Efficacy of nanofibrous conduits in repair of long-segment sciatic nerve defects

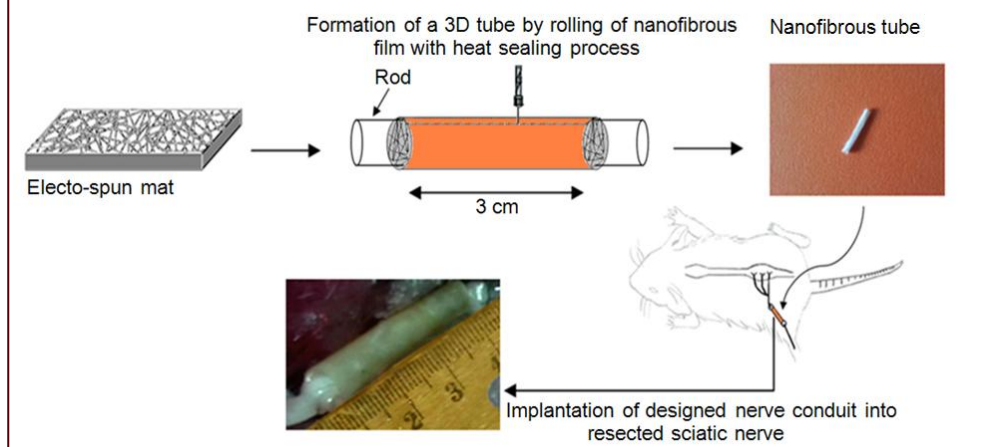**Esmaeil Biazar[1], Saeed Heidari Keshel[2, 3], Majid Pouya[4]**

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Graphical Abstract

Formation of a 3D tube by rolling of nanofibrous film with heat sealing process

Rod

Nanofibrous tube

Electo-spun mat

3 cm

Implantation of designed nerve conduit into resected sciatic nerve

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Abstract

Our previous studies have histomorphologically confirmed that nanofibrous poly(3-hydroxybutyrate-co-3-hydroxyvalerate) conduit can be used to repair 30-mm-long sciatic nerve defects. However, the repair effects on rat behaviors remain poorly understood. In this study, we used nanofibrous poly(3-hydroxybutyrate-co-3-hydroxyvalerate) conduit and autologous sciatic nerve to bridge 30-mm-long rat sciatic nerve gaps. Within 4 months after surgery, rat sciatic nerve functional recovery was evaluated per month by behavioral analyses, including toe out angle, toe spread analysis, walking track analysis, extensor postural thrust, swimming test, open-field analysis and nociceptive function. Results showed that rat sciatic nerve functional recovery was similar after fibrous poly(3-hydroxybutyrate-co-3-hydroxyvalerate) conduit and autologous nerve grafting. These findings suggest that nanofibrous poly(3-hydroxybutyrate-co-3-hydroxyvalerate) conduit is suitable in use for repair of long-segment sciatic nerve defects.

Key Words

neural regeneration; peripheral nerve injury; sciatic nerve; nerve conduit; poly(3-hydroxybutyrate-co-3-hydroxyvalerate); behaviors; motor function; nociceptive function; grants-supported paper; neuroregeneration

INTRODUCTION

Autografts or allografts are commonly used in neurosurgery[1-16]. Unfortunately, autografts have limitations such as body injury, repeated surgeries and disproportion of grafted nerve tissue in terms of size and structure[17-18]. In addition, a similar problem, *i.e.*, stimulation of the immune system, will be encountered in transplantation of allografts or xenografts[19-23]. Some studies used artificial nerve conduits to repair nerve defects[24-26]. Among the artificial nerve conduits used, nanofibrous poly(3-hydroxybuty rate-co-3-hydroxyvalerate) (PHBV) conduits exhibit several advantages. One advantage of these nerve conduits is that they have piezoelectric property and can fabricate electric signal by mechanical pressure. They can be bent to an angle of up to 180˚ and then restore to their original shape, which is necessary for adaptation inside a living system. Moreover, the PHBV conduits have a thin wall and a highly porous structure, which are important determinants for nutrient transport into the conduit[27-38]. A further advantage is that they can be easily fabricated and rolled to any required length and diameter by heat processing. These properties make the nanofibrous PHBV conduits highly suitable for use in artificial nerve scaffolds[39]. Is nanofibrous PHBV conduit suitable for repair of long-segment sciatic nerve defect? How to assess neurofunctional recovery following nanofibrous PHBV conduits grafting? What existing methods are preferred to detect neurofunctional recovery? The walking track analysis is a quantitative method, created by De Medinaceli *et al* [5], for analyzing hind limb performance by examining footprints, known as the sciatic functional index (SFI). De Medinaceli *et al* [5] described a hotplate test for analyzing the nociceptive withdrawal reflex. The extensor postural trust was originally proposed by Thalhammer and collaborators[6] in 1995 as a part of the neurofunctional recovery evaluation in rats after sciatic nerve injury.

