



The Impact of Photobiomodulation Therapy on Enhancing Spermatogenesis and Blood-Testis Barrier Integrity in Adult Male Mice Subjected to Scrotal Hyperthermia

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Abstract

Introduction: Unhealthy lifestyle choices such as alcohol, chemicals, and heat stress can worsen male infertility. Heat stress can cause damage to the essential structure known as the blood-testis barrier (BTB). Photobiomodulation therapy (PBMT) has been employed in various studies to enhance sperm quality in individuals with genital inflammatory conditions in recent times. The current research sought to study how laser therapy affects spermatogenesis and the structure of the BTB in a mouse model of scrotal heat exposure.

Methods: Thirty adult male NMRI mice, 8 weeks old, were divided into three groups: Control, Hyperthermia, and Hyperthermia + Laser 0.03 J/cm². The animals in the hyperthermia group had their testicles exposed to water at 43 °C for 20 minutes five times every other day. Then, the testicles were exposed to laser radiation every other day for 35 days, lasting 3 minutes each time, with an energy density of 0.03 J/cm². Animals were sacrificed, and sperm parameters, reactive oxygen species (ROS) and glutathione (GSH) levels, stereological parameters, and gene expression were assessed in the end.

Results: The study showed that PBMT can significantly enhance sperm quality, quantity of spermatogenic cells, testicular volume, levels of ROS and GSH, and gene expression related to the blood-testis barrier.

Conclusion: Currently, PBMT is a novel approach for addressing male infertility by preserving the integrity of the BTB in Sertoli cells, which in turn supports the growth and specialization of germ cells.

Keywords: Blood-testis barrier; Hyperthermia; Laser therapy; Spermatogenesis.



Introduction

Infertility is an important factor in young couples, affecting 10%-20% of those in reproductive age.¹ Male infertility can be worsened by unhealthy lifestyle choices such as alcohol consumption, chemical exposure, and psychological problems. Additionally, prolonged sitting at work, wearing uncomfortable clothing, and exposure to extreme heat from hot baths can have negative effects on male reproductive health by causing heat stress in the testicles.²

The testes are very sensitive to temperature variations and need to be kept 2 °C cooler than other organs in the body for optimal function.³ Not regulating testicular temperature properly can cause issues with sperm production, lower sex hormone levels, cell atrophy, and

a higher chance of infertility.^{4,5} Heat-induced damage to testicles is identified by the apoptosis of germ cells, damage to DNA, and overproduction of reactive oxygen species (ROS).^{6,7} The precise reason for this vulnerability is not completely understood.

The blood-testis barrier (BTB) is created by intercellular communication between Sertoli cells via cytoplasmic projections. The barrier is formed by peripheral connections, desmosomes, and spaces between Sertoli cells. It is situated near the bottom of the seminiferous tubules and divides the epithelium into basal and luminal parts. The purpose of the BTB is to create a compact environment that facilitates the completion of the meiotic process of germ cells.⁸ Proteins like claudin and occludin

are important for the formation of the BTB. Past research has indicated that environmental toxins, chemotherapy medications, and exposure to heat can lead to higher levels of oxidative stress, resulting in damage to proteins and a breakdown of the barrier's integrity.⁹ In men, structural damage to the BTB can result in reduced sperm count, germ cell loss, and potential infertility.

Low-power laser therapy has emerged as a modern method for restoring various body tissues.¹⁰ The effectiveness of low-power lasers in biological systems stems from their ability to induce photochemical effects, where light absorption leads to chemical changes without causing destructive heat or warming effects. In the realm of male infertility treatment, researchers have demonstrated that low-power laser irradiation can increase sperm motility and viability, increase spermatogonial cell count, and elevate testosterone levels. Laboratory conditions have also shown a reduction in sperm mortality rates.¹¹

In recent studies, photobiomodulation therapy (PBMT), a type of laser therapy, has been utilized to enhance sperm quality in individuals with genital inflammatory conditions like prostatitis and vasculitis.^{12,13} PBMT promotes the growth of different types of cells, such as fibroblasts, endothelial cells, mesenchymal stem cells, and immune cells, leading to healing effects in different parts of the body.

