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Comparison of the effects of ozone therapy and photobiomodulation on sciatic nerve injury in rats

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Abstract
Aim: Studies on drugs or alternative therapies are still the main treatment options for PNI. In this study, we aimed to research the effects of PBM and OT on nerve repair in a rat sciatic injury model.
Material and Methods: 29 Wistar albino rats were divided into four groups: control (n = 2), sham (n = 9), OT (n = 9) and PBM (n = 9). After 30 days of surgery and treatments, tissue specimens and blood samples were taken for histological and biochemical processing. Histological evaluations were performed at light and electron microscopy levels. Myelin basic protein (MBP) and S100 from the rat serum were analysed also.
Results: The OT and PBM groups had a significant increase in regeneration of the sciatic nerve in light microscopic evaluation. In the PBM and OT groups, Schwann cells (SC) around the axons and also axons with a thin myelin sheath were seen, regarded as signs of the myelination process in transmission electron microscopy (TEM) examinations.
Discussion: OT and PBM both resulted in a good healing pattern for sciatic nerve injury in the rat model. Therefore, OT and PBM are considered to be simple and reliable alternative treatment methods for PNI.

Keywords
Ozone, Photobiomodulation, Sciatic Nerve

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Introduction

Peripheral nerves cover a large distance before reaching the end organs and can therefore be damaged in several ways. The main cause of peripheral nerve injury (PNI) is trauma, but ischaemic events, infections, traction, compression, burn injury can also cause PNI. Unfortunately, the response to PNI does not involve mitosis or cellular proliferation as in other tissues of the body. After an injury, SC proliferate, co-migrate and regrow, thus providing a favourable substrate for axonal extension [1,2]. Regeneration differs according to the type of nerve injury or degree of damage. Motor and sensory function defects can lead to unwanted maladaptive clinical situations such as dysaesthesia, hyperreflexia and dystonia [3].

Management of neuropathic pain is a complex clinical condition for both the patient and the physician. Pharmacological treatment is generally the first step, which often includes several drugs. Nerve autografting has been the first choice and the 'gold standard' for repairing peripheral nerve defects. However, this technique has some disadvantages, such as a limited supply of available nerve grafts, permanent loss of the donor nerve function and potential differences in tissue structure and size. Although xenografts and allografts are common alternatives to autografts, they have lower success rates and may be subject to immune rejection [4-5].

Conservative therapies are thought to be useful for milder cases of PNI but a serious crushed nerve may result in Wallerian degeneration of the distal segment [6]. Ozone therapy (OT) and photobiomodulation (PBM) are two of the newer alternative therapies that have an advantage over treatments such as cryotherapy or acapuncture [7]. Ozone is an unstable gas with strong oxidizing power that has good antiseptic, disinfectant and antiviral properties for use on all surfaces. Ozone also works as an immunomodulator and shows long-term anti-inflammatory effects [8,10]. For a long time, OT has been used in numerous different areas of medicine for the treatment of: acute and chronic infections; ischaemic disorders; orthopaedic, dermatological, pulmonary, renal and haematological disorders; and neurodegenerative diseases [10,11

PBM, formerly known as low-level laser therapy (LLLT), has been performed to facilitate the regeneration of peripheral nerves for early recovery of patient functionality. LLLT was first used for this aim in the 1970s, with some inconsistency [12,13]. PBM has been shown to stimulate SC and increase myelin capacity, which is a good marker of axon healing [14]. Also, the positive effects of PBM shown on mental nerve injury and neuropathic pain have been reported in our previous study [7].

In the present study, the primary aim was to evaluate and compare the effectiveness of OT and PBM for treating sciatic nerve damage in rats histologically. A secondary aim was to evaluate the biochemical changes associated with sciatic nerve injury in rats.

Material and Methods

A total of 29 adult Wistar albino rats weighting 250–300 g were included in the study regardless of gender. Animals were given food and water ad libitum. Ethical consent was obtained from the ethical committee of Erciyes University (Erciyes University, Animal Local Etical Comity, 16/011).

