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ORIGINAL RESEARCH

The effect of ozonation of bupivacaine on bupivacaine-induced neurotoxicity in rats

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Abstract

Bupivacaine-induced neurotoxicity (BIN) is common condition associated with free oxygen radicals based degenerative cellular changes. Morever, a apoptotic damages increase the occurences of the BIN. Ozone is a medical gas widely used in the treatment of degenerative diseases, due to its antioxidant and cytoprotective effects. The aim of the study was to investigate the effects of ozonation of bupivacaine in reducing or preventing BIN. Sixty male rats were randomly and equally divided into three groups: control (distilled water), bupivacaine and ozonated bupivacaine. The sciatic nerve was surgically dissected under anesthesia and distilled water, bupivacaine or ozonated bupivacaine was injected perineurally according to the group. The nerve samples were then extracted on 3rd and 7th days, and were examined using immunohistochemical stainings and Terminal Transferase dUTP Nick End Labeling (TUNEL) staining. The effect of ozonation of bupivacaine, on the motor and sensory functions of the sciatic nerves was examined and compated against that of the bupivacaine. H-score analyzes of neuroflament, S100, superoxide dismutase, and Tumor necrosis factor (TNF)-alpha immunostainings, and apoptotic index values after TUNEL staining were used for the assessment of the efficiency levels. The findings in this paper show that ozonation of bupivacaine statistically reduced neurotoxicity in both the 3rd and 7th day groups. No significant differences between bupivacaine and ozonated bupivacaine groups was obtained from the sensory and motor functional tests. No deterioration in the biochemical structure of bupivacaine was induced by the ozonation. The findings in this paper highlight the effectiveness of the, ozonation of bupivacaine in reducing BIN. There were also no significant differences in the resolution times of motor and sensory blocks between ozonated bupivacaine and bupivacaine alone groups.

Keywords

Bupivacaine; Bupivacaine-induced neurotoxicity; Ozonation; Regional anesthesia

1. Introduction

Local anesthetics (LAs) are widely used in daily practice, with sufficient security margins. However, various unwanted side effects such as immunological reactions, cardiotoxicity and neurotoxicity can be associated to the use of LAs. Among those, neurotoxicity is not rare, and may result from the toxic effects of the LA itself or preservative additive agents even at appropriate doses [1]. Serious situations such as transient neurological syndrome, transient radicular irritation, and cauda equina syndrome can also be developed following use of LAs [2]. The frequency of these complications has been reduced with the use of the ultrasonography and nerve simulation techniques. Therefore, in recent years, the clinical researches have focused on reducing the direct chemical toxicity induced by LAs.

Despite cardiotoxic potential, bupivacaine is one of the most preferred LAs due to its potency and sustained action duration [3]. Neurotoxicity, on the other hand, is another important adverse effect related to bupivacaine. However, the development mechanisms of bupivacaine-induced neurotoxicity (BIN) have not been fully elucidated. Free oxygen radicals (ROS), lactate dehydrogenase release, reduction in mitochondrial potential, nuclear condensation, and toxicity mediated by cellular apoptosis were among the mentioned factors for the occurence of BIN accross literatur [4, 5]. Investigations on BIN showed that ROS blasts cause mitochondrial dysfunction and damages to cell organelles and DNA, suggesting that ROS burst might be one of the key points in bupivacaine-induced cytotoxicity and neurotoxicity [6–8]. Besides, cellular injury associated with overproduction of ROS has recently gained great interest [9, 10]. ROS contribute in several critical mechanisms such as cell proliferation, differentiation, migration and host defense. However, excessive production of these substrats can irreversibly destroy or alter the functions of proteins, lipids, nuclear acids, membranes and organelles, resulting in

apoptosis [9, 11]. As a result, these degenerative changes and apoptotic damage seem to be associated with neurological deficits.

Ozone is a known medical gas that has been increasinghly used in the treatment of many degenerative diseases, due to its antioxidant and cytoprotective effects [12, 13]. It is an unstable molecule consisting of three oxygen atoms, and interacts with organic compounds, through oxidation. ROS and lipid peroxidation derivatives, the oxidative products triggered by this reaction, are responsible for the main effects of ozone. These substrates stimulate intercellular antioxidant mechanisms. Morever, Ozone exhibits antibacterial and immunomodulatory effects *via* phagocytosis activation, immunostimulant properties with increasing tumor necrosis factor-alpha (TNF-alpha), analgesic and anti-inflammatory effects through arachidonic acid cascade [14].

Additionally, ozone has a neuroprotective effect by stimulating aerobic respiration in neurons and reduces neuronal damage [15].

The main aim of this experimental study was to assess that whether ozonation of bupivacaine on the reduction of BIN, and evaluate any potential change in the structure and efficacy of the drug.

2. Materials and methods

2.1 Study design and general data

This experimental study was conducted at Eskişehir Osmangazi University Medical and Surgical Experimental Animals Research Center. The budget for all drugs, experimental materials, and animals used in the study was obtained from the Eskişehir Osmangazi University Scientific Research Project (project code: TTU-2021-1581).

2.2 Primary and secondary endpoints

The main objective of this paper is to investigate the efficacy of the ozonation of bupivacaine on reducing or preventing BIN. For this purpose, the potential effects of ozonation in the chemical structure of bupivacaine was investigated. Subsequently, the effect of ozonation of bupivacaine, in comparison to bupivacaine alone, on the motor and sensory functions of the sciatic nerves was investigated and compared to the application of bupivacaine alone.

2.3 Ozonation of bupivacaine

Bupivacaine hydrochloride solution (Sigma-Aldrich B5274-1G) was prepared at 0.5% concentration using bidistilled water. Subsequently, this solution was ozonated for 10 minutes using ozone at 80 µg/NmL concentration in latex-free syringes by bubble creation method (bubbling). Firstly, the potential effects of ozonation on the chemical structure of bupivacaine were investigated. In this context, 1H (Proton) Nuclear Magnetic Resonance (NMR) was performed on both bupivacaine and ozonated bupivacaine. The NMR results have comfirmed the absence of chemical differences between bupivacaine (0.5%, 0.2 mL) and ozonated bupivacaine (0.5%, 0.2 mL), and thus the appropriate drug doses were confirmed.

