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ORIGINAL RESEARCH

# Berberine Enhances Neuroregeneration in a Rat Model of Peroneal Nerve Transection Injury: An Animal Study

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**Background:** Berberine has therapeutic potential in central nervous system disorders, however, few studies have investigated the effect of berberine on axonal regeneration in PNS injury models. Thus, this study aims to assess the effects of berberine on axonal regeneration in a peroneal nerve transection rat model.

**Methods:** Sprague-Dawley rats were divided into two groups: group B, berberine (20 mg/kg) intraperitoneal injection after peroneal nerve transection; and group C, normal saline injection as a control. The sciatic nerve functional index (SNFI) was used to assess functional recovery after nerve injury at 2, 4, and 6 weeks post-transection. Electromyography (EMG) was performed to evaluate quantitative neuromuscular function (latency and amplitude) and the regeneration ratio of the injured nerve was evaluated through histological analysis at 6 weeks post-transection. To analyze the effect of various concentrations of berberine on nerve regeneration, Schwann cell viability was analyzed at 0, 0.1, 0.5, 1.0, 5.0 and 10.0 μM of berberine.

**Results:** At 2 and 4 weeks post-transection, SNFI showed no significant difference between groups B and C. However, at 6 weeks post-transection, the SNFI was significantly higher in group B than in group C. On EMG, the latency and amplitude was significantly lower and higher, respectively, in group B than in group C. Histological analysis showed that the regeneration ratio was significantly higher in group B than in group C. Schwann cell viability was highest when 1.0 μM of berberine was administered (136.7±3.5%), and was significantly higher compared to the groups administered with 0.1 μM (114.5±10.6%) and 0.5 μM (118.5±4.8%).

**Conclusion:** Berberine injections have a therapeutic effect on nerve regeneration after peripheral nerve transection. In in vitro studies, a minimum dose of 1.0 μM berberine was required to obtain optimal nerve regeneration. Further in vivo studies are needed to analyze the optimal concentration.

**Keywords:** rat model, peripheral nerve transection injury, berberine, intraperitoneal injection, nerve regeneration

## Introduction

Peripheral nerve injuries are frequent and can cause loss of function and long-term disability.[1] Unlike the central nervous system, it is known that axons in the peripheral nervous system (PNS) can slowly regenerate following injury.[2] However, in the clinical field, it is reported that even after microsurgical nerve repair it is difficult to gain full functional recovery.[3,4] Willand et al reported that it is difficult to return to the original functional capacity for patients with PNS injury, even with advanced surgical methods and rehabilitation programs.[5]

Drug therapy is widely used to improve injured nerve function.[6] Since Shanbhag et al first reported the pharmacological effects of berberine on the central nervous system in 1970,[7] several studies have reported its therapeutic effects in patients with central nervous system disorders.[8,9] In neurodegenerative diseases such as Alzheimer's and Parkinson's

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disease, berberine is known to alleviate symptoms through its antioxidant, anti-neuroinflammatory, and neuroprotective effects. However, few studies have reported the effects of berberine on the PNS or the mechanism of this effect.

Rahmati et al analyzed the effects of berberine on nerve regeneration by inducing sciatic nerve injury in an animal study.[10] Through histological analysis, they reported that using an alginate/chitosan hydrogel containing berberine had a positive effect on injured nerve regeneration. However, their study did not analyze Schwann cells, which play an important role in axonal regeneration after PNS injury. On the other hand, Han et al reported that more Schwann cells were distributed around regenerated axons in a rat group administered berberine than in the control group using immunocytochemical analysis.[11] However, they did not evaluate the functional recovery of regenerated nerves and did not compare Schwann cell viability using a cell counting assay.

Therefore, we evaluated the effects of berberine on nerve regeneration in a rat model of peroneal nerve transection. Moreover, we investigated the optimal concentration of berberine that promotes nerve regeneration in vitro.

