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Neuroprotective Effects of Halofuginone in Rat's Sciatic Nerve Injury

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■ OBJECTIVE: **The object of the present study was to investigate the neuroprotective effects of halofuginone—with its antifibrotic and anti-inflammatory effects—on peripheral nerve injuries frequently encountered in daily life, at the functional, pathological, and cellular levels through the use of electron microscopy.**

■ METHODS: **Forty Wistar rats were distributed into 4 groups. Group 1 (sham group) consisted of rats with only exposed nerves; group 2 (control group) consisted of traumatized rats; group 3 (methylprednisolone [MP] group) consisted of rats receiving MP treatment after trauma; group 4 (halofuginone group) consisted of rats receiving halofuginone treatment after trauma. The rats were decapitated after one month. During this period, gait track analysis, muscle mass ratio, and pathological and electron microscopic evaluations were performed.**

■ RESULTS: **Significant functional improvement was observed in the animal groups receiving MP and halofuginone compared to the trauma group. It was determined that halofuginone and MP provided improvement in peripheral nerve structure at both the pathological and cellular levels.**

■ CONCLUSIONS: **Halofuginone has peripheral nerve protective effects on a rat peripheral nerve injury model.**

INTRODUCTION

Peripheral nerve injuries are a common clinical problem that may lead to permanent functional impairment.[1] When inadequately treated, sensory, motor, and autonomic functions may be affected.[2] Although many clinical and experimental studies have been conducted examining treatments to improve recovery, no effective standardized treatment currently exists.[3] Studies are being conducted on treatments that will affect the ischemic and inflammatory processes that start after the primary damage that develops following trauma. If we give examples of these studies, we will have to mention many different alternative treatment modalities, including various anti-inflammatory drugs, herbal extracts, neurotrophic factors, hormones, chemicals, lasers, omentum, collagen extracts, and low frequency magnetic fields.[4-6]

Halofuginonehydrobromide {7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone} is an alkaloid obtained from the roots of *Dichroa febrifuga* (Hydrangeaceae), also known as Chinese quinine. This febrifugine analog was originally used as a poultry feed supplement to combat coccidiosis. In recent years, halofuginone has shown potential as an effective treatment for a variety of conditions, including fibrosis, inflammation, malaria, and cancer.[7,8] Animal studies have also revealed its antifibrotic activity in multiple sclerosis.[9] In clinical settings, halofuginone shows promise in the treatment of solid tumors and plasmodium vivax infections.[10]

To our knowledge, no study has been published to date on whether halofuginone has an effect on peripheral nerve trauma. Our study aims to evaluate the neuroprotective effects of halofuginone in a traumatic rat model functionally, pathologically, and electron microscopically.

MATERIALS AND METHODS

In this study, a cohort of 40 male Wistar rats, each weighing between 180 and 220 g, was utilized. The research protocol received approval and oversight from the Local Animal Ethics Committee. The rats were subjected to anesthesia using ketamine hydrochloride at a dosage of 100 mg/kg (Ketalar; Eczacıbaşı Group, Istanbul, Turkey), in conjunction with xylazine at 5 mg/kg

Key words
- Anti-inflammatory
- Halofuginone
- Methylprednisolone
- Peripheral nerve injury

Abbreviations and Acronyms
MP: Methylprednisolone
SFI: Sciatic functional index

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(Rompun; Bayer, Leverkusen, DE). All surgical procedures were carried out by the same surgeon, employing microsurgical techniques and utilizing an operating microscope for enhanced precision.

