

Caffeine attenuates spermatogenic disorders in mice with induced chronic scrotal hyperthermia

Amir Raoofi¹, Omid Gholami¹, Hossein Mokhtari², Fatemeh Bagheri³, Auob Rustamzadeh⁴, Davood Nasiry², Alireza Ghaemi⁵

¹Cellular and Molecular Research Center, Sabzevar University of Medical Sciences, Sabzevar; ²Department of Paramedicine, Amol School of Paramedical Sciences, Mazandaran University of Medical Sciences, Sari; ³Student Research Committee, Sabzevar University of Medical Sciences, Sabzevar; ⁴Department of Anatomy, Faculty of Medicine, Iran University of Medical Sciences, Tehran; ⁵Department of Basic Sciences and Nutrition, Health Sciences Research Center, Faculty of Public Health, Mazandaran University of Medical Sciences, Sari, Iran

Objective: Chronic scrotal hyperthermia (SHT) can lead to serious disorders of the male reproductive system, with oxidative stress playing a key role in the onset of these dysfunctions. Thus, we evaluated the impact of caffeine, a potent antioxidant, on cellular and tissue disorders in mice with chronic SHT.

Methods: In this experimental study, 56 adult male NMRI mice were allocated into seven equal groups. Apart from the non-treated control group, all were exposed to heat stress. Two groups, termed “preventive” and “curative,” were orally administered caffeine. The preventive mice began receiving caffeine immediately prior to heat exposure, while for the curative group, a caffeine regimen was initiated 15 consecutive days following cessation of heat exposure. Each treated group was subdivided based on pairing with a positive control (Pre/ curative [Cur]+PC) or a vehicle (Pre/Cur+vehicle). Upon conclusion of the study, we assessed sperm characteristics, testosterone levels, stereological parameters, apoptosis, antioxidant and oxidant levels, and molecular markers.

Results: Sperm parameters, testosterone levels, stereological parameters, biochemical factors (excluding malondialdehyde [MDA]), and *c-kit* gene expression were significantly elevated in the preventive and curative groups, especially the former, relative to the other groups. Conversely, expression levels of the heat shock protein 72 (*HSP72*) and nuclear factor kappa beta (*NF-κβ*) genes, MDA levels, and apoptotic cell density were markedly lower in both caffeine-treated groups relative to the other groups, with more pronounced differences observed in the preventive group.

Conclusion: Overall, caffeine attenuated cellular and molecular abnormalities induced by heat stress in the testis, particularly in the mice treated under the preventive condition.

Keywords: Apoptosis; Caffeine; Chronic scrotal hyperthermia; Inflammation; Oxidative stress

Received: May 13, 2023 · Revised: July 20, 2023 · Accepted: September 19, 2023
Corresponding author: **Davood Nasiry**
Department of Paramedicine, Amol School of Paramedical Sciences,
Mazandaran University of Medical Sciences, Sari, Iran
Tel: +98-1144026241 Fax: +98-1144026241 E-mail: davood1990nasiry@gmail.com

Co-corresponding author: **Alireza Ghaemi**
Department of Basic Sciences and Nutrition, Health Sciences Research Center,
Faculty of Public Health, Mazandaran University of Medical Sciences, Sari, Iran
Tel: +98-1144026241 Fax: +98-1144026241 E-mail: ghaemi1387@yahoo.com

*This work was supported by Sabzevar University of Medical Sciences in Sabzevar, Iran (Grant no: 400025).

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

Infertility is a multifaceted disorder with substantial medical, psychological, and economic implications [1]. It is particularly prevalent due to male factors, with the environment playing a major role [2,3]. In various mammalian species, a relatively low temperature is necessary for the normal progression of spermatogenesis and sperm fertility [4,5]. Research has demonstrated that the scrotal temperature in most mammals is 2 to 8 °C lower than the internal body temperature [4]. Heat damage to the testes is a primary cause of infertility in men and most mammals [5,6]. Even brief exposure of the testes to heat can halt spermatogenesis, although the induced damage is typically reversible within approximately 73 days [7].

Apoptosis of testicular germ cells plays a crucial role in both testicular development and the destruction of germ cells under physiological and pathological conditions [8,9]. Prior research has demonstrated that hyperthermia triggers cell death in the testis, leading to a noticeable death of primary spermatocytes and spermatogonia [10-12]. Various drugs, with distinct mechanisms, have been employed to mitigate the impact of heat stress on spermatogenic cells in testicular tissue. Concurrently, the use of antioxidants to counteract these adverse effects has been explored [13,14].

Caffeine (1,3,7-trimethylxanthine) is an alkaloid found in the leaves, seeds, or nuts of numerous plants. It is consumed daily by a large number of people worldwide, primarily due to its presence in tea and coffee [15]. Research has demonstrated that in testicular germ cells, uncoupling protein 2 reduces apoptosis caused by hyperthermia [16]. Caffeine has been found to upregulate the expression of this factor [17]. During hyperthermia, endonuclease enzymes are activated, leading to the fragmentation of DNA in germ cells into low-molecular-weight chains. This endonuclease-induced DNA cleavage is recognized as a sign of apoptosis in testicular germ cells, particularly in pachytene spermatocytes and spermatids [9,18]. One potential mechanism through which hyperthermia impacts cells involves heat shock proteins [19]. These proteins are found in various cellular species under normal conditions, but their levels markedly rise under hyperthermic conditions [19-21]. Heat shock proteins prevent other proteins from denaturing and accumulating in cells that are exposed to changes such as high temperature, inflammatory mediators, or infection [21-23]. As the temperature in the testicular tissue rises, the level of lipid peroxidation in germ cells increases, and antioxidant enzyme activity is concurrently compromised [24]. Antioxidant enzymes like catalase (CAT) have been shown to provide significant protection against apoptosis induced by heat or oxidative stress by neutralizing hydrogen peroxide [25-28]. Given the pathogenesis of testicular hyperthermia and the potent free radical scavenging capacity of caffeine, we conducted this study to explore the clinical benefits and molecular mechanisms underlying the use of caffeine in a chronic scrotal hyperthermia (SHT) model.

Methods

1. Ethical approval

The Ethics Committee of Sabzevar University of Medical Sciences in Sabzevar, Iran approved all experimental protocols (Ethic No.: IR.MEDSAB.REC.1400.070). Animals were handled in accordance with the protocol of animal management and welfare of the University of Helsinki, Finland. The animals were housed in standard laboratory hutches and given unrestricted access to rodent food and drinking water. All procedures were conducted in accordance with relevant

guidelines and regulations. The study adhered to the Animal Research: Reporting of *In Vivo* Experiments guidelines. Unless otherwise specified, all materials used in this study were procured from Sigma-Aldrich.

2. Animals and experimental design

In this experimental study, a total of 56 adult male NMRI mice (each weighing 25 to 30 g) were obtained from the Pasteur Institute in Mazandaran, Iran. The animals were housed and fed according to the ethical standards of Sabzevar University of Medical Sciences. The mice were divided into seven groups (with $n=8$ for each group). One group was an untreated control. The remaining mice underwent SHT induction and were randomly assigned to two primary groups: preventive and curative. Each of these was further divided into three subgroups. The preventive subgroups included preventive, in which mice received caffeine starting 2 hours prior to SHT induction and continuing until the end of the 5th week; the positive control for the preventive group (Pre+PC), which did not receive any treatment; and Pre+vehicle, which received distilled water (used as a caffeine solvent) in the same volume and at the same times as the preventive group. The curative subgroups included curative, which received caffeine daily for 15 days following the SHT induction period; the positive control for the curative group (Cur+PC), which did not receive any treatment; and Cur+vehicle, which received distilled water in the same volume and at the same times as the curative group. Given that the damaging effects of heat stress on sperm parameters and DNA become apparent 14 days after the stress, we set the treatment duration at 15 days [29,30]. Figure 1 illustrates the overall study timeline for the experimental groups. We selected the caffeine dose (10 mg/kg) based on previous studies and our pilot experiments [31]. Caffeine was administered via oral gavage, dissolved in 20 μ L of distilled water. At the end of the experiment, the animals were anesthetized with an intraperitoneal injection of ketamine and xylazine. First, blood was collected via cardiac puncture to evaluate testosterone levels. Immediately afterward, the scrotum was opened, and the tails of the epididymis were isolated for semen analysis. Finally, both testicular tissues were harvested. The right testicular tissue was used for stereological and immunohistochemical evaluation, while the left was utilized for biochemical and molecular testing.

