vivo* conditions. Hence, more *in vivo* studies regarding the effects of melatonin on mRNA expression and protein level of the MAPK signaling genes under hypoxic conditions are suggested.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

References

1. Oyedokun PA, Akhigbe RE, Ajayi LO, et al. Impact of hypoxia on male reproductive functions. Mol Cell Biochem 2023; 478(4): 875-885.
2. Reyes JG, Farias JG, Henríquez-Olavarrieta S, et al. The hypoxic testicle: physiology and pathophysiology. Oxid Med Cell Longev. 2012; 2012: 929285. doi: 10.1155/2012/929285.
3. Akhigbe RE, Odetayo AF, Akhigbe TM, et al. Pathophysiology and management of testicular ischemia/reperfusion injury: lessons from animal models. Heliyon 2024; 10(9): e27760. doi: 10.1016/j.heliyon.2024.e27760.
4. Jensen CFS, Østergren P, Dupree JM, et al. Varicocele and male infertility. Nat Rev Urol 2017; 14(9): 523-533.
5. Choudhry H, Harris AL. Advances in hypoxia-inducible factor biology. Cell Metab 2018; 27(2): 281-298.
6. 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.
7. Ayman AH, Anas AO, Yasser AA, et al. Levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone and prolactin at moderate altitude inhabitant male. Pak J Biol Sci 2021; 24(2): 188-192.
8. Wang H, Zhou W, Zhang J, et al. Role of JNK and ERK1/2 MAPK signaling pathway in testicular injury of rats induced by di-*N*-butyl-phthalate (DBP). Biol Res 2019; 52: 41. doi: 10.1186/s40659-019-0248-1.
9. Chai J, Liu S, Tian X, et al. Hypoxia impairs male reproductive functions via inducing rat Leydig cell ferroptosis under simulated environment at altitude of 5000 m. Life Sci 2024; 357: 123076, doi: 10.1016/j.lfs.2024.123076.
10. Liao W, Cai M, Chen J, et al. Hypobaric hypoxia causes deleterious effects on spermatogenesis in rats. Reproduction 2010; 139(6): 1031-1038.
11. Cikutovic M, Fuentes N, Bustos-Obregón E. Effect of intermittent hypoxia on the reproduction of rats exposed to high altitude in the Chilean Altiplano. High Alt Med Biol 2009; 10(4): 357-363.
12. Ajayi AF, Akhigbe RE. Staging of the estrous cycle and induction of estrus in experimental rodents: an update. Fertil Res Pract 2020; 6: 5. doi: 10.1186/s40738-020-00074-3.
13. Nagase T, Kikuno R, Ishikawa K, et al. Prediction of the coding sequences of unidentified human genes. XVII. The complete sequences of 100 new cDNA clones from brain which can code for large proteins *in vitro*. DNA Res 2000; 7(2): 143-150.
14. Bahreinian M, Tavalaee M, Abbasi H, et al. DNA hypomethylation predisposes sperm to DNA damage in individuals with varicocele. Syst Biol Reprod Med 2015; 61(4): 179-186.
15. Akishita M, Yu J. Hormonal effects on blood vessels. Hypertens Res 2012; 35(4): 363-369.
16. De Maddalena C, Vodo S, Petroni A, et al. Impact of testosterone on body fat composition. J Cell Physiol 2012; 227(12): 3744-3748.
17. Morales A, Bella AJ, Chun S, et al. A practical guide to diagnosis, management and treatment of testosterone deficiency for Canadian physicians. Can Urol Assoc J 2010; 4(4): 269-275.
18. Haider SG, Servos G, Tran N. Structural and histological analysis of Leydig cell steroidogenic function. In: Payne AH, Hardy MP (Eds.). The Leydig cell in health and disease. Totowa, NJ, USA: Humana Press 2007; 33-45.
19. Stocco DM, Wang X, Jo Y, et al. Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated than we thought. Mol Endocrinol 2005; 19(11): 2647-2659.
20. Otis M, Gallo-Payet N. Role of MAPKs in angiotensin II-induced steroidogenesis in rat glomerulosa cells. Mol Cell Endocrinol 2007; 265-266:126-130.
21. Manna PR, Stocco DM. The role of specific mitogen-activated protein kinase signaling cascades in the regulation of steroidogenesis. J Signal Transduc 2011; 2011: 821615. doi: 10.1155/2011/821615.
22. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 2011; 75(1): 50-83.
23. Sokanovic SJ, Janjic MM, Stojkov NJ, et al. Age related changes of cAMP and MAPK signaling in Leydig cells of Wistar rats. Exp Gerontol 2014; 58: 19-29.
24. Reiter RJ, Tan DX, Manchester LC, et al. Melatonin and reproduction revisited. Biol Reprod 2009; 81(3): 445-456.
25. Sayed RKA, Mokhtar DM, Fernández-Ortiz M, et al. Lack of retinoid acid receptor-related orphan receptor alpha accelerates and melatonin supplementation prevents testicular aging. Aging (Albany NY) 2022; 12(13): 12648-12668.
26. Pang YW, Sun YQ, Jiang XL, et al. Protective effects of melatonin on bovine sperm characteristics and subsequent *in vitro* embryo development. Mol Reprod Dev 2016; 83(11): 993-1002.
27. Li Z, Zhao J, Liu H, et al. Melatonin inhibits apoptosis in mouse Leydig cells via the retinoic acid-related orphan nuclear receptor α/p53 pathway. Life Sci 2022; 246: 117431. doi: 10.1016/j.lfs.2020.117431.
28. Yu K, Deng SL, Sun TC, et al. Melatonin regulates the synthesis of steroid hormones on male reproduction: a review. Molecules 2018; 23(2): 447. doi: 10.3390/molecules23020447.
29. Niu B, Li B, Wu C, et al. Melatonin promotes goat spermatogonia stem cells (SSCs) proliferation by stimulating glial cell line-derived neurotrophic factor

(GDNF) production in Sertoli cells. Oncotarget 2016; 7(47): 77532-77542.

