The effect of swimming, cells and laser therapy on the expression of Drp1, Murf1 and Mfn2 genes in the testes of azoospermic rats

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Abstract:
Background: One of the causes of infertility is azoospermia. The aim of this study was to investigate the effect of swimming training, cell therapy and laser therapy on the expression of genes involved in mitochondrial dynamics in testicular tissue of azoospermic rats.
Methods: In this experimental study, 40 rats (6-8-week-old) were randomly selected into 8 groups: 1) healthy control, 2) patient, 3) sham, 4) laser, 5) training, 6) cell, 7) cell + laser and 8) cell + training. azoospermia model was induced with busulfan at a dose of 40 mg Stem cells were transplanted once into the vas deferens at the rate of 1000000 cells per mouse. One week after cell transplantation, the laser therapy was applied in three repetitions throughout the study period with an interval of once a week and after wound healing, rats they swam for 30 minutes a day, 5 days a week for 8 weeks. To investigate the expression of the studied genes tissue analysis was performed by Real Time PCR technique.
Results: azoospermia significantly reduced the expression of Mfn2 and significantly increased the expression of Drp1 and Murf1 in testicular tissue. The use of interventional methods increased the expression of Mfn2, and significantly reduced the expression of Murf1 in testicular.
Conclusion: Based on the findings, the best changes were observed in the exercise + laser group. Therefore, it can be said that in rats of azoospermia model, the simultaneous use of regular exercise interventions and laser therapy has the best effectiveness.
Keywords: Azoospermia, Exercise, Laser therapy, Mitochondrial dynamics, Stem cell
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Introduction:

One of the causes of infertility is azoospermia, which refers to the lack of sperm in each ejaculation and affects about 1% of men in the total population (1). Non-obstructive azoospermia is a condition in which no sperm is observed in ejaculation and is related to intra-testicular disorders resulting in impaired spermatogenesis, while in obstructive azoospermia spermatogenesis is normal and the defect is related to obstruction in the ejaculatory ducts (1).

Mitochondria are cellular organs that exist in dynamic networks. Mitochondria make up 30-35% of the cell volume and ATP production is their primary function. Mitochondrial dynamics, especially fusion and fission, are important processes in mitochondrial homeostasis (2). Mitochondrial dynamics are regulated by several different GTPases (1). Mitophyosin 2 (Mfn2), MFN1, and optic atrophy 1 (OPA1) lead to mitochondrial fusion in the outer and inner membranes, respectively. Dynamin related protein 1 (Drp1), on the other hand, is a cytoplasmic protein that, upon activation, translocates to the mitochondrial membrane and promotes cleavage with the interaction of cleft protein (FIS1) (3, 4). Since fusion mediators regulate mitochondrial metabolism in addition to mitochondrial and endoplasmic reticulum binding, their regulatory decline is usually associated with a decrease in mitochondrial oxidative capacity (4).

One of the methods of treating azoospermia is the use of mesenchymal stem cells (1). Bone marrow mesenchymal stem cells have high ability to differentiate into different cell lines (5). The medium used in bone marrow mesenchymal stem cell cultures can induce the return of spermatogenesis in induced azoospermic mice (6).

One of the most important applications of lasers in medical science is the use of low power lasers (LLLT) or Biostimulative laser to treat patients. Low-power laser therapy is a treatment method that uses light radiation with low intensity and causes a change in the permeability of the cell membrane, followed by the production of mRNA and cell division.and it can affect the rate of proliferation and colonization of spermatogonial stem cells (1, 7). Also Numerous studies have also supported exercise as a strategy to reverse the effects of mitochondrial disorders and to prevent or treat disease (1). Physical activity can increase the amount of sex hormones, sperm production and fertility, as well as prevent the testicles from shrinking and increase the amount of semen (8).

Among the aerobic exercises, low-intensity swimming aerobic exercise is one of the exercises that is safe and usable in various physiological conditions and is used in most physiological, biochemical and molecular reactions studies due to its intolerance to water compared to non-water sports. On the other hand, no study has been found to investigate the simultaneous effect of swimming, cell and laser therapy in azoospermic mice. Therefore, this study aims to investigate whether regular aerobic exercise, laser and cell therapy on the expression of genes involved in mitochondrial dynamics of testicular tissue in azoospermic rats.

