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Sleeve bridging of the rhesus monkey ulnar nerve with muscular branches of the pronator teres: multiple amplification of axonal regeneration

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Abstract

Multiple-bud regeneration, *i.e.*, multiple amplification, has been shown to exist in peripheral nerve regeneration. Multiple buds grow towards the distal nerve stump during proximal nerve fiber regeneration. Our previous studies have verified the limit and validity of multiple amplification of peripheral nerve regeneration using small gap sleeve bridging of small donor nerves to repair large receptor nerves in rodents. The present study sought to observe multiple amplification of myelinated nerve fiber regeneration in the primate peripheral nerve. Rhesus monkey models of distal ulnar nerve defects were established and repaired using muscular branches of the right forearm pronator teres. Proximal muscular branches of the pronator teres were sutured into the distal ulnar nerve using the small gap sleeve bridging method. At 6 months after suture, two-finger flexion and mild wrist flexion were restored in the ulnar-sided injured limbs of rhesus monkey. Neurophysiological examination showed that motor nerve conduction velocity reached 22.63 ± 6.34 m/s on the affected side of rhesus monkey. Osmium tetroxide staining demonstrated that the number of myelinated nerve fibers was 1,657 ± 652 in the branches of pronator teres of donor, and 2,661 ± 843 in the repaired ulnar nerve. The rate of multiple amplification of regenerating myelinated nerve fibers was 1.61. These data showed that when muscular branches of the pronator teres were used to repair ulnar nerve in primates, effective regeneration was observed in regenerating nerve fibers, and functions of the injured ulnar nerve were restored to a certain extent. Moreover, multiple amplification was subsequently detected in ulnar nerve axons.

***Key Words:** nerve regeneration; peripheral nerve; rhesus monkey; muscular branches of pronator teres; ulnar nerve; multiple amplification; small gap; sleeve bridging; NSFC grants; neural regeneration*

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Introduction

Peripheral nerve injury is very common in the clinic. With the development of microsurgical techniques and nerve tissue engineering (Haastert and Grothe, 2007; Chang et al., 2008; Biazar et al., 2010; Cunha et al., 2011; Nectow et al., 2012; Hsu et al., 2013), the efficacy of repairing peripheral nerve injury has significantly increased. However, treatment of a large segment of proximal nerve defects and root avulsion (such as brachial plexus injury) remains unsatisfactory due to the lack of a proximal donor nerve (Dahlin et al., 2009; Deumens et al., 2010). Thus, treatment of peripheral nerve injury has been problematic (Gu et al., 2011; Jiang et al., 2013). Peripheral nerve transfer is a common method for treating peripheral nerve injury of proximal donor deficiency and has been extensively used to treat brachial plexus injury (Giuffre et al., 2010; Kachramanoglou et al., 2010). A recent study used the ipsolateral/contralateral C_7 to repair brachial plexus injury (Gao et al., 2013). They also reported that phrenic nerve transfer could be applied to repair brachial plexus injury, as well as to the accessory nerve and suprascapular nerve to repair brachial plexus injury (Lu et al., 2012; Xiao et al., 2014; Yang et al., 2014). For nerve injury of severe limbs (such as large-segment defects of the median nerve, ulnar nerve, radial nerve, or sciatic nerve), when conventional nerve graft is unable to fill the range of neurological defects, or when the donor nerve is deficient, nerve transfer has been used to clinically repair the injured nerve (Siemionow and Brzezicki, 2009). For example, muscular branches of the

musculocutaneous nerve and pronator quadratus have been used to repair median nerve injury and ulnar nerve injury. Follow-up results of the above-mentioned repair methods demonstrated that nerve transfer resulted in regeneration, to a certain extent, as well as some repair of functions of the injured nerve (Dahlin et al., 2009; Fox and Mackinnon, 2011). Several clinical cases have revealed that recovery of impaired nerve function was not satisfactory following nerve transfer to repair brachial plexus injury (Malessy and Pondaag, 2011; Gao et al., 2012). This was likely because the selected donor nerve was relatively small, resulting in less neurons and axons for regeneration and inadequate innervation (Giuffre et al., 2010). Recent studies of peripheral nerve regeneration and repair have focused on novel methods of peripheral nerve repair to fully exploit the potential of peripheral nerve regeneration.