30. Yang M, Guan S, Tao J, et al. Melatonin promotes male reproductive performance and increases testosterone synthesis in mammalian Leydig cells. Biol Reprod 2021; 104(6): 1322-1336.

31. 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.

32. 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.

33. Heidari R, Khosravian P, Mirzaei SA, et al. siRNA delivery using intelligent chitosan-capped mesoporous silica nanoparticles for overcoming multidrug resistance in malignant carcinoma cells. Sci Rep 2021; 11(1): 20531. doi: 10.1038/s41598-021-00085-0.

34. Muñoz-Sánchez J, Chánez-Cárdenas ME. The use of cobalt chloride as a chemical hypoxia model. J Appl Toxicol 2019; 39(4): 556-570.

35. Hwang GS, Wang SW, Tseng WM, et al. Effect of hypoxia on the release of vascular endothelial growth factor and testosterone in mouse TM3 Leydig cells. Am J Physiol Endocrinol Metab 2007; 292(6): E1763-E1769.

36. 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.

37. Nikolaev G, Robeva R, Konakchieva R, et al. Membrane melatonin receptors activated cell signaling in physiology and disease. Int J Mol Sci 2021 31; 23(1): 471. doi: 10.3390/ijms23010471.

38. Valenti S, Guido R, Giusti M, et al. *In vitro* acute and prolonged effects of melatonin on purified rat Leydig cell steroidogenesis and adenosine 3', 5'-mono-phosphate production. Endocrinology 1995; 136(12): 5357-5362.

39. Frungieri MB, Mayerhofer A, Zitta K, et al. Direct effect of melatonin on Syrian hamster testes: melatonin sub-type 1a receptors, inhibition of androgen production, and interaction with the local corticotropin-releasing hormone system. Endocrinology 2005; 146(3): 1541-1552.

40. Rossi SP, Matzkin ME, Terradas C, et al. New insights into melatonin/CRH signaling in hamster Leydig cells. Gen Com Endocrinol 2012; 178(1): 153-163.

41. Zhu Q, Zhou S, Wen Z, et al. Xylene delays the development of Leydig cells in pubertal rats by inducing reactive oxidative species. Toxicology 2021; 454: 152740. doi: 10.1016/j.tox.2021.152740.

42. Li L, Mu X, Ye L, et al. Suppression of testosterone production by nanoparticulate TiO_2 is associated with ERK1/2- PKA-PKC signaling pathways in rat primary cultured Leydig cells. Int J Nanomedicine 2018; 13: 5909-5924.

43. Du Y, Chi X, Wang Y, et al. Advancements in the ERK1/2 signaling pathway affecting male reproduction. Front Biosci (Landmark Ed) 2024; 29(1): 23. doi: 10.31083/j.fbl2901023.

44. Kumar L, Solanki S, Jain A, et al. MAPKs signaling is obligatory for male reproductive function in a development-specific manner. Front Reprod Health 2024; 6: 1330161. doi: 10.3389/frph.2024.1330161.

45. Wei J, Wang B, Chen Y, et al. The immunomodulatory effects of active ingredients from *Nigella sativa* in RAW264.7 cells through NF-κB/MAPK signaling pathways. Front Nutr 2022; 9: 899797. doi: 10.3389/fnut.2022.899797.

46. Li X, Cao X, Zhao H, et al. Hypoxia activates NOTCH4 via ERK/JNK/P38 MAPK signaling pathways to promote lung adenocarcinoma progression and metastasis. Front Cell Dev Biol 2021; 9: 780121. doi: 10.3389/fcell.2021.780121.

47. Emerling BM, Platanias LC, Black E, et al. Mitochondrial reactive oxygen species activation of p38 mitogen-activated protein kinase is required for hypoxia signaling. Mol Cell Biol 2005; 25(12): 4853-4862.

48. Luo F, Shi J, Shi Q, et al. ERK and p38 upregulation versus Bcl-6 downregulation in rat kidney epithelial cells exposed to prolonged hypoxia. Cell Transplant 2017; 26(8): 1441-1451.

49. Li W, Fan M, Chen Y, et al. Melatonin induces cell apoptosis in AGS cells through the activation of JNK and P38 MAPK and the suppression of nuclear factor-kappa B: a novel therapeutic implication for gastric cancer. Cell Physiol Biochem 2015; 37(6): 2323-2338.

50. Zhu C, Jiang H, Deng W, et al. Activation of p38/HSP27 pathway counters melatonin-induced inhibitory effect on proliferation of human gastric cancer cells. J Biomed Res 2019; 33(5): 317-324.