





Sertoli cell-conditioned medium restores spermatogenesis in mice induced by scrotal hyperthermia

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ABSTRACT

Introduction: Spermatogenesis is a complex physiological process susceptible to various influencing factors, among which scrotal hyperthermia is noteworthy. This investigation assesses the impact of conditioned medium derived from Sertoli cells on spermatogenic activity within the testicular tissue of adult male mice after exposure to scrotal hyperthermia.

Methods: A cohort of 40 adult male NMRI mice was employed in this research, and they were allocated randomly into four distinct groups as follows: (1) the control group comprising untreated animals, (2) the hyperthermia group subjected to scrotal hyperthermia through immersion in water at a temperature of 43°C for 20 minutes, administered five times every other day, (3) the DMEM group receiving 10µl of Dulbecco's Modified Eagle Medium (DMEM), and (4) the Sertoli cell-conditioned medium (SCCM) group, administered 10µl of SCCM. After the induction of hyperthermia, intraperitoneal injections of SCCM were administered to the mice daily for 35 days. Samples of sperm were gathered from the epididymal tail, and the testis tissue was subsequently harvested for histological examination and molecular analysis.

Results: Our findings revealed that the administration of SCCM yielded significant increases in the quantities of sperm and germ cells compared to the other groups. Additionally, the relative gene expression levels of receptor tyrosine kinase (C-kit), stimulated by retinoic acid 8 (Stra8), and proliferating cell nuclear antigen (Pcna) exhibited a substantial elevation within the SCCM group compared to the other experimental groups.

Conclusion: The utilization of SCCM holds promise as a therapeutic intervention for addressing infertility concerns, and it presents potential applications within the realms of the field of reproductive and regenerative medicine.

Keywords:

Sertoli cell-conditioned medium

Spermatogenesis

Scrotal hyperthermia

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Introduction

Infertility is one of the most essential problems affecting millions of people of reproductive age worldwide (Maharlouei et al., 2021). Global statistics show that 15% of couples of childbearing age suffer from infertility, of which 50% are related to men (Kazemeini et al., 2017).

Spermatogenesis is a multi-step and complex process that can be affected by various factors, such as congenital anomalies of the urinary system, malignancies, smoking, endocrine disorders, genetic abnormalities, and increased scrotum temperature (Izadpanah et al., 2015; Rockett et al., 2001). In mammals, a relatively low temperature is required for the normal process of spermatogenesis, and the temperature of the testis in most mammals is 4 to 5 °C lower than the basal body temperature (Engel et al., 2021; Sharma and Leslie 2022).

Today, several factors can cause the temperature of the testicles to increase, which can be divided into internal and external factors (Mieusset et al., 1995). Varicocele and cryptorchidism can be mentioned among the internal factors (Cho et al., 2016). Among the external factors involved in this event are the way of sitting and sleeping, frequent use of hot baths and saunas, and jobs in which the ambient temperature is high, which can cause harmful impacts on the process of spermatogenesis and the quality of sperm (Ahmad et al., 2012; Sharma and Leslie 2022).

Different studies have shown that heat stress can lead to the death of germ and somatic cells, decrease the volume of testicular tissue, decrease the quality and quantity of sperm parameters, and finally stop the process of spermatogenesis by inducing the signaling pathways of apoptosis and oxidative stress (Durairajanayagam et al., 2015; Maloy and Hughes 2013). Germ cells are the main component of sexual reproduction in animals, which are more susceptible to heat stress damage than other cells due to frequent divisions and lack of Superoxide Dismutase (SOD) (Mínguez-Alarcón et al., 2018; Widlak and Vydra 2017).

The classical apoptosis pathway (mitochondrial apoptosis pathway) is the first activated mechanism in these cells (Yadav et al., 2018). When this pathway is activated, cytochrome c is released from the pores in the mitochondrial membrane and activates the caspase system by combining with Apaf-1, and finally, by damaging the intracellular structures and DNA, it leads to cell

death (Hengartner 2000; Hikim et al., 2003). Since heat stress leads to a sharp decrease in the level of Superoxide Dismutases (SOD) and Glutathione (GSH), *Reactive oxygen species* (ROS) plays a central role as an intracellular mediator of heat-induced oxidative stress, and the increase in its level with the disruption of the cell's respiratory cycle leads to the induction of mitochondrial apoptosis pathway (Wang et al., 2013).

The division, differentiation, and maturation of germ cells can depend on the expression of intracellular factors, including Stra8 and C-kit (Zhang et al., 2013). Factors whose expression can depend on external factors, including Sertoli cells. Cells that provide microenvironment and structural support for germ cells (Skinner 2005). These cells can provide comprehensive support to germ cells by secreting nutritional, environmental (such as extracellular matrix), and regulatory factors. Regulatory factors affect cell function, growth, or differentiation through receptor-mediated signal binding. Regulatory factors include two main subgroups: growth factors and hormones (Skinner 2005). Growth factors include (IGF-I, TGF- α , TGF- β , NGF, GDNF, and stem cell factor), which, in addition to directly affecting the cell cycle, affect the function and differentiation of cells. Hormones indirectly target cell proliferation by stimulating the production of growth factors (Nazifi et al., 2022).

