

Neuroprotective effects of ultrasound-guided nerve growth factor injections after sciatic nerve injury

Hong-fei Li, Yi-ru Wang, Hui-ping Huo, Yue-xiang Wang*, Jie Tang*

Department of Ultrasound, Chinese PLA General Hospital, Beijing, China

***Correspondence to:**

Jie Tang, M.D. or Yue-xiang Wang, M.D.,
txiner@vip.sina.com or
wangyuexiang1999@sina.com.

orcid:

0000-0002-6882-4044 (Jie Tang)
0000-0003-4108-1161 (Yue-xiang Wang)

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Abstract

Nerve growth factor (NGF) plays an important role in promoting neuroregeneration after peripheral nerve injury. However, its effects are limited by its short half-life; it is therefore important to identify an effective mode of administration. High-frequency ultrasound (HFU) is increasingly used in the clinic for high-resolution visualization of tissues, and has been proposed as a method for identifying and evaluating peripheral nerve damage after injury. In addition, HFU is widely used for guiding needle placement when administering drugs to a specific site. We hypothesized that HFU guiding would optimize the neuroprotective effects of NGF on sciatic nerve injury in the rabbit. We performed behavioral, ultrasound, electrophysiological, histological, and immunohistochemical evaluation of HFU-guided NGF injections administered immediately after injury, or 14 days later, and compared this mode of administration with intramuscular NGF injections. Across all assessments, HFU-guided NGF injections gave consistently better outcomes than intramuscular NGF injections administered immediately or 14 days after injury, with immediate treatment also yielding better structural and functional results than when the treatment was delayed by 14 days. Our findings indicate that NGF should be administered as early as possible after peripheral nerve injury, and highlight the striking neuroprotective effects of HFU-guided NGF injections on peripheral nerve injury compared with intramuscular administration.

Key Words: nerve regeneration; high frequency ultrasound; peripheral nerve injury; nerve growth factor; sciatic nerve; neurotrophic factor; intramuscular injection; medication time; neural regeneration

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Introduction

Peripheral nerve injury is common, accounting for one million injuries per year worldwide (Dahlin, 2008; Siemionow and Brzezicki, 2009). Despite advances in surgical techniques such as microsurgery, restoration of nerve function after injury remains a major challenge in medicine (Zochodne, 2012). The degree of recovery of nerve function after injury depends on axonal regeneration (Siemionow and Brzezicki, 2009), and peripheral nerve injury often involves damage to the axon itself. Therefore, enhancing the rate of axonal regeneration to accelerate repair after peripheral nerve damage has been the focus of many studies to date.

The structural recovery of peripheral nerves is influenced by the microenvironment of the injured nerve (Zochodne, 2012). An important constituent of this microenvironment is nerve growth factor (NGF), which has been studied extensively (Ebendal, 1992; Moges et al., 2011; Pettersson et al., 2011). NGF plays an important role in the nutrition and growth-promotion of axons, and contributes to the regulation of the development, proliferation, differentiation, growth, regeneration and overall function of central and peripheral neurons (Shen et al., 2010a; Barm pitsioti et al., 2011;

Kaiser and Haninec, 2012; Shakhbazou et al., 2012; Euler de Souza Lucena et al., 2014; Martinez et al., 2014). The significant and wide-ranging functions of NGF have led to its use in many preclinical and clinical trials for the treatment of nervous system disorders, such as Alzheimer's and Parkinson's diseases, and peripheral neuropathies (Olson et al., 1991; Apfel, 2002; Mandel, 2010; Wahlberg et al., 2012). The effect of treatment depends largely on two factors: the route and timing of administration, which can be *via* local, intramuscular (i.m.), or systemic (intravenous or oral) routes, or by gene therapy. However, all these methods have disadvantages that limit the clinical success of a treatment. For example, systemic administration cannot ensure an optimal dose at the site of action, and gene therapy is costly and presents a number of risks. Local injections of NGF are used widely in the treatment of nerve injury (Fortun et al., 2009), but this route is not suitable in all cases, such as those in which surgery is not required (Walshe et al., 2001). Moreover, the duration and concentration of NGF cannot be guaranteed (Kubo et al., 2000). Regarding timing, early administration of NGF consistently results in better outcomes. However, its short half-life means that the administration schedule for

NGF still needs further investigation to optimize the treatment effect after peripheral nerve injury.

High frequency ultrasound (HFU) is an ultrasonic diagnostic technique which allows the identification of the fine structure of peripheral nerves and of the exact position of the damaged nerve (Toros et al., 2009; Liu et al., 2012). Used soon after injury, HFU can help to determine the nature and extent of the damage. Furthermore, HFU can be used to guide needle placement when administering drugs directly to the site of action. HFU is therefore increasingly widely used as a tool for the diagnosis and treatment of nerve injury.

The aim of the present study was to determine the efficacy of immediate and delayed (14 days after injury) administration of NGF directly into the damaged area of the sciatic nerve *via* HFU-guided injection, and compare it with immediate and delayed *i.m.* NGF administration, in a rabbit model of sciatic nerve crush injury.

Materials and Methods

Animals and surgery

Sixty male New Zealand white rabbits, aged 4–6 months and weighing 2.5–3.0 kg, were provided by the Laboratory Animal Center, PLA General Hospital, Beijing, China (license No. SCXK (Jing) 2010-0001). Rabbits were anesthetized with Sumianxin (ShengDa Animal Drugs Ltd., Donghua, Jilin Province, China; 0.2 mL/kg, *i.m.*) and fixed on a surgical table. Following routine sterilization, an incision, 3 cm long, was made longitudinally on the posterior thigh of the left hindlimb. The biceps flexor cruris muscle was separated bluntly to expose the sciatic nerve. The hindlimb contracted upon mild stimulation of the sciatic nerve. Intestinal forceps (25 cm; Shanghai Medical Instrument Co., Ltd., Shanghai, China) were used to clamp the nerve vertically at three points, 1.5 cm below the ossa sedentarium (Schmitz and Beer, 2001; Wang et al., 2010). Clamping lasted approximately 5 minutes and was repeated once after a 10 second release. The damaged area was 10 mm wide and the clamped parts were translucent but not broken, and was marked by placing a sterile piece of catheter tubing in the epineurium. The wounds were closed and the rabbits were allowed to recover. Three days after modeling, one rabbit was selected at random and killed using an overdose of Sumianxin. A 5 mm length of spinal cord, with the injury site at the center, was harvested, dehydrated, embedded in paraffin, cut longitudinally into 5 μ m sections, and dyed with toluidine blue to confirm that the model had been established successfully. The experiments were approved by the Animal Ethics Committee of PLA General Hospital, Beijing, China, and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal discomfort and reduce the number of animals used.

