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Experimental Research Article

Conditioned medium of Wharton's jelly-derived mesenchymal stem cells restores spermatogenesis in scrotal hyperthermia-induced azoospermia

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ABSTRACT

Introduction: Azoospermia is a common cause of male infertility. The latest hope for more affordable and safer therapies comes from stem cell research. In this study, the effect of mesenchymal stem cells derived from Wharton's jelly on spermatogenesis in azoospermic mice with scrotal hyperthermia was investigated.

Methods: For the experimental study, 24 adult male mice were divided into four groups: 1. control (Cont), 2. scrotal hyperthermia (Hyp), 3. scrotal hyperthermia + DMEM (10µl) (Hyp/DMEM), 4. scrotal hyperthermia + conditioned medium (10 µl) (Hyp/CM). A temperature of 43 °C was applied for 20 minutes every other day for two weeks to induce hyperthermia. The mice in the experimental group received 10 µl of human Jelly Wharton's Stem Cell-Conditioned Medium (hJWSC-CM) intraperitoneally every other day for 35 days after hyperthermia. Animals were sacrificed after the experiments to perform additional molecular, biochemical, and stereological analyses.

Results: The results showed that CM significantly increased both the total amount of sperm and the number of testicular cells, including spermatids, primary spermatocytes, spermatogonia, Leydig cells, and Sertoli cells, compared with Hyp and Hyp/DMEM; in addition, the biochemical characteristics of testicular tissue were significantly higher in the Hyp/CM group compared with the hyperthermia-induced groups. Further analysis revealed that when the Hyp/CM group was compared with the Hyp and Hyp/DMEM groups, Sox9 protein expression and proliferative gene expression increased significantly and the percentage of apoptotic testicular cells and pro-inflammatory gene expression decreased significantly.

Conclusion: These results suggest that realistic therapeutic approaches for reproductive and regenerative medicine could benefit from hWJSC-CM.

Keywords:
Scrotal hyperthermia
Conditioned medium
Wharton's jelly
Spermatogenesis
Azoospermia

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Introduction

Testicular hyperthermia, which results in genital heat stress, can be caused by various lifestyle factors, including posture, clothing, obesity, and clinical conditions such as varicocele and cryptorchidism (Kumar Roy et al., 2016). Spermatogenesis is temperature-sensitive and occurs optimally at 2–5 °C below abdominal temperature. Therefore, any factor that affects testicular temperature can impair spermatogenesis (Boni 2019). Studies have shown that high testicular temperature can affect male fertility (Moscatelli et al., 2019; Rao et al., 2016). Possible effects on sperm parameters include reduced motility, viability, and cell count. In addition, heat stress can lead to sperm DNA damage, oxidative stress, germ cell death, changes in Sertoli cell structure and function, and autophagy in the testis (Durairajanayagam et al., 2015; Ilacqua et al., 2018; Rao et al., 2015). Several treatments have been used to improve spermatogenesis and preserve human reproductive potential, including gene and cell therapy (Mozafar et al., 2018). Stem cell therapy has attracted a lot of attention. It is known for its potential to restore the structure and function of damaged tissue (Vij et al., 2018). Stem cells can be derived from various sources, including embryonic and mesenchymal stem cells (MSCs). The use of MSCs has attracted considerable due to their ease of derivation and handling. MSCs can be found in numerous locations, e.g. in adipose tissue, bone marrow, umbilical cord, and other areas (Shirazi et al., 2017). Compared to other sources, MSCs from Wharton's jelly are more accessible and do not turn into teratogenic or carcinogenic cells despite their high proliferative capacity (Ranjbaran et al., 2018). Furthermore, MSCs secrete extracellular vesicles (EV) containing growth factors and cytokines with anti-apoptotic, anti-inflammatory, and proliferative properties (Galipeau and Sensébé 2018). Mesenchymal stem cells-conditioned medium (MSCs-CM) contains EVs released by cells (Park et al., 2019). The CM can lead to cell differentiation or tissue regeneration and can also produce factors that stimulate spermatogonial repair (Almeria et al., 2019; Bazoobandi et al., 2020; Jahromi et al., 2017; Shah et al., 2016). Removing cells from this CM can effectively prevent problems associated with cells in the body. These problems include immune responses and tumor formation. The current study aimed to investigate whether the CM of WJ-MSCs, which contains trophic factors and promotes spermatogenesis, can improve spermatogenesis in mice exposed to scrotal hyperthermia.

Methods and Materials

Study design and animals

For this experimental study, 24 adult male NMRI mice weighing 25–30 g and aged eight weeks were used. The animals were purchased from a laboratory animal of the Pasteur Institute in Tehran, Iran, and stored according to standard laboratory procedures. The research protocol was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1400.386). We formed four experimental groups of six mice each by randomizing the animals. 1. control (Cont), 2. scrotal hyperthermia (Hyp), 3. scrotal hyperthermia + DMEM (10µl) (Hyp/DMEM), and 4. scrotal hyperthermia + conditioned medium of hWJ-MSC (10µl) (Hyp/CM).

Induction of scrotal hyperthermia

The first step in the induction of scrotal hyperthermia in mice was the intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) to anesthetize the mice. The lower body, including the testes and hind legs, was then immersed in a 43 °C water bath. This procedure was repeated three times a week at 24-hour intervals for two weeks. To confirm azoospermia, the hyperthermic mice were mated with female mice. We also conducted sperm counting in the semen and performed H&E staining. Subsequently, the experimental groups were treated with the CM of hWJ-MSCs (Ziaeipour et al., 2019b).

