Biological conduit small gap sleeve bridging method for peripheral nerve injury: regeneration law of nerve fibers in the conduit

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Abstract
The clinical effects of 2-mm small gap sleeve bridging of the biological conduit to repair peripheral nerve injury are better than in the traditional epineurium suture, so it is possible to replace the epineurium suture in the treatment of peripheral nerve injury. This study sought to identify the regeneration law of nerve fibers in the biological conduit. A nerve regeneration chamber was constructed in models of sciatic nerve injury using 2-mm small gap sleeve bridging of a biodegradable biological conduit. The results showed that the biological conduit had good histocompatibility. Tissue and cell apoptosis in the conduit apparently lessened, and regenerating nerve fibers were common. The degeneration regeneration law of Schwann cells and axons in the conduit was quite different from that in traditional epineurium suture. During the prime period for nerve fiber regeneration (2–8 weeks), the number of Schwann cells and nerve fibers was higher in both proximal and distal ends, and the effects of the small gap sleeve bridging method were better than those of the traditional epineurium suture. The above results provide an objective and reliable theoretical basis for the clinical application of the biological conduit small gap sleeve bridging method to repair peripheral nerve injury.

Key Words: nerve regeneration; peripheral nerve; small gap; axons; Schwann cells; repair; injury; biological conduit; NSFC grants; neural regeneration

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Introduction
The effective repair of peripheral nerve injury depends on the accurate abutment of nerve fibers with different natures at the proximal and distal ends (Li et al., 2007; Huang et al., 2010a). Epineurium and perineurium sutures commonly used in the clinic cannot achieve the effective accurate abutment of nerve fibers with different natures (Jiang et al., 1994; Jiang and Yoshida, 1998; Jiang and Li, 2003; Mohammadi et al., 2014). The selective regeneration of the peripheral nerve provides some theoretical basis for a biological conduit small gap sleeve bridging method in the repair of peripheral nerve injury (Lundborg, 2000; Jiang et al., 2006, 2010). Previous animal experiments and multi-center clinical trials have suggested that the repair effects of the 2-mm small gap sleeve bridging of the biological conduit in the repair of peripheral nerve injury are better than those in the commonly-used epineurium suture (Zhang et al., 2008, 2009, 2011, 2013). The deacetylated chitin biological conduit used in these studies not only provides a suitable microenvironment for nerve regeneration, but is also non-toxic to organisms, can be completely degraded and absorbed in vivo, and has good biocompatibility (Yu et al., 2009; Zhang et al., 2010). The corresponding production process and repair methods have been granted patents (Yu et al., 2009; Zhang et al., 2010). However, the regeneration law of nerve fibers in the biological conduit is not clear. Thus, this study investigated the changes in the microenvironment of the nerve stump, and the change trend of the nerve fibers and the myelin sheath in the biological conduit.

Materials and Methods
Animals
108 male specific-pathogen-free Sprague-Dawley rats, weighing 250 ± 4.5 g and aged 3 months old, were purchased from the Beijing Institute of Xieerxin Biology Resource, China (Animal license No. SCXK (jing) 2013-0001) and included in this
obtained from each group. Rats were intraperitoneally anesthetized with 2% sodium pentobarbital, and fixed in the supine position. An incision was made in the middle of the chest to expose the thoracic cavity. An infusion needle was inserted in the cardiac apex. After turning on the saline side of the tee pipe and cutting the right auricle, perfusion was conducted until the liver became white. Subsequently, the specimen was perfused with 4% paraformaldehyde for 30 minutes. The right sciatic nerve was obtained after repair, and post-fixed in paraformaldehyde for 4–5 hours. The position of sample collection is shown in Figure 2. The sciatic nerve was divided into three parts: injured segment, proximal injured segment and distal injured segment. Specimens after postfixation were immersed in sucrose solution over night at 4°C, embedded in an optimal cutting temperature embedding medium, and sliced into 7-μm frozen sections. These sections were dried at room temperature for 24 hours and stored in a refrigerator.

Hematoxylin-eosin staining
As shown in Figure 2, paraffin sections of rat sciatic nerve were dewaxed, hydrated, and stained with hematoxylin and eosin, and then observed using a light microscope (Olympus).

Immunofluorescence staining
As displayed in Figure 2, frozen sections of rat sciatic nerve were fixed in acetone at −20°C for 20 minutes, and washed three times with 0.3% Triton X-100/PBS, each for 5 minutes. These sections were then blocked with 10% normal goat serum for 1 hour, incubated with mouse anti-rat glial fibrillary acidic protein monoclonal antibody (1:200; Sigma-Aldrich, St. Louis, MO, USA) and mouse anti-rat NF200 monoclonal antibody (1:200; Sigma-Aldrich) at room temperature overnight, washed three times with 0.3% Triton X-100/PBS, each for 5 minutes. The sections were incubated with Cy2 and Cy3 conjugated rabbit anti-mouse IgG (1:200; Friendship Biotechnology, Beijing, China) at room temperature for 1 hour, and washed three times with 0.3% Triton X-100/PBS.
Cell apoptosis was measured with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis method at 3 days after model establishment (× 400).