The balance of germ cells is controlled by the mitochondrial respiratory chain, and problems with this chain can lead to lower ATP production and higher levels of ROS. Recent research has indicated that PBMT is vital for supporting the health of germ cells as it helps to lower levels of ROS and boost mitochondrial activity in cases of heat stress-induced damage.¹⁴ The BTB is the most vulnerable structure of Sertoli cells to increased ROS levels, and its dysfunction can be associated with male infertility. Since PBMT can modulate ROS levels with its antioxidant properties, it may be able to protect the barrier structure from heat stress. Thus, the study was crafted to explore the impact of PBMT on sperm production and the integrity of the BTB in a scrotal hyperthermia mouse model.

Methods

Animals

Thirty adult male NMRI mice, aged 8 weeks and weighing between 25-30 gr, were chosen and housed in conditions with 12 hours of light and 12 hours of darkness. The mice were split into three different groups by chance: Control, Hyperthermia, and Hyperthermia + Laser 0.03 J/cm², each group consisting of 10 animals. The Research Ethics Committee at Shahid Beheshti University of Medical Sciences approved the protocol for this study under the code (IR.SBMU.AEC.1401.055).

Transient Scrotal Hyperthermia Model

For inducing hyperthermia, animal testicles were

subjected to five 20-minute sessions of water exposure at 43 °C every other day. Subsequently, the animals were anesthetized with ketamine (100 mg/kg) and xylazine (5 mg/kg) via intraperitoneal injection. The lower body, encompassing the scrotum and hind legs, was immersed in the water bath followed by being dried and returned to their cages. The control group animals were anesthetized and maintained at room temperature.¹⁵

Photobiomodulation

The parameters for photobiomodulation (PBM) consisted of a wavelength of 890 nm, a pulse frequency of 80 Hz, a spot size of 1 cm², an exposure duration of 3 minutes for each testis, and an energy density of 0.03 J/cm². The Hyp+Laser group received laser treatment on their testicles every other day for 35 days following hyperthermia induction.^{16,17} Then, one day after the last laser radiation, the animals were deeply anesthetized and finally sacrificed. Sperm samples were taken from the epididymal tail for sperm parameter analysis. The right testicle was transferred to Bouin's fixative for histological studies, and the left testicle was placed in a -80 °C freezer to evaluate the expression of barrier genes and the ROS level of the testis.

Sperm Analysis

Sperm samples were collected from the tail of the epididymis and combined with 1 mL of Ham's F-10 medium from Sigma-Aldrich (Product Number N6635). Afterward, they were placed in an incubator at 37 °C for 20 minutes. A slide was used to place 10 µL of the sample for measuring the sperm count and motility using a counting chamber.

Tissue Preparation

The animals were sedated with ketamine and euthanized in accordance with ethical guidelines at the conclusion of the experiment. Next, the testicles were removed and preserved in Bowen's fixative solution for 48 hours. The testis samples were prepared using a tissue processor and then embedded in paraffin. Serial sections of 10 µm thickness were created with a microtome for histopathological analysis using stereological techniques. A systematic method of selecting 10 sections from each sample using uniform random sampling was used. The process included selecting a random number between 1 and 10 for every sample. The tissue sections were finally stained with H&E (Sigma, USA).

Stereological studies

Total Testis Volume

Cavalieri method was utilized for total testis volume¹⁸:

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

The sum of points hitting the testis sections is denoted as ΣP , the distance between tissue sections is denoted as t , and the area per sample point is indicated as a/p .

Total number of Testicular Cells

Optical dissector method was utilized to measure the total number of testicular cells. By applying the subsequent formula, we assessed the numerical density (N_v) for various cell types.¹⁸

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

The equation incorporates ΣQ to represent the total number of cells, h to represent the thickness of the tissue being counted, a/f to represent the area counted per counting frame, and ΣP to represent the cumulative count of all frames used in all fields. Moreover, t refers to the exact thickness of the section determined by a microcator, BA represents the thickness of the tissue section, and h is the height of the dissector.

