Chemoattractive capacity of different lengths of nerve fragments bridging regeneration chambers for the repair of sciatic nerve defects

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Abstract
A preliminary study by our research group showed that 6-mm-long regeneration chamber bridging is equivalent to autologous nerve transplantation for the repair of 12-mm nerve defects. In this study, we compared the efficacy of different lengths (6, 8, 10 mm) of nerve fragments bridging 6-mm regeneration chambers for the repair of 12-mm-long nerve defects. At 16 weeks after the regeneration chamber was implanted, the number, diameter and myelin sheath thickness of the regenerated nerve fibers, as well as the conduction velocity of the sciatic nerve and gastrocnemius muscle wet weight ratio, were similar to that observed with autologous nerve transplantation. Our results demonstrate that 6-, 8- and 10-mm-long nerve fragments bridging 6-mm regeneration chambers effectively repair 12-mm-long nerve defects. Because the chemoattractive capacity is not affected by the length of the nerve fragment, we suggest adopting 6-mm-long nerve fragments for the repair of peripheral nerve defects.

Key Words
nerve fragment length; nerve regeneration chamber; bridging; long nerve defect; chemotactic ability; neural regeneration

Research Highlights
(1) The 6-, 8- and 10-mm-long nerve fragments, bridging 6-mm regeneration chambers, can effectively repair 12-mm-long nerve defects, and the effect is similar to that of autologous nerve transplantation.
(2) To reduce nerve loss, we suggest adopting 6-mm-long nerve fragments for the repair of peripheral nerve defects.

INTRODUCTION
Autologous nerve grafting is the gold standard in the clinical treatment of long-fragment nerve defects[1]. However, sources for the graft are limited, and the procedure may result in nerve loss-of-function in the donor region. Accumulating evidence indicates that short nerve fragments, harvested from the distal end of the defective nerve, can be used to bridge lesions in a regeneration chamber. This procedure essentially transforms the long defect into two short defects. This exploits the chemoattractive capability of the ends, and nerve repair can be accomplished by neurotropism[2]. The short nerve fragment between the two neural regeneration chambers provides substantial chemotactic signals for the regenerating axons[3], and the most suitable chemotactic distance between the two nerve stumps in the regeneration chamber was found to be 6 mm[4]. Lee et al[5]
demonstrated that distal nerve volume influences the number of regenerating axons. However, it is unclear whether the chemoattractive ability of nerve fragments harvested from the bridging regeneration chamber is also affected by volume. A preliminary study by our research group demonstrated that a 6-mm-long bridging regeneration chamber is equivalent to autologous nerve grafts for the repair of 12-mm-long nerve defects. In the present study, we aimed to explore the differences in chemoattractive ability of different lengths of nerve fragment, and to define the minimal length of nerve fragment capable of promoting a similar degree of regeneration as autologous nerve grafts.

RESULTS

Quantitative analysis of experimental animals
Forty rabbits were used in this study to prepare 12-mm-long sciatic nerve defects, and were randomly divided into four groups (Table 1). All 40 rats were involved in the final analysis.

Behavioral and gross observations
All rabbits exhibited difficulty in walking and hind limb weakness after operation. Different degrees of foot swelling were visible at 1 week, and foot skin ulceration occurred at 4 weeks. Symptoms were not serious in group D, and there were no significant differences among the remaining three groups. At 8 weeks, foot ulcers had essentially healed, and at 12 weeks, muscle atrophy apparently improved, plantar flexion exercises could be performed by all animals, and the feet reacted to skin puncture. These improvements were better in group D than in the other groups. The sciatic nerve was surgically exposed at 16 weeks according to the original incision. In groups A, B and C, the regeneration chamber had degraded and become thinner, but the structure was intact. The regenerating nerve was well-connected with the two ends without apparent expansions, and there was no obvious adhesion with the adjacent tissue. In group D, there was a slight expansion between the regenerating nerve and nerve stumps.

Electrophysiological changes in the injured sciatic nerve
At 16 weeks postoperatively, the injured sciatic nerve was separated, and the nerve conduction velocity (m/s) in groups A, B, C and D were found to be 35.04 ± 3.38, 35.46 ± 3.72, 36.02 ± 4.09 and 37.31 ± 3.21, respectively. One-way analysis of variance showed no significant difference among these groups (P > 0.05).

