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Electrical stimulation does not enhance nerve regeneration if delayed after sciatic nerve injury: the role of fibrosis

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Abstract

Electrical stimulation has been shown to accelerate and enhance nerve regeneration in sensory and motor neurons after injury, but there is little evidence that focuses on the varying degrees of fibrosis in the delayed repair of peripheral nerve tissue. In this study, a rat model of sciatic nerve transection injury was repaired with a biodegradable conduit at 1 day, 1 week, 1 month and 2 months after injury, when the rats were divided into two subgroups. In the experimental group, rats were treated with electrical stimuli of frequency of 20 Hz, pulse width 100 ms and direct current voltage of 3 V; while rats in the control group received no electrical stimulation after the conduit operation. Histological results showed that stained collagen fibers comprised less than 20% of the total operated area in the two groups after delayed repair at both 1 day and 1 week but after longer delays, the collagen fiber area increased with the time after injury. Immunohistochemical staining revealed that the expression level of transforming growth factor β (an indicator of tissue fibrosis) decreased at both 1 day and 1 week after delayed repair but increased at both 1 and 2 months after delayed repair. These findings indicate that if the biodegradable conduit repair combined with electrical stimulation is delayed, it results in a poor outcome following sciatic nerve injury. One month after injury, tissue degeneration and distal fibrosis are apparent and are probably the main reason why electrical stimulation fails to promote nerve regeneration after delayed repair.

***Key Words:** nerve regeneration; peripheral nerve injury; electrical stimulation; bioabsorbable conduit; delayed repair; fibroblast; collagen fibers; transforming growth factor β; Masson staining; neuroprotection; immunohistochemistry; NSFC grants; neural regeneration*

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Introduction

Electrical stimulation has been shown to effectively improve the speed and accuracy of nerve regeneration after peripheral nerve injury. A previous study found that, *in-situ* epineurium suture after femoral nerve transection and subsequent electrical stimulation led to the regeneration in almost all of the motor neuron axons, up to 25 mm at the distal end within 3 weeks after injury. By contrast, without electrical stimulation, the axons in the control group took 8–10 weeks to regenerate (Al-Majed et al., 2000a). In addition, electrical stimulation is also effective for the promotion of sensory nerve regeneration. At 1 hour after nerve injury repair, electrical stimulation significantly increased the number of regenerating nerves in ganglion neurons towards their cutaneous branches and muscular branches (Brushart et al., 2005; Geremia et al., 2007). In preliminary studies, the 1-hour weak transcranial direct current stimulation promoted nerve regeneration through the gap between the stumps, shortened the time of regenerating axons crossing the stump, and ensured that the regeneration nerve reached the target organ as soon as possible (Brushart et al., 2002, 2005; Geremia et al., 2007; Gordon et al., 2009a, b). Whereas these trials performed electrical stimulation immediately after the repairing surgery of the injured nerve, many patients suffering neurological injury are not able to receive immediate surgical treatment and electrical stimulation in the clinical situation (Spinner and Kline, 2000; Siemionow and Sari, 2004). For example, patients with wound infection or severe trauma have to recover their general and local conditions prior to surgery, which results in the delayed repair of nerve injury. Relative to immediate repair, delayed repair is not beneficial for nerve regeneration and recovery of limb function after injury.

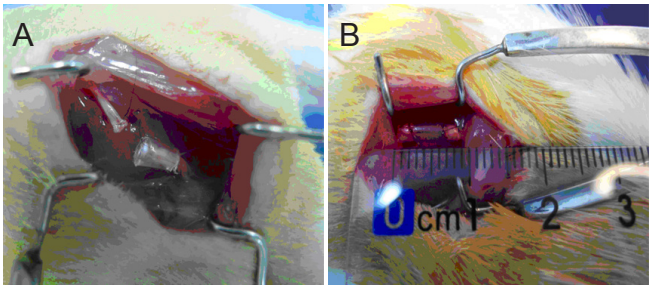

Figure 1 Schematic diagram of delayed repair surgery in rats with nerve injury.
(A) Establishing delayed repair model; (B) repairing nerve stump with bioabsorbable conduit.