HFU-guided NGF administration

All 60 rabbits with sciatic nerve injury were randomly divided into three groups: model (sciatic nerve injury), HFU (HFU-guided NGF injection), and IM (*i.m.* NGF injection).

The normal nerve tissue of the right hindlimb was used as control tissue. The two NGF treatment groups were then divided into two subgroups ($n = 10$) according to the time of treatment: immediately after injury (I-HFU, I-IM) or 14 days after injury (14-HFU, 14-IM). Rabbits in the model group ($n = 20$) received no treatment after injury. An HFU array probe was used to perform bilateral horizontal scans of the sciatic nerves to identify the damaged area, which was then marked by placing a sterile infusion tube in the epineurium 5 mm above the wound. Rabbits in the I-HFU group received injections of NGF (Staidson Biopharmaceuticals Co., Ltd., Beijing, China) into the area surrounding the injured nerve *via* a 22-gauge needle guided by HFU, and rabbits in the I-IM group received *i.m.* NGF (2 μ g/kg), daily from 1 to 14 days after injury. Rabbits in the 14-NGF and 14-IM groups received NGF under the same protocol but from 14 to 28 days after injury.

General observation

Limb movement, wound healing, degree of crus triceps paralysis, foot and ankle anabrosis, and signs of distress were monitored daily after surgery.

Neurological function assessment

Neurological function was assessed at 2, 4, 6, and 8 weeks after injury. Toe spreading (Shen et al., 2010b) was graded from 1 to 4, and modified Tarlov scoring (Matsuyama et al., 2000) from 0 to 4 was also conducted, with higher scores representing better function. The two scores were added to obtain the total neurological function score, and the mean was calculated for the group.

Ultrasound testing

At 2, 4, 6, 8 weeks after injury, the sciatic nerves of all rabbits were examined using HFU diagnostic apparatus (GE Healthcare, Bethesda, MD, USA). The rabbits were anesthetized with an injection of Sumianxin (0.2 mL/kg, *i.m.*) into the neck, and fixed in the prone position. Both hindlimbs were straightened and separated. The HFU array probe was used to conduct bilateral horizontal scans of the sciatic nerves, and the damaged area on the left nerve was identified by the catheter tubing in the epineurium. The inner diameter, continuity, and integrity of the myelinated nerve fibers were observed.

Electrophysiological measurements

At 8 weeks after surgery, we performed electrophysiological measurements to evaluate the recovery condition of triceps surae with an electromyography instrument (Medtronic Keypoint, Skovlunde, Denmark). Two rabbits from each group were chosen and anesthetized with an injection of Sumianxin (0.2 mL/kg, *i.m.*) into the neck, and sciatic nerves were exposed bilaterally. The stimulating electrode was placed on the distal and proximal ends of the injured segment (or equivalent position on the control nerve), and the recording electrode was placed in the triceps surae. Recovery of the triceps surae was evaluated by measuring the peak amplitude and latency of the compound muscle action

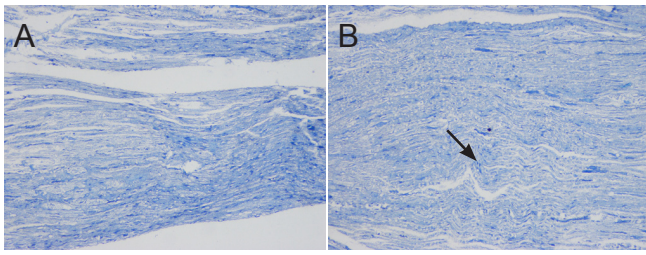


Figure 1 Longitudinal sciatic nerve sections (toluidine blue staining, $\times 200$ magnification). (A) Healthy control tissue; (B) damaged tissue. Compared with control tissue, damaged tissue showed weaker staining and more tortuous fiber tracts (arrow). The nerve axon, myelin sheath, and endoneurium were broken in the damaged nerve. Nerve damage was Sunderland class III.

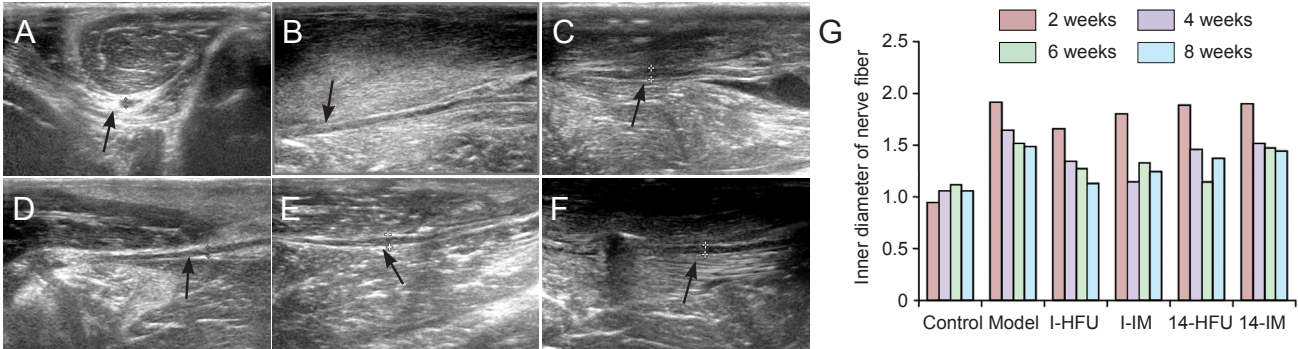


Figure 3 Ultrasonic testing of sciatic nerve injuries.

(A–F) Ultrasound images. Arrows show the sciatic nerve. (A, B) Cross-section (A) and longitudinal section (B) of the normal control sciatic nerve. (C–F) Longitudinal sections of the sciatic nerve for all the rabbits with sciatic nerve injury at 2, 4, 6, and 8 weeks after the treatment. (G) Inner diameter of nerve fiber. The data are expressed as the mean \pm SD and differences were compared by analysis of variance and Student's *t*-test. The inner diameter in the I-HFU group was smallest ($P < 0.05$) and that in the model group was largest ($P < 0.05$) of all treatment groups at the same time. Model: sciatic nerve injury only; I-HFU, 14-HFU: HFU-guided NGF injections immediately or 14 days after injury, respectively; I-IM, 14-IM: intramuscular NGF injections immediately or 14 days after injury, respectively.