Multiple-bud regeneration, *i.e.*, multiple amplification of nerve regeneration, has been shown in peripheral nerve regeneration. Multiple buds have been shown to grow towards the distal nerve stump during proximal nerve fiber regeneration (Bishop, 1982). This kind of regeneration method was verified during the early 20th century, but its clinical application prospects were not used until much later (Jiang et al., 2007). Recent studies have used multiple amplification of peripheral nerve regeneration to treat a multitude of problems, such as peripheral nerve root avulsion and nerve defects (Jiang et al., 2007; Kou et al., 2010; Yin et al., 2013). Based on the phenomenon of using small donor nerves to repair big receptor nerves, donor nerves have been shown to send out a large number of regenerating nerve fibers that grow into receptor nerve (Jiang et al., 2007; Yin et al., 2013). If these regenerating nerve fibers can survive in the receptor nerve and grow into target organs and tissues, functions of the impaired nerve could be restored. Additionally, with the development of the small gap sleeve bridging method and sleeve bridging material for the peripheral nerve, researchers have found that the efficiency of multiple amplification of peripheral nerve regeneration increased under the condition of sleeve bridging (Zhang et al., 2009; Jiang et al., 2010). These results suggested a clinical application value and prospects of multiple amplification of peripheral nerve regeneration. Our team also performed a series of studies on multiple amplification of peripheral nerve regeneration: rodent studies demonstrated that the enlarged limit of the regenerating nerve was about 3.3 in rats after immediate injury. In other words, when the receptor nerve provided sufficient space for growth (ratio of receptor to donor nerve fibers > 4:1), the proximal donor nerve fibers could erupt 3–4 mature lateral buds that grew into the receptor nerve (Jiang et al., 2007; Yin et al., 2013). In the case where there are less donor nerves than receptor nerves, donor nerves could still achieve repair effects similar to receptor nerve fibers, and the optimal proportion of receptor to donor nerve fibers was between 1:1 and 1:2 (Yin et al., 2011). A rodent study showed that donor nerve could repair donor nerve innervated areas and receptor nerves (Yin et al., 2011).

Multiple amplification of the peripheral nerve provides a novel method for treating refractory peripheral nerve injury. To further explore the feasibility of peripheral nerve repair in the clinic, based on multiple nerve amplification, the present study verified previous results from studies on rodents, and evaluated the multiple amplification of primate peripheral nerves and repair effects in rhesus monkey models.

Materials and Methods

Experimental animals

Three specific-pathogen free, healthy, adult, male rhesus monkeys, aged 4–6 years, were purchased and housed in the Experimental Animal Center of Academy of Military Medical Sciences of Chinese PLA. The monkeys were acclimated for 2 weeks at 20°C and humidity of 40–70%. The protocols were conducted in accordance with the Management Measures of Experimental Animal Center of Academy of Military Medical Sciences of Chinese PLA. This study was approved by the Ethics Committee, People's Hospital, Peking University, China.

Establishment of models of ulnar nerve injury

Rhesus monkeys were anesthetized with an intramuscular injection of 1 mL/kg Sumianxin. The right upper limbs were shaved and sterilized. An oblique incision was made 2 cm below the middle of the elbow of the right forearm to expose the median nerve and muscular branches of the pronator teres, and the muscular branches of the pronator teres were dissociated (**Figure 1A**). An incision was made below the sulcus of the ulnar nerve to expose and to dissociate the ulnar nerve. Muscular branches of the pronator teres were transected 1 cm from the bifurcation with a surgical knife. The proximal nerve stump was trimmed, and the distal muscular branches were sutured onto the surrounding muscle tissue. The ulnar nerve was transected 1 cm below the sulcus of the ulnar nerve with a surgical knife (**Figure 1B**). The distal end of the ulnar nerve was dissociated, moved, and sutured to the proximal end of the pronator teres with a cone chitin biological conduit (self-made; patent No. Zl.01134542.X; **Figure 2A**) by small gap sleeve bridging. The gap between the two stumps was 2 mm (**Figure 1C**). The wound was closed and the skin was sutured. At 3 days after model establishment, gentamicin was administered daily to prevent wound infection (Zhang et al., 2008).

