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Sensory reinnervation of muscle spindles after repair of tibial nerve defects using autogenous vein grafts

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Abstract

Motor reinnervation after repair of tibial nerve defects using autologous vein grafts in rats has previously been reported, but sensory reinnervation after the same repair has not been fully investigated. In this study, partial sensory reinnervation of muscle spindles was observed after repair of 10-mm left tibial nerve defects using autologous vein grafts with end-to-end anastomosis in rats, and functional recovery was confirmed by electrophysiological studies. There were no significant differences in the number, size, or electrophysiological function of reinnervated muscle spindles between the two experimental groups. These findings suggest that repair of short nerve defects with autologous vein grafts provides comparable results to immediate end-to-end anastomosis in terms of sensory reinnervation of muscle spindles.

Key Words: *nerve regeneration; peripheral nerve injury; muscle spindle; vein; tibial nerve; gastrocnemius muscle; neurofilament protein H; neural regeneration*

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Introduction

Restoration of function after nerve transection and injuries causing nerve defects requires surgical intervention[1-2]. If untreated, such injuries result in partial or total paralysis. After complete transection of a nerve, good neuroanastomosis is needed to prevent the formation of fibrous connective tissues that form obstacles to nerve regeneration, and to facilitate repair of the injured nerve and reinnervation of its original targets[3-4]. Peripheral nerve defects of more than 10 mm are commonly treated in clinics, and in these injuries a conduit is needed to bridge the gap, prevent the formation of obstacles to nerve regeneration, and guide axonal regrowth[5-6]. During the past 20 years, different conduits have been used to bridge peripheral nerve defects, including veins[7-9], arteries[10-11], muscle fiber tubes[12-13], decalcified bone tubes[14], and artificial nerve conduits[15-18]. Traditionally, the effectiveness of nerve conduits was evaluated by analysis of nerve fiber density in the nerve conduit or the distal nerve stump[19-20]. In 1982, Chiu et al.[7] first used autogenous vein grafts as a conduit for nerve regeneration in rats, and reported that histological examination showed regeneration of nerve fibers within the lumen of the vein graft and in the distal nerve stump. In 1989, Walton et al.[21] reported recovery of two-point discrimination in 12 of 18 digital nerve injuries bridged with vein grafts. Since then, autogenous vein grafts have been used extensively for the repair of nerve defects in experimental and clinical settings, because of their good histocompatibility and ease of harvesting[9-10]. Motor reinnervation of skeletal muscle after repair of nerve defects using vein grafts has been confirmed both structurally and neurophysiologically[7, 22-23]. However, no previous studies have reported on sensory reinnervation of skeletal muscle after repair of nerve defects using vein grafts. Muscle spindles are the most important specialized sensory receptors in skeletal muscle, and primarily detect changes in muscle length and convey this information to the central nervous system *via* sensory neurons in the spinal ganglia. We hypothesized that repair of nerve defects using autologous vein grafts may result in sensory reinnervation of muscle spindles and regaining of muscle spindle function.

After microsurgical reconstruction of injured nerves, the nerves sprout axonal lateral buds that grow along the distal myelin sheath tube to the appropriate end organ[23-24]. Direct anastomosis of the transected nerve is the optimal method of nerve repair when there is low tension at the coaptation site[25-26]. However, if the gap between the nerve ends is too large, a graft is needed to bridge the gap and guide the new axonal lateral buds towards the distal nerve stump, and prevent neuroma formation[27-28]. This study used immunohistochemical and electrophysiological examinations to evaluate reinnervation of muscle spindles in rat gastrocnemius muscles after excision of 10-mm segments of the tibial nerves and bridging with autologous vein grafts.

Results

Quantitative analysis of experimental rats

Forty-eight Sprague-Dawley rats were randomly assigned to two equal-sized groups who underwent different types of