3. SHT model

Chronic SHT was induced in accordance with the study conducted by Ziaepour et al. [32]. To achieve this, the animals were anesthetized through the intraperitoneal administration of ketamine (100 mg/kg) and xylazine (5 mg/kg). Following the induction of SHT, the lower third of the mouse, encompassing the scrotum, tail, and legs, was submerged in a water bath at 43 °C for 20 minutes on alternate

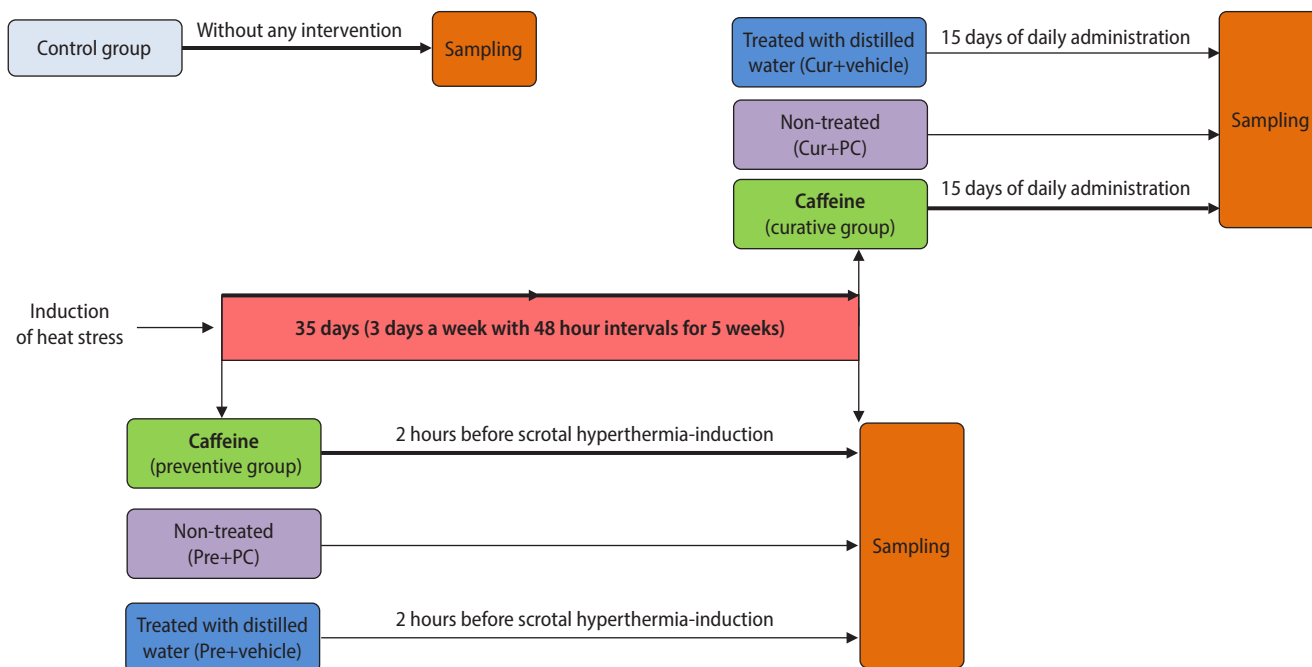


Figure 1. Overall study timeline for the experimental groups. This schematic shows the time points of scrotal hyperthermia induction, caffeine administration, and sampling. Boxes of the same color indicate similarities in the type of intervention between subgroups of the preventive and curative treatment groups. Cur, curative; PC, positive control.

days over a period of 5 weeks.

4. Testosterone evaluation

The collected blood was subjected to centrifugation at $2,500 \times g$ for 15 minutes, resulting in the isolation of blood serum. Testosterone levels were determined using an enzyme-linked immunoassay kit. For this procedure, the samples were introduced into 96-well microplates, and the absorbance was measured at 640 nm using a microplate reader (Biocompare; Agilent Technologies) [33]. All samples were analyzed in triplicate.

5. Semen analysis

After the semen samples were isolated from the tails of the epididymis, they were immediately transferred to 1 mL of Ham F-10 medium and incubated for 20 minutes at 37°C . To obtain the sperm count, sperm suspensions were pipetted and filtered through $80\text{-}\mu\text{m}$ nylon mesh to eliminate tissue fragments. A 0.05-mL aliquot from the 1 mL of sperm suspension was diluted with 1:40 phosphate-buffered saline (PBS, pH 7.2) and thoroughly mixed. A sample of this diluted sperm suspension was then placed into a counting chamber (Sperm Meter; Sperm Processor) after discarding the initial few drops of suspension. The total sperm count was determined by counting the sperm in eight 1-mm^2 squares and multiplying the result by 5×10^6 to estimate the total number of sperm per epididymis [34]. To

assess sperm motility, $10\ \mu\text{L}$ of the sample was separated and examined under an inverted microscope. Sperm viability was evaluated using eosin-nigrosin staining. Additionally, sperm morphology was assessed using a Diff-Quik staining kit [35].

6. Histological and stereological evaluation

At the conclusion of the study, each animal was sacrificed, and the right testicle was extracted and preserved in 10% formalin. Following tissue processing and paraffin embedding, 10 evenly spaced sections were selected and stained with hematoxylin and eosin. The Johnsen test was employed for histological evaluation of the tissue structure [33]. Stereological evaluations included the total volume of testicular tissue, the length density of seminiferous tubules, and the numerical densities of various cells, including spermatogonia, primary spermatocytes, spermatids, Sertoli cells, and Leydig cells [36,37]. To evaluate the tissue structure and total volume, $5\text{-}\mu\text{m}$ -thick sections were prepared, while for the other measurements, $20\text{-}\mu\text{m}$ -thick sections were used.

1) Testicular tissue structure

After the selection and staining of tissue sections, photographs were taken of five distinct areas in each section at a magnification of $\times 400$. These areas included the upper and lower right and left corners, as well as the center of the tissue. Subsequently, the Johnsen

scoring system was utilized to evaluate the degree of tissue structure damage. This scoring system assigns a minimum score of 1 and a maximum score of 10, which correspond respectively to the absence of any epithelium in the seminiferous tubules and the completion of spermatogenesis along with the presence of numerous spermatozoa [33].

2) Total testicular volume

The Cavalieri method was employed for the determination of volume. To this end, all 10 sections obtained from each mouse were superimposed onto a grid of points. Subsequently, to ascertain the volume, all points overlaying each section were counted and analyzed using the following equation.

$$V_{total} = \Sigma P \times \frac{a}{p} \times t$$

Here, ΣP represents the total number of points counted, a/p (in mm^2) indicates the area associated with each square formed between four points, and t (in mm) denotes the distance between the selected sections, as perceived.

3) Numerical density of cells

To determine the numerical densities (N_v values) of spermatogonia, primary spermatocytes, spermatids, Sertoli cells, and Leydig cells, we employed the optical dissector method. This involved using a standard probe, positioned on a monitor connected to a microscope. We examined tissue sections at $\times 400$ magnification and calculated the numerical density using the following formula.

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

Here, ΣQ denotes the total number of nuclei, Σp indicates the total number of counted frames, h (in μm) represents the height of the dissector, a/f (in mm^2) indicates the frame area, t (in μm) denotes the real sectional thickness, and BA (in μm) represents the block advance of the microtome, which was set at 20 μm .

4) Length density of seminiferous tubules

The formula $L_v = \frac{\Sigma Q}{\Sigma P \times \frac{a}{f}}$ was used to determine the length density of the seminiferous tubules. Here, ΣQ indicates the total number of tubules, ΣP denotes the total number of unbiased counting frames across all fields, and a/f (in mm^2) represents the counting frame area.

7. Immunostaining for apoptosis

Apoptosis was evaluated through immunostaining against the caspase-3 protein. For this procedure, 10 tissue sections from each mouse were chosen, as outlined in the description of stereological evaluation. These sections were then deparaffinized and exposed to

goat normal serum to block nonspecific sites. Subsequently, an anti-caspase 3 rabbit polyclonal antibody (1:100 in PBS; Abcam ab4051; Abcam) was applied and left to incubate overnight at 4 °C. Following this, the sections were rinsed with PBS and subjected to a secondary antibody (goat anti-rabbit immunoglobulin G; Abcam). Diaminobenzidine tetrahydrochloride was then added to the sections for 5 minutes to identify cells with a positive reaction. After dehydration, the samples were mounted, and five images of each section were captured [38,39]. Densitometry, using MacBiophotonics ImageJ software version 6 (National Institutes of Health), was employed to calculate the average level of apoptosis.

8. Biochemical evaluation

The harvested samples were washed with sterile PBS to eliminate excess tissue residue, after which they were immediately frozen at -80 °C for subsequent analysis. The biochemical status of the injury site was evaluated by investigating the concentrations of glutathione (GSH), superoxide dismutase (SOD), and CAT as antioxidant biomarkers and malondialdehyde (MDA) as an oxidant factor. All biomarkers were quantified using the method described by Nasiry et al. [33]. In brief, trichloroacetic acid and pyrogallol were utilized to measure the respective levels of GSH and SOD, and their absorption levels were documented via spectrophotometry at the respective wavelengths of 420 and 412 nm. The CAT level was measured using hydrogen peroxide in conjunction with a phosphate buffer, and its absorbance was recorded at a wavelength of 245 nm. Finally, the concentration of MDA was determined using thiobarbituric acid, and its absorbance was documented at a wavelength of 520 nm.

9. Gene expression analysis

Quantitative real-time polymerase chain reaction was employed to quantify the gene expression of the inflammatory cytokine nuclear factor kappa beta ($NF-\kappa\beta$), as well as proto-oncogene *c-KIT* (*c-kit*) and heat shock protein 72 (*HSP72*), which are involved in spermatogonial differentiation, proliferation, and resistance to oxidative stress. In this procedure, tissue samples were promptly homogenized following collection, and total RNA was subsequently extracted using a total RNA extraction kit (Yekta-tajhiz). The complementary DNA (cDNA) MultiScribe kit (Thermo Fisher) was utilized to prepare cDNA [40,41]. Spectrophotometry was used to evaluate the cDNA, and the results indicated that the absorbance ratio for all samples, between the wavelengths of 260 and 280 nm, ranged from approximately 1.8 to 2.01. Ultimately, the levels of gene expression were ascertained using a real-time polymerase chain reaction instrument (StepOne; Applied Biosystems). The sequences of the primers used in this process are presented in Table 1. Furthermore, β -actin was used as an internal control.