# Materials and Methods

## Experimental Design

A total of 44 adult male Sprague-Dawley rats (8 weeks old weighing 200–300g) were obtained from the Orient Bio Institute, Seongnam City, Gyeonggi-do, Republic of Korea. They were bred under controlled conditions (12-h/12-h light/dark cycle, room temperature 20–22°C) with ad libitum access to food and water. All procedures and treatments involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 85–23, revised 1985) and the Animal Care and Use Committee of the Clinical Research Institute. This animal study was approved by the Ethics Committee of Kyung Hee University Hospital in Gangdong (KHNMC AP 2020–016). First, the rats were randomly assigned to two groups. Berberine (20 mg/kg) and normal saline was injected after transection of the peroneal nerve in group B (n=10) and C (n=10), respectively. Moreover, in order to analyze Schwann cell viability according to the concentration of berberine, various concentrations of berberine (0.1 μM, 0.5 μM, 1.0 μM, 5.0 μM and 10.0 μM, n=6 per group) was injected into different groups.

## Peroneal Nerve Transection Model and Berberine Injection

All surgical procedures of peroneal nerve axotomy were performed under a microscope. Under general anesthesia with Zoletil (tiletamine-zolazepam), the skin on the lateral surface of the left thigh was incised, and a section was made directly through the biceps femoris muscle, exposing the sciatic nerve and its three terminal branches: the sural, common peroneal, and tibial nerves. After exposing the peroneal nerve at the peroneal notch, all nerve fibers were cut with a 3 mm gap using iridectomy scissors under a dissection microscope. The artery in the peroneal nerve was maintained for vasa nervorum. Distal and proximal nerve fibers were fixed to nearby soft tissue using nylon to prevent further displacement (Figure 1). Based on a previous study by Han et al, berberine (20 mg/kg) was injected intraperitoneally once daily for one week after transection.[11]

## Sciatic Nerve Functional Index

Sciatic nerve functional index (SNFI) was calculated using the following formula:-38.3 ((EPL-NPL)/NPL) +109.5 ((ETS-NTS)/NTS) +13.3 ((EIT-NIT)/NIT) −8.8.[12] An SNFI value of −100 indicates total crush or transection of the sciatic nerve, while an SNFI value of 0 indicates an intact sciatic nerve. The SNFI was evaluated at 2-, 4-, and 6-weeks post-transection, and the footprints of the hind limbs were obtained for walking track analysis (Figure 2).

## Electromyography

The latency (m/s) and amplitude (mV) were measured using electromyography (EMG) at 6 weeks post-transection. The latency and amplitude of the peroneal nerve were measured and compared between the contralateral and ipsilateral sides. A Sapphire II 4ME electromyograph (Teca Medelec, USA) was used; the intensity ranges for stimulation and the filter were 10–20 mA and 20–2000 hz, respectively.

