

# Pulsed electrical stimulation protects neurons in the dorsal root and anterior horn of the spinal cord after peripheral nerve injury

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## Abstract

Most studies on peripheral nerve injury have focused on repair at the site of injury, but very few have examined the effects of repair strategies on the more proximal neuronal cell bodies. In this study, an approximately 10-mm-long nerve segment from the ischial tuberosity in the rat was transected and its proximal and distal ends were inverted and sutured. The spinal cord was subjected to pulsed electrical stimulation at T<sub>10</sub> and L<sub>3</sub>, at a current of 6.5 mA and a stimulation frequency of 15 Hz, 15 minutes per session, twice a day for 56 days. After pulsed electrical stimulation, the number of neurons in the dorsal root ganglion and anterior horn was increased in rats with sciatic nerve injury. The number of myelinated nerve fibers was increased in the sciatic nerve. The ultrastructure of neurons in the dorsal root ganglion and spinal cord was noticeably improved. Conduction velocity of the sciatic nerve was also increased. These results show that pulsed electrical stimulation protects sensory neurons in the dorsal root ganglia as well as motor neurons in the anterior horn of the spinal cord after peripheral nerve injury, and that it promotes the regeneration of peripheral nerve fibers.

**Key Words:** nerve regeneration; peripheral nerve; pulsed electrical stimulation; spinal cord; neurons; dorsal root ganglion; nerve conduction; neural regeneration

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## Introduction

Most studies on peripheral nerve injury have focused on the site of injury. Nerve grafts (Ding et al., 2009), bridging techniques (Jiang et al., 2006), biopolymer nerve conduits and neuromuscular electrical stimulation (Zhilovupov et al., 2012) have shown clinical effectiveness. However, Wallerian degeneration of nerve fibers distal to the site of injury (Brodal, 1981) and proximal neuronal apoptosis at the corresponding spinal level (Chen et al., 2013; Lu et al., 2014; Preyat et al., 2015) hinder axonal regeneration and limit the effectiveness of repair strategies.

Recently, along with advances in safety (reducing nerve tissue burn and nerve pathway damage) (Rattay et al., 2000) and efficacy (by optimizing the type, frequency, current intensity and duration of electrical stimulation) (Chiba et al., 1998) of electrical stimulation techniques, electrical stimulation of the spinal cord has been increasingly used for rehabilitation, spasm control and analgesia after spinal cord injury (Oakley and Weiner, 1999; Lee et al., 2005; Zhang et al., 2015). Li and Hou (2001) have examined whether electrical stimulation at the corresponding spinal level can reduce neuronal apoptosis and promote axon regeneration after

peripheral nerve injury.

In this study, we investigated the effects of pulsed electrical stimulation at the corresponding spinal level on the survival of dorsal root ganglion (DRG) neurons (sensory neurons) and anterior horn neurons (motor neurons) in a rat model of sciatic nerve injury. This study was performed with the aim of developing novel treatment strategies for peripheral nerve injury.

## Materials and Methods

### Ethics statement

The experiment was approved by the Ethics Committee of Shandong University, China. The animal studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All surgeries were performed under anesthesia, and all efforts were made to minimize the number of animals used and to reduce pain, suffering and death.

### Establishment of a rat model of sciatic nerve injury

A total of 30 male Sprague-Dawley rats, 8 weeks old and weighing 200–230 g, were provided by the Specific-Patho-

gen-Free Animal Laboratory, Center for Animal Testing, Shandong University School of Medicine, China (SCXK (Lu) 2010003). All rats were housed at 20–25°C, at a humidity of 40–70% and a wind velocity of 0.1–0.2 m/s, under a 12-hour light/dark cycle. The rats were equally and randomly divided into the sciatic nerve injury model group and the electrical stimulation group (injury + pulsed electrical stimulation).

The rats were intraperitoneally anesthetized with 10% chloral hydrate (0.3 mL/100 kg). A longitudinal incision was made on the lateral right hind limb to expose the sciatic nerve. An approximately 10-mm-long sciatic nerve segment was obtained from the ischial tuberosity, and its proximal and distal ends were inverted and sutured with three to four stitches with No. 11-0 nylon thread under the microscope (**Figure 1**).