10. Statistical analysis

All data were analyzed using SPSS version 21 (IBM Corp.). The Kolmogorov-Smirnov test was employed to assess the distribution of the data, which were found to be normally distributed. To examine the relationship between groups, one-way analysis of variance and the Tukey *post hoc* test were utilized. Quantitative data are presented as the mean±standard deviation. A *p*-value of less than 0.05 was considered to indicate statistical significance.

Table 1. Primer sequences used for gene expression analysis using quantitative real-time polymerase chain reaction

Gene	Sequence (5' > 3')	Product length (bp)
NF-κβ	F: TGTGAGGACGGGGTATCC	332
	R: AGCTGGCTTTCTAATGTTGAC	
c-kit	F: AGCAAGATGTTCCGAAGGCAA	111
	R: AAGCAGTCTACAGGGACAGTA	
HSP72	F: AGTCCGCATGAAGCACTGG	83
	R: ATCTCCACCGGAAGTACGA	
β-Actin	F: CGCATCTATGATGGTTACGC	141
	R: TTTAATGTCACGCACGATTC	

bp, base pairs; NF-κβ, nuclear factor kappa beta; HSP72, heat shock protein 72.

Results

In all cases, the control mice demonstrated superior results to the other groups. Therefore, we present a comparison of the results among the groups that underwent SHT induction.

1. Administration of caffeine improved sperm characteristics in mice with induced chronic SHT

Figure 2 presents the results regarding sperm characteristics. The preventive and curative groups significantly outperformed the other groups in all measured sperm characteristics, including count, viability, motility, and morphology (all *p*<0.0001). Furthermore, when comparing sperm characteristics between the caffeine-treated groups, the preventive group exhibited significantly higher sperm count (*p*<0.001), viability (*p*<0.001), motility (*p*<0.05), and morphology (*p*<0.001) than the curative group.

2. Administration of caffeine increased testosterone levels in mice with induced chronic SHT

Figure 3 presents the results regarding testosterone levels. Both the preventive (all *p*<0.0001) and curative (all *p*<0.01) groups exhibited significantly higher testosterone levels than the other groups.

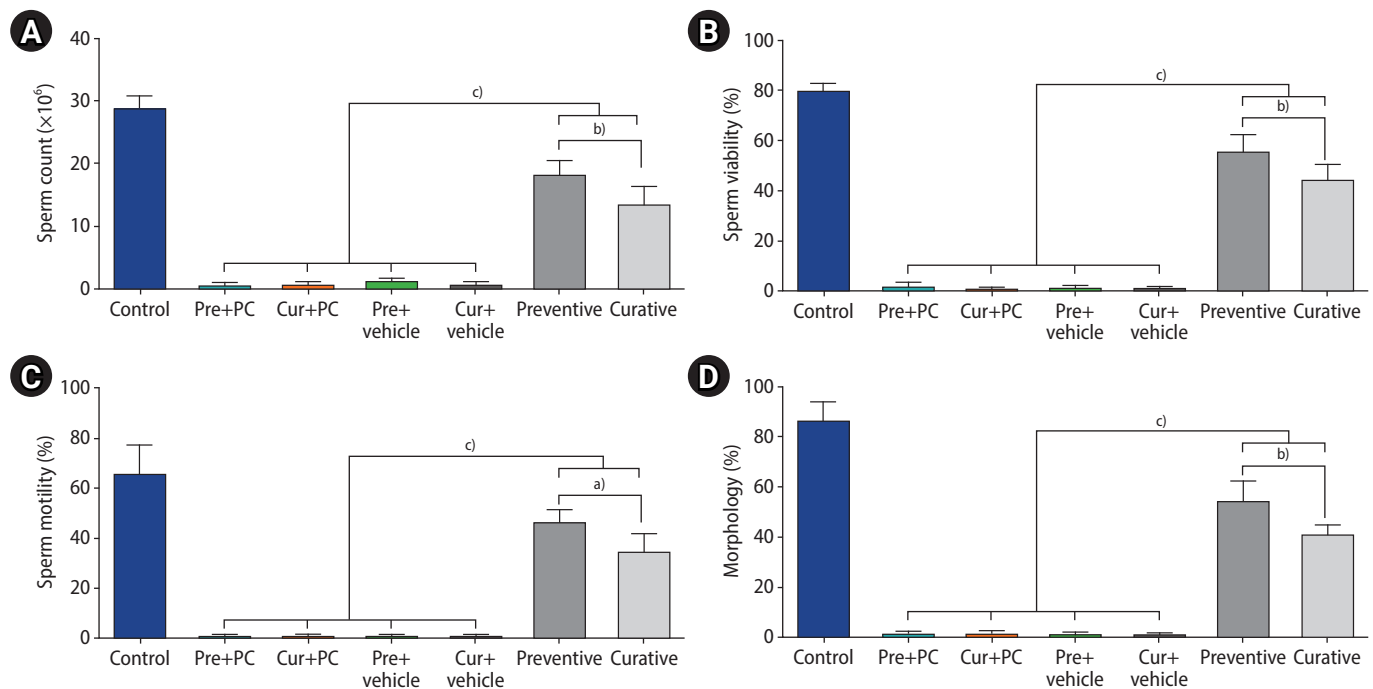


Figure 2. Effects of caffeine on sperm characteristics in mice with induced chronic scrotal hyperthermia. (A) Sperm count, (B) viability, (C) motility, and (D) morphology were compared across the study groups. Data are presented as mean±standard deviation. Pre, preventive; PC, positive control; Cur, curative. ^{a)}*p*<0.05; ^{b)}*p*<0.001; ^{c)}*p*<0.0001.

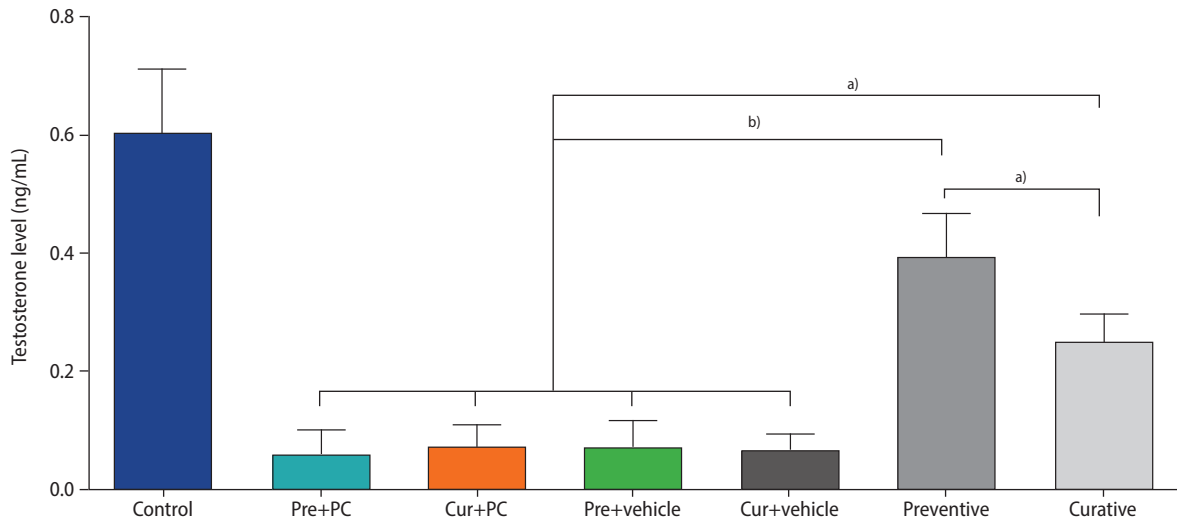


Figure 3. Effects of caffeine on testosterone levels in mice with induced chronic scrotal hyperthermia. Data are presented as mean±standard deviation. Pre, preventive; PC, positive control; Cur, curative. ^{a)} $p < 0.01$; ^{b)} $p < 0.0001$.

Moreover, the testosterone level in the preventive group was significantly greater than that in the curative group ($p < 0.01$).

3. Administration of caffeine prevented structural damage and improved stereological parameters in mice with induced chronic SHT

1) Testicular structure

Under the Johnsen scoring system, the mean±standard deviation for the control, Pre+PC, Cur+PC, Pre+vehicle, Cur+vehicle, preventive, and curative groups were 9.68 ± 0.32 , 2.35 ± 0.49 , 2.7 ± 0.61 , 2.23 ± 0.48 , 2.66 ± 0.7 , 7.54 ± 1.57 , and 5.09 ± 1.29 , respectively. The mean Johnsen scores were significantly higher for the preventive (all $p < 0.0001$) and curative (all $p < 0.01$) groups than for the other groups. Furthermore, the tissue structure status was significantly better in the preventive group compared to the curative group ($p < 0.01$) (Figure 4).

2) Testicular volume

A significantly greater testicular volume was observed in both the preventive (all $p < 0.0001$) and curative (all $p < 0.05$) groups than in the other treatment groups. Additionally, regarding the two caffeine-treated groups, the preventive group exhibited a significantly larger testicular volume than the curative group ($p < 0.01$) (Figure 5A).

