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Original Article

Effects of ozone and methylprednisolone treatment in facial paralysis: An experimental study

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ABSTRACT

Objectives: This study aims to compare ozone with the commonly used methylprednisolone in experimental facial paralysis and to investigate whether combining ozone with methylprednisolone provides additional benefits over methylprednisolone alone.

Materials and methods: The prospective randomized controlled animal study was conducted between November 2023 and January 2024. Thirty-two male Sprague-Dawley rats (mean weight: 300±30 g; range, 270 to 330 g; mean age: 8±1 weeks) with experimentally induced facial nerve injury were divided into five subgroups: control, paralyzed, methylprednisolone (2 mg/kg/day, intramuscular), ozone (1.1 mg/kg/day, intraperitoneal), and combination (ozone+methylprednisolone). Treatments were administered for 14 days. Functional, histologic, and immunohistochemical examinations were made.

Results: Posttreatment comparison of treatment groups showed that whisker movements (p=0.001) and total clinic scores (p=0.023) were significantly higher in the combination group compared to the ozone group. In histologic analysis, vascular congestion was significantly lower in the methylprednisolone group than in the ozone group (p=0.012). On the other hand, apoptosis levels, indicating cell death in the facial nerve, were significantly lower in the combination group compared to the methylprednisolone (p=0.016) and ozone (p=0.036) groups.

Conclusion: The combination of methylprednisolone and ozone lead to the most favorable functional and cellular outcomes. Further studies are required to confirm the benefits of ozone therapy for human facial paralysis, understand the molecular mechanisms behind its effectiveness, and determine the optimal doses for combined treatment.

Keywords: Corticosteroid, facial paralysis, methylprednisolone, ozone, rat.

Facial paralysis can arise from damage along the path of the facial nerve from the brainstem to the neuromuscular junction.[1] Various traumas, surgeries, infections such as herpes zoster, and cold exposure can trigger this condition. Corticosteroids, commonly used for treatment, suppress inflammation, reduce edema, and prevent ischemia and Wallerian degeneration.[2] However, more effective treatments are needed.

Oxidative stress and reactive oxygen species induce neuronal apoptosis after trauma. Antioxidants, which inhibit lipid peroxidation and reactive oxygen species production, can prevent this apoptosis. Ozone (O_3), a gas composed of three oxygen atoms, has notable antioxidant effects. Medical ozone use began with ozone generators, and it also has antimicrobial, analgesic, immunomodulatory, and anti-inflammatory properties.[3] It improves oxygen delivery to tissues, stimulates the immune system, triggers growth factor release, and increases levels of antioxidant enzymes.[4]

The effects of ozone therapy on facial nerve damage are underinvestigated. Studies in rats with experimental facial paralysis showed that intraperitoneal ozone therapy significantly improved vascular congestion, myelin thickness, and axonal

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degeneration compared to saline treatment.[5] Another study found that ozone therapy improved nerve branching, diameters, areas, and axon numbers compared to low-level laser therapy.[6] A human case study reported rapid muscle tone improvement and recovery of oral and ocular functions with ozone therapy.[7]

In sciatic nerve injury, combined treatment with methylprednisolone (MP) and ozone provided more effective recovery than either treatment alone.[8] Positive effects on sciatic nerve sheath atrophy, peripheral tissue inflammation, and perineural vascular proliferation were reported.[8]

Corticosteroids remain the primary treatment for facial paralysis in clinical practice. No studies have compared ozone with corticosteroids in experimental facial paralysis. Given the positive outcomes in sciatic nerve injury with combined treatments,[8] this study hypothesizes that ozone therapy, alone or combined with MP, would enhance recovery in facial nerve paralysis compared to MP alone. This study aimed to investigate the functional, histologic, and immunohistochemical effects of ozone, MP, and combined treatments in a rat model of facial nerve injury.

MATERIALS AND METHODS

The prospective randomized controlled animal study was conducted at the Eskişehir Osmangazi University Faculty of Medicine, Department of Physical Medicine and Rehabilitation with 32 male Sprague-Dawley rats (mean weight: 300±30 g; range, 270 to 330 g; mean age: 8±1 weeks) housed under controlled conditions (45 to 50% humidity, 22±2°C temperature, 12- to 14-h light-dark cycle) between November 2023 and January 2024. Before the study, the normality of facial nerves, corneal reflex, and symmetric whisker movement were assessed in all animals. Animals had unlimited access to food and water. The experimental protocol was approved by the Eskişehir Osmangazi University, Ethics Committee for Animal Experimentation (Date: 07.03.2023, No: 925-1) and supported by Eskişehir Osmangazi University Local Scientific Research Projects Committee (Project Code: TSA-2023-2700).