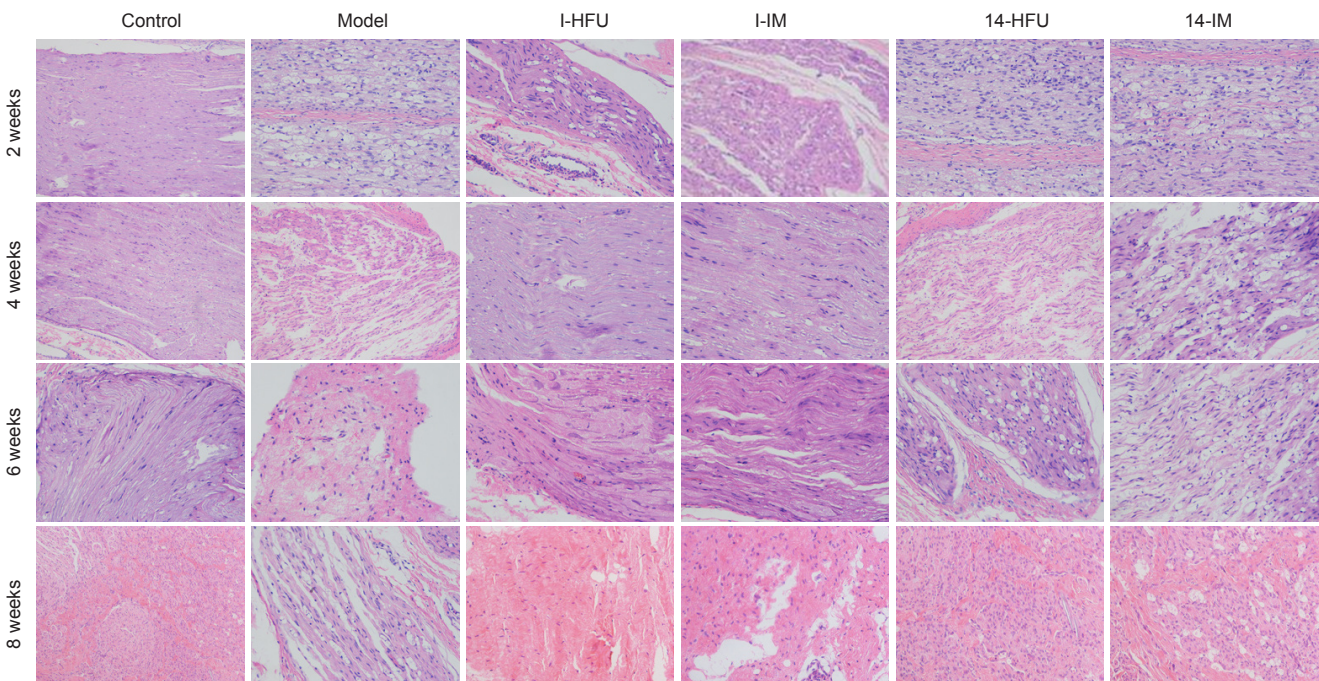


Figure 6 Pathology of rabbit sciatic nerve after injury and treatment with high-frequency ultrasound (HFU)-guided nerve growth factor (NGF) injection (hematoxylin-eosin staining, $\times 200$).

Control: Normal nerve tissue; model: sciatic nerve injury only; I-HFU, 14-HFU: HFU-guided NGF injections immediately or 14 days after injury, respectively; I-IM, 14-IM: intramuscular NGF injections immediately or 14 days after injury. Fewest pathological changes after injury were observed in the I-HFU group, and most in the model group.

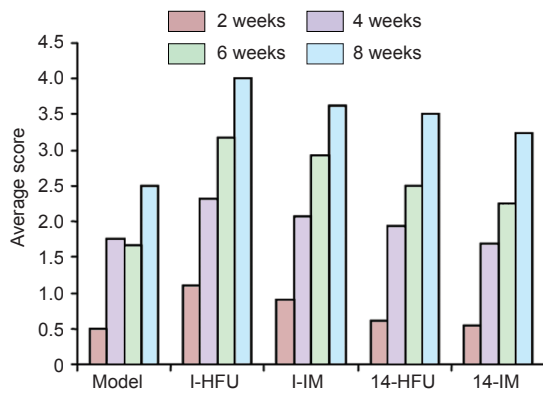


Figure 2 Neurological function in rabbits with sciatic nerve injury after high-frequency ultrasound (HFU)-guided nerve growth factor (NGF) injection.

Total score was the sum of the toe-spreading and modified Tarlov scores. Mean score was calculated as total score divided by the number of rabbits per group. Higher scores represent better neurological function. The model group had the lowest score at each assessment, and the I-HFU group had the highest. Neurological function increased with time after injury in all groups ($P < 0.05$). Scores in all four treatment groups were significantly higher than in the model group ($P < 0.05$), and scores in the I-HFU group were significantly higher than in the 14-IM group ($P < 0.05$). Data are expressed as the mean \pm SD. Differences were compared by analysis of variance and Student's *t*-test. Model: Sciatic nerve injury only; I-HFU, 14-HFU: HFU-guided NGF injections immediately or 14 days after injury, respectively; I-IM, 14-IM: intramuscular NGF injections immediately or 14 days after injury, respectively.

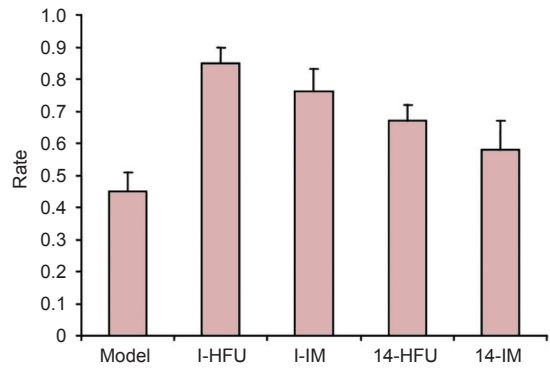


Figure 5 Wet weight recovery rate of the triceps surae after sciatic nerve injury treated by high-frequency ultrasound (HFU)-guided nerve growth factor (NGF) injection.