#### **Pulsed electrical stimulation of the spinal cord**

A midline lumbar incision was made immediately after the sciatic nerve surgery. The vertebral plate was removed after bluntly dissociating the tissue between the T<sub>10</sub> and L<sub>3</sub> laminae and the spinous processes. The silver electrode plate was made in-house and was approximately 4 mm<sup>2</sup> in size, with two side holes, and with silicone rubber wrapping (except for the electrode plate itself). The electrode plate was placed in the epidural space between the T<sub>10</sub> and L<sub>3</sub> laminae and fixed firmly. Electrical stimulation was performed with low-frequency therapy equipment (Nihon Medix, Chiba, Japan) at a current intensity of 6.5 mA and a stimulation frequency of 15 Hz, 15 minutes/each, twice a day for 56 days (**Figure 1**). The rats in the sciatic nerve injury model group had the same electrode implanted, but electrical stimulation was not given.

#### **Sample collection**

Five rats from each group were intraperitoneally anesthetized with 10% chloral hydrate (0.3 mL/100 kg) at 1, 4 and 8 weeks after injury. After the thoracic cavity was opened, the left ventricle was perfused with approximately 200 mL physiological saline containing 500 IU heparin until clear liquid flowed from the right atrium, and then with 0.4% paraformaldehyde (approximately 200 mL). The right L<sub>4-6</sub> DRG, lumbosacral spinal cord (L<sub>5</sub> segment from the fixed spinal cord specimens), and the sciatic nerve were removed through the original incision. Samples used for electron microscopic observation were fixed with 30% sucrose and 4% paraformaldehyde, and stored at 4°C until use. Samples used for hematoxylin-eosin staining were fixed in 4% paraformaldehyde and stored at 4°C until use.

#### **Electron microscopic observation**

The DRG, spinal cord and sciatic nerve were embedded, sliced into ultrathin sections, and stained with lead citrate. Ultrastructural changes in neurons in the DRG and anterior horn of the spinal cord, and the growth of newly formed sciatic nerve fibers were observed by transmission electron microscopy (JEM21200E; Japan Electron Optics Corporation,

Tokyo, Japan). Eight myelinated nerve fibers were randomly selected at 5,000× magnification. The mean thickness of the myelin sheath in the center of the nerve fiber was calculated (Hu and Gu, 2012).

#### **Hematoxylin-eosin staining**

DRG and spinal cord samples were embedded, sliced into transversal sections, and stained with hematoxylin and eosin. Eight 400× fields were randomly selected under the light microscope (Olympus, Tokyo, Japan). The mean number of neurons was calculated.

#### **Determination of nerve conduction velocity (NCV)**

Right sciatic nerves of five rats from each group were dissociated at 4 and 8 weeks post injury. The proximal and distal ends of the injured nerves were stimulated with a hook electrode (JD-4; Shanghai High-Tech Medical Equipment Company, Shanghai, China). The stimulus intensity was increased by 30% over the threshold intensity (frequency: 1 Hz; pulse width: 1 ms). The latencies at the proximal and distal ends were recorded, and the distance between the two stimulation points was measured. NCV (m/s) was calculated as the distance (mm)/[latency at the proximal end (ms) – latency at the distal end (ms)].