Receptor nerve suture using the small gap sleeve bridging method

The nerve stumps were trimmed, and the proximal nerve stump was inserted 2 mm in the biological conduit and sutured 1 mm from the proximal nerve stump. Two suture lines outside the conduit were knotted. The distal nerve stump was sutured to the conduit using the same method. The distal and proximal nerve stumps were separately inserted 2 mm into the conduit, with a 2 mm gap between the two stumps (**Figure 3**).

General morphology of nerve

Wound healing and ulceration in the fingers of rhesus monkey were regularly observed after model establishment. At 6

months after model establishment, all rhesus monkeys were anesthetized to expose the ulnar nerve and muscular branches of the pronator teres. General morphology of nerves, conduit absorption, nerve adhesion and neuroma formation were observed in the suture site.

Evaluation of motor and sensory functions of the ulnar nerve in rhesus monkeys

Feeding actions and activity in the bilateral upper limbs and hands were observed at 1, 3, and 6 months after model establishment, three times for each animal, for 5 minutes each. Evaluation mainly focused on activities of the two fingers on the operated side.

Assessment of passive movement of the ulnar nerve and regional sensory function in rhesus monkeys

All rhesus monkeys were anesthetized to expose the ulnar nerve and muscular branches of the pronator teres at 1, 3, and 6 months after model establishment. Continuous electrical stimulation was administered with a MedlecSynergy electrophysiological instrument (model 04oc003; Oxford Instrument, Abingdon, Oxfordshire, UK) at strength 0.9 mA, pulse width 0.1 ms, and frequency 50 Hz. Stimulation sites included the ulnar nerve at 1 cm from the distal end of suture site and the median nerve at 1 cm from the proximal muscular branches of the pronator teres. During stimulation to the ulnar nerve, finger and wrist flexion on the operated side were observed under continuous electrical stimulation. During stimulation to the median nerve, wrist flexion and pronation of the forearm on the operated side were observed under continuous electrical stimulation. After detection of passive movement, the incision was closed. During restoration of consciousness, a pin was used to sting the right thumb. If reflex retraction or avoidance appeared in the right upper limbs, animals were considered to be in a light anesthetic state. The pin was then used to sting the right little finger, and to observe and to record whether reflex retraction or avoidance appeared.

Neurophysiological measurements

Rhesus monkeys were intramuscularly anesthetized with 1 mL/kg Sumianxin at 6 months after surgery. The sutured nerve was exposed at the original incision. Simultaneously, the distal end of ulnar nerve was exposed through a medial incision of the corresponding upper wrist. A concentric recording electrode was inserted into the hypothenar muscle bellies of the ipsilateral hand. A reference electrode was placed into the non-ulnar nerve muscle of the ipsilateral upper extremity. A stimulating electrode was separately placed into the distal and proximal ends of the nerve trunk. Electromagnetic shielding was ensured in the detection environment, and the region surrounding the nerve stem was coated with paraffin oil to reduce humoral pathway conduction. The stimulation signal was a square wave, with an intensity of 0.9 mA, pulse width of 0.1 ms, and frequency of 1 Hz. Compound muscle action potential was recorded. The latency of compound muscle action potential was recorded after stimulating the distal and proximal ends of the nerve trunk. The difference of latency (dt) was calculated, and the length of nerve trunk between stimulation points of distal and proximal ends (dl) was measured. Motor nerve conduction velocity was calculated by dl/dt. Compound muscle action potential and motor nerve conduction velocity of contralateral normal ulnar nerve were also measured.

Osmium tetroxide staining of the peripheral nerve myelin sheath

For nerve myelin sheath study, the nervous tissue, including the conduit and the 5-mm-segment from the distal and proximal ends of the conduit, was removed en bloc from each rat. For convenience of description, the obtained nerves were referred to as "proximal and distal ends." Moreover, the slicing regions were the sleeve bridging proximal nerve (4 mm proximal to proximal suture point) and the sleeve bridging distal nerve (4 mm distal to distal suture point) (**Figure 2B**). The obtained tissue was fixed in 4% paraformaldehyde for 12 hours, postfixed in 1% osmium tetroxide for 12 hours, washed with running water, immersed in distilled water, dehydrated through a graded alcohol series, permeabilized, embedded in paraffin, and sliced into 5-μm-thick transverse slices with a microtome, dried in an oven, dewaxed, and mounted with neutral resin. The sections were observed under a light microscope (Olympus, Tokyo, Japan), and the number of nerve fibers in the nerve trunk was quantified using Imagetool image analysis software (University of Texas Health Science Center at San Antonio, San Antonio, TX, USA). Each section was quantified three times, and the average value was calculated. The number of myelinated nerve fibers in proximal and distal tissue sections of each animal was quantified. The rate of multiple amplification of nerve regeneration was calculated by the number of myelinated nerve fibers in the proximal end/the number of myelinated nerve fibers in the distal end.