Nowadays, one of the treatment methods used for various diseases is the conditioned medium derived from the stem and somatic cells. Sertoli cells are essential in germ cells' functional and structural support. Today, the conditioned medium derived from these somatic cells is one of the therapeutic solutions to improve the process of spermatogenesis (Afshar et al., 2021; Meng et al., 2005). The secretions of Sertoli cells cultured in laboratory conditions contain induction factors that can be used to improve the spermatogenesis process in patients. It can also have a role to play in stimulating the differentiation of bone marrow-derived mesenchymal stem cells into germ cells (Geens et al., 2011).

The effect of the conditioned medium derived from Sertoli cells on the complications caused by scrotal hyperthermia as a treatment method has not been investigated. Therefore, this study aims to investigate the impact of the conditioned medium derived from Sertoli cells on spermatogenesis in the testicular tissue of adult male mice after scrotal hyperthermia.

Materials and Methods

Animals

This research used 40 adults male NMRI mice with identical body weights (25-30 g) according to typical laboratory conditions. A total of four groups of mice were divided at random: (1) Control (CON), (2) Hyperthermia (HYP), (3) DMEM (10 μ l) (HYP+DMEM), and (4) Sertoli cells-conditioned medium (HYP+SC-CM) (10 μ l). At Shahid Beheshti University of Medical Sciences, the Research Ethics Committee approved the protocol (IR.SBMU.AEC.1402.010).

Transient scrotal hyperthermia model

For hyperthermia induction, the animals' testicles were exposed to water at 43 °C for five sessions of 20 minutes each, administered every other day. Initially, Ketamine (100 mg/kg) and xylazine (5 mg/kg) were intraperitoneally injected into mice to anesthetize them. Following this, the lower portion of their bodies, including the scrotum and hind legs, was immersed in a water bath. Subsequently, they were dried off and placed back in their cages. In the control groups, the mice were anesthetized and maintained at room temperature (Paul et al., 2009; Ziaepour et al., 2019).

Sertoli cells' conditioned medium

Sertoli cells were derived from the testes of mice that were three weeks old. First, the testicular tissue was cut into small pieces and then incubated in 0.25% trypsin (Sigma) and 0.1% collagenase (Sigma, type V) solution for 15 minutes at 37°C for decapsulation and enzymatic digestion. Testis cells were transferred into a cell culture flask and placed in a cell culture incubator. After 24 hours, the cells were washed to remove debris, and the culture medium was replaced with fresh DMEM/F12. Following 48 hours, the cells were subjected to a hypotonic treatment lasting 2.5 minutes using a 20 mM Tris-HCl solution (pH 7.4). This treatment removed any remaining germ cells, isolating Sertoli cells with a purity exceeding 95%. Subsequently, the cells were washed twice with PBS, then serum-free DMEM was added. After 48 hours of incubation with this serum-free culture medium, the media were collected and stored at -80°C for future use (Grootenhuys et al., 1990). To detect Sertoli cells, immunofluorescence of GATA4. Also, to determine the amount of growth factors IGF-1 and GDNF, an ELISA Kit (catalog no. MBS175810) was used (Fig. 1).

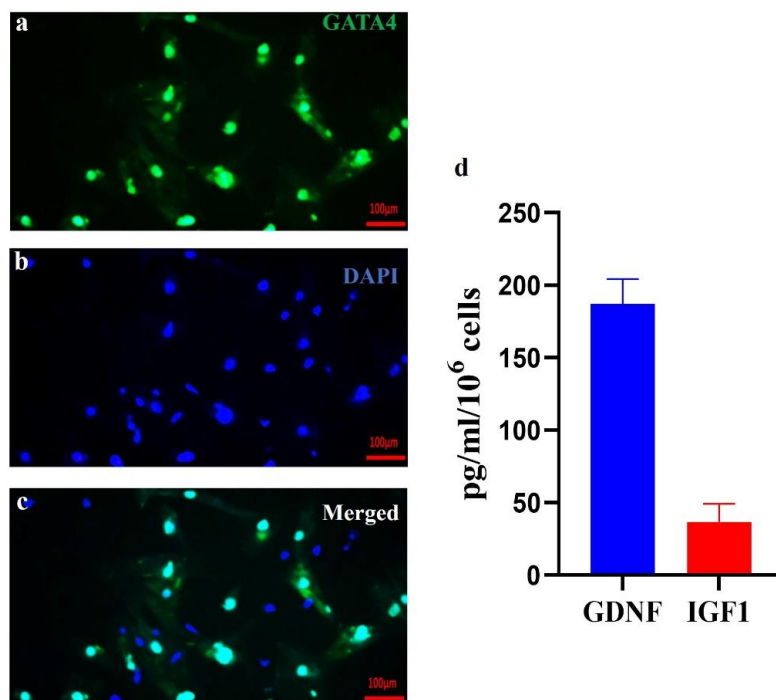


FIGURE 1. (a–c) Photomicrographs showing immunofluorescence of GATA4 expressed in Sertoli cells (green) with nuclei counterstained by DAPI (blue). (d) Serum-free SCCM was analyzed using ELISA for specific growth factors. Concentrations of IGF-1 and GDNF are presented as mean \pm SD.

Injection of SCCM

In the treatment group, after induction of hyperthermia, an SCCM amount of 10 µl intraperitoneal was injected into mice daily for 35 days (Goshadezhn et al., 2023; Panahi et al., 2020).