Intramuscular xylazine was used for anaesthesia induction of the rats: 1 mg/kg (Ketalar, 50 mg/ml; Pfizer, New York, NY) and 0.5 mg/kg (Rompun, 23.32 mg/ml; Bayer, Mefar Ilac San. AS, Istanbul, Turkey). The dorsal third of the sciatic nerve was sutured according to Seltzer et al.'s [15], neuropathic pain model in rats. The skin incision of all rats was covered with resorbable 3-0 suture.

Rats were divided into four groups: in the control group (n = 2), intact nerve tissue from rats whose sciatic nerves were not ligated was collected as normal tissue; in the sham group (n = 9), the sciatic nerve was ligated surgically and no therapy was applied; in the PBM group (n = 9), sciatic nerve treated with PBM was collected; and in the OT group (n = 9), sciatic nerve treated with OT was collected.

PBM therapy

PBM group received the therapy with an OsseoPulse LED (Biolux Research Ltd, Vancouver, Canada) in contact mode to the sciatic nerve. PBM is applied at a 618-nm wavelength and with 20 mW/cm2 output power, during 21 days for 5 minutes daily. Total 6 J/cm2 dosage of the energy amount was used for the treatment of neuropathy which is suggested therapeuticdose for this situations [16].

Ozone therapy

In Group OT, treatment was appliedby a CA probes that contacted to the sciatic nerve area (MIO International Ozonytron GmbH, Munich, Germany) which was connected to the ozone generator (OzonytronXL, MIO International Ozonytron GmbH). The OT, during 60 seconds with 75% power, was applied as recommened. OT was applied once every 3 days, totaly 7 times to the rats' sciatic nerves [16].

The rats sacrified just after taken 5 ml of blood samples from the heart for biochemical evaluations which was expected to support our study results. In the beginning, blood samples were taken to tubes and centrifuged 1200 (Rpm) rates during 12 minutes. The serum of the sample over the tube was taken with a pipet and stored into pellets at -20 oC. Before the ELİSA tests, serum samples which were frozen at -20 °C were defrosted for 2 hours at room temperature.

Histological evaluations:

In order to evaluate the specimens by transmissionelectron microscopy (TEM), the tissue samples obtained from animals were fixed in 2.5% glutaraldehyde solution in 0.1M PBS buffered (pH 7.2) for 4 hours at +4°C. Then the glutaraldehyde solution was washed with PBS and tissues were post-fixed with 1% osmium tetroxide solution during 1 hour at room temperature andpassed through rising alcohol series (%70-90-96-100) for dehydration. The samples were incubated bypropylene oxide/ Epon mixture and embedded in Epon 812. The epon blocks were cut by ultramicrotome (Leica Ultracut R, Wetzlar, Deutchland) in semi-thin sections (900-1000um in thickness) and stained with toluidine blue. Sections were examined by BX-51(Olympus, Japan) light microscope for both semiquantitative analyses and determination of the proper areas which would be analyzed in TEM and then photographed with the DP-72 camera system attached to light microscope.

For Light Microscopic evaluation, photographs taken from at least three areas in x400 magnification were evaluated semiquantitativelyin terms of the presence of axons with

thinmyelin layer and small diameter. The scoring system used as; "0: None, 1: Mild, 2: Moderate, 3: Severe". The observed scoring values were regarded as follows; 'Axons with thin myelin layer and small diameter were accepted as myelinization begins and/or persists' in the samples that had ''3-2: Severe-Moderate'' score leveland 'Although the morphology of some of them is distorted, small-diameter thin myelinated axons are scattered between myelinated axons'. In the samples that had ''0-1: None-Mild'' score level. In regards of the semiquantitaive evaluations, the specimens which had higher score level were accepted as having more regeneration activity than that of having lower score level.