At 8 weeks after injury, recovery rate was lowest in the model group ($P < 0.05$) and highest in the I-HFU group ($P < 0.05$) compared by the other two groups. Amplitude was higher and latency shorter in the I-HFU and 14-HFU groups than in the I-IM and 14-IM groups, respectively (both $P < 0.05$). Data are expressed as the mean \pm SD. Means were compared using analysis of variance and Student's *t*-test. Model: Sciatic nerve injury only; I-HFU, 14-HFU: HFU-guided NGF injections immediately or 14 days after injury, respectively; I-IM, 14-IM: intramuscular NGF injections immediately or 14 days after injury, respectively.

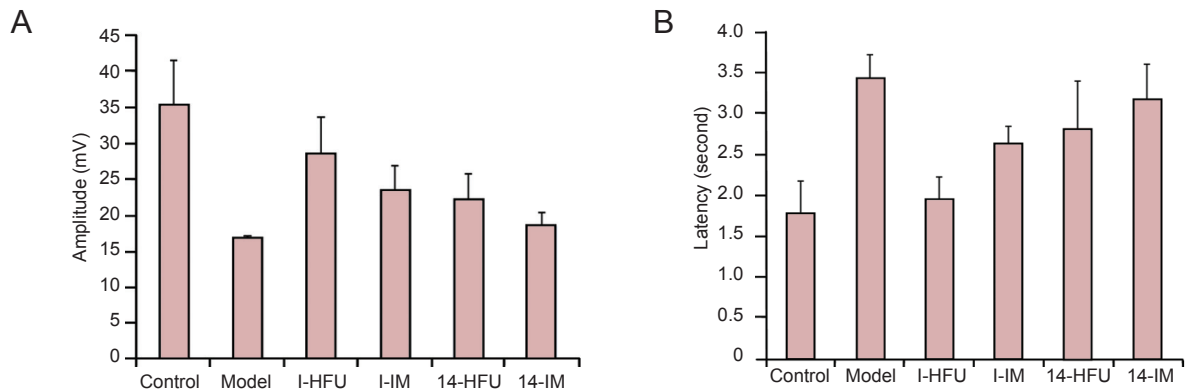


Figure 4 Compound muscle action potential of the triceps surae after sciatic nerve injury treated with high-frequency ultrasound (HFU)-guided injection of nerve growth factor (NGF).

Amplitude (mV) (A) and latency (seconds) (B) of compound muscle action potential 8 weeks after injury. The model group showed the lowest amplitude and longest latency. The control group showed the highest amplitude and shortest latency. Among the four treatment groups, the I-HFU group had the highest amplitude and shortest latency ($P < 0.05$; analysis of variance and Student's *t*-test). Data are expressed as the mean \pm SD. Control: Normal nerve tissue; model: sciatic nerve injury only; I-HFU, 14-HFU: HFU-guided NGF injections immediately or 14 days after injury, respectively; I-IM, 14-IM: intramuscular NGF injections immediately or 14 days after injury, respectively.

potential (CMAP). The rabbits were then sacrificed with an overdose of Sumianxin for histological examination, described below.

Measurement of recovery rate of wet weight of the triceps surae

The triceps surae was dissected out from the two animals per group used for electrophysiological measurements, 8 weeks after injury. Excessive fibrous connective tissues were removed, the tissue was rinsed with physiological saline and

blotted with filter paper, and the wet weight was measured. Recovery rate was calculated as the ratio of wet weight of the tissue from the injured limb to that of the healthy limb in each group. All measurements were carried out under temperature- and humidity-controlled conditions and by the same technician.

Masson trichrome staining

After weighing, the triceps surae tissue was fixed in paraformaldehyde (10%) for 48 hours, dehydrated, and embedded

in paraffin. The paraffin blocks were cut into 10- μ m-thick sections and stained with Masson trichrome. The slices were observed under an optical microscope at 200 \times magnification, and 10 independent muscle fibers were chosen for analysis. The area of muscular fibers was calculated using Image Pro Plus 6.0.

Semi-thin sectioning

The sciatic nerve was dissected out from the two animals per group used for electrophysiological measurements, 8 weeks after injury. A 5 mm length of nerve was removed from the distal stump of the damaged sciatic nerve, fixed in glutaraldehyde (2.5%) for 24 hours, dehydrated, and embedded in epoxy resin. The tissue was cut into sections 1.5 μ m thick, stained with toluidine blue, mounted, and observed under an optical microscope (Olympus). Three visual fields (200 \times magnification) were selected from each slide, and 10 nerve fibers were chosen for analysis. Image Pro Plus 6.0 was used to calculate the density of nerve fibers, diameter of myelinated nerve fibers, and thickness of the myelin sheath.

Transmission electron microscopy

At 8 weeks after injury, one of the rabbits which were tested for electrophysiological measurements was chosen and killed. A 5 mm length of sciatic nerve tissue was removed from the distal stump of the damaged nerve and cut into several sections approximately 1 mm³. The nerve sections were fixed with glutaraldehyde (2.5%), washed by PBS three washes for 15 minutes each, fixed by osmic acid (1%) including 1 \times PBS (pH 7.4) at 20°C for 2 hours, washed by PBS (pH 7.4) three times for 15 minutes each, dehydrated in ethanol gradients (50%, 60%, 70%, 80%, 90%, 100%) in turn for 15 minutes each, treated by acetone plus 812 embedding agent (1:1) overnight, embedded at 60°C for 48 hours, sliced into 60–80 nm thick sections with a HM 360 microtome (Mikrom, Walldorf, Germany), dyed with saturated aqueous uranyl acetate (2%) for 15 minutes, counterstained with citrate for 15 minutes, and finally dried at room temperature overnight. Microscope sections were prepared, observed and analyzed under the electron microscope (FEI Tecnai G2 20 TWIN, USA).

Hematoxylin-eosin (HE) staining

At 2, 4, 6 and 8 weeks after injury, the rabbits were killed with an overdose of Sumianxin. The skin and subcutaneous fascia of the left hindlimb were cut along the original incision, the muscles were separated, and the damaged nerve was exposed. A 5 mm length of nerve tissue was taken from the injury site, fixed, and embedded in paraffin. Sections were cut and stained with HE for observation under an optical microscope (Olympus, Japan). Images were taken and analyzed using Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Immunohistochemistry

At 8 weeks after injury, the remaining animals were killed by an overdose of anesthetic. The damaged nerve was removed,

embedded in paraffin, and dewaxed. Antigen retrieval was carried out in a microwave oven (Galanz, China) for 10 minutes on the low setting, using citric acid buffer (pH 6.0), and the samples were washed three times, for 5 minutes each, in PBS (pH 7.4) on a decoloring shaker (Beijing LiuYi Instrument, Beijing, China). Endogenous peroxidase was blocked with 3% H₂O₂, incubated for 25 minutes in the dark, and then washed in PBS as before. The sections were blocked with 3% bovine serum albumin for 30 minutes, incubated in rabbit anti-mouse S100 β antibody (Abcam, Cambridge, UK) at 1:1,000 dilution overnight at 4°C, followed by goat anti-rabbit IgG H&L (HRP) (Abcam) at 1:500 dilution for 2 hours at room temperature. Proteins were visualized using a 3,3'-diaminobenzidine kit (Dako, Glostrup, Denmark), according to the manufacturer's instructions, and the sections were mounted using neutral gum for viewing under an optical microscope (Olympus). The gray value of each image was calculated using Image Plus Pro 6.0, representing the relative expression of S100 β , and the mean taken for each group.