In this study, a nanofibrous PHBV conduit was used to bridge a 30-mm-long sciatic nerve gap in a rat model of sciatic nerve injury. The neurofunctional recovery in rats with sciatic nerve defect receiving nanofibrous PHBV conduit grafting was evaluated by behavioral analyses.

RESULTS

Quantitative analysis of experimental animals

Fifteen male Wistar rats were included in this study and randomly and evenly assigned to nanofibrous conduit, autograft and injury groups. A 30-mm-long defect was made in the right sciatic nerve in all three groups; in addition, nanofibrous conduit grafting and autografting were performed in the former two groups, respectively. All 15 rats were included in the final analysis.

Structural characterization of nanofibrous conduits

The ultrastructure of nanofibrous conduit is shown in Figure 1. The smooth and homologous nanofibers are clearly shown in Figure 1B. The average diameter (cross- section of nanofiber) obtained for the nanofibers was about 100 nm. The thickness of the designed tube wall was about 30 µm (Figure 1C).

The mechanical and physical properties of the nanofibrous PHBV mat are shown as follows. The porosity of nanofibrous PHBV conduit was calculated to be 95.6 ± 1.2%. The pore size of scaffold was measured as 0.45 ± 0.25 µm. The nanofibrous PHBV conduit showed a contact angle of about 105 ± 3.2 degree. The specific surface area of the nanofibrous conduit was approximately 138 ± 2 m^2/g. The nanofibrous conduit had a high porosity and a high level of specific surface area. The tensile modulus (110 ± 18 MPa) and ultimate tensile stress value (5.9 ± 0.5 MPa) of the nanofibrous PHBV conduit were suitable for mechanical stresses. The designed polymeric conduits were suitable for axon movement and neurogenesis.

Effect of PHBV conduit grafting on behaviors of rats with sciatic nerve defects
Toe out angle
Angle between normal and experimental feet

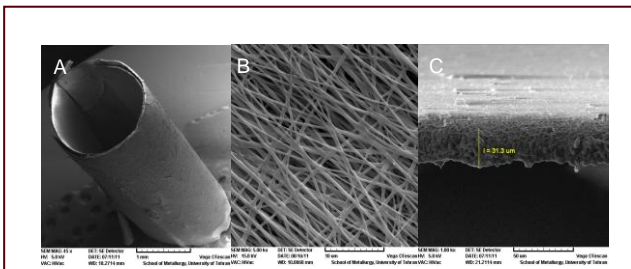calculated by toe out angle analysis is shown in Figure 2.

Figure 1   Ultrastructure of nanofibrous conduit (scanning electron microscope).

(A) The tubular conduit (× 45). (B) The nanofibrous structure of designed conduit (× 5 000). (C) Diameter (about 30 μm) of the tube wall (× 1 000).
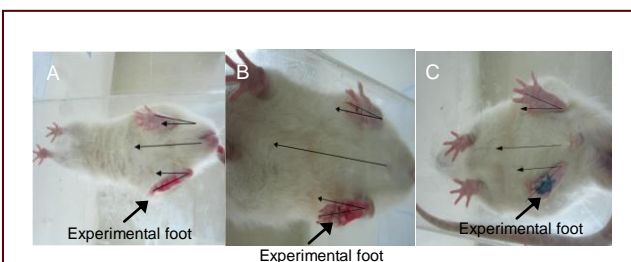

Figure 2   Toe out angle between normal and experimental (injured) feet of rats at 4 months after surgery.

