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The efficiency of ozone therapy and low-level laser therapy in rat facial nerve injury

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ABSTRACT

Purpose: Comparison of low-level laser therapy (LLLT) and ozone therapy (OT) methods for the treatment of facial nerve injury (FNI) in rats, evaluated by histomorphometric measurement analysis.
Materials and methods: Thirty rats were randomly divided into control (C), LLLT, and OT groups. The left facial nerves (FNs) of all rats ($n = 30$) were used in this study. These were held in a surgical clamp for 30 s to create neuropathic damage. The non-injured right FNs of the rats in the control group formed the fourth, sham (S) group in this study ($n = 10$). Therefore the total number of evaluated samples was 40. The injured FNs of rats in the control group were left to heal spontaneously, whereas LLLT was applied for 21 consecutive days (output 100 mW/cm^2 and wavelength 850 nm) and OT (2 ml; 80 μm/ml) once every 2 days for 21 days.
Results: After histomorphological evaluation, the OT group revealed statistically significant outcomes following FNI compared with the OT and control groups in terms of branching of nerve fibers ($p = 0.003$), nerve fiber diameters ($p = 0.0398$), nerve fiber areas ($p = 0.042$), and axon numbers ($p = 0.0327$). Although the LLLT group revealed a better healing process than the control group, the outcome was not statistically significant in terms of branching of nerve fibers ($p = 0.6804$), nerve fiber diameters ($p = 0.7424$), nerve fiber areas ($p = 0.7048$), and axon numbers ($p = 0.7588$).
Conclusions: OT resulted in statistically significant differences in outcome when compared with the LLLT and control groups, and provided a safe and effective treatment for FNI in rats. OT could therefore be considered as an alternative treatment of FNI. Clinical studies should now be performed to establish whether comparable results can be achieved in humans.

1. Introduction

Facial nerve injury (FNI) affects the maintenance and dynamics of facial expression muscles and sensations. Depending on the severity of the injury, the resulting social and functional problems can lead to a significant reduction in quality of life. Facial expressions represent physical and genetic features, but also emotions. By controlling facial expressions, the facial nerves (FNs) have important roles in the determination of identity and interactions with the environment. They therefore not only have motor functions, but also involve a complex interaction of emotional and parasympathetic roles (Proctor and Nager, 1982).

FN damage can lead to Bell's palsy, a specific type of facial paralysis whose symptoms include: paralysis and paresis in the facial muscles on one side, drooping of the lips and eyebrow to one side, and xerophthalmia, due to the inability to seal the eyelid (May and Klein, 1991; Roob et al., 1999).

Treatments for nerve damage include both non-invasive and invasive methods, such as pharmacological approaches, surgery, and alternative treatments. Transcutaneous electrical nerve stimulation (TENS), percutaneous electrical nerve stimulation (PENS), cryotherapy, acupuncture, ozone therapy (OT), and low-level laser therapy (LLLT) are the preferred alternative methods, with LLLT

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considered an effective treatment option (Chong and Bajwa, 2003) and OT recently gaining popularity.

Theories on the possible mechanism of OT are based on stimulation of oxygen metabolism, activation of the immune system, and antibacterial effects/sterilization. Ozone has been shown to reduce fibrosis, vascular congestion, vacuolization, and edema in rodents, and is effective in treating sciatic nerve injury (Somay et al., 2017). On the other hand, the mechanism of action of LLLT is believed to involve the absorption of light (photons) by photoreceptors (such as cytochrome C oxidase), altering the synthesis of ATP in mitochondria through the acceleration of electron transport chains, and thereby modulating cell reactions. LLLT has been shown to generally increase myelin capacity, neural tube formation, and Schwann cell stimulation (Shen et al., 2011; Yamany and Sayed, 2012).

This study aimed to compare LLLT and OT in the treatment of facial nerve injury (FNI) in rats, and to evaluate the outcomes through histomorphometric measurement analysis.