3) Numerical cell density and length density of the seminiferous tubules

The quantitative outcomes of the stereological evaluations are presented in Figure 5B, 5C. In terms of spermatogonia, the numerical density of cells was significantly elevated in both the preventive (all $p < 0.0001$) and curative (all $p < 0.05$) groups relative to the other groups. Furthermore, regarding the two caffeine-treated groups, the

preventive group had a significantly higher density of spermatogonia than the curative group ($p < 0.01$).

When comparing primary spermatocyte density between groups, both the preventive group (all $p < 0.0001$) and the curative group (all $p < 0.05$) had significantly higher numbers of cells than the other groups. Furthermore, the density of primary spermatocytes in the preventive group was significantly greater than that of the curative group ($p < 0.01$).

Evaluation of the spermatids revealed that their numerical density was significantly higher in the preventive group relative to the Pre+PC, Cur+PC, Pre+vehicle, and Cur+vehicle groups (all $p < 0.0001$), as well as the curative group ($p < 0.001$). Moreover, the curative group exhibited a significantly higher cell count compared to the Pre+PC, Cur+PC, Pre+vehicle, and Cur+vehicle groups ($p < 0.05$, $p < 0.01$, $p < 0.01$, and $p < 0.01$, respectively).

The numerical density of Sertoli cells in the preventive group was significantly elevated compared to the Pre+PC, Cur+PC, Pre+vehicle, and Cur+vehicle groups (all $p < 0.001$), as well as the curative ($p < 0.01$) group.

In comparing the numerical density of Leydig cells across groups, both the preventive group (all $p < 0.0001$) and the curative group (all $p < 0.05$) had significantly more cells than the other groups. Additionally, regarding the two caffeine-treated groups, the preventive group had a significantly higher Leydig cell density than the curative group ($p < 0.01$).

A comparison of the length density of the seminiferous tubules revealed that the preventive group had a significantly higher number of tubules than the Pre+PC, Cur+PC, Pre+vehicle, Cur+vehicle (all $p < 0.0001$) groups, as well as the curative ($p < 0.05$) group. Further-

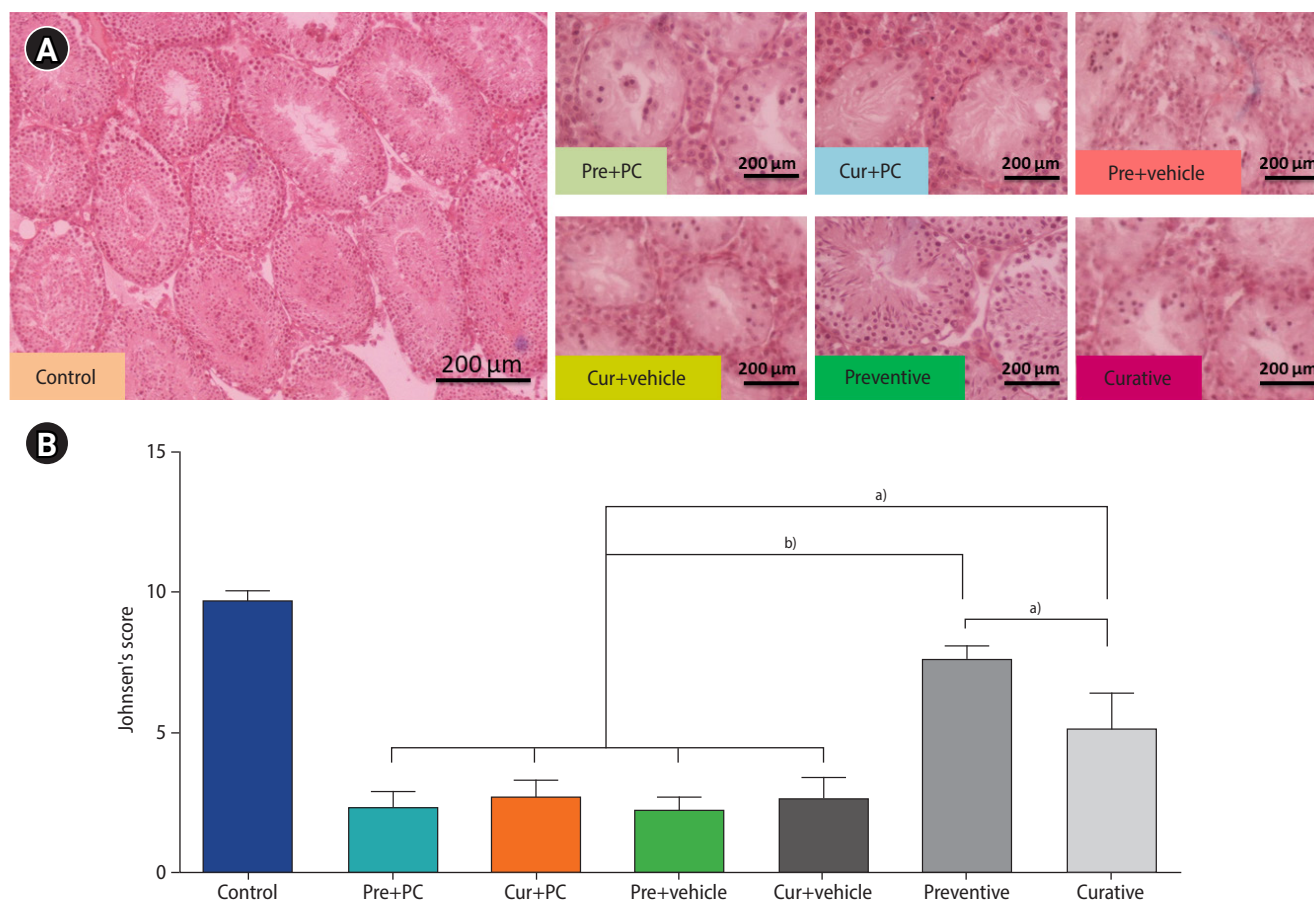


Figure 4. Effects of caffeine on histological structure in mice with induced chronic scrotal hyperthermia. (A) Histological photomicrographs under hematoxylin and eosin staining. (B) Comparison of histological structure across groups based on Johnsen score. Data are presented as mean±standard deviation. Pre, preventive; PC, positive control; Cur, curative. ^{a)} $p < 0.01$; ^{b)} $p < 0.0001$.

more, the curative group exhibited a significantly higher length density of seminiferous tubules compared to the Pre+PC, Cur+PC, Pre+vehicle, and Cur+vehicle groups (all $p < 0.05$).

4. Administration of caffeine inhibits testicular cell apoptosis in mice with induced chronic SHT

Figure 6A presents photomicrographs of immunostaining against the caspase-3 antibody. An examination of the apoptotic cell density in the testicular tissues indicated that the density of apoptotic cells in both the preventive (all $p < 0.0001$) and curative (all $p < 0.05$) groups was significantly lower relative to the other groups. Moreover, the preventive group exhibited a significantly lower degree of apoptotic cells than the curative group ($p < 0.01$) (Figure 6B).

5. Administration of caffeine modulates testicular oxidative biomarkers in mice with induced chronic SHT

Figure 7A presents an analysis of oxidative biomarkers in the testicular tissue, focusing on both oxidative (MDA) and antioxidative

(GSH, SOD, and CAT) factors. Both the preventive (all $p < 0.0001$) and curative (all $p < 0.01$) groups exhibited significantly lower MDA levels than the other experimental groups. Furthermore, the preventive group demonstrated significantly diminished MDA levels relative to the curative group ($p < 0.01$).

Regarding levels of antioxidative biomarkers, the preventive group had significantly higher levels of GSH, SOD, and CAT than the Pre+PC, Cur+PC, Pre+vehicle, Cur+vehicle, and curative groups ($p < 0.001$, $p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively for GSH; $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, and $p < 0.01$, respectively for SOD; and $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively for CAT). Furthermore, relative to the Pre+PC, Cur+PC, Pre+vehicle, and Cur+vehicle groups, the curative group exhibited significantly higher levels of both SOD and CAT (all $p < 0.05$).

