

Neuroprotective Effects of Ozone Therapy After Sciatic Nerve Cut Injury

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Summary: A number of antioxidants have been used to treat peripheral nerve injury. However, there are few definitive experimental studies of ozone therapy for peripheral nerve cut injury. We aimed to examine the effects of mild level ozone therapy on sciatic nerve regeneration. One hundred adult male Wistar albino rats were randomly divided into four groups: group 1 (n=20) no cut injury or therapy; group 2 (n=20) sham; group 3 (n=30) nerve cut injury, no therapy; group 4 (n=30) nerve cut injury and ozone therapy. Sciatic functional index (SFI) and withdrawal reflex (WDR) were measured for all groups before nerve cut, at postoperative day 1, and at weeks 2, 4, 6 and 8. More myelinated (M) nerve fibers were observed after nerve cut injury in the ozone-therapy group. Significant differences were seen in plasma SOD (superoxide dismutase), CAT (catalase) and GPx (glutathione peroxidase) activities ($p<0.05$), and significant functional improvement was observed at postoperative weeks 2 and 4 ($p<0.05$) after ozone treatment. This is the first study conducted for the purpose of examining the effects of ozone therapy on sciatic nerve cut injury.

Key words ozone, ozone administration, peripheral nerve, ozone therapy, nerve cut injury

INTRODUCTION

Peripheral nerve injuries are one of the most important causes of morbidity and loss of economic and social activity [1]. The basic aim of therapy after peripheral nerve injuries is to bring back nerve conduction and lost motor and sensory functions by restoring nerve integrity [1-4]. Several structural and functional changes occur in peripheral nerve injuries. The sensory and motor functions are reduced by optimal surgical peripheral nerve repair. This inadequacy caused by peripheral nerve injuries is related to the type of injury and repair method [1,5]. Oxidative stress can damage peripheral nerve regeneration and the process of repair after nerve cut injury [5-7]. The negative im-

pacts of free oxygen radicals (FOR) on nerve regeneration can be decreased by antioxidants [7-9] Ozone therapy has been developed and it represents a recognized and efficient alternative treatment under specific clinical conditions [10] and has also been suggested as an immunomodulator and activator of cellular metabolism exhibiting long-term anti-inflammatory impacts [11]. Ozone causes a mild level of oxidative stress in endogenous antioxidant systems [12-13] which is beneficial for the increase in tissue oxygenation, suppression of infection, release of cytokines, and regulation of inflammation [14-15]. Moreover, cyclooxygenase can be activated via ozone therapy, and it may cause an increase in free radical (FR) inhibition in an injury

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Abbreviations: A, Axons; CAT, Catalase; C, Collagen Fibers; EM, Electron Microscope; E, Endoneurium; Ep, Epineurium; FOR, Free Oxygen Radicals; FR, Free Radicals; Gpx, Glutathione Peroxidase; LP, Lipid Peroxidation; MDA, Malondialdehyde; MOT, Medical Ozone Therapy; M, Myelinated; P, Perineurium; ROS, Reactive Oxygen Species; SFI, Sciatic Function Index; SOD, Superoxide Dismutase; TBA, Thiobarbituric Acid; TBARS, Thiobarbituric Acid Reactive Substances; TEM, Transmission Electron Microscope; U, Unmyelinated Nerve; V, Vasa Nervorum.

[14-15].

After an injury, ischemic and inflammatory processes start and therapies are utilized to decrease the above-mentioned effects [7,15]. The generation of FR causes lipid peroxidation (LP) leading to oxidative stress and adverse effects on tissues. Cell damage may be increased by FR, which leads to cell death. The cells can be protected by antioxidant enzymes, which reduce the toxic effects of FR. The antioxidant expression can be activated by Reactive Oxygen Species (ROS) in the majority of cells. The activation of ROS-mediated signal cascades leads to an increase in expression of antioxidants, and this is the main mechanism of redox homeostasis. Ozone therapy promotes peripheral nerve regeneration by increasing antioxidant levels and decreasing FOR. Ozone therapy is considered to be an alternative safe and effective therapeutic method for oxygen metabolism, cellular energy, and antioxidant defense [9,14,16]. Ozbay et al. [6], indicated that ozone therapy may be a promising alternative for the improvement of peripheral nerve injury [6]. The primary aim of this study is to determine the reliability of ozone therapy for peripheral nerve cut injury and evaluate its positive impacts by light and transmission electron microscope (TEM), motor, sensorial function tests, and biochemical analysis.