The rats were divided into five groups, with eight rats in each: Group 1 (control), intact right facial nerve tissue from paralyzed group; Group 2 (paralyzed), left facial paralysis induced with no treatment; Group 3 (MP), left facial paralysis treated with MP (2 mg/kg/day, intramuscular) for 14 days;[8] Group 4 (ozone), left facial paralysis treated with ozone therapy (1.1 mg/kg/day, intraperitoneal) for 14 days;[8] Group 5 (combination), left facial paralysis treated with both ozone therapy and MP (same dosages and durations as Groups 3 and 4).[8] In Group 1, the animals were prepared by performing an excision on the right facial nerve of the paralyzed group to prevent nerve damage. This was to avoid unnecessary sacrifice of eight animals. A total of 32 animals were included in the study, and a total of 40 facial nerve examinations were performed.

Surgical technique

All surgical procedures were performed under deep anesthesia in rats, and adequate measures were taken to minimize pain or discomfort. All experiments were carried out in accordance with the guidelines and the local laws and regulations. The scientific and ethical principles for animal experiments were followed throughout all stages of our study. The skin overlying the facial nerve trajectory was shaved and then cleaned with povidone-iodine in all rats. Anesthesia was administered via intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). Experimental facial paralysis was induced in Groups 2, 3, 4, and 5. A 1-cm longitudinal dermal incision was made in the middle of the left side of the neck to locate the facial nerve, which was then clamped with a surgical clamp for 40 min to create neuropathic damage (Figure 1).[9]

On days 1 and 15, after surgery, functional assessments were performed, and the left facial nerves from Groups 3, 4, and 5, as well as both the right and left facial nerves from 8 animals in Groups 1 and 2 (serving as both paralyzed and control groups), were collected, resulting in a total of 40 facial nerves being sent for microscopic examination. At the end of day 15, the animals were sacrificed under deep anesthesia.

Functional assessment

On days 1 and 15, whisker movements and corneal reflexes were observed in response to pressurized air. Whisker movement in rats was assessed by observing vibrissae movement and eye closure through the blinking reflex. Complete functional recovery of the facial nerve branch on the operated side was indicated by normal whisker movement and anterior positioning, which scored 5 points, alongside a full eye closure reflex, also scoring 5 points. The absence of both reflexes and movements was scored as 1 point

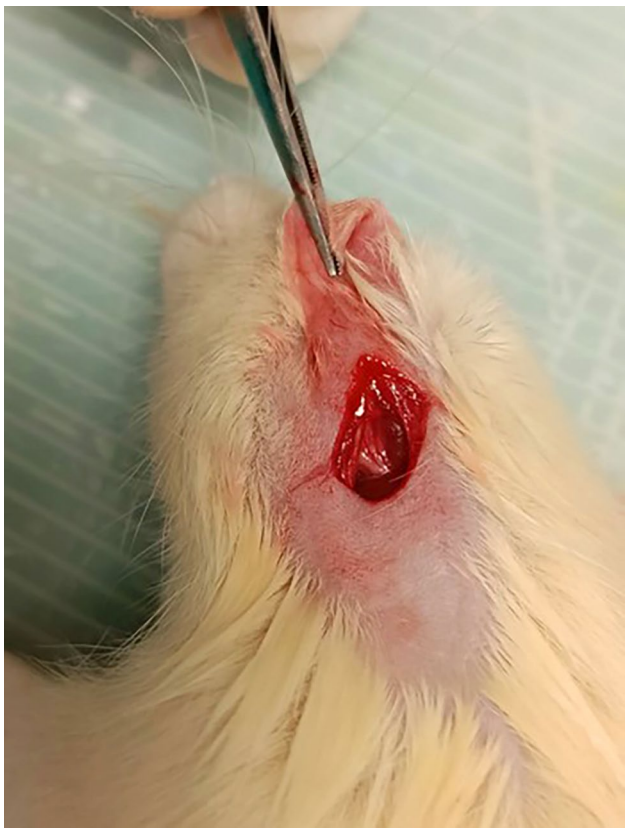

Figure 1. The facial nerve trunk region.

each, indicating total facial paralysis. Intermediate stages of recovery were assigned varying scores based on partial whisker movement and eye closure. The total clinic score was determined (2 to 10 points) following the method described by de Faria et al.[10]

Microscopic assessment

Anesthesia was administered to the animals via intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). The nerve tissue samples from the rats were placed in 10% formalin fixative for 48 h to prepare them for histological examination under a light microscope. After fixation, the samples were washed in tap water for 3 to 4 h to prevent fixative precipitation. The tissue samples were then gradually dehydrated by placing them in a series of alcohol solutions (70%, 80%, 90%, and 96%) for 45 min each. Following dehydration, the samples were cleared by soaking them in xylene twice for 20 min each. The cleared nerve tissue samples were then embedded in paraffin, which was melted in an oven at 65°C and kept in three separate paraffin baths for 60 min each. The paraffin-embedded tissues were subsequently placed into cassettes and prepared for sectioning. The microtome blade used to obtain sections from the paraffin blocks was cooled in a refrigerator, and 5-µm-thick tissue sections were cut from each sample using a microtome.