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

Measurement of total ROS Level

Testicular cells were isolated by trypsin-EDTA treatment, followed by phosphate-buffered saline (PBS) washing and centrifugation at 1200 rpm and 4 °C for 5 minutes. Afterwards, the samples were combined with 20 μ M DCFDA and incubated at 37 °C for 45 minutes in the absence of light. The sample was eventually analyzed at a wavelength of 488 nm with a spectrofluorometer.

Measurement of Glutathione Level

The amount of glutathione (GSH) was determined using the spectrofluorometric technique. Testicular cell suspension (5.0 mL) was stained with N-ethylmaleimide (NEM) and O-phthalaldehyde (OPA). Following one-minute centrifugation at 1000 rpm for the cells, the cell sediment was dissolved in 2 mm of brand-new culture media. To get rid of the fluorescent dye in the medium, the cells underwent two rounds of washing. Lastly, we measured each tissue sample using a 5000 Shimadzu RF spectrophotometer set to 495 nm excitation and 530 nm emission wavelength.¹⁹

Real-Time Polymerase Chain Reaction

With this technique, the amount of fluorescence emission is measured and recorded in a detector so that the reaction may be seen in real time. Using this approach, we conducted a semi-quantitative analysis of the *Ocln*, *Gja1*, and *Cdh2* gene expression.

RNA samples were isolated initially, and any remaining genomic DNA was eliminated by using DNase. cDNA was synthesized in a 20- μ L volume at 42 °C for 60 minutes, following the kit's guidelines.

SYBR Green I dye was used for labeling. This dye will bind to and take the place of the DNA Minor Groove during annealing and DNA replication. The instrument will measure an increased amount of fluorescent light as the double-stranded DNA quantity increases. To examine the genes, we utilized a pair of forward and reverse primers designed by the Primer 3 Plus software, especially for the exon-exon junction. These primers will help us distinguish cDNA from the DNA genome.

We confirmed the polymerase chain reaction (PCR) primers before testing using the Primer-Blast method available at www.ncbi.nlm.nih.gov/tools/primer-blast (Table 1).

Statistical Analysis

Statistical evaluation was done using SPSS version 20. All graphs were designed by GraphPad Prism 9. All data were presented as mean $\pm$ SD. Results were compared by the one-way ANOVA and post hoc Tukey's test. The data reported a significance level of $P < 0.05$.

Results

Impact of PBMT on Sperm Parameters

The sperm quantity in the Hyp group was remarkably lower than in the other groups. In addition, the sperm quantity revealed a higher recovery rate in the treatment group than in the Hyp group ($P < 0.0001$). Moreover, sperm motility in the Hyp group remarkably reduced compared to the other groups. Furthermore, sperm motility revealed a higher recovery rate in the treatment groups as compared to the Hyp group ($P < 0.0001$; Figure 1, Table 2).

Impact of PBMT on Stereological Parameters

Impact of PBMT on Total Testicular Volume

The statistical analysis of the stereological study revealed a notable reduction in the testis volume in the Hyp group compared to the control group. However, after inducing laser therapy, the testis volume was remarkably elevated in the Hyp+Laser group compared to the Hyp group ($P < 0.0001$; Figure 2A, Table 2).