Surgical Technique

The sciatic nerve injury was created using the method described by Apaydin et al.[11] The right sciatic nerve was utilized in surgical procedures. Evaluations were performed double blind. Through a vertical gluteal incision, the muscles were dissected to expose the sciatic notch to the point where the nerve branches. Subsequently, the rats were divided into four groups with 10 animals in each one. Group 1 was the sham group where the sciatic nerve was exposed and the skin was closed. Group 2 was the control group where the right sciatic nerve of the rats was exposed for 30 seconds and then damage was induced by providing continuous pressure using a Yasargil aneurysm clip (Aesculap FT-760 T, closing force: 180g, B. Braun, Bethlehem, PA, USA) to the sciatic nerve. The injury was performed 1nullcm proximal to the bifurcation. Group 3 was the methylprednisolone (MP) group where the crush injury was applied to the sciatic nerve as in the control group; then, 1 mg/kg MP (Prednol, Mustafa Nevzat, Istanbul, Turkey) was given intraperitoneally daily for six days and then tapered for four days. Group 4 was the halofuginone group where crush injuries were applied to the sciatic nerves as in the control group; halofuginone (Halocur; Intervet Ltd., Dublin, Ireland) was then administered by oral gavage at a dose of 0.2 mg/kg/day for 1 week after the procedure.[12] The tissue layers were duly sealed at the end of surgery. All subjects were decapitated on day 30 for pathological and electron microscopic examination.

Hiking Trail Analysis

Motor function was evaluated by free gait pattern analysis as described by de Medinaceli.[13,14] Gait analyses were performed at weeks 2 and 4. The parameters assessed in this study encompassed the length of the toes, defined as the measurement from the heel to the tip of the toe; the width of the toes, characterized by the distance between the first and fifth toes; and the span of the intermediate toes, indicated by the distance from the second to the fourth toe. These measurements were taken on both the surgical and nonsurgical limbs. Additionally, the sciatic functional index (SFI) was computed for each subject, utilizing the methodology established by Bain et al.,[15] with the SFI being represented as a percentage. An SFI of −100% indicates a complete sciatic nerve lesion, while an SFI between −10% and +10% reflects normal function.

Muscle Mass

After the rats were decapitated, both the operated side's and the intact side's gastrocnemius muscles were dissected under a microscope and weighed with the help of an electronic balance. Muscle weights on the operated side were divided by muscle weights on the normal side to get the weight ratio expressed as a percentage. Two independent observers blinded to group assignments performed all measurements.

Pathological Examination

The rats were decapitated at week 4 and the segments, including the sciatic nerve injury site, were removed. The segments were cut in half and divided into two equal parts. One piece was prepared for pathological evaluation and the other was prepared for electron microscopic evaluation. Samples were fixed in 10% formalin. Paraffin blocks were then created from the samples that were followed up in the Leica ASP 300S device. From the blocks, 4 micron sections were prepared with a Leica RM 2255 Microtome. These sections were stained with hematoxylin and eosin. They were examined under an Olympus BX51 microscope. Measurements were performed with imaging software (BS 200 Pro; BAB Soft, Ankara, Turkey). Five random fields were selected for each section at x100 magnification and myelinated fibers were counted. In addition, axon diameters, nerve diameters, myelin diameters, and G ratios (ratio of axon diameter to fiber diameter) were determined.

Evaluation by Transmission Electron Microscopy

Samples were placed in 2.5% glutaraldehyde for fixation for 48 hours followed by postfixation with OsO_3. Postfixation samples were dehydrated in a graded series of alcohol solutions and embedded in Araldite CY212. Sections were taken approximately 2 μm thick. Staining with 1% methylene blue was then performed. Slides were kept on a hot plate at 100–110°C for approximately 40–45 seconds and then washed with tap water and rinsed. For descriptive analysis, sections were first examined under a light microscope. After region of interest identification, tissue blocks were cut to prepare ultrathin sections. Ultrathin sections (60 x 90 nm) taken with a Leica EM UC7 Ultramicrotome were stained with uranyl acetate and lead citrate. The stained sections were visualized and photographed on a Hitachi HT7800 120 KV transmission electron microscope.

The grading system used for quantitative assessment was based on principles similar to those used in the evaluation of different tissue samples and the spinal cord (Table 1). In each group, axon degeneration, axon–myelin cleavage, intracytoplasmic edema, nuclear damage, and vascular endothelial damage were assessed, counted, and presented as mean scores ± standard deviation.[16] The investigator who evaluated the sections was unaware of the group information.

Statistical Analysis

The Mann–Whitney U test was used to compare the results. Statistical significance was determined for P values below 0.05. Variables in the study were reported as mean ± standard deviation.