METHOD

Animals:

A total of 100 adult male 400–450 gr *Rattus norvegicus* Wistar albino rats were randomly divided into 4 groups. Their numbers were determined as follows: Group 1 (normal, without ozone treatment) (n=20), Group 2 (sham, incision only) (n=20), Group 3 (nerve cut injury, no therapy) (n=30) and Group 4 (nerve cut injury with ozone therapy) (n=30) (Table 1). Animals were maintained on a 12 hour light–dark cycle. Food and water were provided *ad libitum*. All procedures were reviewed and approved by the Animal Care and

Usage Committee of Akdeniz University (*protocol no*: 2013.02.0122.002). The sensory and motor function analyses of all groups were evaluated before the operation and on the first day after surgery. Thereafter, evaluations were performed in the 2nd, 4th, 6th and 8th weeks.

Surgery:

Animals were sufficiently anesthetized intraperitoneally with 15 mg/kg XylazinHCl (Rompun®, Bayer, İstanbul, Turkey) and 100 mg/kg Ketamine (Ketarlar®, Pfizer, İstanbul, Turkey). Transverse cut injury to the sciatic nerves (groups 3 and 4) was performed under surgical microscope (Leica, SZGI, Oberhausen, Germany) around the mid-hindlimb. The nerve stumps were repaired (end-to-end repair) with 10.0 suture material. The skin was closed with a 2.0 suture material.

Medical ozone therapy:

Ozone was supplied by Medozon compact ozone generator (Herrmann Apparatebau GmbH, Elsenfeld, Germany). Ozone was administered daily at a concentration of 35-40 ug/ml and volume of 5ml by intraperitoneal method, for 2 months. At the end of the 8th week, evaluation tests were administered, the rats were sacrificed, and their nerve tissues were taken.

Evaluation Tests

Motor Function Test (Walking Track Analysis):

Sciatic Function Index (SFI) was used to monitor the sciatic nerve function. The animals' hind feet were dipped into Indian ink (Winsor & Newton, İstanbul, Turkey) and their footprints were taken. The SFI values were calculated according to the formula modified by Bain et al [17]. SFI was calculated based on the footprints taken on the postoperative 1st day and at the 2nd, 4th, 6th and 8th weeks.

Sensory Function Test (Pinch Test):

Functional sensory recovery was analyzed by pinch test. The rats were handled gently without being

TABLE 1.
Rat Groups

Groups	Numbers	Injury Type	Ozone Therapy
Group 1	20	no	no
Group 2	20	sham (no injury)	no
Group 3	30	cut injury	no
Group 4	30	cut injury + ozone	yes

exposed to stress and the skin of the foot was pinched by forceps (Splinter & Potts-Smith, Leica, Nußloch, GmbH, Germany). The rats in each group were graded on day 1, and at weeks 2, 4, 6 and 8. The levels were graded as no response (Grade 0), weak response (Grade 1), mild response (Grade 2), and severe response (Grade 3).

Tissue harvesting:

The harvesting of sciatic nerve specimens was performed below the lesion and the samples were separated for two evaluations. One half of each nerve sample was immediately prepared for routine electron microscopic examination, and the remainder was used for biochemical analyses: specimens were snap-frozen and kept at a temperature of -80 °C.

Biochemical Analysis:

Measurement of LP, glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) activity:

The thiobarbituric acid (TBA) fluorometric assay was used to measure malondialdehyde (MDA) levels. Briefly, 50 µl of the tissue specimen was put into 1 ml of distilled water and mixed with a volume of 29 mM TBA in acetic acid. The specimens were incubated for 1 hour at a temperature above 95 °C, after which they were cooled, and 25 µl of 5 mM HCL was added. The extraction of the final mixture was performed using 3.5 ml of n-butanol and was centrifuged at 3000×g for 10 min. The determination of MDA levels was conducted fluorometrically at 525 and 547 nm, respectively (LS 45 Fluorescence Spectrometer, Perkin Elmer, Waltham, USA). 1,1,3,3-tetraethoxy-propane standard was taken as a sample and the standard curve was obtained. The amount of Thiobarbituric Acid Reactive Substances (TBARS) in plasma was reported as nmol/g protein.