Methods

In the present experimental research 40 male Wistar rats (6 to 8 weeks old), were purchased from the Center for Research and Reproduction of Laboratory Animals in Tehran and after transferring the subjects to the laboratory and after one week. Adaptation to the new environment was maintained in groups of 5 in transparent polycarbonate cages in an environment with an average temperature of 22 ± 1.4 ° C, humidity of 55% and a dark cycle of 12:12 hours. The care of the animals was carried out in accordance with the guidelines of the International Institute of Health and the protocols of this study, in accordance with the principles of the Helsinki Declaration and

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the rules of medical ethics. Also, during the research, the animals were fed with a food pack made by Behparvar Karaj Company at the rate of 10 g per 100 g of body weight per day (according to weekly weight gain) and had free access to drinking water through bottles.

In order to create azoospermia model, Busulfan at a dose of 40 mg /kg body weight was then injected intraperitoneally to each rat (The waiting time for creating the model was 30 days) (9)
One month after model induction, rats were grouped as follows:
1) healthy control group (kept for 8 weeks), 2) sham group, 3) patient group (remained for 8 weeks until the end of the study, 4) patient+laser group (One month after the model was developed, a low-power laser with a wavelength of 632.8 nm and a power of 10 mW and an energy of 3 joules was applied to the testes of azoospermic mice in three repetitions throughout the study period at weekly intervals and rats were kept for 8 weeks until the end of the study. 5) patient+training group (one month after azoospermic mice, rats swim with low intensity for 30 minutes a day, 5 days a week for 8 weeks, 6) Patient+cell group (one month after azoospermic mice, once stem cells were transplanted in the vas deferens at the rate of one million cells per mouse in the right testicle and the rats were kept for 8 weeks until the end of the study), 7) Patient+training+laser group (one month after azoospermic, low power laser with a wavelength of 632.8 nm and a power of 10 mW and energy of 3 joules was applied in three repetitions throughout the study period with an interval of once a week, then after one week, rats swam for 30 minutes a day, for 5 days a week, which lasted for 8 weeks), and 8) patient+cell+training group (one month after azoospermic mice, once stem cells were transplanted in the vas deferens at the rate of one million cells per mouse, then after one week of transplantation cells, rats swam for 30 minutes a day, for 5 days a week, which lasted for 8 weeks).
Before starting the main protocol, the rats of the training groups were placed in the water pool for 20 minutes each time for one week (5 days) in order to familiarize themselves with the water and reduce the stress of swimming and adapt to the training conditions. Then they swam 5 days a week until the end of the research period in a water tank with dimensions of 50 x 50 x 100 cm with a temperature of 30-32 degrees Celsius during 8 weeks. The duration of training in water was 30 minutes daily until the end of the training period (1).
Tissue sampling of testicular tissue of mice was performed under very similar conditions and in baseline conditions (two days after the end of the training period). To eliminate the acute effect of training, animals were sampled 48 hours after the last swimming training program. For this purpose, the animals were first anesthetized using peritoneal injection of ketamine (50-30 mg / kg) and xylazine (3-5 mg / kg) and then killed, and after killing the transplanted tissues, they were evaluated for genetic studies.
To investigate the expression of the studied genes in each group, tissue analysis was performed by Real Time PCR technique. First, primer design was performed and then total RNA was extracted from tissues and converted to cDNA. The cDNA was then amplified by PCR and examined for the expression of the mentioned genes. For molecular studies on the level of gene expression, RNA was extracted from tissues in all study groups, according to the protocol of the manufacturer (Kiagen, Germany). After extracting RNA with high purity and concentration from all samples, cDNA synthesis was performed according to the protocol of the manufacturer (Fermentas, USA) and then the synthesized cDNA was used for reverse transcription reaction. For the RT-qPCR technique, first, the RNA of all cells was extracted according to the synagen protocol using chiazol solution and exposed to (DNase I Fermentas) to ensure contamination with genomic DNA. Then