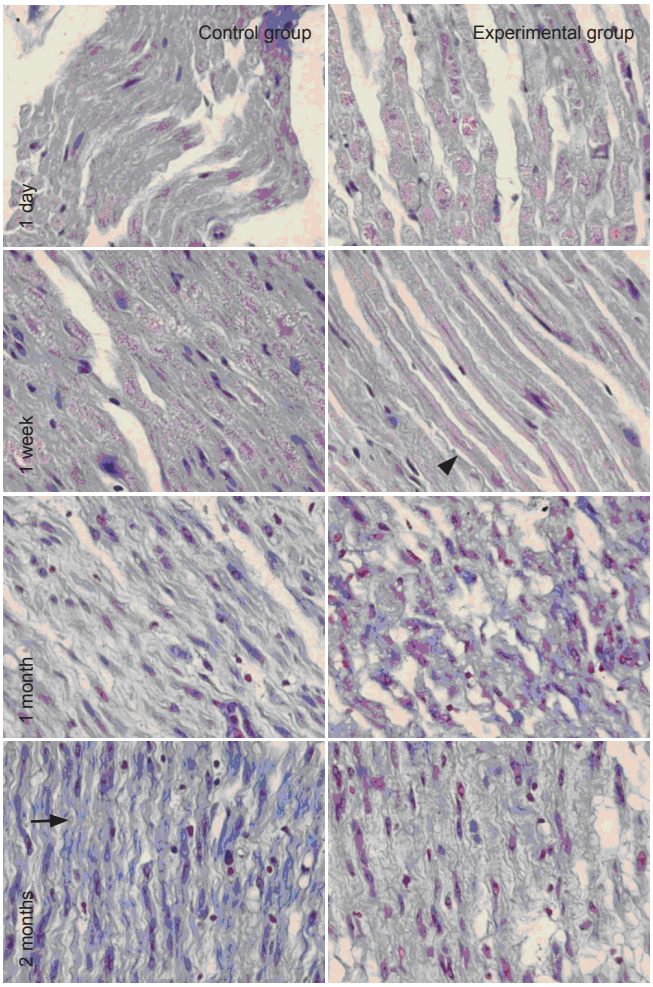

Figure 2 Effect of electrical stimulation on the morphology of regenerating nerve in the distal end of the injured sciatic nerve in rats after delayed repair (Masson staining, light microscope, × 400).
The distal end of the injured sciatic nerve showed normal morphology in both control and experimental groups at 1 day and 1 week after delayed repair, without tissue disintegration and the nerve membrane is blue. At 1 and 2 months after delayed repair, nerve fibers are disarrayed, a large amount of myelin sheaths have degenerated and been lost, and many Schwann cells are damaged and just fragments are observed. Triangular arrowhead points to a normal nerve myelin and the arrow points to a proliferating collagen fiber.

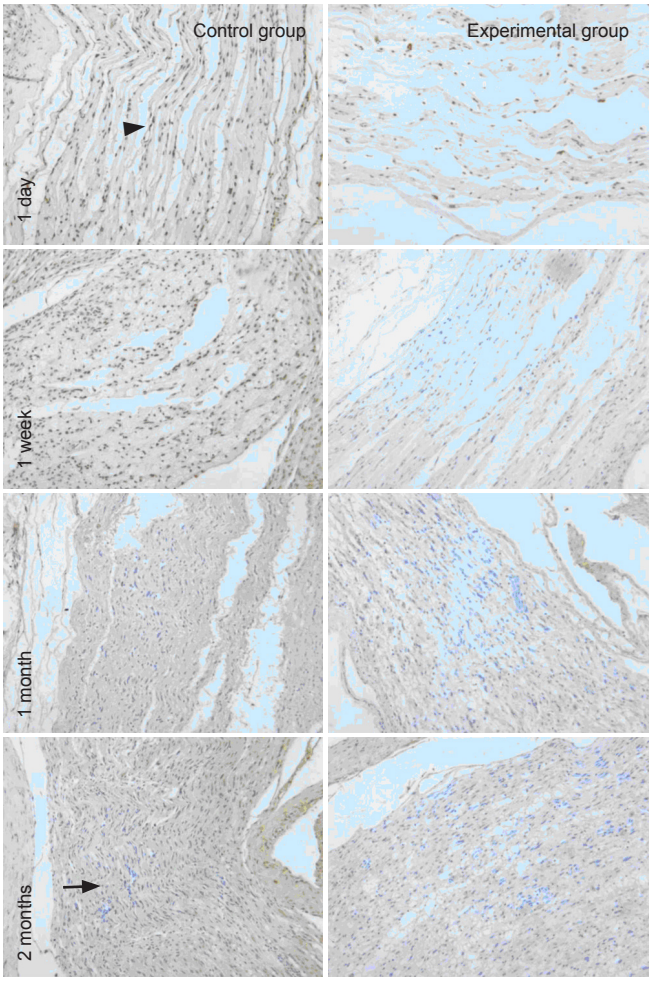

Figure 3 Effect of electrical stimulation on the transforming growth factor β immunoreactivity of regenerating nerve in the distal end of the injured sciatic nerve in rats after delayed repair (immunohistochemical staining, light microscope, × 100).
The density of transforming growth factor β immunoreactivity was enhanced as the time of delayed repair increased in both control and experimental groups, indicating that fibroblasts infiltrate continuously into the distal nerve. The triangular arrowhead and arrow both indicate transforming growth factor β-immunoreactive fibroblasts.

