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Original Article

Specificity of motor axon regeneration: a comparison of recovery following biodegradable conduit small gap tubulization and epineurial neurorrhaphy

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Abstract: Functional recovery is often unsatisfactory after lesions in the peripheral nervous system despite the strong potential for regeneration and advances in microsurgical techniques. Axonal regeneration in mixed nerve into inappropriate pathways is a major contributing factor to this failure. In this study, the rat femoral nerve model of transection and surgical repair was used to evaluate the specificity of motor axon regeneration as well as functional and morphological recovery using biodegradable conduit small gap tubulization compared to epineurial neurorrhaphy. 12 weeks after nerve repair, the specificity was assessed using the retrograde neurotracers TB and DiI to backlabel motor neurons that regenerate axons into muscle and cutaneous pathways. To evaluate the functional recovery of the quadriceps muscle, the quadriceps muscle forces were examined. The quadriceps muscle and myelinated axons were assessed using electrophysiology and histology. The results showed that the specificity of motor axon regeneration (preferential reinnervation) was significantly higher when the nerve transection was treated by biodegradable conduit small gap tubulization and there was no significant difference between the two suture methods with respect to the functional and morphological recovery. This study demonstrated that the quicker and easier biodegradable conduit small gap tubulization may get more accurate reinnervation than traditional epineurial neurorrhaphy and produced functional and morphological recovery equal to traditional epineurial neurorrhaphy.

Keywords: Biodegradable conduit, motor axon regeneration, femoral nerve, small gap, tubulization

Introduction

Peripheral nerve injury is a serious disease that can lead to severe impairment and long-standing disability [1-3]. Misdirection of regenerated axons at the injury site has been long recognized as a major factor to poor functional recovery, not only do these regenerating axons which project incorrectly to the end organs fail to establish functional contacts, they also occupy the pathways of the appropriate axons [4]. In order to improve the clinical effect, many studies focus on improving the accuracy of peripheral nerve regeneration and various types of techniques have been developed to identify sensory and motor fascicles, including intraoperative electro-stimulation, AchE histochemistry, rapid immunostaining technique [5-8]. As each method has its own flaws and shortcomings, these intraoperative diagnostic techniques have limited practical use for most reconstructive nerve surgeons.

Forssman proposed the nerve selective regeneration theory in 1898, the regenerated axons after nerve injury could recognize the distal nerve stumps and selectively grow toward their counterparts. In 1928, Cajal defined this phenomenon neurotropism (chemotropism) [9]. Previous studies in the rodent femoral nerve model, have shown that regenerating motor neurons preferentially regenerate into their original terminal muscle branch as opposed to skin; a phenomenon that has been termed preferential motor reinnervation (PMR) [4, 10]. With the development of nerve selective regeneration theory, nerve conduit bridging has been developed and gradually used for treatment [11-13]. For an extended nerve defect, although tubular conduit or other conduit in the periph-

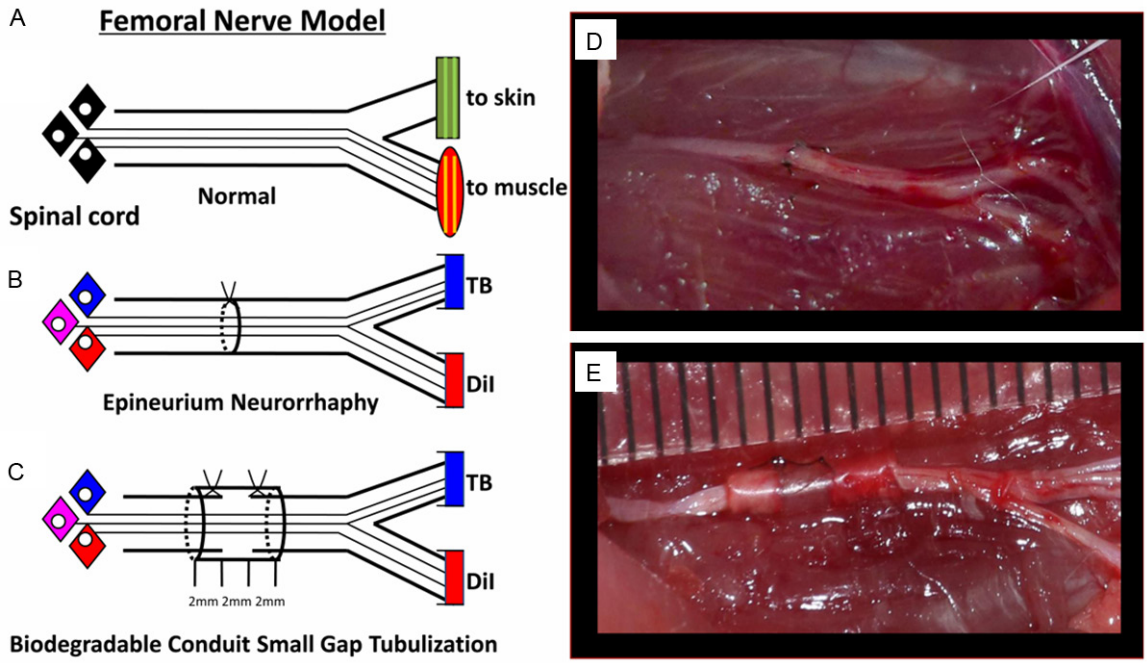

Figure 1. Diagrammatic representations of the following. A. The femoral nerve, the branch to the quadriceps muscles, and the saphenous nerve branch containing sensory nerves to the skin, all motor axons normally project to the muscle branch. B, C. Axonal sprouts from transected axons in the parent nerve can regrow into both distal branches, 12 weeks after transection and repair, axons in the two branches are retrogradely labeled with different colored neurotracers and the motor neurons are counted. B, D. The surgical procedures of repairing the femoral nerve by epineurial neurorrhaphy. C, E. The surgical procedures for repairing the femoral nerve by biodegradable conduit small gap tubulization. The 6 mm biodegradable chitin conduits were placed at the repair site and a 2 mm gap was left between the proximal and distal nerve segments.