Sperm Analysis

Samples of sperm were collected from the tail of the epididymis and placed into 1 ml of Ham's F-10 media (Sigma-Aldrich Product Number N6635). Then, they were incubated at 37°C for 20 minutes. Finally, a 10 µl portion of the sample was placed onto a slide to determine sperm motility and sperm count using a counting chamber.

Tissue preparation

All testis samples were immersed in Bouin's solution for 48 hours. The fixed testis samples were processed in a tissue processor and embedded in paraffin. For histopathological analysis by stereological techniques, serial sections of 10 µm thickness were prepared using a microtome. A systematic uniform random sampling (SURS) was employed to select 10 sections from each sample. This selection involved choosing a random number between 1 and 10 for each sample. Finally, the tissue sections were subjected to H&E staining (Sigma, USA).

Estimating the total testis volume

Based on the Cavalieri formula, the total testis volume was estimated (Gundersen et al., 1988):

$$V_{(total)} = \Sigma p \times a/p \times t$$

Σp is the total number of points hitting the testis sections, a/p indicates the area associated with each sample point, and t represents the distance between tissue sections.

Estimating the somatic and germ cell number

The count of testis cells was calculated utilizing the optical dissector technique in combination with an impartial counting frame. By applying the subsequent formula, we calculated the numerical density (N_v) for various cell types (Gundersen et al., 1988).

$$N_v = \frac{\Sigma Q}{h \times \Sigma P \times a/f} \times \frac{t}{B.A}$$

In the formula, ΣQ represents the overall count of cells, h denotes the thickness of the tissue under consideration for counting, a/f represents the area per counting frame, and ΣP signifies the cumulative count of all the frames used for counting in all fields. Additionally, h corresponds to the height of the dissector, t represents the actual section thickness measured using a microcator, and BA is the thickness of the tissue section.

$$N_{(total)} = N_v \times V_{(total)}$$

ROS level in testicular tissue

After isolating of total testis tissue using trypsin EDTA, we washed the samples with PBS and centrifuged them at 1500 RPM at 4°C for 5 minutes. Then, we added DCFDA at a concentration of 20 µM to a 100 µl portion of the sample and incubated it at 37°C in the dark for 45 minutes. Finally, we used a spectrofluorometer to measure the sample at a wavelength of 488 nm.

Serum Testosterone Measurement

Blood samples were taken from the heart while under deep anesthesia to measure serum testosterone levels. These samples were then centrifuged at 6000×g for 5 minutes at 4°C, and the resulting serum was stored at -80°C. The testosterone concentration was determined using a mouse-specific ELISA kit (catalog no. CSB-E11162r).

TUNEL Assay

The TUNEL assay was utilized to determine the amount of DNA fragmentation. After the deparaffinization of sections, these were washed with PBS (Sigma-P4417). Then, H₂O₂ (Sigma-7722-84-1) was mixed with methanol at a ratio of 1:9 and placed on the sample for 10 minutes. After that, we washed the samples three times with PBS and placed proteinase K (Sigma-21627-M) on the sample for 30 minutes at 37 °C. Then, after washing the sample three times with PBS, we added Triton 0.3% to increase the permeability of the nuclei. In the following, we washed the samples three times with PBS and added TDT enzyme to them for 2 hours at 37 °C. Finally, after washing three times with PBS, we stained the nuclei with DAPI. We used the Olympus BX53 microscope to prepare fluorescent pictures (Pirani et al., 2021; Rosa et al., 2018).

TABLE 1: Primer design

Genes	Primer sequences	Product size (bp)	TM (°C)
<i>C-kit</i>	F: CCTCAAACAAGTCACCTCC R: GCTTTACCTGGGCTATGTG	332 bp	58
<i>Stra8</i>	F: TGTAGAGAGAGAGGTTAGAGGAGT R: ATGTGGAGAGATGATGCTGTT	151 bp	57
<i>Pcna</i>	F: CCTCACCAGCATGTCCAAAAT R: TCACCCGACGGCATCTTTATT	160 bp	53
GAPDH	F: CAGAACATCATCCCAGCCTCC R: TTGGCAGGTTTCTCAAGACGG	293 bp	60

Real-Time PCR

Tissue samples were immediately placed into a stabilized cellular RNA later to preserve cellular RNA and then stored at -80°C. We employed an RNA isolation kit (RNX-Plus; Cinna Gen Co, Tehran, Iran) to extract total RNA, following the manufacturer's guidelines. Subsequently, the RNA was treated with DNase I (Roche) to eliminate any genomic DNA. To synthesize cDNA, we utilized commercial kits (Fermentas, Lithuania), adhering to the manufacturer's instructions, which involved incubation at 42°C for 60 minutes.

To assess the relative gene expression, we employed real-time PCR (TaqMan) with the QuantiTect SYBR Green RT-PCR kit (Takara Bio Inc; Japan). All forward and reverse primer pairs were designed using the Primer 3 Plus software (Qiagen, Hilden, Germany). Primer 3 Plus software (Qiagen, Hilden, Germany) was used to design all forward and reverse primer pairs. A Primer-Blast tool was used to validate these primer designs. (Table 1).