Statistical analysis

All data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and are expressed as the mean \pm SD. Analysis of variance and Student's *t*-test were used to compare means. *P* < 0.05 was considered statistically significant.

Results

Confirmation of successful rabbit model of sciatic nerve injury

Toluidine blue staining confirmed that the model of sciatic nerve injury was established successfully, resulting in a Sunderland grade III lesion (Sunderland, 1951). Damaged sciatic tissue showed weaker staining than control tissue, and more tortuous fiber tracts. The axon, myelin sheath and endoneurium were broken, but the perineurium and perilemma remained intact. Nerve fiber growth was maintained (**Figure 1**).

General observations

All rabbits were subdued after the surgery. Animals dragged the injured leg or avoided putting weight on it. The acrotarium was weak and the toes were notably downturned; these signs were more pronounced when jumping. After 10 days, the model group showed ulceration at the bottom of the left hind foot, blackened toe skin, and self-mutilation of the injured limb; these effects were less severe in the four treatment groups. By 6 weeks after injury, animals in the treatment groups were able to bear more weight on the injured leg than previously, and recovered normal gait by 8 weeks.

Neurofunctional assessments

The mean neurological score was highest in the I-HFU group. Scores in all treatment groups increased with time after injury (*P* < 0.05; **Figure 2**).

Ultrasonic evaluation of the sciatic nerve

In the control group, the echo structure in the cross-sections

was round or oval, and contained scattered hyperechoic spots (**Figure 3A**). By contrast, linear parallel structures were observed in the echo structure of the longitudinal sections (**Figure 3B**). In all groups, the damaged area changed with time after injury. At 2 weeks, the continuity of the linear echo structure was poor, with a prominent, hypoechoic interruption. The nerve tract was tortuous, the inner diameter was notably thicker than in control tissue, and the dividing line between the nerve perilemma and the surrounding tissues was obscure (**Figure 3C**). At 4 weeks, the extent of the variance and the inner diameter was smaller than at 2 weeks, the echogenicity of the interrupted zone was stronger, and the continuity of the linear echo was improved. The dividing line between the nerve perilemma and the surrounding tissue was less obscure than that at 2 weeks within each group (**Figure 3D**). At 6 weeks, the continuity of the echo structure, the inner diameter of the nerve, the echogenicity of the interrupted zone, and the dividing line between the nerve perilemma and surrounding tissue were all greatly improved. Compared with that observed at 4 weeks, the interruptions were less pronounced, the inner diameter was smaller, and the dividing line was clearer (**Figure 3E**). At 8 weeks, the continuity of the linear echo structure was good, the interrupted zone was almost entirely repaired, the inner diameter recovered well and there was a clear dividing line between the nerve perilemma and the surrounding tissue (**Figure 3F**). The inner diameter of the damaged sciatic nerve was smallest in the I-HFU group and largest in the model group, and generally decreased with time after injury. In the I-HFU group, thickness was restored to control measurements by 8 weeks in the I-HFU group. Recovery was best in the I-HFU group, followed by the I-IM and 14-HFU groups, and lastly the 14-IM group (**Figure 3G**).

Electrophysiological function of the triceps surae

At 8 weeks after injury, the functional recovery of the triceps surae on the injured side was evaluated electrophysiologically. CMAP amplitude and latency represent the degree of recovery. The model group showed the lowest amplitude and the longest latency of all groups ($P < 0.05$). Among the treatment groups, I-HFU had the highest amplitude and the shortest latency ($P < 0.05$). The amplitude was higher and the latency shorter in both HFU-mediated treatment groups than in the groups that received i.m. NGF ($P < 0.05$; **Figure 4**).

Recovery rate of triceps surae

At 8 weeks after injury, the triceps surae wet weight recovery rate was lowest in the model group ($P < 0.05$) and highest in the I-HFU group ($P < 0.05$). Of the four treatment groups, recovery rate was highest in the I-HFU group ($P < 0.05$), then the I-IM and 14-HFU groups, and lowest in the 14-IM group ($P < 0.05$) (**Figure 5**).

Pathological changes in injured sciatic nerve

Nerve tissue in the control group showed neatly arranged fibers and a uniform myelin sheath structure (**Figure 6A**). In the model group, sciatic nerve fibers were arranged in a disor-

ganized manner and had swollen or missing axons, and vacuolation. Most of the myelin had disintegrated and inflammatory cell infiltration was observed (**Figure 6B**). In the I-HFU group, compared with the model group, the nerve fibers showed a more orderly arrangement, and less axonal swelling and vacuolation. Part of the myelin had disintegrated and more Schwann cells and fewer inflammatory cells were distributed between the fibers (**Figure 6C**). In the I-IM group, fibers were slightly disordered, axons were swollen, and there was less vacuolation than in the model group, but more than in the I-HFU group. Myelin was partially disintegrated. More Schwann cells and fewer inflammatory cells were distributed between the fibers than observed in the I-HFU group (**Figure 6D**). The pathology of the 14-HFU and 14-IM groups was similar to that in the model group (**Figure 6E, F**).

At 4 weeks after injury, in the control group, the nerve fibers had returned to the control state, showing normal, uniform myelin sheaths and a small number of Schwann cells between the fibers (**Figure 6A**). In the model group, multiple changes were observed in the myelin sheath, including uneven distribution, variable thickness, irregular fiber arrangement, fiber degeneration, and disintegration of axons and myelin. In addition, the normal structure of the myelin sheath had almost disappeared, leaving an irregular outline and notable edema of the nerve. Distribution was sparse, and more inflammatory cells were observed between fibers (**Figure 6B**). Compared with the control group, the I-IM group showed disorganized fibers, less obvious Schwann cell proliferation, better directional arrangement, and less vacuolation (**Figure 6E**). In comparison, the I-HFU group showed a greater number of Schwann cells and even less vacuolation (**Figure 6C**). In the 14-HFU and 14-IM groups, the fibers were arranged in a disorderly manner but less so than in the model group; in addition, compared with the model group, a larger number of Schwann cells were observed between fibers, and more vacuolation was observed (**Figure 6D, F**). Compared with the 14-IM group, far less vacuolation was observed in the 14-HFU group (**Figure 6D**).