Statistical analysis

Data were expressed as the mean ± SD and analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). Because of the small number of experimental animals, statistical comparison was not performed.

Results

General conditions of rhesus monkeys after repair of peripheral nerve based on multiple nerve amplification

General conditions of rhesus monkeys were good after ulnar nerve injury. No infection or obvious rejection was observed in the regional wound.

Motor function of the ulnar nerve in rhesus monkeys following peripheral nerve repair based on multiple nerve amplification

Affected limbs of rhesus monkeys experienced movement disorders at 1 month after model establishment. When eating, two fingers on the ulnar side were in a bent state and did not participate in locomotor activity. At 3 months, the affected limbs still partially suffered from movement disorder, and two fingers moved when eating. At 6 months, the

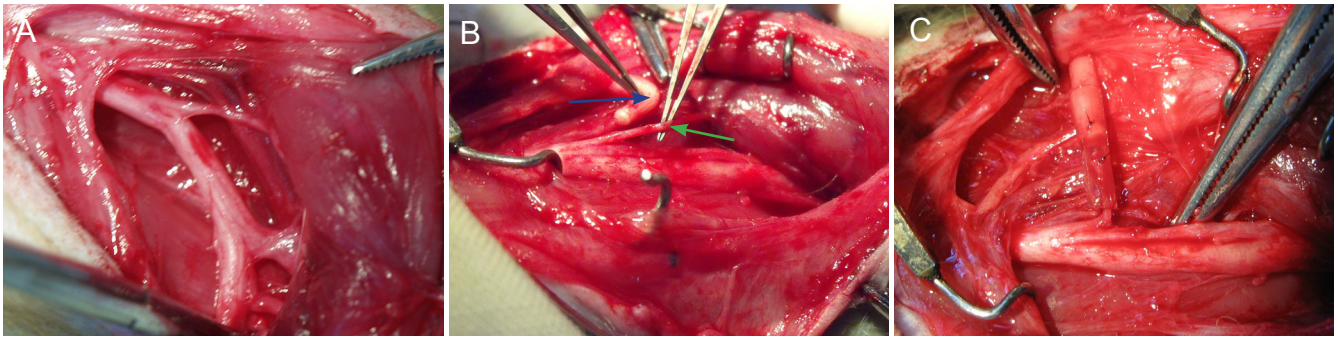

Figure 1 Preparation of rhesus monkey models of ulnar nerve injury.
(A) Exposure of muscular branches of pronator teres and ulnar nerve; (B) dissociation of muscular branches of pronator teres and ulnar nerve (blue arrow shows ulnar nerve; green arrow shows muscular branches of pronator teres); (C) sleeve bridging of proximal muscular branches of the pronator teres and distal end of the ulnar nerve.

Table 1 Evaluation of motor and sensory functions of ulnar nerve in rhesus monkeys

	Month(s) after model establishment		
Item	1	3	6
Active function test			
Grasping	Two ulnar-sided fingers cannot grasp	Two ulnar-sided fingers cannot grasp	+
Wrist flexion	+	+	+
Two-finger flexor on the ulnar side	–	±	+
Sensory function assessment			
Protective response to acupuncture under light anesthesia	–	–	–
Passive motion function test (continuous electrical stimulation)			
Pronation of upper extremity	–	–	–
Two-finger flexor on the ulnar side	–	+	+

"–": Action is not elicited or no related action; "+": action can be elicited or has related action; ±: action can be slightly elicited or has similar related action.

affected limbs presented mild movement disorder. When ulnar nerves received continuous electrical stimulation, the affected limbs showed apparent flexor movement of the two fingers on the ulnar side (**Table 1**).