2.4 Rats

A total of 60 male adult Sprague-Dawley rats, 250–330 g, with normal motor activity, were selected and used for experimental study. Before the experiment, the rats were left in a light environment for 12 hours and a dark environment for 12 hours. The environmental adaptation period was determined as 10 days. There was no change applied to the rats diet during this period. Rats were randomized using a computer modelled lottery method and numbered from 1 to 60. All rats were included in the experiments in numerical order to reduce. Potential bias from the researcher's dexterity. The person who selected the rat and the person who performed the procedure and measurements were different, and both ignored to which group the substance was to be injected (double blind).

2.5 Surgical procedure and sciatic nerve blockade

Anesthesia was achieved by intraperitoneally administering 50 mg/kg ketamine (ketamine hydrochloride, Keta-control®, 1 mL = 100 mg, Doğa drug, İstanbul, Türkiye) plus 10 mg/kg xylazine (Xylazin Bio 2%®, 1 mL = 20 mg, Bioveta). The depth of anesthesia was followed by the skin and toe compression test. After fixing rats in the prone position, the surgical site was cleaned and marked. The sciatic nerve was targeted *via* posterior approach. Thoracantary major, ischial tuberositas, and knee joint were marked. Approximately 1 cm skin incision was applied. Subsequently, the muscles were separated by blunt dissection from the sulcus and between the biceps femoris and the gluteal muscle. The sciatic nerve was reached, and 1 mm proximal to the bifurcation point of the sciatic nerve was selected as the target region. The injections were administered perineurally with a 30 G insulin injector under the fascia on the nerve. Control (C) group received 0.2 mL of distilled water, bupivacaine (B) group was given 0.2 mL of 0.5% bupivacaine, ozonated bupivacaine (OB) group was given 0.2 mL of 0.5% ozonated bupivacaine, and the injection times were recorded. Subsequently, the muscle planes were sutured with 4-0 prolene in accordance with the anatomical structures, and the skin was closed by suturing with 3-0 prolene. All drugs were prepared by an independent operator not involved in the surgical procedure, just five minutes before the procedure. Thus, the operator-related bias were avoided and the procedures were carried out without the prolonged waiting time of ozone.

2.6 Assessment of motor and sensory functions

All functional assessments were performed in both the tested (control, bupivacaine and ozonated bupivacaine) and the untested feet. Sciatic nerve motor functions were assessed using the 4-point scoring system, frequently preferred in routine practice: 0 points (Foot dorsiflexed, able to walk, toes open), 1 point (Foot dorsiflexed, able to walk, toes bent), 2 points (Foot partially flexed from extension to flexion, crawling, toes bent), and 3 points (Foot cannot move from extension to flexion, cannot walk, fingers are bent) [16]. The sensory functions were assessed using termal (hot plate)

and mechanical (claw compression and toe pull) tests. The response to the foot pull test identified with the contraction of the flexor muscles in the hip, knee and ankle. It is a polysynaptic reflex and is triggered by painful stimuli to the extremity of the organs. In this study, pressure was applied to the skin fold on the lateral metatarsal for superficial pain sensation and to the distal phalanx of the fifth toe for deep pain sensation. To avoid user-dependent alterations, a single painful stimulus was applied to designated locations at a constant intensity and for a constant time (1 second) using bulldog forceps. The responses of the rats were scored as follows: 0 points (strong toe pulling and strong vocalization), 1 point (moderate toe pulling and vocalization), 2 points (weak toe pulling and very reduced/no vocalization), 3 points (no foot pulls and no vocalization) [17]. The hot plate test is a thermal response test to widely used evaluate acute cutaneous pain sensitivity. Animals are placed individually on a surface maintained at 50–56 °C and their movements are restricted by a roller. Licking of the hands or feet as well as rapid foot pulling/jumping are recognised as pain response. A delay in licking and/or waving the hind paw or jumping is recorded [18]. In this study, the rats were placed on a hot plate at 52 °C. The times were recorded on the condition that it was kept on the plate for a maximum of 60 seconds in order to prevent damage to the rats. To avoid hyperalgesia, repeated tests were performed at 15-minute intervals. The hot plate test was applied twice in the study. First, in order to determine the thermal pain threshold, three tests were performed on each rat before the surgical procedure and the mean threshold value was recorded. The second test was performed after the surgical procedure. Before starting the hot plate test, motor functions allowed to normal (0 points). Hot plate tests were repeated every 15 minutes until the baseline values were reached. At this stage, the values were recorded separately for both feet, since one foot was the tested one. After the reaction in the untested foot, it was waited for a maximum of 10 seconds to determine whether there would be a reaction in the tested foot. If there was no reaction in the tested foot or if there was a delayed reaction time according to the baseline value, the test was repeated 15 minutes later. To avoid damage, the total time was limited to 60 seconds for each test, including the 10 second waiting time. The test was terminated when the response time to the hot plate test reached the baseline value.

2.7 Obtaining sciatic nerve samples

The rats were anesthetized to obtain sciatic nerve samples on post-procedural 3rd and 7th days, according to their subgroups. The previous incision sutures were removed and the sciatic nerve was reached using the same dissection method. Approximately 3 mm of sciatic nerve tissue (1 mm proximal to the bifurcation where the injection was made was the center of excised tissue) was excised. The rats were then sacrificed by decapitation method.

2.8 Histopathological assessments of the sciatic nerve samples

Tissue samples were first placed in 4% paraformaldehyde solution for light microscopic examination, and subsequently placed in cassettes and washed under running water for two hours. To remove excess water, the samples were passed through a series of increasing degrees of alcohol (60%, 70%, 80%, 90%, 96%, 100%). Finally, the tissues were passed through xylol for polishing and embedded in paraffin. All sections were examined with hematoxylin-eosin (HE), Luxol Fast Blue (LFB) histochemical staining, Terminal Transferase dUTP Nick End Labeling (TUNEL) staining, and immunohistochemical staining.

