The effects of low-power laser on the promotion of spermatogenesis in a mouse model of azoospermia (in-vivo)

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Received: Aug. 10, 2022, Revised: Oct. 2, 2022, Accepted: Nov. 21, 2022, Available Online: Dec. 28, 2022
DOI: 10.30495/ijbbo.2022.1964975.1014

ABSTRACT— In this paper we investigate the effect of low power laser on spermatogenesis in testicular tissue of azoospermia mouse model in-vivo. In this experimental work, 112 adult male Syrian mice were randomly divided into three main groups: negative control group, positive (Azoospermia control) group, and experimental group, but to determine the best dose of laser radiation three experimental groups were tested. To create azoospermia control group, Busulfan was used at a dose of 30mg/kg, for 21 days by intraperitoneal injection. In the experimental groups after Busulfan treatment, they were applied by the low power diode laser (wavelength of 808nm) with three different energy densities of 2, 4, and 8 J/cm^2. The employment of a laser with an energy density of 8 J/cm^2 was shown to be beneficial in boosting germ cell and sperm production.

KEYWORDS: spermatogenesis, azoospermia, laser therapy, infertility

I. INTRODUCTION

Infertility is described as a man or woman's failure to conceive pregnancy following 12 months of unprotected sexual activity. This condition affects up to 15% of the world's population, with a male factor accounting for 50% of cases [1-3].

Male infertility is a multifactorial disorder that encompasses a wide variety of diseases and is a sign of a wide range of pathological conditions affecting both the sexual and other physiological systems, including the endocrine, neurological, blood and immunological systems [4-6].

According to the recommendations of the World Health Organization (WHO) (2000), 16 main nosologies are distinguished, each of which, in turn, includes upwards of several dozen specific pathogenetic factors, 4 of 16 diagnoses are descriptive, without indicating

the true causes: idiopathic oligo-, astheno-, terato- and azoospermia [7, 8].

Diseases such as cancers of the reproductive system in young men, bacterial infections, some genetic syndromes such as praderovirus (PWS), and mutations in fertility genes can cause azoospermia in men [9]. Azoospermia means the lack of sperm in the semen. One percent of all men and ten percent of infertile men have azoospermia. In many of these men, there are no recognizable signs of semen appearance. The volume and shape of semen are normal and the majority of these men have normal sexual desire and sexual functioning except of the fertility problems [10]. There are several factors involved in the development of azoospermia. Non-obstructive genetic azoospermia is caused by hormonal and genetic disorders, trauma to the testicles and blood vessels, varicocele, and so on. Factors that may cause obstructive azoospermia includes vasectomy, congenital absence of the seminal vesicles, obstruction of the epididymis for unknown reasons, or acquired epididymal obstruction that can be due to infections, especially sexually transmitted infections or some inherited diseases such as Cystic fibrosis (CF) noted [11].

Today, various methods are used to treat infertility, including IUI[1], IVF[2], ICSI[3], and laser therapy. However, each of these methods is used for a specific range of infertile people [12].

Laser therapy, which is a modern method of physiotherapy, is an example of interdisciplinary medicine based on research in the fields of physiology, biophysics, and biochemistry [13]. Common methods used to improve and increase fertility in animals and humans are based on the use of drugs or direct injection of sperm into the egg, which is not always effective [14]. Laser light irradiation is an effective alternative to conventional syringe injection because, in addition to being safe and non-invasive, it is also a suitable and practical method for patients who are afraid of syringes [15].

Laser therapy is widely used in modern medicine due to its high efficiency, ease of use and lack of side effects. The natural and biological effect of this method on tissues and attempt to promote tissue repair or reduction of inflammation depend on dose of energy [13, 16] that is consisting of the application of light in continuous or pulsed wave modes within the near-infrared range (600 to 1100 nm) [17]. It is a non-invasive remedy that applies energy densities and wavelength to penetrates different tissue layers thereby leading to activate different cellular mechanism and [18] develop new systems and responses [16].

The use of laser with optimum wavelength and dose of energy in human and animal cell culture and at the in-vivo condition elicits responses at the molecular, cellular, and tissue level. These responses include cell proliferation, tissue repair, increase in metabolism, mitochondrial activity, proliferation, migration, adhesion, differentiation, extracellular matrix secretion, and mineralization, as well as the inhibition of apoptosis that have been reported in the literatures [19-23].

The specific performance of laser irradiation on sperm has been investigated in animal models. In these studies, which used lasers with different powers, different radiation energies, and different radiation durations, the results showed that the use of lasers with low power and low energy density has the most appropriate effect on the quantitative and

[1] Intrauterine insemination
[2] In vitro fertilization
[3] Intracytoplasmic sperm injection

qualitative parameters of sperm. For example, in a study by N. Laffaldano et al. the quality of turkey sperm cells was evaluated. The results showed that exposure to radiation at 3 J/cm^2 centimeters increased the survival rate of turkey sperm compared to the control group [24]. In another study by Corral-Baqués MI et al., dog sperm cells were subjected to a continuous-wave diode laser with a 655-nm at 4, 6 and 10 J/cm^2 energy density. Results showed improvement at the motility features and maintain its functional characteristics [25].