RESULTS

Hiking Trail Analysis

Upon evaluation of SFI measurements, a statistically significant difference was observed between the test groups and the control group ($P < 0.05$). This discrepancy primarily stemmed from the variations noted between the sham and the control groups. However, when the test groups were analyzed in isolation, no statistically significant difference was found in the mean SFI values between the halofuginone and MP groups at weeks 2 and 4 (see Figure 1A, B).

Table 1. Grading System for Quantitative Evaluation of Ultrastructural Findings

Axonal Myelin	
Normal myelin layers	0
Vesiculated myelin	1
Cracked myelin layers	2
Honeycomb & extruded vesicles	3
General axonal	
Normal	0
Light edema	1
Mild edema	2
Severe edema & loss of structure	3
Interaxonal edema	
Absent	0
Light	1
Mild	2
Severe (cell membrane defect)	3
Nucleus	
Normal	0
Clumping	1
Sparse chromatin	2
Severe damage	3
Vascular endothelium	
Normal	0
Light edema	1
Mild edema	2
Severe edema	3

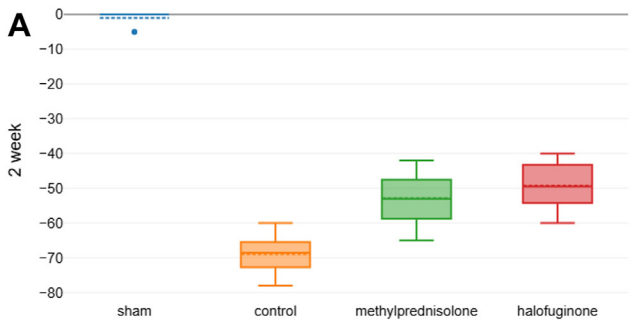
50 axons, 10 schwann cells and 10 capillaries were scored at each group.

Muscle Mass

The findings regarding the mean weight ratios of the gastrocnemius muscles are illustrated in Figure 2. A statistically significant distinction was observed between the muscle weight ratios of the experimental group and the control group ($P < 0.05$). Further analysis revealed that this statistical disparity primarily arose between the sham and the control groups. Conversely, no significant difference was detected between the MP and halofuginone groups ($P > 0.05$).

Pathological Examination

When the light microscope sections of the sham group were evaluated, the perineurium and endoneurium were normal. It was observed that the fibers in the fascicles were homogeneously distributed (Figure 3A). In the trauma group, the nerve fibers were thinner and irregularly distributed, with some fibers exhibiting a lack of continuity. Hemorrhage, fibrosis, inflammatory cells, collagen fibers, and adipose tissue were observed infiltrating the damaged nerve bundles in the trauma area (Figure 3B). Additionally, the integrity of the nerve capsule was compromised and appeared broken. In the MP group, inflammation was less pronounced, and the integrity of the nerve capsule was preserved. However, fibrosis, increased collagen tissue, and vascular proliferation were observed when compared to the halofuginone group. The nerve fibers in this group were irregular and fragmented (Figure 3C). The evaluation of the halofuginone group showed mild irregularity and thinning of nerve fibers in the trauma area. No fibrosis or inflammation was observed. Significant improvement was observed compared to the MP group (Figure 3D). The diameter of nerves, thickness of myelin, diameter of axons, and G ratios of all groups were compared (Table 2). The evaluation exposed a statistically significant difference among all groups ($P < 0.05$). Notably, upon excluding the sham group from consideration, a significant difference remained evident among the drug experiment groups and the traumatized control group ($P < 0.05$). On the other hand, no significant difference was observed between the MP and halofuginone groups ($P > 0.05$). In addition, the results of the



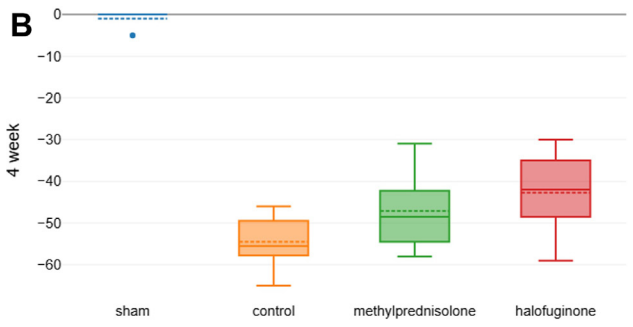


Figure 1. Sciatic function index values. (**A**) There was a statistically significant difference between all groups at week 2 and (**B**) week 4 ($P < 0.05$). No statistical difference existed between the MP and halofuginone groups ($P > 0.05$). MP, methylprednisolone.

