



The Effect of Photobiomodulation on Sperm Parameters and Apoptosis in an Experimental Testicular Torsion Model

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Abstract

Introduction: As a serious urological emergency, testicular torsion (TT) results in ischemia and oxidative stress, negatively impacting sperm function and potentially causing persistent infertility. This research seeks to evaluate the efficacy of photobiomodulation (PBM) therapy as a viable therapeutic intervention for the condition of testicular torsion.

Methods: Male NMRI mice (n=24) were classified into four distinct experimental groups: I) Control, II) laser (L), III) Torsion (T), IV) Torsion accompanied by laser intervention (T&L). We induced testicular torsion by rotating both testes 720° in a counterclockwise direction, and then, after 2 hours, detorsion was performed. In the T&L group, following the torsion induction, the mice's testes underwent laser irradiation at a wavelength of 808 nm (0.03 J/cm²) for 21 days. Post-sacrifice dissection of the testes and epididymis was performed for additional examination.

Results: Sperm motility was significantly higher in the laser-treated group relative to the T group. Furthermore, microscopic evaluation indicated that the T&L group exhibited greater spermatogonia and Leydig cell counts, as well as enlarged seminiferous tubule diameter and thicker epithelium compared to the T group.

Conclusion: On the basis of the present findings, laser therapy can mitigate complications from testicular torsion and improve sperm motility and testicular histopathology.

Keywords: Photobiomodulation; Testicular torsion; Testis; (NMRI) mice.



Introduction

Multiple international studies have reported that testicular torsion affects approximately 3.5 to 4.5 males per 100,000 person-years under the age of 25.^{1,2} This medical condition occurs when the spermatic cord undergoes torsion, which precipitates a compromise in blood circulation to the testis, culminating in acute ischemia manifested by severe pain and edema.³ This condition of acute ischemia has the potential to inflict harm upon the testicular tissue, consequently leading to infertility.⁴ Ischemia severity in testicular torsion is influenced by both twist duration and rotation degree.⁵ Early detection and prompt detorsion surgery are essential for preventing testicular injury. However, it is crucial to recognize that while detorsion surgery restores blood flow to ischemic tissue, it can also potentially increase tissue damage due to the reperfusion process.⁴

Ischemia impairs cellular function by disrupting metabolism and reducing ATP production. Reperfusion leads to an influx of oxygen that causes an increase in

ROS due to rapid tissue reoxygenation.⁶ Spermatozoa's rich content of unsaturated fatty acids renders them especially sensitive to lipid peroxidation resulting from ROS activity. This process can damage their membrane integrity, reduce motility and viability, and ultimately impair their reproductive function.⁷ This oxidative damage also triggers apoptosis in germ cells, negatively impacting spermatogenesis and testosterone synthesis.⁶ Testicular torsion has profound long-term effects on male fertility by impairing sperm quality. A notable percentage of patients suffer from decreased sperm motility and abnormal morphology post-torsion. The severity and duration of ischemia during torsion are key determinants; longer durations correlate with poorer outcomes in terms of motile sperm count. Despite successful surgical interventions like detorsion or orchiectomy, many individuals continue to experience compromised semen quality over time.^{8,9}

Photobiomodulation (PBM) enhances the antioxidant defenses in the testis, effectively reducing oxidative

stress and cellular damage by increasing the levels of protective enzymes like glutathione peroxidase. This action helps reduce lipid peroxidation and shields germ cells from oxidative insults. Additionally, PBM promotes spermatogenic cell proliferation and differentiation, thereby increasing sperm production. Experimental models demonstrate that PBM increases the number of key cell types involved in sperm development, such as spermatogonia and spermatocytes. Furthermore, histopathological assessments indicate that PBM improves the structural integrity of seminiferous tubules after torsion, enhancing such parameters as tubule diameter and epithelial thickness, which are indicative of better testicular architecture and function.^{10,11}

Moreover, PBM modulates apoptosis-related proteins, including Bax and Bcl-2, shifting the balance towards cell survival and reducing germ cell apoptosis induced by ischemia-reperfusion injury.¹² PBM restores spermatogenesis by adjusting the balance between pro-survival (Bcl-2) and pro-apoptotic (Bax) proteins in stressed testicular tissues.¹³

Existing studies have shown that laser therapy significantly decreases apoptotic cell counts, enhances spermatogenic cell numbers, and boosts testosterone levels. It also increases ATP production while reducing reactive oxygen species (ROS). Based on the prior observations, this work explores the role of photobiomodulation in modulating sperm quality and apoptosis in testicular tissue following experimentally induced torsion in NMRI mice.