Statistical Analysis

Statistical evaluation was done using SPSS version 23. Graph Pad Prism 9 was utilized to design the graphs. Mean and standard deviation (SD) were calculated for all data. ANOVA and Tukey's post-hoc test were used to compare the results. The data was significant at $P < 0.05$.

Results

sperm parameters

Our results showed that the quantity of sperm in the hyperthermia group significantly reduced than the other groups. Also, after the injection of SCCM, we indi-

cated a significant increase in the quantity of sperm in the SCCM group than the DMEM and hyperthermia groups ($p < 0.01, 0.0001$, respectively). Our results also showed that sperm motility in the hyperthermia group was significantly reduced than other groups. We indicated a significant increase in sperm motility in the SCCM group than the DMEM and hyperthermia groups ($p < 0.0001, 0.0001$, respectively; Fig.2).

Total testis volume

The results of the stereological study indicated that the volume of the testis in the hyperthermia group was significantly reduced compared to the other groups. However, following SCCM injection, a significant increase in testis volume was observed in the SCCM group compared to the DMEM and hyperthermia groups ($p < 0.001$ and $p < 0.0001$, respectively; Fig. 3A).

Number of testicular cells

The total numbers of spermatogonia, primary spermatocytes, round spermatids, Sertoli cells, and Leydig cells were significantly reduced in the hyperthermia group compared to the other groups. However, following SCCM injection, the numbers of these cells were significantly increased in the SCCM group compared to the DMEM and control groups ($p < 0.0001$ and $p < 0.0001$, respectively; Fig. 3B).

ROS production

ROS production in testicular tissue was measured across the different groups. The hyperthermia group showed significantly elevated levels compared to the other groups. However, following SCCM injection,

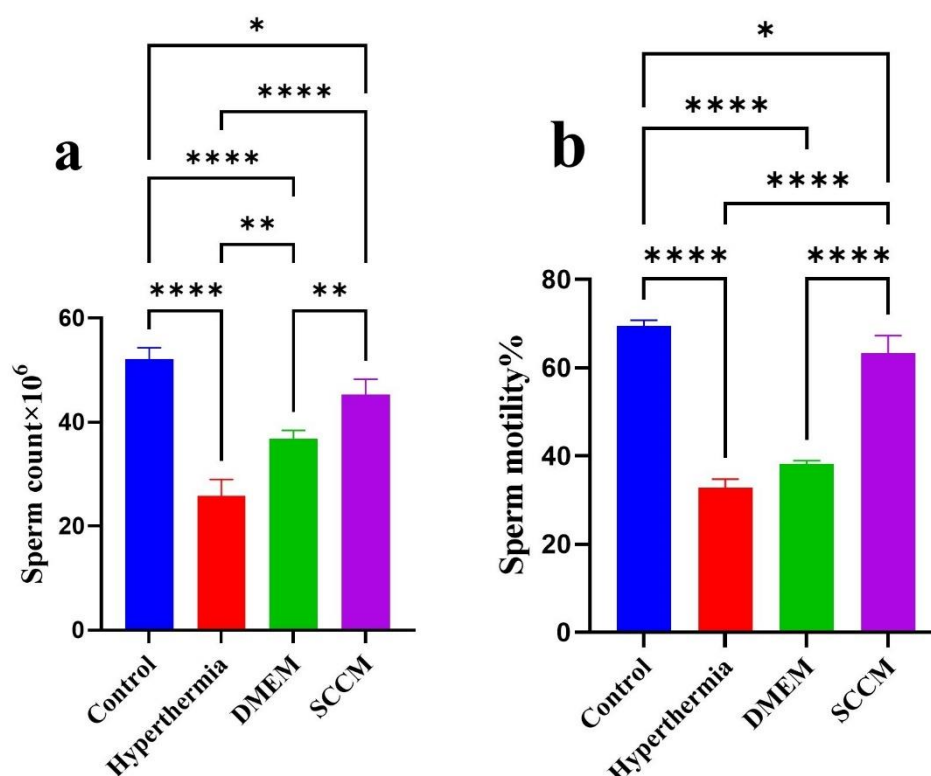


FIGURE 2. (a–b) Mean \pm SD of total sperm counts and sperm motility in the study groups. Groups: (1) Control; (2) Hyperthermia; (3) DMEM; (4) SCCM. p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****)

ROS levels in testicular tissue were significantly reduced compared to the hyperthermia group ($p < 0.001$; Fig. 4).

Serum testosterone level

Measurement of serum testosterone revealed that testosterone levels in the hyperthermia group were significantly decreased compared to the control and SCCM groups. Following SCCM injection, serum testosterone levels in the SCCM group were significantly increased compared to the hyperthermia and DMEM groups ($p < 0.01$ and $p < 0.05$, respectively; Fig. 4).

Number of apoptotic cells

This evaluation showed that the number of apoptotic cells was significantly increased in the hyperthermia group compared to the other groups. However, following SCCM injection, a significant decrease in apoptotic cells was observed in the SCCM group compared to the DMEM and hyperthermia groups ($p < 0.001$ and $p < 0.0001$, respectively; Fig. 5).