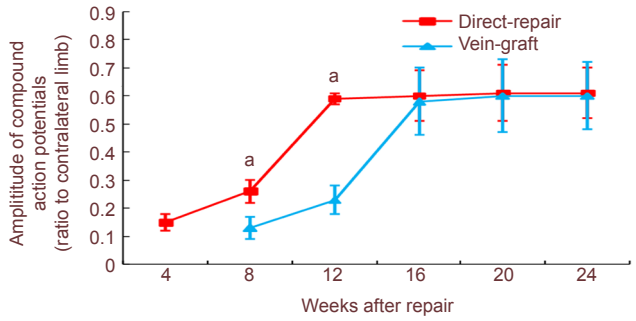

Figure 1 Normalized compound action potential amplitudes after muscle stretch in the direct-repair and vein-graft groups.
The normalized values represent the action potential amplitude in the limb that underwent surgery relative to the action potential amplitude in the contralateral normal limb. Data are presented as mean ± SEM. The significance of differences between groups was analyzed using one-way analysis of variance with a *post hoc* Dunnett's T3 test. [a]$P < 0.05$, *vs.* vein-graft group. Three rats per group at 4, 8, 12, 16, and 20 weeks after surgery, and nine rats per group at 24 weeks after surgery.

surgical intervention: a direct-repair group and a vein-graft group. At 4, 8, 12, 16, and 20 weeks after surgery, functional electrophysiological studies were conducted in three randomly selected rats from each group, with the contralateral limbs used as controls. At 24 weeks after surgery, functional electrophysiological studies and histological examinations were conducted in nine rats from each group, with the contralateral limbs used as controls. All the rats were included in the final analysis.

General condition of rats after model establishment

Before model establishment, all experimental rats were healthy with intact skins and no gait defects. After surgery, some abnormalities appeared. Ulcers were observed on the toes of the limbs that underwent surgery, which gradually started to recover at 3 weeks after surgery. The limbs that underwent surgery lost plantar flexion at the ankle joint and had abnormal movements after surgery, but regained plantar flexion after 16 weeks and subsequently improved further over time. In addition, skeletal muscle atrophy was observed after 4 weeks in the limbs that underwent surgery. No significant resolution of muscle atrophy was observed at 24 weeks after surgery. No significant differences were observed between the direct-repair and vein-graft groups.

Continuous tibial nerves without neuromas were observed in the limbs that underwent surgery in both groups, although the anastomotic sites were attached to surrounding tissues. No collapses of the venous wall were observed in the vein-graft group. The nerves that regenerated in the vein conduits were thinner than the corresponding parts of the contralateral tibial nerves.

Electrophysiological response of the tibial nerve to stretching of the gastrocnemius muscle after surgery

A compound action potential was observed in the tibial nerve after stretching of the gastrocnemius muscle at 4 weeks after surgery in the direct-repair group, and at 8 weeks after surgery in the vein-graft group. The compound action potential amplitude was initially much lower in the limb that underwent surgery than on the contralateral side in the same rat, then increased slowly between 4 and 8 weeks after surgery in the direct-repair group and between 8 and 12 weeks after surgery in the vein-graft group, and increased rapidly to the maximum level in the following 4 weeks in both groups and stayed at the maximum level until 26 weeks (Figure 1A). The maximum compound action potential amplitude was 61% of the control value in the direct-repair group and 60% of the control value in the vein-graft group. The normalized compound action potential amplitude was significantly higher in the direct-repair group than in the vein-graft group at 8 and 12 weeks after surgery ($P < 0.05$). There was no significant difference in the maximum recovery level between the direct-repair and vein-graft groups at 24 weeks after surgery ($P > 0.05$; Figure 1).

Table 1 Number, length, and diameter of muscle spindles in rat gastrocnemius muscles at 24 weeks after nerve transection and repair

Group	Number	Length (μm)	Diameter (μm)
Direct-repair			
Contralateral side	26.7±2.5	324.7±20.5	42.9±5.5
Operated side	19.7±3.2[a]	184.7±17.5[a]	36.7±1.8[a]
Vein-graft			
Contralateral side	28.3±2.5	334.7±22.5	49.7±2.6
Operated side	18.9±2.4[a]	178.7±21.4[a]	27.0±2.5[a]

The number of muscle spindles was counted in each gastrocnemius muscle. Muscle spindle diameter was measured in the equatorial region, which is the thickest part of the fusiform-shaped spindle. Muscle spindle length was measured as the longest portion of the fusiform-shaped spindle. Data are presented as mean ± SEM. Nine rats per group. [a]$P < 0.05$, *vs.* contralateral side in the same group (one-way analysis of variance with *post hoc* Dunnett's T3 test).