Methods

Animals

A total of twenty-four male NMRI mice, weighing between 25 and 30 grams, were utilized for this study. Having unrestricted access to food and water, they were maintained under standardized conditions and kept on a 12-hour light and dark cycle. The experimental protocol received approval from the University Ethics Committee (IR.ARAKMU.AEC.1403.003). To ensure accurate identification and tracking throughout the study, each mouse was marked using a non-invasive tail marking technique with a non-toxic marker. This method was selected to minimize stress and avoid any physiological or behavioral alterations associated with more invasive identification methods. All marking procedures were performed by trained personnel according to institutional and international animal welfare guidelines. The marks were renewed as needed during the study using gentle handling. Afterward, the animals were randomly assigned to four groups, with six mice in each: (I) Control, (II) Laser (L), (III) Torsion (T), and (IV) Torsion combined with Laser (T&L).

Surgical Technique

To anesthetize the animals, ketamine (100 mg/kg, EXIR,

Iran) and xylazine (10 mg/kg, Serumwerk Bernburg, Germany) were administered intraperitoneally. The abdominal area was prepared for surgery by cleaning and shaving the skin. To access the abdominal cavity, incisions were made through the skin and abdominal muscles. For the testicular torsion procedure, both testes were carefully exteriorized from the scrotum, positioned inside the abdominal cavity, and then rotated 720 degrees counterclockwise for a period of two hours. After this duration, the testes were repositioned to their normal location, and the abdominal muscles and overlying skin were carefully sutured to close the incision.¹⁴ Immediately following detorsion, the first dose of laser radiation was administered directly to the left and right testes prior to their repositioning into the scrotum. The testicles were then carefully restored to the scrotum, followed by closure of the abdominal muscles and skin with 4-0 silk sutures. After fully awakening from anesthesia, the animals were transferred back to their housing cages.

PBMT

Following the induction of testicular torsion/detorsion, photobiomodulation therapy (PBMT) was initiated using an Infrared Laser (Unix laser 1600 model, Arman Pooya Co., Isfahan, Iran) with specific parameters: a 1 cm² irradiation area, an 808 nm wavelength, and a radiant exposure of 0.03 J/cm². PBMT was applied thrice weekly over a period of 21 days.¹⁵ The first dose of laser radiation was applied directly to both testes for one minute immediately after detorsion and before returning them to the scrotum. Following recovery, the animals were then placed back into their cages, and the subsequent doses of radiation were given as scheduled.

Sampling

Twenty-one days after the laser treatment, all animals were heavily anesthetized and then sacrificed. The left testis and cauda epididymis were then carefully removed. The epididymal tail was designated exclusively for sperm parameter analysis. While one testis was fixed in 10% formalin for microscopic evaluation, the contralateral testis was quickly snap-frozen in liquid nitrogen and preserved at -70°C for molecular research.

Sperm Parameters

To perform sperm analysis, the epididymal tail was carefully dissected from surrounding tissues and then immersed in a dish containing Ham's F10 solution. The samples were maintained at 35°C for 15–20 minutes to enable the sperm to spread out. A 10 µL aliquot of each sample was transferred onto glass slides for microscopic evaluation, where progressive and non-progressive motility of sperm was observed. Sperm counts were conducted using a Neubauer slide. Furthermore, eosin-nigrosin dye was utilized to evaluate sperm viability.¹⁶