6. Administration of caffeine affected testicular gene expression in mice with induced chronic SHT

The levels of *NF-κβ* gene expression were significantly lower in

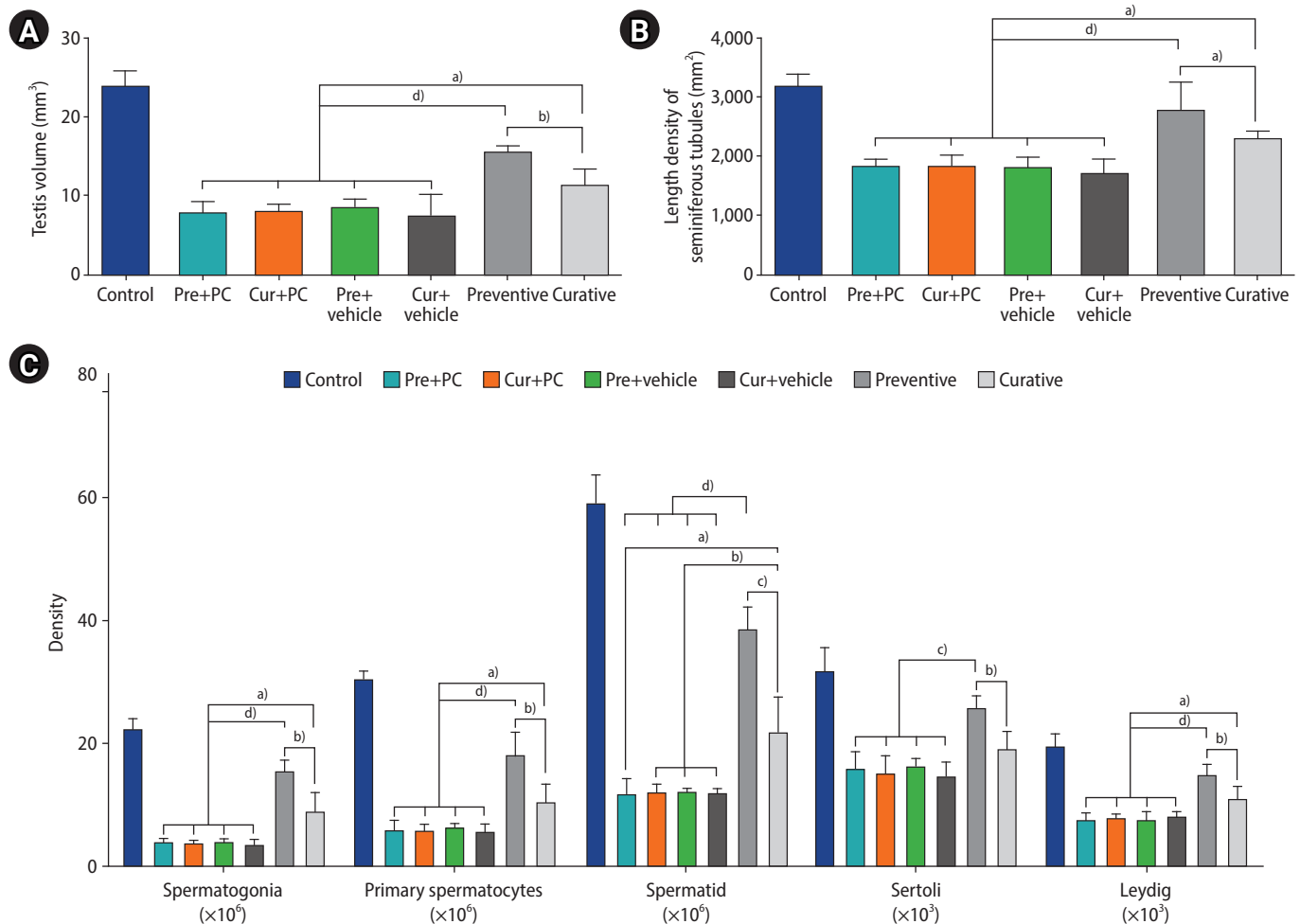


Figure 5. Effects of caffeine on stereological parameters in mice with induced chronic scrotal hyperthermia. (A) Testicular volumes calculated using the Cavalieri method. (B) Length density of the seminiferous tubules. (C) Numerical density of spermatogonia, primary spermatocytes, spermatids, Sertoli cells, and Leydig cells. Data are presented as mean \pm standard deviation. Pre, preventive; PC, positive control; Cur, curative. ^{a)} $p < 0.05$; ^{b)} $p < 0.01$; ^{c)} $p < 0.001$; ^{d)} $p < 0.0001$.

both the preventive (all $p < 0.001$) and curative (all $p < 0.05$) groups relative to the other experimental groups. Moreover, the level of *NF- κ B* gene expression was found to be lower in the preventive than in the curative group ($p < 0.01$) (Figure 7B).

Regarding the *c-kit* gene, both the preventive group (all $p < 0.0001$) and the curative group (all $p < 0.05$) exhibited significantly elevated expression levels relative to the other groups. Furthermore, when compared to the curative group, the preventive group demonstrated significantly greater expression of *c-kit* ($p < 0.01$).

Finally, the level of *HSP72* gene expression was markedly down-regulated in both the preventive (all $p < 0.001$) and curative (all $p < 0.01$) groups when compared to the other groups. Moreover, the preventive group exhibited significantly lower *HSP72* expression than the curative group ($p < 0.05$).

Discussion

Testicular hyperthermia is widely recognized as a primary cause of male infertility, associated with an overproduction of reactive oxygen species (ROS) and the initiation of sperm apoptosis in the testes [42]. In recent years, increasing attention has been paid to the detrimental impact of oxidative stress on male infertility, as well as the potential benefits of antioxidant supplements in enhancing semen parameters among men with infertility [43]. The administration of antioxidant supplements may enhance the body's capacity to inhibit the oxidative chain reaction, thereby improving the spermatogenic process [44]. In the present study, we observed that all evaluated sperm characteristics, including count, viability, motility, and morphology, were significantly superior in the preventive and curative groups compared to the other groups. Numerous studies have high-

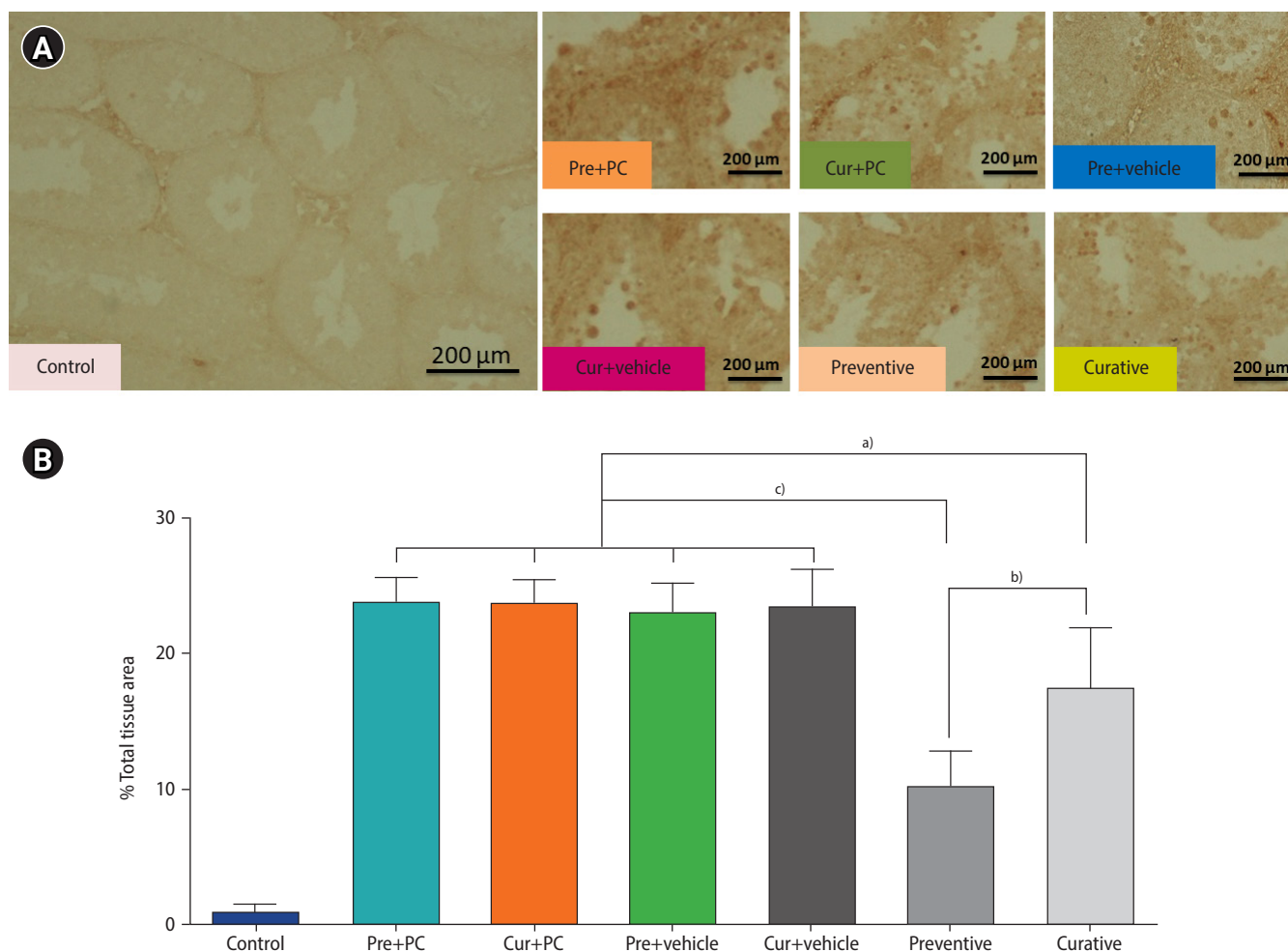


Figure 6. Effects of caffeine on cell apoptosis in mice with induced chronic scrotal hyperthermia. (A) Representative micrographs from immunostaining against caspase 3 protein in the testicular tissue. Caspase 3-positive cells are represented by dark brown cytoplasm. (B) Quantitative analysis of caspase 3-positive cells. Data are presented as mean±standard deviation. Pre, preventive; PC, positive control; Cur, curative. ^{a)} $p<0.05$; ^{b)} $p<0.01$; ^{c)} $p<0.0001$.

highlighted the anti-inflammatory, anticancer, and antioxidant properties of caffeine, even suggesting its potential as a preventative measure against the effects of various drugs and environmental factors on fertility and testicular tissue [15,31,45,46]. Our findings indicate that caffeine mitigates the tissue damage induced by chronic hyperthermia in testicular tissue. This includes key genes involved in germ cell differentiation and inflammation (such as *NF-κβ*, *c-kit*, and *HSP72*), oxidative biomarkers, and sperm parameters, as well as testicular morphology and stereology. The observed protective effect can be attributed to the antioxidant properties of caffeine.