The effects of Low-Level Light Therapy (LLLT) on cellular function arise predominantly from stimulation of ATP production and reduction of oxidative stress. These effects are dose-dependent and a function of beam irradiance and irradiation time. Human sperm motility has been shown to increase with LLLT irradiation [26]. In 2018, C. Philip Gabel et al, examined the effects of low-level laser therapy on the function and motility of human sperm along the DNA integrity of these cells. By comparing the control group and considering the fact that the effects of lasers depended on the amount of radiation and irradiation time, the results showed a four-fold increase in ATP production in spermatozoa [26].

These findings also provide insights into the effect of laser light on increasing the fertility in humans. A 2012 study by Ross S. Firestone examined the effects of the low-level laser light on sperm motility and DNA damage. The collected human specimens were classified as normozoospermia, oligospermia, or asthenospermia. The samples were treated with a 30-second pulse of a 905 nm and 30 MW infrared laser. The results show an increase in the optical sensitivity of cytochrome c oxidase in the electron transport chain in the mitochondria. A significant increase in motility was most characteristic in oligospermia and asthenospermia. The specimens were observed after treatment with the observation of no increase in DNA damage [27].

Although laser therapy is beneficial in the treatment of many tissue damages, little is known regarding its effect on the testis and spermatogenesis. The proper development of germ cells depends on the proper functioning of all the cells in the testicular tissue. Seminiferous tubules, Sertoli, and Leydig cells make up the testis, which produces sperm and testosterone. Sperm cells are generated during a process that is called spermatogenesis. Stem cell spermatogonia, which produces spermatozoa through a series of cell division and differentiation processes known as spermatogenesis, is very sensitive to radiation. When spermatogonia are exposed to radiation, DNA damage occurs, resulting in cell cycle arrest. If the level of DNA damage is severe enough, the entire population of spermatogonia may perish, resulting in infertility [28]. Considering the fact that some studies show that several types of spermatogonia are resistant to radiation [29-31], biochemical and topological analyzes show that spermatozoa exposed to laser have high fertility rate [32]. Therefore, the production of sperm may vary depending on the radiation dose and dose rate.

Due to the importance of fertility in men and the problems caused by azoospermia that causes infertility, this study was performed to determine the effect of low energy laser beam density on seminiferous tubules and spermatogenesis in testicular tissue of in-vivo azoospermic mice.

II. Materials and Methods

In our experiment, adult male mice weighing approximately 35+/-5 g were kept in a constant temperature environment (22+/-2 °

C) with 12 hours of light and darkness periodically. Of course, no restrictions were applied at no time. with the same water and food conditions and without restrictions, were used.

At this stage of the study, 112 mice were randomly divided into 3 groups: negative control group, positive control group (azoospermia control), and experimental group. Negative control mice were not treated under any conditions. To create azoospermia in positive control mice, Busulfan was used at a dose of 30mg/kg [33, 34], 21 days by intraperitoneal injection. In the experimental groups after Busulfan treatment, they were affected by low-power diode lasers at three different energy densities.

The experimental groups were exposed to a diode laser (wavelengths 808nm) with different energy densities (2, 4, 8 J/cm^2). The area in the left testes of mice to be irradiated was shaved and cleaned with alcohol before each treatment. The light beam was applied once every other day for 21 days.

In the next step, the mice of the negative control group, positive control group, and experimental groups were killed by ether anesthesia after 40 days, and sampling was performed on their testes using histomorphometric measurement. testes were prepared in tissue sections.

Finally, the prepared sections were stained, and microscopic studies were performed on negative control group, positive control group, and experimental groups by morphological criteria. Microscopic images were evaluated using software Cellness.

In each group, the diameter of the seminiferous tube, the diameter of the epithelium, the area of the epithelial cell in the seminiferous tubule, and the diameter of the seminiferous duct were measured and statistically compared with magnifications 40x, 100x and 200x respectively. For all groups, mean and standard deviation were calculated. Data were analyzed by one-way ANOVA tests with software SPSS. The p-value was considered less than 0.05.

III. RESULTS

A. *Morphological findings of testicular tissue in the studied groups*

Seminal vesicle degradation and a significant reduction in the number of germinal epithelial cells are seen in the testicular histology of the azoospermic group. A relatively limited number of spermatogenesis cells are observed, which are mainly spermatogonial cells. Interstitial tissue has developed and the diameter of the seminiferous tubules has decreased. The wall of most seminiferous tubules is empty of spermatogenesis cells (Figure1). An increase in the thickness of the epithelial tissue of the tubes was detected in the groups that are exposed to the laser at 2 J/cm^2 and 4 J/cm^2, and repair in these tubes was seen initially (Fig. 2, 3). A small number of spermatogenesis cells are found in the walls of the seminiferous tubules. In the group that is exposed to the laser at 8 J/cm^2, the process of testicular tissue repair was faster, and the diameter of the tubes and the thickness of the tubular epithelium increased, which indicates the improvement of pathological testicular lesions and also the number of germ cells (Fig. 4).

B. *Histomorphometric findings of testicular tissue:*

In the groups which received the laser at 2J/cm^2 and 4J/cm^2, the diameter of the seminiferous tubules and the thickness of the epithelium of the tubes were significantly increased ($P < 0.05$) compare to the azoospermia group. But there were not significant different between the group which

were irradiated by the laser with 2 J/cm^2 and 4 J/cm^2 (Figure 5). In the group receiving the laser at 8 J/cm^2, the diameter of the seminiferous tubules and the thickness of the epithelium of the tubes showed a significant increase compared to the 2 J/cm^2 and 4 J/cm^{2}groups (P <0.05) (Fig. 6).