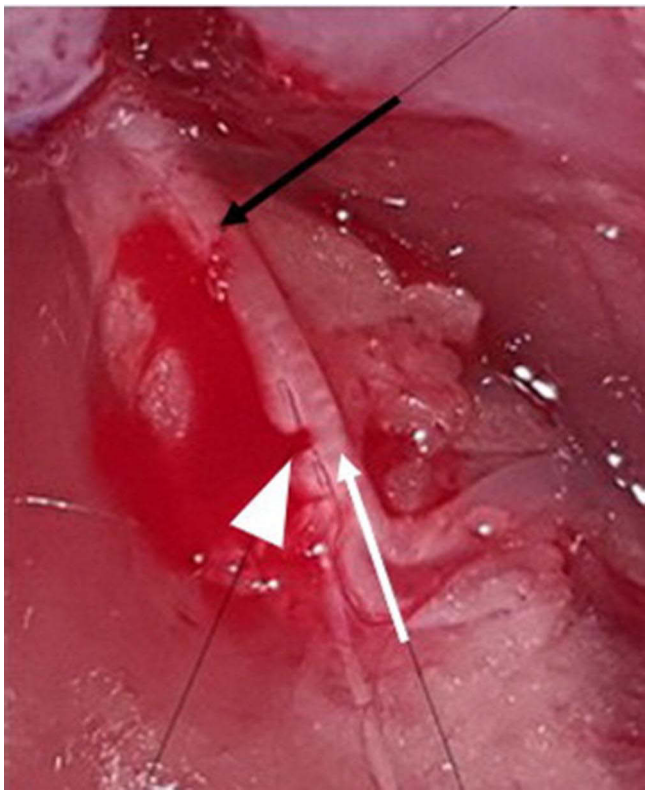

**Figure 1** Peroneal nerve transection model. Sciatic nerve (black arrow), tibial nerve (white arrow) and transected peroneal nerve (white arrowhead) were observed.

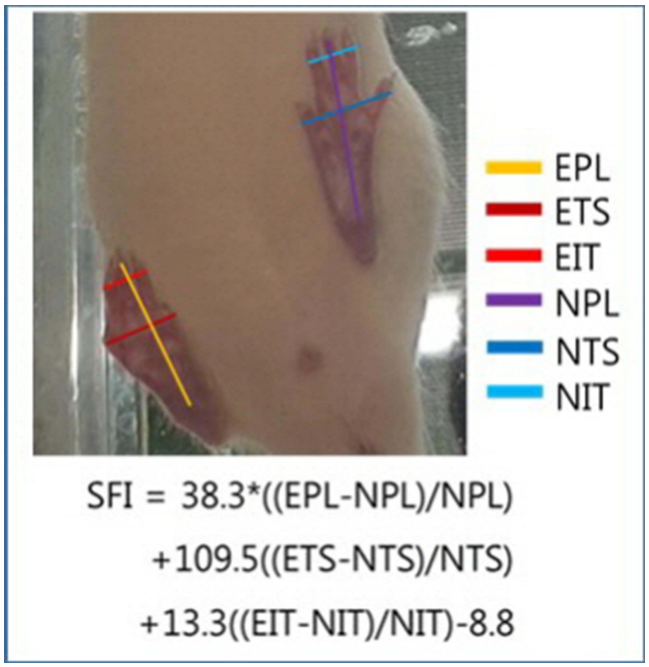


**Figure 2** Sciatic nerve functional index = −38.3 ((EPL-NPL)/NPL) +109.5 ((ETS-NTS)/NTS) +13.3 ((EIT-NIT)/NIT) −8.8. Distance from the heel to the third toe, the print length (PL); distance from the first to the fifth toe, the toe spread (TS); distance from the second to the fourth toe, the intermediary toe (IT). These parameters were measured in both normal (**N**) and experimental (**E**) feet.


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## Histological Analysis

Six weeks post-transection, rats were euthanized with carbon dioxide, and specimens of the transected peroneal nerve were harvested.

### Hematoxylin and Eosin (H&E) Staining

Transverse sectioning of the nerve specimens was performed by H&E staining after fixing with paraformaldehyde, dehydrating with graded ethanol, and embedding in paraffin wax. The paraffin sections were dewaxed with xylene and hydrated with gradient ethanol solutions (100%, 95%, 80%, 70%, 50%). Then, the sections were counterstained in 0.5–1% eosin solution for 10 min, where the cytoplasm was dyed red, and dehydrated twice in 95% ethanol for 1 min each time, and twice in 100% ethanol for 5 min each time. Sections were cleared twice with xylene for 10 min each and sealed with neutral gum. Ten of 100 cross-sections per nerve were photographed at 10×5 magnification (necessary to comprise the entire diameter of the muscle). Five fields per cross-section were obtained under a magnification of 10×40 in the upper left, lower left, upper right, lower right, and central fields of the selected cross-sections of the nerve for quantification using the image analysis software IMAGE J (National Institutes of Health, Bethesda, MA, USA).