Research has demonstrated that carbohydrates, particularly glucose, serve as the primary and essential energy source for mitotic cellular division. The blood-testicular barrier is the main pathway for glucose transfer to the seminiferous tubules [47,48]. Hyperthermia results in a decrease in testosterone synthesis by Leydig cells and a

reduction in the functionality of Sertoli cells [49,50]. Elevated levels of oxidative stress, induced by testicular hyperthermia, adversely impact sperm parameters, egg fusion, sperm, and sperm epigenetic modifications [51,52]. The overproduction of ROS can trigger oxidative stress, leading to impaired spermatogenesis, increased morphological damage, decreased motility, and impaired sperm-oocyte binding [5,53]. Caffeine has been found to mitigate oxidative stress and elevated lipid peroxidation levels in sperm and testicular cell membranes. It does this by enhancing the activity of antioxidant enzymes such as CAT, SOD, and GSH peroxidase, which in turn leads to a decrease in sperm motility and spermatozoon malformation [15]. Furthermore, caffeine has demonstrated its capacity to protect DNA by clearing ROS.

Caffeine has been reported to increase sperm activity and preserve fertility via a cyclic adenosine monophosphate (cAMP)-inde-

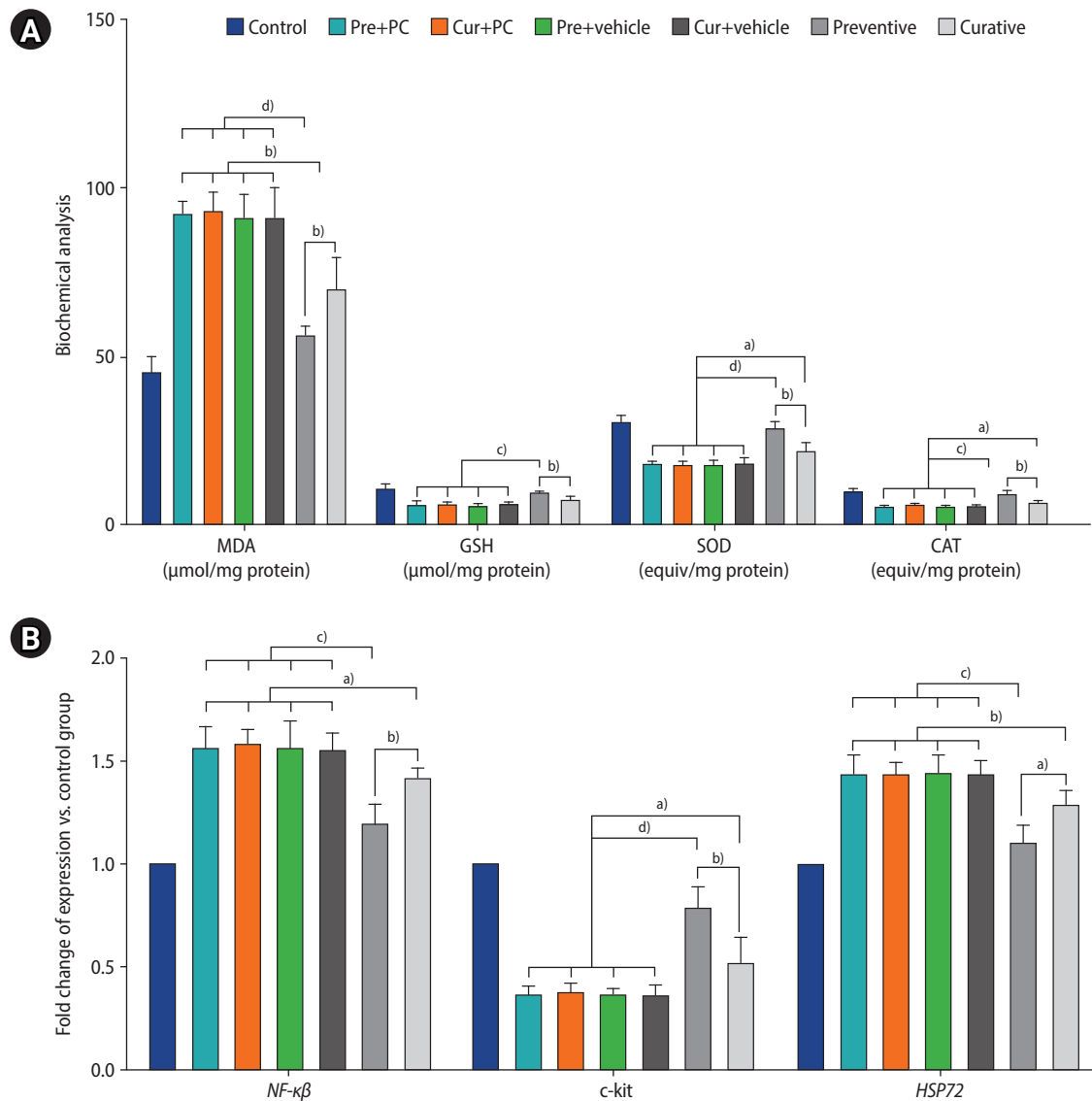


Figure 7. Effects of caffeine on testicular oxidative biomarkers and gene expression in mice with induced chronic scrotal hyperthermia. (A) Concentrations of oxidant (malondialdehyde [MDA]) and antioxidant (glutathione [GSH], superoxide dismutase [SOD], and catalase [CAT]) factors in the testicular tissues were evaluated using the biochemical method. (B) The quantities of transcripts for nuclear factor kappa beta ($NF-\kappa\beta$) (a gene involved in inflammation), as well as c-kit and heat shock protein 72 ($HSP72$) (involved in spermatogonial differentiation, proliferation, and resistance to oxidative stress), were analyzed using quantitative real-time polymerase chain reaction. Data are presented as mean \pm standard deviation. Pre, preventive; PC, positive control; Cur, curative. ^{a)} $p < 0.05$; ^{b)} $p < 0.01$; ^{c)} $p < 0.001$; ^{d)} $p < 0.0001$.

pendent pathway, specifically through a mechanism that relies on protein tyrosine phosphorylation and calcium [54]. Li et al. [55] provided evidence that a single exposure of the scrotum to heat at 42 °C for 25 minutes resulted in an elevated level of MDA, a decrease in antioxidant enzymes (particularly SOD), an increase in germ cell apoptosis through the caspase-3 pathway, and upregulation of nuclear factor erythroid 2-related factor 2 ($Nrf2$). These findings align with several results of our study.

Caffeine inhibits cyclic nucleotide phosphodiesterase, leading to

an increase in intracellular cAMP and intracellular calcium ion levels. These changes enhance sperm viability by inhibiting the caspase-3 apoptotic pathway and preserving the normal structure of the testis [56], potentially helping to explain the findings of our study. Additionally, previous research indicated that 0.4 mg/mL of caffeine enhances sperm motility and vitality *in vitro*, while also boosting the fertilization capacity and the rate of embryo formation in mice [57].

Given that $NF-\kappa\beta$ plays a crucial role in numerous cellular functions—particularly proliferation and apoptosis—and also regulates

certain pro-inflammatory cytokines such as interleukin 1 beta and tumor necrosis factor-alpha, it is plausible that caffeine may regulate inflammation and oxidative stress levels. This regulation could occur through indirect modulation of these cytokines, subsequently enhancing sperm parameters [58,59].

Alterations in membrane proteins and phospholipids can lead to enhanced sperm motility and improved acrosome reaction [60]. Caffeine plays a role in this process by inhibiting cyclic nucleotide phosphodiesterase. This inhibition results in an increase in intracellular cAMP, subsequently inducing the acrosome reaction [56].

Moreover, proteoglycans, including chondroitin and heparin, are crucial for initiating sperm capacitation and the acrosome reaction, signifying a key role of Sertoli cells [61]. Caffeine enhances the production of these proteoglycans and testosterone, which is secreted by Leydig cells. Subsequently, testosterone activates mitogen-activated protein kinase in Sertoli cells [62]. This signaling pathway could partially explain the results of our study, which include an increase in germ cell viability and the upregulation of proliferative factors such as *c-kit* and *HSP72*.

Conversely, nitric oxide is a free radical and a messenger that regulates protein phosphorylation. This modulation affects various physiological processes, particularly spermatogenesis, which exhibits overexpression following hyperthermia [28,63].

The progression of sperm capacitation necessitates an increase in the intracellular concentration of cyclic guanosine monophosphate (cGMP) [64]. Given that cGMP promotes sperm capacitation and the acrosome reaction, caffeine can enhance the acrosome reaction and improve sperm viability by increasing cGMP concentration [65]. Furthermore, caffeine activates protein kinase C [66], a fundamental player in the signaling pathway that contributes to sperm maturation, viability, and motility [67].

The primary mechanism of action for caffeine appears to be the antagonism of adenosine receptors [68]. Given the similar molecular structures of caffeine and adenosine, both of which possess a double-bond ring structure, caffeine can bind to adenosine receptor sites [69]. It has long been suggested that adenosine and its antagonists may influence the male reproductive system [70]. Multiple studies have demonstrated the presence of adenosine receptors in Sertoli cells [71,72] and have shown that these cells can be modulated by adenosine and its analog compounds. Sertoli cells play a crucial role in the functional development of the testis and, consequently, in the expression of the male phenotype [73,74]. They also actively metabolize several substrates, such as glucose, to ensure a steady supply of lactate to the developing germ cells [75]. Therefore, the overall metabolic functioning of Sertoli cells is critical for normal spermatogenesis. Several studies have indicated that caffeine can stimulate Sertoli cells to increase testosterone production [15,76,77].