Examination of interstitial tissue thickness in different groups showed that the groups received irradiation had a significant decrease compared to the azoospermic group, but there was no significant difference between the groups that were exposed to different energy densities (Fig. 7).

C. *Testicular spermatogenesis findings:*

Evaluation of T.D.I coefficient in different groups showed that the regeneration coefficient in the groups that received the laser at 2 J/cm^2 and 4 J/cm^{2}compared to the azoospermia group increased significantly (P <0.05). But there is no significant difference between the groups which were irradiated by the laser with 2J/cm^2 and 4J/cm^2. The group receiving laser at 8 J/cm^2, showed a significant increase compared to the groups that received laser at 2 J/cm^2 and 4 J/cm^2 (P <0.05) (Fig. 8).

Evaluation of the S.I. coefficient in different groups did not show a significant difference between the groups were received laser irradiation (Fig. 9).

On the other hand, the evaluation of R.I in different groups showed that the group received the laser irradiation of 8 J/cm^2 there was a significant increase compared to the other laser irradiated groups (Figure 10).

A comparison of the mean of evaluated parameters in the studied groups is shown in Table 1

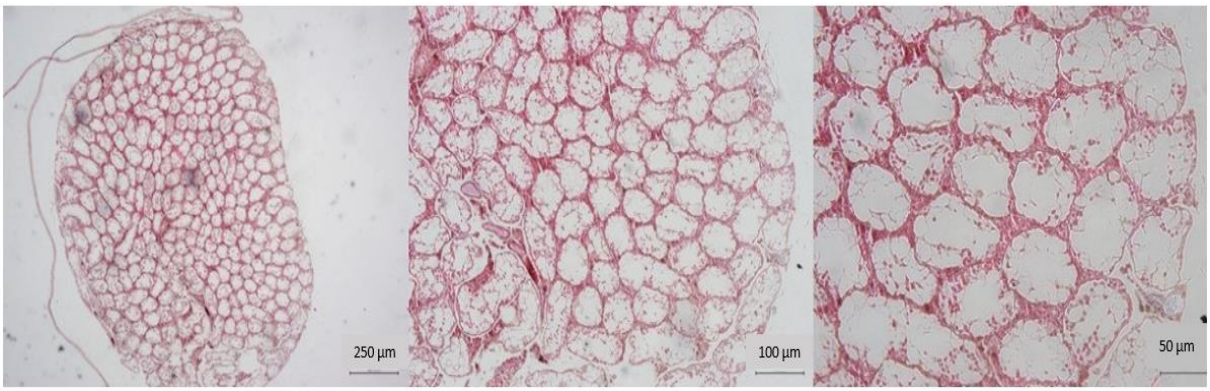

Fig. 1 Microscopic view of testicular tissue in azoospermia group: reduction of seminiferous tubules diameter and severe reduction of tubular epithelial thickness is observed. Spermatogenesis cells are found in very small numbers in the walls of the tubes. (40x,100x and 200x magnification, hematoxylin-eosin staining)

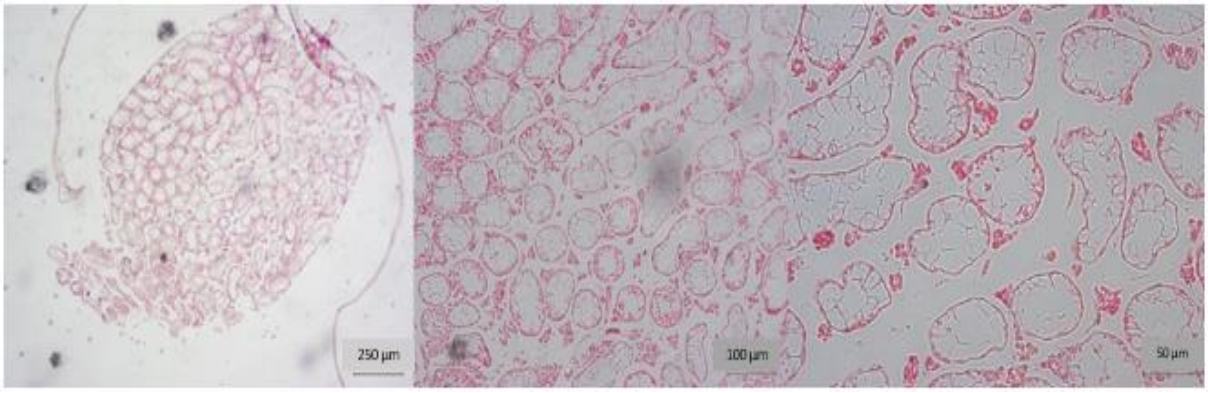

Fig. 2 Microscopic view of testicular tissue in azoospermia group with 2J/cm^2 energy density irradiation: Testicular tissue repair is initially evident, increase in seminiferous tubule diameter and tubular epithelium repair are seen partly. The number of spermatogenesis cells has increased slightly. (40x,100x and 200x magnification, hematoxylin-eosin staining)