(A–C) At 7, 28 and 56 days after model establishment, interstitial edema between the sciatic nerve disappeared and the number of capillary vessels increased with prolonged time. Arrows show capillary vessels.

Each for 5 minutes. These sections were then mounted with a fluorescent mounting medium (Fluoro-Gel; Head (Beijing) Biotechnology Co., Ltd., Beijing, China) and observed with a fluorescence microscope (Olympus). Immunofluorescence images were overlayed using Photoshop software CS2 V9.0 (Adobe, San Jose, CA, USA) (superposition of red and green). Red fluorescence represents axons: the primary antibody is NF, and the secondary antibody is Cy2 conjugated rabbit anti-mouse IgG; green fluorescence represents Schwann cells: the primary antibody is glial fibrillary acidic protein, and the secondary antibody is Cy3-conjugated rabbit anti-mouse IgG. Schwann cells and axons were quantified in nerve specimens within the cross-section range using Image-pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Cell apoptosis

Cell apoptosis was measured with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis method at 3 days after model establishment (× 400).

(A–C) At 7, 28 and 56 days after model establishment, interstitial edema between the sciatic nerve disappeared and the number of capillary vessels increased with prolonged time. Arrows show capillary vessels.
At 3 days after injury, compared with the control group, the rat models after repair by sleeve bridging method showed that the number of apoptotic cells was significantly higher in the sites proximal and distal to the injured sciatic nerve and the region proximal to the conduit in the sleeve bridging group \( (P < 0.05) \). In the sleeve bridging group, compared with the distal end of the injured site, the number of apoptotic cells was significantly less in the sites proximal to the injured sciatic nerve and the conduit \( (P < 0.05; \text{Figure 6}) \).

### Changes in Schwann cells and axons in the region with sciatic nerve injury in rat models after repair by biological conduit small gap sleeve bridging method

**Proximal end of injured sciatic nerve**

Immunofluorescence staining demonstrated that the morphology of Schwann cells and axons was similar at the proximal end of the injured nerve in the control group and sleeve bridging group, with uniform size, regular arrangement and high density at the time of nerve injury. As time increased, the number of Schwann cells and axons gradually decreased at the proximal end of the injured sciatic nerve in the sleeve bridging group, and was significantly different from the control group at 5 and 7 days \( (P < 0.01) \). The number of Schwann cells and axons in the proximal end of the injured sciatic nerve of rats from the control group was increased at 3–5 days, peaked at 5 days, and then decreased, reached its lowest point at 7 days, peaked at 14 days, and then reduced, but it was significantly lower than the sleeve bridging group at 28 days \( (P < 0.01) \). The difference in the change curves of the number of Schwann cells and axons in the proximal end of the injured sciatic nerve is possibly one factor in regeneration differences between the conduit suture and the epineurium suture (Figure 7).

**Distal end of injured sciatic nerve**

Immunofluorescence staining performed at 5 days after injury revealed that the axons and myelin were scattered, and the nerve fibers were in the disintegration stage. The number of complete nerve fibers was few, and nerve fibers were irregular in the distal end of the injured nerve in both the control and sleeve bridging groups. At 28 days, the number of Schwann cells and axons increased and were tightly distributed, and abundant newly born tiny myelinated nerve fibers were observed in the distal end of the injured sciatic nerve in both groups. The number of axons in the sleeve bridging group gradually diminished with prolonged time, reached its lowest point at 5 days, and then increased gradually, and became significantly greater than the control group at 28 days \( (P < 0.01) \). Nevertheless, the number of Schwann cells was lower in the sleeve bridging group than that in the control group at 5 days after injury \( (P < 0.01) \), gradually increased, and was significantly higher than the control group at 28 days \( (P < 0.01; \text{Figure 8}) \).

### Discussion

Common methods for repairing peripheral nerve injury use epineurium or perineurium suture (Schmidt et al., 1997; Al-luin et al., 2009; Singh et al., 2012; Farjah et al., 2013). Functional recovery after peripheral nerve repair mainly depends...
on two factors: (1) effective abutment of the proximal and distal ends of the nerve fibers with different nature (Cheng and Zochodne, 2002; Biazar and Keshel, 2013; Pabari et al., 2014; Sachanandani et al., 2014). (2) Effective reinnervation of distal target organs (Gigo-Benato et al., 2010; Zhao et al., 2013). The outcomes of biological conduit small gap sleeve bridging have been shown to be better than those using epineurium suture (Zhang et al., 2008, 2009, 2011, 2013). This study demonstrated that histocompatibility was satisfactory, and no common neuroma was detected at stumps after repair with the biological conduit. The biological conduit probably plays a mechanical guiding role, guiding the growth of regenerating axons at the proximal end to the distal end, and preventing the escape of regenerating axons. In the sleeve bridging and control groups, the regeneration laws of Schwann cells and axons were slightly different. During the prime nerve fiber regeneration time (2–8 weeks) after repair, the number of Schwann cells and nerve fibers was higher in the sleeve bridging group than in the control group. At 3 days, the number of apoptotic cells was higher at the distal and proximal ends of the conduit and within the conduit than that in the control group, suggesting that the microenvironment of the conduit effectively promoted the removal of necrotic cells and tissues in the early stage of nerve injury. Additionally, the above local microenvironment is more conducive to regenerating the peripheral nerve.