eral nerve lesion site can be used as an alternative to nerve grafts, the gold standard for bridging the proximal and distal stumps is still the nerve autograft [14]. For fresh nerve transections, the use of tubes as an alternative to primary nerve suture has been introduced as a biologic approach to nerve injuries, creating optimal conditions for axonal regeneration over a short empty space intentionally created between the proximal and distal nerve ends. This repair technique is placed on the intrinsic healing capacities of the nerve rather than on the technical skill of the surgeon [11].

Our laboratory has investigated the application of biodegradable conduit small gap tubulization for about 20 years. We have attempted to improve the nerve regenerative effect by determining the optimal conditions for axonal regeneration over a surgically induced short, empty space between the proximal and distal nerve ends. Previous works confirm the benefit of using biodegradable conduit small gap tubulization to substitute traditional epineurial neurorrhaphy. This study was designed to determine if biodegradable conduit small gap tubulization improves the accuracy of motor axon regeneration. We also functionally and morphologically compared the results of nerve anastomosis using biodegradable conduit small gap tubulization and traditional epineurial neurorrhaphy.

Materials and methods

Animal preparation

Female young adult Sprague Dawley rats (200-240 g), obtained from the Laboratory Animal Centre of Peking University (Beijing, China) were deeply anesthetized for all surgical procedures with sodium pentobarbital (30 mg/kg, i.p.). This study was performed in strict accordance with recommendations in the Institutional Animal Care Guidelines and approved ethically by the Administration Committee of Experimental Animals, Peking University People's Hospital, Beijing, China (Permit Number: 2011-16). All efforts were made to minimize suffering.

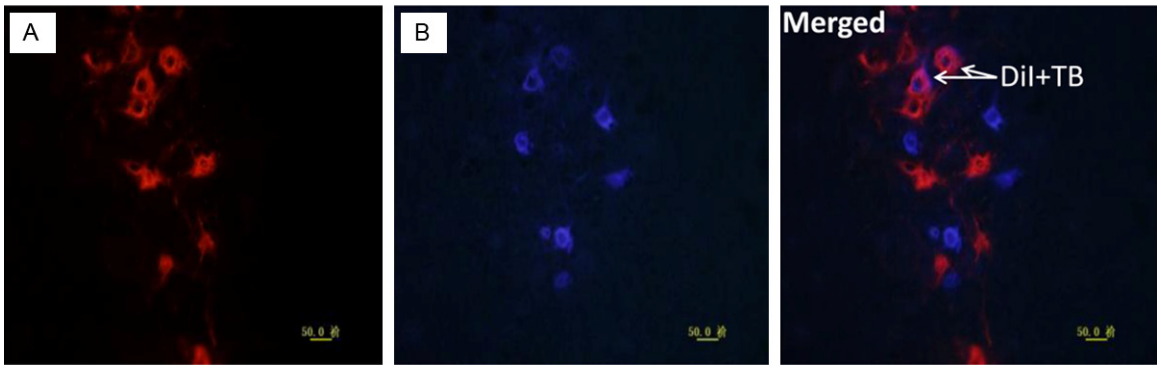

Figure 2. Rat femoral nerve motor neurons 12 weeks after nerve repair. Section demonstrating labeled cells with excitation of DiI in (A) and TB (B). DiI (A) was applied to the muscle branch of the nerve and TB (B) to the cutaneous branch. As the merged image shown here, retrogradely labeled motor neurons that contained only one of the labels (indicating axonal distribution to a single nerve branch) or both labels (DiI+TB) were counted, indicating a simultaneous axonal distribution to both nerve branches (Scale bar=50 uM).

Experimental design

Experiments were performed on the adult rat femoral nerve which contains purely cutaneous sensory fibers growing into the skin via the saphenous nerve and intermingled fibers projecting to the quadriceps muscle via the quadriceps muscle nerve (**Figure 1A**). In the normal femoral nerve, motor axons are found only in the muscle branch so that any motor reinnervation of the sensory branch represents a failure of specificity, which can be identified by retrograde tracing [15].

Thirty-two female rats were randomly divided into the epineurial neurorrhaphy group and the biodegradable conduit small gap tubulization group; when evaluation of the functional and morphological recovery of the two groups, the non-operated side served as the normal control. Regeneration was assessed at 12 weeks after nerve transection and repair.