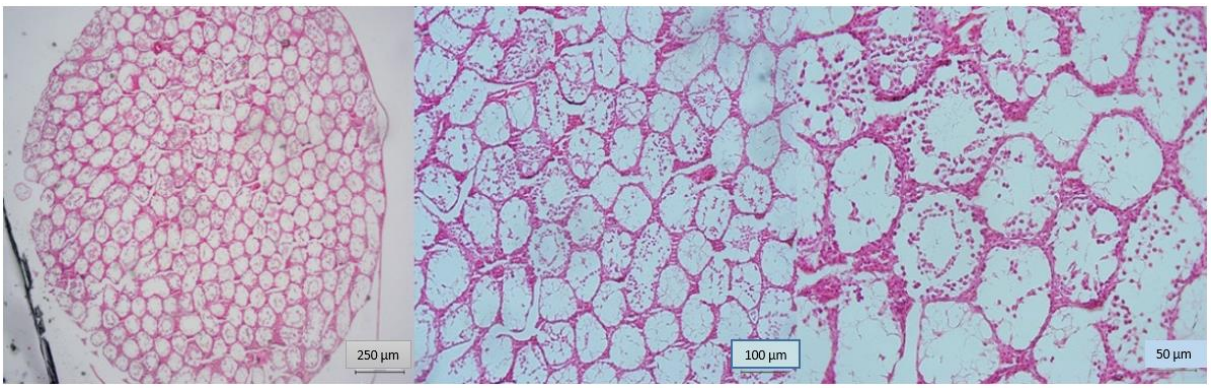

Fig. 3 Microscopic view of testicular tissue in azoospermia group with 4J/cm^2 energy density irradiation Testicular tissue repair is initially evident, increase in seminiferous tubule diameter and tubular epithelium repair are seen partly. The number of spermatogenesis cells has increased. (40x,100x and 200x magnification, hematoxylin-eosin staining)

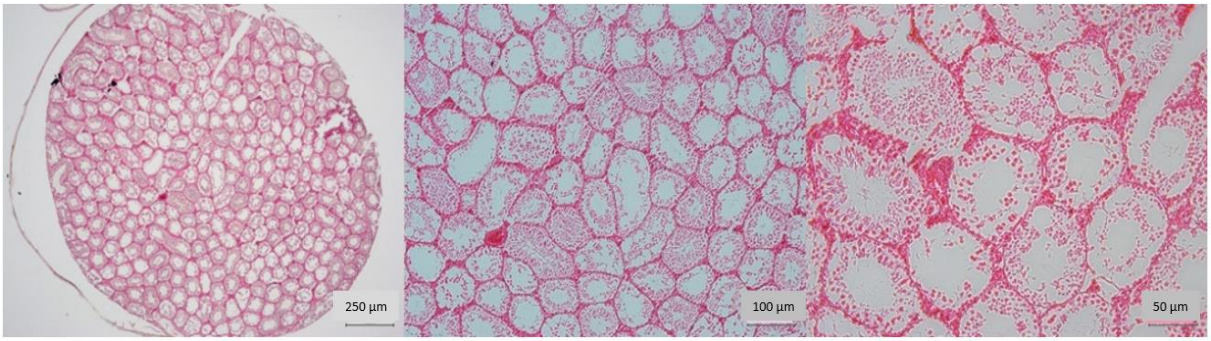

Fig. 4 Microscopic view of testicular tissue in azoospermia group with 8J/cm^2 energy density irradiation: Testicular tissue repair is evident, increase in seminiferous tubule diameter and tubular epithelium repair are seen. The number of spermatogenesis cells has increased sharply. (40x,100x and 200x magnification, hematoxylin-eosin staining)

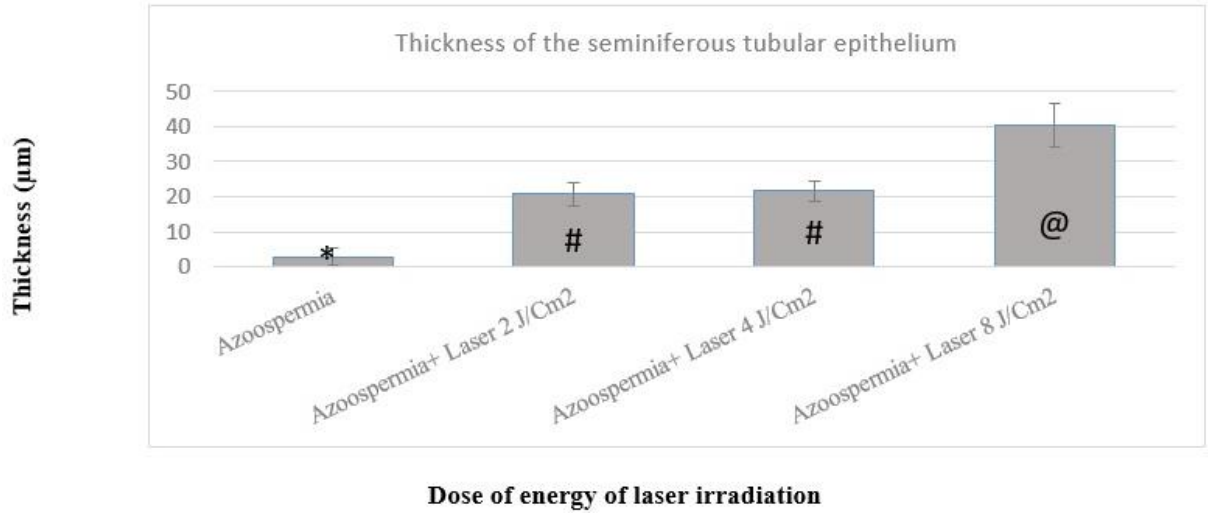

Fig. 5 Comparison of mean ± standard deviation of seminiferous tubular epithelial thickness in different groups

*#@: Different signs indicate significant differences between groups.