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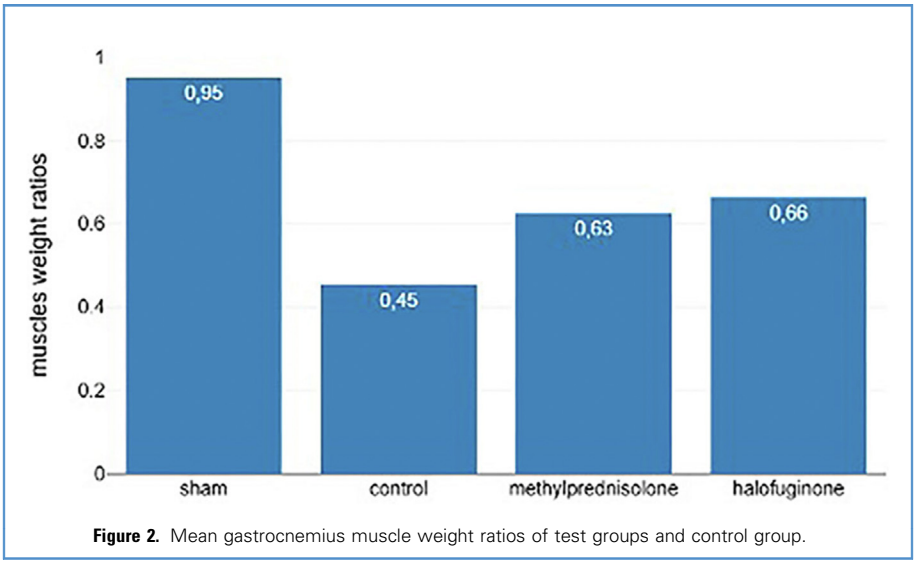


Figure 2. Mean gastrocnemius muscle weight ratios of test groups and control group.

drug experiment groups were found to have larger values when compared with the traumatized control group.

Evaluation by Transmission Electron Microscopy

The sham group had intact myelinated and unmyelinated axons. Myelin layer was continuous and intact (Figure 4A). In the trauma group, edematous areas were detected in myelin and between axons (Figure 4B). In the MP group, some intermediate axons with intact myelin and some large axons with mild edema were seen. Unmyelinated axons were intact and there were no edematous areas between axons (Figure 4C). In the halofuginone group, there were intact myelinated axons and unmyelinated axons. No edema was observed in the axons, but there were edematous areas between some axons (Figure 4D). Ultrastructural examination and scoring showed that surgical trauma caused mild damage to the sciatic nerve, while steroids and halofuginone had a protective effect.

The descriptive characteristics of the electron microscope score variables are outlined in Table 3. A statistically significant difference was detected between all groups in terms of axonal myelin and overall axonal and interaxonal edema scores ($P < 0.05$). However, upon analyzing the overall axonal grades, no significant difference was recognized among the MP and halofuginone groups ($P > 0.05$). In contrast, the other two scores indicated a statistically significant difference between the MP and halofuginone groups ($P < 0.05$). Furthermore, when assessing the nucleus and vascular endothelium, there were no notable differences found among any of the groups ($P > 0.05$).