Determination of the wet weight of the gastrocnemius innervated by the injured sciatic nerve
At 16 weeks after surgery, the bilateral gastrocnemius muscles were removed for wet weight assessment. The muscle weight and size were decreased on the experimental side compared with the normal side. The gastrocnemius wet weight ratio (%) in groups A, B, C and D was 0.653 ± 0.021, 0.656 ± 0.034, 0.659 ± 0.030 and 0.675 ± 0.042, respectively, with no significant difference by one-way analysis of variance (P > 0.05).

Histological changes in the injured sciatic nerve
At 16 weeks post-surgery, hematoxylin-eosin staining and image analysis showed that the number of regenerating nerve fibers was increased, thickened and arranged orderly at 4 ± 2 mm and 10 ± 2 mm from the proximal anastomosis site in groups A, B, C and D. The axons and myelination were in good maturation, and myelin thickness was uniform. At 24 ± 2 mm away from the proximal anastomosis, the number of regenerating nerve fibers and their diameter, as well as myelin sheath thickness, were decreased slightly (Figure 1). There were no significant differences in the average number or diameter of regenerating nerve fibers, or in myelin sheath thickness, in the different groups (P > 0.05; Table 2). At 16 weeks, the nerve fiber quality and myelin thickness of the regenerated nerve at 4 ± 2 mm and 10 ± 2 mm distant from the proximal anastomosis site were better than at 24 ± 2 mm away from the proximal anastomosis site for groups A, B, C and D.

Ultrastructural changes in the injured sciatic nerve
At 16 weeks postoperatively, transmission electron microscopy showed that there were many regenerated myelinated nerve fibers in groups A, B and C (Figure 2). The myelin sheath was uniform in thickness and complete, the lamellar structure was tight, and the axonal membrane was closely apposed to the myelin membranes, and a certain number of mitochondria, microfilaments and microtubules were visible. There was no evident difference compared with group D.

Table 1
| Repairing method for the nerve defect in rabbits (n = 10) |
|----------------------------------------------------------|
| Group | Repairing method                                      |
|-------|-------------------------------------------------------|
| A     | Regeneration chamber 6 mm + autologous nerve fragment 6 mm + regeneration chamber 6 mm |
| B     | Regeneration chamber 6 mm + autologous nerve fragment 8 mm + regeneration chamber 6 mm |
| C     | Regeneration chamber 6 mm + autologous nerve fragment 10 mm + regeneration chamber 6 mm |
| D     | Autologous nerve fragment 12 mm |
Table 2  Number and diameter of regenerating sciatic nerve fibers (n/mm²), the diameter of regenerating nerve fibers (μm) and myelin sheath thickness (μm) at postoperative 16 weeks

| Site                                  | Group | Number of nerve fiber | Diameter of nerve fiber | Myelin sheath thickness |
|---------------------------------------|-------|-----------------------|-------------------------|-------------------------|
| 4 ± 2 mm away from the proximal anastomosis site | A     | 3 358.90±541.95       | 7.29±0.35               | 2.01±0.26               |
|                                       | B     | 3 350.30±518.98       | 7.28±0.50               | 1.98±0.30               |
|                                       | C     | 3 366.40±513.15       | 7.27±0.30               | 1.96±0.21               |
|                                       | D     | 3 412.70±526.97       | 7.30±0.19               | 2.09±0.12               |
| 10 ± 2 mm away from the proximal anastomosis site | A     | 3 210.00±566.18       | 7.26±0.36               | 1.97±0.27               |
|                                       | B     | 3 192.30±599.01       | 7.24±0.51               | 1.95±0.31               |
|                                       | C     | 3 206.30±512.49       | 7.23±0.30               | 1.93±0.23               |
|                                       | D     | 3 375.40±332.57       | 7.45±0.23               | 2.05±0.13               |
| 24 ± 2 mm away from the proximal anastomosis site | A     | 3 097.10±545.74       | 7.21±0.36               | 1.93±0.29               |
|                                       | B     | 2 985.40±490.73       | 7.18±0.51               | 1.89±0.31               |
|                                       | C     | 2 902.40±506.23       | 7.15±0.31               | 1.84±0.24               |
|                                       | D     | 3 250.50±308.66       | 7.41±0.24               | 2.01±0.15               |

Data were expressed as mean ± SD of ten rabbits in each group. One-way analysis of variance showed no significant difference among these groups (P > 0.05). Group A: 6-mm nerve fragment bridging group; group B: 8-mm nerve fragment bridging group; group C: 10-mm nerve fragment bridging group; group D: the autologous nerve grafting group.
DISCUSSION