2. Materials and methods

2.1. Ethical statement

All experimental procedures were performed following the ethical guidelines of the International Association for Study of Pain in Animals and were approved by the ethical committee of the Bezmialem Vakif University (Istanbul, Turkey).

2.2. Surgical procedure

Thirty 10–12-week-old Wistar albino rats of weight 220 ± 20 g were included in our study and randomly divided into three groups: control (C), LLLT, and OT and maintained in an animal facility under an artificial 12-h light-and-dark cycle. The animals had access to food and water ad libitum. The rats were anesthetized with intramuscular xylazine 1 mg/kg (Ketalar 50 mg/ml; Pfizer Inc, New York, NY) or 0.5 mg/kg (Rompun 23.32 mg/ml; Bayer, Mefar Ilaç San AŞ Istanbul, Turkey). Articaine solution 0.2 ml was injected subcutaneously as a local anesthetic agent. The left facial nerves in the control (C), LLLT, and OT groups were detected after making a longitudinal 1-cm dermal incision in the middle of the neck, and were held in a surgical clamp for 30 s to create neuropathic damage. The same surgical procedure was performed to expose the right facial nerve of the C group rats, but without injury to the nerve, to form a fourth sham (S) group. This was to avoid unnecessary animal sacrifice (Fig. 1), a decision taken according to local ethical committee suggestions.

2.3. Treatment procedures

2.3.1. Control and sham Groups

The left and right facial nerves of all rats in the control group were included in this study. The surgical procedure, including neuropathic damage, was performed on the left facial nerves of the rats in the control group ($n = 10$). The right facial nerves of the same rats were left uninjured ($n = 10$). The samples from the left sides (injured sites) were collected for the control group while the samples from the right sides (non-injured sites) were collected for the sham group (Fig. 2). No treatment was administered for these rats.

2.3.2. LLLT group

While leaving the control group to heal spontaneously, a super luminized diode (SLD) was chosen to treat the LLLT group with 4J energy, applied using a Chattanooga Intelect Mobile Laser (DJO UK Ltd.; UK) device for 32 s on 21 consecutive days (output 100 mW/cm^2; wavelength 850 nm). This device allows the application of 1 or 3 MHz, using 20%, 50%, or continuous modes, without any need to change the applicators (Fig. 3).

2.3.3. OT group

Rats in the OT group received an insufflation of an ozone/oxygen (O_3/O_2) gas mixture, comprizing 2.5% O_3 and 97.5% medical O_2. The ozone gas processor MedozonIP (Herrmann Apparatebau, Kleinwallstadt, Germany) was used for the generation and intraperitoneal insufflation of the gas mixture (Fig. 3). The freshly synthesized gas mixture was insufflated into the peritoneum of the rats at a dose of 2 ml (80 μm/ml) for 21 days (once every 2 days) using the specific kit comprizing a plastic tube of 150 mm length with a sterile filter, connected to the outlet of the MedozonIP generator.

2.4. Histological evaluations

After sacrifice, the nerve samples were collected and kept in formaldehyde for histopathological analyses. Histological specimens were sectioned using a microtome (HM 340E; Thermo Scientific, Waltham, MA) and stained for histomorphometric assessment. Measurements and counts were performed using Image J (US National Institutes of Health, Bethesda, MD). An Axio Zoom V16 (Carl Zeiss Microscopy, LLC, Peabody, MA) was used to take photographs of the sectioned nerve tissue at increasing magnifications (50–100–500μm) (Fig. 4a–d).

2.5. Statistical analysis

Statistical analysis was performed using Graph-Pad Prism 5.0 (GraphPad Software, San Diego, CA, USA). One-way ANOVA and Tukey tests were used for statistical analysis, with $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) considered significant (Fig. 5).

3. Results

The existence and continuity of epineurium, perineurium, and endoneurium were evaluated and measured in analyzed transects, along with nerve area, nerve branching, fascicule number, and axon number.