DISCUSSION

Peripheral nerve injury is frequently encountered in daily life, often resulting in significant cosmetic and functional impairment in affected individuals.[17] Peripheral nerve damage leads to the following conditions: the development of both ischemic and inflammatory processes resulting in Wallerian degeneration. In this process, after the initial primary injury, a large number of free radicals and toxic substances begin to accumulate around the site of injury. This cascade of events then stimulates various proteolytic pathways, resulting in cellular destruction and damage, particularly affecting neurofilaments and microtubules. In addition, scar formation at the proximal and distal ends of the trauma site can physically impede axon propagation.[18] Despite advances in technology and surgical techniques, recovery rates after peripheral nerve injury are still not encouraging. This has led to the development of effective therapeutic agents that offer neuroprotective benefits and actively promote regeneration of the injured nerve.[19] The success of peripheral nerve regeneration is closely linked to the balance between Schwann cell proliferation and scar tissue formation.[20] Several cellular components are involved in the regeneration process, including inflammatory cells, Schwann cells and neurotrophic factors. These components work synergistically to promote and maintain axon growth and myelination.[21] It has been previously described in the literature that inhibition of scar formation accelerates axonal regeneration. Studies have exposed that topical usage of melatonin, hyaluronic acid, tacrolimus, and MP can accelerate the healing process. These substances work by limiting fibroblast growth and proliferation, thus reducing scar formation in the affected area.[22]

Halofuginone, an alkaloid derived from the roots of *Dichroa febrifugua*, is utilized as an antiparasitic agent.[23] Beyond its antiparasitic properties, halofuginone demonstrates antifibrotic, anti-inflammatory, and autoimmune regulatory efficiency.[24,25] Two mechanisms have been proposed in the literature to explain the effects of halofuginone: 1) Halofuginone inhibits the phosphorylation of Smad3, which disrupts TGF-β-mediated induction of extracellular matrix components. This action influences genes responsible for tissue inhibitors such as collagen, plasminogen activator inhibitor-1, and metalloprotease-1, ultimately