GPx activity assay kit (Sigma-Aldrich Chemie, Steinheim, Germany) was utilized for the measurement of GPx. The GPx activity assay measures enzyme activity indirectly via a coupled reaction with glutathione reductase (GR). A decrease in absorbance at 340 nm occurs with the oxidation of NADPH to NADP. Spectrophotometric assessment of absorbance kinetics was performed at 340 nm with the NADPH extinction coefficient of 0.00622 µM⁻¹. A unit of enzyme activity was defined as the amount of enzyme leading to the oxidation of 1 µmol NADPH to NADP per minute at 25 °C.

SOD activity assay kit (Cayman Chemical, Ann Arbor, MI, USA) was utilized for the measurement of superoxide activity. Tetrazolium salt is used in the SOD activity assay to determine superoxide radicals produced by xanthine oxidase and hypoxanthine. SOD activity is defined as the amount of enzyme that is required to achieve 50% dismutation of superoxide radical. The SOD assay was used for the measurement of the cytosolic and mitochondrial activity of the enzymes.

CAT activity assay kit (Cayman Chemical) was utilized to measure catalase activity. This kit uses the peroxidatic function of CAT to determine enzyme activity. The reaction of the enzyme with methanol when H₂O₂ is present constitutes the basis of the method. The spectrophotometrical measurement of the formaldehyde generated by the reaction was performed using purpald (4-amino-3-hydrazino-5-marcapto-1,2,4-triazole) as the chromogen. A unit of enzyme activity was defined as the amount of enzyme leading to the formation of 1 µmol formaldehyde per minute at 25 °C.

Electron Microscopic evaluation:

Four percent (4%) glutaraldehyde was used to fix sciatic nerve specimens from the distal part of every lesioned nerve (5 mm distal to the lesion) in 0.1 M Sorensen's phosphate buffer solution (pH 7.3) after the rats had been sacrificed, and 2% osmium tetroxide was used to postfix them in the same buffered solution. The specimens were dehydrated using a graded series of ethanol and then embedded in epoxy resin (Araldite CY212, Agar Scientific Ltd, Stansted, UK). Cutting of semithin (300 nm) and ultrathin (70 nm) sections was carried out on a ultramicrotome (Leica, Ultracut UCT) and photographs were taken by Leo906E TEM.

Data Analysis:

Data from all groups were tested using one-way analysis of variance (post hoc Tukey test), Kruskal-Wallis test (post hoc Dunnett test) and Mann-Whitney test. SPSS 21 (IBM Electronics, USA) software was used for all statistical analyses and a p-value less than 0.05 was considered statistically significant.

RESULTS

Functional tests:

SFI values:

SFI values were significantly decreased in Groups 3 and 4. An obvious decrease was observed in the co-

efficients of negative values starting from the pre-operative period to post-operative week 8 (post-operative 1st day = -85 ± 6.11 ; post-operative 2nd week = -80 ± 5.19 ; post-operative 4th week = -37 ± 6.11 ; post-operative 6th week = -23 ± 5.89 ; post-operative 8th week = -16 ± 5.06) (Fig. 1).

Sensory Function Test (Pinch) Values:

Grade 3 response differed significantly ($p=0.035$, $p<0.05$) from that of the ozone group (group 4) at post-operative week 2 (Fig. 2).

Biochemical Results:

In the control (group 1) and sham-operated group (group 2), GPx activity was 25.07 ± 3.35 , and 24.62 ± 3.35 , respectively. Ozone therapy increased the GPx activity in group 4 (15.83 ± 3.46) ($p<0.05$) and group 3 (5.86 ± 1.12) (Fig. 3.a). In groups 1 and 2, SOD activities were 7.70 ± 1.23 , and 7.06 ± 1.17 , respectively. Ozone therapy increased the SOD activities in group 4 (2.69 ± 0.64) ($p<0.05$) and group 3 (1.39 ± 0.31) (Fig. 3.b). In groups 1 and 2, CAT activity was 24.16 ± 1.73 , and 23.84 ± 1.28 , respectively. Ozone therapy increased the CAT activity in group 4 (15.77 ± 0.93) ($p<0.05$) and group 3 (12.71 ± 1.10) (Fig. 3.c). In groups 1 and 2, MDA activity was 0.18 ± 0.029 , and 0.19 ± 0.03 , respectively. Ozone therapy significantly decreased MDA activity in group 4 (0.28 ± 0.03) ($p<0.05$) compared to group 3 (0.38 ± 0.04) (Fig. 3.d).