Histomorphological characteristics of the muscle spindles after nerve repair

Normally shaped muscle spindles with characteristic annulospiral sensory afferent fibers around intrafusal muscle fibers were observed in the gastrocnemius muscles of the contralateral limbs (Figure 2) and of the limbs that underwent surgery (Figure 3). Compared with the contralateral side, muscle spindles in the direct-repair group (Figure 3A) and vein-graft group (Figure 3C) were thinner and shorter (Figures 2, 3). In addition, a few muscle spindles with abnormal shapes were observed in the limbs that underwent surgery in both the direct-repair group (Figure 3B) and the vein-graft group (Figure 3D). In contrast, only muscle spindles with normal shapes were detected in the contralateral gastrocnemius muscles in both the direct-repair group (Figure 2A) and the vein-graft group (Figure 2B).

The muscles on the contralateral side had significantly greater numbers of muscle spindles than those in the limbs that underwent surgery. Comparison with the contralateral side showed that about two-thirds of the spindles were reinnervated by afferent nerves. Muscle spindles in both the direct-repair and vein-graft groups were significantly smaller in equatorial diameter and shorter on the side that underwent surgery than on the contralateral side. There were no significant differences in the number, length, or diameter of spindles between the direct-repair and vein-graft groups (Table 1).

Discussion

The current gold standard method of bridging nerve defects

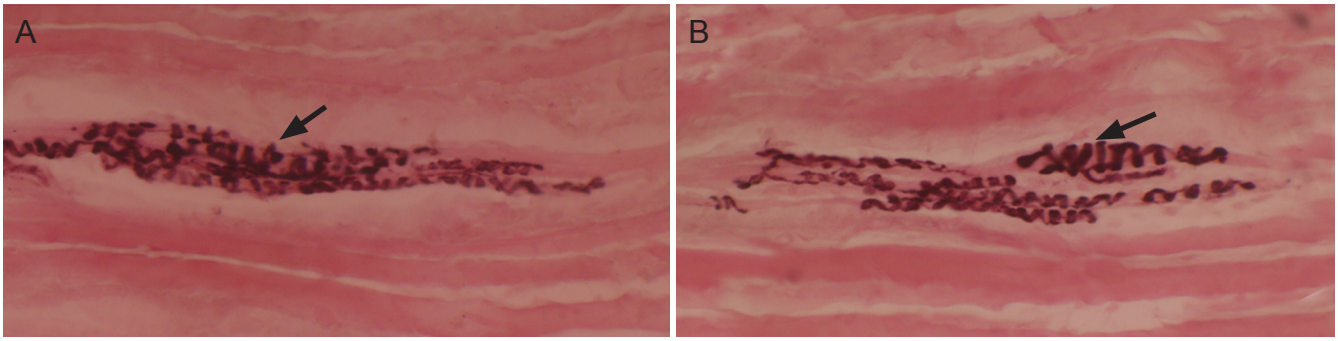

Figure 2 Photomicrographs of muscle spindles in the contralateral gastrocnemius muscles at 24 weeks after tibial nerve transection and repair (immunohistochemical staining, optical microscopy, × 400).
All muscle spindles in the contralateral gastrocnemius muscles were normal, and characteristic annulospiral sensory structures were clearly visible in both the direct-repair group (A) and the vein-graft group (B). The arrows show neurofilament protein H-positive sensory endings in the muscle spindles.