The sections were placed in a 45°C water bath to ensure they spread properly and then mounted onto clean slides. After being incubated in an oven for 1 h, the slides underwent deparaffinization by being held in two separate xylene baths for 1 h each. The sections were then stained using hematoxylin and eosin. The deparaffinized tissue sections were rehydrated by placing them in 96%, 90%, 80%, and 70% alcohol solutions, followed by distilled water, each for 5 min. The sections were stained with hematoxylin for 2 min and eosin for 10 min. Excess stain was removed by rinsing the sections in tap water, followed by rapid dehydration through a series of alcohol solutions. The tissues were then cleared by placing them in two separate xylene baths for 30 min each. The cleared tissues were mounted with Entellan (Merck Merck KGaA, Darmstadt, Germany) and examined under an Olympus BH-2 light microscope (Olympus Corp., Tokyo, Japan). Images of all nerve samples were captured using an Olympus DP-70 digital camera (Olympus Corp., Tokyo, Japan). The photographed samples were scored and statistically analyzed.

To determine apoptotic assessments on the nerve tissue samples, the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) method was applied. All specimens were examined under a microscope and evaluated accordingly.

Statistical analysis

A total of eight rats per group were determined as the required sample size with a sufficient statistical power 0.90 (alpha=0.05, effect size=1.60) using G*Power version 3.1.9.4 software (Heinrich-Heine Universität Düsseldorf, Düsseldorf, Germany).[11]

All analyses were performed using the IBM SPSS version 21.0 software (IBM Corp., Armonk, NY, USA). The distribution of the continuous variables was assessed for normality using the Shapiro-Wilk test. The results were expressed as median values (25-75%). Comparisons of two paired samples were evaluated using the Wilcoxon signed-rank test. Comparison of more than two independent groups were analyzed using the Kruskal-Wallis test. Post hoc Dunn's test was applied to determine which groups had significant differences. A p-value <0.05 was considered statistically significant.

RESULTS

Functional assessments were conducted on days 1 (pretreatment) and 15 (posttreatment) after surgery. No significant differences were found between the paralyzed, MP, ozone, and combination groups at pretreatment regarding corneal reflex; all had lower scores compared to the control group (paralyzed groups, p=0.007; MP, ozone, and combination groups, p<0.001). All groups (control, MP, and combination, p<0.001; ozone, p=0.001) had significantly higher scores than the paralyzed group at posttreatment.

Similar results were found for paralyzed, MP, ozone, and combination groups at pretreatment regarding whisker movements; all lower than the control group (p<0.001). Control and combination groups had significantly higher scores than the paralyzed group at posttreatment (p<0.001). Control and combination groups also scored higher than the ozone group (control, p<0.001; combination, p=0.001). The MP group had lower scores compared to the control group (p=0.017).

Total clinical scores were lower in ozone (p<0.001) and combination (p<0.001) groups, as well as MP (p=0.001) and paralyzed groups (p=0.010), compared to control at pretreatment. Total clinical scores were higher in control (p<0.001), combination (p<0.001), and MP groups (p=0.001) compared to the paralyzed group at posttreatment. The ozone group had lower scores compared to control (p=0.002) and combination groups (p=0.023; Table 1; Figure 2).

On hematoxylin and eosin staining, the control group had a normal histological structure with intact myelinated axons and vascular formations. The paralyzed group had degenerated vacuolar structures, vascular congestion, inflammation, and proliferated Schwann cells. The MP and combination groups had histological structures that were close to normal. The ozone group had mostly normal histological structures, with sparse vascular congestion (Figure 3).

Myelin degeneration scores were lower in control, MP, combination (p<0.001), and ozone groups (p=0.035) compared to the paralyzed group. Myelin degeneration scores were higher in the ozone group compared to the control group (p=0.004).

Vascular congestion scores were lower in the control, MP, and combination (p<0.001) groups compared to the paralyzed group and higher in the ozone group compared to the control (p=0.001) and MP (p=0.012) groups.

Inflammation scores were lower in control, MP (p<0.001), ozone, and combination (p=0.002) groups compared to the paralyzed group.

Schwann cell proliferation scores were lower in the control, MP (p<0.001), and ozone (p=0.004) groups compared to the paralyzed group. The control group also had lower scores compared to combination (p=0.002) and ozone groups (p=0.027).

On toluidine blue staining, the control group had a normal histological structure with mast cells, myelinated axons, and vascular formations. The paralyzed group had numerous mast cells, degenerated vacuolar structures, and vascular congestion. The MP group had sparse vascular congestion, close to normal histology. The ozone and combination groups had histological structure close to normal (Figure 4).

Toluidine blue staining scores were lower in the control (p=0.001), MP (p<0.001), ozone (p=0.001), and combination (p=0.014) groups compared to the paralyzed group. The MP group had lower