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the quality of the extracted RNAs was evaluated by spectrophotometric device (DPI-1, Kiagen). To prepare a single-stranded cDNA from Oligodt primer (MWG-Biotech, Germany) and reverse transcription enzyme (Fermentas) was performed according to the relevant protocol. Each PCR reaction was performed using (PCR master mix Applied Biosystems) and SYBER Green in the device ABI Step One (Applied Biosystems, Sequences Detection Systems Focter City, CA.) according to the manufacturer's protocol. 40 cycles were considered for each Real-Time PCR cycle and the temperatures of each cycle were set including 94 ° C for 20 seconds, 60-58 ° C for 30 seconds and 72 ° C for 30 seconds. Melting diagram was performed to evaluate the accuracy of PCR reactions and was evaluated specifically for each gene and in each reaction with a negative control diagram to check for contamination in each reaction.
To analyze the findings of this study, Kolmogorov-Smirnov test, Fisher's 1-way analysis of variance, and Tukey were used for comparison between different groups. All calculations were performed using SPSS statistical software version 22 at a significant level of $P \leq 0.05$.

Results

Examining the mean and standard deviation of Mfn2 levels in different research groups shows that there was a significant difference between Mfn2 in research groups (F=4.197; P=0.002). Highest Mfn2 index levels belonged to the laser-training group and the lowest levels belonged to the patient group. The results of one-way analysis of variance on the levels of Mfn2 in different groups indicate that there is a significant difference between the levels of Mfn2 in different research groups. Induction of the experimental model of azoospermia decreased the expression of the Mfn2 gene in the testicular tissue compared to the healthy control group. The simultaneous implementation of laser + training significantly increased the expression of this gene compared to the patient group (P=0.004) (Table 1; Figure 1).

Table 1. Results of one-way analysis of variance of Mfn2 levels in different research groups

<table>
<tr><th>Sources of change</th><th>SS</th><th>df</th><th>Average squares</th><th>F</th><th>P</th></tr>
<tr><td>Between groups</td><td>0.000</td><td>7</td><td>0.000</td><td rowspan="3">4.197</td><td rowspan="3">0.002*</td></tr>
<tr><td>Intragroup</td><td>0.000</td><td>32</td><td>0.000</td></tr>
<tr><td>total sum</td><td>0.000</td><td>39</td><td>-</td></tr>
</table>

* Significant level ($P \leq 0.05$)

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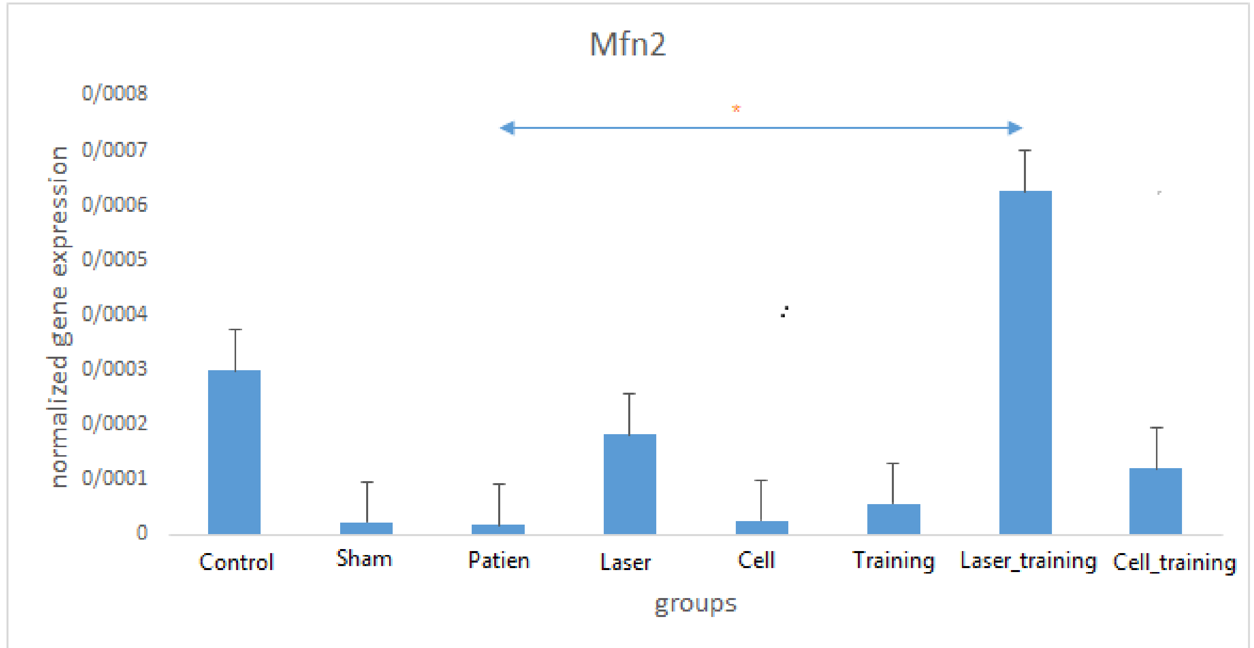

