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Does halofuginone influence regeneration after peripheral nerve injury?

Muzaffer Çaydere[1*] and Ömer Şahin[2]

Abstract

Background Halofuginone is an antiprotozoal drug with antifibrotic and anti-inflammatory properties. The aim of our study was to determine the effects of halofuginone on nerve recovery in sciatic nerve injury and compare it with steroid treatment.

Methods The left sciatic nerves of Sham subjects were exposed without intervention. The nerves of trauma animals were transected and sutured. In the methylprednisolone group and in the trauma group, after nerve transection and repair, 1 mg/kg methylprednisolone per day was administered intraperitoneally for seven days; in the halofuginone group and in the trauma group, after nerve transection and repair, 0.2 mg/kg halofuginone per day was administered orally by gavage for seven days. The rats were functionally evaluated at 4 and 8 weeks through walking path analysis. Pathological-morphometric, immunostaining-quantitative, and muscle weight measurements were performed at 8 weeks.

Results Compared with the trauma group, the methylprednisolone and the halofuginone groups had better functional outcomes ($p < 0.001$). Statistically significant difference was found in comparisons of the pathological and immunostaining results of the methylprednisolone and halofuginone groups (Respectively, nerve diameter ($p = 0.007$) and edema ($P = 0.009$)).

Conclusion Halofuginone positively contributed to recovery after sciatic nerve injury.

Clinical trial number Not applicable.

Introduction

The superficial layers of the human anatomy contain an extensive network of peripheral nerve fibers, rendering them vulnerable to injury from excessive stretching, tearing, and compression [1]. After an incident of peripheral nerve injury (PNI), the distal segment of the injury site sustains Wallerian degeneration, while the proximal segment undergoes degenerative changes [2]. PNI also initiates considerable degenerative alterations in Schwann cells and myelinated nerve fibers [3]. This injury disrupts the communication pathways between the brain or spinal cord and the target cells or tissues. Despite the peripheral nervous system's notable capacity for regeneration, up to 33% of PNIs result in motor and sensory impairments, persistent pain, and muscle wasting, and they can contribute to chronic conditions and permanent disability [4]. Inflammation following peripheral nerve injury triggers fibroblast activity, leading to perineural fibrosis, which inhibits axonal regeneration and can cause neuroma formation lasting 6–12 months. Since Seddon's classification, the mechanisms of axonal

*Correspondence:
Muzaffer Çaydere
muzaffercaydere@gmail.com
[1]Department of Pathology, University of Health Sciences, Ankara Training and Research Hospital, Ankara, Turkey
[2]Department of Neurosurgery, Bestepe State Hospital, Ankara 06560, Turkey

regeneration have been partially understood [5].Surgical techniques like end-to-end and end-to-side anastomosis, as well as nerve grafts, are used for complete injuries. These methods are supported by neurotrophic, neuroprotective, anti-inflammatory, and antifibrotic agents [6]. Some studies have explored unconventional approaches, such as X-rays, amniotic membrane, and glioblastoma cyst contents, to improve recovery [5–7].

Halofuginone (HF), a synthetic derivative of a small quinazolinone alkaloid extracted from Dichroafebrifuga, is commonly employed as a coccidiostat for chickens and turkeys [8]. Recent studies have demonstrated HF's involvement in inflammatory and autoimmune conditions through its selective inhibition of the cluster of differentiation four positive T helper cell subset T helper 17 [9]. Furthermore, research indicates that HF can promote the resolution of existing fibrosis, potentially by decreasing collagen I production and enhancing collagenase activity [10]. To date, the literature has not explored HF's neuroprotective effects on peripheral nerves. Given its anti-inflammatory and antifibrotic properties, we propose that HF may serve as an effective agent for enhancing PNI recovery.

Our research aimed to examine how HF affects nerve regeneration in rats with sciatic nerve injuries. We also sought to compare its efficacy to that of steroids, which are currently the standard clinical treatment for such conditions.

Materials and methods

Surgical procedure

This study utilized 40 female Wistar rats weighing 170–200 g. The Local Animal Ethics Committee approved the study and monitored the research protocol. Anesthesia was administered using a mixture of 95 mg/kg ketamine hydrochloride and 6 mg/kg xylazine. A single surgeon performed all operations. The left sciatic nerve was targeted in the study, and evaluations were conducted by independent pathologists and histologists.