Extraction of human Wharton's Jelly-mesenchymal stem cell (hWJ-MSC)

Umbilical cords (UC) were removed with written consent from patients who had undergone complete cesarean section (n = 6, 25–32 years old) in the Obstetrics Department of Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The hWJ-MSCs were isolated as described below. PBS (GE Healthcare Life Sciences, UT, USA) was used to rinse the UC three times after sterilization with 1% povidone-iodine (Sichuan Kelun Pharmaceutical Co., Ltd., China). Two arteries and one vein were excised from the UC through a 3-cm incision. Subsequently, the gelatinous tissue, now called Wharton's jelly, was cut into 1 mm^2 pieces. Fetal bovine serum (FBS; GE Healthcare Life Sciences, UT,

USA) and an antibiotic mixture were added to Dulbecco's modified Eagle's medium (DMEM; Gibco, USA), and the cells were cultured at 5% CO2 and 37°C. Penicillin G (100 u/ml) and streptomycin (100 mg/ml), both from Gibco, USA, were used to prepare the antibiotic mixture. After reaching 75% confluence, the cells were passaged with trypsin-ethylenediaminetetraacetic acid (EDTA; Sigma, USA). Cells were counted on Neobar slides after passages 3 to 7. The single-cell suspension was seeded in culture flasks at a density of 5×10^5 cells per 25 cm^2 for further studies.

Identification of MSC surface markers using flow cytometry

Flow cytometry was used to verify the isolation of hWJ-MSCs and the absence of hematopoietic stem cells. The presence of specific expression of surface markers on hWJ-MSCs (CD105, CD90, and CD73) in isolated cells was compared with the absence of surface markers specific for hematopoietic stem cells (CD45).

Preparation of human Wharton's jelly stem cell-conditioned medium (hWJSC-CM)

To prepare hWJSC-CM, early four passage hWJ-MSCs were cultured in TP75 flasks at a density of 10,000 cells/cm^2. Before the medium was replaced with serum-free DMEM, the cells were washed three times with PBS at 80 percent confluence. After a 72-hour incubation period, the medium was centrifuged at 300 × g for 5 minutes, filtered through a 0.22 µm filter, and stored at -80 °C for later use.

Growth factor assessment using an enzyme-linked immunosorbent assay (ELISA) in hWJSC-CM

ELISA kit (human FGF ELISA, human TGF ELISA, human IGF-1 ELISA, RayBiotech, Inc., Norcross, GA, USA) was used to determine the type and concentration of growth factors, including fibroblast growth factor (FGF), insulin-like growth factor (IGF-1), transforming growth factor (TGF), and cytokines in hWJSC-CM at the fourth passage.

Administration of CM

All animals in the experimental group received 10µl of hWJSC-CM intraperitoneally every other day after scrotal hyperthermia (Panahi et al., 2020).

Surgery

After intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg), the mice were sacrificed at the end of the study. They were deeply anesthetized to obtain blood via cardiac puncture for analysis of serum testosterone levels. Next, we collected both testis tissues. Stereological and immunohistochemical analyses were performed on the left testicular tissue, while cellular and molecular analyses were performed on the right testicular tissue. In addition, epididymal tails were removed to determine sperm parameters.

Semen collection and analysis

Sperm samples were extracted from the epididymal tail and placed in 1 ml Ham's F-10 medium (Sigma-Aldrich, USA). A 20-minute incubation period at 37°C was then performed. Sperm motility was assessed under an inverted microscope on a slide containing 10 µl of the sample, and sperm were counted in a counting chamber. To assess sperm viability, a smear was placed on a slide, fixed with methyl alcohol, and stained with eosin-nigrosine (Halder et al., 2018). Percentage calculations were performed to determine sperm morphology. Smears that had been air-dried and stained with the Diff-Quik staining kit were used.

SCD test (sperm chromatin dispersion)

A halosperm kit (INDAS Laboratories, Spain) was used to perform the SCD test. In this test, the peripheral halo of the scattered DNA loop could not be generated or visualized in sperm with fragmented DNA but could be visible in healthy sperm. Each sample in this study contained 300 spermatozoa, which were examined at a 100x microscope objective.

Measuring the level of serum testosterone

After separation of the serum by centrifuging the blood samples at 6000 rpm for 5 minutes at 4 °C, the serum was stored at -80 °C until use. The testosterone concentration in the blood serum was measured using a mouse-specific ELISA kit (catalog number. CSB-E11162r).

ROS assay

H2DCFD was used to measure the damaged cell membranes. When these substances enter the cells, de-esterification occurs. Fluorescent 5-chloromethyl-2′,

7′ dichlorofluorescein (DCF) is generated after peroxide reactions. A flow cytometer with a 530-nm bandpass filter and a 488-nm argon ion laser was used to detect the fluorescence signals (Moghimi et al., 2021).

Glutathione disulfide content assessment

Since thiol metabolism is crucial for cell stress defense, GSH concentrations were investigated. The intracellular GSH content was determined using a spectrofluorometric technique. Aliquots of N-ethylmaleimide (NEM) and o-phthalaldehyde (OPA) probe (5 M) were used to stain the testicular cell solution (0.5 ml). The pellet was suspended in 2 ml of fresh incubation medium after the cells were centrifuged at 1000 rpm for 2 minutes. In the following phase, the fluorescent dye was removed from the medium by two rounds of washing the cells. A Shimadzu fluorescence spectrophotometer (RF5000U) was used to measure the samples at 495 nm excitation and 530 nm emission wavelengths in quartz cuvettes (Moghimi et al., 2021).

Tissue preparation and Hematoxylin &Eosin staining

The testes were fixed in Bouin's solution for 48 hours. Hematoxylin and eosin (H&E; Sigma, USA) were used to stain serial sections of 5 and 20 µm thickness to determine the number of testicular cells and the diameter of the seminiferous tubules. The testes were divided into 10 parts with equal distances between each other for each sample (Ghanbari et al., 2016).

Stereology study

Cavalieri method was used to determine testicular volume with a specific formula (Ziaeipour et al., 2019a):

$$V = \sum P \times \frac{a}{p} \times t$$

Where "ΣP" is the total number of points of the testicular sections, "a/p" is the zone associated with each point, and the distance between the sample sections is denoted by "t"

An optical dissector method was used to estimate the number of testicular cells. The following methods were used to calculate the numerical density of testicular cells (Nv):

$$Nv = \frac{\Sigma Q}{\Sigma P \times h \times \frac{a}{f}} \times \frac{t}{BA}$$

"ΣQ" and "ΣP" represent the total number of cells and the counting frame grid fields, respectively, "h" is the height of the dissector, "a/f" is the frame area, "BA" is the thickness of the microtome section, and "t" is the section thickness (Boroujeni et al., 2021).