Figure 1. Comparison of mean Mfn2 levels in different research groups
* Significant sign in relation to the patient group & significant signs compared to the healthy group

Examining the mean and standard deviation of Drp1 levels in different research groups shows that there was no a significant difference between Drp1 in research groups (F=2.577; P=0.057). Highest levels of Drp1 index belonged to the sham group and the lowest levels belonged to the control-healthy group. The results of one-way analysis of variance on Drp1 levels of different research groups show that; there is no significant difference between the levels of Drp1 in different research groups (Table 2; Figure 2).

Table 2. Results of one-way analysis of variance of Drp1 levels in different research groups

Sources of change	SS	df	Average squares	F	P
Between groups	2.748	7	0.393	2.577	0.054
Intragroup	4.417	32	0.152		
total sum	7.165	39	-		

* Significant level (P≤0.05)

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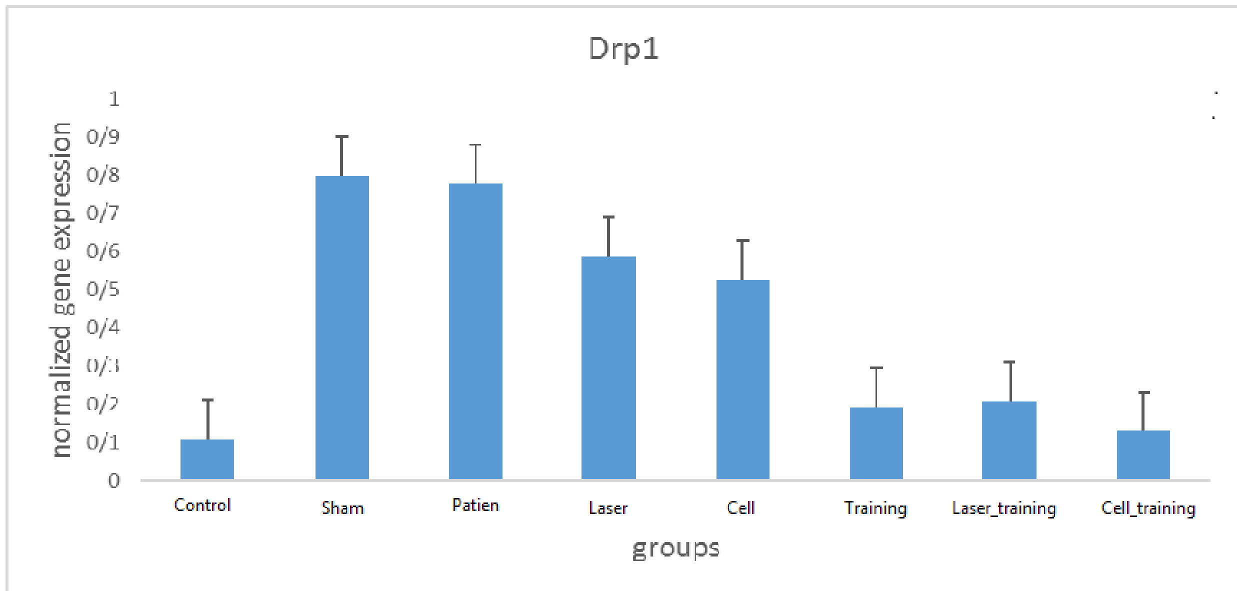