Testis Histopathology

The testes, preserved in 10% formalin (Asia Chem), underwent dehydration and paraffin embedding, and they were then sectioned into 5- μ m-thick slices, which were subsequently stained with hematoxylin and eosin. The spermatogenesis index¹⁷ was evaluated by examining 10 randomly selected microscopic fields per specimen. In at least 10 seminiferous tubules per animal, parameters including the counts of spermatogonia and Leydig cells, seminiferous tubule diameter, and epithelial height were quantified using ImageJ software (version 1.52) at 20 $\times$ magnification.¹⁸

RNA Extraction and cDNA Synthesis

Samples were thawed on ice following retrieval from the freezer. Total RNA extraction was conducted using the RNX Plus kit (CinnaGen, Iran), with RNA resuspended in DEPC-treated water (SinaClon, Iran). Quality and concentration of RNA were assessed by absorbance ratios (260/280 nm) using a NanoDrop device. Two micrograms of RNA were utilized to generate cDNA via a specific kit (Yekta Tajhiz, Iran). The synthesized cDNA was then preserved at -70°C until further processing.

Real-time Polymerase Chain Reaction (Real-time PCR)

The expression of Bax, Bcl-2, and GAPDH (as the internal control) was evaluated by Real-time PCR following RNA isolation and cDNA generation. The Roche LightCycler 96 device was used for amplification. Each reaction mixture volume was 20 μ L, containing 2 μ L of diluted cDNA, 0.5 μ L of primers (outlined in Table 1), 10 μ L of SYBR Green reagent (Yekta Tajhiz Azma, Iran), and 7 μ L of nuclease-free water. Reactions were performed in duplicates. Cycle threshold (CT) data were collected, and relative expression levels were calculated using the $\Delta\Delta$ CT approach.

Statistical Analysis

Using GraphPad Prism software (version 10, GraphPad Prism Software Inc., San Diego, CA), statistical comparisons between the control group and the experimental group over multiple time points were carried out through one-way ANOVA and Tukey's post hoc test. Statistical significance was defined by a p-value of 0.05 or less.

Table 1. Sequences of the primers used for the target genes

Gene	Primer sequences (5'-3')	Product length (bp)
Bax Sense Antisense	CTA CAG GGT TTC ATC CAG CCA GTT CAT CTC CAA TTC G	133
Bcl2 Sense Antisense	CCT GTG GAT GAC TGA GTA CCT G AGC CAG GAG AAA TCA AAC AGA GG	123
GAPDH Sense Antisense	AGC AAG GAC ACT GAG CAA GAG GCA GCG AAC TTT ATT GAT GGT	152

Results

Sperm Parameter Analysis

The data indicated a marked reduction in the total sperm count in the group experiencing torsion in comparison with both the control group and those treated with laser therapy ($P < 0.001$) (Figure 1.a). No notable distinction was observed between the torsion group and the torsion combined with the laser group (Figure 1a). Additionally, sperm motility was notably lowered in the torsion group versus the control group ($P < 0.01$) (Figure 1b), whereas laser treatment significantly improved motility compared with the torsion group ($P < 0.001$) (Figure 1b). Sperm viability was also significantly reduced in the torsion group when compared with the control and laser groups ($P < 0.001$) (Figure 1c); although laser administration improved viability compared to the torsion group, this increase was not statistically significant (Figure 1.c).

Testis Histopathology Evaluation

Under microscopic evaluation, the torsion group exhibited notably fewer spermatogonia and Leydig cells than both the control and laser treatment groups ($P < 0.001$) (Figures 2, 3a-b). Alongside these findings, the torsion group showed a significant decline in the diameter of seminiferous tubules, epithelial height, and spermatogenic index relative to the other groups ($P < 0.001$) (Figures 2, 4a-c). Following laser therapy, there was a marked elevation in spermatogonia and Leydig cell populations (Figures 2, 3a-b), coupled with improvements in the seminiferous tubule diameter, epithelial layer height, and spermatogenic index compared with the torsion group without the laser. ($P < 0.001$) (Figures 2, 4a-c).