Gene expression

Gene expression levels of C-kit, Stra8, and Pcn were normalized and quantified across the groups. The relative mRNA expression of C-kit was significantly reduced in the hyperthermia group compared to the SCCM and control groups, while the SCCM group showed significantly higher expression than the DMEM group ($p < 0.05$). Similarly, Stra8 expression was significantly reduced in the hyperthermia group compared to the SCCM and control groups. Following SCCM injection, the SCCM group exhibited significantly higher Stra8 expression than the DMEM group ($p < 0.01$). The expression of Pcn was significantly reduced in the hyperthermia group compared to the SCCM and control groups; however, no significant difference was observed between the SCCM group and the other groups. Notably, a significant difference was found between the control and DMEM groups ($p < 0.01$; Fig. 6).

Discussion

Sertoli cells play a pivotal role in overseeing spermatogenesis. They support the transition of fetal germ

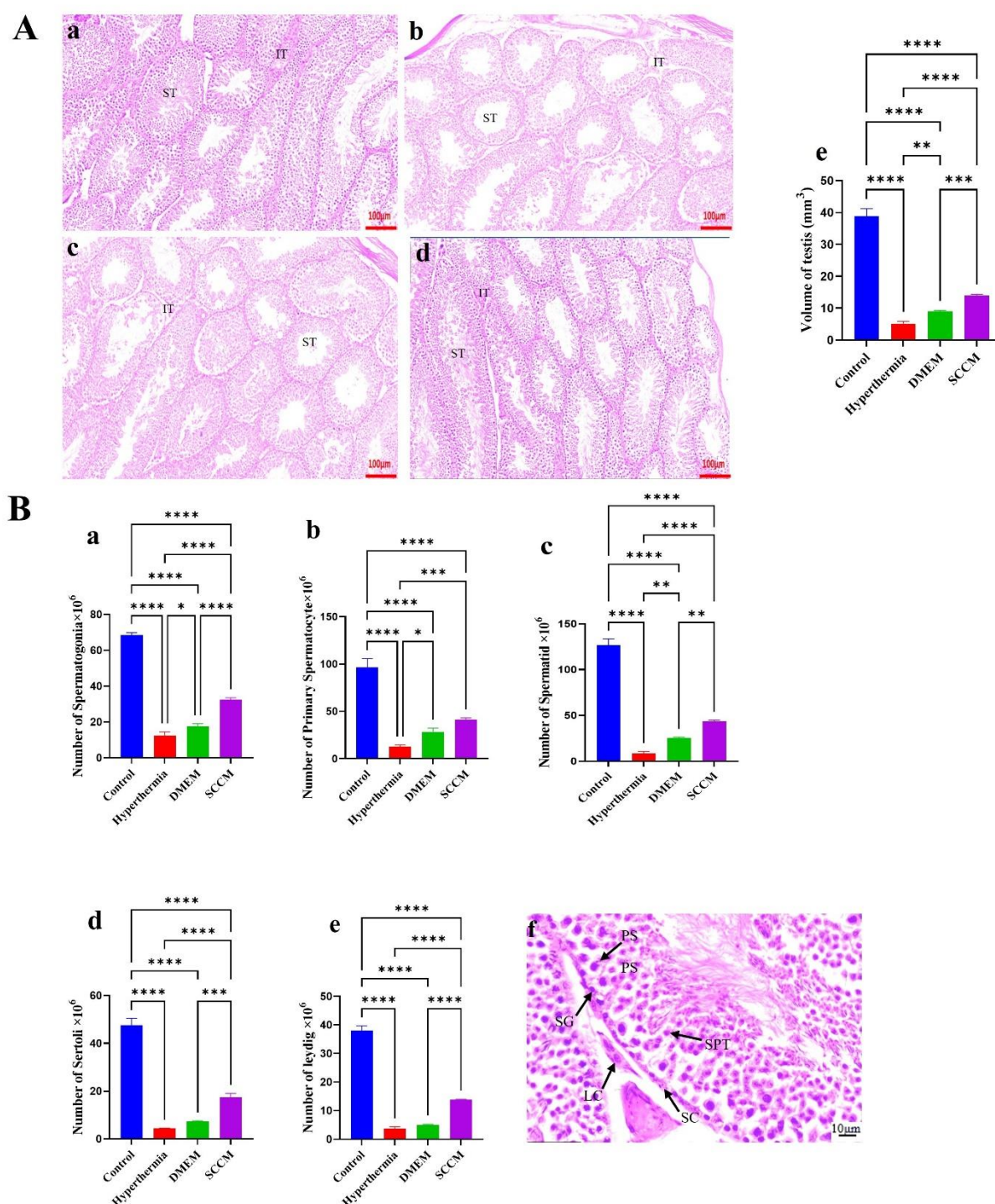


FIGURE 3. (A) a-d Photomicrograph of the testis stained with H&E, $\times 10$. a Control group. b Hyperthermia group. c DMEM group. d SCCM group. Interstitial tissue (IT), Seminiferous tubules (ST). Mean \pm SD of the total volumes of testes in different groups. Groups: 1. Control; 2. Hyperthermia; 3. DMEM; and 4. SCCM. p values < 0.05 (*), p values < 0.01 (**), and p values < 0.0001 (****). (B) a-e Mean \pm SD of the total number of Spermatogonia, primary Spermatocytes, Spermatids, and Sertoli and Leydig cells in study groups. Groups: 1. Control; 2. Hyperthermia; 3. DMEM; and 4. SCCM. p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****). f Photomicrograph of the testis stained with H&E, $\times 40$. SG (spermatogonia), PS (primary spermatocyte), SPT (round spermatid), SC (Sertoli cell), LC (Leydig cell). Scale bar = 10 μ m.