Electron and light microscopic analysis:

Sciatic nerve ultrastructure was normal in groups 1 and 2. Myelin sheaths showed good organization, and no myelin residues or edema were present. No deformation was encountered in the myelinated (M) nerve fibers. In the semi-thin specimens taken from group 3, M nerve sheaths of different diameters and thicknesses were also observed, along with many regenerating fibers. In group 4, schwann cells with a thick layer of cytoplasm around the M nerve fibers were observed. All nerve fibers were encircled tightly by collagen fibers (C). In group 3, it was found that the entire nerve contained remyelinated axons with different diameters. The improvement of the above-mentioned ultrastructural properties was more notable in group 4 (cut + ozone) than in group 3 (cut) (Fig 4).

The epineurium (Ep) had a normal structure in the control group (group 1), but its connections with the perineurium (P) had been cut due to separation in some fields. The same separation was observed between the P and E. These separations were evaluated as preparation-related artifacts because there was no significant nerve fiber atrophy or degeneration. There were M sciatic fibers of different diameters and vasa nervorum in the sections. The EP encircled the nerve trunk integrally and covered all bundles. No deformation was encountered in the M nerve fibers (Figure 4.a). The M and unmyelinated (U) fibers showed a normal ultrastructure in the group 1 electron micrographs. Schwann cells had a normal ultrastructure with the usual cell membrane and the cytoplasmic