Femoral nerve injury and repair animal model: epineurial neurorrhaphy

Experiments were performed under aseptic conditions on the right femoral nerves. The surgical procedures were performed under a surgical microscope using standard microsurgical techniques. The femoral nerve was exposed using an inguinal approach and was separated by gentle dissection to minimize tension on the subsequent repair site. The parent nerve was transected with microscissor about 7 mm proximal to the bifurcation point of the nerve into muscle and cutaneous branches, a level reported to have randomly distributed muscle and cutaneous fibers. For the epineurial neurorrhaphy group (n=16), the proximal and distal stumps were then carefully aligned and repaired by an epineurial neurorrhaphy with 10-0 nylon suture (**Figure 1B** and **1D**).

Femoral nerve injury and repair animal model: biodegradable conduit small gap tubulization

For the biodegradable conduit small gap tubulization group (n=16), the right femoral nerve was exposed using the same approach as for epineurial neurorrhaphy repair, 6 mm biodegradable chitin conduit (0.1 mm thick , 1 mm inner diameter) was used to bridge the gap at the repair site, a 2 mm small gap was left between the proximal and distal portion of the repaired nerve (**Figure 1C** and **1E**) and the nerve was fixed with a 10-0 nylon suture to the conduit (this surgical procedure has been described by Jiang Baoguo, 2006) [16]. Biodegradable chitin conduits (patented by our lab and authorized by the State Intellectual Property Office of the People's Republic of China No. ZL01 136314.2; this conduit is now in a preclinical study) using in this study are artificial nerve grafts consisting of a polysaccharide shell that demonstrates satisfactory biocompatibility and degradation characteristics. Finally, the surgical site was then closed in layers with 4-0 nylon sutures.

Retrograde labeling and counting of motor neurons

For the two groups (10 rats in each group), the right femoral nerve was re-exposed 12 weeks after nerve repair, the muscle and cutaneous

Table 1. Retrogradely labeled motor neurons: individual rat data

Muscle branch	Cutaneous branch	Both branches	Total neurons	Percentage to muscle branch (%M)
Epineurial neurorrhaphy				
211	165	61	437	48.28
106	69	21	196	54.08
205	116	30	351	58.40
172	97	25	296	58.11
295	193	72	560	52.68
151	84	32	267	56.55
133	100	19	252	52.78
98	54	23	175	56.00
200	122	47	369	54.20
295	118	22	435	67.82
Biodegradable conduit small gap tubulization				
207	63	15	285	72.63
240	78	46	364	65.93
186	46	14	246	75.61
330	156	61	547	60.33
105	62	22	189	55.56
246	75	15	336	73.21
279	96	55	430	64.88
358	84	51	493	72.62
168	46	18	232	72.41
96	31	13	140	68.57

Note. The percentage to muscle branch (%M) identify the percentage of these neurons which project correctly and only to muscle and are thus conservative estimates of specificity.

branches were isolated, cut, and backlabeled with neurotracers to identify the motor neurons innervating each branch (**Figure 1B** and **1C**). The muscle and cutaneous branches were cut 5 mm distal to the femoral bifurcation. In each rat, one branch was labeled with TB (True Blue diaceturate salt; Sigma-Aldrich T5891) and the other with DiI (1,1'-Dioctadecyl-3, 3, 3',3'-tetramethylindocarbocyanine perchlorate; Sigma-Aldrich 468495) (in practice, the dye application was alternated between animals to control for possible differences in retrograde uptake and transport of the dyes). Backlabeling with TB was done by exposing the tip of the severed branch to 2% TB in distilled water for 2 hr in a small polyethylene tube, the tube was sealed with a mixture of silicone grease and Vaseline to prevent leakage, the tube was then removed, the tip of the severed branch was extensively irrigated, sealed with silicone grease and reflected to a distant portion of the wound. The same way, backlabeling with DiI was done by exposing the tip of the severed branch to 15% DiI in 100% ethanol for 2 hr, and then irrigating the nerve and placing it in the opposite corner of the wound to prevent cross-contamination by diffusion of tracers [17]. Animals were kept for 7 days after tracer application to allow the retrograde tracers to travel back to the neuronal cell bodies.

Rats were deeply anesthetized and perfused through the left ventricle. A warm saline flush was followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After perfusion, the lumbar spinal cord (T11-L1) that includes all the femoral motor neurons [18] was removed and post-fixed for several hours in 4% paraformaldehyde and then cryoprotected in 20% sucrose overnight. The cord was frozen on dry ice and stored at -80°C until being sectioned with a cryostat. Serial 25-um frozen longitudinal sections were mounted onto glass slides, air dried, and coverslipped with Prolong (P-7481, Molecular Probes) according to the manufacturer's instructions.

The spinal cord sections were viewed by independent observers unaware of the experimen-

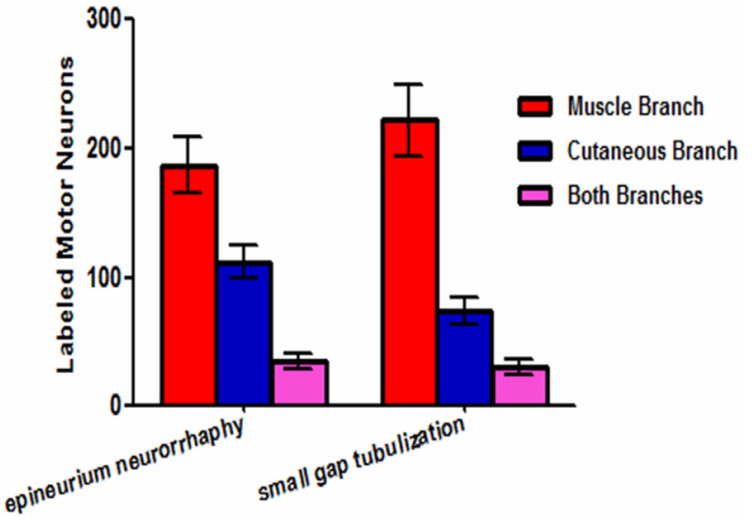

Figure 3. Average motor neuron counts of the two groups including the motor neurons correctly to the muscle branch, motor neurons incorrectly to the cutaneous branch, or motor neurons simultaneously to both branches. The two groups both reflect the preferential motor reinnervation (PMR), significant specificity of motor axon regeneration is seen after biodegradable conduit small gap tubulization. n=10, (Bars=SE).