The sciatic nerve was exposed from the sciatic notch to bifurcation through a transverse gluteal incision and muscle separation. The rats were then allocated into four groups:

1. Sham group ($n = 10$): The nerve was isolated without additional surgery.
2. Trauma group ($n = 10$): The nerve underwent primary epineural repair after transection, using four 8 – 0 Ethilon sutures.
3. Methylprednisolone (MP) group ($n = 10$): Following nerve transection and repair identical to that in the trauma group, 1 mg/kg/day MP as administered intraperitoneally for seven days post-procedure.
4. HF group ($n = 10$): Following nerve transection and repair identical to that in the trauma group, 0.2 mg/kg/day HF was given orally via gavage for seven days post-procedure [11].

All animals were housed individually. Standard laboratory food and water were provided to the rats ad libitum. Rats were euthanized at the end of the experimental time (8 weeks) for pathological assessment.

Walking track analysis

The evaluation of motor function involved an analysis of the unrestricted ambulation pattern, a methodology originally articulated by de Medinacelli et al. [12]. Walking track assessments were performed prior to surgery and at 4 and 8 weeks after the procedure. Three essential metrics were recorded as previously Medinacelli outlined. These metrics were documented for both the experimental and control groups. Subsequently, the sciatic functional index (SFI) was computed for each subject utilizing the formula established by Hare et al. [13]. The SFI serves to quantify impairment as a percentage, with – 100% denoting a complete injury to the sciatic nerve and values ranging from – 10% to + 10% indicating normal function.

Muscle mass

The assessment of recovery considered the weight ratio of the gastrocnemius muscles. After euthanasia, careful extraction of the gastrocnemius muscles from both the affected and unaffected sides was performed using an operating microscope. Once adequately moistened, the excised muscles were weighed utilizing an electronic balance. To determine the weight ratio (expressed as a percentage), the mass of the muscle from the side that had sustained a nerve injury was divided by the mass of the muscle from the healthy side. All measurements were conducted by two independent examiners who were unaware of the assigned groups.

Pathological examination

Following the euthanasia procedure, a 1.5-cm segment of the sciatic nerve—particularly around the sutured area—was obtained from the rats. The collected samples were fixed in a 10% formalin solution; after that, routine follow-up procedures were performed on the LEICA ASP 300 S device. Subsequently, paraffin blocks were made, from which 4-micron-thick sections were taken using the LEICA RM 2255 microtome. The sections were then stained with hematoxylin and eosin, Masson trichrome, and reticulin; additionally, anti-S100 (4C4.9) and anti-Neurofilament (NF) (2F11) antibodies were processed using the Ventana Roche device (Ventana Roche, USA).

The examination was performed using an OLYMPUS BX51 microscope, and the morphometric measurements

were facilitated by the BABSOFT BS200Pro imaging system. For each analyzed section, five fields were randomly selected at ×100 magnification to count myelinated fibers. Additional evaluations were made regarding axon diameters, nerve diameters, myelin thickness, and G ratios (which represent the ratio of axon diameter to fiber diameter).

The immunostaining grading system used for quantitative assessment was based on principles similar to those previously reported by Bostan et al. [14] in the evaluation of sciatic nerve (Table 1). For each group, myelin degeneration, axon degeneration, epineurium degeneration, fibrosis, epineurium thickening, perineurium thickening, lymphocytic infiltration, vacuolization, and edema were assessed, counted, and presented as median scores (25th and 75th percentiles) [14]. The examiner who evaluated the sections was unaware of the group information of the samples.

Table 1 Grading system for quantitative evaluation of pathological and immunostaining

	Pathological Changes Level	
Myelin degeneration	None	0
	Mild	1
	Moderate	2
	Severe	3
	Very severe	4
Median (Min-Max) Axon degeneration	None	0
	Mild	1
	Moderate	2
	Severe	3
	Very severe	4
Median (Min-Max) Epineurium degeneration	None	0
	Mild	1
	Moderate	2
	Severe	3
Median (Min-Max) Fibrosis	None	0
	Mild	1
	Moderate	2
	Severe	3
Median (Min-Max) Epineurium thickening	None	0
	Mild	1
	Moderate	2
	Severe	3
Median (Min-Max) Perineurium thickening	None	0
	Mild	1
	Moderate	2
	Severe	3
Median (Min-Max) Lymphocyte infiltration	None	0
	Mild	1
	Moderate	2
	Severe	3
	Very severe	4
Median (Min-Max) Vacuolisation	None	0
	Mild	1
	Moderate	2
	Severe	3
	Very severe	4
Median (Min-Max) Edema	None	0
	Mild	1
	Moderate	2
	Severe	3
	Very severe	4