2.9 TUNEL method and apoptotic activity

The TUNEL method (TdT Fragel DNA Fragmentation Kit, Cat No QIA33, Calbiochem, San Diego, CA, USA) provided the staining of apoptotic cells in the determination of cell death. Initially, the sections were washed in distilled water and then with Phosphate Buffer solution (PBS) for 15 (3 × 5) minutes following the deparaffinization process. Subsequently, 20-µg/mL Proteinase-K diluted 1/500 with PBS solution was added at room temperature for 15 minutes. After washing with PBS, sections were treated with 3% H_2O_2 for 5 minutes, and then washed again 3 × 5 min with PBS. All samples were kept at room temperature for 5 minutes with Equilibration buffer and then kept for 1 hour at 37 °C in a humid environment with TdT-enzyme. At the end of an hour, samples treated with Stop Wash Buffer for 10 minutes and then with Antidigoxigenin Peroxidase Conjugate for 30 minutes and then were washed 3 × 5 minutes with PBS again. It was then stained with 3,3′-diaminobenzidine (DAB), and washed with distilled water. Background was painted with Mayer's Hematoxylin.

Tissue samples were collected 2 times for 2 min. After soaking in deionized water for a period of time, it was placed in Mayer's Hematoxylin for 5 minutes for counterstaining and kept in tap water for 10 minutes. Afterwards, it was passed through xylene and graded alcohols and covered with a coverslip using entellan (BioMount, Bio-Optica).

TUNEL staining were scored semi-quantitatively to determine the number of positive staining, ranked as none (−), weak (+), moderate (++), high (+++), very high (++++). These analyses were performed on two sections for each animal, at a rate of ×40 magnification for at least 10 different regions per section. Finally, apoptotic index was calculated as follow; (the number of apoptotic cell/the number of observed cell) × 100%. The obtained data were subject to the One Way-ANOVA Tukey statistical analysis, and $p < 0.05$ results were considered statistically significant [19, 20].

2.10 Immunohistochemical analysis

In the immunohistochemical study, neurofilament, S100, superoxide dismutase (SOD), and TNF-alpha immunoreactive cells were detected. Neurofilament biology is important for understanding the structural features of axons, such as the generation of axon diameter by radial growth. Neurofilament level in peripheral axons was determined by anti-neurofilament staining. To assess neuroinflammation, the concentration of TNF-alpha, a proinflammatory cytokine, was measured. In addition, the enzymatic activity of superoxide dismutase (SOD) was measured to assess the level of the oxidative stress. Finally, schwann cell density was determined by S100 antibody

staining, which is an important protein in myelin sheath formation. Immunoreactivity was evaluated with the H-score method based on the ratio of immunopositive cells to all cells in selected areas. Immunoreactive cell counting was performed by a blinded observer and graded as follows: 0 (no staining); 1 (weak staining), 2 (moderate staining) and 3 (strong staining in a particular area). The overall score was calculated using the following formula: H-score = (% of cells stained at 0) × 0 + (% of cells stained at 1+) × 1 + (% of cells stained at 2+) × 2 + (% of cells stained at 3+) × 3. The H-score ranges from 0 to 300 [21].

2.11 Statistical analysis

A power analysis was performed to determine the number of animals required for the study, based on the study by Cohen [22]. A sample size of 60 achieved 80% power to detect an effect size of 0.4 using a 2 degrees of freedom Chi-Square Test at a significance level (alpha) of 0.05. Continuous data were obtained as mean ± standard deviation (SD), and categorical data as percentage (%). Shapiro Wilk's test was used to assess the normality of the data ahead of ANOVA test. One-Way ANOVA test was used in comparison of normally distributed groups when the number of groups was three or more. Two-Way Analysis of Variance method was used to test group effects and interaction effect. IBM SPSS Statistics 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp., USA) program was used in the analysis. A value of $p < 0.05$ was accepted as a criterion for statistical significance.

3. Results

A total of 60 rats were included in the study without any loss. The hot-plate test was discontinued after one rat has undergone the experiment. Bleeding in the forenails was observed as a result of attaching rats firmly attaching nails to the plate as response to the hot plate. The sciatic nerve samples of each group were successfully. Extracted data was recorded in the workbook.

3.1 Morphological findings (electron microscopic findings)

In the HE histological staining, inflammatory findings such as axonal swelling, a small amount of vascular proliferation, and lymphocyte infiltration in the epineurium were found in the sciatic nerve tissues of C-3 and C-7 subgroups. It was observed that the epineurium, perineurium and endoneurium connective tissue surrounding the nerve fiber bundles of the nerve tissue were normal (Fig. 1a,d). In bupivacaine subgroups, axonal degeneration, vascular proliferation, demyelination and mononuclear cell infiltration in the epineurium were observed. Neurotoxicity was found to be more especially in the B-7 subgroup due to demyelination. Demyelination and inflammation were observed more intensely in this group than in others (Fig. 1b,e). In the samples taken on the 7th day in the OB subgroup, the integrity of the nerve fiber bundle and the regression in axonal degeneration were among the important findings although the inflammation continued (Fig. 1c,f).

When the results of LFB staining were evaluated, myelin loss was very low in the C-3 and C-7 subgroups as suggested by the findings from the LFB staining shown in (Fig. 2a,d). While Demyelination increased in the B-3 and B-7 subgroups (Fig. 2b,e), its occurrence was found to lessen in OB subgroups (Fig. 2c,f).

3.2 Immunohistochemical findings

Neurofilament biology is important for understanding the structural features of axons, such as the generation of axon diameter by radial growth. Neurofilament level in the axons of peripheral nerves was determined by anti-neurofilament staining. In the anti-neurofilament staining, the immune reactivity in the C-3 and C-7 subgroups was severe high, and a statistically significant difference was found between the bupivacaine and ozonated bupivacaine groups ($p < 0.05$). There was no significant difference between the bupivacaine subgroups ($p > 0.05$). The highest statistical significance was found between the C-7 subgroup and the bupivacaine subgroups ($p < 0.001$).