cells into mature adult germ cells. They are essential for germ cell proliferation and maintaining the spermatogonial stem cell population (O'Donnell et al., 2022). Many

studies have shown that the secretion of these cells, including GDNF and FGF 2, can affect the process of spermatogonial stem cell proliferation and differentia-

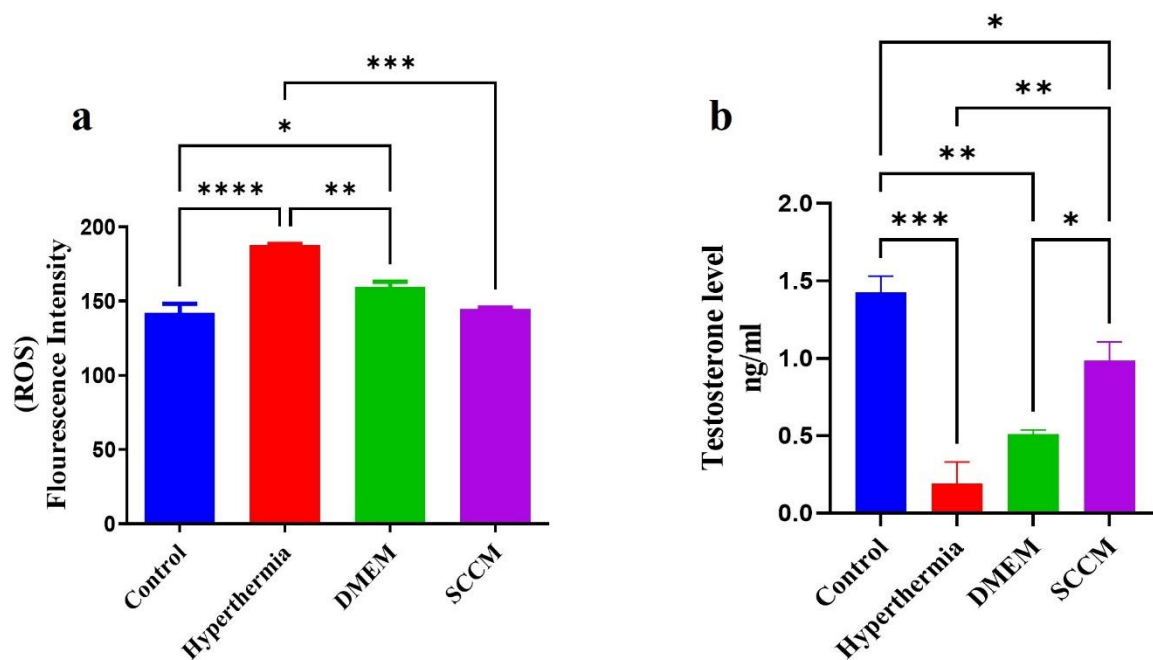


FIGURE 4. (a) Mean \pm SD of ROS generation in testicular tissue across study groups. (b) Mean \pm SD of serum testosterone levels across study groups. Groups: (1) Control; (2) Hyperthermia; (3) DMEM; (4) SCCM. p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****)

tion (Kanatsu-Shinohara et al., 2012; Takashima et al., 2015).

Our research indicated that SCCM can significantly increase the quantity of round spermatids, spermatogonia, primary spermatocytes, Sertoli cells, and Leydig cells compared to the hyperthermia group. Interaction between germ cells and Sertoli cells is essential for spermatogenesis. The differentiation and proliferation of spermatogonial cells, as well as their entry into meiosis, depend on the secretion of growth factors from Sertoli cells. These growth factors regulate the number of germ cells in the seminiferous epithelium (Feng et al., 2014; Khanehzad et al., 2021).

The SCCM group showed significantly more volume improvement than the hyperthermia group in this study. The testis volume improvement may be related to the proliferation of germ cells and the maintenance of spermatogonial stem cells (Panahi et al., 2020). In the research conducted by Rebourcet et al., it was demonstrated that Sertoli cells have the capacity to regulate the creation of seminiferous tubules, the development of Leydig cells, the functioning of peritubular myoid cells, and the survival and growth of germ cells. This study also revealed a fundamental connection between

the quantity of Sertoli cells, the number of Leydig cells, and the overall quantity of germ cells. This relationship is likely a fundamental factor influencing the establishment and development of the adult testis (Rebourcet et al., 2017).

This study showed that serum testosterone levels in the SCCM group were significantly elevated compared to the hyperthermia group. Testosterone is released by Leydig cells in response to LH stimulation. This hormone influences spermatogenesis through androgen receptors (ARs) found in the Sertoli cells (Li et al., 2021).

Wu et al. have demonstrated that Sertoli cells release some growth-promoting factors that stimulate Leydig cells' proliferation (Wu and Muroso 1994). Testosterone synthesis can relate to the Leydig cell population (Monageng et al., 2023). Our stereological study has also confirmed the increase in the Leydig cell population.