The toe out angle in the nanofibrous conduit (A) and autograft (B) groups was significantly less than in the injury group (C). There were five rats in each group.

At 4 months after surgery, toe out angle of the normal foot was significantly less than that of the experimental foot in each group (Table 1). The autograft and nanofibrous conduit groups showed a better neurofunctional recovery than the injury group ($P < 0.01$, $P < 0.05$). The neurofunctional recovery was similar between autograft and nanofibrous conduit groups.

Table 1   Toe out angle in rats with sciatic nerve defect in each group

Foot (degree)	Nanofibrous conduit group	Autograft group	Injury group
Normal	13.0±0.2	12.0±0.4	17.0±0.6
Experimental	30.0±0.7[a]	19.0±0.3[b]	40.0±0.8

[a]$P < 0.05$, [b]$P < 0.01$, *vs*. injury group. Data were expressed as mean ± SD from five rats per group.

Toe spread analysis

Toe spread was analyzed by measuring the toe spread factor and the intermediate toe spread factor of the left (contralateral to the lesion site) and right (ipsilateral to the lesion site) paws of animals. At 4 months after surgery, the injury group showed loss of toe spread. During

the observation period, rat toe spread improved significantly in the autograft and nanofibrous conduit groups compared to the injury group ($P < 0.01$, 0.05; Table 2), but there was no significant difference in toe spread between autograft and nanofibrous conduit groups ($P > 0.05$; Table 2).

Table 2   Toe spread factors in rats with sciatic nerve defect in each group

Index	Nanofibrous conduit group	Autograft group	Injury group
TSF	−0.6±0.01	−0.4±0.04	−0.7±0.09
ITSF	−0.5±0.06[a]	−0.2±0.01[b]	−0.8±0.03

Toe spread factor (TSF) = [operated side toe spread (OTS) − non-operated side toe spread (NTS)]/NTS. Intermediate toe spread factor (ITSF) = [operated side intermediate toe spread (OITS) − non-operated side intermediate toe spread (NITS)]/NITS. [a]$P < 0.05$, [b]$P < 0.01$, *vs*. injury group. Data are expressed as mean ± SD from five rats per group.

Walking track analysis

Neurofunctional recovery was assessed by walking track analysis, and SFI was obtained. At 4 months after surgery, SFI was 40.1 ± 1.3, 66.2 ± 1.5, and 91.4 ± 1.7 in the autograft, nanofibrous conduit and injury groups, respectively. SFI analyses showed that neurofunctional recovery was better in the autograft and nanofibrous conduit groups than in the injury group ($P < 0.05$). Representative toe prints obtained for SFI analysis (Figure 3).

Figure 3   Representative toe prints obtained when animals walked along a runway for sciatic functional index (SFI) analysis.

(A–C) The samples harvested from the nanofibrous conduit, autograft and injury groups, respectively at 4 months after surgery. $n = 5$ rats in each group at each time point.

SFI = −38.3[(EPL−NPL)/NPL] + 109.5[(ETS−NTS)/NTS] + 13.3[(EIT−NIT)/NIT] − 8.8 based on analysis of walking tracks[19]. SFI = 0 and 100 indicate normal function and complete dysfunction, respectively. EPL: Experimental print length; NPL: normal print length, ETS: normal toe spread; NTS: normal toe spread; EIT: experimental intermediary toe spread; NIT: normal intermediary toe spread.

Extensor postural thrust

At 1 month after surgery, the extensor postural trust was similar and high in the nanofibrous conduit and autograft groups (about 85% and 100%). The extensor

postural trust was recovered significantly in the following months except in the injury group. At 4 months after surgery, motor deficits were decreased to about 55% and 40% in the nanofibrous conduit and autograft groups, respectively (Figure 4).

Figure 4   The extensor postural thrust (EPT) test (percentage of motor deficits) results of rats with sciatic nerve defect in the nanofibrous conduit and autograft groups at 1–4 months after surgery.