In the sciatic nerve samples taken on the 3rd day, the control group, as expected, had significantly positive results in total H-scores for all stainings than the other two groups. The ozonated bupivacaine group, on the other hand, had significantly ($p < 0.001$) positive results across all staining compared to the group tha received bupivacaine on the 3rd day (Table 1).

In the sciatic nerve samples taken on the 7th day, the control group, as excepted, exhibited significantly higher results in H-scores calculated from neurofilament, SOD, and TNF-alpha stainings than the other two groups. In addition, in the H-scores calculated from the S100 staining in the 7th day compared to that on the 3rd day, the control group and the ozonated bupivacaine group had statistically similar results and showed significantly more higher results than the bupivacaine group. The ozonated bupivacaine group had significantly ($p < 0.001$) positive results in all staining compared to the bupivacaine group (Table 2).

In each group, the immunohistochemical findings of the 3rd and 7th days were compared (Table 3). In the control group, the H-score of TNF-alpha staining was found to be statistically significant, indicating that neuroinflammation was higher on the 7th day compared to that on the 3rd day. There were no statistically significant changes in the other stainings of the control group.

In the bupivacaine group, the H-score of S100 staining was found to be statistically significantly decreased, suggesting that myelination loss was higher on the 7th day. There was no statistically significant change in other stainings in the bupivacaine group.

In the ozonated bupivacaine group, the H-scores of neuroflament and S100 stainings were found to be statistically significantly increased, indicating that both myelination were incresed and neurotoxicity was decreased on the 7th day. There was no statistically significant change in other stainings in the ozonated Bupivacaine group.

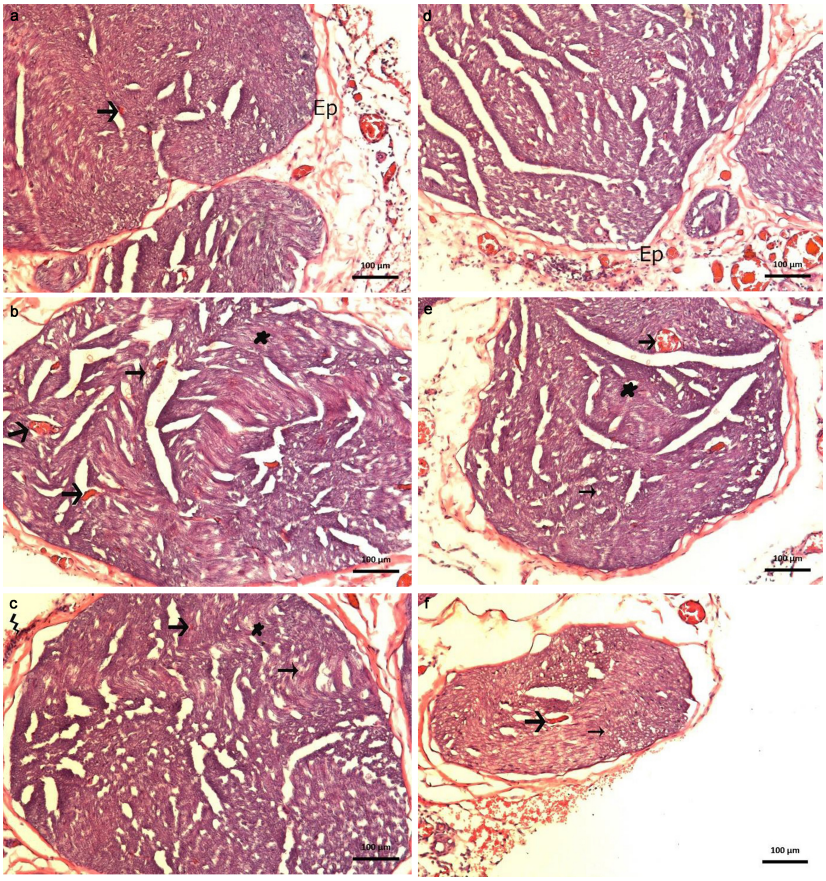

FIGURE 1. HE stainings. (a) C-3, (b) C-7, (c) B-3, (d) B-7, (e) OB-3, (f) OB-7.