Seminiferous epithelium undergoes apoptosis as a physiological process to regulate germ cell numbers. Numerous factors are involved in regulating this apoptosis, including growth factors and cytokines (Fujioka et al., 2001). In this study, the quantity of apoptotic cells in the SCCM group significantly reduced in comparison to the hyperthermia group.

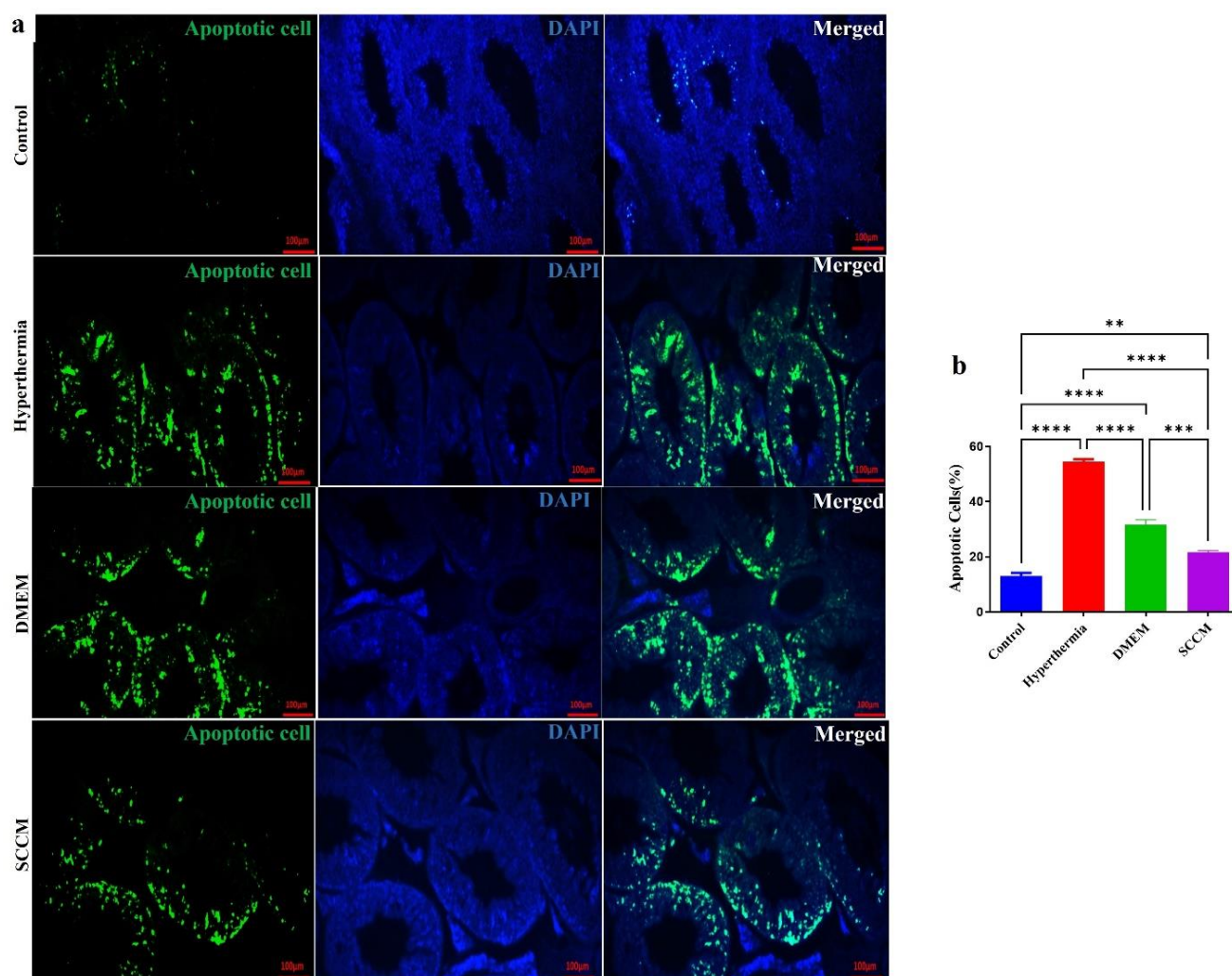


FIGURE 5. (a) TUNEL detection of apoptosis in testis tissue. Photomicrographs show apoptotic cells (green) de-tected by immunofluorescence, with nuclei counterstained by DAPI (blue), across the study groups: (1) Control; (2) Hyperthermia; (3) DMEM; (4) SCCM. (b) Mean \pm SD of apoptotic cell counts in the study groups. p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****).

Sertoli cells produce growth factors, including stem cell factor (SCF) and c-kit. The c-kit receptor of germ cells plays an essential role in primordial germ cell survival and proliferation, as well as spermatogonial adhesion, proliferation, and survival (Hakovirta et al., 1999; Yan et al., 2000). The expression of c-kit in spermatogonia is a marker to determine differentiating spermatogonia and plays essential roles in the proliferation, migration, survival, and apoptosis suppression of the germ cells (Zhang et al., 2011). Moreover, another study has shown that FSH and testosterone maintain spermatogenesis homeostasis by inhibiting germ cell apoptosis (Shaha et al., 2010).