For TEM examinations, the epon blocks were trimmed to get the proper areas. The thin sections in 80-100um thickness were cut by ultramicrotome (Leica Ultracut R,Wetzlar, Deutchland) on 200-mesh copper grids. Air-dryed grids were stained with uranyl asetate and lead citrate and evaluated in the TEM and photographed with SIS Morada camera system.

Biochemical Evaluations:

Rat MBP (Myelin basic protein S) Test and Rat S100 tests were performed as below with Wuhan Fine Biological Technology ELISA Kits. Absorbance values were obtained by reading in a 450 nm wavelength filter on an ELISA reader.

Statistically Analysis:

To summarize the biochemical data obtained from the study, descriptive statistics were given as a median-quarterly width for continuous variables. The normality test of numerical variables was checked with Kolmogorov Smirnov test. In independent group comparisons more than two groups, Kruskal Wallis H test was used in cases where the numerical variables did not show normal distribution. Statistical analysis was performed by Jamovi project (2018). Jamovi (Version 0.9.1.5) [Computer Software]. (Retrieved from https://www.jamovi.org) (open source), and the statistical analysis of the level of significance (p-value) was considered as $p< 0.05$.

Results

Clinical findings and observations

In this study, we observed that all of the animals for 24 hours after operation, showed that the rats were not completely paralysed.

Histological outcomes

Data from semi-quantitative evaluations obtained at the light microscopy level (Figure 1) showed that the OT and PBM groups had a significant increase in regeneration of the sciatic nerve. Regular and normal myelin sheath morphology was seen in the control group and irregular myelin sheath morphology was detected in the sham group. Furthermore, axons with a thin myelin sheath were observed in both therapy groups.

Table 1. Biochemical results

Groups	MBP	p	S100B	p
Control	0,716 (0,6-0,832)		56,895 (56,27-57,52)	
Sham	1.02 (0.903-0.675)		107,54 (62,2-148,24)	
Ozone	0,88 (0,646-2,337)	0,534*	140,15 (76,9-198,85)	0,401*
Laser	1,091 (0,252-1,261)		47,115 (42,465-103,955)	

* Kruskal-Wallis H test was performed. Descriptive statistics are given as median (Q1-Q3).

In transmission electron microscopy (TEM) examinations, myelinated axons with regular morphology were seen in the control group whereas the structure of the myelin sheath was degenerated in the sham group. In the PBM and OT groups, SC around the axons and also axons with a thin myelin sheath were seen, regarded as signs of the myelination process (Figure 2).

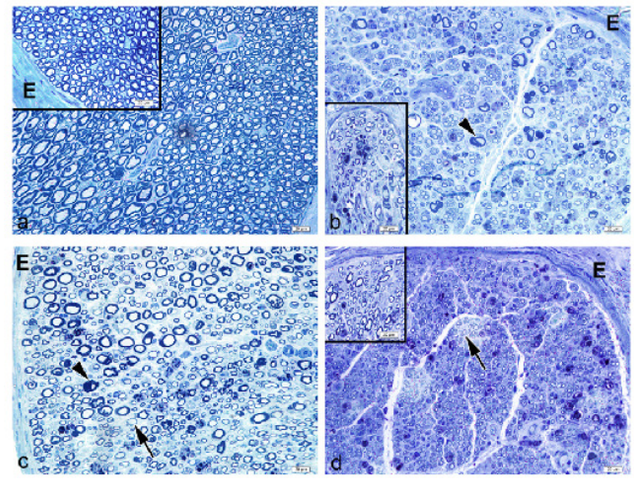

Figure 1. Demonstrative micrographs at light microscopic level from each experimental groups. a. Control group, b. Sham-operated group, c. Laser-treated group, d. Ozone-treated group. E: Epineurium, arrow: axon with thin myelin sheet, arrowhead: myelin sheeth with irregular morphology. Toluidine blue staining.