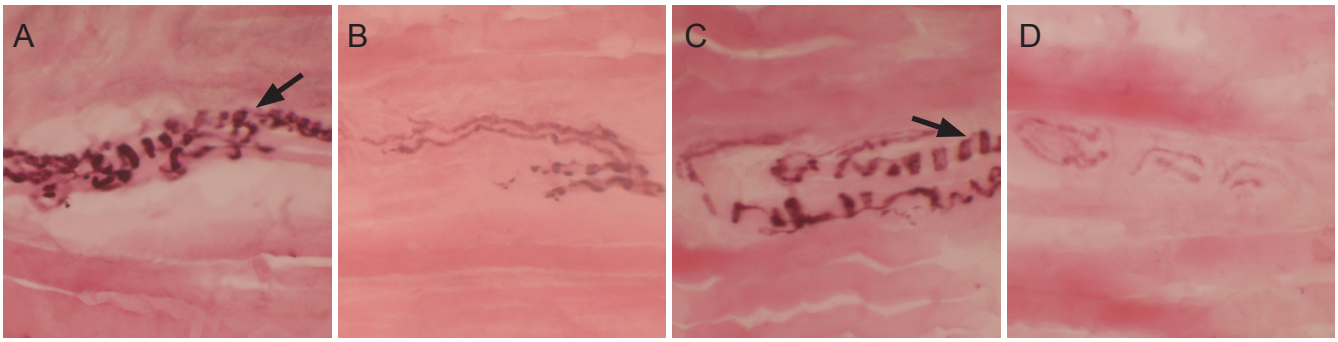

Figure 3 Photomicrographs of neurofilament protein H-positive regeneration in the sensory nerve endings of the gastrocnemius muscles in the limbs that underwent surgery, at 24 weeks after tibial nerve transection and repair (immunohistochemical staining, optical microscopy, × 400).
Most regenerated muscle spindles had normal morphology with characteristic annulospiral sensory structures in both the direct-repair group (A) and the vein-graft group (C). However, there were a few regenerated muscle spindles with abnormal morphology and disorderly regeneration of sensory structures in both the direct-repair group (B) and the vein-graft group (D). The arrows show neurofilament protein H-positive sensory endings in the muscle spindles.

of more than 5 mm is by nerve autograft. However, nerve autografts have significant disadvantages including neuroma formation, donor-site morbidity, nerve site mismatch, and the limited amount of donor tissue available[27, 29-30]. Some alternative grafting techniques have been developed over recent decades[31-32]. Among them, autogenous vein grafts are a good option for bridging defects of less than 30 mm[33]. Over the last 30 years, autogenous vein grafts have been used both experimentally and clinically to bridge peripheral nerve defects, because of their ready accessibility and minimal donor-site morbidity compared with nerve grafts[34-38]. Excellent nerve regeneration and functional recovery were observed in rats[4] and rabbits[39] who underwent autogenous vein grafting for repair of peripheral defects of less than 30 mm. Autogenous vein grafts were also effective in clinical studies, with recovery of two-point discrimination in patients with short digital nerve defects that were bridged by vein grafts[21, 40-42]. Currently, autogenous vein grafts are mainly used to repair nerve defects of less than 30 mm because veins have little mechanical resistance to kinking and collapse[43-44]. We conducted a comparative experiment that evaluated the sensory reinnervation of muscle spindles after nerve repair using vein grafts, in which the length of the nerve defects was limited to 15 mm. We found that many muscle spindles were reconstructed in both the vein-graft and direct-repair groups, which is consistent with previous results that afferent nerve fibers can regenerate and reinnervate muscle spindles after nerve crush and transection injuries[45-47]. There were no significant differences in the number, diameter, or length of muscle spindles in the limbs that underwent surgery between the vein-graft and direct-repair groups. Electrophysiological examination showed that the regenerated muscle spindles had partially restored ability to respond to muscle stretch, which was similar in the vein-graft and direct-repair groups. Although the onset of recovery of the ability to respond to muscle stretch was recorded 4 weeks later in the vein-graft group than in the direct-repair group, the ultimate degree of recovery was not significantly different between the two groups. Plantar flexion of the ankle joint was lost immediately after surgery in both groups, and was then gradually regained. Vein-graft repair of short nerve defects therefore had comparable results to direct repair of transected nerves without defects.

In this study, the muscles in the contralateral limbs had a significantly greater number of spindles than the muscles in the limbs that underwent surgery in both the vein-graft and direct-repair groups. These results suggest that the reinnervation of muscle spindles was incomplete in both the vein-

graft and direct-repair groups. Outcomes after nerve repair or grafting procedures depend on a number of factors[48-49]. In the present study, we controlled as many factors as possible, such as the type and location of the lesion, the type and timing of repair, the surgical technique used for repair, the type and length of the graft, and the age of the rats. Because these factors have been clearly demonstrated to be strongly correlated with peripheral nerve regeneration, we controlled the conditions to encourage nerve regeneration, and to enable accurate assessment of the effectiveness of vein grafting. For example, the length of the vein graft was limited to 15 mm, because vein grafting is not suitable for nerve defects of more than 30 mm[33]. We therefore concluded that other factors that were not controlled might play a role in the degree of reinnervation of muscle spindles. Some improved vein grafts, such as veins filled with muscle graft, veins treated with nerve growth factor, and veins with stem cell transplants have been shown to enhance nerve regeneration[50-53]. Veins treated with nerve growth factor and vein conduits with interposition of nerve tissue have been used to repair nerve defects of more than 30 mm[52, 54]. Future studies of nerve regeneration should also evalaute these interventions for acceleration of axon growth from the lesion into the distal nerve stump.

