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Experimental Research Article

Upregulation of heat shock proteins 70 and 90 induced by transient scrotal hyperthermia in mice

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ABSTRACT

Introduction: It is now accepted that scrotal heat stress could adversely affect spermatogenesis. This high thermal condition can cause a reduced male fertility potential. Nowadays, insufficient research exists on the impact of transient scrotal hyperthermia on heat shock proteins 70 and 90 in murine subjects. In the current study, we investigated the effects of scrotal hyperthermia on the expression of heat shock proteins, stereological parameters, and semen quality in mice.

Methods: In this examination, a total of 18 healthy adult male NMRI mice were divided equally into two groups: control and scrotal hyperthermia. Scrotal heat stress was induced by placing the lower parts of mice bodies into the water bath for three consecutive days (43°C, 20 min/day). Then, epididymis and testicular samples were collected for evaluation of sperm parameters, stereological study, mRNA, and protein expression of HSP70 and HSP90.

Results: Our results revealed that scrotal hyperthermia could strikingly increase the level of mRNA and protein expression of HSP70 and HSP90 in the samples. In addition, stereological parameters and semen quality significantly decreased in transient scrotal hyperthermia-induced mice compared to the control group.

Conclusion: Our research indicates that transient hyperthermia on the scrotum can lead to increased expression of HSP70 and HSP90 at both mRNA and protein levels, subsequently affecting male fertility.

Keywords:
Hyperthermia
Heat Shock Protein
Testis
Male fertility
Heat stress

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Introduction

Infertility is one of the biggest challenges facing medical science and almost 15% of couples suffer from this problem (Abd El-Emam et al., 2023). Based on recent reports, male-related factors are responsible for 20 to 70% of infertility cases (Agarwal et al., 2014). It is well-documented that male infertility is a multifactorial disorder that various systems, such as endocrine, nervous, blood, and immune systems, may affect (Ghuman and Ramalingam 2018; Hasani et al., 2020). It has been proven in multiple studies that elevated testicular temperature can adversely affect the normal functioning of testicular cells such as Sertoli cells, Leydig cells, and spermatogonia, which ultimately could lead to disrupting the spermatogenesis process and decrease semen quality (Bedford 2015; Li et al., 2013; Qiao et al., 2021). Hence, to protect testicular cells from high temperatures, they are located in the scrotum, outside the body cavity (roughly 2-8 °C below core body temperature) (Hess and De Franca 2009; Ilkha ni et al., 2020).

A bulk of investigations have revealed job or lifestyle-related factors that could increase scrotal temperatures, such as driving, sitting, or cycling for a long time, sauna use, and wearing tight clothes that could induce adverse effects on male fertility (Aldahhan and Stanton 2021; Khorsandi et al., 2013; Setchell 2018). Furthermore, several studies have also reported the destructive impacts of thermal stress on normal spermatogenesis following various pathophysiological situations, including Varicocele, Cryptorchidism, and Fever (Aldahhan and Stanton 2021; Durairajanayagam et al., 2014).

The process of transforming spermatogonia into spermatid is called spermatogenesis (Sharma and Agarwal 2011). This process is highly complex, regulated, and temperature-dependent and occurs in the mammalian species' testes after puberty (Hou et al., 2015; Panggalih et al., 2021). There is growing evidence illustrating that high scrotal temperatures can adversely affect various stages of spermatogenesis (Aldahhan and Stanton 2021). Consistent with the results of previous studies, the researchers suggested that scrotal hyperthermia could cause various histological and molecular changes in testicular cells (germ cells, Leydig cells, Sertoli cells), including increased germ cell apoptosis, blood-testis barrier disruption, sperm DNA damage, ROS production, autophagy-related genes, and mitochondrial dysfunction (Asadi et al., 2017; Bozhedomov et al., 2013; Panggalih et al., 2021). In addition, experimental studies have shown that sperm quality and quantity decrease after scrotal heat stress (Jerng et al., 2014; Zhu et al., 2004). Therefore, the researchers proposed that hyperthermia of the scrotum is one of the most critical factors in male infertility (Abd El-Emam et al., 2023).