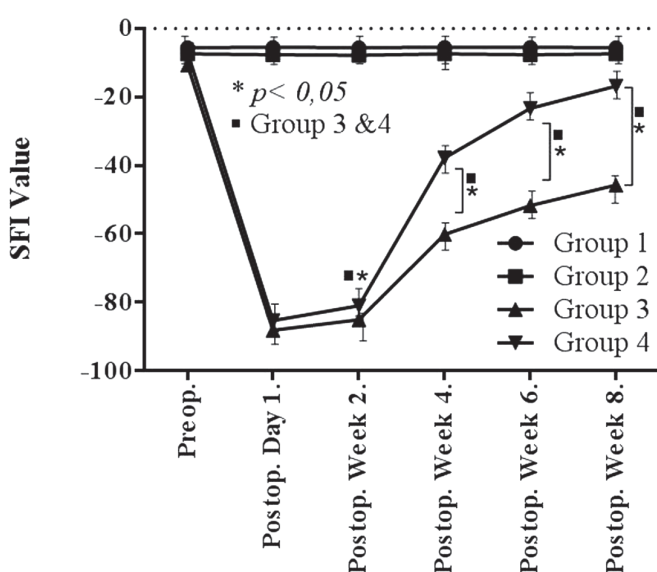


Fig. 1. SFI values and operation time

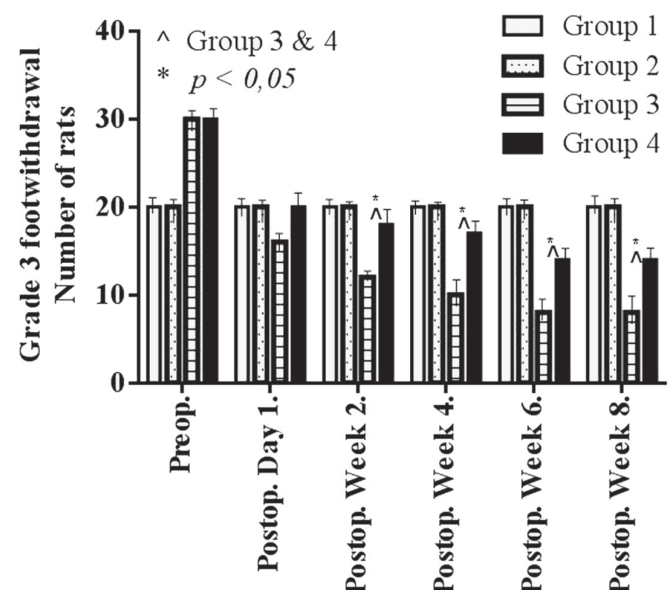


Fig. 2. Number of animals showing grade 3 withdrawal reflex

composition. The structure of the myelin sheath was normal and no expansion or local dissolution was encountered among the membrane layers of the Schwann cells. The relationship of myelin sheath and the mesaxon structures was normal in axolemma and no separation was found in the samples. The organelles showed a normal morphology in axoplasm. Neurotubules and neurofilaments could be monitored clearly and mitochondria and smooth endoplasmic reticulum had a normal structure (Figure 4.b). Also, the endoneurial capillary, endothelium and pericytes were normal in terms of both structure and ultrastructure. Endothelial tight junctions and uninterrupted basal lamina were seen in the control group samples (Figure 4.a, b). Similar characteristics were observed in group 2. All the sections showed M nerve fibers of various diameters and endoneurial vasa nervorum (Figure 4.c). The sciatic nerve ultrastructure was similar to that of the

control group in the electron micrographs (Figure 4.d).

Sciatic nerve regeneration was seen in the light and electron micrographs of group 3. Different diameters of M nerve sheaths and different thicknesses were also observed in the nerve trunk. There were many regenerative fibers and a small amount of myelin residue and deformative M axons in the nerve trunk (Figure 4.e). Schwann cells with a thick layer of cytoplasm were observed around the M nerve fibers related to regeneration. Samples had thicker cytoplasm and more organelles as compared to the control and sham groups. The cytoplasm generally formed a thin line around the M nerves. The Schwann cells in this group also contained abundant mitochondria, granular endoplasmic reticulum, lysosomes and vesicles. Endoneurium (E) contained dense collagen bundles. All nerve fibers were encircled tightly by C. Among the bundles