At 6 weeks, in the control group the tract and structure of the nerve were normal and uniform. Few Schwann cells were observed between the fibers (**Figure 6A**). In the model group, the distribution of fibers was uneven, the thickness variable, and the arrangement of the myelin sheath irregular. Nerve fiber degeneration was observed, and the disintegrated axons and myelin sheath were partially absorbed. The myelin sheath was sparse and misshapen, and there were few regenerating nerve fibers. Some fibers were pyknotic, and notable spongiform change was observed. There was no evidence of axonal regeneration (**Figure 6B**). In the I-HFU and I-IM groups, compared with the model group, Schwann cell proliferation was more obvious, cells were distributed more uniformly, and the arrangement of nerve fibers was also more uniform. There was considerably less spongiform change than in the model group. There was better nerve fiber recovery in the I-HFU group than in the I-IM group (**Figure 6C, E**). In the 14-HFU and 14-IM groups, arrangement of fibers was considerably more disordered but more regular than in the model group. Some of the disintegrated material

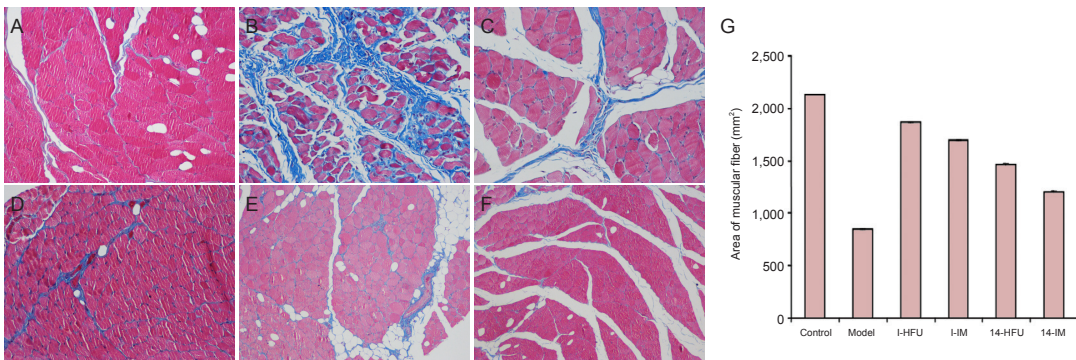


Figure 7 Muscle fibers of rabbits with sciatic nerve injury after high-frequency ultrasound (HFU)-guided nerve growth factor (NGF) injections. (A–F) Muscle fiber histology at 8 weeks after injury (Masson trichrome staining, $\times 200$). (A) Control; (B) model; (C) I-HFU; (D) I-IM; (E) 14-HFU; (F) 14-IM. In the model group (B), muscle cells were sparse and fiber structure was markedly damaged. In the I-HFU group (C), bonds between muscle cells were tight and the structure was regular. The structure was not clear in the I-IM group (D). Recovery of muscle fiber structure in the 14-HFU and 14-IM groups was better than in the model group, but best in the I-HFU and I-IM groups. (G) Muscle fiber area. Treatment groups showed larger muscle fiber area than the model group ($P < 0.05$). Muscle fiber area in I-HFU and I-IM groups was significantly larger than in the I-IM and 14-IM groups ($P < 0.05$). Data are expressed as the mean \pm SD. Means were compared using Student's *t*-test and analysis of variance. Control: Normal nerve tissue; model: sciatic nerve injury only; I-HFU, 14-HFU: HFU-guided NGF injections immediately or 14 days after injury, respectively; I-IM, 14-IM: intramuscular NGF injections immediately or 14 days after injury. Fewest pathological changes after injury were observed in the I-HFU group, and most in the model group.

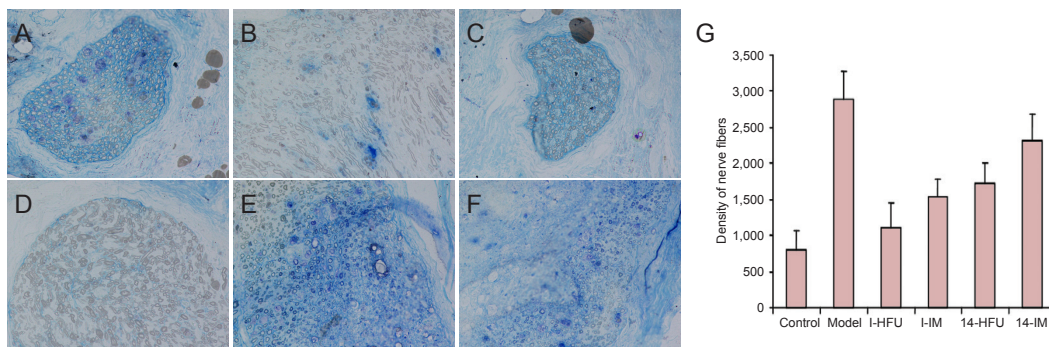
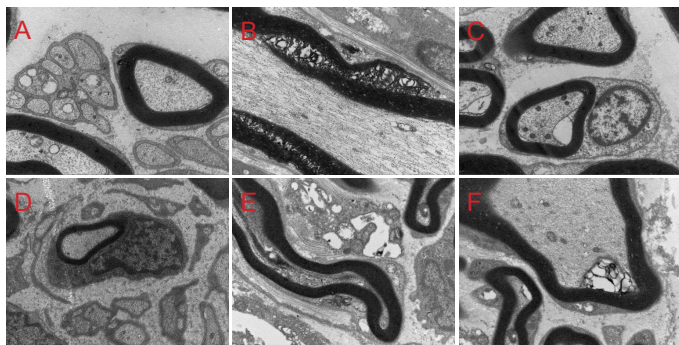


Figure 8 Effects of high-frequency ultrasound (HFU)-guided nerve growth factor (NGF) injection on injured sciatic nerve tissue. (A–F) Morphology of sciatic nerve fibers at 8 weeks after injury ($\times 200$, semi-thin sections, toluidine blue staining). (A) Control; (B) model; (C) I-HFU; (D) I-IM; (E) 14-HFU; (F) 14-IM. (G) Nerve fiber density. Myelinated nerve fiber density was markedly lower when NGF was injected immediately, especially in the I-HFU group ($P < 0.05$; analysis of variance and Student's *t*-test). Data are presented as the mean \pm SD. Control: normal nerve tissue; model: sciatic nerve injury only; I-HFU, 14-HFU: HFU-guided NGF injections immediately or 14 days after injury, respectively; I-IM, 14-IM: intramuscular NGF injections immediately or 14 days after injury. Fewest pathological changes after injury were observed in the I-HFU group, and most in the model group.