Chaperons and co-chaperons are a group of proteins that assist other proteins in folding into the correct three-dimensional structure, which is essential for normal cell functions. They are expressed in different stressful environmental situations, such as inflammation, oxidative stress, infection, starvation, and heat stress (Bohush et al., 2019; Shen et al., 2019). A body of literature now shows that heat shock factor-1 and heat shock proteins (HSP70, HSP90) are known as chaperon proteins. They act similarly to chaperons and are upregulated in response to heat stress, which can inhibit protein denaturation in various cells (Bohush et al., 2019; Jha et al., 2013). Hence, investigators proposed that the expression of these factors could reduce the destructive effects of heat stress on normal cell functions by suppressing P-53 activity as one of the critical regulators of apoptosis (Gu et al., 2015; Rizzoto et al., 2020). Besides, based on recent studies, researchers indicated that HSP families play an essential role in regulating testis-specific serine/threonine kinases (TSSKs) in germ cells, one of the crucial factors in male fertility, by affecting their stability and activity (Jha et al., 2013). In addition, another paper illustrated evidence that HSPs have an essential role in the meiotic division of male germ cells (Grad et al., 2010). Nowadays, there is limited data regarding the effect of transient scrotal hyperthermia on HSP 70 and 90 in mice. In this study, we investigated the effects of scrotal hyperthermia on the expression of heat shock proteins, stereological parameters, and semen quality in mice.

Material and Methods

Animals

In this investigation, we utilized 18 healthy male NMRI mice with equal weight (15-20g) and 3 weeks of age. All experimental animals were purchased from the Pasteur Institute (laboratory animal center) in Tehran, Iran. Throughout the entire study process, animals were sustained in individual cages, provided with unrestricted access to water and food, and maintained under standard laboratory conditions. Then, healthy animals were

randomly and equally divided into control and scrotal hyperthermia. All experimental procedures in this study were assessed and approved by the Ethics Committee (IR.SBMU.MSP.REC.1400.1169).

Transient scrotal hyperthermia model

In the scrotal hyperthermia group, to induction heat stress, firstly, animals were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) administration (i.p). The lower body regions of the mice, including the scrotum and hind legs, were immersed in a water bath for three days in a row (43°C, 20 min/day). After that, the animals were dried and reverted to their cages. Additionally, the animals in the control group were administered anesthesia and were maintained at room temperature(Khosravi et al., 2021).

sperm Analysis

To collect sperm samples, epididymal tails were scraped and placed in 1 mL of Ham's F-10 medium (Sigma-Aldrich Product No. N6635). A 20-minute incubation period was then conducted at 37°C. Next, 10 µ of the sample was placed on a slide and examined under an inverted microscope for sperm motility. In this study, approximately 100 sperm were tallied in each counting chamber. The evaluation of sperm motility adhered to the criteria outlined by WHO (1999; 2010), encompassing a) progressive motility b) non-progressive motility, and c) no observable movement. A counting chamber was used to measure the sperm count. To assess sperm viability and morphology, sperm samples were stained with Eosin Nigrosine (Aghajanpour et al., 2024).

Sampling and Tissue Preparation

At the study's endpoint, all animals were deeply anesthetized by administration of ketamine and xylazine. Then, both testicles were extracted for histological (left testis) and molecular (right testis) examinations. For the histological goal, all samples were fixed by Bouin's solution for 48 h. After that, the routine histological passage was carried out, and paraffin blocks were obtained. Serial sections (5 µm thickness) were made by using a microtome and were placed on the poly-l-lysine coated slides. The right testis was immediately transferred to RNA protecting solution (RNA-later) and stored at -80°C for molecular evaluations(Tabatabaee et al., 2024).