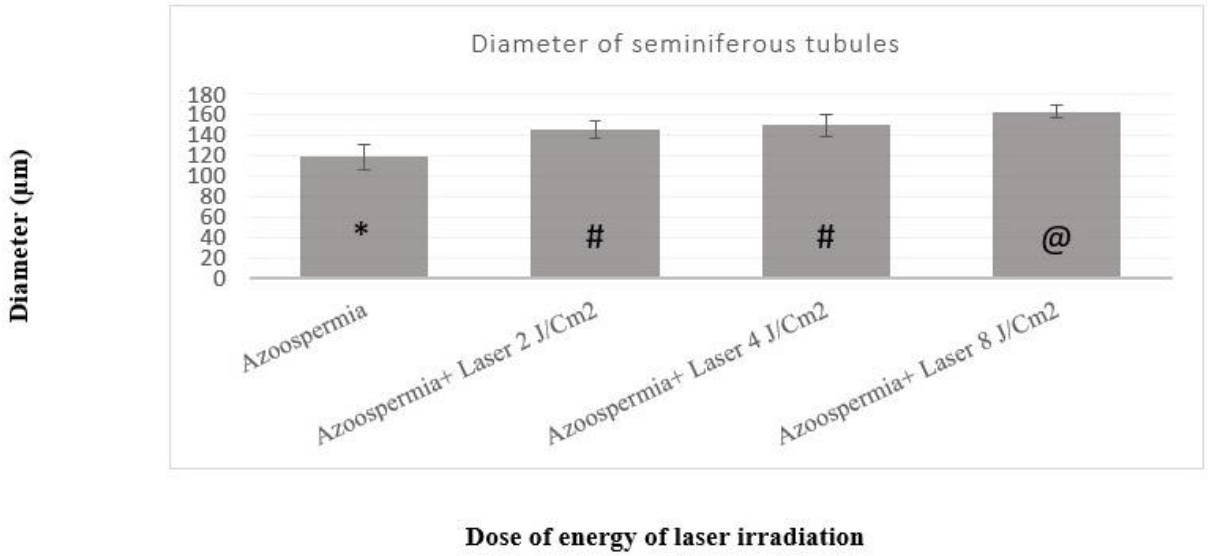

Fig. 6 Comparison of mean ± standard deviation of seminiferous tube diameters in different groups

*#@: Different signs indicate significant differences between groups.

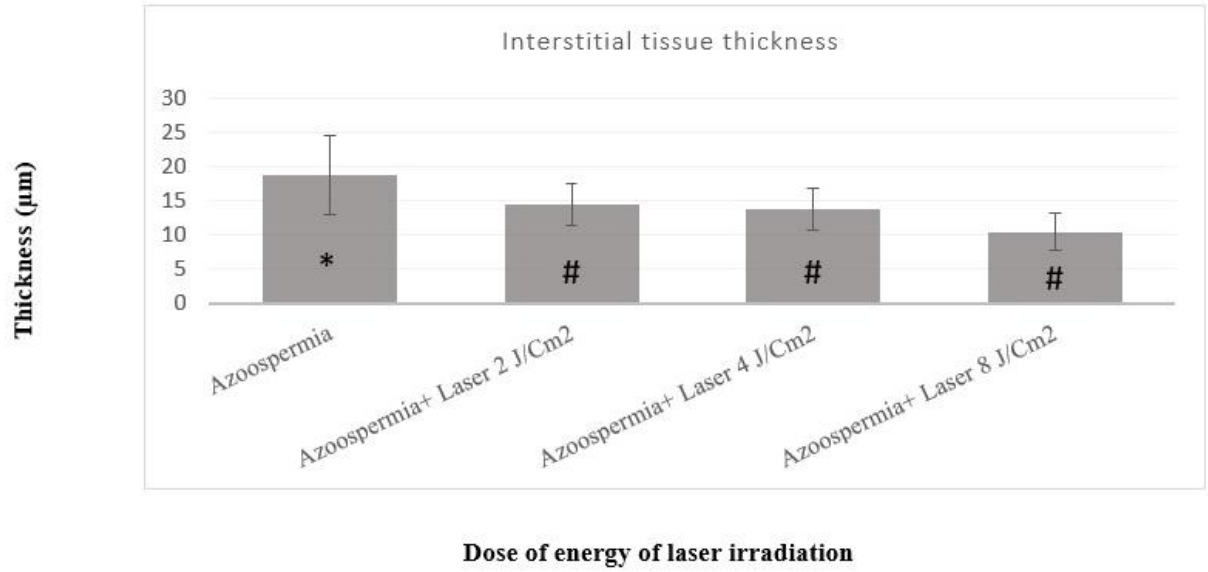

Fig. 7 Comparison of mean ± standard deviation of interstitial tissue thickness in different groups

*#: Different symbols indicate significant differences between groups.