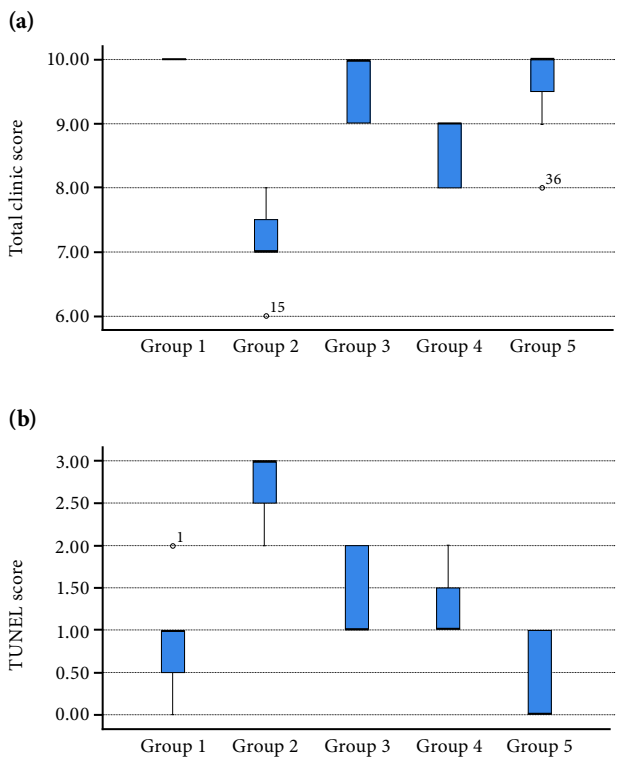

Figure 2. Box-plot distribution of **(a)** total clinical scores and **(b)** TUNEL staining scores, and their comparison between groups after treatment.

TABLE 1
Comparison of functional assessment data before and after treatment within and between groups

Variables	Group 1		Group 2		Group 3		Group 4		Group 5		*p*	Post hoc Dunn's test
	Median	25^{th}-75^{th} percentile	Median	25^{th}-75^{th} percentile	Median	25^{th}-75^{th} percentile	Median	25^{th}-75^{th} percentile	Median	25^{th}-75^{th} percentile		
Corneal reflex (BT)	5	5-5	2	1-2.8	1	1-3.25	1	1-1	1	1-1	<0.001	1-2†, 1-3* 1-4*, 1-5*
Corneal reflex (AT)	5	5-5	3	3-3.8	5	5-5	5	4-5	5	4.3-5	<0.001	1-2*, 2-3* 2-4°, 2-5*
p	>0.050		0.047		0.008		0.010		0.010			
Whisker movement (BT)	5	5-5	1	1-1.8	1	1-2.5	1	1-1	1	1-1	<0.001	1-2*, 1-3* 1-4*, 1-5*
Whisker movement (AT)	5	5-5	4	4-4	4	4-5	4	4-4	5	5-5	<0.001	1-2*, 1-3[1], 1-4*, 2-5*, 4-5°
p	>0.050		0.015		0.010		0.007		0.008			
Total clinic score (BT)	10	10-10	3	2.3-3.8	2	2-5	2	2-2	2	2-2	<0.001	1-2[2], 1-3°, 1-4*, 1-5*
Total clinic score (AT)	10	10-10	7	7-7.75	9	9-10	9	8-9	10	9.3-10	<0.001	1-2*, 1-4[3], 2-3°, 2-5*, 4-5‡
p	>0.05		0.015		0.011		0.010		0.010			

BT: Before treatment; AT: After treatment; * p<0.001; † p=0.007; ° p=0.001; [1] p=0.017; [2] p=0.010; [3] p=0.002; ‡ p=0.023.