The length of seminiferous tubules and their density were determined using the following equation (Ziaeipour et al., 2019a):

$$Lv = \frac{2\Sigma Q}{\Sigma P \times \frac{a}{f}}$$

The total number of seminiferous tubules was represented as "ΣQ". The area per frame was indicated as "a/f". The total number of points superimposed on the testicular tissue is indicated as "P" The length density (Lv) multiplied by the testicular volume provided the total length of seminiferous tubules.

Immunohistochemical Staining for SOX9 as a Sertoli Cell Marker

Testis tissues were fixed in 10% buffered formalin for 48 hours, embedded in paraffin, and sectioned at a thickness of 5 µm using a microtome. The sections were mounted on Superfrost Plus slides, deparaffinized in xylene, and rehydrated through a graded ethanol series into water. To block endogenous peroxidase activity, sections were incubated with hydrogen peroxide for 10 minutes at room temperature. Antigen retrieval was performed using Tris-EDTA buffer (pH 9.0) in a microwave for 20 minutes. Following this, sections were treated with a protein block for 15 minutes at room temperature. Primary antibodies against SOX9 (Sigma-Aldrich, USA) were diluted in PBS containing 1% BSA and 0.3% Triton X-100, and the sections were incubated with these antibodies for 1 hour at room temperature. After washing in PBS, sections were incubated with HRP-conjugated secondary antibodies (Mousespecific HRP/DAB Detection IHC Kit) for 30 minutes at room temperature. The SOX9 signal was visualized using the DAB Substrate Kit according to the manufacturer's instructions, with optional counterstaining performed using hematoxylin. Finally, the sections were dehydrated through graded ethanol to xylene and mounted with a suitable mounting medium. Imaging was conducted using a Nikon E200 light microscope, and images were captured with an attached camera.

TUNEL assay

TUNEL staining, an important method for studying apoptosis, was used to determine the extent of DNA damage in the cells. We then stained tissue sections using the TUNEL method (Roche Basel, Switzerland) to stain tissue slices. Finally, we calculated the proportion of TUNEL-positive cells in the testicular tissue sections.

Analysis of gene expression

The tissue fragments were extracted and stored at 80 °C to stabilize the cellular RNA. After RNA sample extraction with TRIzol (Invitrogen®, US), genomic DNA contamination was removed with DNase I (Roche, Basel, Switzerland). Using a commercial kit (FIREScript®, Tartu, Estonia), cDNA was generated at 42 °C for 60 minutes following the manufacturer's guidelines. Specific primers for proliferation and pro-inflammatory markers were designed for real-time quantitative RT-PCR (RT-qPCR), and GAPDH was used as an internal reference gene to standardize the results. Reactions were performed using the SYBR® Premix Ex TaqTM II (Takara Bio Inc., Japan). 45 cycles of primer-specific denaturation at 95 °C for 10 s and extension at 72 °C for 20 s formed the first 15 minutes of denaturation at 95 °C. The Primer 3 Plus tool was used to generate all pairs of forward and reverse primers using the exon-exon junction approach. We previously assessed the PCR primers using the primer-blast program. The primer sequences used for the target genes were as follows (Table 1).

Method of statistical analysis

The results were expressed as the Mean ± standard deviation. We used One-way ANOVA and Tukey's post hoc analysis to compare the data between groups after confirming the normality of the data with the Kolmogorov-Smirnov test. SPSS software (version 23, USA, IBM Corp) was used for statistical analysis, with the significance level set at $p<0.05$.

Results

Verification of mesenchymal stem cell characteristics

Graph generated using FlowJo software to analyze MSC cell surface markers showed that hW-MSCs lacked CD45 but had CD90, CD73, and CD105 (Figure 1A).

Growth factor assessment using an ELISA in hWJSC-CM

An ELISA kit was used to determine the levels of transforming growth factor (TGF-β1), insulin-like growth factor (IGF-1), and fibroblast growth factor (FGF2) in hWJSC-CM (Figure 1B). The findings of this study showed that hWJSC-CM had elevated levels of IGF-1, TGF-β1, and FGF2 (Figure 1B).

TABLE 1: primer design

Genes	Primer sequences	Product size (bp)	TM (°C)
c-kit	F: GCATCACCATCAAAAACGTG R: GATAGTCAGCGTCTCCTGGC	332	58
STRA8	F: GTTTCCTGCGTGTTCCACAAG R: CACCCGAGGCTCAAGCTTC	151	57
PCNA	F: GATGTGGAGCAACTTGGAAT R: AGCTCTCCACTTGCAGAAA	160	53
IL1-α	F: GTCGGGAGGAGACGACTCTA R: GCAACTCCTTCAGCAACACG	107	59
IL6	F: GCCTTCTTGGGACTGATGCT R: TGCCATTGCACAACTCTTTTCT	181	59
TNF-α	F: CCTCACACTCACAAACCACCA R: ACAAGGTACAACCCATCGGC	132	60
GAPDH	F: ATCACTGCCACTCAGAAGACTG R: TGGATGCAGGGATGATGTTCTG	293	60