### Toluidine Blue Staining

One-μm-thick sections were deparaffinized and immersed in 1% Toluidine Blue (Sigma-Aldrich, USA) for 5 min, and washed twice with double distilled water. Finally, the slides were mounted and observed under a light microscope at 40 x magnification.

In previous studies, peripheral nerve regeneration was evaluated by the length of nerve regeneration per day (mm/day).[13] In this study, in order to analyze the regeneration ratio more quantitatively, the ratio was calculated using the area rather than the length. To evaluate the regeneration ratio, the distance between the two neuroma ends was set as the initial transected distance. Then, based on the length of the transected distance, the area occupied by the tibial nerve and the area occupied by the regenerated peroneal nerve were calculated (Figure 3).

## Cell Viability Counting Assay

Primary rat Schwann cells were isolated from the peroneal nerves of adult Sprague-Dawley rats 3 days after nerve transection. The cells were expanded on collagen (type I from rat tail; Sigma)-coated plates in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma). For purification, the cells were treated with cytosine arabinoside (10 μM; Sigma) twice for 24 h and subjected to immunopanning with an

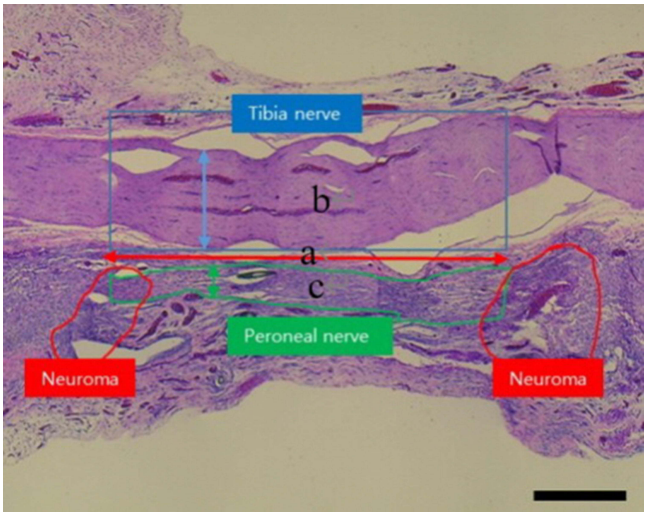


**Figure 3** Calculating the regeneration ratio through histologic analysis of longitudinal sections. The transected nerve's full length is set as the distance between neuromas (double-headed red arrow, (**a**). Using "a" as a reference, tibia and peroneal nerves within the length of "a" were selected. The surface area of selected tibia nerve (**b**) and surface area of selected peroneal nerve (c) were measured, and the ratio was calculated by (c/b x 100).

antibody against THY1.1 (Sigma). Schwann cell cultures was obtained with >95% purity by these procedures. For differentiation experiments, the purified cells were cultured in DMEM supplemented with 10% FBS for 3 days then in serum-free medium for another 2 days before treatment with c-AMP (10 μM; Sigma) The purified rat Schwann cells were fixed with 4% paraformaldehyde, incubated with a primary antibody against S100 β, followed by a secondary antibody conjugated to a green fluorophore, and the cell nuclei were counterstained with DAPI, resulting in green fluorescence indicating the presence of S100 β protein and blue fluorescence for the nuclei, allowing for the visualization of Schwann cell-specific protein expression and the identification of individual cell nuclei.[14]

To evaluate the viability of Schwann cells treated with various concentrations of berberine, the cell counting kit-8 (CCK-8) assay was used. Primary cultured Schwann cells were administered with berberine at concentrations of 0.1 μM, 0.5 μM, 1.0 μM, 5.0 μM and 10.0 μM, and a CCK-8 assay was implemented after 24 hours according to the manufacturer's instructions (Dojindo Molecular Technologies, Kumamoto, Japan). An Emax Plus Microplate Reader (Molecular Devices Emax, CA, USA) was used to measure the absorbance of each well at 540 nm.