Values are expressed as mean ± SD. $^{a}P < 0.05$, *vs.* injury group. $n = 5$ rats in each group at each time point. Motor deficit = (NEPT−EEPT)/NEPT × 100%. NEPT: Normal (unaffected limb) EPT value; EEPT: experimental EPT value.
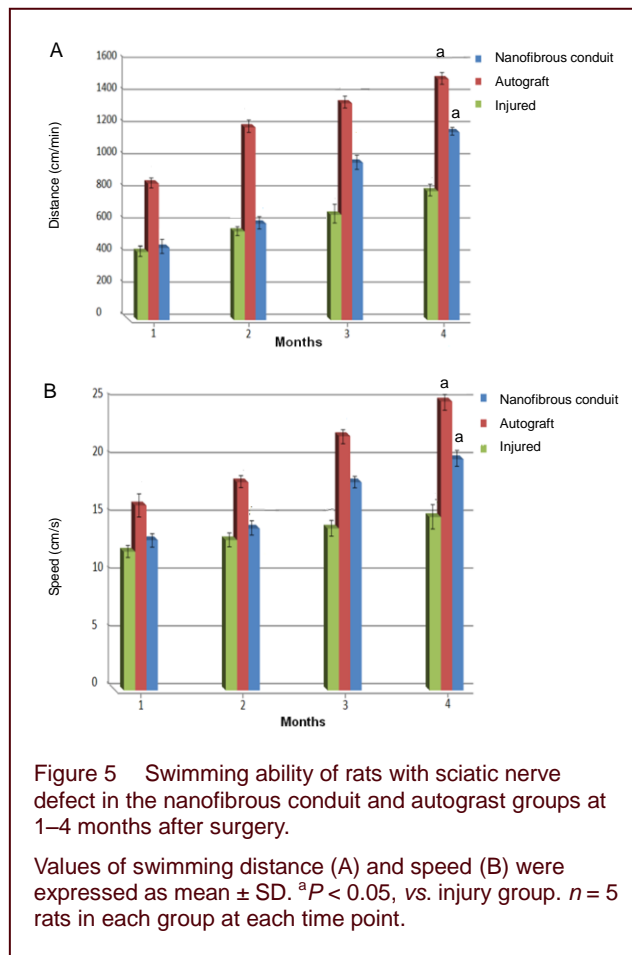
Swimming ability

Swimming ability of rats was evaluated at 1–4 months after surgery. Factors such as traveled distance in a given time period (1 minute), and their navigation were investigated. At 1 month after surgery, there were no significant differences between groups; but at 4 months after surgery, swimming ability in the autograft and nanofibrous conduit groups was significantly better than in the injury group ($P < 0.05$; Figure 5).

Walking ability

Walking ability of rats was evaluated by open field analysis during 4 months after surgery. Factors such as walking distance in a given time period (300 seconds) and walking speed were investigated. There was no significant difference in walking ability between groups at 1 month after surgery. At 4 months after surgery, waking ability in the autograft and nanofibrous conduit groups was significantly improved than in the injury group ($P < 0.05$); but there was no significant difference between autograft and nanofibrous conduit groups (Figure 6).

No significant difference was seen between the groups at first month after sciatic nerve transection. The results showed significantly better walking ability in the autograft and nanofibrous conduit groups compared to the

injury group ($P < 0.05$). Statistical significance was not seen between the autograft group and nanofibrous conduit groups during 4 months (Figure 6). Walking routes of rats at 4 months after surgery are shown in Figure 7.

Figure 5   Swimming ability of rats with sciatic nerve defect in the nanofibrous conduit and autograst groups at 1–4 months after surgery.

Values of swimming distance (A) and speed (B) were expressed as mean ± SD. $^{a}P < 0.05$, *vs.* injury group. $n = 5$ rats in each group at each time point.

Nociceptive function

Nociceptive function was assessed by withdrawal reflex latency (WRL) analysis. At 1 month after surgery, latency was similar to and high in the nanofibrous conduit and autograft groups (7–12 seconds). In the following months, nociceptive function was significantly recovered in both the nanofibrous conduit and autograft groups. At the end of the 4th month, nociceptive function recovered more obviously in both nanofibrous conduit and autograft groups compared to the injury group ($P < 0.05$). However, the injury group rats still presented severe loss of nociceptive function at 4 months after surgery (Figure 8).