tal treatment under a fluorescence microscope (Olympus, BX51TR) equipped with a CCD camera (Olympus, DP70). The following Olympus mirror units were used: U-MWG2 (exciter filter 510-550 nm, dichroic beamsplitter 570 nm, barrier filter 590 nm) for DiI, U-MWU2 (exciter filter 330-385 nm, dichroic beamsplitter 400 nm, barrier filter 420 nm) for TB. Motor neurons were observed as either single labeled (DiI or TB only) or double labeled (both DiI and TB). Counting variation among the independent observers was approximately 2%. The presence of split cells in adjacent sections was corrected for by the method of Abercrombie [19]. Motor neurons were scored as projecting axons (1) correctly to the muscle branch, (2) incorrectly to the cutaneous branch, or (3) simultaneously to both branches.

Preliminary experiments in control animals using DiI and TB applied to previously uninjured nerves (n=12) were used to establish the number of motor neurons normally supplying the quadriceps muscle and the parity between retrograde tracers, the animals' spinal cords were prepared and analyzed as described above.

Electrophysiological tests and muscle force

The operated and non-operated femoral nerves of two groups (6 rats in each group) were re-exposed and carefully isolated from the surrounding tissue at 12 weeks after nerve repair. The non-operated side served as the normal control. The stimulating bipolar electrodes were placed proximal and distal to the repair site in each group sequentially. The recording electrode was placed in the quadriceps muscle, while the ground electrode went subcutaneously, between the stimulating and recording electrodes. The electrical stimuli (5 V in intensity, 0.1 ms in duration, 1 Hz in frequency) (MedlecSynergy; Oxford Instrument Inc, United Kingdom) was applied to the repaired femoral nerves. The latency of Compound Muscle Action Potentials (CMAP) was recorded on the quadriceps muscle belly. The distance between the distal and proximal stimulated sites was measured to calculate the motor nerve conduction velocity (NCV) of the experimental side and control side.

Recovery of the muscle strength was determined by measuring a twitch tension and tetanic tension in the quadriceps muscle. The muscle from the experimental side was freed from its surroundings, leaving the proximal origin intact. The knee and femur were fixed with clamps. The distal tendon of the quadriceps muscle was connected to force transducers (MLT500/D; Force Transducer, ADInstruments) using a nylon ligature. Hook-shaped stimulating electrodes were placed on the femoral nerve trunk proximal to the repair site. While the single maximal stimulus was then delivered to the femoral nerve, the twitch tension of the entire quadriceps was recorded at the optimal muscle length. Tetanic tension was subsequently determined with a 50-Hz electronic stimulation. The monitoring data were recorded and analyzed using the Scope software (version 3.6.12). The muscle strength of the control side was measured as well.

Evaluation of the quadriceps muscle

After the electrophysiological tests, the quadriceps muscles of the experimental side and the control side were harvested and their wet

Table 2. Measurements of contraction force on quadriceps muscles and motor nerve conduction velocity of femoral nerves (Mean ± SD)

Measurement	Normal control (n=12)	Epineurial neurorrhaphy (n=6)	Biodegradable conduit small gap tubulization (n=6)
Twitch tension (N)	1.72±0.07	0.94±0.06*	1.00±0.09*
Tetanic tension (N)	4.44±0.30	2.68±0.28*	2.99±0.67*
MNCV (m/s)	47.06±6.22	24.83±3.12*	28.60±2.15*

*$p < 0.05$ versus normal control.

Table 3. Measurements of wet weight and cross-sectional area on quadriceps muscles (Mean ± SD)

Measurement	Normal control (n=12)	Epineurial neurorrhaphy (n=6)	Biodegradable conduit small gap tubulization (n=6)
Wet weight (g)	2.30±0.20	1.60±0.12*	1.64±0.08*
Cross-sectional area (um^2)	1515.66±57.24	1294.58±116.81*	1360.14±62.13*

*$p < 0.05$ versus normal control.

weights were measured. Muscle samples were cut from the midbelly of the harvested quadriceps muscle and fixed in a buffered 4% paraformaldehyde solution. Afterwards, the muscle samples were cut and subsequently washed in water, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraffin and cut into 5 mm thick transverse sections. Following the H&E staining, the sample was photographed with a DFC 300FX color digital camera (Leica, Heidelberg, Germany) to measure the cross-sectional area of muscle fibers. For each in four H&E stained sections of every specimen, the images were taken from four random fields and analyzed with a Leica QWin software package Q550 IW image analysis system (Leica Imaging Systems Ltd., Cambridge, England).