## Statistical Analysis

The Mann–Whitney *U*-test and Wilcoxon signed-rank test were used to compare the variables (SNFI, latency, amplitude, and latency) to evaluate the functional recovery of injured nerve. Based on the concentration of berberine, one-way analysis of variance and post hoc analyses were performed to evaluate the differences. Statistical significance was set p=0.05, with a 95% confidence interval. SPSS version 21.0 software (SPSS, Inc., Chicago, Illinois, USA) and GraphPad Prism version 5.01 software (Windows, San Diego, CA, USA) were used for all statistical analyses.

# Results

## In vivo Analysis

### Comparison of Functional Recovery Using SNFI

SNFI of Group B and C did not show statically significant differences at 2 (−46.1±6.5 vs −58.0±23.5, p=0.440) or 4 (−29.6±8.7 vs −43.6±21.5, p=0.096) weeks post-transection. At 6 weeks post-transection, group B showed a significantly higher SNFI than group C (−19.4±9.1 vs −39.4±27.5, p=0.019) (Table 1).

### Comparison of Neuromuscular Function Using Electromyography

Six weeks post-transection, EMG was performed to evaluate the functional recovery of the myelinated axons. There was no significant difference in latency (3.1±0.7 m/s vs 3.8±1.8 m/s, p=0.346) or amplitude (3.9±1.8 mV vs 3.2±2.1 mV, p=0.418) on the contralateral side between the two groups. The latency of the ipsilateral side after transection was lower in group B than group C (3.2±0.7 m/s vs 4.4±1.7 m/s, p=0.043), and amplitude was significantly higher in group B than group C (2.9±1.0 mV vs 1.9±1.1 mV, p=0.025) (Figure 4).

## In vitro Analysis

### Comparison of Regeneration Ratio Using Histologic Analysis

At 6 weeks post-transection, the regeneration ratio was significantly higher in group B, compared to group C (57.1 ±18.7% vs 33.1±14.6%, p=0.036) (Figures 5 and 6).

**Table 1** Comparison of SNFI in Group B and C

| Post-transection | Group B Injected with Berberine | Group C Injected with Normal Saline | p-value |
|---|---|---|---|
| 2 weeks | −46.1±6.5 | −58.0±23.5 | 0.440 |
| 4 weeks | −29.6±8.7 | −43.6±21.5 | 0.096 |
| 6 weeks | −19.4±9.1 | −39.3±27.5 | 0.019 |


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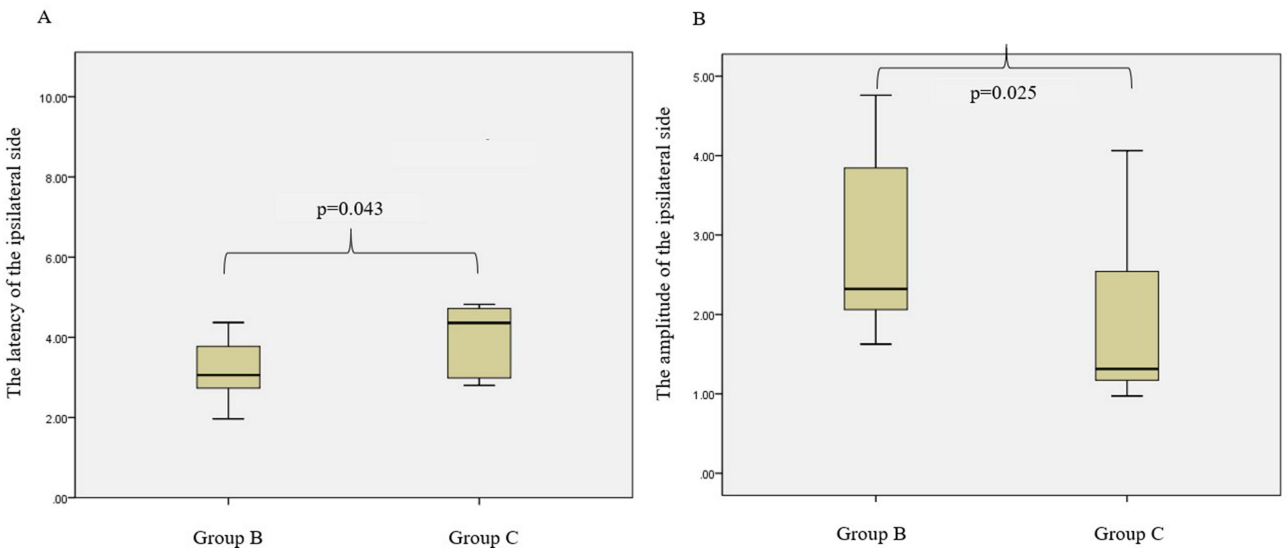