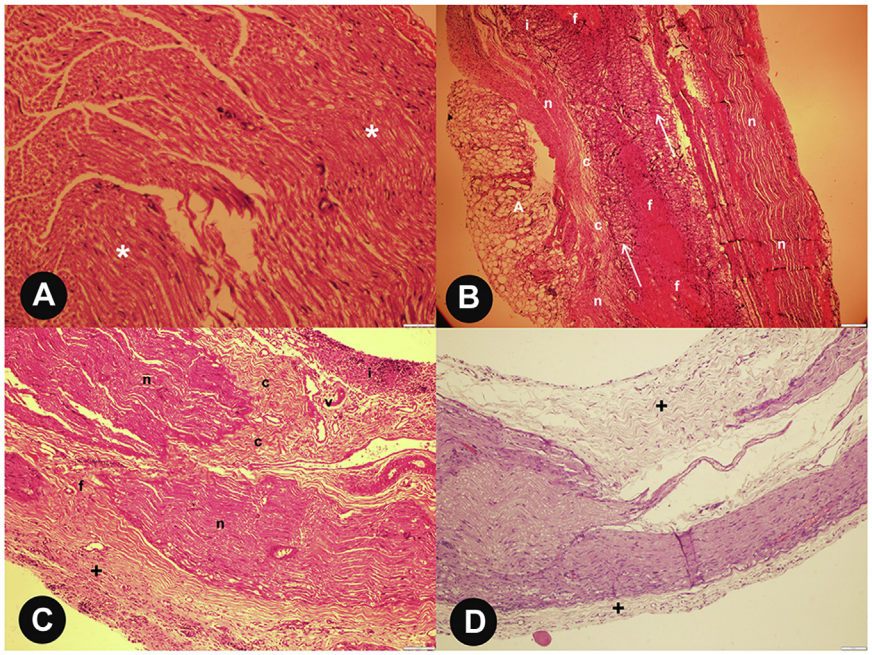


Figure 3. In pathological sections (**A**) nerve fibers are observed regularly. (H&E staining; *: uniform and regular nerve sheets) (**B**) In the trauma group, nerve continuity is disrupted, and the nerve fibers exhibit an irregular structure. Adipose tissue infiltration, fibrosis, and clusters of inflammatory cells are observed between the nerve bundles. Additionally, fibrin deposition, hemorrhage, inflammation, and adipose tissue are present in this area. Collagen fiber formation was detected within the fibrotic tissue. (H&E staining; white arrow: adipose tissue infiltration, F: fibrosis, C: collagen, I: inflammatory cells, A: adipose tissue, n: nerve tissue). (**C**) In the MP group, nerve tissue integrity was preserved, and the nerve fibers appeared more regular compared to those in the trauma group. Furthermore, inflammation was reduced in the MP group relative to the trauma group. A greater amount of collagen fibers and fibrotic tissue was observed between the nerve bundles in the MP group compared to the halofuginone group. (H&E staining; +: capsule, n: nerve tissue, F: fibrosis, C: collagen, v: vascular proliferation, I: inflammation). (**D**) In the halofuginone group, inflammation is minimal in the trauma area and there is no increase in connective tissue and no granulation tissue formation. (H&E staining; +: capsule). MP, methylprednisolone.

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leading to the suppression of epithelial-mesenchymal transition and antifibrotic effects. 2) Halofuginone also inhibits prolyl-tRNA synthetase activity during the blood stage of malaria. Additionally, it suppresses T-helper 17 cell differentiation, thereby reducing inflammation and autoimmune responses. This occurs through the activation of amino acid starvation pathways and the integrated stress response.[8] The anti-inflammatory properties of halofuginone, combined with its potent inhibitory effects on fibrosis, suggest it could be an effective therapeutic option for peripheral nerve injury.

MP is a well-established anti-inflammatory and antioxidant agent, known for its role in mitigating secondary damage in central nervous system injuries. It works by reducing free radical production, lowering malondialdehyde levels, and protecting enzymes like glutathione peroxidase and superoxide dismutase,

Table 2. Morphometric Parameters of the Nerve in the Test and Control Groups; D-Nerve Diameter, D-Myelin Diameter, D-Axon Diameter, G-Ratio - Ratio of Axon Diameter to Fiber Diameter

	Sham	Control	Methylprednisolone	Halofuginone
D-Nerve	22,97 ± 5,06 μm	6,44 ± 1,79 μm	15,76 ± 2,63 μm	16,55 ± 2,57 μm
D-Myelin	7,67 ± 2,96 μm	1,9 ± 0,75 μm	4,17 ± 1,28 μm	5,17 ± 0,86 μm
D-Axon	15,3 ± 2,58 μm	4,54 ± 1,39 μm	11,59 ± 2,45 μm	11,38 ± 1,86 μm
G-Ratio	0,68 ± 0,07	0,71 ± 0,08	0,73 ± 0,08	0,69 ± 0,02