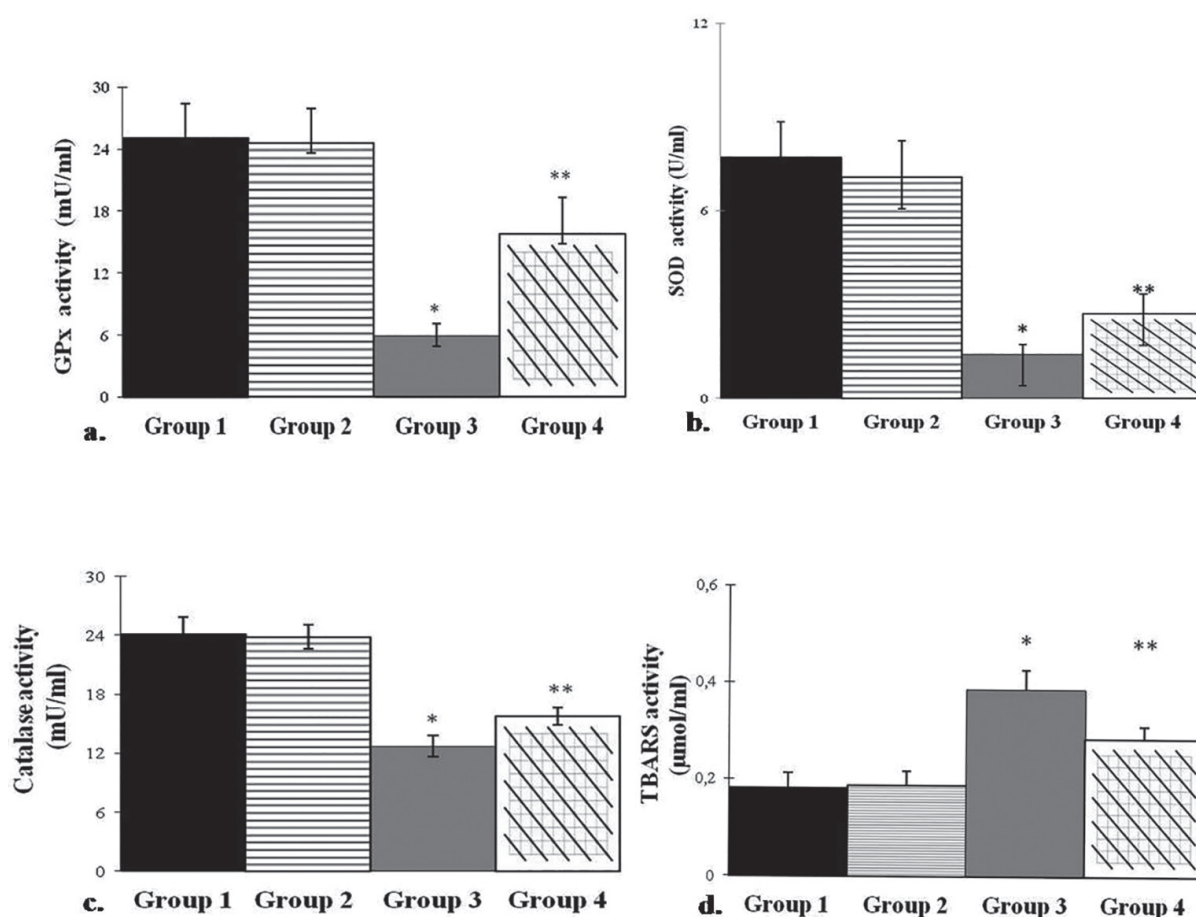


Fig. 3. **a** Diagram showing plasma GPx activity in sciatic nerve samples of animal groups. * group 1, group 2, group 4 ($p < 0.05$), ** group 1, group 2, group 3 ($p < 0.05$). **b** Diagram showing plasma SOD activity in sciatic nerve samples of animal groups. * group 1, group 2, group 4 ($p < 0.05$) and ** group 1, group 2, group 3 ($p < 0.05$). **c** Diagram showing plasma CAT activity in sciatic nerve samples of animal groups. * group 1, group 2, group 4 ($p < 0.05$) and ** group 1, group 2, group 3 ($p < 0.05$). It was also seen that increased plasma CAT activity in group 4. **d** Diagram showing plasma TBARS activity of animal groups. * group 1, group 2, group 4 ($p < 0.05$) and ** group 1, group 2 and group 3 ($p < 0.05$).

were thin extensions of fibroblasts formed by C (Figure 4.f). The sciatic nerve samples of group 3 had similar characteristics to those of group 4 (Figure 4.g). The integral nerve trunk contained different diameters of regenerative remyelinated axons. There were also a few deformed M fibers in the sections. A relatively increased amount of endoneurial connective tissue filled the gaps between the fibers in this group. There was active cytoplasm containing developed granular endoplasmic reticulum, abundant mitochondria and lysosomes in the Schwann cell ultrastructure (Figure 4.h). The group 4 samples did not show a striking ultrastructural difference from group 3 except for an increase in Schwann cells and cytoplasmic organelles.

DISCUSSION

Oxygen and nutrients are accumulated as a result of reperfusion, which consequently increases the num-

ber of FOR. FR cause LP, which has destructive effects on tissues. The cumulative effects of ischemic and mechanical processes is more significant than the effects of FR [7,18]. Promising results in terms of the regeneration of peripheral nerves were obtained as a result of ozone therapy [18]. The mechanism of the pharmacological activation and biological effects on antioxidant systems by ozone therapy were described by Bocci et al. [16,19] who administered ozone intraperitoneally or in combination with other methods [16]. In the present study we administered ozone intraperitoneally because this activated antioxidant systems more rapidly in the systemic circulation.

In a study that evaluated the effect of different concentrations of ozone on the peripheral nerve in rats, Lin Q et al and Dianzani reported that ozone concentrations higher than 50 ug/ml could cause pain or severe injury in the peripheral nerve. When the ozone