DISCUSSION

The rat sciatic nerve is a widely used model for evaluation of motor and sensory nerve function at the same time[40].

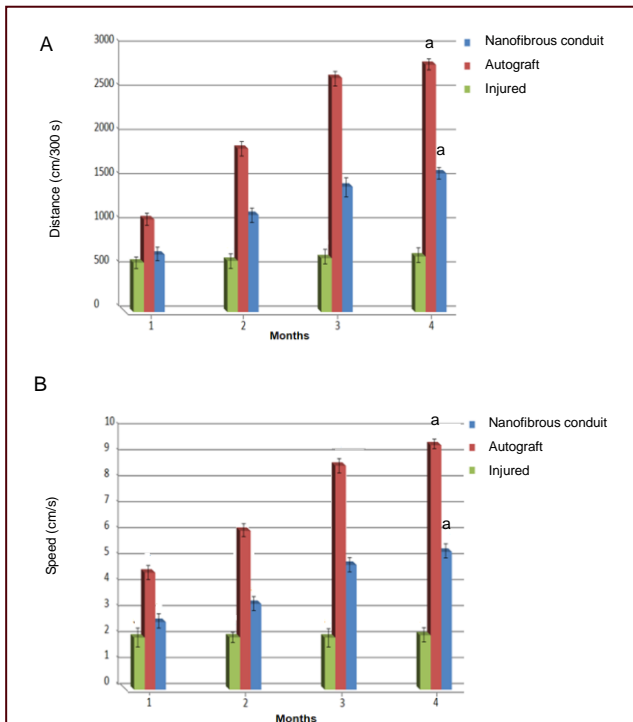One of the most addressed issues in experimental nerve repair research is represented by entubulation[41].

Figure 6　Walking ability of rats with sciatic nerve defect in the nanofibrous conduit and autograft groups by the open field test at 1–4 months after surgery.

Values of walking distance (A) and speed (B) were expressed as mean ± SD. [a]*P* < 0.05, *vs.* injury group. *n* = 5 rats in each group at each time point.
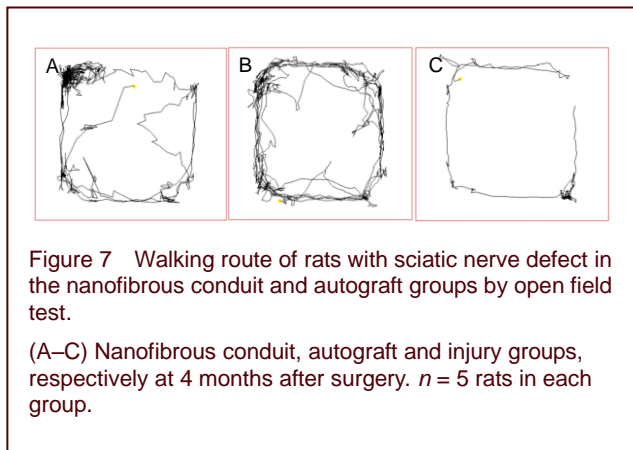

Figure 7　Walking route of rats with sciatic nerve defect in the nanofibrous conduit and autograft groups by open field test.

(A–C) Nanofibrous conduit, autograft and injury groups, respectively at 4 months after surgery. *n* = 5 rats in each group.

Early related studies are more directed towards biological entubulation[42]. Recent studies have focused more on synthetic entubulation[43]. In the present study, we used a nanofibrous PHBV conduit for nerve regeneration and neurofunctional recovery in the rat sciatic nerve transection model. The walking track, swimming ability and open field analyses have frequently been used to reliably determine functional recovery following nerve repair in rat models[5-6]. In our study, the SFI in walking track analysis was obtained about –60 and –40 in the