Counting of Testicular Cells

The quantity of testicular cells was determined using the optical dissector method equation(Gundersen et al., 1988; Howard et al., 1992). The formula employed for this calculation was $Nv = \sum Q / \sum P \times h \times a/f \times t \times BA$, where ΣQ represents the quantity of testicular cells, h denotes a microcator connected to the microscope stage for measuring dissector height, ΣP is the total number of fields counted, a_f is the probe area divided by the magnification, BA is the thickness of the tissue section, and t represents the actual thickness of the tissue section. N (total) = Nv × V (final).

Immunofluorescence Assay

We utilized immunofluorescence (IF) staining to evaluate the distribution of HSP70 and HSP90 proteins in testicular specimens. To achieve this purpose, briefly, all slides were dewaxed, rehydrated, and endogenous peroxidase blocked. Subsequently, testicular sections were incubated with the primary antibodies against HSP70 (SC-80607, Santa Cruz Biotechnology) and HSP90 (orb67311, Biorbyt) for 30 min at room temperature. After that, slides were washed with Tris-buffered saline (TBS; 0.1 M Tris-HCl, pH 7.4 and 0.9 NaCl) and then incubated again with secondary antibodies (HSP70: orb688925, HSP90: orb688924, Biorbyt) at room temperature for 2 hours. Finally, we used 40,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) for nuclear counterstained, then all sections were mounted (Im et al., 2019).

Molecular assessment

To explore HSP70 and HSP90 gene expression Real-time PCR technique was performed based on the method explained formerly. In summary, after total RNA extraction from collected testicular samples, to eliminate genomic contamination, DNase I (Roche, Basel, Switzerland) was used. After that, cDNA was synthesized by the commercial kits (Fermentas, Lithuania) according to the manufacturer's instructions. Real-time PCR (TaqMan) was conducted following the protocol of the QuantiTect SYBR Green RT-PCR kit (Takara Bio Inc., Japan) for the quantification of relative gene expression. Primer sets (both forward and reverse) were designed using the exon-exon junction method with sequences sourced from the NCBI database, utilizing the Primer 3 Plus software. The specificity of the primers