Impact of PBMT on the Total Number of Testicular Cells

The quantity of spermatogonia notably declined in the Hyp group than in the Control group. The finding also revealed

Table 1. Primer Design

Genes	Primer sequences	Product Size (bp)	TM (°C)
<i>Ocln</i>	F: AGTTGTGGGAGAAGGGAGAGG R: ACTGGAGATAGGAAAGTGATGGA	156	60
<i>Gja1</i>	F: TGCTATGACAAGTCCTTCCCC R: TGCCGTGTTCTTCAATCCCAT	238	57
<i>Cdh2</i>	F: CCACCTCCGTATCCTCCATCC R: GCGCGAGTGTGTGTATGTG	551	53
<i>GAPDH</i>	F: CAGAACATCATCCCAGCCTCC R: TTGGCAGTTTCTCAAGACGG	152	60

Table 2. Data Presented as Mean $\pm$ SD in Different Groups

Variable	Groups (Mean $\pm$ SD)			P Value
	Control	Hyperthermia	Hyper + Laser	
Sperm count	72.5 $\pm$ 3.30	23.20 $\pm$ 3.32	56.41 $\pm$ 3.53	≤ 0.0001
Sperm motility	69.51 $\pm$ 1.14	32.73 $\pm$ 1.14	61.52 $\pm$ 1.06	≤ 0.0001
Testis volume	33.95 $\pm$ 0.56	5.12 $\pm$ 0.54	13.95 $\pm$ 0.38	≤ 0.0001
Number of spermatogonia	48.94 $\pm$ 1.72	12.47 $\pm$ 1.34	21.20 $\pm$ 1.73	≤ 0.0001
Number of primary spermatocytes	96.54 $\pm$ 4.84	12.65 $\pm$ 4.68	28.39 $\pm$ 4.36	≤ 0.0001
Number of spermatids	105.8 $\pm$ 1.23	8.24 $\pm$ 0.98	37.67 $\pm$ 1.34	≤ 0.0001
Number of sertoli cells	42.65 $\pm$ 1.26	4.42 $\pm$ 0.36	17.73 $\pm$ 1.69	≤ 0.0001
Number of leydig cells	38 $\pm$ 1.11	3.74 $\pm$ 1.02	10.01 $\pm$ 1.16	≤ 0.0001
ROS	142.3 $\pm$ 5.77	187.8 $\pm$ 5.34	159.3 $\pm$ 5.91	0.0006
GSH	98.36 $\pm$ 5.21	10.77 $\pm$ 3.41	45.52 $\pm$ 5.49	≤ 0.0001

Comparison between the groups was done by using the One-Way ANOVA test.

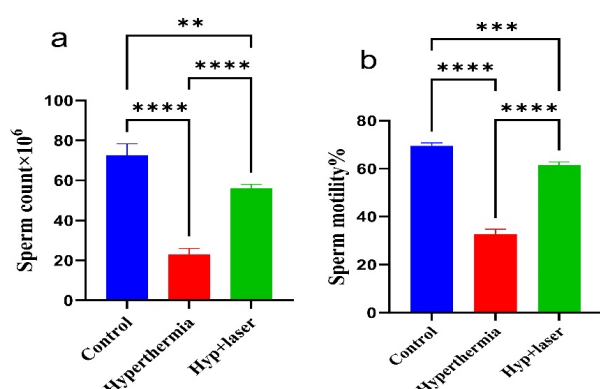


Figure 1. The Effect of PBMT on Sperm Parameters in The Scrotal Hyperthermia Model. (a-b) Mean $\pm$ SD of the total sperm count and sperm motility of the testis in the study groups (five animals per group). * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ and **** $P < 0.0001$

a notable recovery in the quantity of spermatogonia in the Hyp + Laser group than in the Hyp group ($P = 0.0059$; Figure 2B, Table 2).

The quantity of primary spermatocytes exhibited a notable reduction in the Hyp group than in the control group. The data also showed that the quantity of spermatids notably reduced in the Hyp group compared to the control group. However, after inducing laser therapy, we revealed an increase in the quantity of the primary spermatocytes and spermatids in the Hyp + Laser group than in the Hyp group ($P = 0.04$ & $P < 0.0001$, respectively) (Figure 2B, Table 2).

This study also revealed that the quantity of Sertoli and Leydig cells notably declined in the Hyp group than in the control group. But, after inducing laser therapy, there was a notable increase in the quantity of Sertoli and Leydig cells in the Hyp + Laser group than in the Hyp group ($P = 0.0001$ & $P = 0.0033$, respectively) (Figure 2B; Table 2).