nanofibrous conduit group and autograft group, respectively. In the swimming ability and open field analyses, neurofunctional recovery was improved significantly with time in the nanofibrous conduit and autograft groups compared to the injury group. Neurofunctional assessment showed that there were no significant differences in motor deficits and nociceptive function between the nanofibrous conduit and autograft groups.
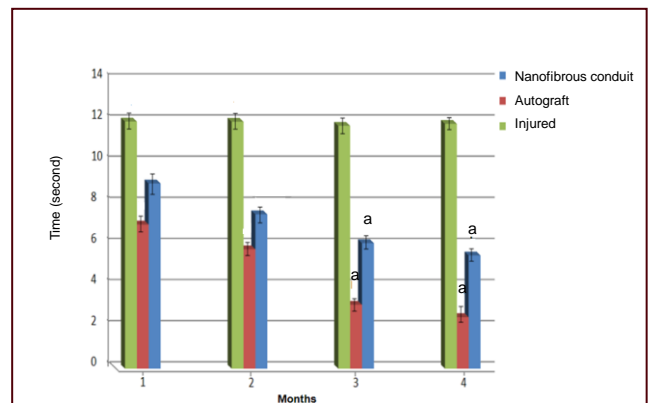

Figure 8　Nociceptive function of rats with sciatic nerve defect in the nanofibrous conduit and autograft groups by withdrawal reflex latency at 1–4 months after surgery.

Values were expressed as mean ± SD. [a]*P* < 0.05, *vs.* injury group. *n* = 5 rats in each group at each time point.

The extensor postural thrust was proposed by Thalhammer and colleagues[6]. The extensor postural trust results were initiated by a stretching of the spindles in the interosseous muscles and stimulation of sensory receptors of the foot in the nanofibrous conduit and autograft groups. A steady recovery of motor deficits occurred throughout the 4th month after surgery in the nanofibrous conduit and autograft groups. The nociceptive function in the withdrawal reflex analysis was recovered to a significantly larger extent in the nanofibrous conduit and autograft groups compared to the injury group. At the end of the 4th month, mean latency was satisfactory in the nanofibrous conduit and autograft groups. Interestingly, when the nerve was transected and again regenerated, sensory neurons exhibit a faster regenerative pattern than motor neurons[44]. This study again supports the idea that these analyses are more comprehensive than histomorphometrical methods.

In this study, grafted nerves with nanofibrous conduit promoted neurofunctional recovery over a 30-mm-long nerve gap. Rats were evaluated by toe out angle, toe spread, walking track, extensor postural thrust, nociceptive function, open field and swimming ability analyses after 4 months of surgery. The grafted samples with nanofibrous conduit showed promising results. Recovery of

motor and sensory function was observed in the grafted samples with nanofibrous conduit and the autograft samples. These nanofibrous conduits can be a good alternative to autografts in neural regeneration. Further studies are encouraged to investigate the potential use of this type of nerve conduits in bigger animal models.

MATERIALS AND METHODS

Design
A randomized, controlled, animal experiment.

Time and setting
This study was performed at Department of Biomaterial Engineering, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran, between November 2011 and October 2012.

Materials
Fifteen male Wistar rats, aged 4–8 weeks, weighing 180–220 g, were included in this study. Animals were managed according to the guidelines established for animal care at the Proteomics Research Center, Beheshti University of Medical Sciences, Iran.

Methods
Design of nanofibrous scaffold
A poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) was purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2,2-trifluoroethanol used to prepare PHBV solution was also purchased from Sigma-Aldrich and used as received, without further purification.

Electro-spinning apparatus was purchased from Fanavaran Nano-Meghyas Company (Iran). PHBV was dissolved at determined concentration in 2,2,2-trifluoroethanol. The PHBV solution (2% w/v) was contained in a glass syringe controlled by a syringe pump. A positive high-voltage source through a wire was applied at the tip of a syringe needle. In this situation, a strong electric field (20 kV) was generated between PHBV solution and a collector. When the electric field reached a critical value with increasing voltage, mutual charge repulsion overcame the surface tension of the polymer solution and an electrically charged jet was ejected from the tip of a conical shape as the Taylor cone. Ultrafine fibers were generated by narrowing the ejected jet fluid as it underwent increasing surface charge density due to the evaporation of the solvent. An electro-spun PHBV mat was carefully detached from the collector and dried in a vacuum for 2 days at room temperature to remove solvent