In this study, we used Masson staining and immunohistochemical staining methods to explore whether fibrosis was a reason for electrical stimulation failing to promote nerve regeneration after delayed repair.

Materials and Methods

Experimental animals

Sixty-four 10-week-old female Sprague-Dawley rats weighing 200–250 g were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China; license No. SYXK (Jing) 2011-0010). Experiments were approved by the Ethics Committee of Peking University People's Hospital in China. Rats were housed in the specific-pathogen-free laboratory, under 12-hour light/dark cycle and allowed free access to food and water.

Bioabsorbable conduits

The conduit was a hollow cylindrical sleeve of chitosan (deacetyl chitin; co-invented by Peking University People's Hospital and China Textile Academy, Beijing, China; patent No. 01136314.2), 1 mm in length, 0.1-mm thick and had an internal diameter of 1.5 mm (Zhang et al., 2008).

Establishing a rat model of sciatic nerve transection injury and grouping

Sixty-four Sprague-Dawley rats were randomly divided into four groups (n = 16 per group): delayed repair at 1 day, 1 week, 1 and 2 months. Sixteen rats in each of the four groups were treated with sleeve bridging using a bioabsorbable chitin conduit at 1 day, 1 week, 1 and 2 months after sciatic nerve transection injury. Subsequently, rats in each group were further assigned into two subgroups, one experimental and the other as control, with eight rats in each subgroup. In the experimental subgroups, rats were given electrical stimulation immediately after nerve repair, while rats in the control subgroups received no electrical stimulation.

The sciatic nerve transection injury model was established in the specific-pathogen-free laboratory. In brief, rats were anesthetized with an intraperitoneal injection of 2% sodium pentobarbital (30 mg/kg body weight; Sigma, St. Louis, MO, USA). The sciatic nerve on one side was exposed and then transected with a sharp blade at 1 cm lateral to the proximal end at the bifurcation point. To prevent the growth of the proximal nerve into the distal end after transection, the proximal nerve was embedded in the adjacent muscles and sutured using 10-0 thread. The stump at the distal end was cannulated with a small polyethylene cap (**Figure 1**). The incisions were sutured and the rats were returned to their cages. Rats in each group received secondary surgery at the corresponding time points (1 day, 1 week, 1 and 2 months). In brief, the sciatic nerve was exposed through the original route and was carefully separated from the adhesive tissue. The proximal stump of the sciatic nerve that had been sutured to the muscle and the distal stump of the sciatic nerve cannulated in the cap were identified and isolated, respectively. The neuroma at the nerve stump was removed using micro-scissors. Approximately 1 mm of both the distal and proximal ends of the nerve were inserted in the bioabsorbable chitin conduit with a distance between nerve stumps in the conduit of 2 mm.

Electrical stimulation

At each of the time points, rats in the experimental subgroups had an electrode placed on the proximal nerve, after the nerve had been inserted into the conduit, and the other end of the electrode was connected to an electrical stimulator through the bioabsorbable conduit. The parameters of the electrical stimulator (MedlecSynergy; Oxford Instrument Inc., Oxfordshire, UK) were adjusted as follows: voltage 3 V, frequency 20 Hz, duration 100 ms, for 1 hour. During electrical stimulation, the surgical incisions were covered with wet gauze, to avoid body fluid evaporation. After electrical stimulation was completed, the surgical incision was sutured using a 4-0 suture. In the control group that received no electrical stimuli, the muscle and skin were sutured immediately after the repair surgery. Following surgery, all rats were housed in cages.

At 5 days after the secondary surgery, six rats in each group were randomly selected and killed by decapitation. The nerves in the proximal and distal ends of the bridging sleeve conduit were harvested and stored in liquid nitrogen or 4% paraformaldehyde for further use.

Masson staining

Sciatic nerve tissues were fixed in 4% paraformaldehyde and prepared into longitudinal paraffin sections. Then sections were dewaxed to distilled water, counterstained with Weigert's iron hematoxylin for 5–10 minutes, and differentiated in a mixture of ethanol with hydrochloric acid, followed by rinsing in lotic environment. The blue was developed using dilute ammonia solution for 15 seconds, nerve tissues were stained with acid fuchsin-ponceau de xylidine for 5–10 minutes, then terminated by glacial acetic acid treatment for 1 minute. Last, they were dehydrated with 95% ethanol, then absolute ethanol, cleared and then mounted. Sciatic nerve sections were observed under a light microscope (DM 4000B; Leica, Heidelberg, Germany).