Statistical analysis

The sample sizes of the groups were 10; thus, median (25th and 75th percentiles) values were reported and non-parametric tests were applied as recommended in literature [15]. For overall group comparisons, the Kruskal-Wallis test was applied, and following detection of significant differences between the groups, pairwise independent subgroup comparisons were conducted by using Mann-Whitney U test. Type 1 error was set 5% and to minimize the of type I error inflation caused by multiple subgroup comparisons, p-values underwent adjustment via the Bonferroni correction method. We used IBM SPPS version 23 for analysis (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp).

Results

Walking track analysis

The SFI values of the groups were evaluated, and statistically significant differences were found between all groups ($p < 0.001$): in group comparisons, the median SFI values of the HF group showed statistically better results than the MP and trauma groups ($p < 0.001$) in the 4th week and in the 8th week (Fig. 1).

Muscle weight measurement

The median gastrocnemius muscle weight ratios were evaluated (Fig. 2). In double and triple comparisons, HF group showed statistically better results than the MP and trauma groups (respectively $p = 0.002$ and $p < 0.001$).

Pathological examination

When the sections of the Sham group were evaluated with a light microscope, it was observed that the endoneurium, perineurium, and epineurium were normal and that the fibers in the fascicles were homogeneously and regularly distributed (Fig. 3). In the trauma group, the nerve fibers were thinner and more irregularly distributed, and some fibers did not have continuity. Hemorrhage, fibrosis, inflammatory cells, foreign body giant cells, increased collagen tissue, and vacuolization and neovascularization between the nerve fibers were observed in the trauma area (Fig. 4a, b,c). With reticulin staining, it was observed that the reticular roof in the