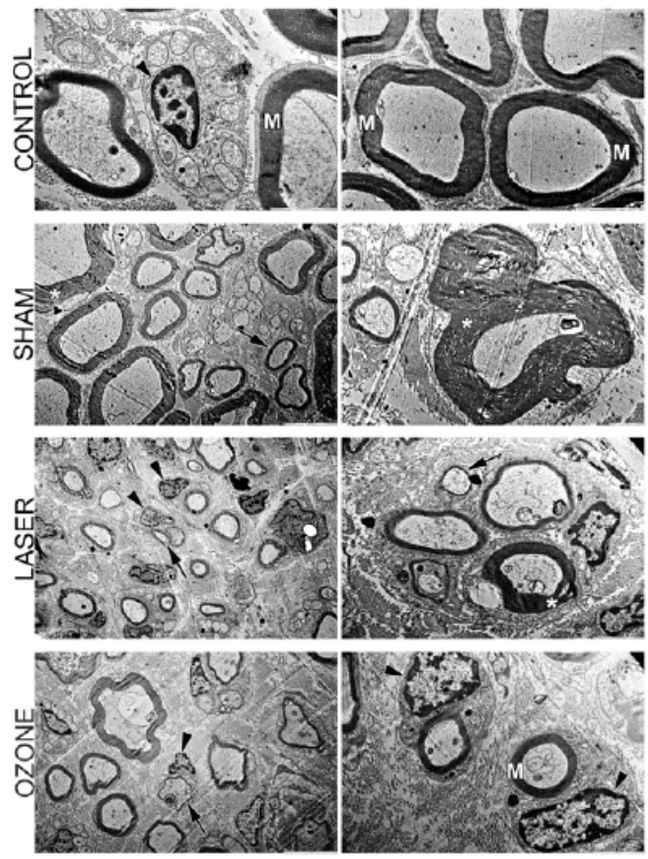

Figure 2. Demonstrative micrographs at electron microscopic level from each experimental groups. M: Myelin sheeth with regular morphology, asterisk (*): Myelin sheeth with irregular morphology, arrow: axon with thin myelin sheet, arrowhead: Schwann cell.

Biochemical outcomes

There was no statistically significant difference between the groups when comparing the median values of two biochemical markers: myelin basic protein (MBP; p = 0.534, Table 1) and S100 calcium-binding protein B (S100B; p = 0.401, Table 1).

Discussion

According to our study results, PBM and OT were found to be reliable, promising and alternative treatment methods for PNI by improving regeneration of the sciatic nerve.

Several studies were performed to explain the pathophysiological mechanisms in PNI and histological differentiation. The goal of nerve damage treatment is to provide nerve integrity and conduction, along with restoration of the primary function of the nerve [17,18]. Actually, the human sciatic nerve does not damage easily. Rats are often preferred for studies into peripheral nerve regeneration because of the similarity of rat nerve branches to those of humans. Also, rat sciatic nerve includes axons of different sizes and types and is thus a multi-fascicular mixed-type nerve [19]. Although there are several behavioural tests for researching neuropathic pain in the literature, difficulty using these tests in practice for rats and also differences in sensibility of the researchers may affect the reliability of the measures [20,21]. Thus, we did not prefer behavioural tests in this study and aimed to show whether treatment-related improvement histologically could be supported by biochemical results.

Several treatment approaches have been applied to solve neuropathic pain, such as drug therapy alone or in different combinations or with interventional therapies [22]. Unfortunately, treatment success for neuropathic pain requires an interdisciplinary approach and this is not always possible. Pain reduction of at least 30% is generally accepted to be a clinically meaningful result [23]. PBM and OT are considered to be enhanced alternative therapy modalities [6]. In our study, PBM and OT provide healing in sciatic nerve damage histologically.