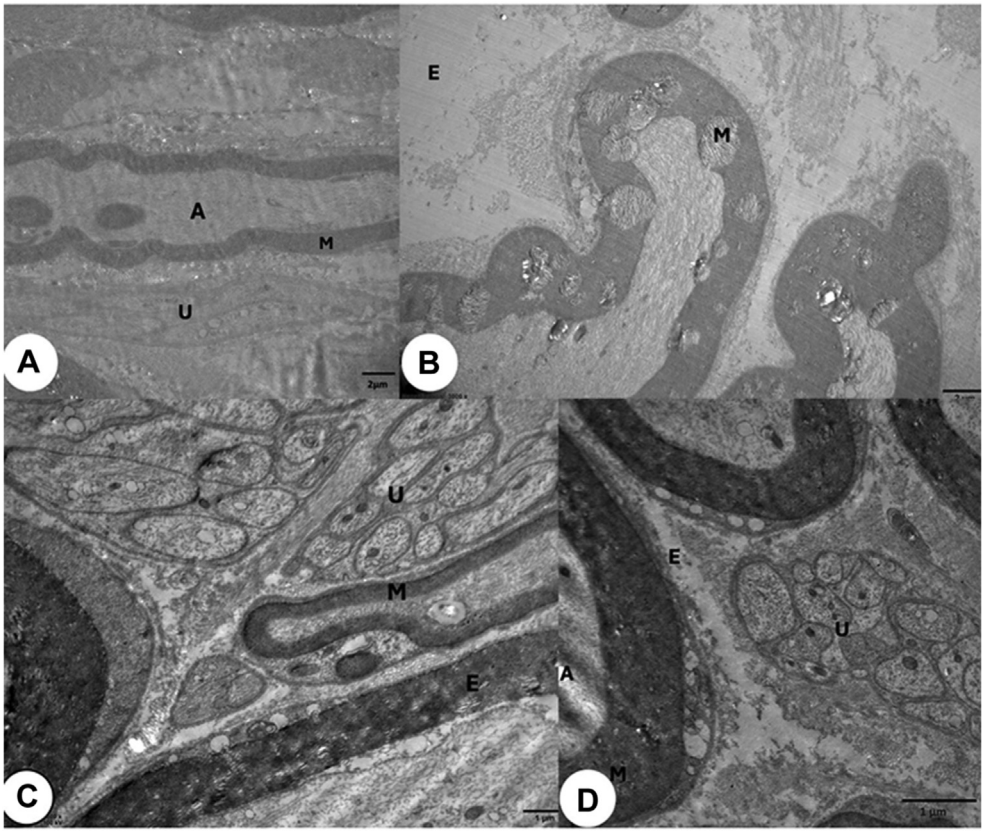


Figure 4. (**A**) Sham group demonstrated intact myelinated (**A**) and unmyelinated (U) axons. Myelin layer (M) is continuous and intact. (**B**) Trauma group demonstrated edematous areas within the myelin (M) and between the axons (**E**). (**C**) MP group shows some intermediate axons with intact myelin (M) and some larger axons with mild edema (**E**). Unmyelinated axons (U) are intact and there are no edematous areas between the axons. (**D**) Halofuginone group shows intact myelinated axons (M) and unmyelinated axons (U). No edema is observed in the axons (**A**) but some interaxonal edematous areas (**E**) are present. MP, methylprednisolone.

thereby supporting neurological recovery. Given its clinical use in peripheral nerve injury, MP was included in our study for comparison with halofuginone. After sciatic nerve injury, the halofuginone group showed better outcomes than the trauma group and the MP group in terms of functional evaluation, pathological measurements, and electron microscopic morphometric analysis. However, there was no statistically significant difference between the MP and halofuginone. Pathological sections revealed that

Table 3. Mean Injury Grading Scores in Samples (Values are Presented as the Mean ± SD of the Mean)

Score	Sham	Control	Methylprednisolone	Halofuginone	
Axonal myelin score	0.04 ± 0.2	1.9 ± 0.46	0.54 ± 0.5	0.22 ± 0.42	*,§
General axonal score	0.04 ± 0.2	0.44 ± 0.5	0.18 ± 0.39	0.24 ± 0.43	*,‡
Intracytoplasmic edema	0.02 ± 0.14	0.98 ± 0.51	0.34 ± 0.48	0.66 ± 0.59	*,§
Nucleus	0 ± 0	0.2 ± 0.42	0 ± 0	0 ± 0	†
Vascular endothelium	0 ± 0	0.2 ± 0.42	0 ± 0	0 ± 0	†

*Significant difference between all groups ($P < 0.05$).
†No significant difference between all groups ($P > 0.05$).
‡No significant difference between methylprednisolone and halofuginone groups ($P > 0.05$).
§Significant difference between methylprednisolone and halofuginone groups ($P < 0.05$).

halofuginone reduced inflammation and fibrotic tissue formation in the injured nerve section, similar to the effects observed with MP. However, a clear difference has been observed between them in terms of fibrosis and collagen fiber formation. MP treatment affected the irregular fibrotic tissue present in the trauma group, promoting more regular collagen fiber formation. In contrast, halofuginone inhibited both fibrosis and collagen formation. Subsequent electron microscopic analysis also demonstrated that halofuginone exhibited neuroprotective effects distinct from those of MP at the cellular level. Specifically, halofuginone preserved the structure of both myelinated and unmyelinated axons (interstitial edema) but was unable to prevent edema in the interaxonal region (vasogenic edema). In contrast, MP failed to protect myelinated axons but showed protective effects on unmyelinated axons and the interaxonal region. These differences in therapeutic efficacy may stem from the varying anti-inflammatory mechanisms and potencies of the two agents.