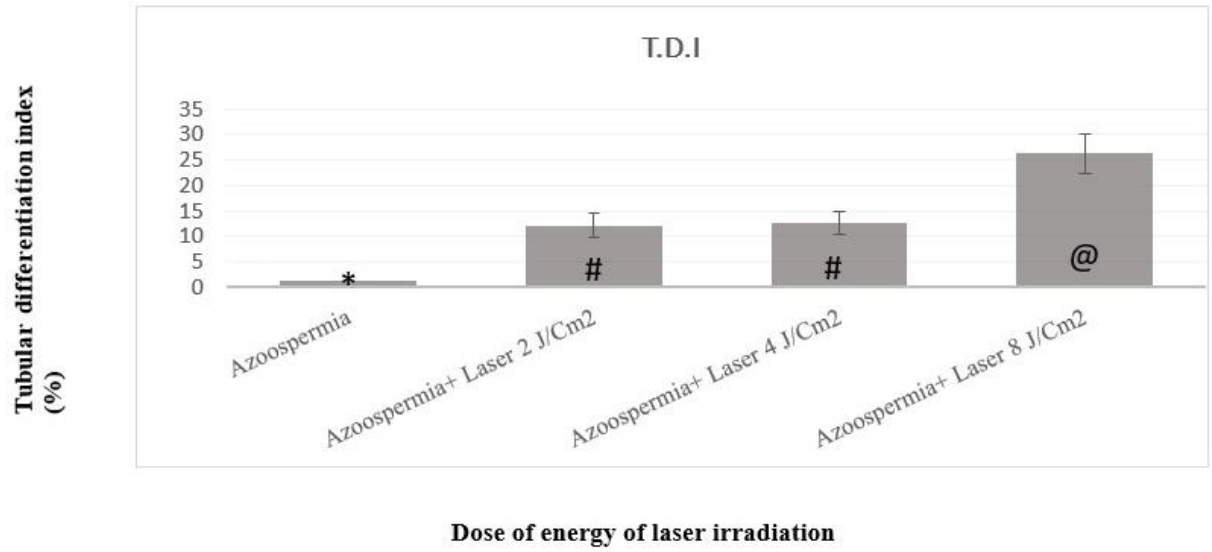

Fig. 8 Comparison of mean ± standard deviation of T.D.I coefficient in different groups

*#@: Different signs indicate significant differences between groups

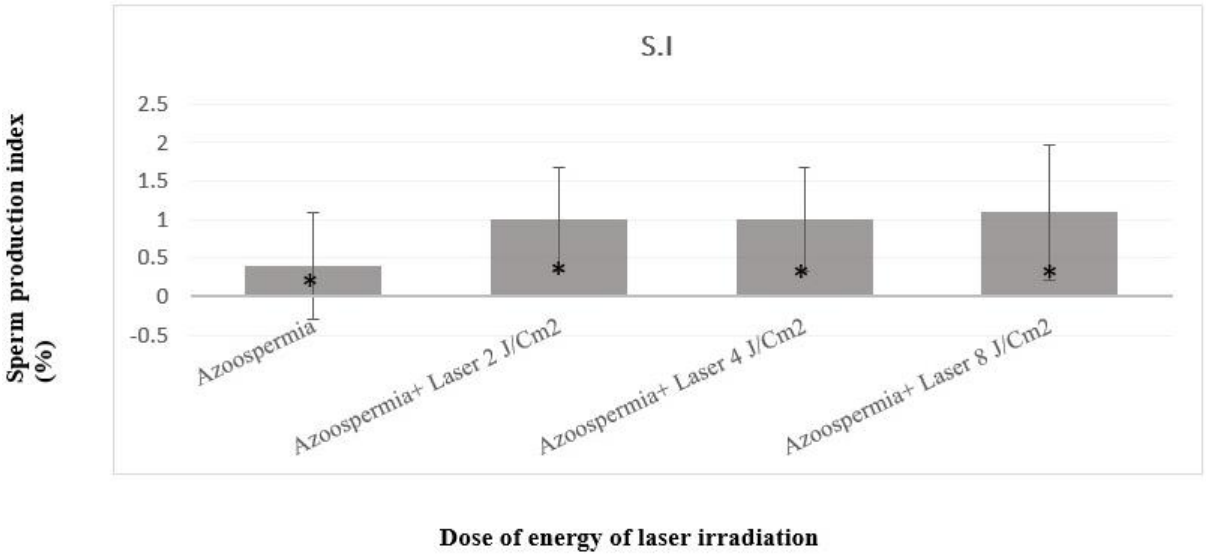

Fig. 9 Comparison of mean ± standard deviation of S.I. coefficient in different groups

*#@: Different signs indicate significant differences between groups.