Figure 2. Comparison of mean Drp1 levels in different research groups

Examining the mean and standard deviation of Mfn2 levels in different research groups shows that there was a significant difference between Mfn2 in research groups (F=4.303; P=0.002). Highest Murf1 index levels belonged to the patient group and the lowest levels belonged to the control-healthy group. The results of one-way analysis of variance on the levels of Murf1 in different research groups indicate that there is a significant difference between the levels of Murf1 in different research groups (Table 4). The induction of the experimental model of azoospermia significantly increased Murf1 gene expression compared to the healthy control group (p=0.003), the implementation of each of the interventions; cell (p=0.046), training (p=0.003), laser+training (p=0.003) and cell+training significantly decreased the expression of this gene compared to the patient group (p=0.004) (Table 3, Figure 3).

Table 3. Results of one-way analysis of variance of Murf1 levels in different research groups

Sources of change	SS	df	Average squares	F	P
Between groups	0.000	7	0.000	4.303	0.002*
Intragroup	0.000	32	0.000		
total sum	0.000	39	-		

* Significant level (P≤0.05)

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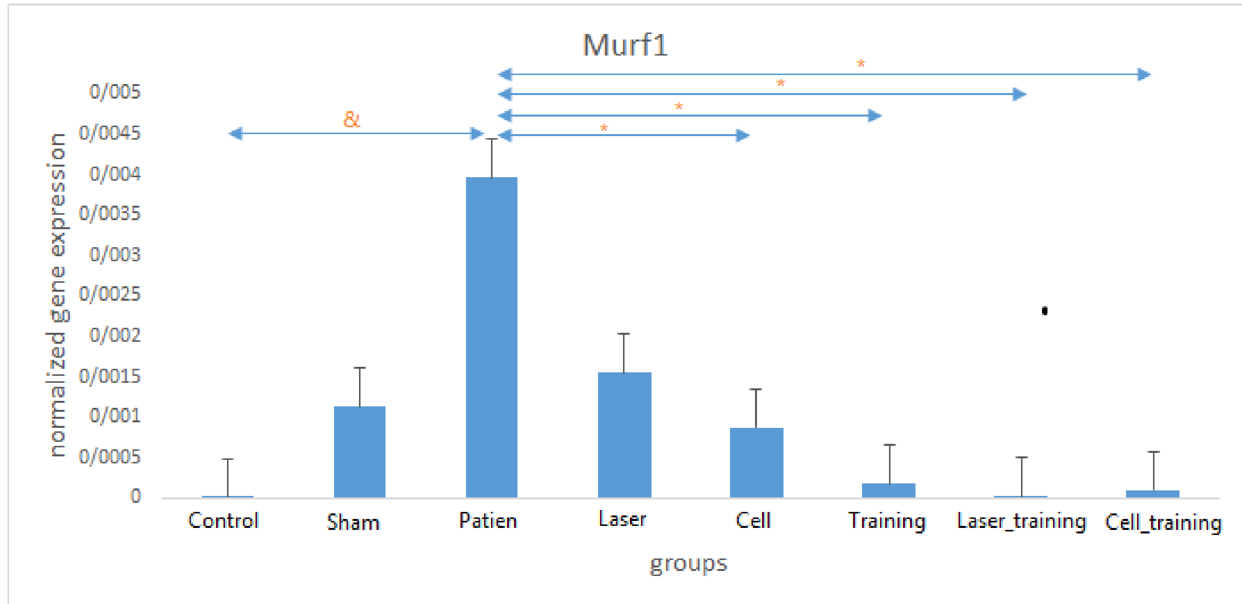

Figure 3. Comparison of mean Murf1 levels in different research groups

* Significant sign in relation to the patient group & significant signs compared to the healthy group

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Discussion

Induction of azoospermia model caused a significant decrease in testicular tissue Mfn2 gene, which was enhanced by training, laser, cell and combination methods in testicular tissue, which was only significant in the training+laser group. Regarding Drp1 gene expression in testicular tissues of rats, the induction of azoospermia model increased the expression of this gene, the treatment methods used decreased the expression of this gene, which was not significant in any of the groups. Also, the induction of azoospermia model significantly increased the expression of the Murf1 gene in the testicular tissue, the treatment methods decreased the expression of this gene, which was significant in the training, cell, training+laser, training+cell and cell+laser groups.