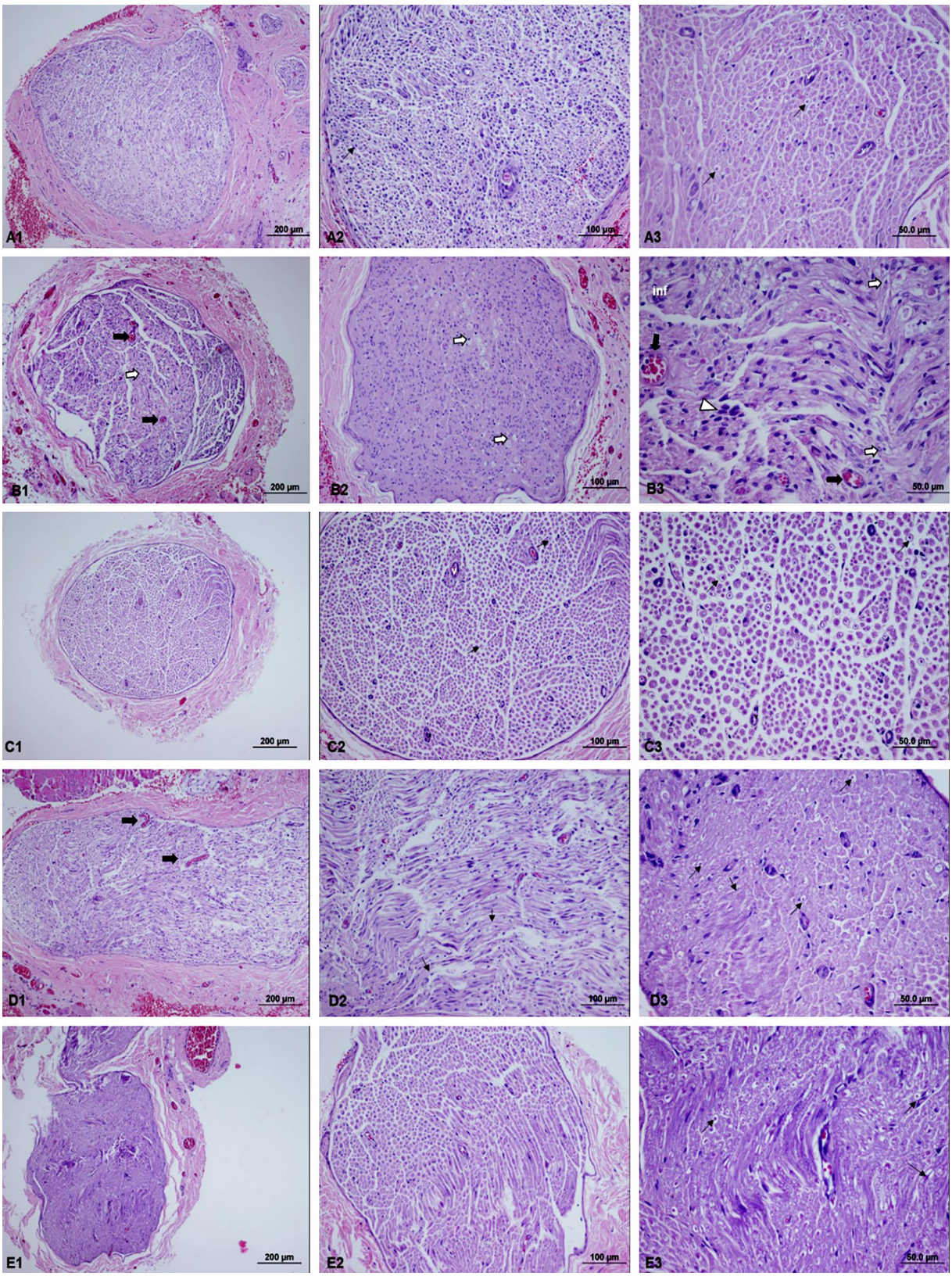

Figure 3. Light microscopy examination images of facial nerve samples from each group.

A1-A3: Control group; B1-B3: Paralyzed group; C1-C3: Methylprednisolone group; D1-D3: Ozone group; E1-E3: Combination group. H&E, scale bar: 200 µm-×10, 100 µm-×20, 50.0 µm-×40) normal myelinated axon structures (→), degenerated vacuolar structures observed in myelinated axons (⇒), vascular structures (v), vascular congestion (➡), inflammation (inf), proliferating Schwann cells (▷).