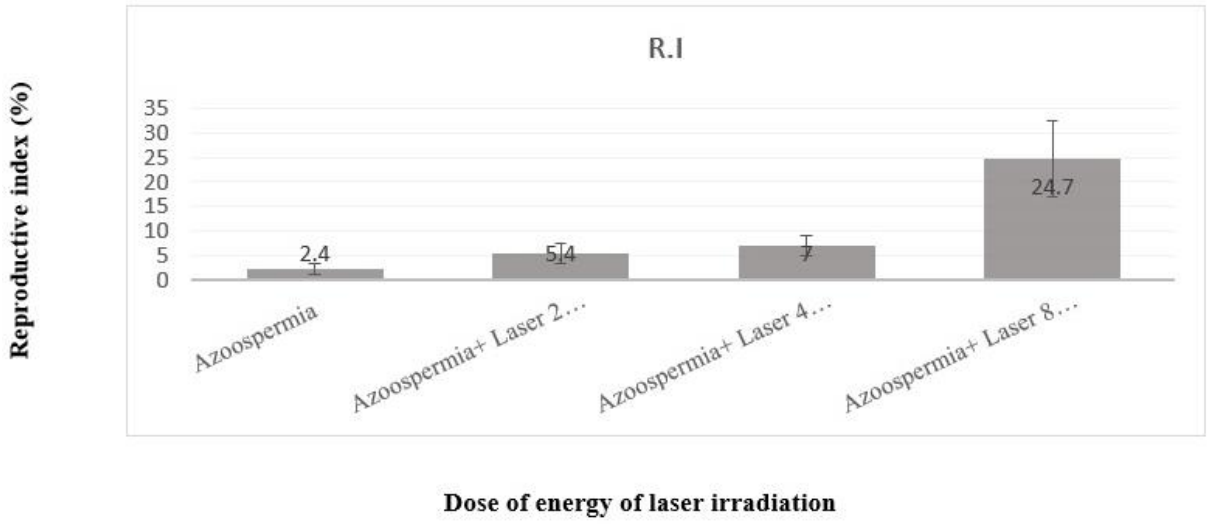

Fig. 10 Comparison of mean ± standard deviation of R.I coefficient in different groups

*#: Different symbols indicate significant differences between groups.

Table 1. Comparison of the mean of the evaluated parameters in the testicular tissue of the studied groups

parameter	Azoospermic group	Azoospermic group+ 2J/cm^2 irradiation	Azoospermic group+4J/cm^2 irradiation	Azoospermic group+8J/cm^2 irradiation
Epithelial thickness (micrometers)	2.85±2.30^a	20.67±3.43^b	21.60±2.89^b	40.54±6.14^c
Diameter of seminiferous tubules (micrometers)	119.09±12.43^a	145.83±9.14^b	149.99±10.51^b	163.27±6.16^c
Interstitial tissue thickness (micrometers)	18.71±5.78^a	14.47±3.02^b	13.84±3/13^b	10.48±2.75^b
T.D.I.[4]	1.20±0.92^a	12.20±2.30^b	12.60±2.32^b	26.30±3.89^c
S.I.[5]	0.40±0.70^a	1.00±0.67^a	1.00±0.67^a	1.10±088^a
R.I.[6]	2.40±1.18^a	5.40±2.12^a	7.00±2.06^a	24.70±7.86^b

Different abc letters in each row show a significant difference ($p < 0.05$)

IV. DISCUSSION

This study, which aimed to evaluate the effectiveness of laser light on male infertility, showed that using the right dose of laser can be a good treatment for Busulfan-induced azoospermia. According to other reports [33, 34], our findings confirm that Busulfan with an effective dose of 30mg/kg reduces the number of sperm cells to create an azoospermic model. In this study we examined three experimental groups exposed to laser radiation with a density of 2,4,8 J/cm^2. All three groups presented satisfactory results of testicular repair compared to the group treated with Busulfan.

Busulfan is a chemotherapy drug used to treat some diseases such as leukemia, although it can cause male infertility. It is a powerful chemical agent that preferentially destroys spermatogonia stem cells and disturbs the spermatogenesis process by affecting germ cells and sertoli cells[35-37], The morphological and histomorphological findings of our study on the azoospermic control group showed substantial atrophy of the seminal vesicle, a severe decrease of reproductive epithelial cells, and the presence of a very small number of spermatogonia cells.

[4] Tubular differentiation coefficient
[5] Spermiogenesis coefficient
[6] Reconstruction coefficient

Many investigations have been conducted so far to modify and abolish the infertile effect of Busulfan. In a study, the therapeutic effect of bone marrow-derived mesenchymal stem cells on Busulfan-induced azoospermia demonstrated that the seminiferous tubules' epithelial tissue was normal and that spermatogenesis was detectable in the majority of these tubes. These stem cells, which have differentiation potential, are a good choice for therapeutic applications due to the secretion of anti-inflammatory cytokines and growth factors [38] . In another study, a condition medium from these stem cells was used to treat Busulfan-induced azoospermia. Histomorphological findings showed an increased thickness of the azoospermic seminiferous tubules compared to the control group [36]. Overall, these studies show that the anti-infertility effect of Busulfan is treatable and provides a way to use easier and less risky treatments such as photobiomodulation.