Anatomic form of rhesus monkey nerves after peripheral nerve repair based on multiple nerve amplification

At 6 months after model establishment, the muscular branches of the pronator teres near the median nerve were connected to the ulnar nerve in the rhesus monkey. The regionally repaired nerve had adhered to the surrounding tissue. The chitin biological conduit at the suture site had been absorbed. Additionally, mild adhesion was detected in the suture site, but no neuroma was formed. The diameter of the ulnar nerve on the operated side was slightly smaller than the ulnar nerve on the normal side (**Figure 4A**).

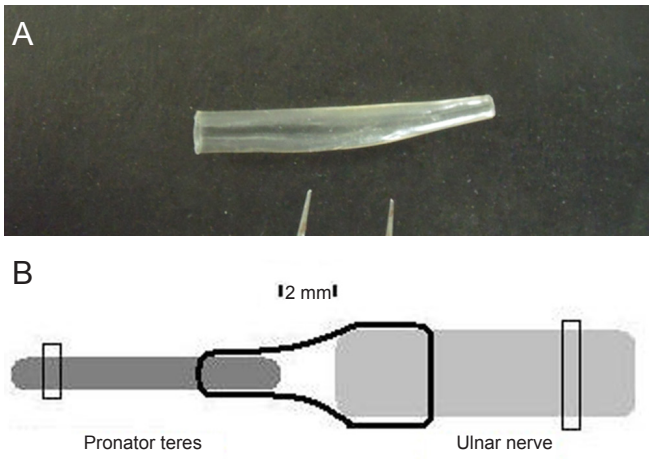

Figure 2 Shape of cone chitin biological conduit and position of peripheral nerve collection.
(A) Shape of cone chitin biological conduit; (B) sleeve bridging proximal nerve (4 mm proximal to proximal suture point) and sleeve bridging distal nerve (4 mm distal to distal suture point).

Motor nerve conduction of the ulnar nerve of rhesus monkeys following peripheral nerve repair based on multiple nerve amplification

At 6 months after model establishment, motor nerve conduction velocities of repaired ulnar nerves and contralateral ulnar nerves were 22.63 ± 6.34 m/s and 45.64 ± 9.81 m/s, respectively.

Recovery of myelinated nerve fibers of the injured ulnar nerve of rhesus monkeys following peripheral nerve repair based on multiple nerve amplification

At 6 months after model establishment, osmium tetroxide staining results revealed that myelinated nerve fibers in the proximal muscular branches of the pronator teres were uniform and densely arranged, and their morphology and structure were similar to that of normal nerve tissue (**Figure 4B**). Regenerating nerve fibers on the distal end mainly exhibited a round or elliptical shape, and had a scattered distribution. Nerve fiber density was apparently smaller than the donor nerve (**Figure 4C**). Additionally, the diameter and myelin sheath thickness of nerve fibers were less than in the proximal

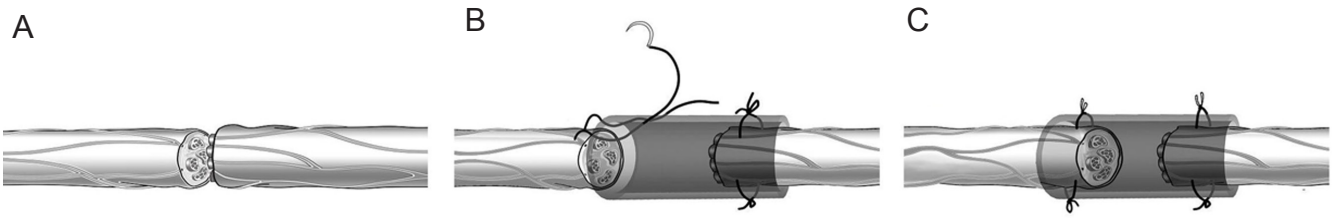

Figure 3 Pattern of small gap sleeve bridging to repair peripheral nerve injury.
(A) Trimming nerve stump; (B) the proximal nerve stump was inserted 2 mm into the conduit and sutured 1 mm from the proximal nerve stump. Two suture lines outside the conduit were knotted. (C) Distal and proximal nerve stumps were separately inserted 2 mm into the conduit, with a 2-mm-gap between the two stumps.