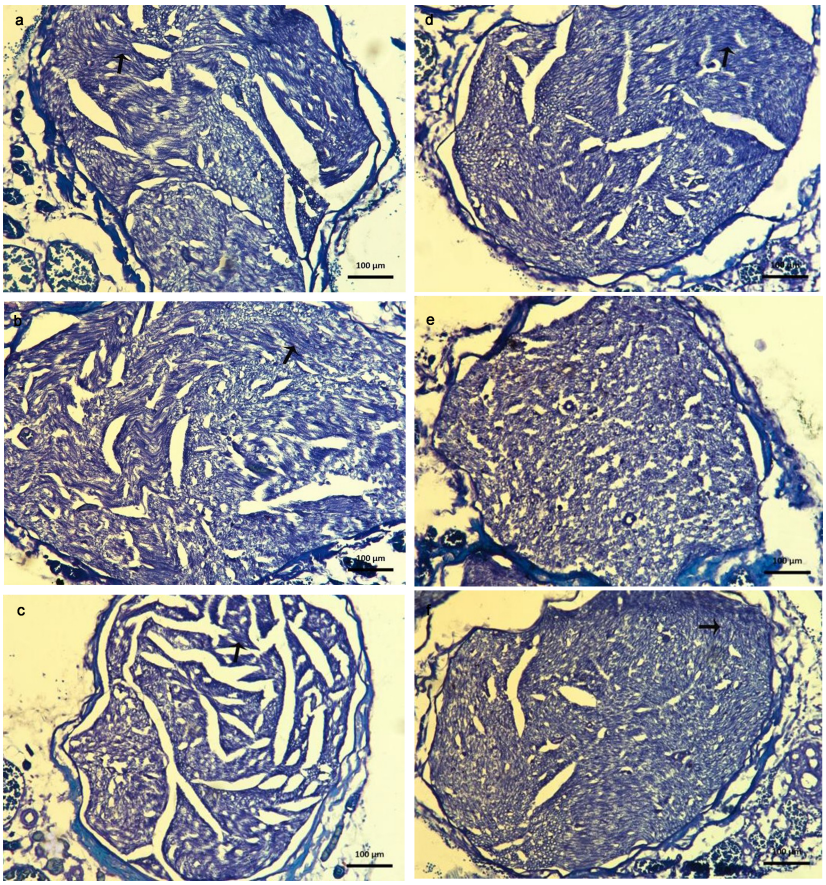

FIGURE 2. LFB stainings. (a) C-3, (b) C-7, (c) B-3, (d) B-7, (e) OB-3, (f) OB-7.

TABLE 1. H-scores of sciatic nerve immunohistochemical stainings on 3rd day.

	C-3	B-3	OB-3	*p* value
Neurofilament	230 ± 3.3	161 ± 3.1	187 ± 4.5	<0.001 (between all groups)
S100	245 ± 2.5	183 ± 2.5	221 ± 2.0	<0.001 (between all groups)
SOD	164 ± 2.7	132 ± 1.5	148 ± 1.7	<0.001 (between all groups)
TNF-alpha	176 ± 1.9	244 ± 1.6	204 ± 1.8	<0.001 (between all groups)

High neurofilament H-score indicates the presence of neurofilament-rich axons. Low values are indicative of neurotoxicity.
High S100 H-score indicates myelination of schwann cells. Low values are indicative of neurotoxicity.
High SOD H-score indicates low SOD activity. Low values are indicative of high oxidative stress and neuroinflammation.
High TNF-alpha H-score indicates high neuroinflammation. Low values indicate low neurotoxicity.
OB: ozonated bupivacaine; SOD: superoxide dismutase; TNF-alpha: tumor necrosis factor-alpha.

TABLE 2. H-scores of sciatic nerve immunohistochemical stainings on 7th day.

	C-7	B-7	OB-7	*p* value
Neurofilament	237 ± 3.3	151 ± 3.5	207 ± 2.2	<0.001 (between all groups)
S100	242 ± 2.8	173 ± 2.1	246 ± 2.4	<0.001 (between C-7 and B-7) <0.001 (between B-7 and OB-7) 0.092 (between C-7 and OB-7)
SOD	163 ± 2.1	124 ± 1.6	154 ± 2.1	<0.001 (between C-7 and B-7) <0.001 (between B-7 and OB-7) 0.034 (between C-7 and OB-7)
TNF-alpha	187 ± 2.4	247 ± 2.5	209 ± 1.8	<0.001 (between all groups)

High neurofilament H-score indicates the presence of neurofilament-rich axons. Low values are indicative of neurotoxicity.
High S100 H-score indicates myelination of schwann cells. Low values are indicative of neurotoxicity.
High SOD H-score indicates low SOD activity. Low values are indicative of high oxidative stress and neuroinflammation.
High TNF-alpha H-score indicates high neuroinflammation. Low values indicate low neurotoxicity.
OB: ozonated bupivacaine; SOD: superoxide dismutase; TNF-alpha: tumor necrosis factor-alpha.

TABLE 3. Comparison of the H-scores of the immunohistochemical stainings in each groups.

	C group			B group			OB group		
	C-3	C-7	*p*	B-3	B-7	*p*	OB-3	OB-7	*p*
Neurofilament	230 ± 3.3	237 ± 3.3	0.594	161 ± 3.1	151 ± 3.5	0.341	187 ± 4.5	207 ± 2.2	0.002
S100	245 ± 2.5	242 ± 2.8	0.973	183 ± 2.5	173 ± 2.1	0.030	221 ± 2.0	246 ± 2.4	<0.001
SOD	164 ± 2.7	163 ± 2.1	0.991	132 ± 1.5	124 ± 1.6	0.087	148 ± 1.7	154 ± 2.1	0.271
TNF-alpha	176 ± 1.9	187 ± 2.4	0.008	244 ± 1.6	247 ± 2.5	0.883	204 ± 1.8	209 ± 1.8	0.430

OB: ozonated bupivacaine; SOD: superoxide dismutase; TNF-alpha: tumor necrosis factor-alpha.

3.3 TUNEL findings and apoptotic index (%)

Across all the investigated groups, apoptosis was triggered and TUNEL reactivity was high-particularly in the groups administered that received bupivacaine. Apoptosis was milder in the control groups and appeared to occur predominantly in stellate cells. Apoptosis was observed to regress in ozonated Bupivacaine groups. Statistical significance was determined between the groups according to the apoptotic index values. The highest significance between the groups in TUNEL positivity was between the control groups and bupivacaine groups ($p < 0.0001$). p value less than 0.05 was considered statistically significant. While the least significance was observed among the control subgroups ($p > 0.05$), the significance was determined as $p < 0.001$ between the bupivacaine and ozonated bupivacaine groups. Apoptotic index values of all subgroups were presented in Fig. 3.

3.4 Findings of motor and sensory functions

In order to avoid errors regarding anesthesia-related delay, motor and sensory functions were evaluated in both tested and untested feet. As expected, there was no statistical difference between the three groups (control, bupivacain and ozonated bupivacaine group), in terms of resolution times of both motor and sensory blocks in untested feet.

Among the feet with sciatic nerve block, the bupivacaine and ozonated bupivacaine groups had significantly longer motor and sensory block times compared to the control group. The findings in Table 4 show that the mean motor block time was longer in the ozonated bupivacaine group, whereas bupivacaine group had longer mean sensory block time; however, these differences were not statistically significant.