Impact of PBMT on the Level of ROS and GSH

The results of this evaluation revealed that the level of ROS in the Hyp group exhibited a significant increase than in the control group. However, after inducing laser

therapy, the Hyp + Laser group demonstrated a notable reduction in ROS level than in the Hyp group ($P = 0.0063$). On the other hand, the level of GSH remarkably reduced in the Hyp group than in the Cont group. However, after inducing laser therapy we observed a notable growth in the Hyp + Laser group than in the Hyp group ($P < 0.0001$; Figure 3, Table 2).

Impact of PBMT on Normalized Gene Expression

Following the outcomes of this investigation, the normalized gene expression levels of *Ocln*, *Gja1*, and *Cdh2* in the Hyp group were notably reduced in comparison with in the control group. However, after inducing laser therapy, the expression levels of *Ocln*, *Gja1*, and *Cdh2* notably elevated in the Hyp + Laser group compared to those in the Hyp group ($P = 0.03$, $P = 0.0013$, & $P = 0.0036$, respectively; Figure 4, Table 3).

Discussion

Our research showed that PBMT can enhance the expression of genes related to the BTB, boost antioxidant levels, decrease free radicals, enhance testicular tissue volume and the number of germ and somatic cells, and improve sperm parameters, leading to an improved spermatogenesis process.

The production of ROS has an inverse relationship with the activity of the electron transport chain, and the decrease in the rate of electron transport in the respiratory chain will be associated with the increase in ROS levels.²⁰ Different conditions, including heat stress, can cause an increase in ROS levels with the decreased synthesis of ATP and the increasing leakage of electrons in the respiratory chain. Our study found that PBM increases ATP and cAMP levels in germ cells, leading to reduced ROS levels.²¹ In this regard, Hasani et al. revealed that treating mice with PBM can cause a decrease in ROS levels.²²

Sertoli cells provide nutrients and support for germ cells during spermatogenesis.²³ Cells that form a BTB shield the germ cells from immune response and harmful substances.