In the control group, smaller nerve fibers, with an irregular array in the fibrous scar tissue, were observed. Numerous capillary and connective tissue hemorrhages were also detected among the

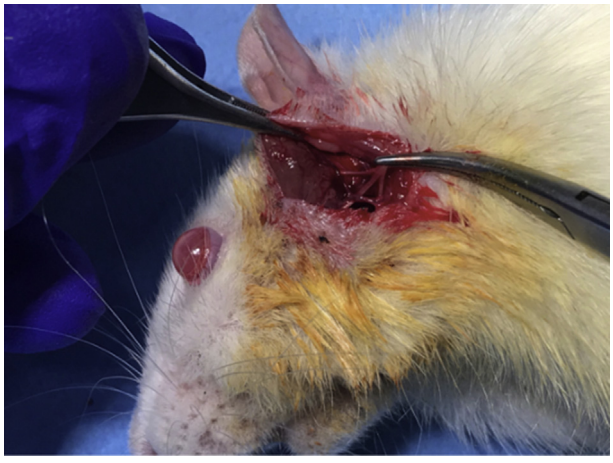

Fig. 1. The facial nerve trunk region: clamp damage for 30 s.

Fig. 2. Timeline diagram for the study.

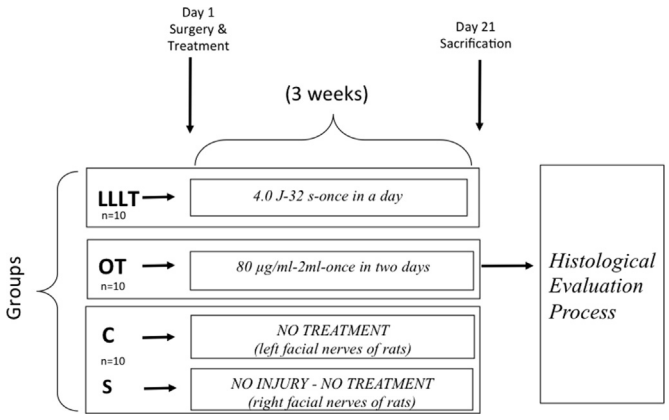

Fig. 3. (A) Administration of low-level laser therapy. (B) Administration of ozone therapy.

nerve fibers. Endoneurial fibrosis and capillaries were found, along with thickening of the epineurium and perineurium. A decrease in the diameter of nerve fibers and a loss of myelinated axons was also observed (Fig. 4A).

In the LLLT group, the morphology of the peripheral nerve fibers was similar to that in the control group. In some samples, many small-diameter nerve fibers were observed in fibrotic connective tissue. A low rate of nerve fiber regeneration after injury was observed. Although myelinated axons were seen in the nerve fibers, unmyelinated axons were more common (Fig. 4B and C).

In the OT group, a significant increase was observed in the diameter of the nerve fibers, while the connective tissue between these nerve fibers and the thickness of the epineurium were normal in appearance. There was a significant increase in the number of myelinated axons, with increased regeneration of nerve fibers. Myelinated axons were arranged neatly and parallel to each other in the nerve fibers (Fig. 4D).

3.1. Nerve branching number

Mean nerve branching number was 6 ± 1.7 in the sham group, 2.9 ± 1.5 in the control group, 3.6 ± 2 in the LLLT group, and 5.2 ± 2.3 in the OT group. A significant decrease was observed in the control group ($p = 0.0003$) and LLLT groups ($p = 0.0102$) when compared with the sham group. A significant increase was observed in the OT group when compared with the control group ($p = 0.003$) (Fig. 5A).