Histological analysis for nerve regeneration

After the quadriceps muscles were dissected out, the segments of the distal femoral nerve at 2 mm distal to the repair site and the normal femoral nerve at the same level were harvested. After these segments were fixed in 1% osmium tetroxide for 24 hours, they were dehydrated with ethanol and embedded in paraffin. The specimen blocks were cross-sectioned at a thickness of 5 um using an ultramicrotome. All sections were then transferred to adhesion microscope slides. The nerve sections were examined and digitized images were obtained using a DFC 300FX color digital camera (Leica, Heidelberg, Germany). The number of myelinated fiber; diameters of the myelinated axons and the thickness of myelin sheaths were examined from the digitized images.

Statistical analysis

The specificity of motor axon regeneration (the percentage to muscle branch (%M)) was calculated by dividing the number of motor neurons which project to muscle by the number of total labeled motor neurons [20]. The SPSS 17.0 software package (SPSS Inc., USA) was used for statistical analysis. Experimental data were compared using the Student's t test and One-Way ANOVA followed by Student-Neuman-Keuls test. Differences were considered statistically significant when $P<0.05$.

Results

General observations

All of the rats used in this study survived in the experiments. None of rats showed signs of systemic or regional inflammation and serious surgical complications following the surgeries. The right hind limbs and feet of the animals in both two groups showed mild swelling. Significant muscle atrophy was observed in all rats. The biocompatibility of biodegradable chitin conduit in rats was quite good. The regenerated nerve in conduit grew smoothly, without neuroma formation.

Specificity of motor axon regeneration

12 weeks after repair of the femoral nerve, motor neurons were identified in the spinal cord as being labeled from just one terminal branch or from both branches simultaneously (**Figure 2**). The results of retrogradely labeled

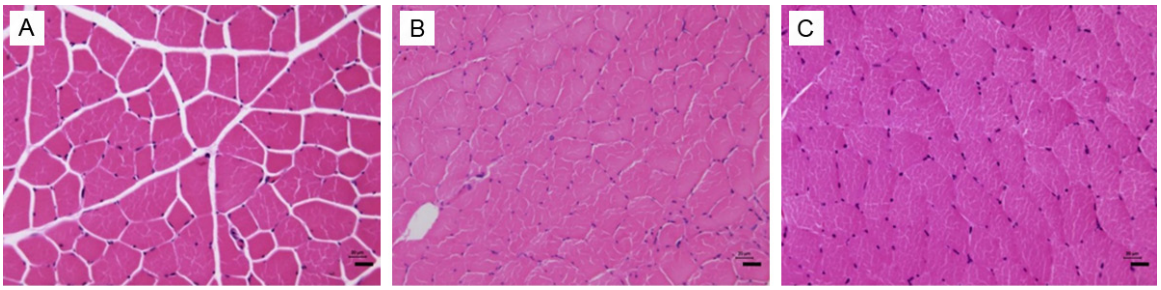

Figure 4. Light microscopy images of transverse sections of the quadriceps muscle, A. Normal control; B. Epineurial neurorrhaphy group; C. Biodegradable conduit small gap tubulization group (Scale bar=20 uM).

motor neurons were shown in **Table 1** and **Figure 3**. There was no statistical difference in the number of total labeled motor neurons (single- and double-labeled) of two groups (biodegradable conduit small gap tubulization group: 326±42 vs. epineurial neurorrhaphy group: 334±38, P>0.05). The tendency of motor neurons to preferentially reinnervate the muscle as compared with the cutaneous branch was reflected within individual animal data (**Table 1**). Although there was no statistical difference in the number of motor neurons labeled from the muscle nerve branch between small gap tubulization group (222±28) and epineurial neurorrhaphy group (187±22) (P>0.05), the number of motor neurons which project incorrectly to cutaneous nerve branch in small gap tubulization group (74±11) was significantly lower than epineurial neurorrhaphy group (112±13) (P<0.05). This observation is reflected in a significant difference in the specificity of motor axon regeneration between the two groups (small gap tubulization group: 68.18± 2.04% vs. epineurial neurorrhaphy group: 55.89±1.63%, P<0.05) (**Figure 3**).

Preliminary experiments in normal animals were used to value the efficacy of the retrograde tracers DiI and TB as retrograde labels of motor neurons projecting to the terminal femoral nerve branches. No significant differences were seen between the number of motor neurons labeled by the TB (347±14, n=6) and DiI (356±18, n=6), and these counts are in agreement with previous rat femoral nerve work [4, 21].

Electrophysiological tests and muscle force

The results of nerve conduction velocity were indicated in **Table 2**. The motor nerve conduction velocities of small gap tubulization group (24.83±3.12 m/s) and epineurial neurorrhaphy group (28.60±2.15 m/s) were significantly slower than that of the normal control group (47.06±6.22 m/s) . From **Table 2**, it is demonstrated that the recovery level of electrophysiological properties in small gap tubulization group was a bit higher than that in epineurial neurorrhaphy group, there was no statistically difference between the two groups (P>0.05).

The quadriceps muscle's contraction force, containing twitch and tetanic tension, of the animals were shown in **Table 2**. The mean twitch and tetanic tensions in small gap tubulization group (I.00±0.09 N, 2.99±0.67 N, respectively) and epineurial neurorrhaphy group (0.94±0.06 N, 2.68±0.28 N, respectively) were significantly lower than that in the control group (1.72±0.07 N, 4.44±0.30 N, respectively). The mean twitch and tetanic tensions in small gap tubulization group were slightly higher than those in epineurial neurorrhaphy group, this differences were not statistically significant (P> 0.05).