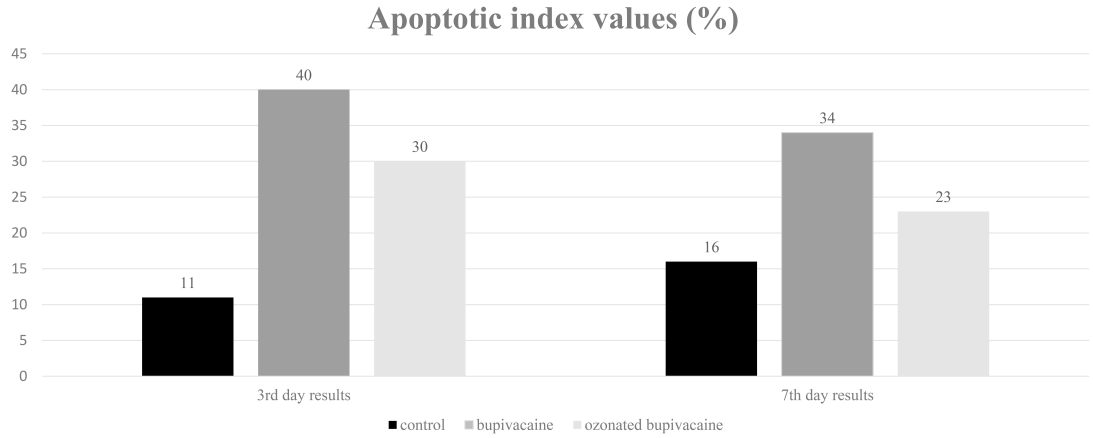

FIGURE 3. Apoptotic index values of all subgroups.

TABLE 4. The comparison of resolution times of motor and sensory blocks between the groups.

Motor block				
4-point scoring	C group	B group	OB group	*p*
Untested foot				
Time to reach to 2 point	55.6	54.4	52.1	1.00 (Between C–B, C–OB, B–OB)
Time to reach to 1 point	76.3	75.3	91.7	1.00 (Between C–B: 1.00) 0.98 (Between C–OB, B–OB: 0.98)
Time to reach to 0 points	101.6	94.2	113.6	1.00 (Between C–B, C–OB) 0.92 (Between B–OB)
Tested foot				
Time to reach to 2 point	60.2	145.7	170	<0.0001 (between C–B, C–OB) 0.65 (between B–OB)
Time to reach to 1 point	76.9	172.2	199.1	<0.0001 (between C–B, C–OB) 0.69 (between B–OB)
Time to reach to 0 points	93.3	212.9	230.8	<0.0001 (between C–B, C–OB) 0.98 (between B–OB)
Sensory block				
4-point scoring	C group	B group	OB group	*p*
Untested foot				
Time to reach to 2 point	27.2	26	24.3	1.00 (Between C–B, C–OB, B–OB)
Time to reach to 1 point	46.9	45.5	50.8	1.00 (Between C–B, C–OB, B–OB)
Time to reach to 0 points	71.6	61.6	70.1	1.00 (Between C–B, C–OB, B–OB)
Tested foot				
Time to reach to 2 point	23.8	161.8	153.3	<0.0001 (between C–B, C–OB) 1.00 (between B–OB)
Time to reach to 1 point	36.5	198.2	189.6	<0.0001 (between C–B, C–OB) 1.00 (between B–OB)

OB: ozonated bupivacaine.

4. Discussion

LA-related neurotoxicity is a complex phenomenon, related to the dose, concentration, duration and structure of the LA [23]. To date, no single dominant pathway has been identified in its pathophysiology [24].

Although especially high doses of lidocaine were found to be most often responsible for this undesirable situation, further studies showed that all LAs can cause similar effects [22]. LA-related neurotoxicity was demonstrated to develop through various mechanisms in animals, cell lines and human studies [23, 25].

In this paper, the effects of ROS, myelin loss, and four inflammation markers (neurofilament, S100, SOD and TNF-alpha) to BIN was investigated. First, the neurotoxic effects of bupivacaine were identified through histopathological examinations and functional tests. Second, bupivacain was ozonated, and then the neuroprotective effect of ozonation on BIN was

assessed.

Peripheral nerve fibers consist of neuronal axons, myelin sheaths synthesized by schwann cells, and collagen-rich extracellular matrix.

One of the most reliable methods used for the identification of axonal degeneration and demyelination in histochemical studies is LFB staining. In this paper, demyelination was evaluated using both HE and LFB stainings. The findings, clearly confirmed that ozonated bupivacaine caused less neuronal demyelination compared to the sole application of bupivacaine.

In a study conducted on rats, myelin degeneration and axanol damage were detected in sciatic nerve samples taken on the 2nd and 7th days after perineural injection of bupivacaine [26]. Comparable findings in other animal studies reported similar maximum levels of axonal degeneration and structural changes on the 2nd and 7th days [27]. Morever, Mueller *et al*. [27] noted that acute, subacute, and chronic effects were observed on 1st–3rd, 7th and 14th–21st days, respectively. In our study, the main objective was to evaluate the acute and subacute effects. In this context, rat sciatic nerve samples were extracted at two different times, 3rd and 7th day after the procedure. Demyelination was assessed by S100 staining, with low S100 H-scores indicating demyelination and neurotoxicity. According to the results obtained on the 3rd day, the H-scores of the S100 staining were lower in both bupivacaine group and ozonated bupivacaine groups compared to the control group. However, the H scores on the 7th day were significantly lower in the bupivacaine group, suggesting higher levels of myelination loss on the 7th day. In the ozonated bupivacaine group, the H-score was found to be increased significantly. In addition, a significant difference was found between the two groups in both the 3rd and 7th day S100 H-scores, which showed that ozonation reduced the severity of neurotoxicity.

In the pathophysiology of LA-associated neurotoxicity, some studies have associated these drugs, especially at high doses, with cell death and necrosis [28]. LAs were shown to increase the activity of many pro-apoptotic enzymes such as caspase, p38 mitogen-activated protein kinase (MAP-Kinase) and Jun N-terminal kinase [23]. In our study, TUNEL staining was used to monitor the apoptotic process. Apoptosis was found to be higher in the bupivacaine group compared to the rest of investigated. There was a significant difference in apoptotic index between ozonated bupivacaine and bupivacaine group, meaning that ozonation reduced the apoptotic effect of LA. Moreover, there was also a significant difference between the control group and ozonated bupivacaine groups. However, this statistical difference decreased in the 7th day samples in comparison to the 3rd day samples.