In this study, most of the reconstructed muscle spindles had normal morphology, but were smaller and thinner than on the contralateral side in both the vein-graft and direct-repair groups. In addition, a few reconstructed muscle spindles with abnormal appearance were detected in both groups. Previous studies have reported abnormal reinnervation of spindles after nerve transection and direct anastomosis[47, 55]. Abnormal innervation was also detected in the muscle spindles of diabetic mice after the afferent axons underwent degeneration and subsequent regeneration[56]. Atypical afferent reinnervation of muscle spindles may be a common phenomenon during nerve regeneration and reconstruction.

In conclusion, autogenous vein grafts are effective conduits for regeneration of short nerve defects. Reinnervation and maturation of motor nerves have been reported in previous studies of autogenous vein grafting to repair nerve defects of less than 30 mm[34-35]. The results of this study show that use of autogenous vein grafts to bridge small nerve defects results in reinnervation and functional recovery of muscle spindles, confirming the clinical usefulness of autogenous vein grafts for bridging short nerve defects.

Materials and Methods

Design

A randomized, controlled animal experiment.

Time and setting

This study was conducted at the Department of Anatomy and K.K. Leung Brain Research Center, Fourth Military Medical University, China from January to September 2012.

Materials

Forty-eight healthy 9-week-old Sprague-Dawley rats of both sexes, weighing 250 ± 50 g, were provided by the Laboratory Rat Center of the Fourth Military Medical University of Chinese PLA (Permission No. SCXK2002-006). All rats were housed in rooms with controlled temperature (23±1°C), humidity (25%), and light/dark cycle (12 hours/12 hours), and were fed a standard rat diet with free access to water. Experiments were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals* by the Ministry of Science and Technology of China[57], and were approved by the Animals Ethics Committee, Fourth Military Medical University, China.

Methods

Repair of rat tibial nerve injuries

All operations were performed under sterile conditions using microsurgical techniques and an operating microscope (Olympus, Tokyo, Japan). Rats were anesthetized with intraperitoneal injection of 3% sodium pentobarbital (1 mL/kg). The contralateral (left) gastrocnemius muscles and tibial nerves were used as normal controls. In each rat, the right tibial nerve was exposed in the posterior thigh, approximately 15 mm from its point of entry into the lateral head of the gastrocnemius muscle.

In the direct-repair group, the tibial nerve was transected 5 mm from its point of entry into the lateral head of the gastrocnemius muscle and repaired immediately by end-to-end anastomosis using 10-0 nylon thread. The anastomotic site was covered with adjacent muscles.

In the vein-graft group, the tibial nerve was transected 15 mm from its point of entry into the lateral head of the gastrocnemius muscle and a 10-mm length was removed from the distal nerve stump. Automatic retraction of the proximal and distal nerve stumps resulted in a 15-mm gap between the nerve ends. The saphenous vein was exposed and all its branches were ligated. A 15-mm segment of the saphenous vein was then harvested and rinsed with normal saline. The epineurium of the proximal and distal nerve stumps was stripped for 1–2 mm, and the saphenous vein graft was reversed and sutured to the nerve ends using 10-0 nylon thread.

The surgical wound was closed and antibiotics were administered. The rats were kept warm with a heating pad for 6 hours after surgery, and then housed for 4–24 weeks with free access to food and water.

Electrophysiological assessment of nerve response to stretching of the gastrocnemius muscle

At 4, 8, 12, 16, 20, or 24 weeks after surgery, rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (2 mL/kg) and pretreated with atropine to prevent excessive salivation. The core temperature of anesthetized rats was maintained at 36.5 ± 0.5°C using a heating pad and infrared radiation. The right and left tibial nerves were exposed and tested by electromyography, according to previously described methods[58]. An incision was made on the lateral side of each hind limb to expose the tibial, peroneal and sural nerves. The sural nerve, peroneal nerve and branches of the tibial nerve to the medial head of the gastrocnemius muscle were transected to eliminate their input. Another incision was made on the medial side of each hind limb proximal to the ankle joint to expose and transect the descending branches of the tibial nerve innervating the foot and ankle, to eliminate proprioceptive input from these nerves. The animal was mounted in a frame, and the limbs were immobilized by supporting clamps that were fixed firmly to the lower femur and lower tibia. The branches of the tibial nerve

to the lateral head of the gastrocnemius muscle were kept intact. Spiral cuff electrodes were placed around the tibial nerve approximately 5 mm above the proximal repair site. The muscles and the skin were then closed. Finally, the foot was secured in a cradle that was coupled to a computer controlled motor shaft to produce repeatable flexion-extension movements.