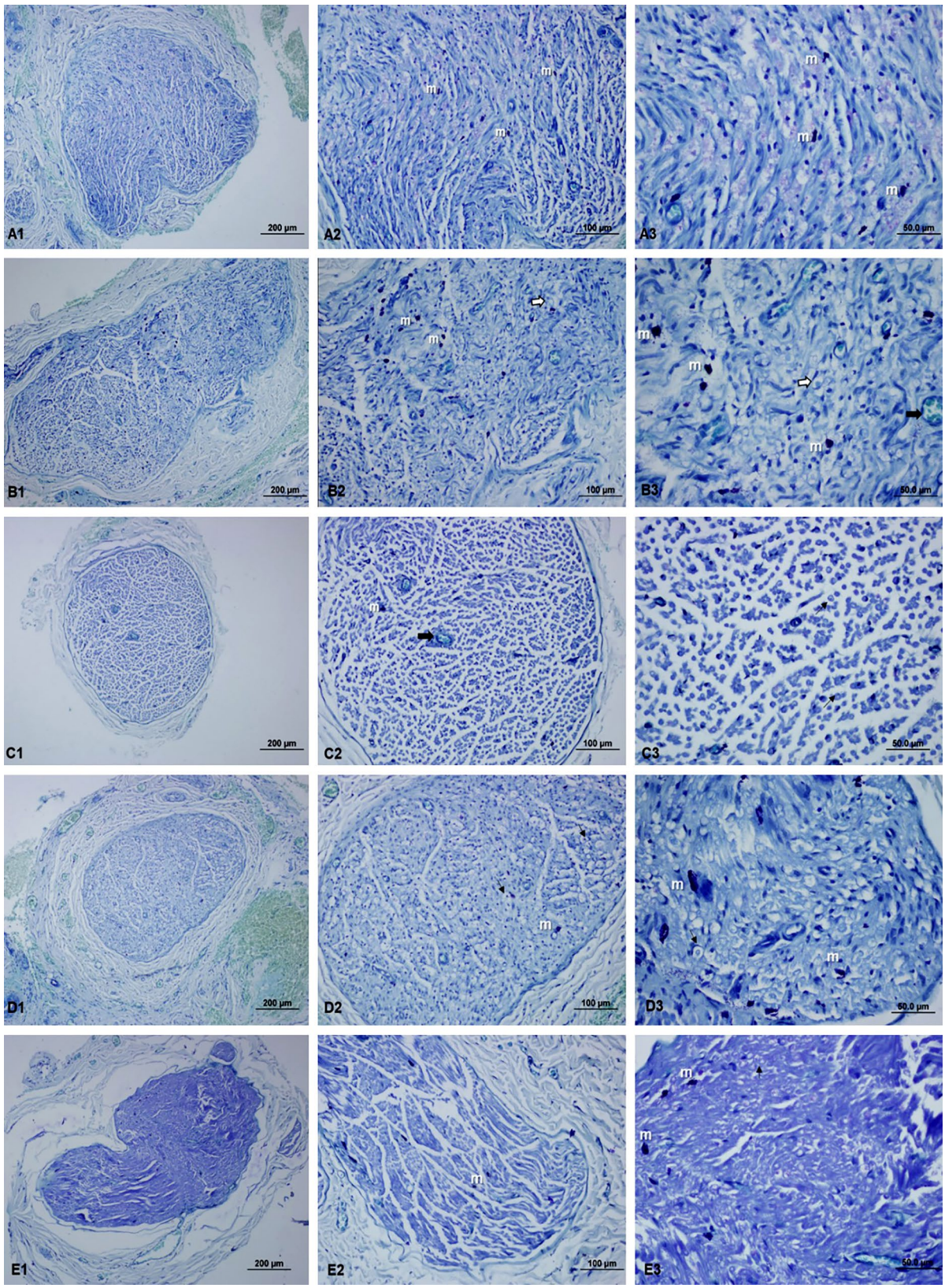

Figure 4. Light microscopy images of toluidine blue-stained preparations of facial nerve samples from each group.

A1-A3: Control group; B1-B3: Paralyzed group; C1-C3: Methylprednisolone group; D1-D3: Ozone group; E1-E3: Combination group. Toluidine blue, scale bar: 200 µm-×10, 100 µm-×20, 50.0 µm-×40). Mast cells (m), normal myelinated axon structures (→), degenerated vacuolar structures observed in myelinated axons (⇒), vascular congestion (➡).

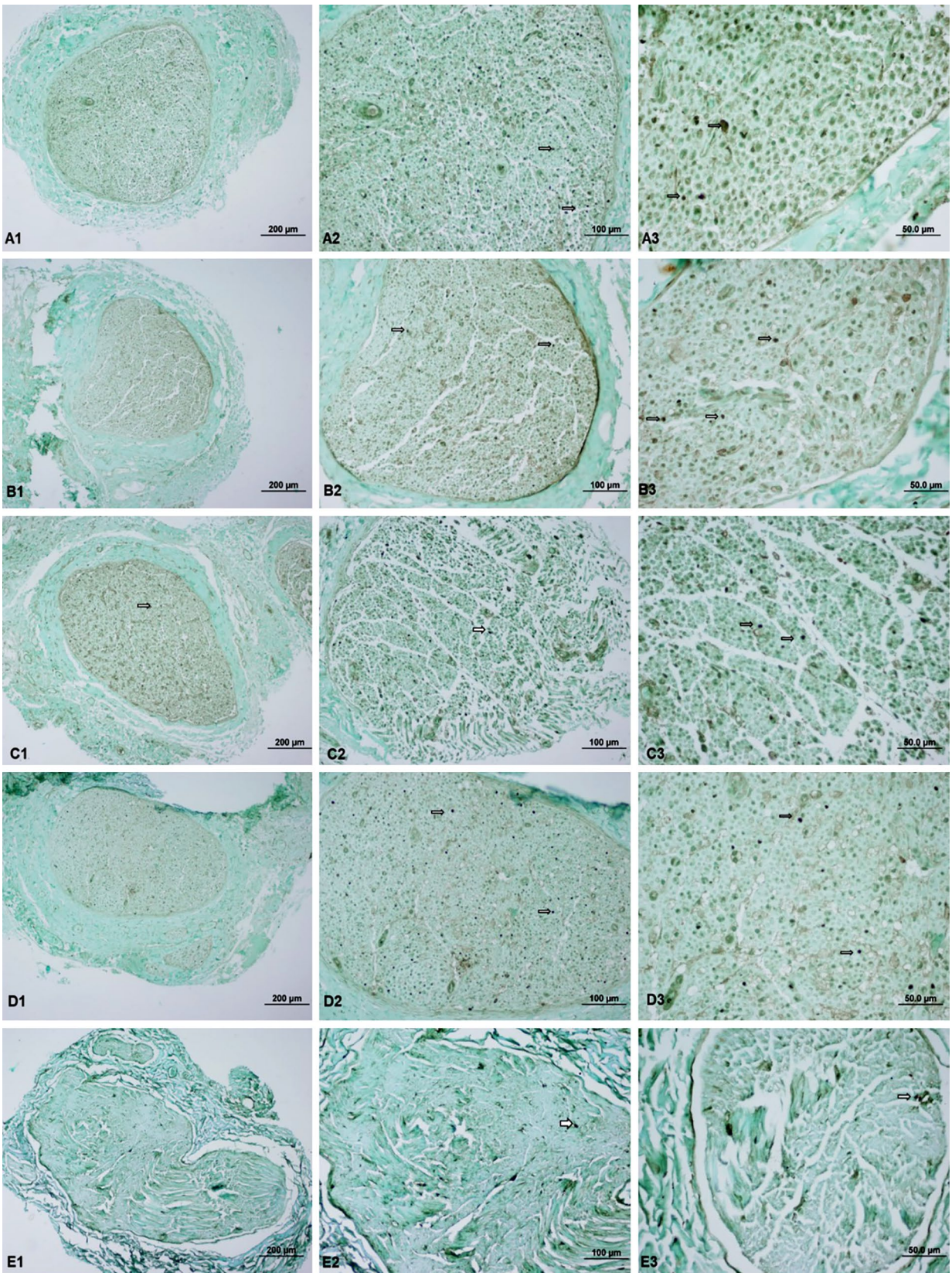

Figure 5. Immunohistochemical examinations following TUNEL staining of facial nerve samples from each group