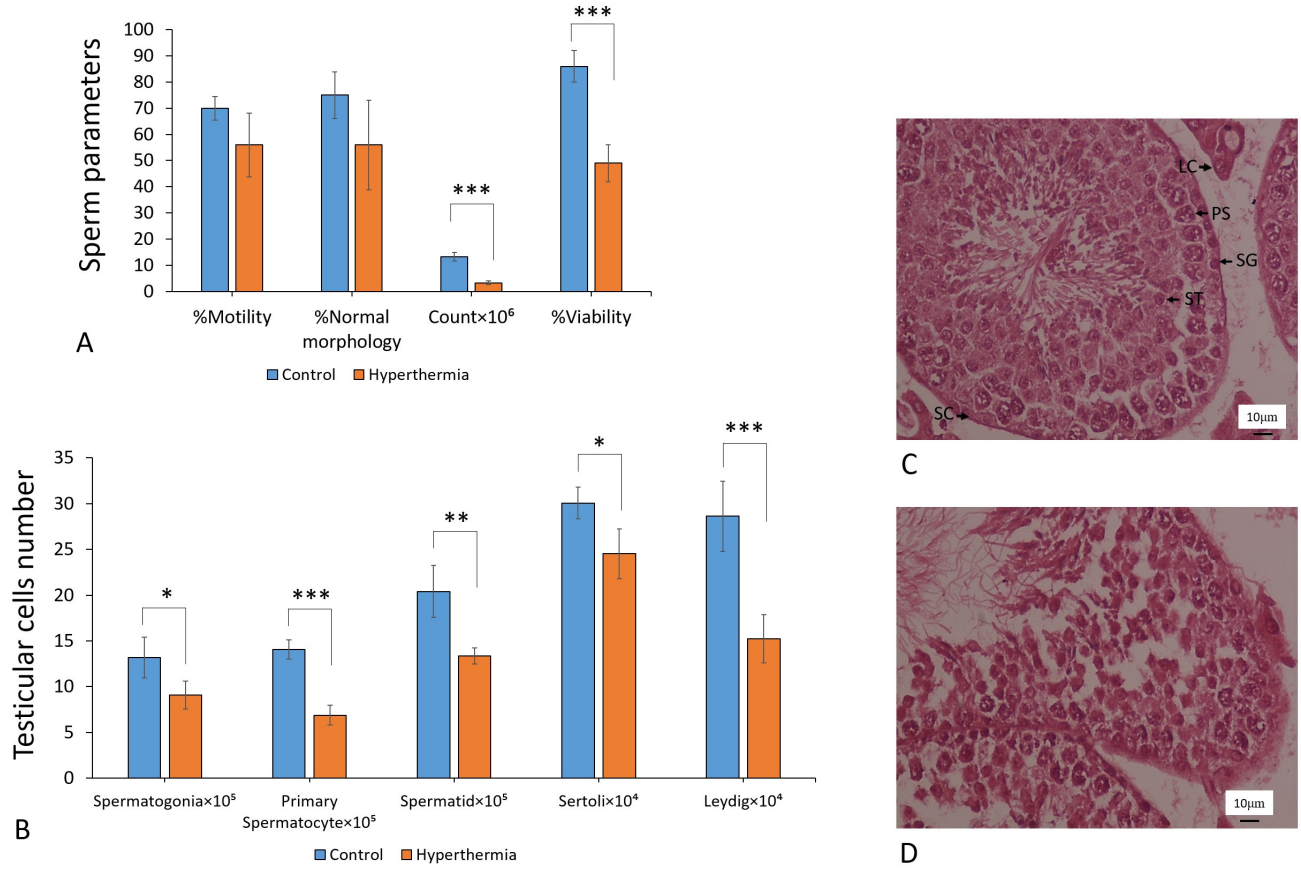

FIGURE 1. (A) Mean ± SD of the total sperm count, sperm motility, sperm viability, and sperm normal morphology in the all-experimental groups .p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****)
(B) Mean ± SD of the total number of Spermatogonia, primary Spermatocytes, Spermatids, and Sertoli and Leydig cells in the all-experimental groups. p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****).
(C,D) Photomicrograph of the testis stained with H&E, × 40. SG (spermatogonia), PS (primary spermatocyte), ST (round spermatid), SC (Sertoli cell), LC (Leydig cell). Scale bar = 10 µm.

was validated through the Primer-Blast tool, accessible on the website www.ncbi.nlm.nih.gov/tools/primerblast (Table. 1) (He et al., 2016).

Statistical analysis

In this research, we utilized GraphPad Prism Version 9 for all statistical analysis. All data were expressed as means ± *standard deviation* (Mean±SD), and an Unpaired t-test was performed. Significant differences were determined at $P \leq 0.05$.

Results

Changes in Sperm Parameters Induced by Heat Stress

The total sperm quantity was remarkably lower in the hyperthermia group compared to the control group ($P < 0.001$; Figure 1). Moreover, the percentage of sperm viability was also significantly lower in the hyperthermia group compared to the control group ($P< 0.001$; Figure 1). However, there was no significant difference in sperm motility and sperm morphology between the study groups (Figure 1).

Changes in Stereological Parameters Induced by Heat Stress

As indicated by stereology results, the quantity of testicular cells exhibited a significant reduction in the hyperthermia group compared to the control group (Figure 1B). Mice in the control group displayed intact germinal epithelium, whereas the hyperthermia group showed degenerative alterations in the seminiferous tubules, leading to impaired spermatogenesis (Figure 1C and D).