**Figure 4** Electromyography of the ipsilateral side at 6 weeks post-transection. (**A**) The latency; (**B**) Amplitude.

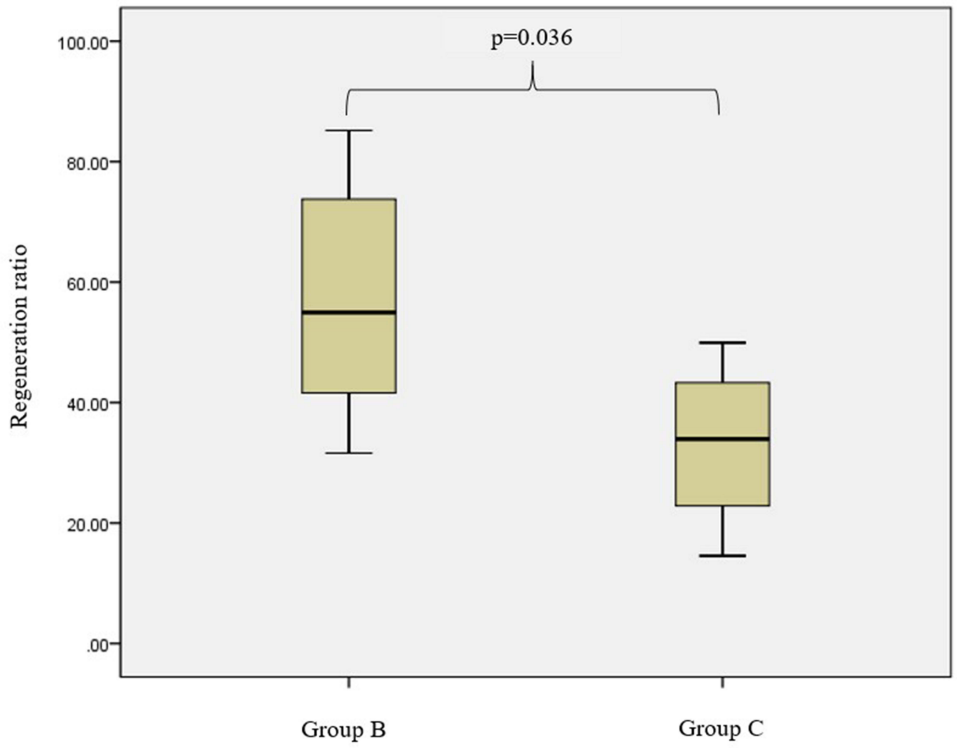


**Figure 5** Regeneration ratio at 6 weeks post-transection.

## Comparison of Berberine Concentration on Schwann Cell Viability

Among the groups administered with various concentrations of berberine, the groups with 0.5 μM, 1.0 μM, 5.0 μM and 10.0 μM administered had significantly higher viability compared to the group with normal saline injection ($p=0.025$, $p<0.001$, $p=0.001$ and $p=0.012$, respectively). No significant differences were observed between the groups injected with 0.1 μM and normal saline ($p=0.096$). Schwann cell viability was highest when 1.0 μM (136.7±3.5%) berberine was administered, which was significantly higher than the 0.1 μM (114.5±10.6%) and 0.5 μM administered groups (118.5 ±4.8%) ($p=0.007$, $p=0.027$, respectively). When compared to the 5.0 μM (129.2±5.7%) and 10.0 μM (120.7±5.9%)