The Effects of Laser Therapy on Bax and Bcl-2 Gene Expression

The investigation focused on how laser therapy influenced the expression of the apoptosis-related genes Bax and Bcl-2 in testicular tissues. The findings demonstrated that Bax expression was markedly higher in the torsion group compared to both the control and laser-treated groups ($P < 0.001$ and $P < 0.05$, respectively) (Figure. 5). Although Bcl-2 expression was lower in the torsion group, this reduction did not reach statistical significance (Figure. 5). Furthermore, laser therapy resulted in a decrease in Bax gene expression that was not statistically significant, but it significantly increased Bcl-2 expression in the group receiving both torsion and laser treatment relative to all other groups ($P < 0.001$) (Figure. 5).

Discussion

Testicular injury triggered by ischemia-reperfusion (I/R) results from a complex cascade of events that ultimately damage the tissue. Initially, ischemia, caused by interrupted blood supply to the testis, leads to oxygen and nutrient deprivation, triggering cellular damage and death.¹⁹ In

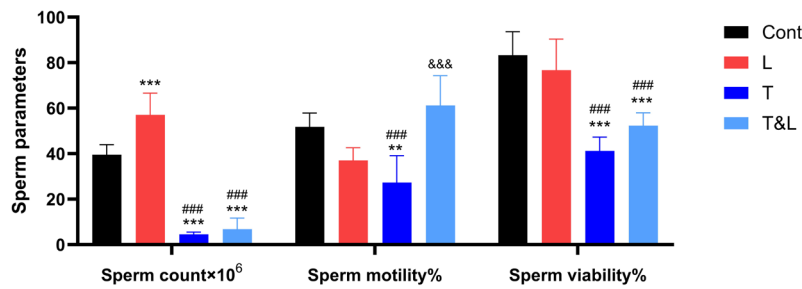


Figure 1. The impact of PBMT on sperm parameters, including sperm count, sperm motility, and sperm viability. Data are presented as mean $\pm$ SD based on measurements from the study groups (* P <0.05, ** P <0.01, and *** P <0.001). * Indicates comparisons with the control group, # indicates comparisons with the Laser group, and & indicates comparisons with the Torsion group

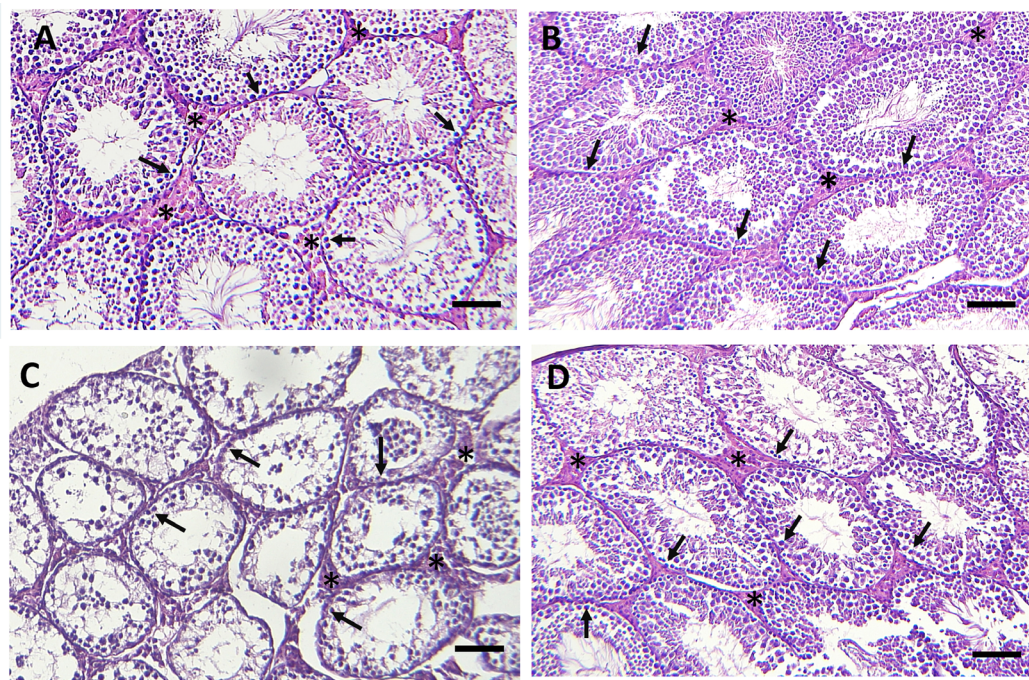


Figure 2. The effects of Laser on testis histopathology of different experimental groups. The bar represents 50 μ m.; arrowhead, spermatogonia; star, Leydig cells. A. Control, B. Laser C. Torsion, D. Torsion + Laser