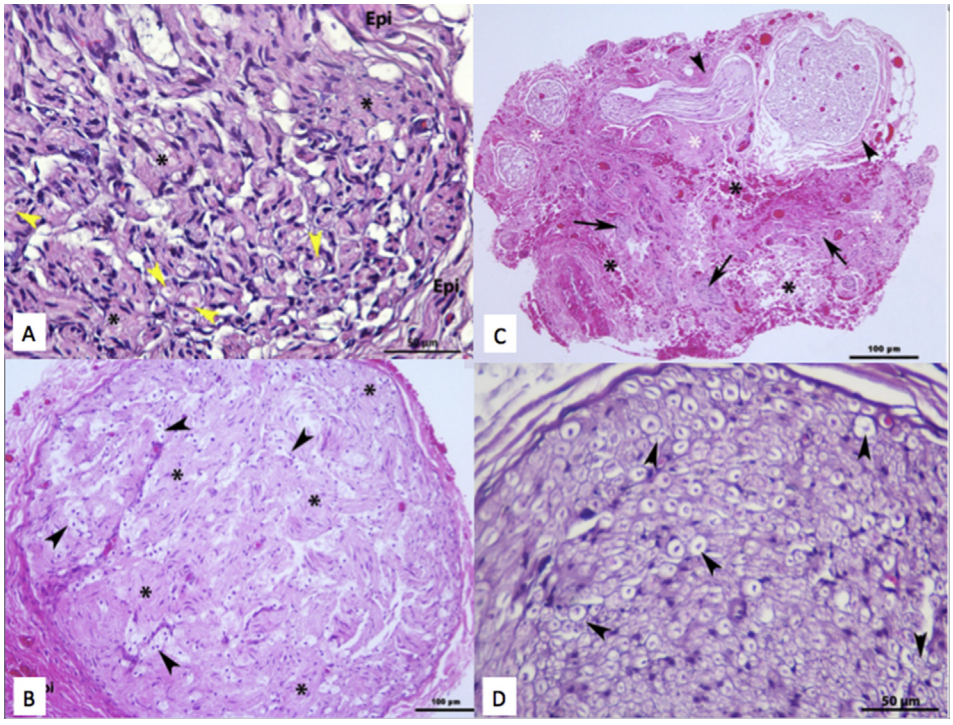

Fig. 4. (A) C group myelinated axons (yellow arrows). (B) LLLT group; unmyelinated axons are seen in nerve fibers. Black arrows indicate myelinated axons and black stars indicate unmyelinated axons. (C) LLLT group; nerves, branching, epineurium, and perineurium. A wide variety of small-diameter nerve fibers was observed within the fibrotic connective tissue. (D) Increased number of myelinated axons (black arrows), showing a uniform alignment in parallel with each other in the OT group.