Evaluation of the quadriceps muscle

The wet weight of quadriceps muscle in small gap tubulization group (1.64±0.08 g) and epineurial neurorrhaphy group (1.60±0.12 g) were significantly smaller than that in the control group (2.30±0.20 g). The wet weight in small gap tubulization group was slightly higher than that in epineurial neurorrhaphy group which was not significant (P>0.05) (**Table 3**).

The transverse sections of the quadriceps muscle were displayed in **Figure 4**. The quadriceps muscle fiber boundary in the control group was clear. Sections from small gap tubulization group and epineurial neurorrhaphy group displayed unclear boundary. Significant muscle

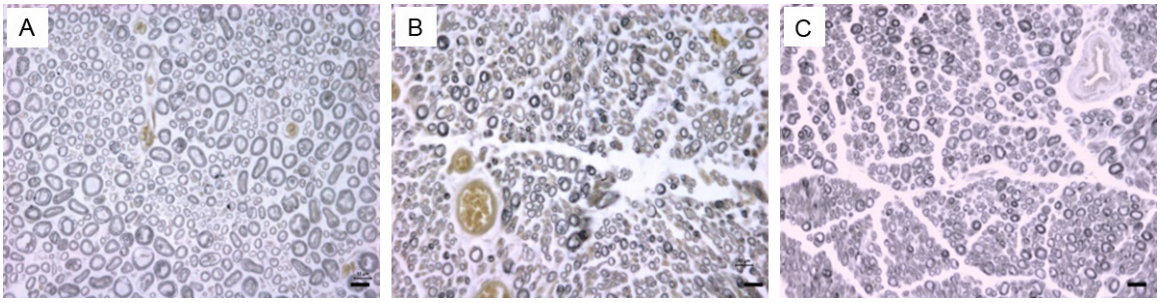

Figure 5. Light microscopy images of transverse sections of the femoral nerve. A. The nerve segment in the normal control; B. The distal femoral nerve segment in epineurial neurorrhaphy group; C. The distal femoral nerve segment in biodegradable conduit small gap tubulization group (Scale bar=20 uM).

atrophy was observed in small gap tubulization group and epineurial neurorrhaphy group. The fiber cross-sectional area in small gap tubulization group (1360.14±62.13 um^2) and epineurial neurorrhaphy group (1294.58±116.81 um^2) was significantly smaller than that in the control group (1515.66±57.24 um^2). The fiber cross-sectional area in small gap tubulization group was a bit higher than that in epineurial neurorrhaphy group, there was no statistically difference between the two groups (P>0.05) (**Table 3**).

Histological analysis for nerve regeneration

12 weeks after surgery, the sectioned nerves from each group were stained with osmium tetroxide. The micrographs of transverse sections of the femoral nerve were displayed in **Figure 5**. The distal femoral nerve segments at 2 mm distal to the repair site in small gap tubulization group and epineurial neurorrhaphy group revealed that the regenerated myelinated fibers occurred, with a higher density but smaller fiber size compared to the control group. For both small gap tubulization group and epineurial neurorrhaphy group, the regenerated motor axons were evenly distributed and the diameter of the fibers was similar.

The results of the morphological analysis are summarized in **Figure 6**. The myelinated fiber count of the distal femoral nerve segments in small gap tubulization group (4539±205) and epineurial neurorrhaphy group (4374±159) were both significantly higher than those in the control group (2396±226). The myelin sheath thickness of the distal femoral nerve segments as well as the axonal diameter and axonal area of myelinated nerves in small gap tubulization group (1.03±0.04, 2.27±0.20, 4.08±0.69, respectively) and epineurial neurorrhaphy group (0.96±0.07, 2.16±0.15, 3.68±0.48, respectively) were significantly lower than those in the control group (1.53±0.10, 3.57±0.11, 10.04±0.60, respectively). The morphological outcomes of small gap tubulization group were a bit higher than that of epineurial neurorrhaphy group, this differences were not statistically significant (P>0.05).

Discussion

This femoral nerve model was chosen to explore sensory/motor specificity because of its anatomical characteristics. The femoral nerve divides just distal to the inguinal ligament into one cutaneous branch and a muscle branch to the quadriceps. At the site of nerve repair, proximally within the femoral trunk, axons destined for skin and muscle intermingle. Regenerating motor axons will thus have equal access to Schwann cell tubes that lead to both skin and muscle as they reinnervate the distal nerve stump. Distally, the nerve bifurcates into distinct muscle and cutaneous branches that are well-matched as targets for regenerating axons. In 1945, Weiss and Edds originally introduced the rat femoral nerve as a model system to study the fate of axons that originally innervated muscle or skin when they were forcibly misdirected into the inappropriate nerve branch [22]. In the normal femoral nerve, no motor axons project into the cutaneous branch, thus, reinnervation of this distal branch by regenerating motor axons represents a failure of specificity, which can be quantified by retrograde tracing [15]. In our study, the results suggest that, for the two different suture methods, the regenerating motor neurons preferen-