A1-A3: Control group; B1-B3: Paralyzed group; C1-C3: Methylprednisolone group; D1-D3: Ozone group; E1-E3: Combination group. TUNEL, scale bar: 200 µm-×10, 100 µm-×20, 50.0 µm-×40). TUNEL staining (⇒).

TABLE 2
Comparison of microscopic analysis results between groups

Variables	Group 1		Group 2		Group 3		Group 4		Group 5		*p*	Post hoc Dunn's test
	Median	25^{th}-75^{th} percentile	Median	25^{th}-75^{th} percentile	Median	25^{th}-75^{th} percentile	Median	25^{th}-75^{th} percentile	Median	25^{th}-75^{th} percentile		
Myelin degeneration	0	0-0	3	3-3	0.5	0-1	1	1-2	0.5	0-1	<0.001	1-2*, 1-4[2] 2-3*, 2-4[1], 2-5*
Vascular congestion	0	0-0	3	2.3-3	0	0-0.8	1	1-2	0.5	0-1	<0.001	1-2*, 1-4° 2-3*, 2-5* 3-4[e]
Inflammation	0	0-0	2.5	2-3	0	0-0	0.5	0-1	0.5	0-1	<0.001	1-2*, 2-3*, 2-4[3], 2-5[3]
Schwann cell proliferation	0	0-0	3	3-3	0.5	0-1	1	0.3-1	1	1-1	<0.001	1-2*, 1-4± 1-5[3], 2-3*, 2-4[2]
Toluidine blue	1	1-1	3	2.3-3	0	0-0.8	1	1-1	1	1-2	<0.001	1-2°, 1-34 2-3*, 2-4° 2-5^, 3-44, 3-5[3]
TUNEL	1	0.3-1	3	2.3-3	1	1-2	1	1-1.8	0	0-1	<0.001	1-2[3], 2-3[‡], 2-4[†], 2-5*, 3-5[£], 4-5[€]

The scoring system for myelin degeneration, vascular congestion, inflammation and Schwann cell proliferation; 0: Hone, 1: Minimally present, 2: Moderately present, 3: Severely present. The scoring system for toluidine blue and TUNEL; 0: None, 1: Minimal staining, 2: Moderate staining, 3: Severe staining. * p<0.001; ° p=0.001; [1] p=0.035; [2] p=0.004; [3] p=0.002; [±] p=0.023; [e] p=0.012; [±] p=0.027; ^ p=0.014; [4] p=0.038; † p=0.005; ‡ p=0.012; [£] p=0.016; [€] p=0.036.

scores than control (p=0.038), ozone (p=0.038), and combination (p=0.002) groups.

On TUNEL staining, the control group had minimal positive staining. The paralyzed group had advanced positive staining. The MP group had moderate positive staining. The ozone group had moderate positive staining. The combination group had minimal positive staining (Figure 5).

The TUNEL test scores were lower in control (p=0.002), ozone (p=0.005), combination (p<0.001), and MP (p=0.012) groups compared to the paralyzed groups. The combination group had lower scores than MP (p=0.016) and ozone (p=0.036) groups (Table 2, Figure 2).

DISCUSSION

This study is the first to compare ozone and MP treatments in experimental facial paralysis. Our results showed that whisker movements and total clinical scores were highest in the combination group, and the combination group had lower apoptosis levels than the MP and ozone groups.

Facial nerve motor fibers can regenerate their injured axons, but not all patients with facial paralysis achieve complete recovery. Our study confirmed this; the untreated paralyzed group showed minimal functional improvement and had the worst histological outcomes. Effective treatment of facial paralysis should address the direct effects of trauma, ischemia, edema, oxidative stress, and inflammation that cause structural nerve changes.[12]

Corticosteroids, the traditional treatment, have antioxidant and anti-inflammatory effects,[13] but their benefits are debatable.[14] There is a need for superior treatments to achieve better functional and histologic recovery in facial nerve injury.