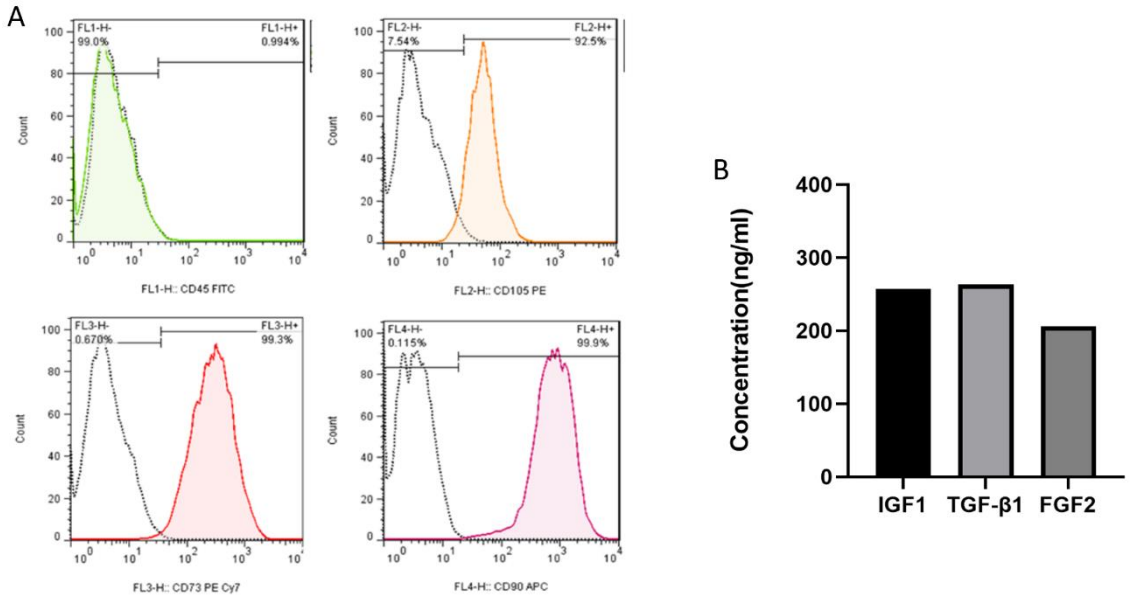

FIGURE 1. (A) According to the flow cytometry results, these cells expressed the MSC markers, including CD105, CD73, and CD90, in significant concentrations but not the hematopoietic cell marker (CD45). (B) The levels of insulin-like growth factor (IGF-1), FGF2, and transforming growth factor (TGF-β1) were measured in hWJSC-CM at passage 4 using an ELISA kit protocol.

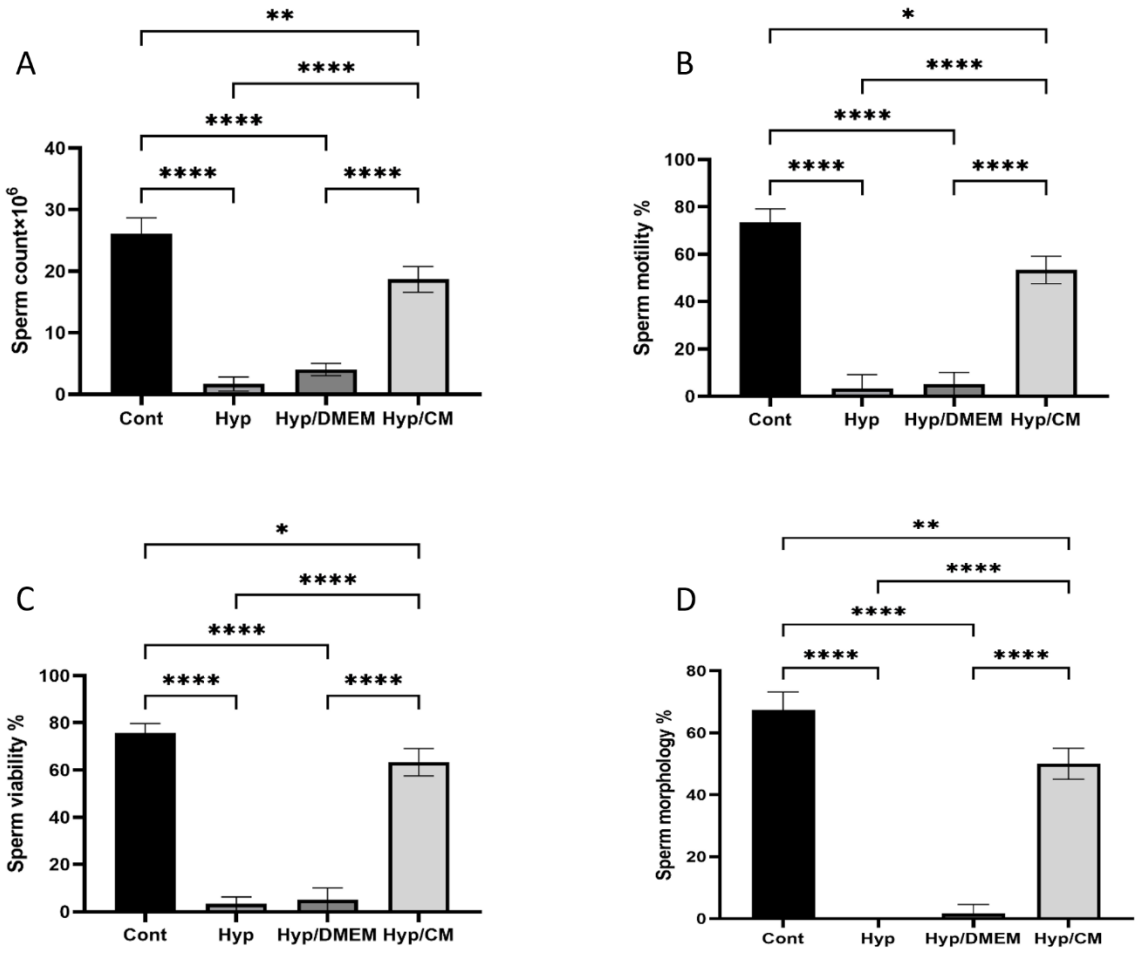

FIGURE 2. Effects of hWJSC-CM on sperm parameters in the study groups. (A-D); Mean ± SD of the total sperm count, percentage of sperm motility, percentage of sperm viability, and percentage of normal sperm morphology (*p<0.05, **p<0.01, ***p<0.001, and **** p<0.0001).