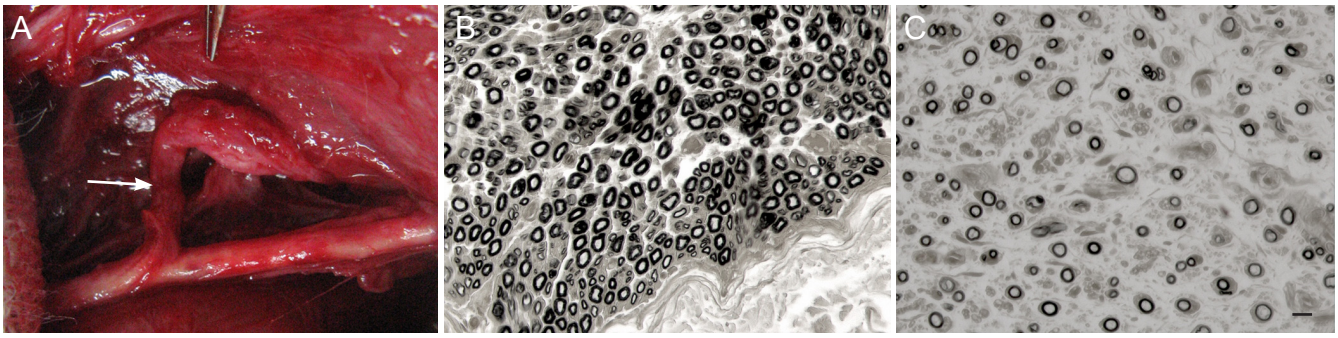

Figure 4 General appearance and myelinated nerve fibers of the injured nerve in the rhesus monkey after peripheral nerve repair based on multiple nerve amplification at 6 months after model establishment.
(A) Proximal muscular branches of the pronator teres were connected to the distal end of the ulnar nerve (arrow). The biological conduit had been absorbed. No noticeable tissue adhesion appeared surrounding the nerve. No neuroma was visible in the suture site. (B) Donor nerve proximal to the ulnar nerve; (C) receptor nerve distal to the ulnar nerve. Osmium tetroxide staining. Scale bar: 10 μm.

end. The number of myelinated nerve fibers of the proximal donor nerve was 1,657 ± 652, and the number of myelinated nerve fibers in the distal receptor nerve was 2,661 ± 843. The rate of multiple amplification of axonal regeneration was 1.61.

Discussion

Multiple amplifications (bifurcation) exist in peripheral nerve regeneration. In other words, proximal nerve fibers regenerate and erupt multiple buds that grow towards the distal nerve stump. Therefore, when small donor nerves are used to repair larger receptor nerves, the donor nerve can erupt more regenerating nerve fibers than donor nerves towards the receptor nerve, resulting in the recovery of injured nerve function. Our previous study confirmed that the amplification limit of a rat regenerating nerve was about 3.3 (Jiang et al., 2007). In the present study, quantification of myelinated nerve fibers demonstrated that the rate of multiple amplifications was less in primate (about 1.61) than in rodents. Moreover, the maturity of myelinated nerve fibers in primates at 6 months after repair was less than in rodents at 3 months after surgery. The reasons could be attributed to the following: (1) species difference: the ability of neuronal regeneration is stronger in rodents than in primates. (2) Peripheral nerve axons are longer in primates, so transport energies of neurons-synthetized nutritional factors and renewable materials are not sufficient after injury; speed and capacity of nerve regeneration are poorer in primates than in rodents. (3) The low proportion of donors to receptors: muscular branches of the pronator teres are mainly composed of motor nerve fibers, but the ulnar nerve contains only 30–40% motor nerve. Although the proportion of donors to receptors is about 1:5, the proportion of donors to receptors in the motor nerve is about 1:2. (4) Distal nerve degeneration: the distal nerve length is 5–10 times longer in primates than in rodents. During proximal nerve regeneration, the distal nerve experiences longterm degeneration, which causes a reduction in nerve regeneration in the distal end. Therefore, partial lateral buds cannot successfully grow into the distal nerve and ultimately become mature after multiple amplifications. It may take a long time of nerve regeneration in primates for nerves to grow into target organs. Additionally, some amplified axons cannot successfully grow into target organs and become mature during regeneration. These results suggest that it is necessary to use drugs to promote nerve regeneration following peripheral nerve repair based on multiple nerve amplification. These findings also indicate that distal neural degeneration probably affected peripheral nerve repair in the clinic. Nerve transfer based on multiple amplification delayed the degeneration of injured nerve and elevated the effects of nerve repair.