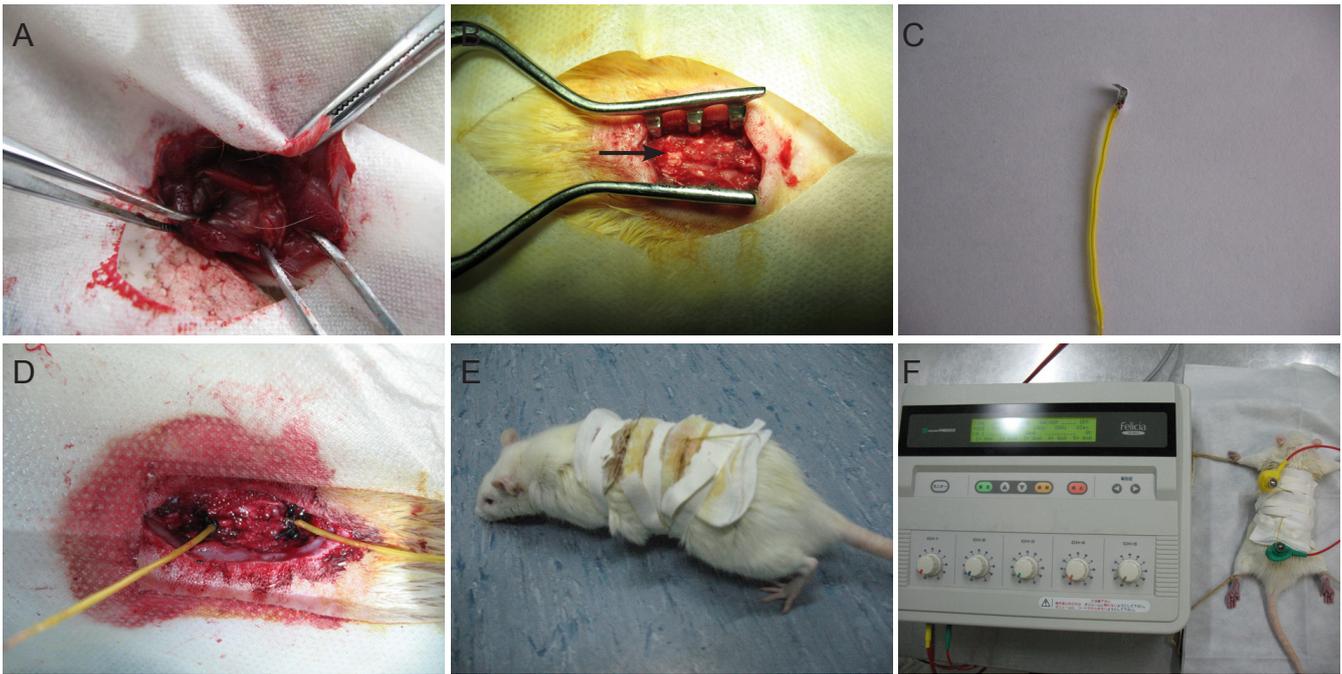
#### **Statistical analysis**

The data are expressed as the mean ± SD, and were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). The difference between groups was compared using independent two-sample *t*-test. A value of *P* < 0.05 was considered statistically significant.

## **Results**

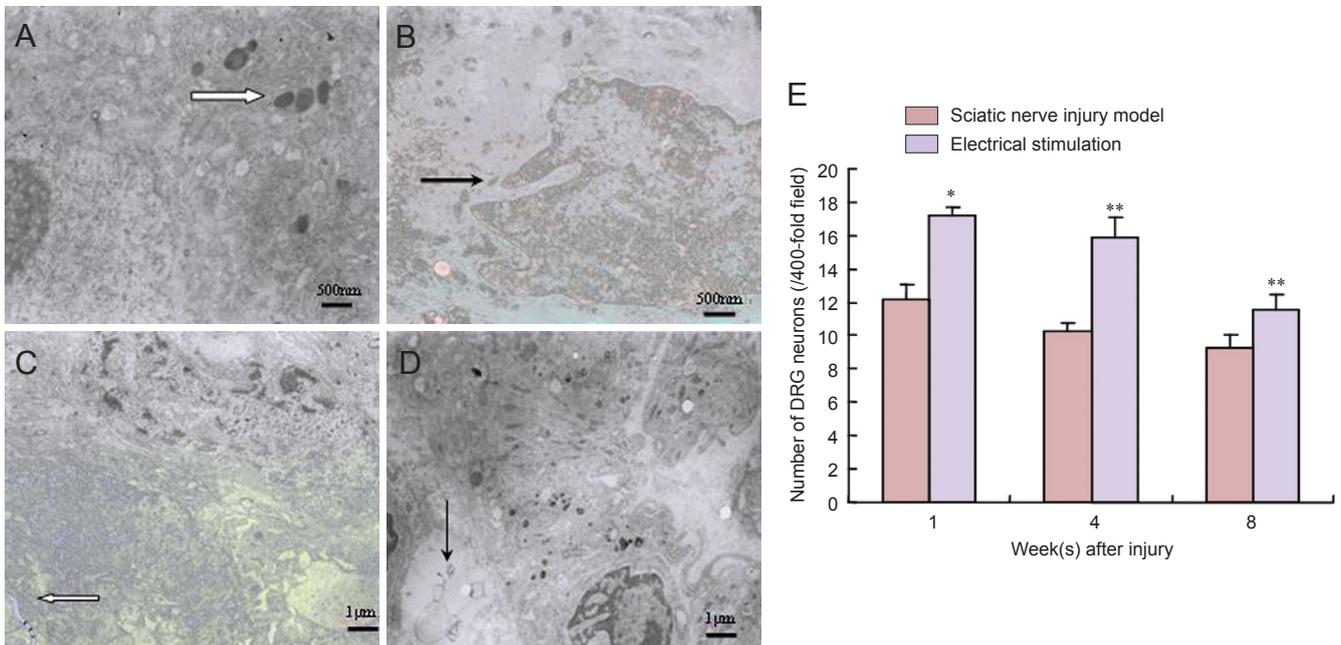
### **Pulsed electrical stimulation improved the ultrastructure of DRG and anterior horn neurons in rats with sciatic nerve injury**