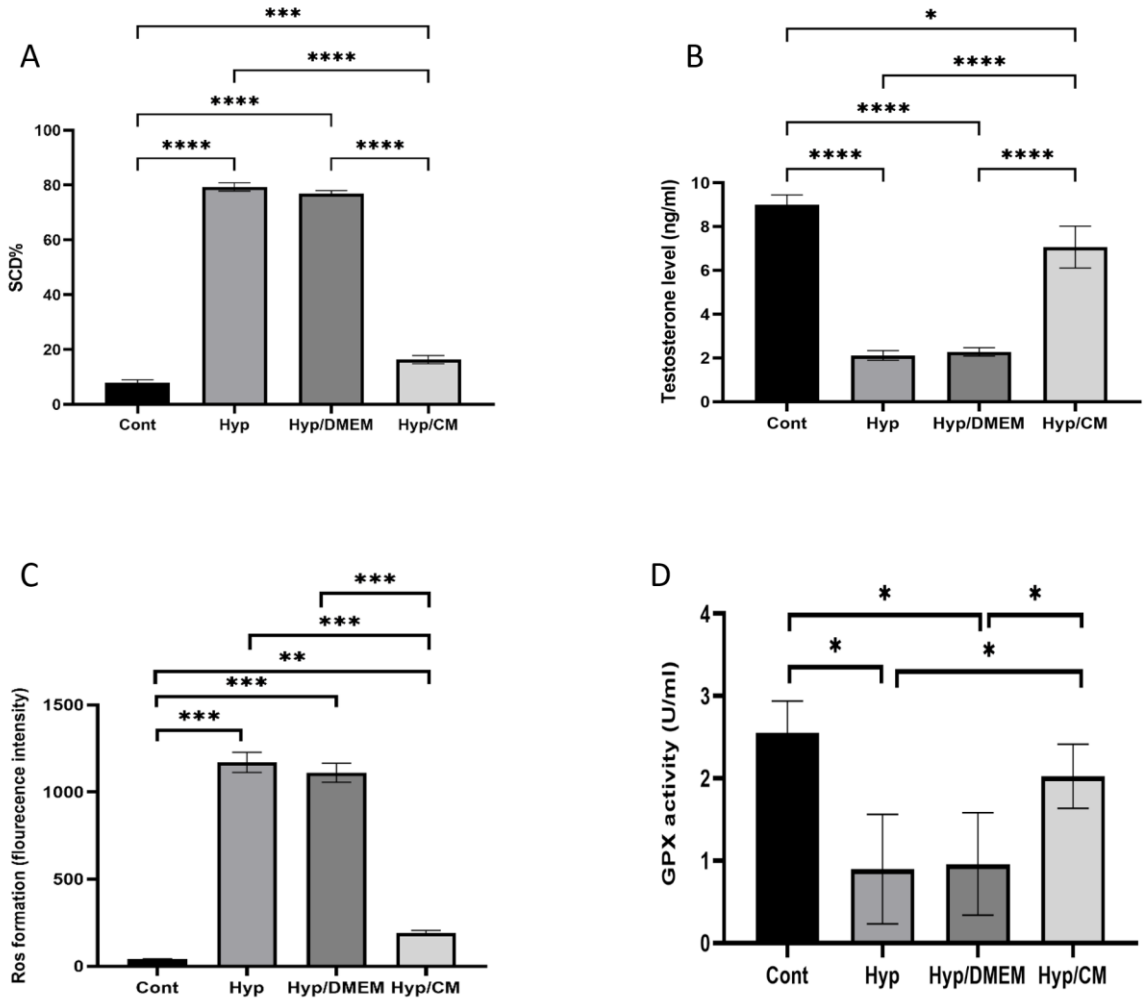

FIGURE 3. Effects of hWJSC-CM on SCD, serum testosterone levels, ROS production, and GSH activity in the study groups. (A-D) Mean ± SD of SCD, serum testosterone levels, ROS production, and GSH activity of testes in different groups (*p<0.05, **p<0.01, ***p<0.0001, and ****p<0.0001).

Confirming the azoospermia

To confirm azoospermia, mice induced by scrotal hyperthermia and healthy mice were naturally mated with female mice. After mating, two mice in the hyperthermia group became pregnant and were removed from the study, while all female mice in the healthy group became pregnant. We also performed sperm counting in the semen and conducted H&E staining.

Semen analysis and SCD index

In contrast to the Cont group, motility, viability, sperm count, and morphology were significantly reduced in the Hyp and Hyp/DMEM groups, as shown in Figure 2 A-D. However, the corresponding parameters improved significantly when the mice were treated with hWJSC-CM compared to the Hyp and Hyp/DMEM groups. Moreover, the percentage of DNA fragmentation detected by the SCD assay was higher in the Hyp group than in the Cont group (p<0.0001); however, the percentage of SCD-positive cells decreased significantly after administration of hWJSC-CM compared to the Hyp group (p<0.0001). The Hyp/CM and Cont groups differed significantly from each other (p<0.001) (Figure 3A).

Testosterone levels

Serum testosterone levels were significantly lower in the Hyp and Hyp/DMEM groups than in the control group (p<0.0001). Our results showed that serum testosterone level was significantly increased by the administration of hWJSC-CM. Figure 3B shows significant differences (p<0.05) between the Hyp/CM and control groups.