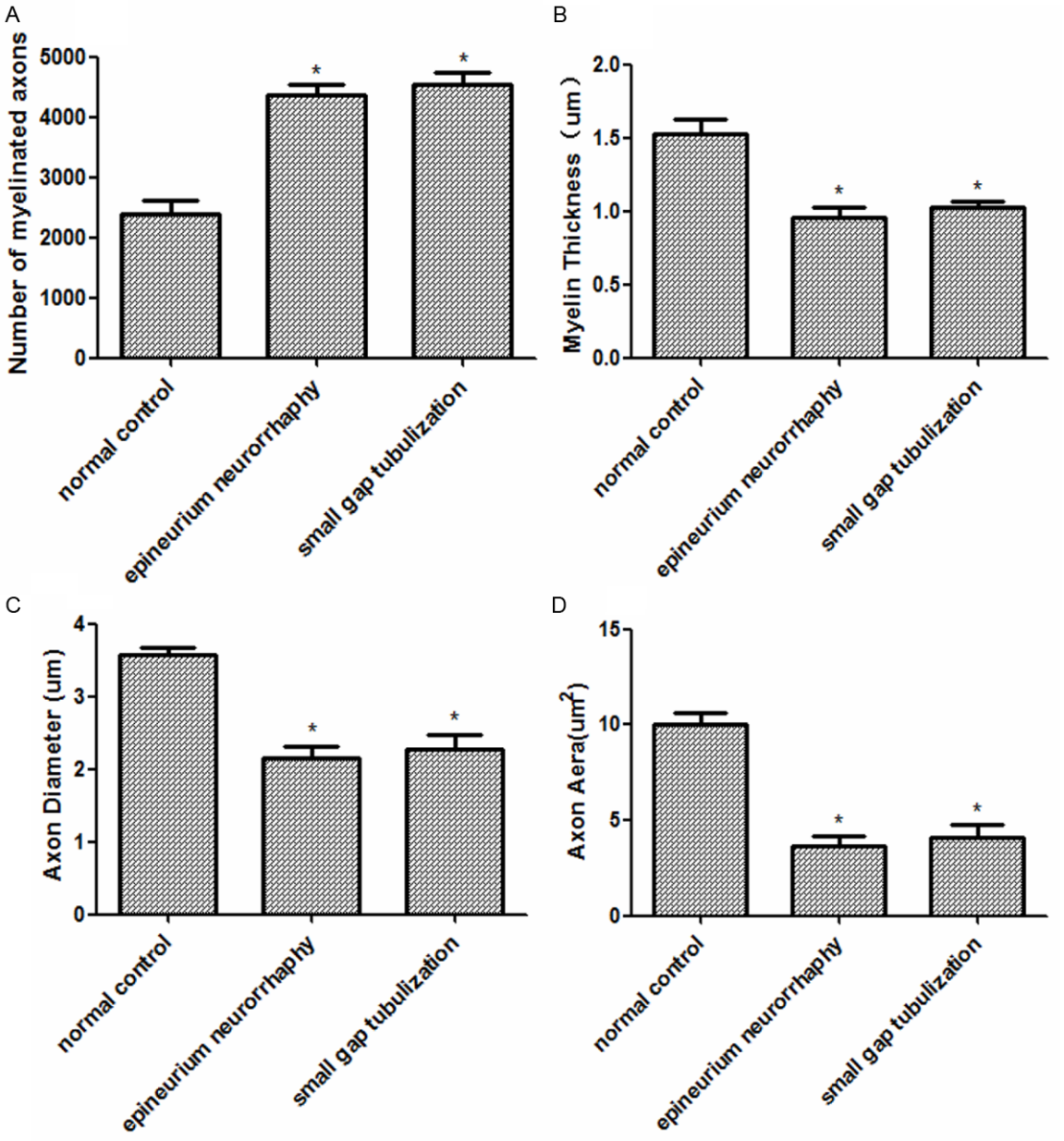

Figure 6. The femoral nerve morphological parameters of the normal control (n=12), epineurial neurorrhaphy group (n=6) and biodegradable conduit small gap tubulization group (n=6). A. The number of myelinated axons; B. The myelin sheath thickness; C. The axon diameter; D. The axon area. *P<0.05 versus normal control (Bars=SD).

tially reinnervated a terminal nerve branch to the muscle as opposed to the skin, which are consistent with previous studies. This process is also known as preferential motor reinnervation (PMR) [4, 10]. The percentage of regenerating motor neurons that project correctly to muscle (%M) is a conservative measure of specificity, as it includes double-labeled motor neurons without scoring them as correct projections [20].

Fluorescent retrograde tracers are frequently used in the rat femoral nerve model to evaluate the specificity of regeneration. It is especially important that the tracers being used have similar labeling efficacies and label the same population of studied neurons. The labeling efficacy of different fluorescent tracers was found to be related to the method of its application and the survival time of experimental animals after tracer application, as well as intrinsic proper-

ties of the tracers. The retrograde tracers DiI and TB using in our study have the highest and similar labeling efficacy and their combinations are also most suitable for double retrograde labeling studies [23]. The number of DiI labeled motor neurons in normal animals was not statistically significantly different from the number of TB labeled motor neurons in preliminary experiments, in addition, the recovery period of 7 days was chosen in this study to allow enough time for retrograde transport and tracer accumulation in the neuron body.