Increased in HSP70 and HSP90 Protein Expression Induced by Heat Stress

We performed IF staining to evaluate the amount of HSP70 and HSP90 protein expression in testicular sam-

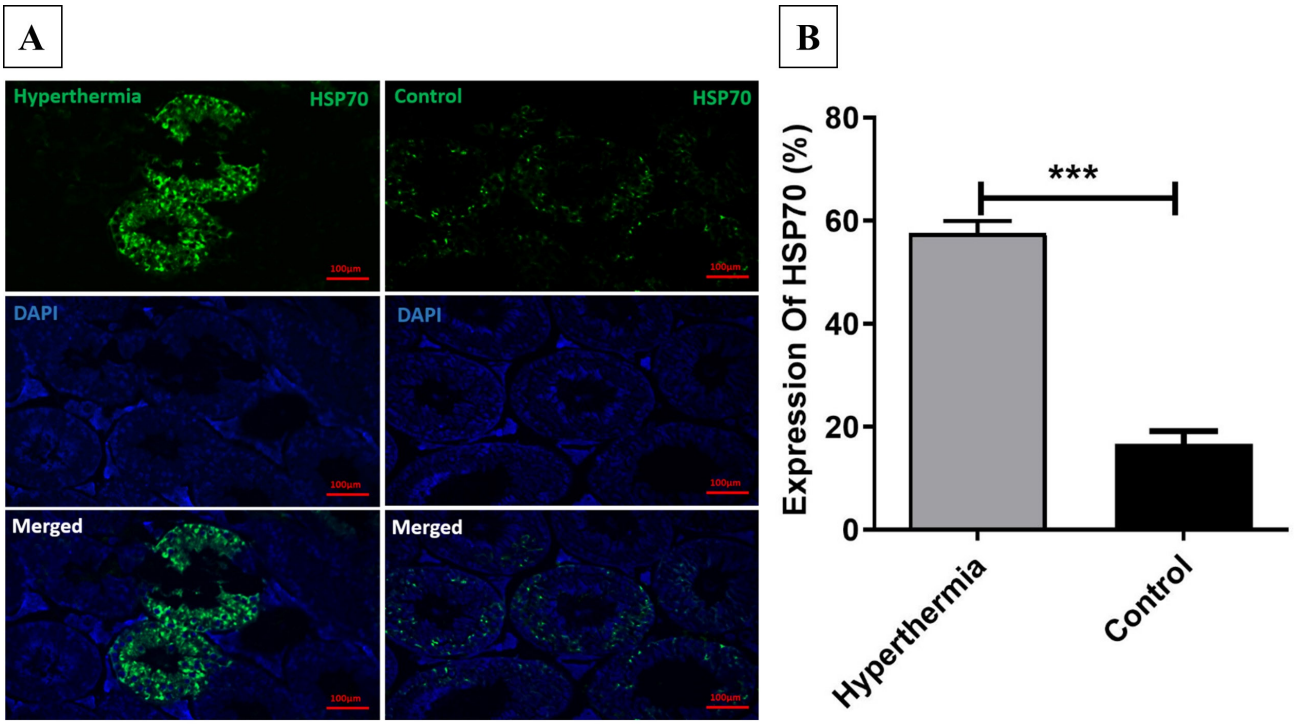

FIGURE 2. (A) Immunofluorescence staining against HSP70 proteins in the all-experimental groups. Scale bare: 100 µm. (n= 9, in each group). (B) The effects of heat stress on HSP70 proteins in testicular samples. (Mean ± SD). p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****).