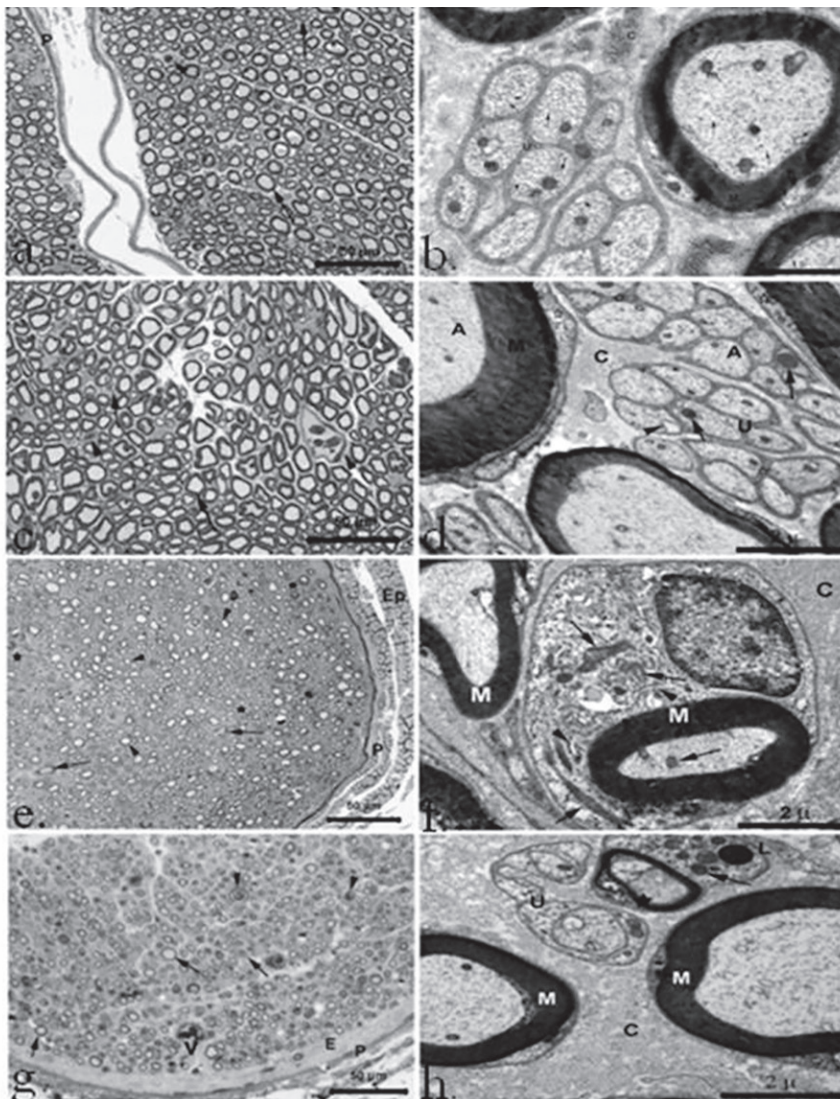


Fig. 4. Representative light and electron micrographs of the sciatic nerve samples. **a, b** group 1; **c, d** group 2; **e, f** group 3; **g, h** group 4. **a** Perineurium (P), myelinated (M) nerve (arrows) and capillary section (arrow head), **b** Mitochondria (m), smooth endoplasmic reticulum (arrow heads) and neurotubuli (arrows) can be seen in the axoplasm within the M and unmyelinated (U) nerve, collagen fibers (C) **c** M (arrows) and vasa nervorum (arrow heads). **d** Schwann cell cytoplasm surrounding the M and U nerve and mitochondria (arrows) and smooth endoplasmic reticulum (arrow heads) and C, axons (A) in the axoplasm. **e** Regenerated M nerves (arrow heads). Vasa nervorum, Endoneurium (stars), P, Epineurium (Ep). **f** Regenerated and remyelinated axons, in the cytoplasm of the Schwann cell, mitochondria (arrows) and granular endoplasmic reticulum (arrow head). **g** E, P, vasa nervorum (V), M nerve (arrows) and capillary section (arrow head), **h** Schwann cell containing M and U axons among the endoneurial C a remyelinated axon (full star) showing regeneration in cytoplasm, mitochondria (arrow).

concentration was below 20 µg/ml; most of the erythrocytes in the plasma were hemolytic and also the antioxidant systems were inhibited [20,27]. Therefore 20-50 µg/ml was administered in several clinical studies and this dosage range gave the best results in the treatment of neurodegenerative diseases and peripheral nerve injury [16,22]. For this reason in the present study we used a dose of 30-50 µg/ml (mild level) as a clinically suitable concentration of ozone to stimulate the antioxidant enzyme defense system.