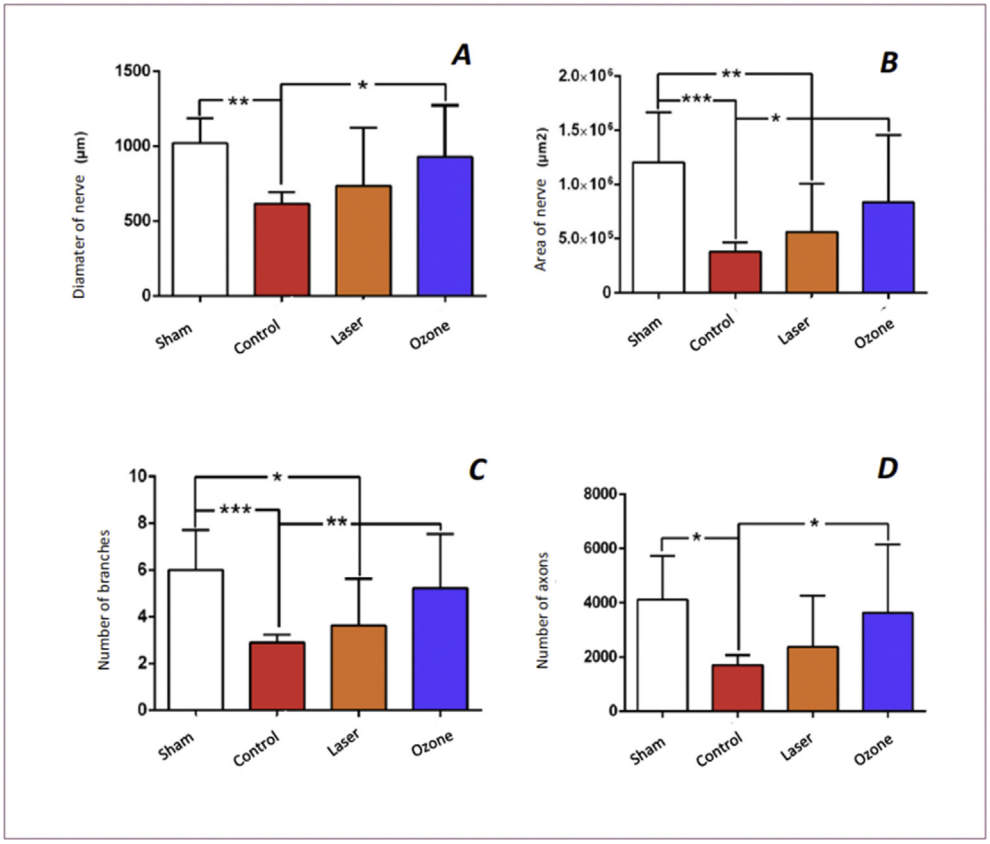

Fig. 5. Statistical analysis of (A) Nerve fiber diameters, (B) Nerve fiber areas, (C) Nerve branching numbers, and (D) Axon numbers among the groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.2. Diameter of nerve fibers (μm)

Mean nerve fiber diameter was 1021 ± 165 μm in the sham group, 617 ± 306 μm in the control group, 732 ± 391 μm in the LLLT group, and 929 ± 344 μm in the OT group. Compared with the sham group, a decrease was observed in all groups, but this was significant in the control group ($p = 0.0091$). Compared to the control group, there was an increase in the LLLT group, but a significant increase in the OT group ($p = 0.0398$) (Fig. 5B).

3.3. Area of nerve fibers (μm^2)

The mean area of nerve fibers was $12 \pm 4.6 \times 10^5$ μm^2 in the sham group, $3.8 \pm 3.3 \times 10^5$ μm^2 in the control group, $5.6 \pm 4.4 \times 10^5$ μm^2 in the LLLT group, and $8.4 \pm 6.2 \times 10^5$ μm^2 in the OT group. Compared with the sham group, the mean values decreased significantly in the control group ($p = 0.0002$) and the LLLT group ($p < 0.044$). When compared with the control group, a significant increase was observed in the OT group ($p < 0.042$) (Fig. 5C).

3.4. Number of axons

The mean number of axons was 4114 ± 1608 in the sham group, 1702 ± 1511 in the control group, 2379 ± 1898 in LLLT group, and 3640 ± 2514 in the OT group. Compared with the sham group, a decrease was observed in all groups, but this was significant in the control group ($p = 0.0103$). Compared with the control group, a small increase was observed in the LLLT group, while the number of axons increased significantly in the OT group ($p = 0.0327$) (Fig. 5D).

4. Discussion

The facial nerve should be treated immediately after the injury. Otherwise, atrophy and fibrosis may develop in the muscles as the duration of denervation increases. In the literature, muscle biopsies were performed periodically in patients who underwent anastomosis immediately after denervation and 4 weeks after anastomosis. Irreversible pathologies were detected in late surgery, while emergency surgery facilitated axonal regeneration (Constantinidis et al., 2003). Neurotrophic activity peaks within the first 3–6 h after nerve incision and returns to normal within 1–7 days. If this contact is provided by early coaptation, faster and better-quality regeneration can be achieved. Therefore it is suggested that nerve repair in the peripheral nerve section is an urgent surgical procedure and should be performed as early as possible (Schwarting et al., 1984; Constantinidis et al., 2003) For this reason, in our study treatments of the injured rat facial nerves were started immediately.