Transmission electron microscopy revealed that 4 weeks after injury, in the electrical stimulation group, DRG neurons had normal looking intracellular organelles, with intact nuclear membranes, distinct nucleoli, and uniform nuclear chromatin. Only a few endosomes were observed in the cytoplasm in the electrical stimulation group. Atrophy of some DRG neurons, nuclear condensation and an increase in heterochromatin were seen in the sciatic nerve injury model group. At 8 weeks, slight atrophy of DRG neurons, endosomes and pseudo-inclusion bodies in the cytoplasm were observed in the electrical stimulation group. In comparison, in the sciatic nerve injury model group, substantial atrophy of DRG neurons, small vacuoles and electron-dense granules in the cytoplasm, and ciliated cells were seen. The nuclear membranes were not intact. Nuclear precession, uneven nuclear chromatin and an increase in heterochromatin were observed as well in the sciatic nerve injury model group (**Figure 2A–D**). At 1, 4 and 8 weeks, hematoxylin-eosin staining demonstrated that the number of DRG neurons was higher in the electrical stimulation group than in the sciatic nerve injury model group (1 week: *P* < 0.05; 4 and 8 weeks: *P* < 0.01; **Figure 2E**).



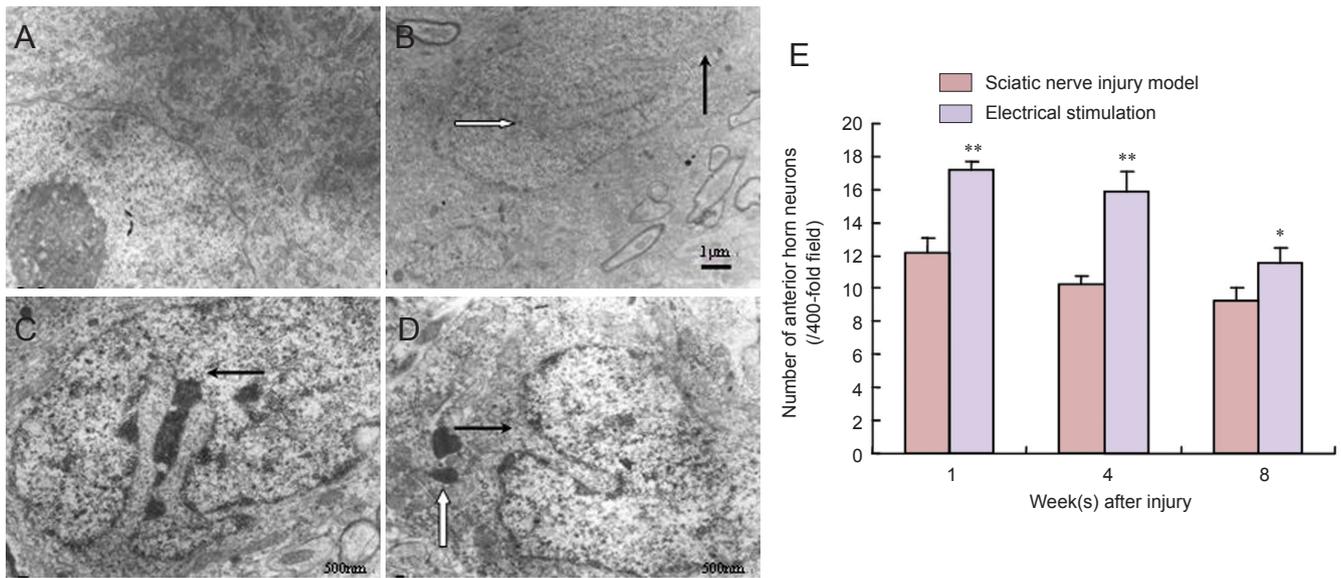
**Figure 1 Establishment of the rat model of sciatic nerve injury and pulsed electrical stimulation.**

(A) Preparation of the rat model of sciatic nerve injury. (B) Exposure of the rat spinal cord (arrow). (C) Electrode plate (made in-house). (D) Electrode plate was placed within the epidural region and fixed firmly. (E) Physical activity in rats after inserting the electrode plate. (F) The rat receiving pulsed electrical stimulation.