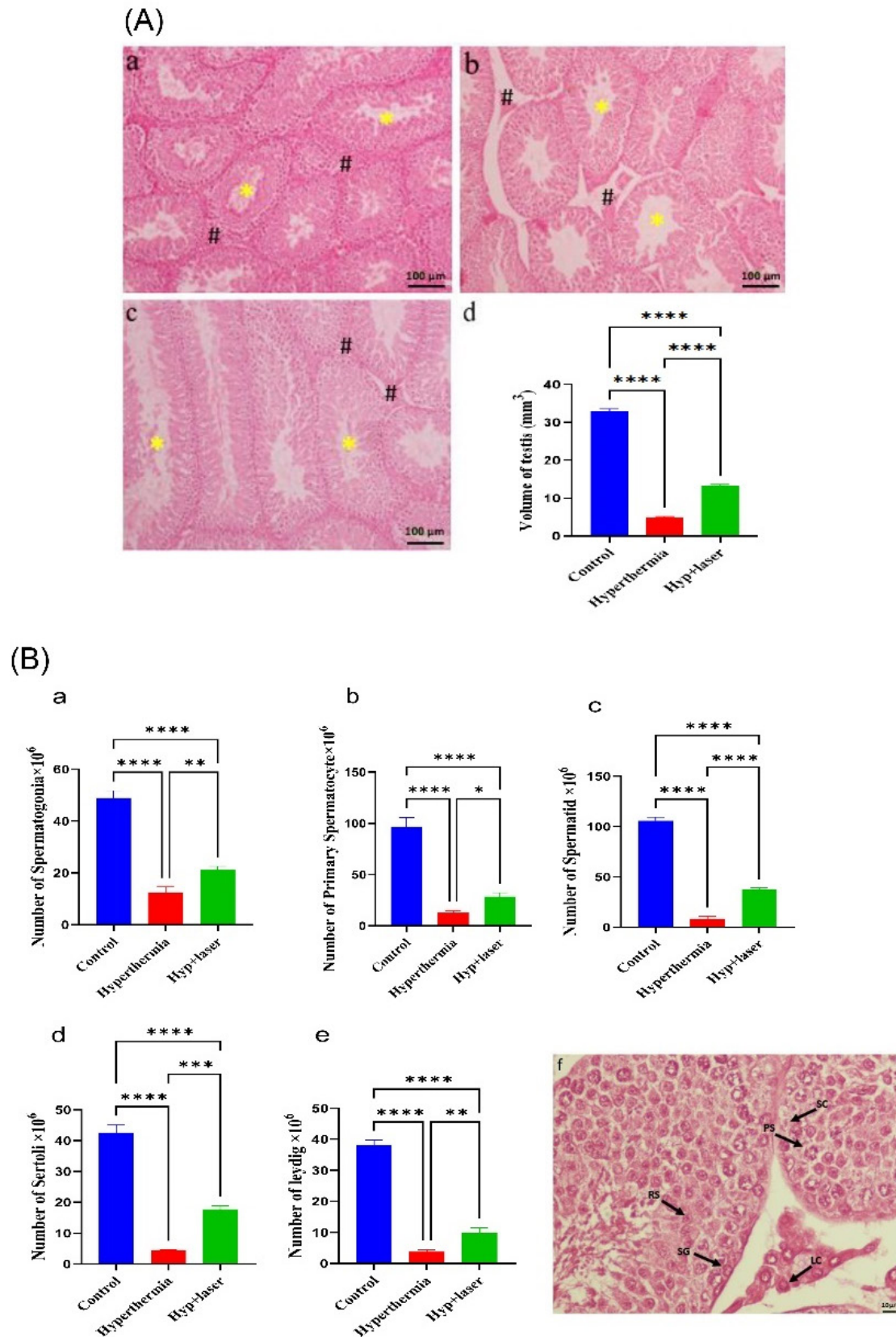


Figure 2. (A) a-c) Photomicrograph of the Testis Stained With H&E, $\times 10$. (a) Control group. (b) Hyperthermia group. c) Hyp+Laser group. Interstitial tissue (#), Seminiferous tubules (*). (d) Mean $\pm$ SD of the total testicular volume in the study groups (five animals per group). * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ and **** $P < 0.0001$. (B) The Effect of PBMT on Total Testicular Cells in the Scrotal Hyperthermia Model. (a-e) Mean $\pm$ SD of the total number of spermatogonia, primary spermatocytes, spermatids, Sertoli and Leydig in the study groups (five animals per group). * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$ and **** $P < 0.0001$. (f) Photomicrograph of the testis stained with H&E, $\times 40$. SG (Spermatogonia), PS (Primary spermatocyte), RS (Round spermatid), SC (Sertoli cell), LC (Leydig cell). Scale bar = 10 μ m