Figure 9 Sciatic nerve ultrastructure 8 weeks after injury and treatment with high-frequency ultrasound (HFU)-guided nerve growth factor (NGF) injections (transmission electron microscope, $\times 1,500$).



(A) Control tissue showing regular fiber arrangement with uniform axon diameters, myelin sheath thicknesses and dye uptake, and oval-shaped Schwann cell nuclei. (B) Model group. Myelin sheath thicknesses were not uniform. Breakage and dispersal of the myelin layer was observed, dye uptake was poorly distributed, and Schwann cell nuclei were irregularly shaped. (C–F) NGF-treated groups. Nerve fiber regeneration was better in the model group than in the four treatment groups including I-IM and 14-IM. Regenerating axons developed well, and showed orderly arrangement, uniform axon diameters and myelin sheath thickness, and well-distributed staining in the I-HFU (C) and I-IM (D) groups. Schwann cell nuclei were oval in the I-HFU group but irregularly-shaped in the I-IM group. Similarly, myelin sheath thicknesses were uniform in the I-HFU group and variable in the I-IM group. In the 14-HFU (E) and 14-IM (F) groups, axons developed poorly and were arranged irregularly, myelin sheath thicknesses varied, and Schwann cell nuclei were misshapen. Model: Sciatic nerve injury only; I-HFU, 14-HFU: HFU-guided NGF injections immediately or 14 days after injury, respectively; I-IM, 14-IM: intramuscular NGF injections immediately or 14 days after injury.

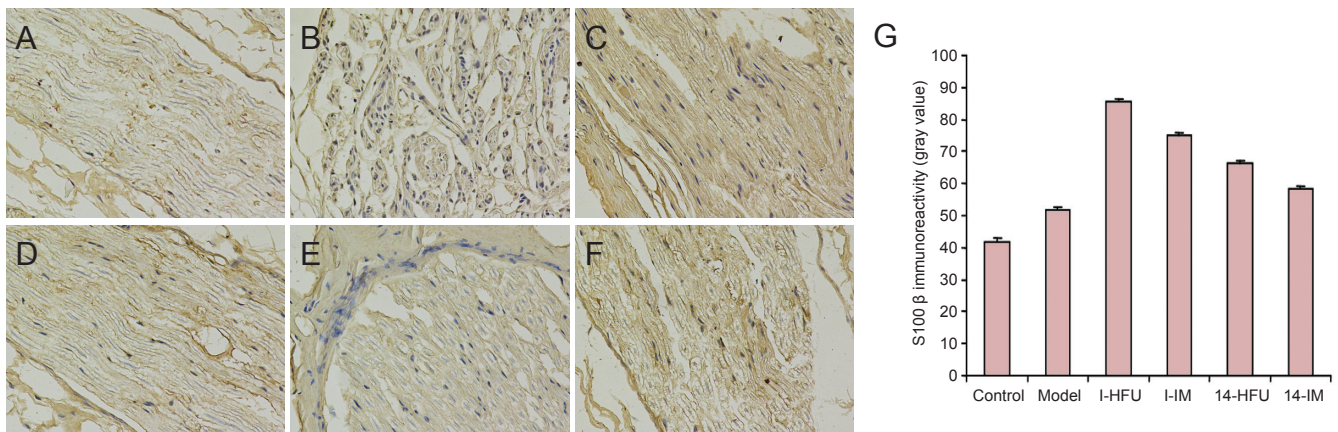


Figure 10 S100 β immunoreactivity in the sciatic nerve after injury and treatment with high-frequency ultrasound (HFU)-guided nerve growth factor (NGF) injection.

(A–F) Immunohistochemistry ($\times 200$) in control (A), model (B), I-HFU (C), I-IM (D), 14-HFU (E), and 14-IM (F) groups. There was some sporadic expression of S100 β in the control group. S100 β immunoreactivity in the model group was a little stronger, but the four treatment groups showed the strongest immunoreactivity. (G) Quantitation of S100 β immunoreactivity. Gray value (intensity of S100 β) was greater in the I-HFU group than in the 14-HFU group ($P < 0.05$), and greater after HFU-guided NGF injections than intramuscular injections ($P < 0.05$). Data are expressed as the mean \pm SD. Means were compared by analysis of variance and Student's *t*-test. Control: Normal nerve tissue; model: sciatic nerve injury only; I-HFU, 14-HFU: HFU-guided NGF injections immediately or 14 days after injury, respectively; I-IM, 14-IM: intramuscular NGF injections immediately or 14 days after injury.

had been absorbed, and more Schwann cells were observed between the fibers. The nerve fibers had regenerated but were fewer in number than in the I-HFU and I-IM groups, and the spongiform changes were more evident. More regenerated fibers and Schwann cells were observed in the I-HFU group than in the I-IM group (Figure 6C, E).

At 8 weeks after injury, in the control group, nerve fiber tracts were normal and uniform in structure. Axons were also uniform in diameter and arranged in a regular manner. Small Schwann cells were visible (Figure 6A). In the model group, the regenerating nerve fibers were sparse and dispersed, and with an uneven distribution. Myelin thickness was uniform, but the structure was loose and disorganized. Mesenchyme was evident. Spongiform changes were clear and inflammatory cell infiltration was observed (Figure 6B). In the I-HFU and I-IM groups, fibers were orderly. Compared with the model group, there were more regenerating fibers, and more evident proliferation and distribution of Schwann cells. Nerve fibers were arranged in a more orderly manner, and there was little spongiform change and few connective tissues between the fibers. Nerve fibers recovered better in the I-HFU group than in the model group (Figure 6C, E). In the 14-HFU and 14-IM groups, the disintegrated products were partially absorbed and Schwann cells were observed between the fibers. Fewer regenerating nerve fibers, more spongiform change and mesenchyme between fibers, and more infiltrated inflammatory cells, were observed in the 14-HFU and 14-IM groups than in the I-HFU and I-IM groups. The number of regenerated fibers was greater in both HFU groups than in both IM groups (Figure 6D, F).