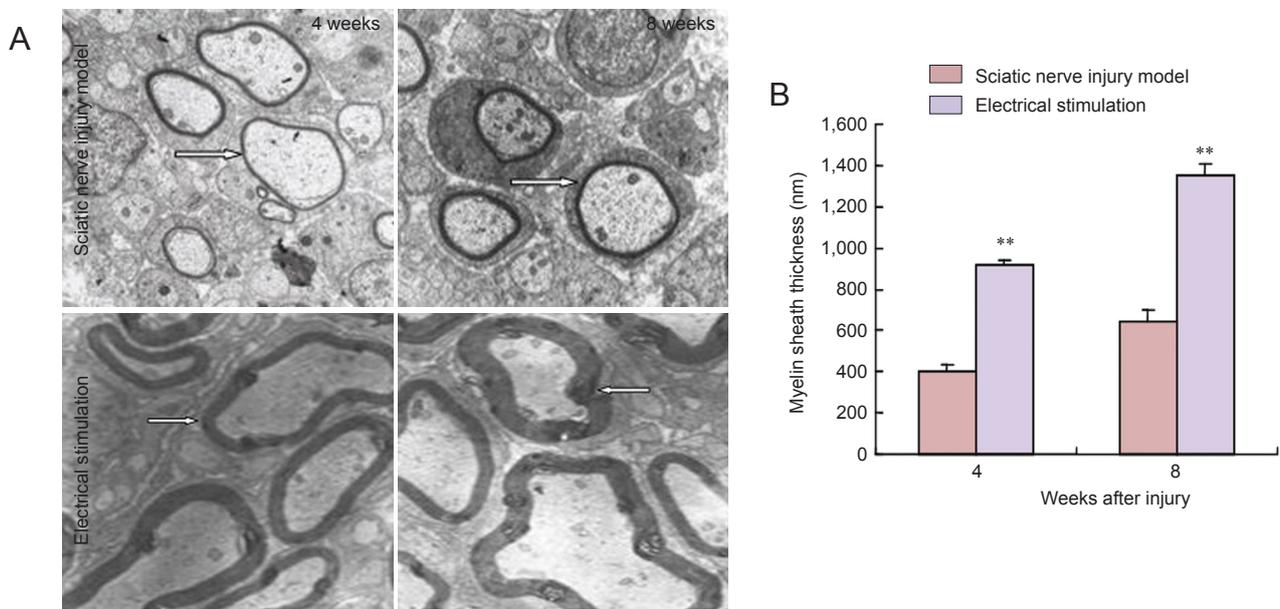
**Figure 2 Effects of pulsed electrical stimulation on the ultrastructure of DRG neurons in rats with sciatic nerve injury.**

(A) Electrical stimulation group at 4 weeks: endosomes (arrow) in DRG neurons (transmission electron microscope,  $\times 10,000$ ). (B) Sciatic nerve injury model group at 4 weeks: nuclear precession (arrow) (transmission electron microscope,  $\times 10,000$ ). (C) Electrical stimulation group at 8 weeks: pseudo-inclusion body (arrow) (transmission electron microscope,  $\times 5,000$ ). (D) Sciatic nerve injury model group at 8 weeks: ciliated cells (arrow) (transmission electron microscope,  $\times 5,000$ ). (E) Number of DRG neurons. Data are expressed as the mean  $\pm$  SD, with five rats in each group. \* $P < 0.05$ , \*\* $P < 0.01$ , vs. sciatic nerve injury model group (independent two-sample  $t$ -test).



**Figure 3** Effects of pulsed electrical stimulation of the spinal cord on the ultrastructure of neurons in the anterior horn of the spinal cord in rats with sciatic nerve injury.