There are several alternative treatment options for peripheral nerve injury (PNI). However, for this study LLLT and OT were performed because of their effectiveness and safety (Yucesoy et al., 2017).

4.1. LLLT on facial nerve injuries

One of the first PNI studies was performed by Rochkind et al. in the 1980s (Rochkind et al., 1987; Rochkind et al., 1988) to evaluate the effect of different wavelengths of LLLT. Other studies have followed in recent decades (Hamilton et al., 1992; Anders et al., 1993; Anders et al., 2004; Chen et al., 2005; Barbosa et al., 2010; Yucesoy et al., 2017). For LLLT, a light-emitting diode (LED), the application of low-power lasers (via gases), and super luminized diodes (SLD) were the preferred options. Although the application methods are different, the results and efficacy have been similar (Telemeco and Schrank, 2013). The mechanism of action is believed to involve the absorption of light (photons) by photoreceptors (such as cytochrome C oxidase), altering the synthesis of ATP in mitochondria through the acceleration of the electron transport chains, thereby modulating cell reactions. In terms of peripheral nerve injury treatments, LLLT generally increases myelin capacity, neural tube formation, and Schwann cell stimulation (Hashmi et al., 2010; Farivar et al., 2014; Yucesoy et al., 2017).

In 2011, Kim et al. reported a very important study in which they stated that LLLT does indeed work, and has many useful aspects in clinical practice for practitioners in many surgical specialties when specific criteria are met, such as the correct wavelength for the target cells or chromophores, a suitable photon intensity, and an adequate dose or fluence (Kim and Calderhead, 2011). However, although we report an increased healing process for all parameters in the histological evaluations in our study, the results for the LLLT group were not statistically significant when compared with the control group. This surprising outcome was likely to have occurred because the treatment area was not specifically indicated by a marker (suturing, staining, etc.), which could result in the probe being positioned away from the injury area, negatively affecting efficiency.

Various parameters of LLLT, such as wavelength, dosage, and photon intensity, have also been discussed in the literature (Kim and Calderhead, 2011). Other parameters, such as the LLLT applicator, nerve type (motor or sensorial), PNI injury type, and operator effects have not been adequately discussed, given that LLLT has been shown to either inhibit or stimulate tissue metabolism depending on a range of factors (Kim and Calderhead, 2011). For example, SLD was preferred in our study, whereas it was recently reported that photobiomodulation by LED was highly effective on rat mental nerve injury, and that photobiomodulation produced better outcomes than OT (Yucesoy et al., 2017).

LED radiation is monochromatic and almost infrared, providing a less coherent scattering pattern, so it can be used safely on large areas of the body (Karu et al., 2005). Controversially, Chaves et al. reported that LED (non-coherent) would be less efficient than laser (coherent), or even unable to produce therapeutic effects, and that the cellular response to photostimulation was not associated with specific properties of laser light, such as coherence (Chaves et al., 2014). Moreover, Karu et al. reported that the property of coherence is lost during the interaction of light with biological tissue, thus not being a prerequisite for the process of photostimulation or photoinhibition (Karu, 1987).

While discussing the different parameters of LLLT in different studies, one of the most crucial problems appears to be the difficulty in the standardization of LLLT for PNI. Andreo et al. stated that the determination of these parameters is important for the standardization of an LLLT protocol to enhance the regeneration process following a PNI (Andreo et al., 2017). Oliviera et al. stated that there were many conflictions, which could be attributed to several variables such as wavelength, radiation dose, and type of radiation (de Oliveira et al., 2015).