In line with these findings, our study results demonstrated significantly elevated testosterone levels in the group that received caffeine compared to the other groups.

In contrast, Sertoli cells are known to produce lactate at high rates. Any disruption in related processes could potentially result in increased levels of oxidative stress, which may subsequently lead to male subfertility or infertility [78]. Intriguingly, caffeine has been identified as a protective agent against cellular damage, offering beneficial antioxidant effects [79]. The results of our study similarly demonstrated a significant reduction in the oxidative stress level within the testicular tissue of the groups that received caffeine. These findings align with the results of previous studies.

In conclusion, our findings indicate that SHT leads to a decrease in sperm parameters and testicular dysfunction, primarily through the induction of inflammation, oxidative stress, and apoptosis. Nevertheless, caffeine, which possesses potent antioxidant and anti-apoptotic properties, has the potential to enhance sperm parameters and address infertility issues associated with heat-induced oxidative stress.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

The authors express their gratitude for the support of Mazandaran University of Medical Sciences in Sari, Iran.

ORCID

Amir Raoofi	https://orcid.org/0000-0001-7402-1726
Davood Nasiry	https://orcid.org/0000-0003-0249-4115
Alireza Ghaemi	https://orcid.org/0000-0001-6132-1068

Author contributions

Conceptualization: AR, DN. Data curation: AR, HM, FB. Formal analysis: AR, OG. Funding acquisition: AR, DN. Methodology: AR, HM, DN, AG. Project administration: AR, DN, AG. Visualization: AR, DN, AG. Writing-original draft: AR, DN, AG. Writing-review & editing: AR, DN, AG.

References

1. Sharma A. Male infertility; evidences, risk factors, causes, diagnosis and management in human. *Ann Clin Lab Res* 2017;5:188.

2. Miyamoto T, Tsujimura A, Miyagawa Y, Koh E, Namiki M, Sengoku K. Male infertility and its causes in human. *Adv Urol* 2012;2012:384520.
3. Chavoshi Nezhad N, Vahabzadeh Z, Allahveisie A, Rahmani K, Raoufi A, Rezaie MJ, et al. The effect of L-carnitine and coenzyme Q10 on the sperm motility, DNA fragmentation, chromatin structure and oxygen free radicals during, before and after freezing in oligospermia men. *Urol J* 2021;18:330-6.
4. Banks S, King SA, Irvine DS, Saunders PT. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. *Reproduction* 2005;129:505-14.
5. Durairajanayagam D, Agarwal A, Ong C. Causes, effects and molecular mechanisms of testicular heat stress. *Reprod Biomed Online* 2015;30:14-27.
6. Setchell BP. The effects of heat on the testes of mammals. *Anim Reprod* 2006;3:81-91.
7. Abdelhamid MHM, Walschaerts M, Ahmad G, Mieusset R, Bujan L, Hamdi S. Mild experimental increase in testis and epididymis temperature in men: effects on sperm morphology according to spermatogenesis stages. *Transl Androl Urol* 2019;8:651-65.
8. Murphy CJ, Richburg JH. Implications of Sertoli cell induced germ cell apoptosis to testicular pathology. *Spermatogenesis* 2015;4:e979110.
9. Shaha C, Tripathi R, Mishra DP. Male germ cell apoptosis: regulation and biology. *Philos Trans R Soc Lond B Biol Sci* 2010;365:1501-15.
10. Zhang M, Jiang M, Bi Y, Zhu H, Zhou Z, Sha J. Autophagy and apoptosis act as partners to induce germ cell death after heat stress in mice. *PLoS One* 2012;7:e41412.
11. Paul C, Teng S, Saunders PT. A single, mild, transient scrotal heat stress causes hypoxia and oxidative stress in mouse testes, which induces germ cell death. *Biol Reprod* 2009;80:913-9.
12. Khosravi A, Hasani A, Rahimi K, Aliaghaei A, Pirani M, Azad N, et al. Ameliorating effects of curcumin-loaded superparamagnetic iron oxide nanoparticles (SPIONs) on the mouse testis exposed to the transient hyperthermia: a molecular and stereological study. *Acta Histochem* 2020;122:151632.
13. Shahat AM, Rizzoto G, Kastelic JP. Amelioration of heat stress-induced damage to testes and sperm quality. *Theriogenology* 2020;158:84-96.
14. Sukhotnik I, Nativ O, Ben-Shahar Y, Bejar IN, Pollak Y, Coran AG, et al. Antioxidant treatment ameliorates germ cell apoptosis induced by a high-dose ionizing irradiation in rats. *Pediatr Surg Int* 2019;35:137-43.
15. Akomolafe SF, Akinyemi AJ, Oboh G, Oyeleye SI, Ajayi OB, Omonisi AE, et al. Co-administration of caffeine and caffeic acid alters some key enzymes linked with reproductive function in male rats. *Andrologia* 2018;50:e12839.
16. Zhang K, Shang Y, Liao S, Zhang W, Nian H, Liu Y, et al. Uncoupling protein 2 protects testicular germ cells from hyperthermia-induced apoptosis. *Biochem Biophys Res Commun* 2007;360:327-32.
17. Kogure A, Sakane N, Takakura Y, Umekawa T, Yoshioka K, Nishino H, et al. Effects of caffeine on the uncoupling protein family in obese yellow KK mice. *Clin Exp Pharmacol Physiol* 2002;29:391-4.
18. Xu YR, Dong HS, Yang WX. Regulators in the apoptotic pathway during spermatogenesis: killers or guards? *Gene* 2016;582:97-111.
19. Adly MA, Assaf HA, Hussein MR. Heat shock protein 27 expression in the human testis showing normal and abnormal spermatogenesis. *Cell Biol Int* 2008;32:1247-55.
20. Purandhar K, Jena PK, Prajapati B, Rajput P, Seshadri S. Understanding the role of heat shock protein isoforms in male fertility, aging and apoptosis. *World J Mens Health* 2014;32:123-32.
21. Naaby-Hansen S, Herr JC. Heat shock proteins on the human sperm surface. *J Reprod Immunol* 2010;84:32-40.
22. Ferlin A, Speltra E, Patassini C, Pati MA, Garolla A, Caretta N, et al. Heat shock protein and heat shock factor expression in sperm: relation to oligozoospermia and varicocele. *J Urol* 2010;183:1248-52.
23. Swelum AAA, Hashem NM, Abo-Ahmed AI, El-Hack MEA, Abdo M. The role of heat shock proteins in reproductive functions. In: Asea AAA, Kaur P, editors. *Heat shock proteins in inflammatory diseases*. Springer; 2020. p. 407-27.
24. Turner TT, Lysiak JJ. Oxidative stress: a common factor in testicular dysfunction. *J Androl* 2008;29:488-98.
25. Xiong Y, Yin Q, Li J, He S. Oxidative stress and endoplasmic reticulum stress are involved in the protective effect of alpha lipoic acid against heat damage in chicken testes. *Animals (Basel)* 2020;10:384.
26. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. *Adv Exp Med Biol* 2008;636:154-71.
27. Baskaran S, Finelli R, Agarwal A, Henkel R. Reactive oxygen species in male reproduction: a boon or a bane? *Andrologia* 2021;53:e13577.
28. De Luca MN, Colone M, Gambioli R, Stringaro A, Unfer V. Oxidative stress and male fertility: role of antioxidants and inositols. *Antioxidants (Basel)* 2021;10:1283.
29. Perez-Crespo M, Pintado B, Gutierrez-Adan A. Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Mol Reprod Dev* 2008;75:40-7.
30. Shadmehr S, Fatemi Tabatabaei SR, Hosseinifar S, Tabandeh MR, Amiri A. Attenuation of heat stress-induced spermatogenesis complications by betaine in mice. *Theriogenology* 2018;106:117-