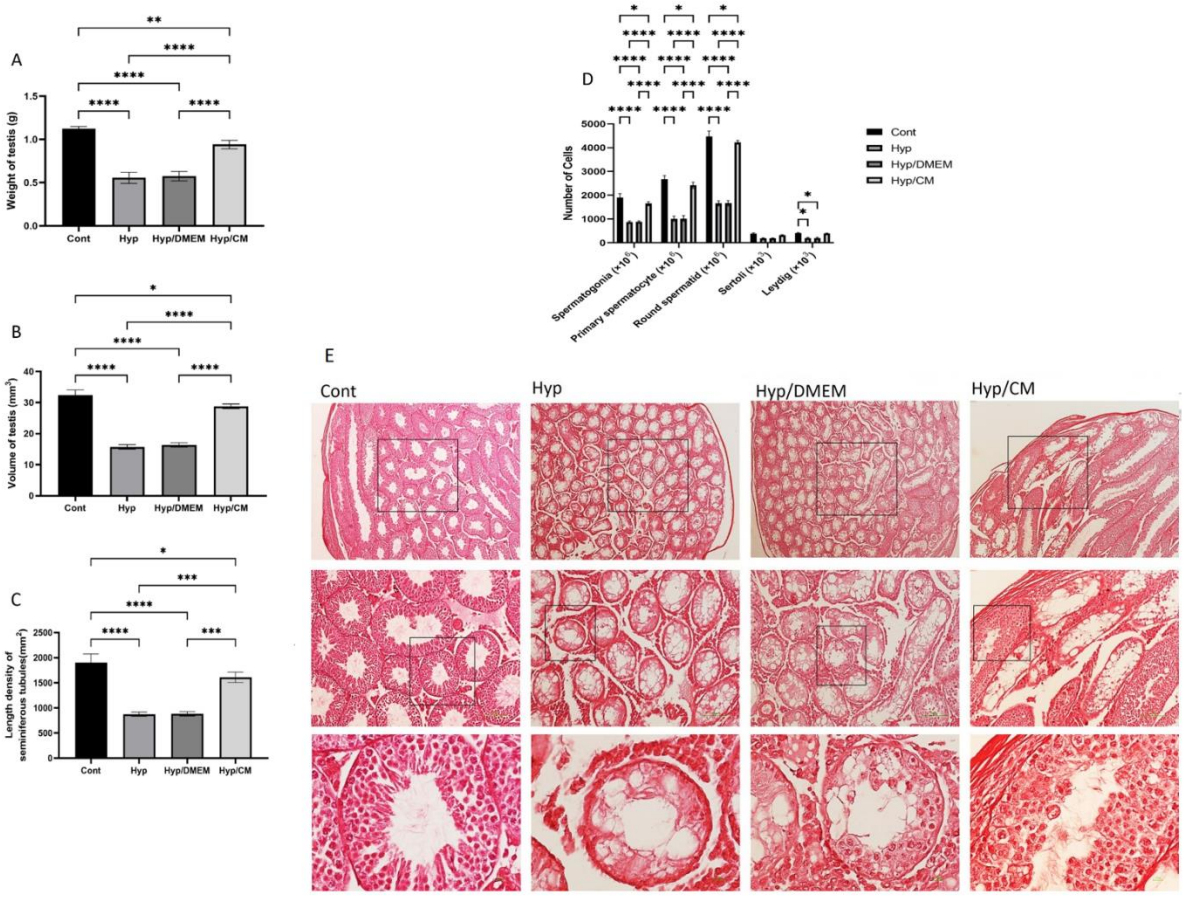

FIGURE 4. Effects of hWJSC-CM on stereological parameters in the study groups. (A-D) Mean ± SD of the weight of the testis, volume of the testis, length density of seminiferous tubules, number of spermatogonia, primary spermatocytes, round spermatids, and Leydig and Sertoli cells in the testes (*p<0.05; **p<0.01, ***p<0.001, ****p<0.0001). (E) Photomicrograph of testis tissue stained with hematoxylin and eosin (4X, and 40X, respectively) in different groups. Empty seminiferous tubule (star). SG (spermatogonia), PS (primary spermatocytes), ST (round spermatids), SC (Sertoli cell), and LC (Leydig cell).

ROS production and GSH activity

The Hyp group showed significantly higher ROS production (fluorescence intensity) than the control group. However, after the administration of hWJSC-CM, ROS production was significantly reduced in the Hyp group (p<0.0001) (Figure 4C). GSH levels in the testes were measured and showed a significant reduction in the Hyp and Hyp/DMEM mice compared to the Cont and Hyp/CM groups. Furthermore, our data showed that treatment with hWJSC-CM significantly increased testicular GSH levels compared to the Hyp and Hyp/DMEM groups (p<0.05). (Figure 3D).

The stereological analysis

After the study, the assessments of testicular weight revealed that the Hyp and Hyp/DMEM groups were significantly lower than the Cont and Hyp/CM groups (p<0.0001). Testicular weight was restored to the usual level in the control group by hWJSC-CM treatment; however, there was a significant difference (p<0.01) between the two groups (Figure 4A). Testicular volume in the Hyp group was significantly lower than in the Hyp/CM and Cont groups (p<0.0001), as determined by stereological examination of histological investigation. Testicular volumes in the Hyp/CM group were significantly lower than in the control group (p<0.05) (Figure 4B). In addition, the length of the seminiferous tubule was significantly shorter in the Hyp group compared to the Hyp/CM and Cont groups (p<0.001 and p<0.0001, respectively) (Figure 4C). The number of spermatogonia, primary spermatocytes, and round spermatids increased significantly in the treatment group after scrotal hyperthermia stimulation, as shown by the data (Figure 4D). A significant difference (p<0.05) was observed