Another proposed mechanism in LA-induced neurotoxicity is the oxidative stress mediated by reactive oxygen products [29]. In the present study, enzymatic activity of SOD was measured to assess the oxidative stress levels in sciatic nerve samples. Low values of this inflammatory marker are related to high oxidative stress and neuroinflammation. In both 3rd day and 7th day samples, bupivacaine group exhibited the lowest SOD values compared to other groups. Statistically, ozonated bupivacaine group had lower SOD value than bupivacaine group, confirming the neuroinflammation-reducing effect of ozonation.

In previous experimental studies, various agents were investigated for potential reducing effect of BIN. For instance, in a study conducted in the cloned mouse neuroblastoma Neuro2a cell line, activation of the threonine-serine protein kinase B pathway was targeted with dexamethasone, and BIN was reported to be reduced [4]. In a study with 18 healthy Sprague-Dawley rats, neurotoxicity was demonstrated first with intrathecally administered bupivacaine. Subsequently, intrathecal rapamycin was found to reduce neuronal damage by decreasing caspas-3 levels [30]. In another study with the SH-SY5Y cell line, cell viability was decreased and apoptosis increased with bupivacaine. In that study, Artemisia capillaris treatment was applied to suppress ROS production and inactivate the phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) pathway. Capillaris was shown to protect SH-SY5Y cells against bupivacaine-induced apoptosis by inhibiting ROS-mediated oxidative stress, mitochondrial damage, and endoplasmic reticulum stress [31]. In our study, ozonation, which is one of the strong antioxidant mechanisms, was applied to investigate its neuroprotective against BIN. In all immunohistochemical staining H-Score analyses; higher neurotoxic effects were observed in the bupivacaine groups compared to the ozonated bupivacaine groups. The effectiveness of ozone has been corroborated by in a study, particularly through lipid peroxidation, reactive oxygen radicals and antioxidant mechanisms [32]. A recent review reported, the potential of Ozone was shown to improve the cytotoxic effects in neurological disorders through reduction of oxidative stress and lipid peroxidation [33]. In that study, ozone was also shown to be histopathologically protective in parkinson models of rats created with rotenone, especially in the midbrain. In a recent work, ozone therapy was examined in the improvement of sciatic nerve damage caused by incision in rats, and histopathological improvement was observed with significant improvement [34].

The study was conducted in a single center, which can potentially incur limitations for the generalizability of the statistical results. The small sample sizes of the subgroups may be another limitation of this study. Although neurotoxic effect was assessed by immunohistochemical stainings of neuroflament, S100, superoxide dismutase, and TNF-alpha, and apoptotic index values in TUNEL staining, other indicators that can contribute to neurotoxicity were not investigated.

5. Conclusions

The findings in this paper have confirmed that Ozonation did not cause a negative effect on the biochemical structure of bupivacaine. There were also no significant differences in the resolution times of motor and sensory blockades between ozonated bupivacaine and bupivacaine alone groups. As the primary finding of the study, ozonation of bupivacaine significantly reduced BIN. These results are promising for future studies in terms of the use of ozonation in other neurotoxic drugs.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

MAH and YK—conception, manuscript writing, materials collection, and literature review; YK and MSG—study design, data analysis and/or interpretation, critical review; MAH—data collection and/or processing; MAH, YK and MSG—supervision and final approval of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Experimental Animals Local Ethics Committee of Eskişehir Osmangazi University Faculty of Medicine (date/number: 10.10.2019/771).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1] Zhang Y, Feng S, Cheng X, Lou K, Liu X, Zhuo M, *et al*. The potential value of exosomes as adjuvants for novel biologic local anesthetics. Frontiers in Pharmacology. 2023; 14: 1112743.

[2] Xiang Y, Wang W, Jing S, Zhang Z, Wang D. Vocal fold paralysis and cauda equina syndrome following spinal-epidural anesthesia: a case report. Medicine. 2021; 100: e24374.

[3] Cherobin ACFP, Tavares GT. Safety of local anesthetics. Anais Brasileiros De Dermatologia 2020; 95: 82–90.

[4] Sun L, Yuan R. LncRNA SNHG12 ameliorates bupivacaine-induced neurotoxicity by sponging miR-497–5p to upregulate NLRX1. Human & Experimental Toxicology. 2022; 41: 096032712210890.

[5] Chen L, Zhuang K. Kaempferol counteracts bupivacaine-induced neurotoxicity in mouse dorsal root ganglia neurons by regulating TRAF6-dependent NF-κB signaling. The Kaohsiung Journal of Medical Sciences. 2023; 39: 710–717.

[6] Li L, Ye X, Lu A, Zhou S, Liu H, Liu Z, *et al*. Hyperglycemia magnifies bupivacaine-induced cell apoptosis triggered by mitochondria dysfunction and endoplasmic reticulum stress. Journal of Neuroscience Research. 2013; 91: 786–798.

[7] Zhao W, Liu Z, Yu X, Lai L, Li H, Liu Z, *et al*. ITRAQ proteomics analysis reveals that PI3K is highly associated with bupivacaine-induced neurotoxicity pathways. Proteomics. 2016; 16: 564–575.

[8] Zhang L, Zhang L, Guo F. MiRNA-494-3p regulates bupivacaine-induced neurotoxicity by the CDK6-PI3K/AKT signaling. Neurotoxicity Research. 2021; 39: 2007–2017.

[9] Bedard K, Krause K. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiological Reviews. 2007; 87: 245–313.

[10] Li Y, Zhao W, Yu X, Li F, Liu Z, Li L, *et al*. Activation of p47phox as a mechanism of bupivacaine-induced burst production of reactive oxygen species and neural toxicity. Oxidative Medicine and Cellular Longevity. 2017; 2017: 8539026.