Nerve transfer is often considered a passive repair method and is selected when other repair methods cannot be implemented (Dahlin et al., 2009; Irintchev, 2011; Tung, 2014). Donor nerves selected for nerve transfer are tiny, but the

receptor nerve is relatively thick (Lu et al., 2012). In previous nerve transfers, the number of donor nerve fibers was less, which resulted in insufficient innervation and impacted neurological function. With a better understanding of multiple amplification of the peripheral nerve (Jiang et al., 2007), the clinical significance and application prospects of nerve transfer have been recognized and assessed. Based on multiple amplification of the peripheral nerve, receptor nerve fibers were able to reinnervate a certain proportion of donor/receptor nerve fibers. Theoretically, if nerve fibers with multiple amplification were completely mature and were able to form effective innervation, the majority of nerve functions could be restored, which has been verified in animal experiments (Jiang et al., 2007; Kou et al., 2010; Yin et al., 2013).

A review of research progress of peripheral nerves over nearly a hundred years demonstrated that histological and functional restoration could be found in experimental animals with peripheral nerve injury (Kuffler, 2009). However, recovery of injured peripheral nerves was not clinically ideal, especially injury to the proximal nerve (Madduri and Gander, 2012; Nectow et al., 2012). The main reason for the difference in recovery of injured peripheral nerves between experimental animals and patients is the long distance from injured the peripheral nerve site to target organs in human. Additionally, the speed of nerve regeneration is relatively slow. Regenerating axons often require a few months, even up to ten months, to grow into target organs. This phenomenon is most obvious following hand surgery (Chuang, 2009; Siemionow and Brzezicki, 2009). After upper limb nerve repair, the recovery of upper arm or forearm muscle function following proximal nerve injury is good, but the recovery of intrinsic muscle function of the hand is poor, which is associated with the long reinnervation of hand muscle, resulting in irreversible atrophy of the intrinsic hand muscles. Based on nerve transfer techniques and multiple amplification of nerve regeneration, experts in related fields have proposed that the nerve foster method could be used to prevent muscle denervation and atrophy, and is possibly an effective tool for addressing poor recovery of hand function following upper extremity nerve injury.

Results from this study confirmed that muscular branches of the pronator teres small gap sleeve bridging to repair the ulnar nerve could restore motor function of the ulnar nerve to a certain degree. The sleeve bridging method was applied, because there is a difference in diameter between donor and receptor nerves. The epineurium peel method was previously used in the clinic for this kind of nerve suture. However, different diameters between donor and receptor nerves led to tension in the suture site, and finally resulted in suture failure. Thus, the present study used a conical biological conduit small gap sleeve bridging method, and the biological conduit was matched with the diameter of the proximal and distal nerves (Zhang et al., 2008; Kou et al., 2010; Yin et al., 2013). The suture was simple, and the method avoided tension at the regional suture site. This biological conduit was developed and made by our team. Systematic studies confirmed that this conduit exhibited good mechanical strength and biosecurity. The regeneration chamber in the bridging site reduced escape of partial nerve fibers, provided a good microenvironment for multiple amplification of nerve regeneration, and elevated the effects of nerve regeneration (Zhang et al., 2009; Jiang et al., 2010; Kou et al., 2010).

Rhesus monkeys have been extensively applied in the study of nerve regeneration (Hu et al., 2007; Lin et al., 2012; Hu et al., 2013). They are physiologically similar to humans and ideal experimental animals for nerve regeneration. However, rhesus monkeys are precious, so they are only used in some special experiments and confirmatory studies (Zhang et al., 2009; Wang et al., 2010; Yuan et al., 2010). In combination with previous rodent studies, as well as years of clinical, the present study investigated multiple amplification of axonal regeneration using muscular branches of the pronator teres sleeve bridging to repair the ulnar nerve in rhesus monkeys, and further verified previous results. Therefore, the number of experimental animals was few. We re-repaired the obtained injured nerve again after the experiments were finished. Considering ethics, analyses, such as muscle function and histological index, were not performed in this study. Due to a limitation of sampling, electron microscopy and immunohistochemistry were not conducted. Nevertheless, these limitations did not impact the aim and results of this study. The motor nerve of the muscular branches of the pronator teres was selected for this study, so the indices detected in this study were related to motor nerve functions.

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Conflicts of interest: *None declared.*

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