4.2. OT on facial nerve injuries

The administration of OT has been performed safely and effectively on different peripheral nerves in rats. One of the very first studies was performed by Fuccio et al., in 2009, who injected ozone subcutaneously in a single dose and studied healing in the orbitofrontal cortex of neuropathic mice. The results were very promising because the OT prevented allodynia and decreased the overexpression of proinflammatory caspases (Fuccio et al., 2009). Some 2 years later Lin et al. studied the effects of ozone on the sciatic nerve in rats. They also performed a study on 30 rats, injecting ozone intraperitoneally at different doses. Their initial study suggested that ozone concentrations from 10 μg/ml to 80 μg/ml

injected around the rats' peripheral nerve did not cause serious sequelae or significant damage to the structure and function of the peripheral nerve. This finding provides evidence for the safety of ozone injected around the peripheral nerve (Lin et al., 2011). In 2017, Lu et al. also concluded that a single injection of ozone prevented neuropathy in a chronic constriction injury (CCI) rat model (Lu et al., 2017). Moreover, it was reported that the administration of ozone at a dose of 0.5 mg/kg after PNI in rats reduced injury to the myelin and axons (Kızılay et al., 2019). According to Somay et al. (2017) OT reduces fibrosis, vascular congestion, vacuolization, and edema in rodents and might be used to assist in sciatic nerve injury. Ozbay et al. revealed that OT exerted a beneficial effect on the regeneration of crushed facial nerves in rats (Ozbay et al., 2017).

On the other hand, although changes in the ocular surface induced by ozone have received limited research attention, some authors have concluded that ozone exposure interfers with ocular surface integrity and induces inflammation (Lee et al., 2013). Furthermore, Avcı et al. reported on a case of ischemic stroke after oxygen-ozone therapy, a condition also known as 'Anton syndrome' (Avcı et al., 2015). However, Valacchi et al. concluded that the correlation between ozone exposure and the development of skin pathologies needed further discussion (Valacchi, 2016).

In 2015, the World Federation of Ozone Therapy (WFOT) published a very important declaration, reporting that 'the ozonation of saline solution' introduces a very low amount of desolving of ozone in the body when you compare it with major autohemotherapy. And this amount is not deemed enough of a treatment to call it a 'therapy'. The WFOT also added that this technique induces the generation of dangerous oxidized chlorine derivatives, which have been shown to demonstrate mutagenicity and toxicity in clinical reports. For these reasons, WFOT could not admit this technique as a therapy (Schwartz-Tapia et al., 2015). Thus, we did not opt for ozonization of saline solution and instead carried out intraperitoneal ozone injections (80 μm/ml) for the treatment of FNI in rats, with results found to be statistically more significant than with LLLT in our study.

In other OT and LLLT comparison studies on different tissues, both therapies were found to be significantly superior, but the durations of treatment and methods of LLLT and OT application were different from our study (Alan et al., 2015; Yucesoy et al., 2017; Isler et al., 2018). We report that LLLT did not lead to a significant difference when compared with the OT and control groups. Although it has been commonly reported in the literature that the use of energy between 1 J and 6 J is sufficient, the presence of only one OT and LLLT comparison study of PNI in rats (Yucesoy et al., 2017) led us to conclude that further comparative studies with different parameters were required to understand the nerve regeneration process, and to produce improved and clearer standardization of treatment protocols.

The limitations of the study were that the histological evaluations were performed only under the light microscope, and not in the electron microscope, and that no Schwann cell count was carried out as part of the histological measurements.

5. Conclusions

This study concluded that OT therapy provides a safe and effective treatment, offering insight into the use of OT and LLLT for the treatment of FNI, and encouraging clinicians to use OT in their routine practice and to perform clinical studies.

OT should not be performed with a mixture of gas ozone and NaCl serum. However, with proper usage, OT outcomes in this study were was statistically better than those for the LLLT and control groups. Although LLLT showed better outcomes than the control group, the differences were not statistically significant ($p > 0.05$).

For standardization in future treatments, the parameters should be more precise and specific to the injured tissue. Further comparative studies on peripheral nerve regeneration are essential.

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Declaration of Competing Interest

All authors declare that they have no conflicts of interest.

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