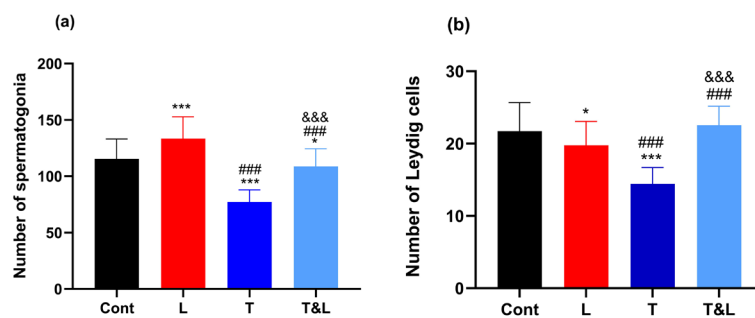


Figure 3. Comparison of the number of spermatogonia (a) Leydig cells (b) in different experimental groups. Laser treatment considerably increased the number of spermatogonia and Leydig cells. Data are presented as mean $\pm$ SD based on measurements from the study groups (* P <0.05, ** P <0.01, and *** P <0.001). * Indicates comparisons with the control group, # indicates comparisons with the Laser group, and & indicates comparisons with the Torsion group

the present study, spermatic cord twisting was induced by turning both testes 720 degrees counterclockwise, followed by detorsion after 2 hours. As anticipated, the

quality of sperm and the condition of testicular tissue were damaged after the surgical procedure.

Our findings indicate that (I/R) injury in the testes

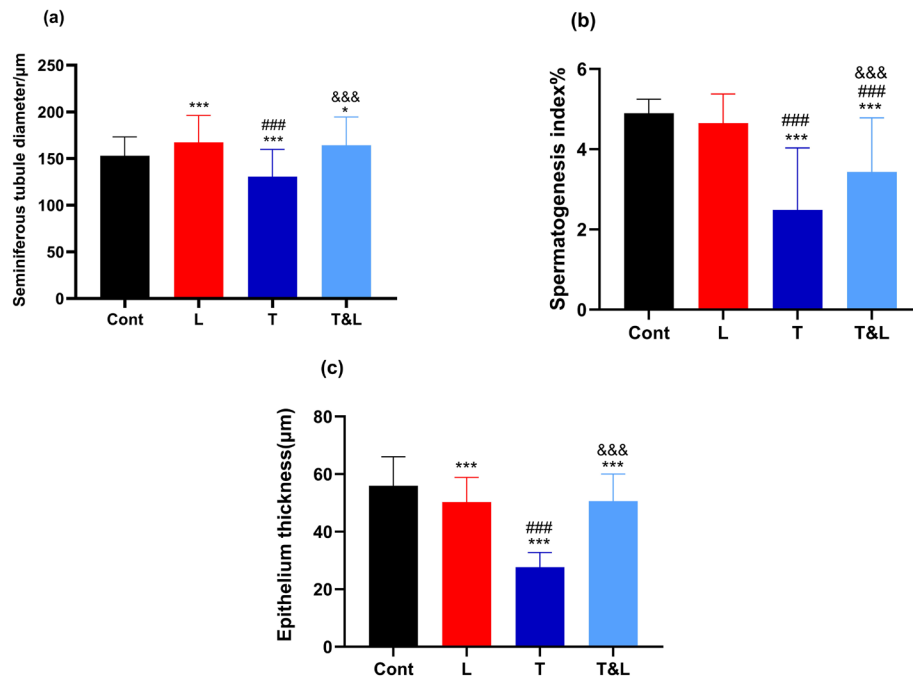


Figure 4. Seminiferous tubules diameter (a), spermatogenesis index (b) and epithelium thickness (c) are presented in different experimental groups. Data are presented as mean $\pm$ SD based on measurements from the study groups (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). * Indicates comparisons with the control group, # indicates comparisons with the Laser group, and & indicates comparisons with the Torsion group