(A) Electrical stimulation group at 4 weeks: Normal neurons in the anterior horn of the spinal cord (transmission electron microscope,  $\times 5,000$ ). (B) Sciatic nerve injury model group at 4 weeks: Nuclear precession (black arrow) and pseudo-inclusion body (white arrow) (transmission electron microscope,  $\times 5,000$ ). (C) Electrical stimulation group at 8 weeks: Nuclear precession (black arrow) (transmission electron microscope,  $\times 10,000$ ). (D) Sciatic nerve injury model group at 8 weeks: Nuclear precession (black arrow) and liposome (white arrow) (transmission electron microscope,  $\times 10,000$ ). (E) Number of neurons in the anterior horn of the spinal cord of rats (light microscope). Data are expressed as the mean  $\pm$  SD, with five rats in each group. \* $P < 0.05$ , \*\* $P < 0.01$ , vs. sciatic nerve injury model group (independent two-sample  $t$ -test).



**Figure 4** Effects of pulsed electrical stimulation of the spinal cord on regenerated sciatic nerve fibers in rats with sciatic nerve injury (transmission electron microscope).

(A) Morphology of regenerated sciatic nerve fibers ( $\times 5,000$ ). Arrows show regenerated nerve fibers. (B) Myelin sheath thickness of regenerated nerve fibers. Data are expressed as the mean  $\pm$  SD, with five rats in each group. \*\* $P < 0.01$ , vs. sciatic nerve injury model group (independent two-sample  $t$ -test).

Under the transmission electron microscope, 4 weeks after injury, in the electrical stimulation group, intracellular organelles in neurons of the anterior horn of the spinal cord were regular, without abnormal mitochondria, and no particles or vacuoles in the cytoplasm were observed. Intact distinct nuclear membranes, uniform nuclear chromatin and distinct nucleoli were visible. Normal mitochondria and endoplasmic reticulum were seen in the neurons of the anterior horn of the spinal cord. In comparison, while intracellular organelles appeared normal, and nuclear membranes were intact, nuclear precession and an increase in heterochromatin were visible in the sciatic nerve injury model group. At 8 weeks, in the electrical stimulation group, nuclear precession and an increase in heterochromatin were seen in neurons of the anterior horn of the spinal cord. The nuclear membranes were intact, and mitochondria and endoplasmic reticulum were normal in the electrical stimulation group. Neuronal atrophy was evident in the anterior horn of the spinal cord. Vacuoles and endosomes were visible in the cytoplasm. Mitochondrial cristae were ruptured, the nuclear membrane was discontinuous, nuclear condensation was noticeable, and heterochromatin was increased in the sciatic nerve injury model group (Figure 3). At 1, 4 and 8 weeks after injury, light microscopy showed that the number of neurons in the anterior horn of the spinal cord was higher in the electrical stimulation group than in the sciatic nerve injury model group (1 and 4 weeks:  $P < 0.01$ ; 8 weeks:  $P < 0.05$ ; Figure 3E).

#### Pulsed electrical stimulation of the spinal cord improved the ultrastructure of regenerated sciatic nerve fibers in rats with sciatic nerve injury

Under the transmission electron microscope, regenerated nerve fibers were visible in both groups. The number of myelinated nerve fibers was less, the myelin sheath was thin, and mild edema was observed in portions of the interstitium in the sciatic nerve injury model group. The number of myelinated nerve fibers was higher in the electrical stimulation group than in the sciatic nerve injury model group ( $P < 0.01$ ). In contrast, the myelin sheath was thick and uniform, without lamellar separation or vacuoles in the electrical stimulation group (Figure 4).

#### Pulsed electrical stimulation of the spinal cord elevated nerve conduction velocity in rats with sciatic nerve injury

Electrophysiological testing revealed that nerve conduction velocity was higher in the electrical stimulation group than in the sciatic nerve injury model group at 4 and 8 weeks after injury ( $P < 0.01$ ; Figure 5).