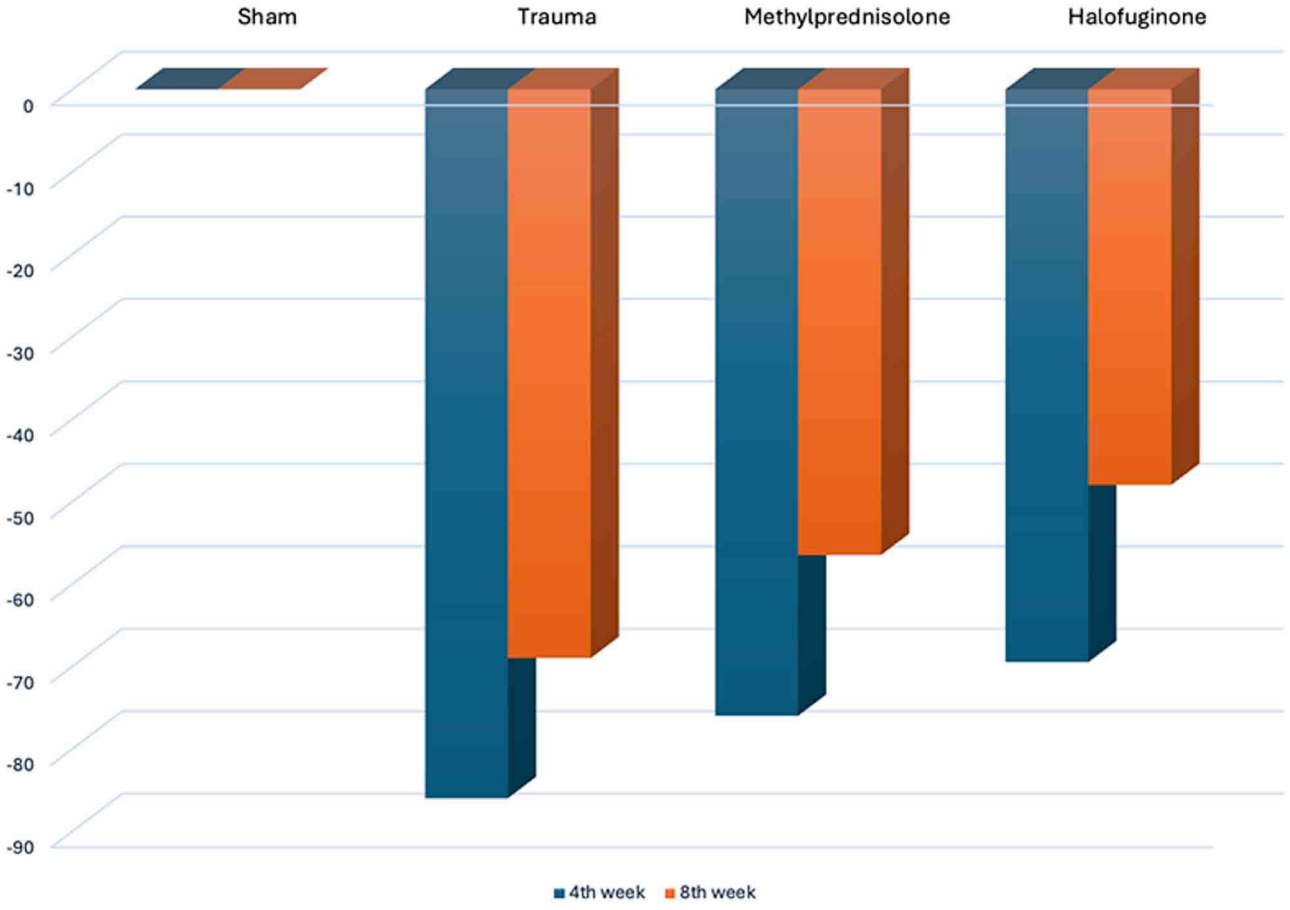

Fig. 1 The SFI values for the fourth weeks and eighth weeks in all groups

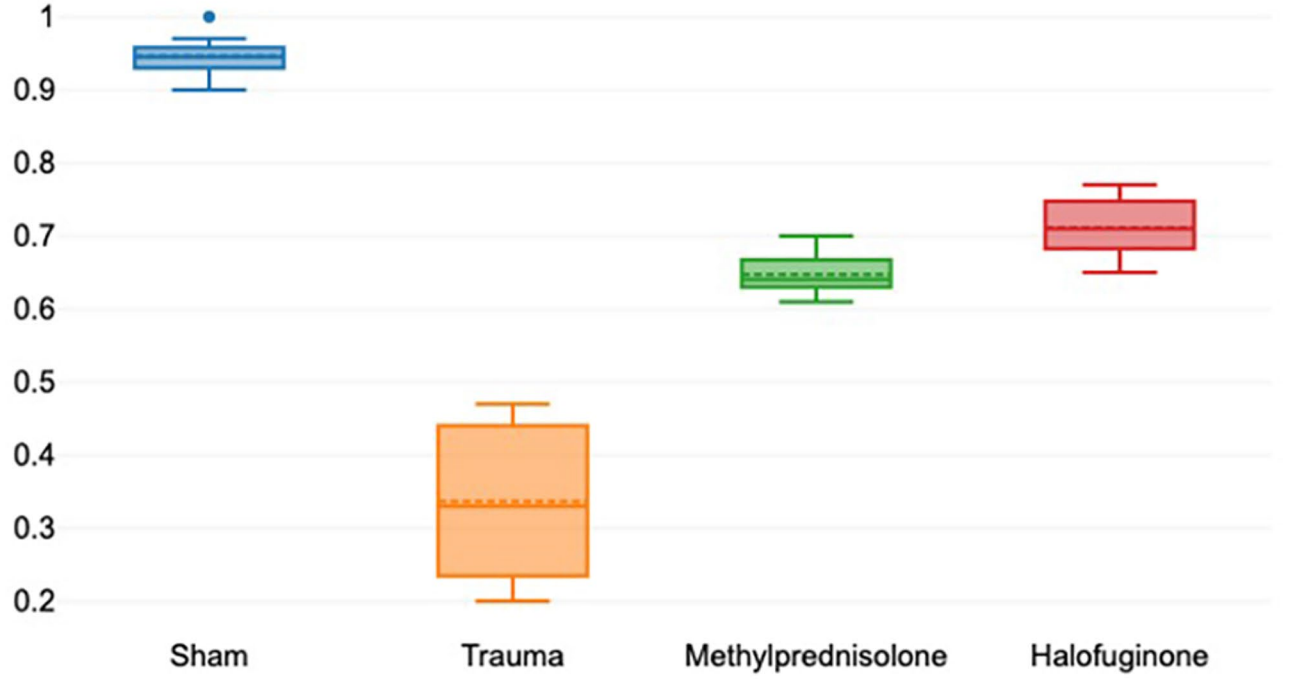

Fig. 2 Box-plot demonstrating median gastrocnemius muscle weight ratios of all groups