Lin et al., reported that no obvious damage was present in nerve fibers in P in ozone groups [20]. Ozbay et al. found less vascular congestion and macrovacuolation in the ozone group. Similarly, Somay H et al found less vacuolization, vascular congestion, edema, hemorrhage and fibrosis after ozone therapy [6,21]. In the present study the characteristics of sciatic nerve specimens in the untreated group were similar to those of the treated group (Figure 4.g) except for an increased abundance of schwann cells and cytoplasmic organelles in the treated group. Both groups contained regenerative remyelinated axons of various diameters, and there were a few degenerated M fibers in the sections. There was a relatively higher amount of endoneurial connective tissue filling the gaps between these fibers (group 4). Schwann cell ultrastructure in the ozone group had active cytoplasm containing developed granular endoplasmic reticulum, with abundant mitochondria and lysosomes (Figure 4.h). Treated samples did not show any striking ultrastructural differences from the untreated group, but a greater abundance of organelles indicates that ozone therapy is a reliable method. According to Bocci et al. [16], the levels of oxidized glutathione and the antioxidants were increased after ozone therapy [16]. Travagli et al. [22], also reported that ozone increased the total antioxidant status. Somay H. [21] et al reported that ozone therapy increased blood circulation and oxygenation by releasing growth factors and cytokines and reducing chronic oxidative stress, and that it can stimulate adaptation of oxidative stress and protects against cellular damage caused by FR [21]. Ozone treatment plays an important role in the peripheral nerve injury regeneration process. FR can cause damage to the peripheral nervous system, due to its high oxygen consumption, rich lipid content, and the paucity of antioxidant enzymes. It has been suggested that oxygen FR significantly decreased neural tissue degeneration after nerve cut injury. Plasma ozone levels triggered a number of biochemical responses in the above-mentioned specimens, and the regenerative capacity after

nerve cut injury was improved as a result of an increase in SOD, CAT, GPx and antioxidant enzymes in plasma [23]. Ozone therapy can be a safe and efficient method due to its effects on oxygen metabolism, cellular energy, and antioxidant defense mechanisms [6]. In this study, SOD, CAT, and GPx activities increased and MDA levels decreased in the ozone group. We also observed a significant increase in plasma antioxidant levels in the ozone group. These results suggest that ozone increased plasma antioxidant levels by triggering various biochemical pathways. Therefore, it is suggested that ozone therapy can have positive impacts on nerve cut injury.

According to Vogelaar et al. [24], sensory and motor functions were improved at the 3rd postoperative week and the SFI and withdrawal reflex were also improved on the 30th day after surgery [24]. Other studies on ozone therapy have found effective anti-inflammatory properties and reduced inflammation in rats [6,21,25]. In the early post-traumatic period, the number of Schwann cells increases rapidly, then decreases slowly. This difference can be seen differently in various trauma models. Gupta and Steward reported an increase in the number of schwann cells in a chronic sciatic nerve compression model [26]. In contrast, Somay et al. indicated a decrease in the number of schwann cells in their sciatic nerve model [21]. In the present study we observed an increased number of schwann cells, cytoplasmic organelles and M axons in nerve cut injury after ozone therapy, and SFI values and pinch tests were improved at postoperative 4th week in the ozone group ($p < 0.05$) compared to group 3 (postoperative 8th week) ($p < 0.05$). Based on these results we can say that ozone accelerates the regeneration of nerve fibers through the interaction of various factors. Ozone may show neuroprotective effects by increasing plasma antioxidant levels. Another factor is the activation of the schwann and glial cells, which affect the myelin sheath thickness and development of the rat nervous system. Our functional, biochemical, and ultrastructural data showed that ozone therapy has positive and beneficial effects in treating sciatic nerve cut injury. We believe that ozone increased plasma antioxidant levels by triggering different biochemical pathways without causing peripheral nerve degeneration.

CONCLUSION

This is the first study to examine the impacts of ozone therapy on sciatic nerve cut injury. Our findings

suggest that ozone therapy may be a safe alternative method and a new therapeutic agent for peripheral nerve cut injury. Further studies are needed to elucidate which biochemical pathways are triggered by ozone therapy in the nerve regeneration process.

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