PBM has many constructive effects, such as reducing edema, inflammation and pain, and also has anti-inflammatory and analgesic effects with wound healing and bioactive properties. Furthermore, PBM (i.e. LLLT) has some of the healing effects on the nerve that are reported to increase myelin capacity and provide neural tube formation with SC stimulation [12]. LLLT is an energy that does not exceed 36.5°C is produced. This application is mainly non-thermal and biostimulatory due to its low energy output and density [22]. In this recent study, we preferred LED-mediated monochromatic infrared LLLT for obtaining the regenerative and biostimulant effect, at a wavelength of 618 nm and output power of 20 mW/cm2 for 5 min in each session over 21 days in rat sciatic nerve. In light microscopy semi-quantitative evaluations, regeneration findings were found. Also, in TEM examinations the structure of the myelin sheath was degenerated in the sham group where surgery was performed without any therapy. This result was an indication of the nerve damage that can be produced. We observed SC around the axons, and axons with a thin myelin sheath.

OT is thought to be based on the conversion of oxygen atoms in the environment to ozone following contact of a special type (probe) with the tissue [23]. Although the wound healing capacity of OT is known, studies in which neuronal regeneration has been followed with OT are rare in the literature. No clear information was found about the frequency of OT application, the power of the device or the duration of application in the literature. We administered OT once every 3 days over 21 days (totalling seven times) at 75% density and 60 s, as in our previous study performed in the mental nerve of rats, and found success for neuropathic pain [7]. In our previous study we found a higher number of SC after OT. In the present study we similarly observed SC around the axons as a myelination process in both light microscopy and TEM views.

As a result, PBM and OT were found to be effective for sciatic nerve injury in this study. At the beginning of the study we wondered if biochemical markers such as S100B and MBP may change with these treatment methods, which determined the basis of this study. In vitro and in vivo studies have shown that S100B has a neurotrophic effect on the regeneration of neurons after neuron damage and increases the neuronal protection properties during development [23]. S100B is a member of the S100 protein family and is responsible for the regulation of energy metabolism in brain cells. S100B is found not only in brain tissue and SC in the peripheral nervous system, under physiological and pathological conditions [24]. Iwasaki et al. [25] observed that neurons could maintain viability and that S100B reduced motor neuron loss. In this study, we researched S100B levels in control and other groups from blood. We detected that S100B was higher in the sham and treatments groups than the control group, although this result was not statistically significant. An increase in the level of MBP, the other main protein in SC in the peripheral nervous system, was also expected during nerve regeneration [26]. A meaningful increase or difference between the control and the sham or treatment groups didn't observed. Healing in the sciatic nerve did not supported by biochemical findings. Perhaps if these markers were evaluated immunohistochemically from histological sections a different result would be obtained, because in some situations S100B cannot be detected in blood serum due to other cytokines released from T cells.

In current anaesthesiology settings, the sciatic nerve may be damaged during anaesthesiology application or with different surgeries, therefore improving new alternative therapeutic strategies for sciatic nerve damage and related neuropathic results is really important for anaesthesiologists and surgeons. There are many studies that have researched OT or PBM on sciatic nerve in the literature but this is the first original study to compare the effects of OT and PBM in rats with sciatic nerve injury using light microscopy and TEM.

A limitation of this study could be that we did not use behavioural tests; these may point out neuronal disorders in animal models by showing abnormal responses to sensory stimuli and supported neuropathy [27]. However, such stimulatory-mediated methods, applied with cold, warm or mechanical stimuli, are applicable to nerves that have more motor functions and also evaluation of the response of animals to physical or chemical stimuli can often be very subjective [27]. Thus, we did not use behavioural tests in this study, preferring more objective evaluations such as light and electron microscopy.

In conclusion, PNI that causes neuropathic pain is an undesirable, uncomfortable condition. Both OT and PBM are considered to be simple and reliable alternative treatments in the PNI model by partially suturing the sciatic nerve in the subject animals, although the superiority of OT and low-dose PBM is not proven in this study.

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Scientific Responsibility Statement
The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement
All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest
None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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