Sciatic nerve injury causes functional impairments characterized by a decrease in the SFI in rats, which has been previously demonstrated.[19] Our research has shown that both MP and halofuginone effectively reduce sciatic nerve injury and promote nerve regeneration, with statistically significant improvement in SFI during behavioral tests. In addition, another result proving this regeneration is that our statistically significant gastrocnemius muscle ratio results in the experimental group are compatible with the SFI results. In our study, the trauma group showed a significant decrease in SFI at 2 and 4 weeks after surgery compared to the sham group. Although it was four weeks after surgery, trauma group's SFI could not reach normal levels but showed a partial recovery. This suggests axonotmesis, as supported by the pathology and electron microscopy results. In contrast, the MP-treated groups showed significant improvements in SFI four weeks after surgery compared to the trauma group, consistent with previous findings reported by Yüce et al. in their study on MP groups.[4] Although the trauma group retained the ability to walk, they exhibited an unsteady, rolling gait throughout the follow-up period. In contrast, the halofuginone-treated group showed a stable gait after four weeks, similar to preinjury conditions. The halofuginone group showed results similar to the MP group's and there was no statistical difference between them.

It has been previously reported in the literature that electron microscope scoring of spinal cord injury is a useful method in pharmacological studies.[26,27] Thus, ultrastructural evaluation not only provides ultrastructural information about the lesion site but also allows quantitative comparison between control and trauma groups. Furthermore, comparison of drug effects on different subcellular organelles was also possible.[27] This study also provides ultrastructural information about the protective effect of halofuginone on traumatized peripheral nerves. We found that halofuginone protected neurons, myelin, axons, and subcellular organelles such as the nucleus and mitochondria. It also reduced cytoplasmic edema. Evaluation according to the grading system (Table 1) showed the neuroprotective effects of halofuginone. Compared with MP, halofuginone provided much more neuroprotection. Its effect on peripheral nerve axons, myelin, and interaxonal edema was found particularly prominent. In cases where treatment is difficult, such as its potential application to clinical conditions like brachial plexus injury, halofuginone might be more effective for specific nerve sizes.

Hematoxylin, eosin, and electron microscopy sections of sciatic nerves from the trauma group showed significant nerve tissue degeneration, including Wallerian degeneration and Schwann cell hyperplasia as prominent features. There were also edematous areas within the myelin and between axons. These degenerative changes were ameliorated by both halofuginone and MP treatment, consistent with previously published reports, resulting in less inflammation and fewer fibrotic areas.[24] The improvement observed in the halofuginone-treated group may be attributed to halofuginone's ability to normalize the myelination process in peripheral nerves by inhibiting fibrosis and granulation tissue formation. Halofuginone treatment may accelerate axonal regrowth and sprouting, nerve reconstruction and neural plasticity after peripheral nerve injury. This was statistically demonstrated in our study with both pathological and electron microscopic findings and additional qualitative measurement results. The missing parts of our study are the lack of biochemical and immunohistochemical investigations. The mechanism of action can be examined in detail in new studies with larger subjects and different dosages.

CONCLUSION

The present study showed that both MP and halofuginone caused healing in sciatic nerve injury in rats. Both MP and halofuginone showed anti-inflammatory and nerve proliferative activities suggesting neuroprotective effects. Our study contributes to the research on the possible use of MP and halofuginone in the treatment of sciatic nerve injury and may lead to further studies for detailed evaluation of possible underlying mechanisms.

CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Ömer Şahin: Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing — original draft. **Tuncer Taşcıoğlu:** Conceptualization, Investigation, Methodology, Visualization, Writing — review & editing. **Ayşegül Fırat:** Formal analysis, Investigation, Project administration, Resources, Software, Supervision, Writing — original draft, Writing — review & editing. **H. Selçuk Sürücü:** Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing — original draft, Writing — review & editing. **Muzaffer Çaydere:** Investigation, Methodology, Project administration, Supervision, Validation, Writing — original draft, Writing — review & editing. **Duran Berker Cemil:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing — original draft, Writing — review & editing.

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