Muscle fiber changes after sciatic nerve injury

At 8 weeks after injury, muscle fibers were plump on the

control side but thin on the injured side (Figure 7A). In the model group, bonds between the muscle cells were loose and the structure was completely damaged (Figure 7B). In the I-HFU group the bonds between cells were relatively tight, with a regular structure (Figure 7C). In the I-IM group, the structure was not clear (Figure 7D). The recovery of the muscle fiber structure in the 14-HFU and 14-IM groups was better than in the model group, but not as good as in the I-HFU and I-IM groups (Figure 7E, F). Muscle fiber area was calculated on the cross-section of the triceps surae, and was greater in the four treatment groups than in the model group ($P < 0.05$). Muscle fiber area was greater in the I-HFU and I-IM groups than in the 14-HFU and 14-IM groups, greater in the HFU groups than in the IM groups, and greatest in the control group (Figure 7G).

The density of myelinated nerve fibers

In the control group, myelinated nerve fibers and myelin sheath thickness were uniform, and the arrangement was regular (Figure 8A). In the model group, myelinated fibers on the injured side were disorganized, with irregular form and sheath thickness (Figure 8B). In the I-HFU and 14-HFU groups, the myelinated nerve fibers were more regular than in the model group, but less so than in the control group. Early NGF administration, in particular with HFU guiding, resulted in more regular myelinated nerve fibers, and more uniform myelin sheath thickness, than later or i.m. administration (Figure 8C–F). The diameter of the myelinated nerve fibers and thickness of the myelin sheath showed greatest improvement in the I-HFU group ($P < 0.05$; data not shown). Nerve fiber density was highest in the model group and lowest in the control group. The density of myelinated nerve fibers was much lower if NGF was administered early, especially with HFU guiding ($P < 0.05$) (Figure 8G).

Ultrastructure of the injured sciatic nerve

Control tissue showed regular axonal arrangement, uniform myelin sheath thickness, and well-distributed staining, with oval Schwann cell nuclei (Figure 9A). In the model group, the thickness of the myelin sheath was not uniform; breakage and dispersion of the myelin layer was observed, staining was not well distributed, and Schwann cell nuclei were irregularly shaped (Figure 9B). Nerve fiber regeneration was better in the four treatment groups than in the model group. Immediate treatment resulted in good axonal regeneration, orderly axon arrangement, uniform axon diameters and myelin sheath thicknesses, and uniform dye uptake (Figure 9C, D). Schwann cell nuclei were oval in the I-HFU group but irregularly shaped in the I-IM group; similarly, myelin sheath thickness was uniform in the I-HFU group but varied in the I-IM group. In the 14-HFU and 14-IM group, axons were poorly developed and irregularly arranged, with variable myelin sheath thicknesses and irregularly shaped Schwann cell nuclei (Figure 9E, F).

S100 β immunoreactivity in the sciatic nerve

At 8 weeks after injury, the immunoreactivity of S100 β in the sciatic nerve varied with time after injury, and administration method. S100 β immunoreactivity was observed as brown granules in the cytoplasm. Some sporadic expression (+) was observed in the control group (Figure 10A). There was less S100 β immunoreactivity in the model group (+/-) than in the control group (Figure 10B). S100 β immunoreactivity was strongest in the I-HFU group (++++), weaker in the I-IM (++) and 14-HFU (++) groups, and weakest in the 14-IM group (++) (Figure 10C-F).

Gray value was used to quantify S100 β expression. Immediate treatment resulted in higher S100 β expression than delayed treatment ($P < 0.05$), and HFU-guided NGF injections resulted in higher NGF expression than i.m. injection ($P < 0.05$; Figure 10G).

Discussion

With advances in surgery, including microsurgery, treatment of peripheral nerve injury is becoming increasingly successful (Battiston et al., 2000; Martinez et al., 2014). Regeneration after peripheral nerve injury is affected by many factors, and the prognosis depends on the location and extent of the damage. It is difficult to improve treatment by modifying surgical techniques alone (Euler de Souza Lucena et al., 2014; Hsueh et al., 2014). Regeneration after peripheral nerve injury is greatly influenced by the microenvironment. Neurotrophic factors contribute to this microenvironment, and of these, NGF has been accepted as the most effective, with striking treatment effects obtained in clinical and preclinical studies (Wang et al., 2009). NGF promotes the survival of neurons and regeneration axons (Kaiser and Ianinec, 2012). Therefore, maintaining a microenvironment rich in NGF is key to repairing damaged nerves (Fumagalli et al., 2008). Treatment effect depends largely on the route and timing of administration. To date, studies of NGF administration have mainly focused on i.m., intravenous, or local administration.

NGF cannot pass through the blood-brain barrier owing to its high molecular weight and short half-life. To maintain an effective concentration of NGF, repeated and long-term administration is needed, which limits its use in the clinic (Madduri et al., 2010). It therefore remains a priority to explore new methods of NGF use. Silicone tube implants have previously been used to infuse NGF continuously into the site of nerve damage, and the method can promote nerve regeneration (Scheidegger et al., 2011). However, the tubes cannot be implanted in the body for a long time because of histocompatibility problems. HFU can be used to detect peripheral nerve injury in the early period and accurately guide injection of the drug into the damaged area. This use of HFU has drawn considerable attention in the medical field, including a report of a clinical trial in which HFU was used in the treatment of Hansen's disease (Bathala et al., 2012). In the present study, we used HFU to guide NGF injections into the damaged area to treat nerve injury. We compared two administration times and performed a series of experiments to determine a suitable administration schedule. Our results show that HFU-guided injection of NGF leads to better structural and functional outcomes in nerve and muscle recovery than i.m. injection.

Histological examinations and electron microscopy also revealed that HFU-guided NGF administration was more effective than i.m. injection in promoting recovery of the myelinated fibers and myelin sheath in the injured nerve, and in the surrounding muscle tissue. HFU-guided NGF injection was also more effective when administered immediately after injury than after 14 days.

In conclusion, HFU-guided injections of NGF, administered soon after the injury, leads to good recovery of nerve and muscle after nerve injury. Further studies exploring treatment schedules, doses, and administration sites are necessary to improve prospects for the clinical application of this promising technique.

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