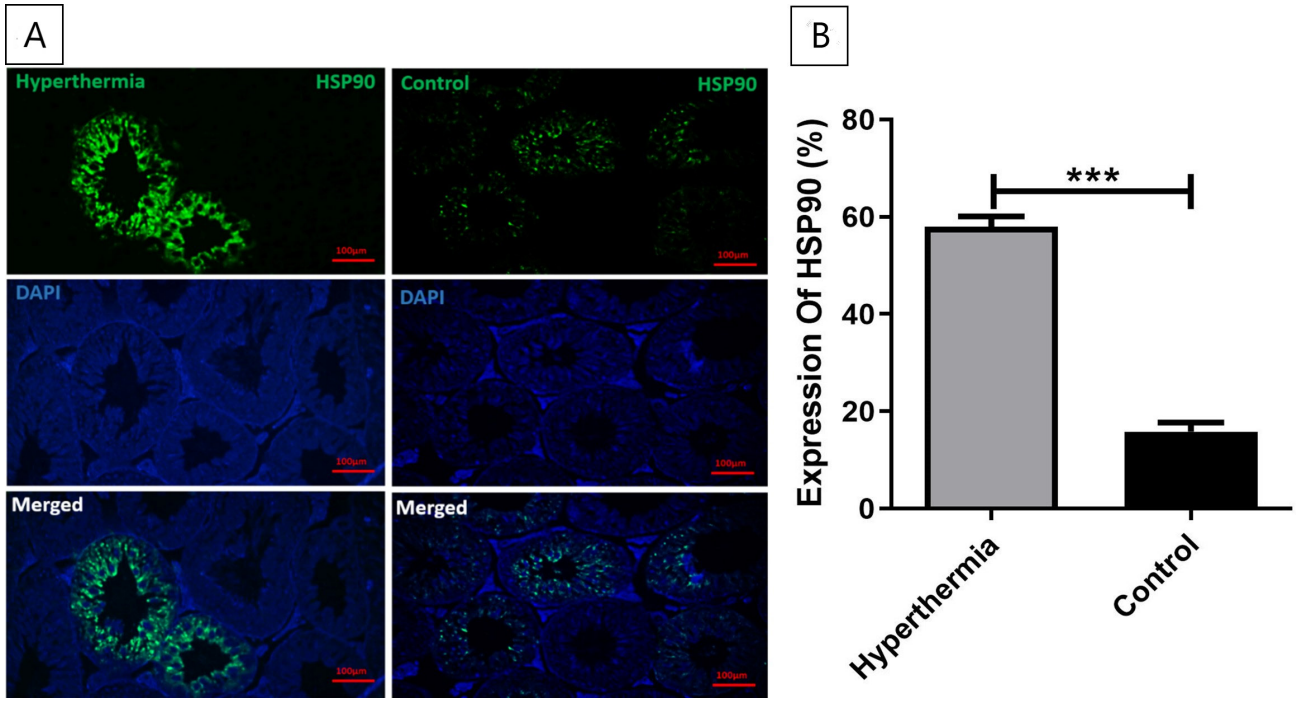

FIGURE 3. (A) Immunofluorescence staining against HSP90 proteins in the all-experimental groups. Scale bare: 100 µm. (n= 9, in each group) (B) The effects of heat stress on HSP90 proteins in testicular samples. (Mean ± SD). p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****).

ples. According to our findings, there was a dramatic elevation in the protein expression of HSP70 in the hyperthermia group compared to the control groups ($p<0.001$) (Figures 2 and 3). In addition, as illustrated, similar to