[11] Liu M, Wu X, Cui Y, Liu P, Xiao B, Zhang X, *et al*. Mitophagy and apoptosis mediated by ROS participate in AlCl3-induced MC3T3-E1 cell dysfunction. Food and Chemical Toxicology. 2021; 155: 112388.

[12] Khan SA, Re K, Gandhi J, Liang R, Patel S, Joshi G, *et al*. Clinical utility of ozone therapy and hyperbaric oxygen therapy in degenerative disc disease. Medical Gas Research. 2023; 13: 1–6.

[13] Serra MEG, Baeza-Noci J, Mendes Abdala CV, Luvisotto MM, Bertol CD, Anzolin AP. The role of ozone treatment as integrative medicine. An evidence and gap map. Frontiers in Public Health. 2023; 10: 1112296.

[14] Schwartz A, Sánchez GM, Sabah F, Avilés MH. Madrid declaration on ozone therapy. 3rd edn. International Scientific Committee of Ozone Therapy (ISCO3): Madrid. 2020.

[15] Yanchu L, Rong P, Rong C, Li Z, Xiaoyan Y, Feng W. Ozone therapy for high-grade glioma: an overview. Frontiers in Oncology. 2023; 13: 1161206.

[16] Ali Erdogan M, Polat A, Yucel A, Aydogan MS, Parlakpinar H, Tekin S, *et al*. Effects of perineural administration of dexmedetomidine in combination with levobupivacaine in a rat sciatic nerve block. Current Therapeutic Research. 2013; 74: 74–78.

[17] Brummett C, Norat M, Palmisano J, Lydic R. Perineural administration of dexmedetomidine in combination with bupivacaine enhances sensory and motor blockade in sciatic nerve block without inducing neurotoxicity in rat. Anesthesiology. 2008; 109: 502–511.

[18] Langford DJ, Mogil JS. Pain testing in the laboratory mouse. Anesthesia and Analgesia in Laboratory Animals. 2008; 83: 549–560.

[19] Gürpınar T, Ekerbiçer N, Uysal N, Barut T, Tarakçı F, Tuglu MI. The effects of the melatonin treatment on the oxidative stress and apoptosis in diabetic eye and brain. The Scientific World Journal. 2012; 2012: 498489.

[20] Öztürk Ş, Sönmez PK, Özdemir I, Topdağı YE, Tuğlu MI. Antiapoptotic and proliferative effect of bone marrow-derived mesenchymal stem cells on experimental Asherman model. Cukurova Medical Journal. 2019; 44: 434–446.

[21] Numata M, Morinaga S, Watanabe T, Tamagawa H, Yamamoto N, Shiozawa M, *et al*. The clinical significance of SWI/SNF complex in pancreatic cancer. International Journal of Oncology. 2013; 42: 403–410.

[22] W. Cohen Jacob. Statistical power analysis for the behavioral sciences. 2nd edn. Lawrence Erlbaum Associates: Hillsdale, New Jersey. 1988.

[23] Verlinde M, Hollmann MW, Stevens MF, Hermanns H, Werdehausen R, Lirk P. Local anesthetic-induced neurotoxicity. International Journal of Molecular Sciences. 2016; 17: 339.

[24] Nouette-Gaulain K, Capdevila X, Rossignol R. Local anesthetic '*in-situ*' toxicity during peripheral nerve blocks. Current Opinion in Anaesthesiology. 2012; 25: 589–595.

[25] Koo CH, Baik J, Shin HJ, Kim JH, Ryu JH, Han SH. Neurotoxic effects of local anesthetics on developing motor neurons in a rat model. Journal of Clinical Medicine. 2021; 10: 901.

[26] An K, Elkassabany NM, Liu J. Dexamethasone as adjuvant to bupivacaine prolongs the duration of thermal antinociception and prevents bupivacaine-induced rebound hyperalgesia *via* regional mechanism in a mouse sciatic nerve block model. PLOS ONE. 2015; 10: e0123459.

[27] Mueller M, Wacker K, Ringelstein EB, Hickey WF, Imai Y, Kiefer R. Rapid response of identified resident endoneurial macrophages to nerve injury. The American Journal of Pathology. 2001; 159: 2187–2197.

[28] E Q, Wu Y, Liang X, Chen M, Peng J, Zhou Z, *et al*. Establishment of an animal model of sciatic nerve injury induced by local anesthetics. Human & Experimental Toxicology. 2023; 42: 096032712311733.

[29] Wang T, Zheng L, Zhang W. Hesperidin alleviates bupivacaine anesthesia-induced neurotoxicity in SH-SY5Y cells by regulating apoptosis and oxidative damage. Journal of Biochemical and Molecular Toxicology. 2021; 35: e22787.

[30] Xue X, Lv Y, Leng Y, Zhang Y. Autophagy activation attenuates the neurotoxicity of local anaesthetics by decreasing caspase-3 activity

in rats. Brazilian Journal of Anesthesiology. 2020; 70: 627–634. (In Portuguese)

[31] Zhao T, Wang Q. Capillarisin protects SH-SY5Y cells against bupivacaine-induced apoptosis *via* ROS-mediated PI3K/PKB pathway. Life Sciences. 2020; 259: 118279.

[32] Hidalgo-Tallón FJ, Torres-Morera LM, Baeza-Noci J, Carrillo-Izquierdo MD, Pinto-Bonilla R. Updated review on ozone therapy in pain medicine. Frontiers in Physiology. 2022; 13: 840623.

[33] Masan J, Sramka M, Rabarova D. The possibilities of using the effects of ozone therapy in neurology. Neuroendocrinology Letters. 2021; 42: 13–21.

[34] Ogut E, Yildirim Fb, Sarikcioglu L, Aydin Ma, Demir N. Neuroprotective effects of ozone therapy after sciatic nerve cut injury. The Kurume Medical Journal. 2018; 65: 137–144.

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