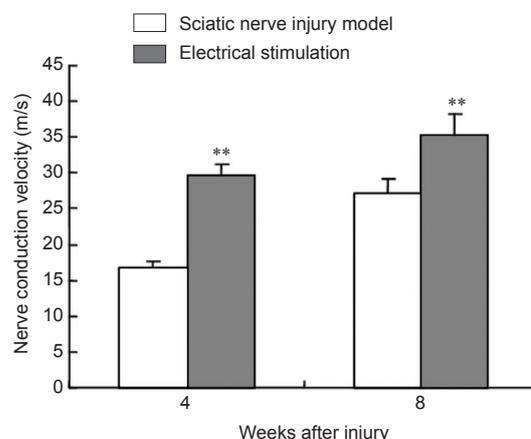
### Discussion

Peripheral nerve injury may induce apoptosis in sensory and motor neurons in the corresponding segments, because retrograde transport is interrupted after axonal injury, and neuronal bodies (especially sensory neurons) cannot obtain neurotrophic factors from the target organs (Tong and Rich, 1997; Nakamura and Myers, 2000). Simultaneously, electro-

physiological changes cause  $\text{Ca}^{2+}$  influx, thereby perturbing intracellular  $\text{Ca}^{2+}$  homeostasis, and activating proteolytic enzymes and endonucleases. This triggers a series of immune responses and results in neuronal apoptosis (Preyat et al., 2015). Mitochondrial changes play an important role in apoptosis. Abnormal expression of the *bax* and *bcl-2* genes alters mitochondrial membrane permeability and triggers apoptosis (Chen et al., 2013).

The aim of treatment strategies for peripheral nerve injury is to restore the continuity and integrity of nerves, to promote axonal regeneration and remyelination, to inhibit neuronal apoptosis, to prevent atrophy of innervated muscles and organs, and to promote functional recovery. A reduction in the number of cell bodies will undoubtedly affect functional recovery after nerve injury. Therefore, effectively preventing damage to central neurons following peripheral nerve injury is the key to promoting nerve regeneration and functional recovery.

In this study, we found that pulsed electrical stimulation of the spinal cord protects DRG neurons and neurons in the anterior horn of the spinal cord following sciatic nerve injury. This indicates that pulsed electrical stimulation of the spinal cord effectively contributes to the regeneration of injured nerve fibers, and improves the recovery of nerve conduction following nerve repair. The mechanisms that may underlie the therapeutic efficacy of pulsed electrical stimulation include the following: (1) The electric field recruits neurotrophic substances, in particular, nerve growth factor. Bandtlow et al. (1993) showed that mature neurons, especially DRG sensory neurons, require trophic support from nerve growth factor. Moreover, pulsed electrical stimulation increases the number of nerve growth factor-positive neurons in spinal lamina II, the dorsal nucleus and the posterior root ganglion of the spared dorsal root. (2) The electric field leads to ion movement, affecting the distribution of intracellular and extracellular ions, reducing  $\text{Ca}^{2+}$  influx, stabilizing the intracellular environment, and blocking neuronal



**Figure 5** Effects of pulsed electrical stimulation of the spinal cord on nerve conduction velocity in injured rat sciatic nerve. Data are expressed as the mean  $\pm$  SD, with five rats in each group. \*\* $P < 0.01$ , vs. sciatic nerve injury model group (independent two-sample *t*-test).

apoptosis (Lee et al., 2012). (3) The electric field expands arterioles and improves local blood flow (Gyawali et al., 2011). The electric field can cause vasodilatation by axon reflex and segmental reflex, by electrolyzing tissue protein, releasing vasoactive peptides, expanding arterioles and improving local blood flow, which improves the intracellular and extracellular microenvironment after neuronal injury. (4) The electric field regulates gene expression. Electric fields enhance *bcl-2* gene expression and decrease *bax* gene expression after sciatic nerve transection (Liang et al., 2013). Furthermore, pulsed electrical stimulation regulates immune, hematopoietic and endocrine functions. For example, it promotes mast cell survival, enhances histamine release, and affects the proliferation and differentiation of lymphocytes (Singh et al., 2011). Together, these varied effects of pulsed electrical stimulation mitigate damage to central neurons following peripheral nerve injury.

In summary, peripheral nerve injury causes neuronal apoptosis in the proximal nerve stump. Pulsed electrical stimulation effectively prevents neuronal apoptosis, maintains the normal structure and function of sensory and motor neurons, and promotes axonal regeneration and functional recovery following peripheral nerve transection. Our findings suggest that pulsed electrical stimulation may have clinical efficacy for treating peripheral nerve injury.

**Author contributions:** BAP and LSW participated in study concept and design. BAP performed experiments, collected data, and wrote the paper. JHZ participated in data processing and statistical analysis. LSW and CHZ were in charge of paper authorization. YZC provided technical or material support, and served as a principle investigator. All authors approved the final version of the paper.

**Conflicts of interest:** None declared.

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