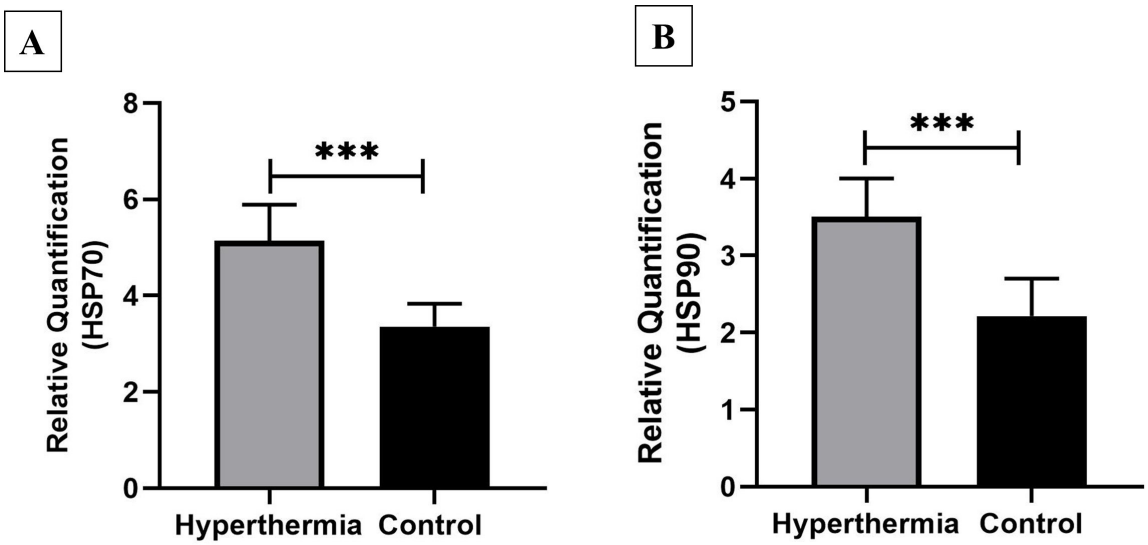

FIGURE 4. Graphs illustrate effects of hyperthermia on mRNA level of HSP70 and HSP90 in both experimental groups (n= 9, in each group). (Mean ± SD). p values < 0.05 (*), p values < 0.01 (**), and p values < 0.001 (***), p values < 0.0001 (****).

HSP70, the level of HSP90 protein expression also showed a considerable increase in animals that received heat stress compared to the control group ($p<0.001$) (Figures 2 and 3).

Increased in HSP70 and HSP90 mRNA Expression Induced by Heat Stress

The effect of heat stress on testicular mRNA levels of HSP70 and HSP90 was assessed. As depicted in Fig 4, the level of both HSP70 and HSP90 mRNA expression substantially increased in the hyperthermia group compared to the control group ($p<0.001$).

Discussion

It is now well-documented that spermatogenesis is a tightly regulated process strongly sensitive to heat stress(Hirano et al., 2022; Kong et al., 2000). Therefore, the male gonads are located in the scrotum, which provides an appropriate temperature to ensure normal spermatogenesis (Einer-Jensen and Hunter 2005). The results of several studies have confirmed that heat stress has various negative impacts on spermatogenesis, including structural and molecular changes in testicular tissue and cells that can lead to impaired male fertility (Abd El-Emam et al., 2023; Aldahhan and Stanton 2021; Gu et al., 2015; Hasani et al., 2020; Ilkhani et al., 2020). Therefore, to mitigate the harmful effects of temperature increase on spermatogenesis, understanding the mechanisms by which heat stress affects normal testes functions is essential.

This study aimed to investigate the impact of scrotal hyperthermia on various aspects of male reproductive function, including sperm parameters, histopathological patterns, and the expression levels of HSP70 and HSP90 genes and proteins. Our findings revealed significant reductions in sperm quantity, sperm viability, and the number of testicular cells. Additionally, scrotal heat stress significantly increased the expression of mRNA and protein levels of HSP70 and HSP90. These findings strongly suggest that scrotal hyperthermia has detrimental effects on male reproductive health, affecting both sperm quality and the expression of crucial heat shock proteins.

Recent studies have described the effects of scrotal heat stress on structural and cellular changes in the testicles(Aldahhan and Stanton 2021; Setchell 2018). For instance, Ilkhani et al. (2020) assessed structural alterations in the testes using an animal hyperthermia model and found that scrotal hyperthermia not only causes a dramatic reduction in the total volume of testicles and interstitial tissue but also significantly diminishes the number of germ cells and somatic cells. Other studies have reported that the length and lumen diameter of seminiferous tubules showed a marked decrease due to scrotal hyperthermia (Hasani et al., 2020). Additionally, it is well-documented that sperm parameters and serum testosterone levels significantly dwindle in mice subjected to scrotal heat stress (Khosravi et al., 2021;

Moscatelli et al., 2019; Setchell 2018).