Today, photobiomodulation is widely used in the treatment of infertility disorders. According to other reports that have been presented so far about the use of laser therapy in the treatment of infertility [8, 26, 32], our results also confirmed the restorative effect of low-power laser on testicular tissue. In our study, using a diode laser having a wavelength of 808nm, three experimental groups with three different doses of, Laser irradiations namely, 2, 4, and 8 J/cm^2 were considered. Histological criteria and testicular spermatogenesis showed a significant change in experimental groups compared to the Busulfan control group where spermatozoa cells were well detectable after laser irradiation. According to previous studies, light modulation can increase cellular metabolism after receiving energy and modulated cellular processes such as proliferation, differentiation, and tissue repair [8]. Therefore, energy and optical power, if given in the right dose, will have a natural and biological effect on the tissue and lead to the initiation of some processes in the living cell [39]. In our study, comparing the effectiveness of three doses of energy, the energy dose of 8 J/cm^2 was more efficient compared to the doses of 2 and $4J/cm^2$. It means that with laser irradiation of $8J/cm^2$, the largest increase in testicular volume was observed and the presence of germ cells in the seminiferous tubules were observed after extensive degradation due to the effect of Busulfan, which indicate stimulation of spermatogenesis and balance between proliferation and cell differentiation.

There are still unanswered questions about what range of wavelength lasers and in what range of power and radiation energy can have the most effect. In general, studies have shown that light modulation using laser can increase sperm motility [24-27]. These studies showed that laser light stimulates the production of ATP and reduces oxidative stress, leading to increased sperm motility. The laser light affects the bipolar lipid layers of the cell membrane and the membrane of the organelles inside the cell, therefore, by transferring calcium and other ions from the ion channels on the mitochondrial membrane, it causes the activation of the electron transport chain and increase ATP and energy production needed for sperm motility [40]. For example, Daryl Precee and colleagues 2017 [14] measured the motility and chromatin structure of sperm using a 633nm red-wave He-Ne laser with a radiation power of $31mW/cm^2$. They found that laser radiation increases sperm motility. However, this amount of radiation power causes damage, albeit minor, to the DNA structure. Therefore, the primary importance in the therapeutic application of laser is to investigate the lack of damage to the DNA structure due to laser light irradiation [14]. In a recent study, the results show that the Ga.Al.As infrared laser with a wavelength of 808nm and an energy density of 28 J/cm^2 not only did not affect increasing testosterone concentration, sperm quality, and motility but also caused the appearance of lesions in the sperm [41]. The reason for this result is that increasing the radiation dose from an acceptable level, by over-stimulating aerobic phosphorylation leads to an unregulated increase in ROS production and oxidative DNA damage resulting in cell death due to oxidative stress [42, 43]. According to the Moskvin paper, the kinetics of ROS release are indirectly dependent on the radiant energy rather than the radiant power [8]. In 2014, R Salman Yazdi et al. continuously

irradiated the sperm of patients with asthenospermia with a wavelength of 830nm with different radiation energies. Their results showed that sperm were able to move due to radiation of 4J/cm^2 and 6J/cm^2 [44]. Therefore, for a more effective laser treatment, it is necessary to check the appropriate energy dose. In this study, three low-energy doses were used, all three of which showed significant results by examining the degree of cell differentiation and sperm fertility. However, the effects of energy doses of 2 J/cm^2 and 4 J/cm^2 were the same and there was no significant difference between these two energy doses. On the other hand, measuring SI, which indicates sperm production, was almost equally effective in all three groups. In general, the results of our research show that the reduction of energy dose has a better effect on improving the process of spermatogenesis and thus the treatment of azoospermia.

In a similar study to ours, Busulfan-induced infertile rats were treated with an energy density of 0.03 and 0.2 J/cm^2. In this study, a pulsed infrared laser with a wavelength of 890nm was used. Examination of sperm parameters, measurement of serum testosterone level, TUNEL assay, and histomorphological evaluation showed that the energy density of 0.03 J/cm^2 with a wavelength of 890nm caused a significant increase in the number of germ cells and Leydig cells and decreased apoptosis [40]. This shows that in addition to radiation energy, a parameter such as the radiated wavelength is also effective in terms of the effectiveness of laser treatment. In the present study, which used a diode laser with a wavelength of 808 nm, a higher energy dose of 8 J/cm^2 showed the best results. Therefore, increasing the energy dose in the range defined as low level laser irradiation can be more effective. The majority of studies, together with the results of our study, confirm that low-energy laser radiation has the best effect on activating cellular mechanisms and improving metabolism, and this non-invasive method is the best option in the treatment of infertility-related diseases such as azoospermia.

To use this non-invasive and safe in-vivo treatment on the human testicle requires further research and evaluation of the reproducibility of these findings in vitro as well as measuring the harmful effects on DNA integrity.

V. Conclusion

In this investigation, the employment of a laser with an energy density of 8J/cm^2 was shown to be beneficial in boosting germ cell and sperm production. Although the two different groups with lower energy density than 8J/cm2 were examined and had positive effects on sperm production, but the group that received the laser at 8J/cm2 had the best effect on spermatogenesis process and male infertility treatment.

Authors' Contributions

Conceptualization, designing project, data curation: H.T, M.M and E.S… Project administration, Material preparation, Data collection: H.T, A.S, F.F, Z.A… Statistical analysis: R.Sh… writing original draft: Z.A and F.F… writing-review-editing: all authors.

Acknowledgement

The authors acknowledge the Biophotonics and Biotechnology Research Center of Azad University of Tabriz for its scientific support.

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