Correlation between the length of nerve fragment and chemoattractive ability

Following nerve injury, Schwann cells at the nerve stump exhibit hyperplasia and proliferate, and various neurotrophic factors are expressed, thereby promoting axonal ingrowth into the nerve distal stump. Similarly, proliferating Schwann cells migrate into the regeneration chamber, forming the myelin for the regenerating axons. In this study, the harvested nerve fragment was of the same thickness, i.e., having a similar cross-sectional area, and the number of surviving Schwann cells was similar. In contrast, when nerve fragments are cut into different lengths, such as 6, 8 or 10 mm, the total number of Schwann cells also differs. In the present study, we found no significant difference in the number of regenerating nerve fibers in the distal nerve stumps in groups A, B, C or D. This is evidence that the chemoattractive ability of the regeneration chambers is similar, revealing that chemoattractive ability is not affected by the length or size of the nerve fragment used to bridge the regeneration chambers, or by Schwann cell quality within the nerve fragment. Whether the chemoattractive ability affects the thickness of the nerve fragment or the number of Schwann cells is unclear and requires further study.

Feasibility of cutting nerve fragments less than 4 mm in length

The epineurium should be incised if the nerve graft diameter is greater than 2 mm, to permit the penetration of nutrients and to reduce the death of Schwann cells. A 6-mm-long nerve fragment bridging the regeneration chambers was inserted into the chambers 1 mm on each side. Consequently, these 1-mm portions of nerve become impermeable. Only nerve fragments greater than 4 mm in length can connect with soft tissue. If the nerve is thicker than 2 mm in diameter, the outer membrane is opened and the nerve is flattened, thus reducing the diameter and providing better access to nutrients for Schwann cells, thereby promoting their survival. If 4-mm-long nerve fragments are inserted into the regeneration chamber an additional 1 mm, only a 2-mm-long portion of nerve is exposed. Consequently, the nerve section cannot be flattened, although the outer membrane is opened. The 2-mm section of exposed nerve is too close to the regeneration chambers and has a poor chemoattractive ability. The internodes of type A nerve fibers are 1–2 mm wide. Thus, a 4-mm-long nerve fragment is likely to induce Schwann cell damage and necrosis during the cutting process. Therefore, 4-mm nerve fragments were excluded from comparisons between groups.

Clinical benefits of long versus short nerve fragments

In this study, there was no significant difference in the number of axons between groups A, B and C, indicating similar repair of the nerve defect. For the treatment of long nerve injuries or defects, we recommend using short (6-mm) nerve fragments for clinical treatment. This helps maintain sufficient length of nerve graft at the distal stump and facilitates cutting of the nerve into two or three nerve fragments for bridging three or four regeneration chambers. The bridging of more than three regeneration chambers for the repair of long nerve defects requires further study.

In summary, different lengths of nerve fragments bridging 6-mm regeneration chambers were used for the repair of a 12-mm-long nerve defect. The chemoattractive capacity of the nerve fragment was independent of nerve fragment length, size or the number of Schwann cells within the nerve fragment. Therefore, we recommend using the shortest nerve fragment assessed in our study (6-mm) for nerve repair.

MATERIALS AND METHODS

Design

A randomized, controlled animal experiment.

Time and setting

Experiments were performed from March 2011 to August 2011 in the Histology and Embryology Laboratory of Liaoning Medical University and the Animal Laboratory of First Affiliated Hospital of Liaoning Medical University, China.

Materials

Forty healthy, clean, Japanese big ear rabbits, aged 4 weeks, irrespective of gender, weighing 750 ± 50 g, were provided by the Animal Experimental Center of Liaoning Medical University, China (license No. SCXK (Liao) 2003-0007). Experimental procedures were in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China.

Methods

Preparation of chitosan-collagen-ciliary neurotrophic factor regeneration chamber

Chitosan powder (Sigma, St. Louis, MO, USA) was dissolved in 0.2 M acetic acid solution, to prepare 1% solu-
tion and mixed with type I collagen (Sigma) at a ratio of 3:1. The mixture was stirred using a magnetic stirring device (Jintan Scientific Analysis Instrument Company Limited, Jiangsu Province, China) for 6 hours. The chitosan-collagen solution was poured into a self-made mold, maintained in place for 3-5 hours to remove excess air bubbles, and then refrigerated at -80°C for 2 hours. The frozen chitosan-collagen regeneration chamber was removed from the mold and placed in a -80°C freezer overnight. The regeneration chamber was transferred to a pre-cooled freeze dryer (Shanghai Pudong Frozen Drying Equipment Co. Ltd., Shanghai, China) for 24 hours. The regeneration chamber was de-acidified with 2% NaOH and rinsed with triple-distilled water. Following natural drying and 60Co irradiation (10 kGy) for disinfection, the chitosan-collagen regeneration chamber was immersed into sterile PBS containing 80 μg/mL ciliary neurotrophic factor (Sigma) overnight.