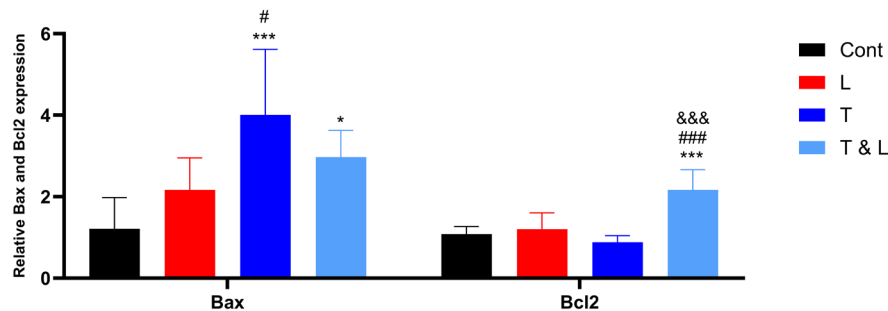


Figure 5. Real-time PCR analysis of mRNA expression levels of Bax and Bcl2 across various experimental groups. The data are presented as mean $\pm$ SD, based on measurements from the study groups (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). * indicates comparisons with the control group, # indicates comparisons with the Laser group, and & indicates comparisons with the Torsion group

significantly reduces sperm count, motility, and viability, as well as a decrease in the count of spermatogonia and Leydig cells. Furthermore, there was a notable reduction in the epithelium's thickness and the seminiferous tubules' diameter. Additionally, the expression levels of Bax were elevated. However, PBM therapy was able to mitigate these histological damages and improve some sperm parameters. Testicular torsion, recognized as a prevalent urological emergency in pediatric populations, induces a severe restriction of blood flow to the testicular tissue. Prior investigations demonstrate that ischemia-reperfusion (I/R) events within the testes can induce morphological injuries to the tissue, which may ultimately lead to testicular atrophy and subsequent male infertility.⁴

Our findings corroborate reports by Hussain et al who linked hypoxia and elevated reactive oxygen species

(ROS) levels to reduced testicular metabolic activity and diminished sperm viability. Accordingly, sperm counts were markedly lower in the torsion-detorsion models. ROS-mediated oxidative stress damages cellular organelles and impairs mitochondria in spermatozoa, negatively affecting motility.²⁰

Microscopic analysis indicated a decline in spermatogonia and Leydig cell populations, as well as reductions in the epithelial height and the seminiferous tubule size in the torsion group. The reperfusion phase, characterized by sudden oxygen and nutrient influx, exacerbates pre-existing tissue damage.²¹ Germ cells exhibit a heightened vulnerability to ischemic injury owing to their elevated metabolic demands and reliance on a consistent supply of oxygen and nutrients for the process of spermatogenesis. Thus, the interruption of blood flow

and oxygen delivery during ischemia can precipitate the demise of these cells, resulting in testicular damage and impaired fertility.²² Conversely, the deficiency of oxygen during ischemia contributes to the accumulation of ROS, which can instigate lipid peroxidation within cellular membranes, induce DNA damage, and promote protein denaturation.²³

Immediately following the induction of testicular torsion/detorsion, PBMT was initiated. Our study demonstrated that three weeks of laser irradiation significantly improved sperm motility in the ischemia/reperfusion group, corroborating previous research showing the positive influence of laser irradiation on sperm quality.²⁴ Low-level laser therapy (LLLT) enhances mitochondrial activity, improving ATP generation and possibly enhancing sperm motility in asthenozoospermia.²⁵ The uptake of photons by cytochrome c oxidase (CCO) accelerates electron transfer reactions, boosting ATP synthesis.^{26,27} Nitric oxide (NO) is essential for regulating sperm motility and acrosome activity,^{28,29} with light exposure stimulating NO production through nitric oxide synthase (NOS) activation.³⁰ Visible light also increases cellular Ca²⁺ levels, further activating NOS and raising NO concentrations in sperm and endothelial cells. Additionally, light reverses the inhibitory effects of NO binding to CCO, promoting higher respiration rates in both isolated mitochondria and whole cells.³¹