- 26.
31. Aprioku JS, Boms R, Ife-Tubiyeye DO. Effect of simultaneous coffee/caffeine and ethanol administration on sperm quality and reproductive hormones: an experimental study in Sprague Dawley rats. *Drug Chem Toxicol* 2022;45:1012-20.
32. Ziaeipour S, Rezaei F, Piryaei A, Abdi S, Moradi A, Ghasemi A, et al. Hyperthermia versus busulfan: finding the effective method in animal model of azoospermia induction. *Andrologia* 2019;51:e13438.
33. Nasiry D, Khalatbary AR, Ahmadvand H, Talebpour Amiri FB. Effects of *Juglans regia* L. leaf extract supplementation on testicular functions in diabetic rats. *Biotech Histochem* 2021;96:41-7.
34. Vega SG, Guzman P, Garcia L, Espinosa J, Cortinas de Nava C. Sperm shape abnormality and urine mutagenicity in mice treated with niclosamide. *Mutat Res* 1988;204:269-76.
35. Tavares RS, Silva AF, Lourenco B, Almeida-Santos T, Sousa AP, Raimalho-Santos J. Evaluation of human sperm chromatin status after selection using a modified Diff-Quik stain indicates embryo quality and pregnancy outcomes following in vitro fertilization. *Andrology* 2013;1:830-7.
36. Howard CV, Reid MG. Unbiased stereology: three-dimensional measurement in microscopy. Garland Science; 2004.
37. Raoofi A, Delbari A, Mahdian D, Mojadadi MS, Akhlaghi M, Dadashizadeh G, et al. Effects of curcumin nanoparticle on the histological changes and apoptotic factors expression in testis tissue after methylphenidate administration in rats. *Acta Histochem* 2021;123:151656.
38. Nilforoushzadeh MA, Afzali H, Raoofi A, Nouri M, Naser R, Gholami O, et al. Topical spray of Wharton's jelly mesenchymal stem cells derived from umbilical cord accelerates diabetic wound healing. *J Cosmet Dermatol* 2022;21:5156-67.
39. Seyed Sharifi SH, Nasiry D, Mahmoudi F, Eteazadpour M, Ebrahimzadeh MA. Evaluation of *Sambucus ebulus* fruit extract in full-thickness diabetic wound healing in rats. *J Mazandaran Univ Med Sci* 2021;31:11-25.
40. Nasiry D, Khalatbary AR, Abdollahifar MA, Bayat M, Amini A, Kazemi Ashtiani M, et al. SDF-1 α loaded bioengineered human amniotic membrane-derived scaffold transplantation in combination with hyperbaric oxygen improved diabetic wound healing. *J Biosci Bioeng* 2022;133:489-501.
41. Nilforoushzadeh MA, Raoofi A, Afzali H, Gholami O, Zare S, Nasiry D, et al. Promotion of cutaneous diabetic wound healing by subcutaneous administration of Wharton's jelly mesenchymal stem cells derived from umbilical cord. *Arch Dermatol Res* 2023;315:147-59.
42. Kokubu D, Ooba R, Abe Y, Ishizaki H, Yoshida S, Asano A, et al. *Angelica keiskei* (Ashitaba) powder and its functional compound xanthoangelol prevent heat stress-induced impairment in sperm density and quality in mouse testes. *J Reprod Dev* 2019;65:139-46.
43. Ahmadi S, Bashiri R, Ghadiri-Anari A, Nadjarzadeh A. Antioxidant supplements and semen parameters: an evidence based review. *Int J Reprod Biomed* 2016;14:729-36.
44. Asadi N, Bahmani M, Kheradmand A, Rafeieian-Kopaei M. The impact of oxidative stress on testicular function and the role of antioxidants in improving it: a review. *J Clin Diagn Res* 2017;11:IE01-5.
45. Al-Megrin WA, El-Khadragy MF, Hussein MH, Mahgoub S, Abdel-Mohsen DM, Taha H, et al. Green *Coffea arabica* extract ameliorates testicular injury in high-fat diet/streptozotocin-induced diabetes in rats. *J Diabetes Res* 2020;2020:6762709.
46. Labib H, Galal A. Caffeine versus antioxidant combination (Antox) and their role in modifying cadmium-induced testicular injury in adult male albino rats. *Andrologia* 2021;53:e13948.
47. Fok KL, Chen H, Ruan YC, Chan HC. Novel regulators of spermatogenesis. *Semin Cell Dev Biol* 2014;29:31-42.
48. Neto FTL, Flannigan R, Goldstein M. Regulation of human spermatogenesis. *Adv Exp Med Biol* 2021;1288:255-86.
49. Kim JH, Park SJ, Kim TS, Kim JM, Lee DS. Testosterone production by a Leydig tumor cell line is suppressed by hyperthermia-induced endoplasmic reticulum stress in mice. *Life Sci* 2016;146:184-91.
50. Hwang EC, Min KD, Jung SI, Ryu SB, Ahn KY, Lee K, et al. Testicular steroidogenesis is decreased by hyperthermia in old rats. *Urol Int* 2010;84:347-52.
51. Barratt CL, Aitken RJ, Bjorndahl L, Carrell DT, de Boer P, Kvist U, et al. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications: a position report. *Hum Reprod* 2010;25:824-38.
52. Boni R. Heat stress, a serious threat to reproductive function in animals and humans. *Mol Reprod Dev* 2019;86:1307-23.
53. Dutta S, Sengupta P, Slama P, Roychoudhury S. Oxidative stress, testicular inflammatory pathways, and male reproduction. *Int J Mol Sci* 2021;22:10043.
54. Colas C, Cebrian-Perez JA, Muino-Blanco T. Caffeine induces ram sperm hyperactivation independent of cAMP-dependent protein kinase. *Int J Androl* 2010;33:e187-97.
55. Li Y, Cao Y, Wang F, Li C. Scrotal heat induced the Nrf2-driven antioxidant response during oxidative stress and apoptosis in the mouse testis. *Acta Histochem* 2014;116:883-90.
56. Rossi M, Gonzalez-Castro R, Falomo ME. Effect of caffeine and pentoxifylline added before or after cooling on sperm characteristics of stallion sperm. *J Equine Vet Sci* 2020;87:102902.
57. Nabavi N, Todehdehghan F, Shiravi A. Effect of caffeine on motility

- and vitality of sperm and in vitro fertilization of outbreed mouse in T6 and M16 media. *Iran J Reprod Med* 2013;11:741-6.
58. Hwang JH, Koh EJ, Lee YJ, Chio J, Song JH, Seo YJ, et al. Anti-inflammatory effect of caffeine by regulating NF- κ B activation in murine macrophage. *FASEB J* 2016;30(S1):lb256.
 59. Conti F, Lazzara F, Romano GL, Platania CBM, Drago F, Bucolo C. Caffeine protects against retinal inflammation. *Front Pharmacol* 2022;12:824885.
 60. Sheriff DS, Ali EF. Perspective on plasma membrane cholesterol efflux and spermatozoal function. *J Hum Reprod Sci* 2010;3:68-75.
 61. Bae J, Choi H, Choi Y, Roh J. Dose- and time-related effects of caffeine on the testis in immature male rats. *Exp Anim* 2017;66:29-39.
 62. Fix C, Jordan C, Cano P, Walker WH. Testosterone activates mitogen-activated protein kinase and the cAMP response element binding protein transcription factor in Sertoli cells. *Proc Natl Acad Sci U S A* 2004;101:10919-24.
 63. Owumi SE, Irozuru CE, Arunsi UO, Faleke HO, Oyelere AK. Caffeic acid mitigates aflatoxin B1-mediated toxicity in the male rat reproductive system by modulating inflammatory and apoptotic responses, testicular function, and the redox-regulatory systems. *J Food Biochem* 2022;46:e14090.
 64. Staicu FD, Martinez-Soto JC, Canovas S, Matas C. Nitric oxide-targeted protein phosphorylation during human sperm capacitation. *Sci Rep* 2021;11:20979.
 65. Yang R, Wang J, Chen Y, Sun Z, Wang R, Dai Y. Effect of caffeine on erectile function via up-regulating cavernous cyclic guanosine monophosphate in diabetic rats. *J Androl* 2008;29:586-91.
 66. Sun JH, Zhu PH. The accumulation of diacylglycerol and activation of protein kinase C by caffeine treatment in frog skeletal muscle. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai)* 1997;29:533-40.
 67. Ickowicz D, Finkelstein M, Breitbart H. Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases. *Asian J Androl* 2012;14:816-21.
 68. Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci* 2001;24:31-55.
 69. Fisone G, Borgkvist A, Usiello A. Caffeine as a psychomotor stimulant: mechanism of action. *Cell Mol Life Sci* 2004;61:857-72.
 70. Casali EA, da Silva TR, Gelain DP, Kaiser GR, Battastini AM, Sarkis JJ, et al. Ectonucleotidase activities in Sertoli cells from immature rats. *Braz J Med Biol Res* 2001;34:1247-56.
 71. Rivkees SA. Localization and characterization of adenosine receptor expression in rat testis. *Endocrinology* 1994;135:2307-13.
 72. Stiles GL, Pierson G, Sunay S, Parsons WJ. The rat testicular A1 adenosine receptor-adenylate cyclase system. *Endocrinology* 1986;119:1845-51.
 73. Mackay S. Gonadal development in mammals at the cellular and molecular levels. *Int Rev Cytol* 2000;200:47-99.
 74. Rato L, Alves MG, Socorro S, Duarte AI, Cavaco JE, Oliveira PF. Metabolic regulation is important for spermatogenesis. *Nat Rev Urol* 2012;9:330-8.
 75. Alves MG, Neuhaus-Oliveira A, Moreira PI, Socorro S, Oliveira PF. Exposure to 2,4-dichlorophenoxyacetic acid alters glucose metabolism in immature rat Sertoli cells. *Reprod Toxicol* 2013;38:81-8.
 76. Dias TR, Alves MG, Bernardino RL, Martins AD, Moreira AC, Silva J, et al. Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile: relevance for male fertility. *Toxicology* 2015;328:12-20.
 77. Oluwole OF, Salami SA, Ogunwole E, Raji Y. Implication of caffeine consumption and recovery on the reproductive functions of adult male Wistar rats. *J Basic Clin Physiol Pharmacol* 2016;27:483-91.
 78. Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod* 2010;25:2415-26.
 79. Grucka-Mamczar E, Zalejska-Fiolka J, Chlubek D, Kasperczyk S, Błaszczyk U, Kasperczyk A, et al. The influence of sodium fluoride and caffeine on the activity of antioxidative enzymes and the concentration of malondialdehyde in rat liver. *Fluoride* 2009;42:105-9.