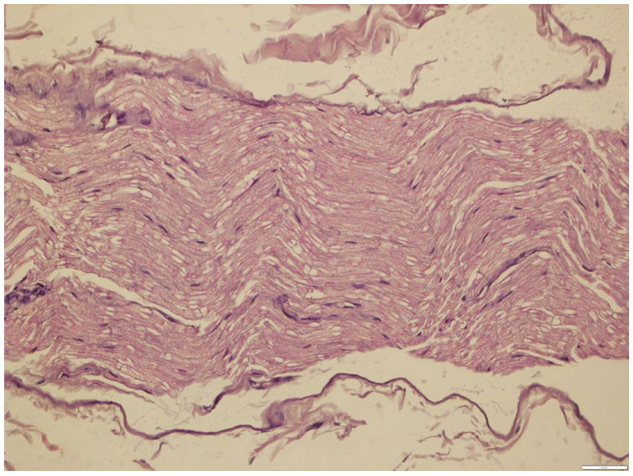

Fig. 3 Sham group: hematoxilen-eosine staining section demonstrate fibers in the fascicles were homogeneously and regularly distributed

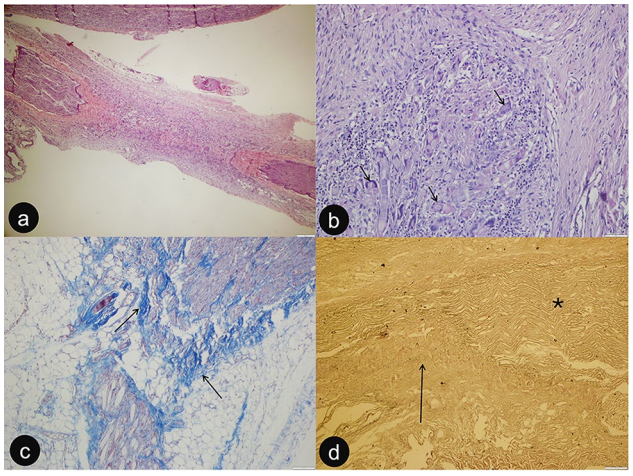

Fig. 4 Trauma group: **a**) Hematoxilen-eosine staining section demonstrates the nerve fibers were thinner and more irregularly distributed. Also, some fibers did not have continuity. **b**) A high magnification region of Hematoxilen-eosine staining section shows inflammatory cells, neovascularization and foreign body giant cells (*Black arrow*). **c**) Masson trichrome staining section demonstrates increased collagen tissue around the nerve fibers (*black arrow*). **d**) Reticulin staining shows irregular and scattered reticular network in the nerve tissue (*black arrow*). Asterix shows regular reticular network

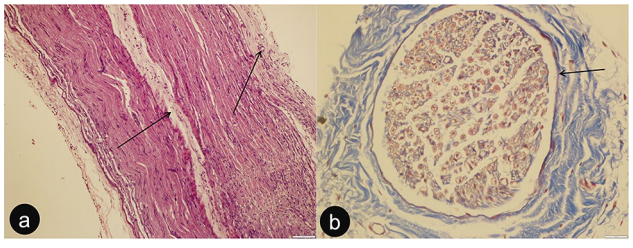

Fig. 5 Methylprednisolone group: **a**) Hematoxilen-eosine staining section demonstrates less inflammation area than trauma group. Black arrow point outs inflammation and neovascularisation. **b**) Masson trichrome staining section shows less visible collagen fibers in the nerve tissue. Black arrow point outs thickening of the perineurium

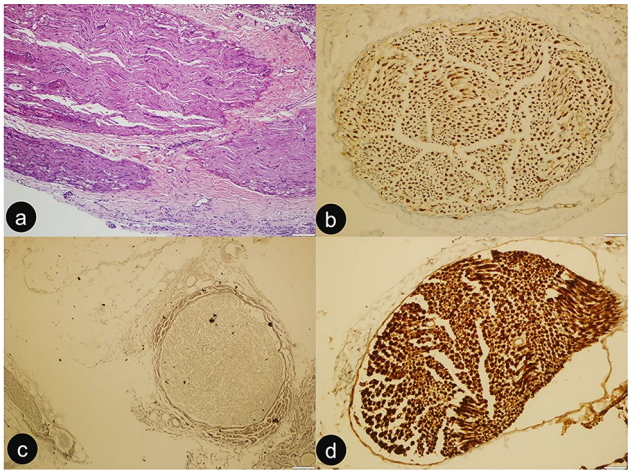

Fig. 6 Halofuginone group: **a**) Hematoxilen-eosine staining section demonstrates minimal irregularity and thinning of the nerve fibers in the trauma area. **b**) Neurofilament antibodies staining section shows nerve fibers are uniform. **c**) Reticulin staining shows regular reticular network in the nerve tissue. **d**) s100 antibodies staining section shows regular schwann cells in the nerve tissue