Muzaffar et al. showed a significant improvement in spermatogenesis and testicular tissue indices in samples treated with culture medium obtained from bone marrow mesenchymal stem cells compared to the induced azoospermic group (6). Other studies in animals such as hamsters and mice have shown a significant improvement in spermatogenesis due to stem cell therapy (10, 11). On the other hand, studies have shown that many cellular pathways are regulated by cell redox status. Modulation of cellular redox status by NF-KB transcription factor and phospholipase A2 increases DNA synthesis. Therefore, low power laser accelerates the rhythm of mitotic and meiotic divisions in spermatogenesis and increases the number of germ cells, especially primary spermatocytes (7). In addition to affecting oxidative phosphorylation in mitochondria and increasing ATP production, low-power laser beams increase microcirculation (microscopic circulation). Laser causes vasodilation by releasing chemicals such as histamine, which increases cellular metabolism along with increasing oxidative phosphorylation of cells (1, 7). Increased microcirculation of the testis in the testis improves the metabolic status, which after laser irradiation justifies the important biological stimulatory effect on spermatogenesis (12).

Also based on the results of previous research, exercise has positive effects on mitochondrial health (13). Zeng et al. showed a significant increase in Mfn2 protein related to skeletal muscle mitochondrial fusion of elderly mice after exercise interventions (14). Also, comparing the mitochondrial fusion stress response to exercise activity, studies have shown that the expression of genes associated with mitochondrial cleavage increases significantly after a period or prolonged exercise (15). Fealy et al. Also reported in a study that exercise reduces the activity of Drp1 protein in skeletal muscle (16). One of the properties of Drp1 is apoptosis in mitochondria, so exercise can protect tissue against oxidative stress and reduce Drp1 levels by reducing fat peroxides and activating antioxidant defenses. Decreased Drp1 reduces cleavage and apoptosis in cells, and improves apoptotic-dependent markers (17)). Reduction of Drp1 is essential for the development and maintenance of proper mitochondrial function and improves cell survival by reducing oxidative stress (18).
The ubiquitin proteasome pathway is applied to the entire cell and involves many substrates and reaction processes in vivo, including the ubiquitination of the substrate protein and the degradation of ubiquitinized proteins. Ubiquitin E3 ligases, such as Atrogin-1 and Murf1, are essential for protein ubiquitin (19). In Zheng research, exercise significantly reduced Murf1 gene expression in the skeletal muscle of older rats, which may subsequently inhibit the process of protein ubiquitination (14). Regulation of Murf1, including the NF-κB and FoxO3a signal pathways, has a cumulative effect on skeletal muscle atrophy, with NF-κB being one of the most important transcription factors for the regulation of Murf1 involved in senile skeletal muscle atrophy (20).Eexercise can reduce the expression of Murf1 proteins mediated by inhibiting oxidative stress (21, 22).
In this study, each of the treatment methods was effective in mitochondrial dynamics, but in each of the combined methods, a better result was obtained and they had a synergistic effect, and this synergy was more in the laser+exercise group than other combined methods.

Conclusion

According to the results of the research, it is possible that the combination of swimming exercise with cell therapy and laser therapy by changing some of the genes involved in the mitochondrial dynamics of the testicular tissue of experimental rats with azoospermia may cause the rats to become fertile, but a definite opinion needs more research in this regard.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethical statement

All steps of the present research have been approved by the Research Ethics Committee of Islamic Azad University, Sari branch (IR.IAU.SARI.REC.1398.149).

Author contributions

All authors have contributed equally in writing this article.

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