Standardized muscle stretches were administered to the lateral gastrocnemius muscle using the cradle, which allowed only movement of the ankle in the sagittal plane. The nerve action potential was recorded in the repaired tibial nerve (ABR recorder; Dantec, Denmark). Control recordings were also obtained from the contralateral tibial nerves of all animals. At least six nerve action potentials were captured and recorded in each animal. The mean amplitude of the compound action potential on the repaired side was normalized relative to the amplitude on the contralateral side of the same rat.

Harvesting materials

At 24 weeks after surgery, all rats were deeply anesthetized, and underwent transcardial infusion of 100 mL of 25 mmol/L PBS (pH 7.3), followed by 500 mL of 0.1 mol/L PBS (pH 7.3) containing 4% (w/v) paraformaldehyde and 75% (v/v) saturated picric acid. The lateral gastrocnemius muscles were then removed and immersed in 20% sucrose solution overnight at 4°C. Frozen lateral gastrocnemius muscles were sectioned longitudinally at 20 μm intervals using a Leica cryostat (RM2235, Nussloch, Germany). Sections were divided into two groups of alternating slices, and were mounted onto gelatin-coated slides.

Immunohistochemical staining for neurofilament protein H in the muscle spindles of the lateral gastrocnemius muscles

The sections were immunostained with neurofilament protein H antibody to label the sensory fibers, and then counterstained with eosin to show the intrafusal and extrafusal muscle fibers. Sections were washed three times with PBS and blocked with 5% normal goat serum for 1 hour, and then sequentially incubated at room temperature in rabbit anti-neurofilament H IgG (1:500; Chemicon Temecula, CA, USA) overnight followed by biotinylated donkey anti-rabbit IgG antibody (1:200; Jackson, West Grove, PA, USA) for 4 hours. The incubation medium was 0.05 mol/L PBS (pH 7.3) containing 0.3% (v/v) Triton X-100, 0.25% (w/v) γ-carrageenan, 0.02% (w/v) NaN_3, and 5% (v/v) normal donkey serum. The sections were incubated at room temperature in avidin-biotinylated peroxidase complex (1:100; Vector, Burlingame, CA, USA) for 3 hours. The incubation medium in step 3 was 0.05 mol/L PBS (pH 7.3) containing 0.3% (v/v) Triton X-100. After staining with diaminobenzidine and nickel ammonium sulfate, and counterstaining with eosin, samples were examined under an optical microscope (AX80; Olympus, Osaka, Japan) with a 40 × objective lens, and images were captured using a digital camera (DP70; Olympus) and adjusted (15–20% contrast enhancement) in Photoshop CS2 (Adobe Systems, San Jose, CA, USA). The second series of sections was used for control immunohistochemical staining, in which one of the primary antibodies was omitted or replaced with normal IgG. The number of positive muscle spindles was counted on every third section for all animals. The diameter and length were measured for each neurofilament protein H-positive muscle spindle that was counted. For neurofilament protein H-positive muscle spindles that were intact on the transverse sections, the maximum diameter at the equatorial region was measured on photographs using the analysis program. At the same time, the length of neurofilament protein H-positive muscle spindles was measured across the maximum transverse axis of the extrafusal fibers.

Statistical analysis

All measurement data are presented as mean ± SEM. The significance of differences between groups was analyzed using one-way analysis of variance with a *post hoc* Dunnett's T3 test. Statistical analyses were performed using SPSS software, version 11.0 (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

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Author contributions: *Pang YW and Hong QN participated in experimental implementation, data generation, data analysis, integration of statistical analysis, literature review and manuscript writing. Zheng JN and Hong QN were responsible for funds and study design. All authors approved the final version of the paper.*

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