nerve tissue was irregular and scattered (Fig. 4d). Irregularity in the nerve fibers was noted with S100 and NF staining. In the MP group, less inflammation and fibrosis were observed; in addition, there was no neovascularization, and the increase in collagen fibers was less (Fig. 5a, b). The reticular roof was preserved. With S100 and NF staining, it was observed that the nerve fibers and Schwann cells were more regular compared to the trauma group. Finally, in the HF group, there was minimal irregularity and thinning of the nerve fibers in the trauma area (Fig. 6a). No fibrosis, inflammation, or collagen increase was observed, and the reticular roof was preserved. With S100 and NF staining, it was observed that the nerve fibers showed significant improvement compared to the MP group (Fig. 6b, c,d).

Morphological measurements of the nerve diameter, myelin thickness, axon diameter, and G ratios are given in Fig. 7. Comparisons revealed statistically significant differences between all groups ($p < 0.001$)exceptG ratio value ($p = 0.617$); if the Sham group was excluded from the evaluation to determine which group was different from the others, a significant difference was observed between the drug treatment groups and the trauma group ($p < 0.001$). In a comparison of the MP and HF groups, significant difference was observed between the two (D-Nerve $p = 0.007$; D-Axon $p = 0.012$; D-Myelin $p = 0.015$; G-Ratio $p = 0.684$).

In comparisons of the myelin degeneration, axon degeneration, epineurium degeneration, fibrosis, epineurium thickening, perineurium thickening, lymphocytic infiltration, vacuolization, and edema grades obtained from immunostaining sections (Table 2), statistically significant difference was found between the MP and HF

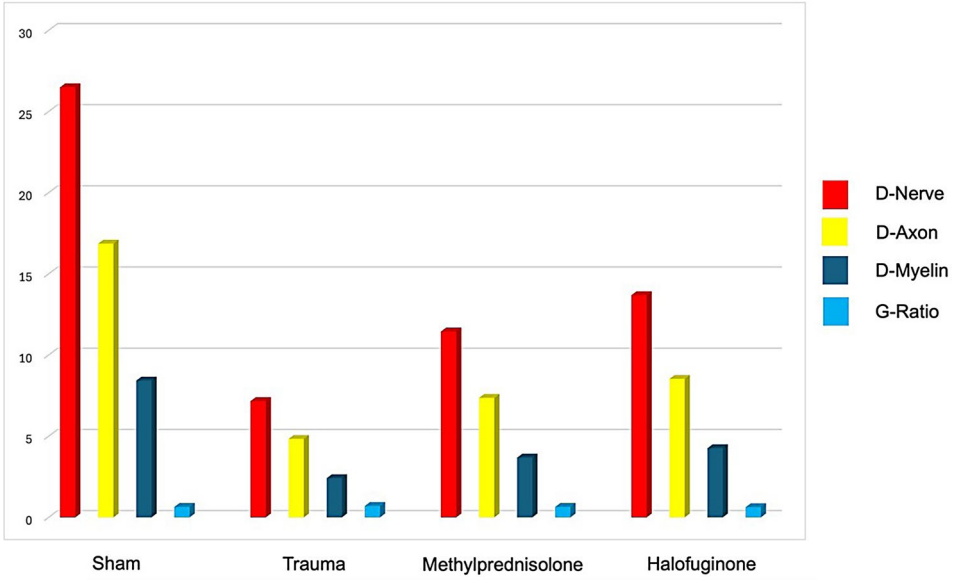

Fig. 7 Morphometric parameters all groups; D-Nerve diameter, D-Myelin diameter, D-Axon diameter, G-Ratio - ratio of axon diameter to fiber diameter

Table 2 Pathological and immunostaining scores in samples (Values are presented as the median scores (25th and 75th percentiles))