PBM treatment also induced an augmentation in spermatogonia and Leydig cell counts, along with the thickening of the seminiferous tubule epithelium, which collectively suggests an enhancement in spermatogenic activity. These changes suggest that PBM improves testicular health by stimulating cell proliferation and differentiation and by reinforcing the blood-testis barrier to support sperm development.^{32,33} Histological analyses further demonstrate enhancements in testicular structure, with increases in Leydig and Sertoli cells—both crucial for sperm production.¹⁶ Additionally, laser treatment has anti-inflammatory properties that reduce edema and promote recovery following testicular injury³⁴. Also, PBMT induces vasodilation through the release of chemicals like histamine, which enhances cellular metabolism by increasing oxidative phosphorylation.³⁵

The absence of significant changes in the sperm count following testicular torsion with laser treatment can be attributed to the incomplete coverage of the spermatogenic cycle by the therapy. Spermatogenesis in mice requires approximately 35 days to progress through various stages, from spermatogonia to mature spermatozoa.³⁶ However, since laser therapy was administered for only 21 days after testicular torsion/detorsion, it did not encompass the entire spermatogenic process. As a result, the treatment primarily influenced the earlier stages of germ cell development, which explains why its effects on mature sperm production were not yet observable within this

timeframe.

Real-time PCR analysis confirmed elevated Bax and reduced Bcl-2 expression three weeks following torsion, consistent with ischemic hypoxia leading to oxidative damage. Reperfusion further increases ROS production, a key mechanism underlying testicular I/R injury.³⁷ Excess ROS leads to DNA damage and protein misfolding, and it triggers lipid peroxidation.^{37,38} During I/R, cells can become overwhelmed by the stress, leading to cell death. Apoptosis is the primary mechanism by which this occurs. This apoptosis process is initiated by signaling mechanisms that operate via both extrinsic and intrinsic pathways.³⁹ The apoptotic pathway functions through a series of caspase-mediated events that progress from initiation to execution stages. In this process, the Bcl-2 protein family plays a central role, with Bax promoting apoptosis and Bcl-2 acting to inhibit it.⁴⁰ Our results confirm increased Bax and decreased Bcl-2 post-torsion, echoing earlier reports.^{4,41}

Bcl-2 stability and activity are influenced by reactive oxygen species, which promote its degradation through the ubiquitin–proteasome mechanism. Conversely, nitric oxide counteracts this effect by preventing Bcl-2 breakdown, thereby contributing to the regulation of apoptosis.^{42,43}

Following three weeks of laser irradiation after torsion/detorsion, Bax gene expression decreased, though not significantly, while Bcl2 expression was markedly higher than that of the torsion/detorsion group. This suggests that laser light, by activating mitochondrial photoreceptors, modulates cellular signaling and may improve outcomes in stressed cells.⁴⁴ PBM's ability to reduce ROS levels and improve cellular functions supports its role in promoting recovery from oxidative stress.⁴⁵

One proposed mechanism involves nitric oxide (NO) generated during stress binding to mitochondrial cytochrome c oxidase, impairing electron transport and inducing respiratory chain inhibition that may cause cell death.⁴⁶ PBM may alleviate this by releasing NO from these binding sites, restoring mitochondrial respiration, and maintaining ROS within non-toxic levels.⁴⁷

Conclusion

Our study reveals that three weeks of laser irradiation significantly enhances sperm motility in ischemia/reperfusion models while promoting testicular health through improved cellular processes. Despite the lack of significant changes in the sperm count after testicular torsion, the positive effects on spermatogenesis and anti-apoptotic gene expression indicate potential therapeutic benefits of PBM therapy. We recommend that future research in this area concentrate on inflammatory markers and oxidative stress.

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Competing Interests

The authors confirm that no conflicts of interest exist in relation to this research.

Ethical Approval

This study has been approved under the ethical code IR.ARAKMU.AEC.1403.003.

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