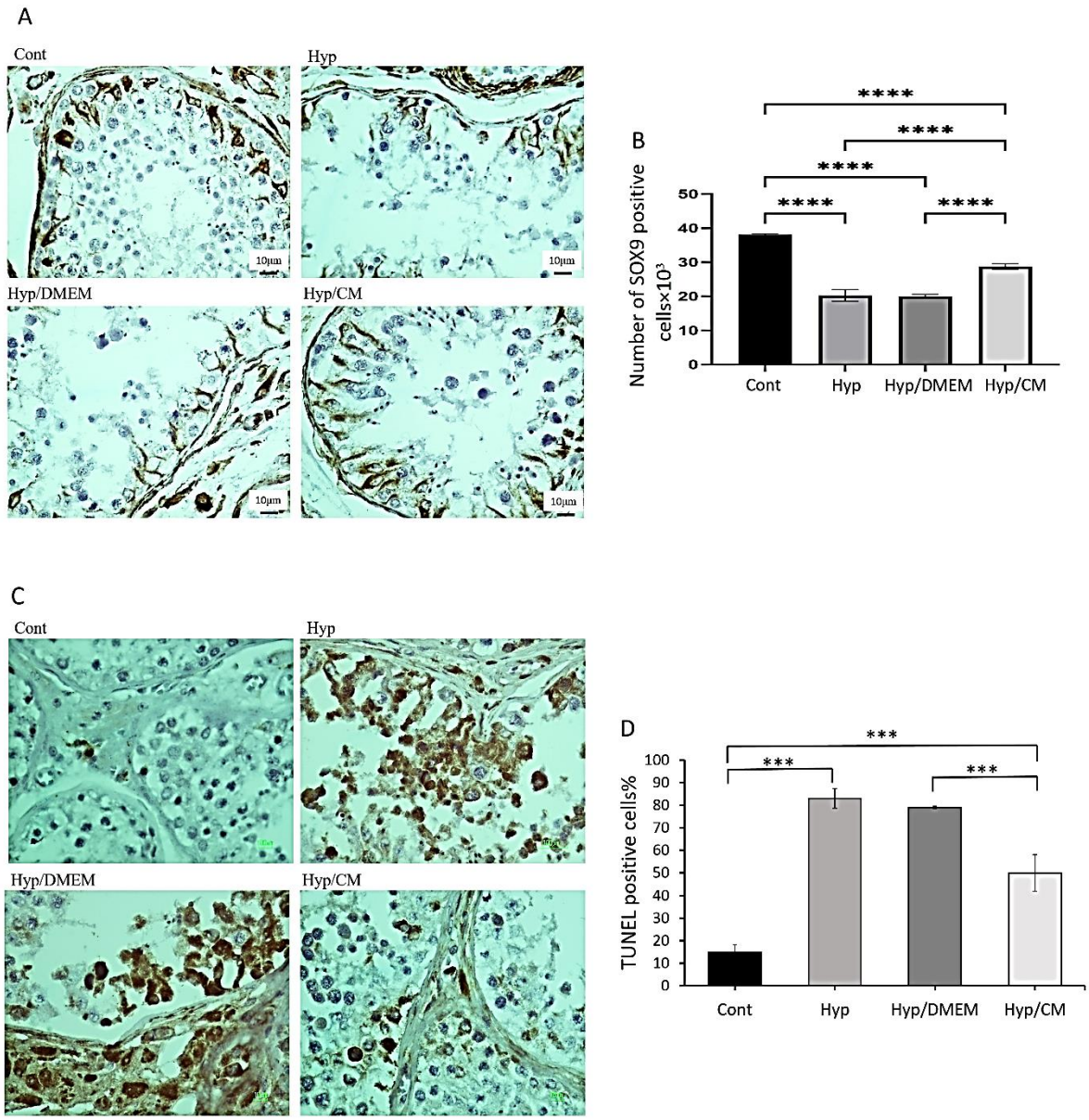

FIGURE 5. Effects of hWJSC-CM on the number of SOX9 positive cells in the study groups. (A) Photomicrograph of immunohistochemical staining for SOX9 in the testes of the study groups. Expression of SOX9 in Sertoli cells according to immunoreactivity (brown color). (B) Data are represented as mean ± SD (****p<0.0001). (C) TUNEL staining of mouse testes tissue showing TUNEL-positive cells inside seminiferous tubules. Brown cells indicate TUNEL-positive cells. (D) Data are represented as mean ± SD (***p<0.001).

between the Hyp/CM and Cont groups. The number of Leydig cells was significantly lower in the Hyp group than in the Cont group, while Sertoli cells demonstrated resistance to elevated testicular temperatures (Figure 4D, $p<0.05$). Stereological analysis of histological tests showed a significant improvement after treatment with hWJSC-CM (Figure 4E).

Immunohistochemistry of SOX9

Immunohistochemical analyses indicated a significant difference in the expression of target proteins involved in Sertoli cell biomarkers. In addition, testicular expression of Sox9 was significantly decreased in the Hyp and Hyp/DMEM groups compared to the Cont and Hyp/CM groups ($p<0.0001$; Figure B). Moreover, treatment with hWJSC-CM restored the levels observed in the Cont and Hyp/CM groups (Figure 5A and 5B). The brownish color was considered evidence of the positive expression of Sox9 in the testes.

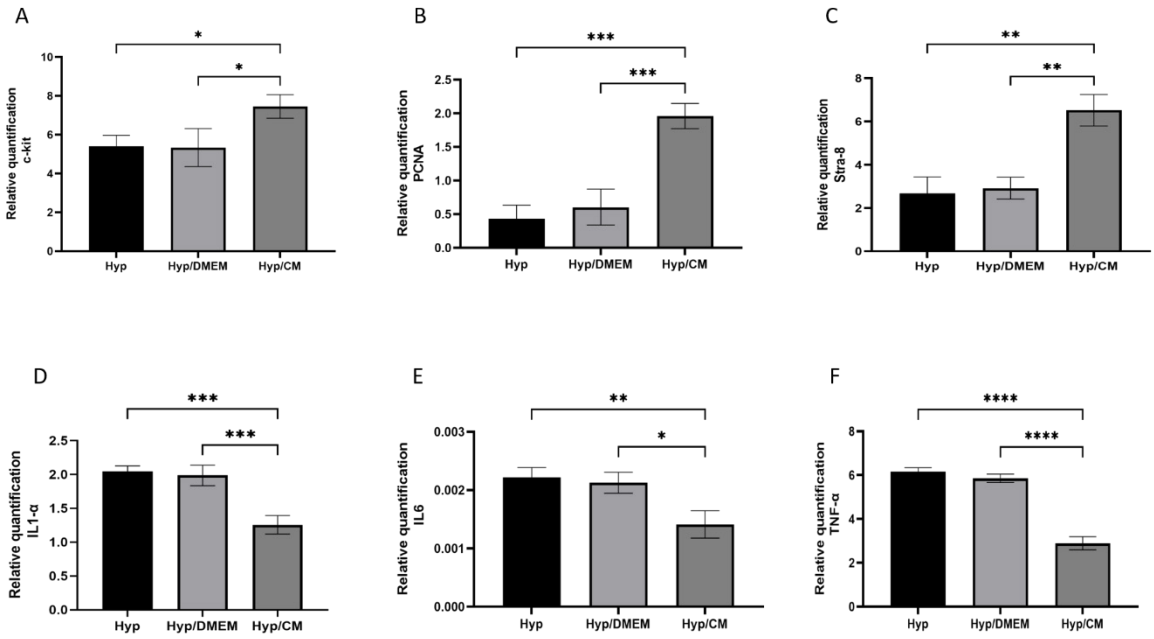

FIGURE 6. Effect of hWJSC-CM on gene expression in the study groups. A-F: The quantity of transcripts for three genes contributing to the proliferation and differentiation of spermatogonia cells (c-Kit, PCNA, and Stra-8) (a-c) and three genes involved in pro-inflammation (Il-6, Il-1α, and TNF-α) (d-f) were analyzed using RT-qPCR. Data are represented as mean ± SD (*p<0.05; **p<0.01, ***p<0.001, ****p<0.0001).