Score	sham	trauma	methylprednisolone	halofuginone	
Myelin degeneration	0 (0–0)	3 (3-3.75)	2(1–2)	1(1–1)	†($p<0.001$) ‡ ($P=0.029$)
Axon degeneration	0 (0–0)	3 (2.25-4)	2(1.25–2.75)	1(1–1)	†($p<0.001$) ‡($P=0.012$)
Epineurium degeneration	0 (0–0)	3 (2.25-3)	2(2–2)	1(1–1)	†($p<0.001$) ‡($P=0.023$)
Fibrosis	0 (0–0)	3 (3–3)	2(2–2)	1(1–1)	†($p<0.001$) ‡($P=0.029$)
Epineurium thickening	0 (0-0.75)	3 (3–3)	2(1.25-2)	1(0.25-1)	†($p<0.001$) ‡($P=0.019$)
Perineurium thickening	0 (0–0)	3 (3–3)	2(2–2)	1(0.25-1)	†($p<0.001$) ‡($P=0.002$)
Lymphocyte infiltration	0 (0-0.75)	2.5 (2–3)	2(1.25-2)	1(0–1)	†($p<0.001$) ‡($P=0.001$)
Vacuolisation	1(1–1)	4(3–4)	3 (2–3)	1.5 (0.25-2)	†($p<0.001$) ‡($P=0.002$)
Edema	0 (0–0)	3 (2.25-3)	2(1.25-2)	1(1–1)	†($p<0.001$) ‡($P=0.009$)

Note

† Significant difference between all groups

‡ Significant difference between methylprednisolone and halofuginone groups

groups (p values are in the table). In the triple and quadruple comparisons of these two groups with the other groups, statistically significant differences were found between all groups ($p<0.001$).

Discussion

PNI result in disrupted mechanical transmission and microcirculation. Subsequent reperfusion causes an accumulation of oxygen and nutrients, leading to free radical formation, which in turn trigger lipid peroxidation in tissues, causing destructive effects [16]. The combined impact of ischemic and mechanical factors is greater than their individual effects [17]. While the peripheral nervous system possesses remarkable regenerative capabilities, managing nerve injuries remains challenging, and the effectiveness of treatments largely depends on the injury's location, severity, and classification. For complete nerve transections, various surgical techniques and pharmacological interventions have been proposed to enhance functional recovery; natural tubulization options include vascular grafts [18], local administration of brain derived neurotrophic factor with

silicone conduit [19], local administration of laminin and fibronectin with chitosan conduit [20], denatured muscle tissue [21], and fascial or synovial membranes [22]. However, almost none of these have successfully made their way into clinical use. PNI remains a significant unresolved challenge, profoundly affecting patients' daily lives and carrying substantial economic implications for society [7]. Non-steroidal anti-inflammatory drugs are currently used to treat chronic nerve injuries, but they often come with serious side effects. Consequently, there is a pressing need for alternative therapies that can promote nerve repair while minimizing adverse reactions [23].

PNI significantly impacts Schwann cells in the affected region, which play a critical role in regulating axonal sprouting and facilitating nerve regeneration. The success of peripheral nerve regeneration largely depends on maintaining a balance between Schwann cell proliferation and scar tissue formation [19]. Various cellular components, including inflammatory cells, Schwann cells, and neurotrophic factors, work synergistically to support axon growth and myelination [20]. Research has shown that minimizing scar formation can significantly enhance axonal regeneration, and topical application of agents such as melatonin, hyaluronic acid, tacrolimus, and MP has been found to promote healing by inhibiting fibroblast growth and proliferation, thereby reducing scar tissue formation in the injured area [24]. This fibrosis at the repair site may lead to neuromas and perineural fibrosis, which hinder axonal regeneration by causing traction, ischemia, and tissue adherence [6]. Such complications can result in Wallerian degeneration, muscle atrophy, and permanent nerve damage. Despite extensive research, achieving full functional recovery and seamless nerve repair remains a significant challenge [5, 6].