molecules completely. The nanofibrous mat was designed with certain parameters: syringe size: 17 mm; collector speed: 1 000 r/min; injection speed: 2 mL/min; syringe tip distance to collector: 75 mm; voltage: 20 kV; temperature: 30°C; time: 7 hours. The electrospinning set-up and the designed nanofibrous mat are shown in Figure 9.
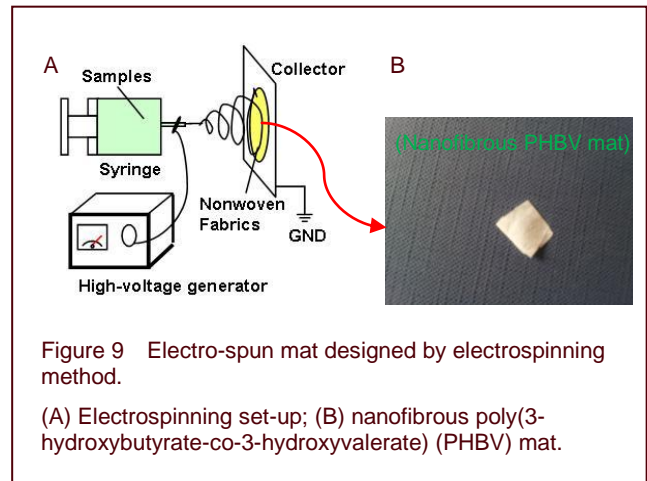

Figure 9   Electro-spun mat designed by electrospinning method.

(A) Electrospinning set-up; (B) nanofibrous poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) mat.

Structural characterization of nanofibrous conduits
The surface characteristics of electro-spun mats were investigated by a scanning electron microscope (Stereo-scan, Model S-360, Cambridge instruments, Wetzlar, Germany) to analyze the changes in the surface morphology. The mats were first gold sputtered for 2 hours (Joel fine coat) to provide surface conduction before scanning. The static contact angles were investigated by a contact angle measuring apparatus (KRUSS G10, Matthews, NC, Germany) according to the sessile drop method. For mechanical investigations, the mats were subjected to stress-strain analysis[45] using a universal testing machine under an extension rate of 5 mm/min and 100 N load cell. The specific surface area of nanofibrous mats was determined by the surface area and pore size analyzer (BEL Japan).

Preparation of three-dimensional nerve conduits
The electro-spun mat (33 mm in length and 5 mm in width) was rolled around the cylindrical rod to form a three-dimensional tubular structure and was maintained in this form using a thermal agent (temperature 60˚C).

Surgical procedure
A long segment of right sciatic nerve was then resected, leaving a gap of about 30 mm caused by the retraction of nerve ends. In the autograft group, a 33 mm segment of sciatic nerve was excised, reversed and sutured back in place. In the nanofibrous tubes of 33 mm in length were used so that the two nerve ends could be slided for

1.5 mm into the tube and anchored with two epi-perineural sutures. A 30 mm gap was thus maintained between the nerve stumps inside the tube. All animals had free access to standard rat food and water. The right lateral foot was not fixed and no drugs were administered during the postoperative period.

Behavioral analyses
Toe out angle
The toe out angle is the angle in degrees between the direction of progression and a reference line, which is defined anatomically from the calcaneus to the tip of the third digit. To determine toe out angle, the rats were placed into acrylic glass containers (100 cm × 15 cm) with a mirror below it. A camera (Nikon-Japan) was positioned underneath the transparent base plate in order to photograph the plantar surface of the animal's paws. Angles were measured and recorded[46].

Toe spread
For the determination of toe spread, the rats were placed into acrylic glass containers (20 cm × 12 cm × 9 cm) on a transparent base plate, then a camera (Nikon-Japan) was positioned underneath the plate in order to photograph the plantar surface of the animal's paws. Toe-spread factors were determined by measuring the distance between the first and fifth toes (1–5, toe spread) and between the second and fourth toes (2–4, intermediate toe spread) as previously described by Bervar[47].

Toe spread factor (TSF) = [operated side toe spread (OTS) – non-operated side toe spread (NTS)]/NTS

Intermediate toe spread factor (ITSF) = [operated side intermediate toe spread (OITS) – non-operated side intermediate toe spread (NITS)]/NITS

Walking track analysis
Deficits in descending fine motor control were quantified using walking track or footprint analysis. Footprints were evaluated by three parameters: (1) distance from the heel to the third toe, print length (PL); (2) distance from the first to the fifth toes, toe spread (TS); and (3) distance from the second to the fourth toes, intermediary toe spread (IT). All three measurements are taken from the experimental (E) and normal (N) sides. Functional recovery was assessed by calculating the SFI value[48].