Previous studies have also illustrated that following hyperthermia, blood-testis barrier (BTB) integrity and the spatial arrangement of Leydig and Sertoli cells are disrupted, leading to inadequate support for germ cells and causing cell death and impairment in the normal spermatogenesis process (Hu et al., 2021; Ilkhani et al., 2020). Furthermore, transient scrotal hyperthermia can induce mitochondrial dysfunction, which leads to a significant elevation in the synthesis of reactive oxygen species (ROS) in the scrotal cells (Gundersen et al., 1988; Hasani et al., 2020; Qiao et al., 2021). Oxidative stress is suggested to promote apoptosis and DNA damage of testicular cells, which has destructive effects on spermatogenesis (Aldahhan and Stanton 2021; Gu et al., 2015; Kanter et al., 2013).

The molecular mechanisms underlying testicular heat stress involve several pathways. Heat stress activates the hypothalamic-pituitary-gonadal axis, leading to altered hormonal levels and impaired spermatogenesis (Durairajanayagam et al., 2014).

Specifically, heat stress reduces luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels, which are crucial for the stimulation of testosterone production and spermatogenesis, respectively. Additionally, androgen receptors are affected by heat stress. Shen et al. (2019) found that heat stress markedly elevates mRNA and protein levels of androgen receptors and HSP70 in boars' testes. HSP70 may enhance androgen receptor inhibition, disrupting normal spermatogenesis, reducing sperm quality, and decreasing androgen sensitivity of testicular cells.

HSP70 and HSP90 are expressed in response to heat stress in various cells and can act as chaperone proteins(Bohush et al., 2019; Jha et al., 2013). These proteins help prevent protein denaturation during heat stress(Chen et al., 2008; Shen et al., 2019; Zhao et al., 2010) and play pivotal roles in cell cycling and meiosis in testicular germ cells. Gu et al. (2015) found that increased HSP70 due to heat stress could suppress programmed cell death by affecting the TrP53 gene. Pei et al. (2012) indicated that heat stress increased the expression levels of HSP60, HSP70, and HSP90 in rabbits, although the localization patterns did not change. Similar to previous studies, our results also exhibited a significant elevation in the mRNA and protein expression levels of HSP70 and HSP90 due to transient scrotal hyperthermia.

In the context of stereological parameters, heat shock proteins such as HSP70 and HSP90 have been shown to play a role in maintaining the structural integrity of testicular tissue. Their increased expression in response to heat stress helps in stabilizing and refolding denatured proteins, thus protecting the cells from stress-induced damage. This protective mechanism, however, is not entirely sufficient to prevent the reduction in the volume and number of testicular cells observed in our study and others. The disruption of the BTB and the spatial arrangement of Leydig and Sertoli cells further exacerbates the detrimental effects, leading to compromised spermatogenesis and reduced semen quality.

There are several limitations to our study that must be acknowledged. First, our study was conducted on a mouse model, which may not fully replicate the complexities of human reproductive physiology. Second, the duration and intensity of heat exposure in our experimental design were chosen based on preliminary studies; different parameters might yield varying results. Third, while we measured the expression levels of HSP70 and HSP90, other heat shock proteins and molecular markers may also play significant roles and were not investigated in this study. Lastly, the long-term effects of transient scrotal hyperthermia on reproductive health were not assessed, which could be crucial for understanding the full impact of heat stress.

Conclusions

Given the importance of infertility issues in men and the significant changes in lifestyles and working conditions, the possibility of disrupting sperm production due to scrotal hyperthermia is a growing concern. Extensive research is being conducted in this field. The results of our study showed that transient increases in scrotal temperature in mice significantly increased the production of heat shock factors, including HSP70 and HSP90. This increase likely represents a defense mechanism of the male reproductive system against transient scrotal hyperthermia. However, the upregulation of these heat shock proteins, while protective to some extent, is insufficient to fully mitigate the adverse effects on spermatogenesis and semen quality. Understanding the precise molecular mechanisms, including the role of androgen receptors and the detailed pathways by which HSP70 and HSP90 exert their effects, is crucial for develop-

ing targeted interventions to protect male reproductive health from the effects of heat stress.

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Conflict of interest

The authors declare that they have no competing interests.

Ethics approval

All experimental procedures in this study were reviewed and approved by the Ethics Committee at Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1400.1169).

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