HF exhibits multiple interconnected beneficial biological effects. It has received approval as an antiprotozoal agent for use in poultry and ruminant animals [25]. In addition, as an analog of febrifugine, HF has demonstrated efficacy in experimental malaria treatment [26]. It functions as a powerful inhibitor of angiogenesis in various tumors, and tumor angiogenesis accompanied by suppression of fibroblast-to-myofibroblast transition and reduction of tumor extracellular matrix [27, 28]. Additionally, HF contributes to anti-fibrosis, inflammation reduction, and autoimmune regulation [29, 30]. The literature offers two models that elucidate these effects. First, by inhibiting Smad3 phosphorylation, HF impacts transforming growth factor beta mediated extracellular matrix induction and genes for collagen, plasminogen activator inhibitor-1, and tissue inhibitor of metalloprotease-1, resulting in Epithelial–mesenchymal transition-inhibition and anti-fibrotic action. Second, HF inhibits prolyl-transfer ribonucleic acid synthetase activity during the blood stage of malaria and impedes Th17 cell differentiation, thereby reducing inflammation and autoimmune responses through activation of amino acid starvation and integrated stress responses [31].

MP is widely recognized as an effective anti-inflammatory and antioxidant agent, particularly in the context of secondary damage following injuries to the central nervous system. Numerous studies have demonstrated its therapeutic properties—particularly its ability to inhibit the production of free radicals generated by trauma, thereby contributing to the enhancement of neurological function. Biochemically, MP reduces levels of malondialdehyde and protects critical enzymes, such as glutathione peroxidase and superoxide dismutase, from inhibition in affected tissues [32]. Following sciatic nerve injury, the Halo group demonstrated superior outcomes compared to both the trauma group and the MP group across pathological measurements, immune-chemical analyses, and functional evaluations. Pathological examinations indicated that HF group reduced inflammation and fibrotic tissue formation in the injured nerve section, as to be MP group. However, immunostaining analysis showed that, notable differences were also observed between the HF and MP groups in terms of Schwann cell regularity, nerve fiber uniformity, reticular network organization, fibrosis, and collagen fiber formation. While MP treatment influenced myelin-axon-epineurium degeneration, fibrosis, epineurium-perineurium thickening, lymphocytic infiltration, vacuolization, and edema after the injury; HF group showed statistically better results than MP group. Immune-chemical analysis further highlighted that HF exhibited neuroprotective effects structurally distinct from those of MP, particularly in preserving the reticular network and promoting regular Schwann cell development, which contributed to the uniform growth of nerve fibers.

Our findings demonstrate that HF effectively reduces sciatic nerve injury and enhances nerve regeneration, as evidenced by statistically significant improvements in the SFI during behavioral tests. Additionally, the gastrocnemius muscle ratio results, which align with the SFI outcomes, further support this regenerative effect. In our study, the trauma group exhibited the poorest SFI results at both 2 and 4 weeks' post-injury. Even four weeks after surgery, the trauma group's SFI did not return to normal levels, indicating only partial recovery and limited nerve regeneration, as corroborated by pathological and immune-chemical analyses. In contrast, the MP-treated group showed significant SFI improvements four weeks' post-surgery compared to the trauma group, consistent with previous findings by Yüce et al. [33]. in their research on MP. However, the HF-treated group displayed a stable gait after four weeks, resembling pre-injury conditions, and outperformed the MP group, with a statistically significant difference between the two. These results

underscore HF's potential as a superior therapeutic agent for nerve regeneration compared to MP.

Morphological analysis of Hematoxylin-eosin stained sciatic nerve sections from the trauma group revealed the Wallerian degeneration process in the nerve tissue. Both HF and MP treatments showed therapeutic effects by effectively reducing these degenerative changes. This improvement was evident by decreased inflammation and reduced fibrotic areas, consistent with the findings from previous studies [34].

Although our study indicated the neuroprotective effect of HF in peripheral nerve damages, findings regarding the molecular mechanisms resulting in the neuroprotective effect are still lacking. We have not given the electrophysiological, biochemical and electron microscopic evidence for neuroprotective action of HF. This may be considered as a limitation to our study.

Conclusion

The functional advantages (SFI, muscle mass) of HF suggest a potential benefit to recovery after sciatic nerve injury but these findings require further validation. In addition, no adverse effects were noted in the study's scope. Thus, we believe that it can be used to support the treatment of nerve injuries.

Abbreviations

PNI	Peripheral nerve injury
HF	Halofuginone
MP	methylprednisolone
SFI	sciatic functional index

Author contributions

M.Ç and Ö.Ş. wrote the main manuscript text and M.Ç. prepared figürs. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethical approval

The study was conducted with the approval of Ankara Training and Research Hospital Animal Experiments and Local Ethics Committee decision with permission number 773.

Competing interests

The authors declare no competing interests.

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