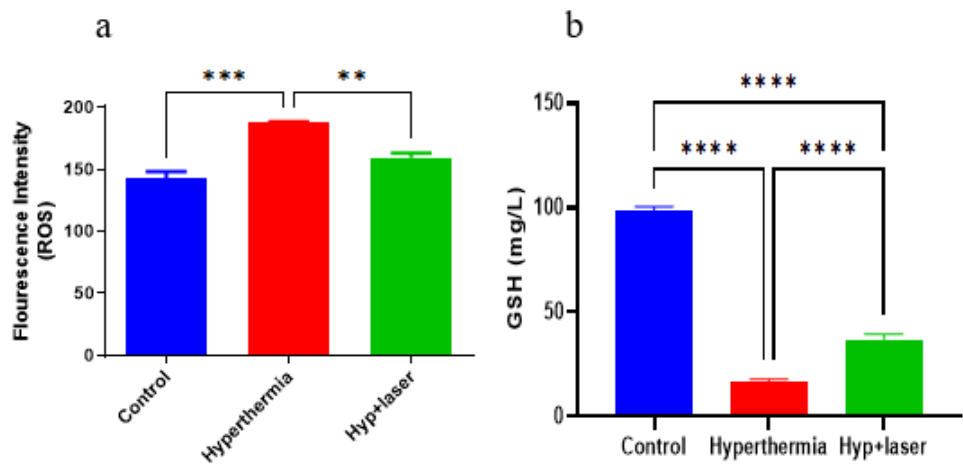


Figure 3. The effect of PBMT on ROS and GSH levels in the scrotal hyperthermia model. (a-b) Mean $\pm$ SD of the ROS and GSH levels of the testis in the study groups (five animals per group). * P <0.05, ** P <0.01 *** P <0.001 and **** P <0.0001

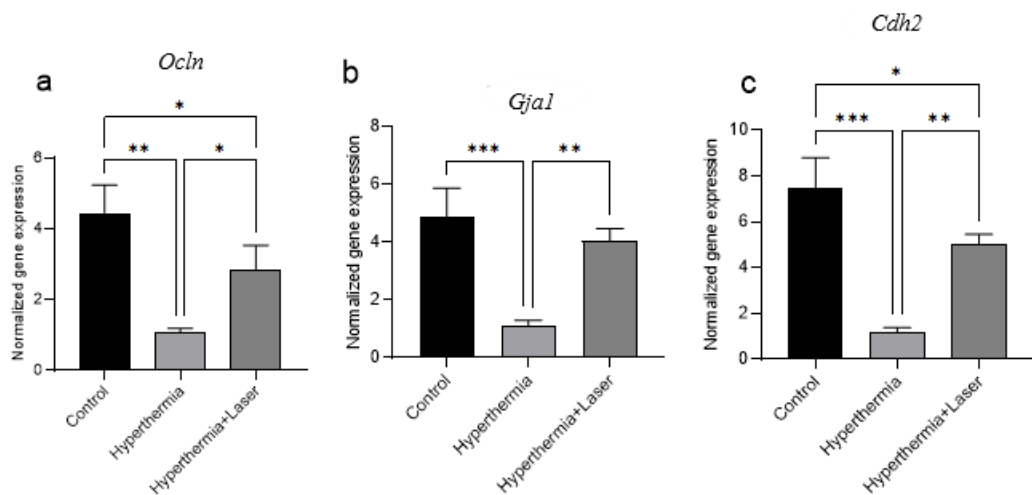


Figure 4. The Effect of PBMT on Normalized Gene Expression Levels in the Scrotal Hyperthermia Model. (a-c) Mean $\pm$ SD of the Gene expression levels of *Ocln*, *Gja1* and *Cdh2* of the testis in the study groups (five animals per group). * P <0.05, ** P <0.01 *** P <0.001 and **** P <0.0001

Table 3. Data Presented as Mean $\pm$ SD of Genes in Different Groups

Genes	Groups (Mean $\pm$ SD)			P Value
	Control	Hyperthermia	Hyper + Laser	
<i>Ocln</i>	4.45 $\pm$ 0.49	1.07 $\pm$ 0.31	2.85 $\pm$ 0.36	0.001
<i>Gja1</i>	4.87 $\pm$ 0.51	1.12 $\pm$ 0.14	4.03 $\pm$ 0.23	0.0008
<i>Cdh2</i>	7.48 $\pm$ 0.66	1.17 $\pm$ 0.08	4.99 $\pm$ 0.81	0.0002

Comparison between the groups was done by using the One-Way ANOVA test.