Distal stumps of injured nerves go through a series of cellular and biomolecular changes, including elimination of axons and marrow, proliferation of SCs, and Wallerian degeneration. Proliferated but not yet differentiated SCs form a "band of Bungner" in the inner membrane conduits and produce various neurotrophic factors. The basal lamina scaffolds of Schwann cells in the distal stump serve as effective pathways for the elongation, maintenance, and maturation of regenerating axons. During early stages of regeneration (2 and 3 weeks), an equal number of motor neurons project correctly to muscle and incorrectly to skin, with many projecting collaterals to both. It is not until later stages of regeneration (8 and 12 weeks) that incorrect collaterals are pruned and the majority of motor neurons project their axons to muscle [4, 10]. There was a small decline in the number of motor neurons that regenerated into both muscle and cutaneous branches with time, the "pruning" is a relatively minor contributor to the emergence of PMR in adult rat nerve regeneration, motor axons that project only to the cutaneous branch early on did not change after 2 weeks [24]. When regenerating motor axons enter Schwann cell tubes in the distal stump that lead to sensory nerve branches, they are directed to sensory end organs. Not only do these axons fail to establish functional contacts, they also exclude appropriate axons from entering the pathways that they occupy [4]. Thus, an initial correct projection is important for functionary recovery. In this study, the specificity of motor axon regeneration (the percentage of motor neurons which project correctly and only to muscle) were significantly higher when the nerve transection was treated by biodegradable conduit small gap tubulization, which indicated that biodegradable conduit small gap tubulization may improve the accuracy of peripheral nerve selective regeneration.

The higher specificity of regeneration that was obtained using biodegradable conduit small gap tubulization is an important discovery possibly leading to a more functional recovery over epineurial neurorrhaphy. Zhang et al. demonstrated that using the biodegradable conduit small gap tubulization to substitute for the traditional epineurial neurorrhaphy can achieve enhanced recovery of morphology and electrophysiology in monkeys [25, 26]. Zhang et al. also reported that the operation procedure time of conduit small gap tubulization was more convenient and timesaving compared with traditional epineurial neurorrhaphy, the clinical regeneration effect of conduit small gap tubulization was better than traditional epineurial neurorrhaphy [27]. However, using silastic tube with 0-mm gap, 2-mm gap and 5mm gap to repair the transected sciatic nerve, Weber RA et al. demonstrated that there was an insignificant functional influence of regeneration specificity [28]. Romano et al. reported that when compared traditional suture, collagen tubs with small gap were equally as effective in their recovery [29]. These differences may be because of the differences of tubs and animal models as well as different assessment methods. Our results suggesting that nerve repair with biodegradable conduit small gap tubulization may be slightly better than epineurial neurorrhaphy at 12 weeks after nerve repair will require further experimentation. Technically, when compared to using traditional epineurial suture under the microscope, nerve repair is considered less traumatic, quicker and easier using conduit small gap tubulization [29]. This could mean increased clinical use of repair technique requiring less skilled training.

The mechanism of higher specificity of motor axon regeneration (PMR) by using biodegradable conduit small gap tubulization is not completely understood from the data presented here. The chitin conduits may create a microenvironment that is suitable for the diffusion of target-derived neurotrophic factors and allow for better interaction between target-derived and proximally generated biochemical signals. Lundborg et al. and Mackinnon et al. using silicone chambers demonstrated that axons will grow toward nerve rather than tendon, muscle,

or granulation tissue. This phenomenon is referred to as neurotropism (chemotropism) [30, 31]. Several previous assessments of reinnervation specificity in the adult femoral nerve model demonstrated that preferential motor reinnervation may be influenced by subtle changes in the regeneration state of the motor neuron, the permissiveness of the repair site, or the environment within the distal nerve stump [32]. Madison and Robinson reported that there are two explanations for the specificity of motor axonal regeneration, the most important one is that regenerating motor axons evaluate the relative levels of trophic support (including many growth factors such as brain-derived neurotrophic factor (BDNF), glia-derived neurotrophic factor (GDNF), neurotrophin-3 (NT3), ephrins, hepatocyte growth factor (HGF), fibroblast growth factors, etc.) in each pathway and preferentially regenerate into the one that provides the greater amount of support and the other is that the Schwann cell tubes of the respective pathways keep a specific identity which can be recognized by regenerating motor axons [33]. With a small gap between the ruptured mixed nerve, the proximal sensory and motor nerve fibers grow through the gap and selectively find their counterparts in the distal nerve stumps. The chitin conduits may provide an effective nerve regenerating chamber, neurotropic (substance exerting attraction at distance on growing axons) and neuronotropic (factor important for the survival and maturation of the neuron) factors may accumulate in the tube [34, 35]. This could create an optimal environment for orientation and guidance of the regenerating axons. The specificity of traditional epineurial neurorrhaphy is lower possibly because it does not provide an effective nerve regenerating chamber for the regenerating axons to find their counterparts in the distal nerve stumps.

In conclusion, the major determinant of functional recovery after lesions in the peripheral nervous system is the accurate regeneration of axons to their original target end-organs. Small gap tubulization with biodegradable chitin conduits can create optimal conditions for axonal regeneration and improve the accuracy of reinnervation. The functional and morphological results in small gap tubulization group were slightly better than that in epineurial neurorrhaphy group at 12 weeks after nerve repair in our study. This repair technique is placed on the intrinsic healing capacities of the nerve. In future studies, because of the enhanced initial accuracy, less traumatic, quicker and easier technique, a peripheral nerve injury may be repaired by biodegradable conduit small gap tubulization as an alternative to conventional repair techniques.

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Disclosure of conflict of interest

None.

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