Apoptotic cells percentage

The effects of hWJSC-CM on testicular apoptotic cells were examined using the TUNEL assay. Significantly more apoptotic cells were observed in the Hyp and Hyp/DMEM groups than in the Hyp/CM and Cont groups ($p<0.001$), as shown in Figure 5C and D. In addition, hWJSC-CM significantly reduced testicular cell apoptosis in the Hyp/CM group. However, significant differences were observed between the Hyp/CM and Cont groups ($p<0.001$) (Figure 5C and D).

Gene expression profile

In the study, transcript levels involved in the proliferation and differentiation of spermatogonia (*c-Kit*, *PCNA*, and *Stra-8*) and three genes involved in inflammation (*Il-1α*, *TNF-α*, and *Il-6*) were examined to evaluate the molecular effects of hWJSC-CM treatment in hyperthermic mice. In contrast to the Hyp/CM group, the expression of *c-Kit*, *Stra-8*, and *PCNA* was significantly decreased in the Hyp group (Figure 6A-C). Treatment of mice with hWJSC-CM resulted in significantly lower expression of *Il-6*, *Il-1α*, and *TNF-α* genes in the Hyp/CM group than in the other groups (Figure 6D-F).

Discussion

Testicular hyperthermia, which is associated with the destruction of sperm in the testes, is one of the main causes of male infertility. Therefore, it is crucial to find safe therapies to treat testicular dysfunction caused by hyperthermia. MSC and CM therapies are considered potential treatment options for non-obstructive azoospermia. Although several studies have been conducted on mesenchymal stem cell transplantation for the treatment of infertility and restoration of spermatogenesis, no study has yet investigated the effect of hJWSC-CM on infertility caused by scrotal hyperthermia. The application of MSC-CM in the azoospermia model resulted in spermatogenesis in mice, which is consistent with our results (Mahiddine et al., 2020; Payehdar et al., 2017; Ziaeipour et al., 2019b). WJ-MSCs are frequently used in stem cell therapy because they can be easily obtained from the umbilical cord and cultured in vitro. WJ-MSCs were initially promising due to their multipotent differentiation capacity or their paracrine activities. However, their therapeutic effect was diminished by poor MSC retention and low survival rates in injured tissue. This suggests that the replacement of damaged cells could be significantly improved by the paracrine action of MSCs (Chudickova et al., 2019; Vizoso et al., 2017). Many growth factors are produced by WJMSC-CM, including IGF-I, TGF-β, IL-1, GDNF, and FGF2. Spermatogonial stem cells are dependent on several stimuli including

IGF-1 and FGF2, for their proliferation, differentiation, and maturity. Numerous studies have shown that these factors affect cells inside or outside the seminiferous tubules (Shen et al., 2015; Takashima et al., 2015).

Consistent with other studies on how hyperthermia affects spermatogenesis in animal models (Ilkhani et al., 2020; Park et al., 2017), mice treated with hWJSC-CM in the current study showed significantly higher levels of sperm parameters (motility, viability, sperm count, and normal morphology), number of spermatogonia, primary spermatocytes, and round spermatids, as well as a decrease in sperm DNA fragmentation and a reduction in inflammatory cell infiltration into the testicular interstitium. hWJSC-CM may be responsible for the observed increase in parameters by stimulating the proliferation of Leydig and spermatogenic cells that remain inactive. The results of our study show that hWJSC-CM can enhance the proliferation of spermatogonia through the secretion of growth factors. Based on previous studies, our results indicate that scrotal hyperthermia causes morphological damage to Leydig cells and impairs steroidogenesis (Afshar et al., 2021; Khosravi et al., 2021). A study has shown that Leydig stem cells are found in the interstitial compartment of the testis as mesenchymal stem cells. It has been shown that these multipotent cells can differentiate into Leydig cells, which are critical for Leydig cells enhancement as well as for the role of regulatory steroidogenesis in the interstitium (Li et al., 2022). The growth factors secreted by hWJ-MSCs can stimulate these cells to improve Leydig cells' function and regeneration.

In addition, this study examined the mRNA expression levels of pre-meiotic (*Stra-8, c-Kit, and PCNA*) and inflammatory (*IL-1α, IL-6, and TNF-β*) markers in testicular tissue. Both the onset of meiosis and the differentiation of spermatogonial stem cells are regulated by Stra-8. A widely recognized marker for germ cell differentiation is the c-kit receptor. Maghami et al. have shown that co-culturing stem cells from Wharton's jelly with a Sertoli cell-conditioned medium can lead to the differentiation of germ-like cells. After MSC differentiation, they also show improved Stra-8 expression (Ghaem Maghami et al., 2018). In agreement with previous studies on the effects of testicular hyperthermia on *c-Kit* expression, our study found a significant downregulation of c-Kit transcript levels in the Hyp groups, while it was significantly upregulated in the CM groups (Boroujeni et al., 2021; Pirani et al., 2021). The mRNA expression of inflammatory markers in testicular tissue was analyzed. There was also a significant transcriptional difference between the animals treated with CM and the animals subjected to scrotal hyperthermia. In addition, we observed a decrease in ROS generation when we compared the Hyp/CM group with the Hyp group. The generation of ROS can lead to cell death by causing lipid peroxidation in the mitochondria, a decrease in mitochondrial metabolism, and a decrease in ATP production (Ziaeipour et al., 2021). Our results showed that administration of hWJSC-CM to mice reversed the negative effects of scrotal hyperthermia, including decreased ROS production, pro-inflammatory markers such as *TNF-α, IL-6*, and *IL-1β*, cell apoptosis, and increased GSH levels. It can be concluded that hWJSC-CM can reduce oxidative stress, which reduces cell death.

Conclusion

The current findings show that the stimulation of growth factors and the release of cytokine by the conditioned medium of hWJ-MSCs improves the proliferation, differentiation, and maturation of germ cells into spermatozoa. In reproductive and regenerative medicine, the conditioned medium of hWJ-MSCs could be used as a novel technique for the treatment of male infertility.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

The experimental protocol and all procedures performed in studies involving the use of animals were reviewed and approved by the Ethical Committee at Shahid Beheshti University of Medical Sciences (IR. SBMU.MSP.REC.1400. 676).

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