Immunohistochemical staining

The sciatic nerve tissue was fixed in 4% paraformaldehyde and prepared into longitudinal paraffin sections, *i.e.*, parallel to the traveling of the nerves. These sections were then deparaffinized and rehydrated to distilled water and incubated with 3% H_2O_2 for 10 minutes. After PBS washes, antigen retrieval was performed and sections were blocked with normal goat serum for 15 minutes. The sciatic nerve tissues were incubated with rabbit anti-rat TGF-β polyclonal antibody (1:50, Sigma) at 4°C overnight and rinsed with PBS three times. Finally, the sections were incubated with biotin-labeled working solution at 37°C for 30 minutes, rinsed with PBS, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) for 15 minutes. After three PBS washes, sections were developed with DAB for 5 minutes and counterstained with hematoxylin for 2 minutes, followed by tap water washes for 10–15 minutes. The stained sections were dehydrated, cleared, mounted and observed under a light microscope (Leica).

Depending on the number of TGF-β immunoreactive cells, TGF-β immunoreactivity can be classified as: weak ("+", the number of immunoreactive cells is less than 25% of the total cells), moderate ("++", the number of immunoreactive cells is between 25% and 49% of the total) or strong ("+++", the number of immunoreactive cells is more than 50% of the total).

Results

Effect of electrical stimulation on the general condition of rats with sciatic nerve injury after delayed repair

At 5 days after delayed repair, rats appeared to experience

varying degrees of ankle sagging and reduced mobility whether electrical stimulation was given or not. These phenomena such as ankle sagging and reduced mobility were possibly related to the recovery time.

Effect of electrical stimulation on the morphology of regenerating nerve in the distal end of the injured sciatic nerve in rats after delayed repair

Masson staining results showed that the distal end of the injured sciatic nerve had normal morphology and tight arrangement in both of control and experimental groups at 1 day and 1 week after delayed repair; there was no tissue disintegration, the nerves were parallel to each other and their axons were continuous and surrounded by a thick layer of reddish myelin. Schwann cells were stained red-brown in the nuclei and blue in the membrane, with normal morphology. At 1 and 2 months after delayed repair, some nerve fibers had disappeared and the remaining fibers were disorganized; a large amount of myelin sheath had degenerated and been lost and many Schwann cell fragments were observed. In addition, massive collagen fibers were seen scattered between the disintegrating nerve fiber tissues, the ratio of collagen fibers to nerve fibers gradually increased as the time before the delayed repair increased. Wallerian degeneration and deinnervated Schwann cells were found around collagen fibers (**Figure 2**).

Effect of electrical stimulation on the TGF-β immunoreactivity of regenerating nerve in the distal end of the injured sciatic nerve in rats after delayed repair

At 5 days after delayed repair, the distal end of the injured sciatic nerve at the operated side was prepared into longitudinal paraffin sections. Immunohistochemical staining showed that the density of TGF-β immunoreactivity was enhanced as the time of delayed repair increased in both control and experimental groups. At 1 day after delayed repair, the TGF-β immunoreactivity was weak, while the TGF-β immunoreactivity was moderate in these groups at 1 week after delayed repair. TGF-β immunoreactivity at 1 and 2 months after delayed repair was strong. However, at each time point, no difference in numbers of TGF-β-immunoreactive cell between the subgroups was observed. This evidence indicates that, fibroblasts infiltrated continuously into the distal nerve before the delayed repair. This result was consistent with the results of Masson staining, which revealed less infiltration of fibroblasts, less synthesis and expression of collagen fibers within a short term after nerve transection. The number of fibroblasts was increased with time and a large amount of collagen fibers were found within nerve tissue (**Figure 3**).