SFI = –38.3[(EPL–NPL)/NPL] + 109.5[(ETS–NTS)/NTS] + 13.3 [(EIT–NIT)/NIT] – 8.8 based on analysis of walking tracks[49].

Postoperatively, the rats were assessed once per month within 4 months after surgery. The investigators were blinded to the animal groups during walking track analysis. SFI 0 and 100 indicate normal function and complete dysfunction, respectively.

Extensor postural thrust
All rats were evaluated in 4 months by extensor postural thrust analysis. For this test, the entire body of the rat, except the hind limbs, was wrapped in a surgical towel. The foot extensors or force were measured by a digital balance (DE 12K1N model, KERN Co., Germany). As the animal was lowered to the platform, it extended the hindlimbs, anticipating the contact made by the distal metatarsus and digits. The force in grams (g) applied to the digital platform balance (DE 12K1N model, KERN Co., Germany) was recorded. The motor deficit difference between normal and experimental feet was calculated as follows as described by Koka and Hadlock, in 2001[50]:

Motor deficit: (NEPT – EEPT)/NEPT × 100% (NEPT: Normal (unaffected limb) extensor postural thrust; EEPT: experimental extensor postural thrust values)

Swimming analysis
A tank (stainless steel; diameter: 150 cm, height: 60 cm, volume: 300 L) filled with water to a depth of 30 cm was used in this test. The ability of rat to swim the length of the tank based swimming speed and distance at 1 minute was assessed by motion sensors in the tank's path[51].

Open-field analysis
Neurofunctional outcome was assessed using the Basso, Beattie and Bresnahan locomotor rating scale for rat hindlimb motor function[51]. Rats were assessed during the course of a 5-minute exposure to an open-field area consisting of a metal circular enclosure (100 cm diameter, 18 cm height). The ability of rat to walk in the length of the tank based walking speed and distance was assessed by motion sensors[51].

Evaluation of nociceptive function
For evaluation of regenerated sensory nerves, the rats were wrapped in a surgical towel above their waist and then positioned to stand with the affected hind paw on a hot water bath at 50°C, the legs were inserted into the warm water to contact the bottom of the hot water tank. Nociceptive withdrawal reflex was defined as the time elapsed from the onset of hot warm contact to withdrawal of the hind paw and measured with a stopwatch (Stopwatches model , Mainland, China)[52].

Statistical analysis

Data were analyzed using SPSS 16.0 (SPSS, Chicago, IL, USA) and were expressed as mean ± SD. All data were analyzed by one-way analysis of variance with Duncan's multiple range tests. A level of $P < 0.05$ was considered statistically significant.

Research background: Nanofibrous nerve conduits exhibit excellent mechanical and physiological properties and the culture medium of nanofibrous nerve conduits has been confirmed to promote neuronal growth.

Research frontiers: Nanofibrous PHBV conduit has been confirmed to promote rat peripheral nerve regeneration, which is consistent with the morphological findings from our previous studies.

Clinical significance: Whether rat sciatic nerve functions can be improved by repair of over 30-mm-long sciatic nerve gaps with nanofibrous PHBV conduits? Whether the effect of nanofibrous PHBV conduit was better than, similar to or worse than that of autologous nerve graft in repair of over 30-mm-long sciatic nerve defect? This study provides experimental data and new thoughts for clinical treatment of sciatic nerve injury with artificial nerve conduits.

Academic terminology: Extensor postural trust – The entire animal body was lifted away from the ground by grasping its forelimbs and then slowly put on the ground. When touching the ground, the animal will straighten its hind limbs and present a retrusive action during descending its forelimbs. If there are some abnormalities during this process, then the hind limb motor nerve will be considered abnormal.

Peer review: The present findings are preliminary, and histological evidence of implanted nanofibrous PHBV conduit is needed to understand the compatibility of this nanofibrous conduit with the sciatic nerve. In addition, a sciatic nerve without nanofibrous PHBV conduit implantation should be used as a control to better clarify this issue.

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