Peripheral nerve injury causes complicated changes in the proximal and distal ends of the nerve. Wallerian degeneration was observed at the distal end. Schwann cell proliferation formed Büngner’s band, provided channel for the axonal growth of regenerating motor neurons, and secreted various extracellular matrix components related to nerve regeneration (Huang et al., 2010b; Wang et al., 2010). In the control group (traditional epineurium suture), the proliferation time of Schwann cells in the proximal and distal ends of the conduit appeared early (day 5), and the number of Schwann cells was noticeably greater than in the sleeve bridging group. Nevertheless, 2 weeks later, the number of Schwann cells at the proximal and distal ends of the conduit was higher than in the epineurium suture group. Based on previous research findings and clinical experience analysis, there is a significant delay of about 1 week in early regeneration, and ≥2 weeks is the prime regeneration time after peripheral nerve repair. The number of Schwann cells and axons was significantly higher in the biological conduit group than in the epineurium suture group within 3–8 weeks of repair (Sun et al., 2002a, b, c, d), which may be the reason why the effects of nerve regeneration at the distal end in the biological conduit group was better than that in the control group (Jiang et al., 2007).

The results of this study confirmed that during the prime time of regeneration (2–8 weeks), not only the number of Schwann cells, but the number of regenerating axons was significantly higher in the biological conduit group than in the epineurium suture group. Previous studies verified that the number of axons and the axonal cross-sectional area were greater than that at the proximal end of the injured nerve, which has also been called multiple-bud regeneration, i.e., multiple amplification, in peripheral nerve regeneration (Jiang et al., 2007; Wang et al., 2014; Zhang et al., 2014). The relatively close regenerative microenvironment constructed by the 2-mm small gap sleeve bridging of the biological conduit was adequate to allow multiple-bud axonal regeneration (Li et al., 2014; Yan et al., 2014), which may be a reason why repair effects are better than when using the traditional epineurium suture.

In summary, this study first compared the change trends of the number of Schwann cells and axons at the distal and proximal ends after peripheral nerve repair using a biological conduit sleeve bridging method, which provides a solid theoretical foundation for the use of biological conduit small gap sleeve bridging to treat peripheral nerve injury.

Author contributions: NH and YHK participated in study concept and design. PXZ and BGJ were responsible for fund-raising. LYA provided the data and performed experiments. PXZ wrote the manuscript. FX was in charge of manuscript authorization. XPY and TBW participated in statistical analysis. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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Figure 5 Alterations in sciatic nerve in the rat sciatic nerve bundle at 7 days after repair using 2-mm small gap sleeve bridging of the biological conduit (hematoxylin-eosin staining).
(A, B) Proximal and distal ends of sciatic nerve (× 100); (C) nerve in the conduit (× 50). A few nerve fibers extended to the distal end across the biological conduit.

Figure 7 Effects of 2-mm small gap sleeve bridging of the biological conduit on Schwann cells and axons in the proximal end of the rat models of sciatic nerve injury.
(A, B) Morphology of Schwann cells and axons at the proximal end of the injured nerve in rats at 5 and 14 days after model establishment (immunofluorescence staining, × 400). Positive expression of neurofilament shows red fluorescence, stain is Cy2, representing axons; positive expression of glial fibrillary acidic protein shows green fluorescence, stain is Cy3, representing Schwann cells. (C, D) Alterations in the number of Schwann cells and axons in the proximal end of the injured nerve of rats. Data are expressed as the mean ± SD. Six rats were used in each group. The difference was compared using one-way analysis of variance and independent-sample t-test. *P < 0.05, **P < 0.01, vs. control group.
Figure 8 Effects of 2-mm small gap sleeve bridging of the biological conduit on Schwann cells and axons in the distal end of the rat models of sciatic nerve injury.

(A, B) Morphology of Schwann cells and axons in the distal end of injured nerve in rats at 5 and 28 days after model establishment (immunofluorescence staining, × 400). (C1, 2) Superposition of Schwann cells and axons in the distal end of injured nerve in sleeve bridging group and control group rats at 56 days after modeling (immunofluorescence staining, × 400). Positive expression of neurofilament shows red fluorescence, stain is Cy2, representing axons; positive expression of glial fibrillary acidic protein shows green fluorescence, stain is Cy3, representing Schwann cells. (D, E) Alterations in the number of Schwann cells and axons in the distal end of injured nerve of rats. Data are expressed as the mean ± SD. Six rats were used in each group. The difference was compared using one-way analysis of variance and independent-sample t-test. **P < 0.01, vs. control group.
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