The initiation of meiosis strongly depends on the expression of Stra8, the absence of which can be associat-

ed with sterility in both males and females (Anderson et al., 2008; Endo et al., 2015). In our study, Stra8 expression in the SCCM group was significantly elevated compared to the hyperthermia group. Sertoli cells synthesize retinoic acid (RA), which is essential for spermatogonial differentiation, entry into meiosis, and spermatid elongation (Endo et al., 2017; Helsel and Griswold 2019). This factor can induce Stra8 expression in germ cells (Endo et al., 2015). Shen et al. have shown that high expression of stra8 can inhibit spermatogenic cell apoptosis (Shen et al., 2018).

Moreover, our results showed that the relative mRNA expression of PCNA was significantly increased following SCCM injection. PCNA plays a crucial role in several cell-cycle pathways, including DNA replication,

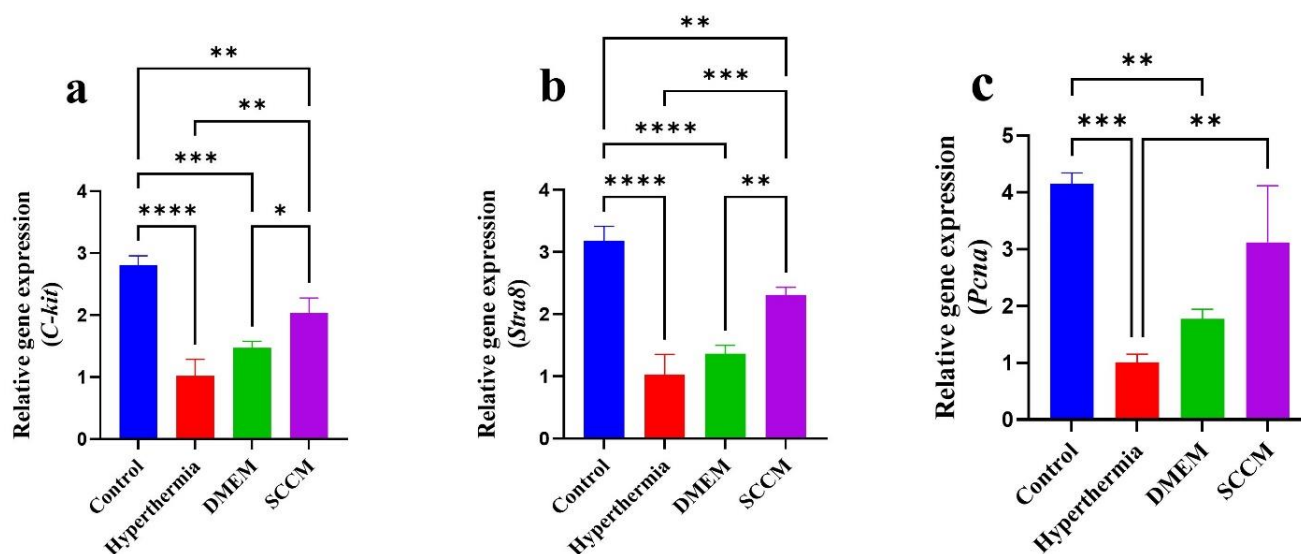


FIGURE 6. (a–c) Mean \pm SD of the relative gene expression of C-kit, Stra8, and Pcnq in testis tissue across the study groups: (1) Control; (2) Hyperthermia; (3) DMEM; (4) SCCM. p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****).

elongation, and apoptosis (Tousson et al., 2011). Studies have shown that SDF-1, a member of the CXC chemokine, is synthesized by Sertoli cells. This factor can increase spermatogonial stem cell markers, including PCNA and TP2, which are important factors in normal sperm morphology (Ghaem Maghami et al., 2018).

Animal studies have shown that low total sperm count and low motility can relate to Sertoli cell dysfunction (Monfared et al., 2016). Moreover, Panahi et al. have also shown that a Sertoli cell-conditioned medium can increase sperm count and motility, which aligns with our results (Panahi et al., 2020).

In various cell types, hypoxia and oxidative stress are essential in the intrinsic apoptosis pathway and cell death. The high level of electron leakage from sperm mitochondria can result in extensive oxidative damage, including DNA damage, lipid peroxidation, and motility loss, as demonstrated in this study (Aitken 2020). However, this study showed that the decrease in oxidative stress could be related to growth factors secreted by Sertoli cells.

Conclusion

In conclusion, our study highlights the essential role of Sertoli cells in spermatogenesis and their potential therapeutic application in azoospermia induced by scrotal hyperthermia. Sertoli cells not only provide structural and functional support to germ cells but also secrete

various growth factors that independently regulate intrinsic factors and hormone synthesis, ultimately inhibiting apoptosis in germ cells. While our findings suggest that spermatogenesis may be restored, future research must explore the specific mechanisms underlying germ cell death and other potential apoptotic pathways. Nonetheless, the use of Sertoli cell-conditioned medium is a promising approach for addressing male infertility associated with scrotal hyperthermia-induced azoospermia. This therapeutic approach holds the potential to unlock new avenues for treating this condition and improving male reproductive health through further investigation.

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Ethics Statement

The authors declare that all experiments protocols were approved by the Ethics Committee, deputy of

research, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.AEC.1402.010). The procedures were conducted following the appropriate guidelines and regulations.

Conflict of Interest

The authors declare that they have no competing interests.

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