Increasing levels of ROS caused by heat stress can interfere with the function and structure of Sertoli cells, leading to a decrease in gene expression related to the BTB and affecting the growth and maturation of germ cells.^{24,25}

The Occludin protein, encoded by the *Ocln* gene, plays a key role in the initial formation of the BTB, while the Connexin43 protein, encoded by the *Gja1* gene, helps regulate the integrity of the BTB by controlling the production of proteins involved in tight junctions.²⁶ The

Cdh2 gene encoded N-cadherin strengthens the structure of this barrier by creating an adherence junction and helps the spermatogenesis process and germ cell survival by the intermediation of Sertoli cells with the germ cells. Therefore, the expression of genes related to these proteins regulates the formation and maintenance of the BTB structure.²⁷ We showed for the first time in this study that after PBM, gene expression including *Ocln*, *Gja1* and *Cdh2* remarkably elevated in the treatment group due to heat stress. In line with this study, Aghajanjpour et al also revealed that heat stress can cause a decline in genes related to BTB structure.²⁸

Prior research has demonstrated a link between the integrity of the BTB and sperm quality, especially sperm motility and count. The decreased expression of BTB-related proteins can hinder sperm maturation.²⁸ According to our research, Zhao et al found that heat stress can disrupt the structure of the BTB, leading to a decrease in sperm count and sperm motility.²⁹

Mitochondria are one of the important organelles of the cell, and their regulation of the respiratory chain can be affected by external factors, including PBMT. In this regard, Ott et al have demonstrated that light stimulation can cause a change in function and cell signaling by elevating mitochondrial metabolism, releasing growth factors and cytokines, and reducing ROS generation, and through this, it can cause the improvement of sperm parameters.^{30,31}

Our stereological studies revealed that the total testis tissue volume has a notable increase in the treatment group compared to the hyperthermia group. It is likely that PBM increases the proliferation activity of germ cells through antioxidant activity and reduces the apoptosis of germ cells, preventing the testis tissue volume reduction. In this regard, Ziaei et al also exhibited that PBM, through reducing the apoptosis of germ cells, can cause an increase in the testis tissue volume.³²

After laser therapy was administered, there was a significant increase in the number of germ cells and somatic cells, as indicated by other stereological findings. A decrease in ROS levels due to PBM leads to an increase in mitochondrial metabolism, higher ATP levels, and lower lipid peroxidation, ultimately reducing the death of germ cells.³³ In this regard, Poorhassan et al. revealed that PBM can cause a reduction of germ cell apoptosis.³⁴ Moreover, Tabatabaee et al also exhibited that PBM through-increasing antioxidant activity, can cause a reduction of germ cell death.³¹

Our studies, in confirmation with previous studies, show that the reduction of oxidative stress due to PBM is associated with maintaining the integrity of BTB. Light stimulation, by increasing the expression of genes involved in BTB, can protect germ cells from damage caused by immune cells and improve the spermatogenesis process.³⁵ One of the limitations of this study was the lack of evaluation of the proteins involved in BTB, as well as the lack of examination of cellular apoptosis.

Conclusion

Nowadays, PBMT is a new method for treating men's infertility that can maintain the integrity of BTB in the Sertoli cells, thereby helping the growth and maturation of the germ cells. By increasing the gene expression of *Ocn*, *Gja1* and *Cdh2*, PBMT can maintain the integrity of BTB through signaling pathways related to ROS and can cause the maturation of the sperm. This treatment method, along with other treatment methods such as antioxidants and growth factors, can protect Sertoli cells from environmental damage and thus improve the process of spermatogenesis.

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Supervision: Mohsen Norouzian and Fatemeh Fadaei Fathabadi.

Validation: Hassan Akbari, Hamid Nazarian and Hojjat-Allah Abbaszadeh.

Visualization: Ibrahim Mohammadzadeh, Hassan Akbari and Faraj Tabeie.

Writing—original draft: Reza Soltani and Ibrahim Mohammadzadeh.

Writing—review & editing: Fakhroddin Aghajanzpour, Reza Soltani and Mohsen Norouzian.

Competing Interests

The authors declare that they have no competing interests.

Ethical Approval

All experimental procedures in this study were reviewed and approved by the Ethics Committee at Shahid Beheshti University of Medical Sciences (IR.SBMU.AEC.1401.055).

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