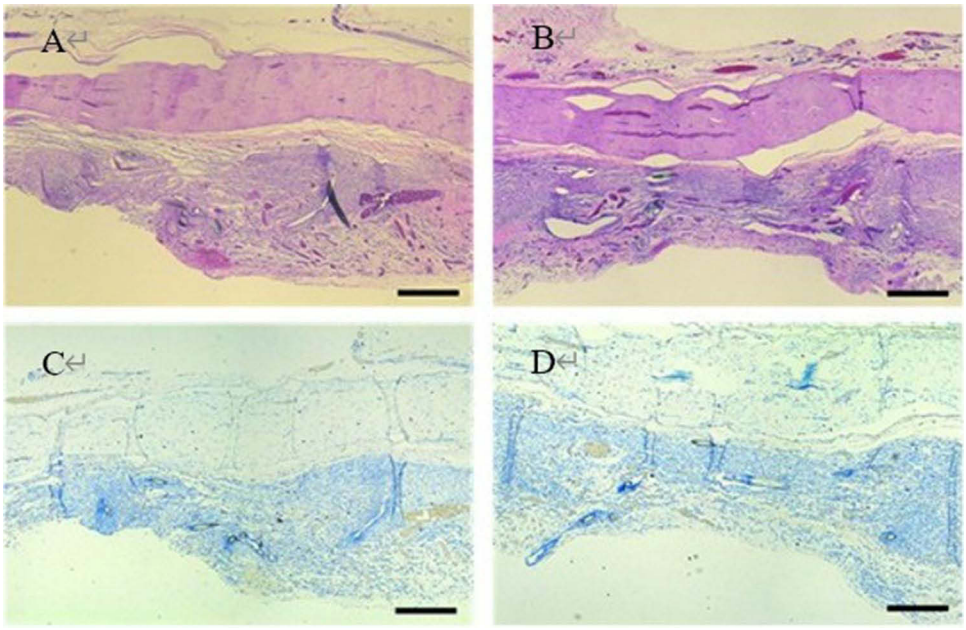


**Figure 6** Higher regeneration ratio was calculated in Group B in comparison to Group C at 6 weeks post-transection (**A** and **B**), H&E staining; (**C** and **D**), toluidine blue staining, scale bar = 500 μm). A. Group C; B. Group B; C. Group C; D, Group B.

administered groups, the 1.0 μM administered group did not show any significant differences in Schwann cell viability (p=0.665, p=0.140, respectively) (Table 2).

## Discussion

In this study, functional recovery and regeneration of the injured nerves were improved in the group administered berberine (group B), compared with the control group (group C). To analyze functional recovery, the SNFI performed 6 weeks post-transection was significantly higher in group B than in group C. In EMG, lower latency and higher amplitude were observed in group B. Furthermore, group B showed significantly higher regeneration ratio of peroneal nerve compared to group C in histologic analysis.

When peripheral nerve injury occurs, neurological deficits occur at the distal part of the transection area.[15] When reinnervation is delayed, atrophy progresses to the denervated muscle, resulting in decreased functional capacity.[16] Despite continuous advances in surgical techniques, such as acellular nerve allograft implantation,[17] functional deficits may still occur after nerve injury. Therefore, electrical stimulation of growth hormones and drug therapy are constantly being studied to enhance reinnervation.[4–6,18,19] Berberine has been reported to exert therapeutic effects on neuronal diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease by reducing neuronal damage and apoptosis.[20] However, only a few studies have reported on PNS disorders and none on central nervous system disorders.