Before treatments, all experimental groups (paralyzed, MP, ozone, and combination) had similar functional assessment scores, all of which were lower than those of the control group, indicating established facial paralysis. After treatment, all groups showed significant functional improvements. Corneal reflex scores in MP, ozone, and combination groups were higher than in the paralyzed group and similar to controls. Whisker movements improved most in the combination group, followed by the MP group, with the ozone group scoring lowest among the treatment groups. Total clinical scores were highest in the MP and combination groups, similar to controls, which had normal tissue, while the ozone group had lower scores among the treatment groups. There is limited literature on the functional effects of ozone therapy in facial paralysis.[5,7] Ozbay et al.[5] found that ozone-treated rats had a lower stimulation threshold, indicating better functional improvement. Valença et al.[7] reported significant functional improvement in a Bell's palsy case treated with laser and ozone therapy. Comparing ozone and MP, other nerve studies found better motor distal latencies with ozone therapy by six months.[15] Ozone enhances tissue oxygenation, promotes cytokine secretion, and improves angiogenesis and nutrient delivery, facilitating healing. Previous studies showed reduced vascular congestion, edema, and hemorrhage in nerves with ozone therapy.[5,16] Histological analysis of our study revealed similar myelin degeneration and inflammation across all treatment groups, with lower scores than the paralyzed group. Vascular congestion was lower in all treatment groups than in the paralyzed group but not statistically significant in the ozone group. Schwann cell proliferation was similar across all treatment groups. Schwann cells are crucial for nerve regeneration by secreting neurotrophic factors and myelinating neurons.[17] Histological analysis after 15 days showed near-normal myelinated axon structures and vascular formations in the MP, ozone, and combination groups. We believe that the low level of Schwann cell proliferation indicates that healing has been completed at a level close to normal. If this proliferation had been examined in the nerve tissue between days 7 and 10, it might have been high in all treatment groups, similar to the untreated the paralyzed group.

Corticosteroids are essential for reducing swelling and capillary permeability in nerve injury and are recommended within the first day.[13,18] Ozone therapy also has antioxidant, anti-inflammatory, and immunomodulatory effects.[8] No prior study compared MP and ozone in experimental facial paralysis. Our study showed higher vascular congestion in the ozone group than in the MP group. Similar to our study, in an experimental mental nerve injury, perineural infiltration of inflammatory cells and vascular proliferation were reported to be lower in the MP group than in the ozone group.[19] Ozturk et al.[8] reported the efficacy of ozone therapy in comparison with MP in a previous rat study with experimental sciatic nerve injury. Forty Sprague-Dawley rats were divided into four groups.

The first group received intraperitoneal ozone at a dose of 20 µg/mL, the second group received 2 mg/kg MP with the same dosage in our study, the third group received a combination of ozone and MP, and the fourth group received saline only. All groups underwent a 14-day treatment period. Only histomorphological assessments were made without functional assessments. It was reported that combined therapy had lower degeneration and higher perineural vascular proliferation and inflammation in peripheral tissue. Different from that study our study results showed similar myelin degeneration and inflammation scores between ozone, MP, and combination groups. Furthermore, the MP group showed significantly lower scores than the ozone group in terms of vascular congestion. The smaller size of the facial and mental nerves compared to the sciatic nerve enables a faster healing process. Both our study and Ozturk et al.'s[8] study evaluated the outcomes after 14 days of treatment. Our results show improved facial nerve healing, whereas their results may be attributed to the ongoing healing process of the sciatic nerve due to its larger size. The effect of ozone therapy on facial nerve healing may differ due to the nerve size and recovery speed compared to the sciatic nerve.

Apoptotic changes evaluated with the TUNEL test showed advanced positive staining in the untreated paralyzed group, indicating significant apoptosis and neuron death. All treatment groups had lower TUNEL staining, with minimal positive staining in the combination group, indicating less apoptosis. Comparing facial paralysis groups, the combination group had significantly lower apoptosis than MP and ozone groups. If the basal plate of the neural tube is preserved in a crushing injury that damages axons and disrupts nerve continuity, new axons can rapidly regrow and reconnect along the basal plate of the neural tube, resulting in complete functional recovery.[20] Preventing the additional nerve damage caused by the reactive oxygen species by inhibiting apoptosis pathways is very important for nerve recovery. Both ozone[3,5,6,12,21] and MP[22] have antioxidant, antiapoptotic, and anti-inflammatory effects. Our study suggests that combined MP and ozone therapy synergistically reduces apoptosis and promotes histological recovery.

The limitations of our study include the absence of electrophysiological examinations and electron microscopy. The strengths of our study lie in the comparative functional, histological, and immunohistochemical examination. Furthermore, the similarity of the functional and microscopic assessment data in the combination group, excluding Schwann cell proliferation, with the increased activity of the right control side due to left-sided paralysis highlights a significant level of recovery in the combination group, further enhancing the strength of the study.

In conclusion, combining ozone with MP yields the best outcomes in apoptosis reduction and whisker movements compared to each treatment alone. This suggests ozone could complement corticosteroids in treating facial paralysis. Further studies are needed to confirm the benefits of ozone therapy in humans, elucidate underlying mechanisms, and determine optimal dosages for combined treatment.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Supervision: B.A., O.A., F.B.; Data collection or processing: F.B., B.A., O.A., S.Ö., A.O., D.B.D.; Analysis: F.B., F.Ş.M.; Literature search: F.B., B.A., A.O.; Writing manuscript: F.B., D.B.D., F.Ş.M., A.O. Concept, critical review: All authors.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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