Preparation of sciatic nerve injury models and repair method
Rabbits were anesthetized using 3 mL/kg of 10% chloral hydrate via abdominal injection, and a sciatic nerve defect was prepared 6 mm away from the piriform muscle inferior margin. In groups A, B and C, a 12-mm-long nerve defect was obtained, and 6, 8 or 10-mm nerve fragments were cut from the distal end of the damaged nerve. Then, two 8-mm-long regeneration chambers were sheathed in autologous nerve fragment, each 1 mm, to form a bridge. Then the bridging regeneration chamber was transplanted into the lesioned nerve, so that the distance between the two regeneration chambers was 6 mm. All incisions were fixed with three needles under a surgical microscope, using 10-0 non-invasive injury suture (Shanghai Yuanhong Medical Instrument Factory, Shanghai, China; Figures 3, 4). In group D, a 12-mm-long nerve fragment was turned 180° in situ, and the neural epineurium was anastomosed under the surgical microscope. All rabbits were conventionally fed for 16 weeks postoperation.

Gross observation
Rabbit foot ulceration, wound healing and physical activities were postoperatively assessed. At 16 weeks after surgery, the sciatic nerve was excised under anesthesia via the primary incision, and regeneration was evaluated.

Neural electrophysiological determination
At 16 weeks postoperatively, the exposed sciatic nerve was examined with a BL420 electrophysiology apparatus (Chengdu Technology and Market Co., Ltd., Chengdu, China) set at 5 mA current, 0.2 ms duration and 1.9 Hz frequency. The recording electrode was inserted into the gastrocnemius muscle belly, and the stimulating electrode was placed in the proximal sciatic nerve for nerve stimulation. The nerve conduction velocity was measured.

Measurement of gastrocnemius wet weight
After the electrophysiological measurement was com-
plete, the bilateral gastrocnemius muscles were excised and weighed on an electronic balance (accurate to 0.000 1 g), and the wet weight ratio was calculated according to the following formula: muscle wet weight at the experimental side / muscle wet weight at the control side × 100%.

**Counting of regenerating nerve fibers**

At postoperative 16 weeks, a 4-mm segment of sciatic nerve was cut 4 ± 2 mm, 10 ± 2 mm or 24 ± 2 mm away from the proximal anastomosis site for each group. Then, the nerve fragments were fixed in 4% paraformaldehyde for 24 hours for dehydration, embedded in paraffin, and prepared into continuous cross-sectional slices, ten slices for each fragment, 5-µm-thick. Hematoxylin-eosin staining and light microscopy (HB-2 optical microscopy and imaging system; Olympus, Tokyo, Japan) were performed to observe the structure and morphology of the regenerated nerve. The number and diameters of regenerated nerve fibers, as well as myelin sheath thickness, were determined using a CIAS-1000 cell image analysis system (Beijing Daheng Visual Images Inc., Beijing, China).

**Electron microscopy observations**

Nerve fragments were harvested from the distal sciatic nerve, fixed in 2.5% glutaraldehyde for 24 hours, dehydrated, embedded with EPDN-812, and cut into transverse sections with a thickness of 50–70 nm using a LKB-V type microtome. The sections were stained with uranyl acetate and lead citrate for 20 minutes, and the ultrastructure of the regenerated nerve fibers, myelin sheath and axons was observed under a H7500 transmission electron microscope (Hitachi, Tokyo, Japan).

**Statistical analysis**

Data were expressed as mean ± SD and statistical analysis was performed using SPSS 13.0 software (SPSS, Chicago, IL, USA) by one-way analysis of variance. P value less than 0.05 was considered statistically significant.

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**Author contributions:** Jiren Zhang was in charge of funds, had full access to study concept and design, drafted the manuscript and validated the research. Yubo Wang and Jincheng Zhang provided and integrated experimental data, and performed statistical analysis.

**Conflicts of interest:** None declared.

**Ethical approval:** This pilot was approved by the Experimental Animal Ethics Committee of Liaoning Medical University in China.

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