Among the studies that analyzed the neuroregenerative effects of berberine on peripheral nerve injury in a rat model,[10,11] Han et al reported that remyelinated axon thickness was most improved by berberine administration and that it promoted axonal regeneration.[11] In this study, to evaluate nerve regeneration in injured nerves more precisely, the regeneration ratio was evaluated based on the tibial nerve, and the regeneration ratio was highest in the group

**Table 2** Comparison of Schwann Cell Viability According to Various Berberine Concentrations

| Berberine Concentration | Control | 0.1μM | 0.5μM | 1.0μM | 5.0μM | 10.0μM |
|---|---|---|---|---|---|---|
| Relative cell viability (%) | 100 | 114.5** | 118.5*,** | 136.7* | 129.2* | 120.7* |

**Note**: *P <0.05 vs control, **P <0.05 vs 1.0μM.


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administered berberine. Furthermore, to analyze the functional recovery of the transected peroneal nerve, both in vitro and in vivo analyses using SNFI and EMG were performed. At 6 weeks post-transection, the berberine administered group had a significantly higher SNFI, lower latency, and higher amplitude in EMG. Therefore, the results of the in vitro analysis were confirmed by the in vivo analysis.

It has been reported that proliferation and migration of Schwann cells are important for the regeneration of injured peripheral nerves.[2,11,21–23] A previous animal study reported that more Schwann cells were distributed in the regenerated axonal area in the berberine group.[11] Min et al have reported that Schwann cells migrate to the nerve bridge from the proximal and distal stumps after peripheral nerve transection, affecting axon regeneration.[2] However, no studies have compared the degree of Schwann cell proliferation based on the amount of berberine administered. Therefore, the extent to which berberine should be administered to have a significant effect on nerve regeneration in peripheral nerve injuries is unknown. In this study, the proliferation of Schwann cells after administration of various concentrations of berberine was analyzed using a CCK-8 assay. At least 0.5 μM berberine was administered to obtain a significant therapeutic effect in peripheral nerve injury. Schwann cell proliferation was the highest when 1.0 μM berberine was administered. Since no significant difference was observed between the group administered with 1.0 μM and the group administered with more than 1.0 μM, 1.0 μM could be set as the optimal concentration for the therapeutic effect in vitro analysis.

The limitations of this study were as follows. First, when peripheral nerve transection occurs in a clinical setting, end-to-end neurorrhaphy is commonly performed.[24] However, in this study, the proximal and distal ends of the nearby soft tissue were repaired to prevent further displacement without neurorrhaphy after transection. This may lead to complications such as neuroma in clinical settings. Second, even though berberine administration is effective in nerve regeneration, the optimal concentration was determined in this study, and only in vitro analysis was performed. Additional in vivo studies including bromodeoxyuridine (BrdU) proliferation analysis are required to apply this in the clinical field in future. Third, functional recovery after peroneal nerve transection was evaluated using SNFI, not an index that specifically evaluates the function of the peroneal nerve. Fourth, EMG and histologic analysis were not performed at 2 and 4 weeks post-transection. Fifth, since the peroneal nerve contains sensory fibers, sensory recovery was not evaluated. However, despite these limitations, this study evaluated the effect of berberine on peripheral nerve injury using both in vitro and in vivo studies, and is meaningful in that it is the first animal study to analyze the optimal concentration.

## Conclusion

Berberine injections have a therapeutic effect on nerve regeneration after peripheral nerve transection. In in vitro studies, a minimum dose of 1.0 μM berberine was required to obtain optimal nerve regeneration. Further in vivo studies are needed to analyze the optimal concentration.

## Abbreviations

DMEM, Dulbecco's Modified Eagle's medium; FBS, Fetal bovine serum; H&E, Hematoxylin and Eosin; IT, Intermediary toe; PL, Print length; PNS, Peripheral nervous system; SNFI, Sciatic nerve functional index; TS, Toe spread.

## Data Sharing Statement

The datasets used and/or analysed during the current study are available from the corresponding author (Myung-Seo Kim) on reasonable request.

## Ethics Approval and Consent to Participate

This animal study was approved by the Ethics Committee of Kyung Hee University Hospital in Gangdong (KHNMC AP 2020-016).

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no competing interests in this work.

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