Discussion

Peripheral nerve injury may lead to the complex physiological and pathological changes, including morphological changes (Cajal, 1928) and cell metabolism (Fu and Gordon, 1997). After injury, there was retrograde decomposition of the proximal nerve stump to the node of Ranvier nearest to the injury, and then a growth cone grew several axonal buds toward the distal nerve (Fawcett and Keynes, 1990). It is estimated that the growth rate of regenerating buds is 1–3 mm every day, which may vary slightly among different species. However, the growth of regenerating buds may be delayed in the area of the nerve injury (Gutmann et al., 1942). Previous studies found that it is about 1 month before all the motor nerve fibers grew and crossed over the nerve suture site (Witzel et al., 2005). During this period (1 month), the distal nerve and muscles degenerated continuously. The function of Schwann cells in the distal end gradually attenuated as the time of denervation is prolonged and the Schwann cells become apoptotic. Scarring appears at the motor endplate at the nerve-muscle junction, acetylcholine receptors disappear and the muscle tissue begins to degenerate after denervation. Therefore, the delayed time of the regenerating nerve across the injury site is one of the main causes leading to long-term denervation of the distal target organ and to poor recovery of limb function. Methods to shorten the time of the regenerating nerve crossing through the injury site have gained increasing attention in the field of nerve regeneration. Previous studies found that, the 1-hour weak transcranial direct current stimulation promoted nerve regeneration across the gap between the stumps and shortened the time of regeneration, ensuring the regenerating nerve reached the target organ as soon as possible (Brushart et al., 2002; Gordon et al., 2009a, b). However, these trials performed electrical stimulation immediately after nerve injury, unlike the majority of nerve injury patients who receive surgical treatment at 5–7 days post-injury (Brushart et al., 1983). Frequently physicians have to postpone the repair surgery of peripheral nerve injury because of other factors such as a wide range of soft tissue defects caused by injury, wound pollution, priority to treat life-threatening injuries, extensive avulsion and crush injuries (Terry Canale and James Beaty, 2009). In these situations of delayed repair it is important to know whether electrical stimulation would promote nerve regeneration. The preliminary studies of our research group found that the promotion effect of electrical stimulation on nerve regeneration was associated with the time of delayed repair after peripheral nerve injury (Xu et al., 2014). We confirm that electrical stimulation given within 1 month post-injury is effective to promote nerve regeneration, but intervention at 1 month post-injury is ineffective.

The validity of electrical stimulation after delayed repair of nerve injury has to be assessed for two reasons. On the one hand, the nerve cell bodies in spinal cord and dorsal root ganglia are prone to die as the denervation time increases. Growing evidence shows that neurotrophic factors, such as brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5, can reduce retrograde death of motor nerve fibers, maintain its cholinergic phenotype and promote motor nerve fiber regeneration after peripheral nerve transection injury (Yan et al., 1994; Novikov et al., 1995; Wang et al., 1997; Fernandes et al., 1998). The survival of motor neurons and the resultant expression of nerve growth factors underlie the mechanism of peripheral nerve regeneration by electric stimulation (Al-Majed et al., 2000b, 2004). On the other hand,

Schwann cells become apoptotic and only react slightly to the signal expression of axonal regeneration after long-term denervation in the distal stump (Li et al., 1997). Destruction of the Schwann cells' environment is unfavorable for nerve regeneration after delayed repair of nerve injury.

In this study, we established animal models of nerve injury after delayed repair to observe the promotion effect of electrical stimulation on the nerve regeneration. The rat sciatic nerve was transected and the repair surgery was delayed for various durations (1 day, 1 week, 1 and 2 months). Results showed that, the area ratio of Masson stained collagen fibers was less than 20% in two of the groups, at 1 day and 1 week after delayed repair, and that the collagen fiber area was gradually increased along with the time of repair post-injury but the nerve fibers were still visible, with abundant myelin sheath and the infiltration of TGF-β-stained fibroblasts was slight. After 1 month of delayed repair, nerve fibers had completely disintegrated, continuous nerve structures were replaced by the scattered Schwann cells and collagen fibers, and the density of TGF-β-stained fibroblasts had increased. Previous studies have shown that TGF-β is a multifunctional cytokine that is involved in the regulation of cell proliferation and differentiation, stimulation of the synthesis and secretion of various cytokines and inflammatory mediators, as well as an increase in the areas of extracellular matrix. TGF-β plays a crucial role in tissue fibrosis and is representative of the cytokines that contribute to induce the fibrosis of various tissues (Brushart et al., 1983). At 1 and 2 months after delayed repair, TGF-β expression was upregulated compared with that at 1 day and 1 week after delayed repair, as detected by immunohistochemical staining. This evidence indicates that, the degree of fibrosis in the distal nerve tissue clearly increased as the repair time prolonged. After 1 month of peripheral nerve injury, Schwann cell apoptosis and tissue fibrosis in the distal nerve could be the main causes of electrical stimulation failing to promote nerve regeneration after delayed repair.

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Author contributions: *PXZ and FX designed the study. NH, CGX, YHK and XFY implemented experiments. TBW and FX evaluated the study. NH and CGX collected the data. YHK analyzed the data. NH and CGX wrote the paper. PXZ revised the paper. NH was responsible for the paper. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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